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Ovitek: Uporaba termovizijske kamere za oceno učinkov masaže na telesne regije konja (Foto: Andrej Mergeduš)
*Cover: Use of a thermal imaging camera to assess the effects of massage on the horse's body regions (Photo: Andrej Mergeduš)*

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## Review Article / Pregledni znanstveni članek

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# Evaluation of salinity tolerance in seedlings of *Hyppeastrum reticulatum* (L'Hér.) Herb.

Mohammad Ali KHALAJ [1, 2], Mohammad Hossein AZIMI [1, 2], Pegah SAYYAD-AMIN [3]

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**Evaluation of salinity tolerance in seedlings of** ***Hyppeastrum reticulatum*** **(L'Hér.) Herb.**

**Abstract:** Amaryllis (*Hyppeastrum* Herb.) is one of the bulbous ornamental plants that is distributed around the world. Regarding the cultivation of ornamental plants in landscaping, it is essential to use salinity-resistant ornamental species. Less research has been done on the impact of salt irrigation on the growth and development of bulbous ornamental plants like this plant. So, in order to investigate salinity tolerance in amaryllis, the experiment was done with five salinity concentrations [control (distilled water) with EC = 0dSm$^{-1}$, and electrical conductivity (EC) at 2, 4, 6 and 8 dSm$^{-1}$] with four replication on leaf freshness, leaf length and width, proline, nitrogen (N), potassium (K), phosphorous (P) content, and peroxidase enzyme activity. Results showed that increasing salinity led to decreased leaf nutrients and growth parameters like plant height, shoot mass, leaf length, width, mass, and corm mass, and increased proline and peroxidase activity.

**Key words:** leaf, nitrogen, phosphorous, potassium, proline, peroxidase

**Ovrednotenje sejancev križancev amarilisa** (*Hippeastrum reticulatum*) **(L'Hér.) Herb. na slanost**

Izvleček: Amarilis (*Hippeastrum* Herb.) je ena izmed čebulastih okrasnih rastlin, ki je razširjena po vsem svetu.. Pri uporabi okrasnih rastlin v ozelenjevanju je pomembno, da se uporabljajo na slanost odporne okrasne rastline, pri čemer je bilo opravljenih le malo raziskav o vplivu zalivanja s slano vodo na rast in razvoj čebulastih okrasnih rastlin. Z namenom preučiti toleranco amarilisa na slanost je bil izveden poskus s petimi slanostmi in štirimi ponovitvami. Obravnavanja s slanostmi so bila: kontrola (distilirana voda) z električno prevodnostjo EC = 0 dSm$^{-1}$ in obravnavanja z električno prevodnostjo (EC) 2, 4, 6 in 8 dSm$^{-1}$.V rastlinah so bili ocenjeni/izmerjeni naslednji parametri: svežost listov, dolžina in širina listov, vsebnost prolina, dušika (N), kalija(K), fosforja in aktivnost peroksidaze. Rezultati so pokazali, da se je s povečevanjem slanosti zmanjševala vsebnost hranil v listih, zmanjševali so se tudi rastni parametri kot so višina rastlin, masa poganjkov, dolžina, širina in masa listov, masa celotnih rastlin, povečali sta ste vsebnost prolina in aktivnost peroksidaze.

**Ključne besede:** nadzemni del rastline, list, dušik, fosfor, kalij, prolin, peroksidaza

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## 1 INTRODUCTION

Amaryllis (*Amaryllis* Herb.) is one of the bulbousornamental plants that is distributed around the world. This plant belongs to the Amaryllidaceae family and *Hippeastrum* genus. Amaryllis (*Hippeastrum x hybridum* Hort.) are used as flowering plants, pot plants, and cut-flowers or limitedly in landscape designing. In Iran, they are mostly grown in the northern regions (the provinces of Mazandaran, Gilan, and Golestan). In Persian, amaryllis is called *Nasrin* (Azimi, 2024)

Salinity is an abiotic stress that usually occurs in semi-arid and arid areas, influencing plant growth and agricultural productivity (Porcel et al., 2012). Ionic toxicity is caused by an accumulation of Na+ and Cl- ions at high salt concentrations, which harms plant growth and development and interferes with the uptake of potassium, phosphorus, calcium, and nitrogen ions, leaving the plant with insufficient quantities of those components (Ulczycka-Walorska et al., 2020) and causing physiological changes (Fatma et al., 2016). As a result, these physiological changes reduce cell division, expansion, or promotion of cell death and induce a decrease in growth rate and yield. They also destroy chlorophyll in leaves, which leads to leaf senescence (Rahemi et al., 2017).

Additionally, it was mentioned that osmotic stress is induced by an increase in sodium and chlorine ions. Furthermore, it was mentioned that oxidative stress (secondary stress) is brought on by an increase in reactive oxygen species (ROS), such as superoxide, hydroxyl radicals, and peroxide, which are ROS that have a negative impact on normal cell growth and metabolism (Aroca et al., 2013).

It is essential to use ornamental species that are tolerant to increased salinity or to develop a resistance trait through plant breeding and physiological techniques when growing attractive plants for landscaping (Bayat et al., 2013). This researcher also reported that the flower number and diameter of *Gerbera aurantiaca* Sch. Bip. exposed to salinity decreased compared to control plants.

The references state that compared to other horticultural products, less research has been done on how salt irrigation affects the growth and development of ornamental plants, particularly bulbous plants. Therefore, due to the salinity problem, which is considered a limiting factor for landscape development, the physiological and morphological study of *Hippeastrum* is important.

## 2 MATERIALS AND METHODS

The seeds of amaryllis (*Hyppeastrum reticulatum* (L'Hér.) Herb.) were obtained from the "Ornamental Plants Research Center (OPRC) of Mahallat, Iran". The seeds were cultivated in a cultivation tray and kept in a greenhouse with 70 ± 5 % relative humidity and 25 ± 5 °C conditions. The seedlings were transplanted at the three-leaf stage into the pots. Then, the uniform seedling genotypes were selected and transplanted into the pots filled with loamy soil, rotten animal manure, and compost (1:1:1); then transferred to open space. The experiment consisted of five salinity concentrations [control (distilled water) with EC = 0 dSm$^{-1}$, and electrical conductivity (EC) at 2, 4, 6, and 8 dSm$^{-1}$] with four replicas. In order to make experimental solutions, 1.28, 2.56, 3.84, and 5.12 g.l$^{-1}$ of NaCl were used for EC = 2, 4, 6, and 8 dsm$^{-1}$.

For two months (July-August), salinity treatments were used twice a week. The volume of applied saline water was 300 ml for each treatment. To prevent salt accumulation, the pots were leached twice a week. Leaf freshness, leaf length and width, fresh and dry mass shoots, crown diameter, bulb mass, proline, nitrogen (N), potassium (K), and phosphorous (P) content, and peroxidase enzyme activity were measured.

### 2.1 MEASUREMENTS OF GROWTH

Digital callipers and rulers were used to measure leaf length and width, plant height, bulb diameter, and crown diameter. Fresh and dry shoots and crowns were assayed by digital balance.

Nitrogen, phosphorous, and potassium were measured using the Khejeldal device, a spectrophotometer and a falme photometer, respectively (Tekaya et al., 2014).

### 2.2 PEROXIDASE ACTIVITY

The Guaiacol technique was used to measure the peroxidase (POD) activity (Oraee et al., 2020). For three minutes, the variations in 470 nm absorbance were used to track how well guaiacol was being oxidised. 50 ml 100 mM PBS (pH 6.0), 19µl 30 % $H_2O_2$, 28µl guaiacol comprised the reaction mixture solution. The enzyme extract was added to the solution of the reaction mixture to begin the reaction.

The following equation was used to calculate POD activity:

*POD activity* ($\Delta A470/\mathrm{min{\cdot}g\ FM}$) $=\Delta A470\times VT/M\times VS\times t$. *$\Delta A470$: the changes of absorption; were $VT$: total volume of the extracted solution; $VS$: volume of enzyme solution for testing; M: the mass of samples*".

### 2.3 PROLINE CONTENT

The method developed by Oraee et al. (2020) was used to measure the proline content in the leaves. In 10 ml of 30 ml $l^{-1}$ sulfosalicylic acid, fresh leaves (1.0 g) from each of the four replications were homogenized and the extract was used to spectrophotometrically measure proline.

### 2.4 STATISTICAL ANALYSIS

Eight different seedling genotypes were planted in each of the three replicates of the experiment's factorial, complete randomised block design.

Using the SAS statistical programme, data were examined by variance mean comparison and the Duncan multiple range test.

## 3 RESULTS AND DISCUSSION

### 3.1 GROWTH CHARACTERISTICS

The highest and the lowest plant heights were related to control (36.97 cm) and EC = 8 dsm-$^{1}$ (22.33 cm) (Figure 1). By increasing salinity stress, plant height was reduced by 9.8, 18.87, 26.68, and 39.6 %, respectively. This trend was the same for other vegetative traits such as leaf number, width, and length (Figure 1). The highest decrease was obtained with EC = 8 dsm$^{-1}$ at 50, 44 and 25 % for leaf number, width, and length as compared to control, respectively. Plants treated with EC = 0 dsm$^{-1}$(control) to EC =8 dsm$^{-1}$ showed a decreasing trend in fresh and dry shoot mass, corm mass, and crown length in comparison with control (Figures 1 and 2). The highest and the lowest values were attributed to EC = 8 dsm$^{-1}$ and control in all the traits.

Reduced growth traits are one of the earliest impacts of salt stress on plants. According to Sarvandi et al. (2020), plants' reduced ability to absorb water as a result of osmotic stress brought on by salt is the reason why their leaf surface area is decreasing (Sarvandi et al., 2020). Additionally, it was claimed that the synthesis and transportation of hormones between roots and shoots are impacted by the absorption of chloride and sodium ions, which reduces leaf area and plant dry biomass and lowers specific leaf area (SLA). In addition to lowering leaf area (LA), salinity inhibits the growth of the root system, delays the production of apical buds, and induces chlorosis with subsequent necrosis on the leaf edge (Oliveira et al., 2017). Dry matter reduction under stress conditions has also been reported due to decreased leaf area index, photosynthesis rate, growth of aerial organs, and the relative growth rate of the plant (Soheili-Movahed et al., 2017). In response to elevated salt concentrations in *Poa pratensis* L., fresh and dry mass of roots and shoots decreased (Esmaeili and Salehi, 2016).

Vegetative growth, including leaf width and length, number of leaves, and number of shoots, decreased as the concentration of sodium chloride increased (Naseri Moghadam et al., 2020) and salinity stress has more detrimental effects than drought stress on the development, aesthetic, and physiological aspects of *N. tazetta* L. flowers (NaseriMoghadam et al., 2020). Regarding salinity's impacts on leaf area, salinity inhibits the root system, causes a large increase in Na+ content across all plant tissues with growth, delays in the development of apical buds, and raises the concentration of NaCl in the nutritional solution (Dlamini et al., 2019). A restriction in leaf expansion followed by a reduction in leaf area is one of the first signs of plants exposed to excessive salinity. It can be explained by alterations in the cells and a decline in leaf turgor. Reduced cell elongation and cell division cause leaves to appear more slowly and to grow to a smaller size in the end. Leaves become smaller and thicker as a result of a shift in cell size that reduces area more than thickness (Go´mez-Bellot et al., 2013).

According to our results, the growth characteristics decreased with the increased salinity. These findings were similar to above finding. This means that in ornamental plants, salinity stress reduces growth, flower size, flower turnover, and visual quality (Toscano et al. 2020). It is well known that salinity reduces photosynthesis and carbohydrate levels, which are useful for flower production and development. The consequence of this is a reduction in biomass accumulation, as observed in plants and flowers. These results were also observed and confirmed in amaryllis in our research (Trivellini et al., 2023). In fact, stunted growth is an adaptive mechanism for survival, which allows plants to combat salt stress. Salt stress might reduce the expression of key regulatory genes involved in cell cycle progression (e.g., cyclin and cyclin-dependent kinase), leading to decreased cell numbers in the meristem and a growth inhibition which impacts the plant's ability to absorb nutrients and water efficiently and to a lesser extent, cell division, is affected, resulting in a lower root and leaf growth rate. After the occurrence of salinity stress, the lateral shoot enlargement is affected, leading to apparent differences in overall growth. This response is due to changes in the cell–water relation resulting from osmotic changes outside the root. The osmotic effect leads to a reduction in the capability of plants to absorb water (Balasubramaniam et al., 2023).

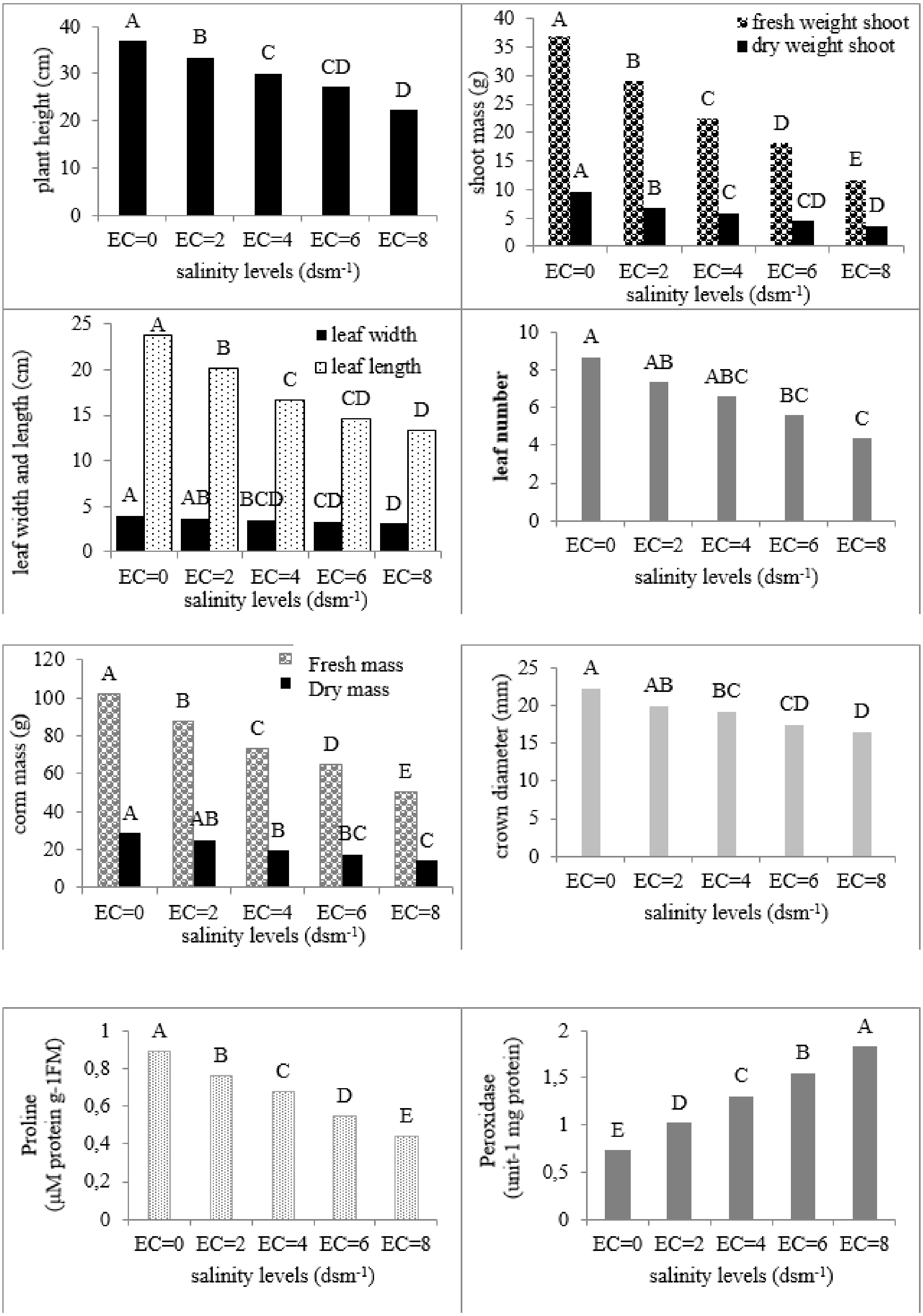

**Figure 1:** Effects of salt stress on morpho-physiological and biochemical traits

## 3.2 PROLINE CONTENT AND PEROXIDASE ACTIVITY

Considering proline and peroxidase contents in leaves, they increased by 102 and 151 % for EC = 8 $dsm^{-1}$ as compared to control, respectively (Fig 1).

Proline content increases when the water potential of the leaf decreases, which leads to the maintenance of cellular turgor and reduces the damage to the membrane in the plant; therefore, with osmotic adjustment, tolerance to water stress increases (Rahdari and Hosseini, 2012). It also serves as an enzyme and membrane protector, as well as a reservoir of energy and nitrogen for utilisation (García-Caparrósand Lao, 2018). Proline accumulation is a well-known adaptive mechanism in plants against salt stress. Additionally, because the rise in proline content may be positively linked with the degree of tolerance, proline accumulation has been proposed as a selection criterion for salt tolerance (García-Caparrósand Lao, 2018). The rate of proline synthesis depends on the development of stress, the age of the plant organ, and genetic variation (Bajji et al., 2001). The proline concentration changes at different levels of salinity showed that with increasing salinity, the proline content of genotypes increased (García-Caparrós et al., 2016).

## 3.3 PEROXIDASE ACTIVITY

The maximum peroxidase activity was present in plants tend to counteract the reactive oxygen species produced by stress (Kaya et al., 2013). Plants subjected to salt stress exhibited up-regulation of the antioxidant defense system (Hussain et al., 2016). According to these studies, salinity increased the activity of peroxidase enzymes in salt-sensitive cultivars. One of the typical responses of plants to saline circumstances is the acceleration of the production of reactive oxygen species (ROS), which include the lethal superoxide radical ($O_2$), singlet oxygen ($1O_2$), hydroxyl radical (OH-), and hydrogen peroxide ($H_2O_2$). Peroxisomes, chloroplasts, and mitochondria are the key cell components that generate ROS. These reactive oxygen species are involved in a variety of activities, including protein oxidation, lipid peroxidation, and DNA damage (Shams and Khadivi, 2023).

In order to overcome the negative effects of ROS at the cellular level, plants show a mechanism of scavenging of these species through the antioxidative machinery composed by enzymatic and non-enzymatic components such as superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POX) and catalase (CAT) (García-Caparrós and Lao, 2018).

Regarding the status of macro elements in leaves, nitrogen, phosphorous and potassium decreased with increase in salinity level by 56.7, 44 and 58 % as compared to control (Fig 2). These results were in agreement with Ulczycka-Walorska et al. (2020) who stated that high salt concentration in plants disturbs the absorption of potassium, phosphorus, calcium and nitrogen ions leading to insufficient levels of those elements in the plant. The results of our study were in agreement with previous studies that excess salt, restricting plants' ability to absorb water and minerals such as $K_+$ and $Ca^{2+}$ (Mircea et al., 2025).

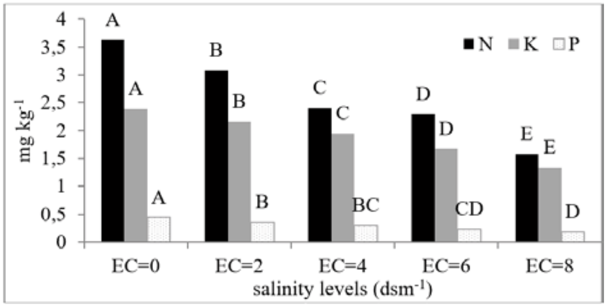

**Figure 2:** Effects of salt stress on leaf nitrogen, potassium and phosphorous

## 3.4 MULTIVARIATE ANALYSIS

In order to group the salinity levels based on increasing dissimilarity, a hierarchical agglomerative cluster assessment was performed (Fig 3). The first group

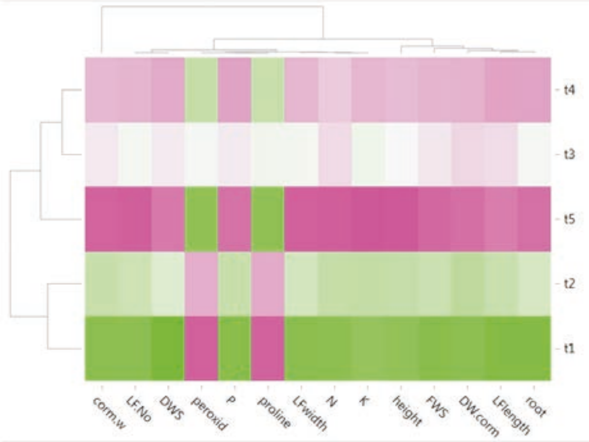

**Figure 3:** Cluster analysis of salinity stress on amaryllis based on physical and chemical properties of leaf (gradient from low (pink), white (medium) to high (green)).Abbreviations: corm.w: corm mass, LFNo: leaf number, DMcorm: Corm dry mass, FMS: leaf fresh mass, LFwidth: leaf width, LF: leaf length and t1 to t5: EC = 0,2,4,6 and 8 ds/m, respectively.

(Cluster I, Figure 5), which included t4 and t3, t4 had the highest levels of peroxidase and proline. The second cluster (Cluster II, Figure 5), which included t5, showed low values of leaf nutrients (N, P and K), corm, root, corm dry mass, leaf length, width, and number, and high values for proline and peroxidase. The third group (Cluster III) included t1 and t2, within this cluster, t1 had the highest vegetative traits and nutrient contents and the lowest values for proline and peroxidase

## 4 CONCLUSION

Amaryllis is described as a plant with low water requirements, with water surpluses being detrimental to the development of the crop. The level of tolerance to salinity in *Hippeastrum* hybrids showed that this ornamental plant was susceptible to EC. Increasing salinity led to decreased leaf nutrients and growth parameters like plant height, shoot mass, leaf length, width, mass, and corm mass, and increased proline and peroxidase activity.

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**Author contributions:** Conceived and designed the experiments: Performed the experiments: Azimi, M.H, Khalaj, MA, Analyzed and wrote the paper: Sayyad-Amin P, Revised and edited the paper: Hosseinpour, N

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# Efficacy and phytotoxicity of post emergence herbicides in garlic

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**Efficacy and phytotoxicity of post emergence herbicides in garlic**

**Abstract:** The incidence of weeds in the garlic crop interferes with the quality and quantity of the harvest. This study aimed to evaluate the efficiency of herbicides in different combinations of sequential applications during the cultivation of garlic. The experiment was carried out in a randomized block design, with ten treatments and four replications. The treatments consisted of sequential applications of the herbicides pendimethalin, pyroxasulfone/flumioxazin, ioxynil, and pendimethalin. The doses of Herbadox® (pendimethalin) were 4.0 l·ha$^{-1}$, Totril® (ioxynil) 1.5 l·ha$^{-1}$, and Kyojin® (pyroxasulfone/flumioxazin) 0.15 l·ha$^{-1}$. Treatments consisted of sequential applications of herbicide combinations. Applications were performed 7, 58, 97, and 128 days after planting. Phytotoxicity on the crop, efficacy in weed control, bulb classification, and productivity were evaluated. The results were submitted to ANOVA, and the means were compared by the Scott-Knott test. The sequential application of pyroxasulfone/flumioxazin at 7 days after planting (DAP), ioxynil at 58 DAP, pendimethalin at 97 DAP, and pyroxasulfone/flumioxazin at 128 DAP (T7) stood out (96–100 % control of weeds, average productivity of 11.42 t·ha$^{-1}$), which presented the best results relative to phytotoxicity, weed control, bulb classification, and crop productivity.

**Key words:** phytotoxicity, efficacy, weed control, post-emergence.

**Učinkovitost in fitotoksičnost herbicidov uporabljenih po vzniku česna**

**Izvleček:** Pojavnost plevelov v nasadih česna vpliva na količino in kakovost pridelka. V raziskavi je bila ovrednotena učinkovitost herbicidov v različnih kombinacijah v zaporednih nanosih med gojenjem česna. Poskus je bil zasnovan v naključni bločni obliki, z desetimi obravnavanji in štirimi ponovitvami. Obravnavanja so obsegala zaporedno uporabo herbicidov kot so pendimetalin, piroksasulfon/flumioksazin, ioksinilin pendimetalin. Odmerki Herbadox-a® (pendimetalin) so bili 4,0 l·ha$^{-1}$, Totril-a® (ioksinil) 1,5 l·ha$^{-1}$in Kyojin-a® (piroksasulfon/flumioksazin) 0,15 l·ha$^{-1}$. Obravnavanja so bila izvedena v zaporednih kombinacijah herbicidov. in sicer 7, 58, 97 in 128 dni po sadnji. Ovrednotene so bile fitotoksičnost za posevek, učinkovitost zatiranja plevelov, klasifiklacija čebulic in produktivnost. Rezultati so bili obdelani z ANOVA, povprečja so bila primerjana s Scott-Knottovim testom. Zaporedna uporaba piroksasulfona/flumioksazina 7 dni po sadnji (DAP), ioksinila 58 DAP, pendimetalina 97 DAP in piroksasulfona/flumioksazina 128 DAP (T7) je izstopala (96–100 % nadzor plevelov, poprečna produktivnost 11,42 t·ha$^{-1}$), kar je predstavljalo najboljše rezultate glede na fitotoksičnost, nadzor plevelov, klasifikacijo čebulic in produktivnost česna.

**Ključne besede:** fitotoksičnost, učinkovitost nadzora plevelov po vzniku

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## 1 INTRODUCTION

Garlic cultivation in Brazil is a profitable source for both small-family farmers and large producers. This crop is very important in generating jobs (Meneguzzo et al., 2022). In 2021, 167,102 t of this crop were harvested, with an average yield of 12.8 t·ha$^{-1}$, mostly in small farms (about 1–2 ha), totaling an acreage of 13,544 ha (IBGE, 2023). The garlic crop presents a reduction in development when there is the simultaneous presence of weeds due to its slow initial growth, with low height and narrow and erect leaves that do not cover the soil surface. The critical period of garlic competition with weeds occurs between the first 20 days after emergence and during bulb maturation. Despite not interfering so much with maturation, weeds make manual harvesting difficult and mechanical harvesting unfeasible (Patil et al., 2016; Sidhu et al., 2018).

The primary weeds in colder periods in southern Brazil are the lesser swinecress (*Coronopus didymus* (L.) Sm.), wild radish (*Raphanus* spp.), chickweed (*Stellaria media* (L.) Vill.), broad-leaved dock (*Rumex obtusifolius* L.), and Italian ryegrass (*Lolium multiflorum* Lam.) (Uzo & Currah, 1990). In warmer periods, crabgrass (*Digitaria* spp.), alexandergrass (*Urochloa plantaginea* (Link) R.D.Webster), barnyardgrass (*Echinochloa* spp.), beggartick (*Bidens pilosa* L.), wild poinsettia (*Euphorbia heterophylla* L.), arrowleaf sida (*Sida* spp.), starbur (*Acanthospermum* spp.), potato weed (*Galinsoga parviflora* Cav.), and pigweed redroot (*Amaranthus* spp.) occur (Abouziena & Haggag, 2016). Weeds that grow in summer and spring seasons do not tolerate frost (Guerra et al., 2020).

Pre-emergence herbicides are widely used in preplanting and, in some cases, incorporated into the soil. Post-emergence herbicides control weeds that are in full physiological development. At post-emergence, the management of weeds becomes problematic as they grow (Das & Mondal, 2014; Wilkinson et al., 2015).

Pre-emergence herbicides, such as pendimethalin (Herbadox®), which inhibits cell division, and linuron (Afalon®), a photosystem II inhibitor, are widely used by garlic producers in Brazil. These active principles have a good residual in the soil, keeping the area clean of weeds. Also, there is a large offer of these products on the market (Guerra et al., 2020).

Kyojin® is a selective, contact, and systemic herbicide composed of pyroxasulfone, a cell division inhibitor, and flumioxazin, a Protox inhibitor. It is broad-spectrum effective against many weeds of the garlic cultivation (Agrofit, 2022). Flumioxazin successfully controlled weeds in garlic crops (Guerra et al., 2020).

This work aimed to evaluate the efficiency of different herbicide combinations in the garlic crop, applied sequentially, with varying application intervals.

## 2 MATERIALS AND METHODS

The experiment was conducted on a farm in the municipality of São Marcos, Rio Grande do Sul State (geographical coordinates: 28°54’ S, 51°07’ W, and an altitude of 679 m above sea level). The local climate is classified as Cfa (subtropical humid climate with mild summer), characterized by an average yearly rainfall of 1,688 mm, an average minimum temperature of 13 °C, and an average maximum temperature of 23 °C (Alvares et al., 2013). During the experiment, the average temperature was 17.3 °C, varying between 0.6 °C and 33.4 °C, and the accumulated rainfall in the period was 571.8 mm (INMET, 2023).

The experimental design used was randomized blocks with four replications. The plots consisted of six rows spaced 0.2 m apart, 5 m long, and 1.2 m wide, totaling an area of 6 m$^2$. The two central lines of each plot were considered useful for evaluation and harvesting, the remainder being considered borders.

The garlic cultivar ‘San Valentin’ was planted in the experiment. The bulbs underwent viral cleaning and were in their third generation, coming from the 2020/2021 harvest. The bulbs were selected and classified, subsequently submitted to vernalization, where they remained for 48 days in a cold chamber at a temperature of 2 ºC.

The area was prepared with a duck foot plow, harrowing and filling. A marker roller coupled to a rotary hoe was used for planting the bulbil, helping with the planting work. The fertilization was carried out before planting with 60 kg·ha$^{-1}$ of N, 375 kg·ha$^{-1}$ of P, and 180 kg·ha$^{-1}$ of K (750 kg of NPK fertilizer Topmix® 4-25-12, with 4 wt.% of nitrogen compounds, 25 wt.% of phosphorus compounds and 12 wt.% of potassium compounds) before planting and two topdressing applications with a total of 36 kg·ha$^{-1}$ of N and 96 kg·ha$^{-1}$ of K (100 kg per hectare with NPK 9-00-24, containing 9 wt.% of nitrogen compounds and 24 wt.% of potassium compounds) during crop development.

The bulbils underwent phytosanitary treatments, in which the fungicides captan and abamectin were used at doses of 2.0 ml·l$^{-1}$ and 2.5 ml·l$^{-1}$, respectively, before vernalization. Planting was carried out on July 20, 2022, and microsprinklers provided irrigation at the beginning of the crop cycle. The tested treatments, active ingredients, and doses are compiled in Table 1.

Herbicide application started seven days after planting (DAP) of the crop, in pre-emergence of weeds; the second application occurred at 58 DAP; the third ap-

**Table 1:** Compilation of treatments applied and their composition, days after planting, active ingredient, and doses of commercial product. São Marcos, UCS, 2022.

| Treatment | DAP* | Active ingredient | Dose (g·ha$^{-1}$) | Dose (l·ha$^{-1}$) |
|---|---|---|---|---|
| Treatment 1 (Infested control) | - | - | - | - |
| Treatment 2 (Weeded control) | - | - | - | - |
| Treatment 3 | 7 | Pendimethalin[1] | 1,600 | 4.0 |
| | 58 | Pyroxasulfone/flumioxazin [2] | 45/30 | 0.15 |
| | 97 | Ioxynil[3] | 375 | 1.5 |
| | 128 | Pendimethalin | 1,600 | 4.0 |
| Treatment 4 | 7 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| | 58 | Pendimethalin | 1,600 | 4.0 |
| | 97 | Ioxynil | 375 | 1.5 |
| | 128 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| Treatment 5 | 7 | Ioxynil | 375 | 1.5 |
| | 58 | pendimethalin | 1,600 | 4.0 |
| | 97 | Pyroxasulfone/flumioxazin | 45/30 | 0.150 |
| | 128 | Ioxynil | 375 | 1.5 |
| Treatment 6 | 7 | pendimethalin | 1,600 | 4.0 |
| | 58 | Ioxynil | 375 | 1.5 |
| | 97 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| | 128 | pendimethalin | 1,600 | 4.0 |
| Treatment 7 | 7 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| | 58 | Ioxynil | 375 | 1.5 |
| | 97 | pendimethalin | 1,600 | 4.0 |
| | 128 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| Treatment 8 | 7 | Ioxynil | 375 | 1.5 |
| | 58 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| | 97 | pendimethalin | 1,600 | 4.0 |
| | 128 | Ioxynil | 375 | 1.5 |
| Treatment 9 | 7 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| | 58 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| | 97 | Ioxynil | 375 | 1.5 |
| | 128 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| Treatment 10 | 7 | Ioxynil | 375 | 1.5 |
| | 58 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| | 97 | Pyroxasulfone/flumioxazin | 45/30 | 0.15 |
| | 128 | Ioxynil | 375 | 1.5 |

* – Days after planting. [1] – Herbadox®; [2] – Kyojin®; [3] – Totril®. Source: Authors (2025)

plication occurred at 97 DAP, and the last application was performed at 128 DAP. The herbicides were always applied in the morning. The equipment used to apply the treatment was a knapsack sprayer pressurized with $CO_2$, calibrated with a constant pressure of approx. 2.5 kPa, which provided a spray volume of 500 l·ha$^{-1}$. Other aspects of cultural management, such as phytosanitary treatments and sprinkler irrigation, were carried out during the crop cycle when necessary, according to farmer's expertise.

The evaluation of weed control was carried out at the end of the cycle, in the pre-harvest period. The assessment of the classification of the bulbs occurred at the end of the crop cycle, quantifying, and classifying the bulbs of the two central lines. The bulbs were measured using an analog caliper and classified according to Mercosul GMC resolution 05/21 (Mercosul, 2021), considering the largest cross-sectional diameter of the bulb, expressed in millimeters (mm). Thus, bulbs > 56 mm were classified as number 7, > 47–56 mm as number 6, > 42–47 mm as number 5, > 37–42 mm as number 4, and > 32–37 mm as number 3 (Brazil, 1992). Bulbs smaller than 32 mm do not fit the regulation. Therefore, they were classified as industrial garlic.

The productivity evaluations were performed with a digital electronic scale (ALC500, Marte, Brazil), where ten random bulbs were weighed, harvested in each block, and divided by the number of bulbs present in the weighing. Per repetition (block), 1.1 kg were planted. With this, the average productivity was estimated considering 1,000 $kg \cdot ha^{-1}$ of planted bulbils.

Phytotoxicity symptoms were evaluated seven days after each application (DAA). Phytotoxicity scores were based on visual criteria, based on control, considering a scale from zero to 100 %, where zero represents the absence of injuries caused by the herbicides and 100 % (one hundred) corresponded to the death of the plants. For the visual evaluations of the infested plants, percentages of control provided by the herbicides were applied relative to the control (T1 – infested control) (SBCPD, 1995).

The results were evaluated for homoscedasticity (Levene's test) and normality (Shapiro-Wilk's test), being submitted to the Analysis of Variance (ANOVA). The means of the treatments were compared by the post hoc test of multiple comparison of means of Scott- Knott at a 5 % error probability ($\alpha = 0.05$). The statistical analyses were performed using the AgroEstat® software (Brazil).

## 3 RESULTS AND DISCUSSION

For the evaluated variables, there was a statistical difference between the herbicide treatments tested (Tables 2 to 6). The results of phytotoxicity to garlic crops caused by each herbicide treatment are presented in Table 2.

The phytotoxicity caused by the different herbicides in the garlic crop was presented as follows: the combinations started seven days after planting (DAP) with pyroxasulfone and flumioxazin at a dose of 0.15 $l \cdot ha^{-1}$ and ioxynil at 1.5 $l \cdot ha^{-1}$ caused a reduction in development relative to the weeded control (T2). In contrast, pendimethalin at a 4.0 $l \cdot ha^{-1}$ dose did not cause crop phytotoxicity.

At 58 DAP, garlic was very susceptible to injuries caused by the herbicides tested after the second application. Yellowing symptoms were quite noticeable, where all herbicides showed percentages of phytotoxicity higher than 7 DAP. The active ingredient ioxynil (Totril®) caused less phytotoxicity than the other ingredients at a 1.5 $l \cdot ha^{-1}$ dose.

The combination in which the association pyroxasulfone/flumioxazin was applied at 97 DAP caused phytotoxicity symptoms of up to 33 % of the leaf area with signs of yellowing and whitish lesions caused by pyroxasulfone and flumioxazin. It is worth mentioning that there was a higher incidence of bacterial diseases in the plots

**Table 2:** Results of visual assessment of phytotoxicity symptoms in the garlic crop, seven days after the application (DAA) of herbicide combinations, at different development crop stages. São Marcos, UCS, 2022.

| Treatment | Phytotoxicity (%) in 7 DAA | | | |
|---|---|---|---|---|
| | 7 DAP | 58 DAP | 97 DAP | 128 DAP |
| T1 – Infested Control | 0 c | 0 f | 0 c | 0 c |
| T2 – Weeded Control | 0 c | 0 f | 0 c | 0 c |
| T3 – Pen[1], Pyr/Flu[2], Iox[3], Pen | 0 c | 20 c | 0 c | 10 b |
| T4 – Pyr/Flu, Pen, Iox, Pyr/Flu | 7 a | 17 d | 0 c | 16 a |
| T5 – Iox, Pen, Pyr/Flu, Iox | 4 b | 17 d | 33 a | 5 c |
| T6 – Pen, Iox, Pyr/Flu, Pen | 0 c | 10 e | 20 b | 0 c |
| T7 – Pyr/Flu, Iox, Pen, Pyr/Flu | 5 b | 17 d | 0 c | 11 b |
| T8 – Iox, Pyr/Flu, Pen, Iox | 4 b | 36 a | 0 c | 0 c |
| T9 – Pyr/Flu, Pyr/Flu, Iox, Pyr/Flu | 5 b | 28 b | 0 c | 17 a |
| T10 – Iox, Pyr/Flu, Pyr/Flu, Iox | 4 b | 30 b | 20 b | 16 a |
| Coefficient of variation (%) | 26.7 | 11.1 | 49.3 | 39.9 |

[1] – pendimethalin; [2] – pyroxasulfone/flumioxazin; [3] – ioxynil. The adopted doses were 4.0 $l \cdot ha^{-1}$ for pendimethalin®, 0.15 $l \cdot ha^{-1}$ for pyroxasulfone/flumioxazin, and 1.5 $l \cdot ha^{-1}$ for ioxynil. DAA – days after application of treatments; DAP – days after crop planting. Means in column followed by the same letter do not differ statistically by the Scott-Knott test at a 5 % error probability. Source: Authors (2025).

where pyroxasulfone/flumioxazin was applied at 0.15 $l\cdot ha^{-1}$ compared to the weeded control (T2), consequently, due to the lesions caused by the herbicide.

Pyroxasulfone and flumioxazin, when applied at 128 DAP, caused greater phytotoxicity than the other herbicides tested. However, the damage caused was lower than that seen at 97 DAP, not leading to the death of the culture of interest at the dose used. On the other hand, the ioxynil and pendimethalin principles caused a lower degree of injuries caused by phytotoxicity in the crop when applied at 128 DAP.

The damage capacity of the pyroxasulfone/flumioxazin association at a dose of 0.15 $l\cdot ha^{-1}$ was noticed on garlic plants at different stages of crop development, where significant damage to the leaves stood out. Among the best combinations, which caused the lowest percentages of phytotoxicity, those that started with applying pendimethalin at a dose of 4.0 $l\cdot ha^{-1}$ stood out.

The main weeds occurring in the experimental area were the wild carrot (*Daucus carota* L.), speedwell (*Veronica* spp.), potato weed (*Galinsoga parviflora*) and lesser swinecress (*Coronopus didymus*). The results of the

**Table 3:** Control (%) of potato weed (*Galinsoga parviflora*), lesser swinecress (*Coronopus didymus*), wild carrot (*Daucus carota*), and speedwell (*Veronica* spp.) in garlic crop due to different herbicides applied in the pre-harvest period. São Marcos, UCS, 2022.

| Treatment | Control (%) | | | |
|---|---|---|---|---|
| | Potato weed | Lesser swinecress | Wild carrot | Crabgrass |
| T1 – Infested Control | 0 b | 0 b | 0 d | 0 c |
| T2 – Weeded Control | 100 a | 100 a | 100 a | 100 a |
| T3 – Pen[1], Pyr/Flu[2], Iox[3], Pen | 100 a | 97 a | 58 b | 96 b |
| T4 – Pyr/Flu, Pen, Iox, Pyr/Flu | 98 a | 97 a | 68 b | 98 a |
| T5 – Iox, Pen, Pyr/Flu, Iox | 85 a | 87 a | 31 c | 93 b |
| T6 – Pen, Iox, Pyr/Flu, Pen | 100 a | 100 a | 68 b | 100 a |
| T7 – Pyr/Flu, Iox, Pen, Pyr/Flu | 96 a | 97 a | 100 a | 100 a |
| T8 – Iox, Pyr/Flu, Pen, Iox | 98 a | 97 a | 58 b | 100 a |
| T9 – Pyr/Flu, Pyr/Flu, Iox, Pyr/Flu | 96 a | 100 a | 79 b | 100 a |
| T10 – Iox, Pyr/Flu, Pyr/Flu, Iox | 96 a | 100 a | 68 b | 100 a |
| Coefficient of variation (%) | 9.8 | 9.6 | 20.5 | 2.6 |

[1] – pendimethalin; [2] – pyroxasulfone/flumioxazin; [3] – ioxynil. The adopted doses were 4.0 $l\cdot ha^{-1}$ for pendimethalin®, 0.15 $l\cdot ha^{-1}$ for pyroxasulfone/flumioxazin, and 1.5 $l\cdot ha^{-1}$ for ioxynil. DAA – days after application Means in column followed by the same letter do not differ statistically by the Scott-Knott test at a 5 % error probability. Source: Authors (2025).

**Table 4:** Classification of garlic bulbs, following the MAPA regulation 242, treated with different combinations of herbicides during the crop cycle. São Marcos, UCS, 2022.

| Treatment | Classification - Class (%) | | | | | |
|---|---|---|---|---|---|---|
| | C7 | C6 | C5 | C4 | C3 | Industry |
| T1 – Infested Control | 0 b | 0 d | 0 b | 5 b | 57 a | 37 a |
| T2 – Weeded Control | 0 b | 81 a | 18 b | 0 b | 0 c | 0 b |
| T3 – Pen[1], Pyr/Flu[2], Iox[3], Pen | 2 b | 25 b | 52 a | 20 a | 0 c | 0 b |
| T4 – Pyr/Flu, Pen, Iox, Pyr/Flu | 0 b | 30 b | 50 a | 17 a | 2 c | 0 b |
| T5 – Iox, Pen, Pyr/Flu, Iox | 0 b | 2 c | 47 a | 32 a | 17 b | 0 b |
| T6 – Pen, Iox, Pyr/Flu, Pen | 0 b | 32 c | 47 a | 20 a | 0 c | 0 b |
| T7 – Pyr/Flu, Iox, Pen, Pyr/Flu | 7 a | 50 b | 32 a | 5 b | 2 c | 0 b |
| T8 – Iox, Pyr/Flu, Pen, Iox | 0 b | 37 c | 45 a | 15 a | 2 c | 0 b |
| T9 – Pyr/Flu, Pyr/Flu, Iox, Pyr/Flu | 0 b | 52 b | 40 a | 5 b | 0 c | 0 b |
| T10 – Iox, Pyr/Flu, Pyr/Flu, Iox | 0 b | 10 d | 32 a | 37 a | 12 b | 2 b |
| Coefficient of variation (%) | 33.8 | 52.6 | 43.1 | 16.4 | 78.9 | 84.7 |

[1] – pendimethalin; [2] – pyroxasulfone/flumioxazin; [3] – ioxynil. The adopted doses were 4.0 $l\cdot ha^{-1}$ for pendimethalin®, 0.15 $l\cdot ha^{-1}$ for pyroxasulfone/flumioxazin, and 1.5 $l\cdot ha^{-1}$ for ioxynil. DAA – days after application Means in column followed by the same letter do not differ statistically by the Scott-Knott test at a 5 % error probability. Source: Authors (2025).

control percentage of each weed species relative to the treatments tested are compiled in Table 3.

For the control of potato weed and lesser swinecress (Table 3), all combinations showed excellent control (> 85 %), with emphasis on the combination that started with pendimethalin at doses of 4.0 l·ha$^{-1}$ at 7 DAP, ioxynil 1.5 l·ha$^{-1}$ at 58 DAP, pyroxasulfone/flumioxazin 0.15 l·ha$^{-1}$ at 97 DAP and pendimethalin again at 128 DAP, showing total control (100 %) of both evaluated weeds.

The association pyroxasulfone/flumioxazin, ioxynil, pendimethalin, and pyroxasulfone/flumioxazin (T7) stood out, which promoted total control (100 %) of these invasive species.

The combination of ioxynil, pendimethalin, pyroxasulfone/flumioxazin, and ioxynil (T5) proved to be the least effective concerning the other treatments, showing 93 % control of crabgrass, but only 31 % of control for wild carrot (Table 3). These differences are due to the specific sensitivity of each species to herbicides, resulting in greater control of crabgrass when compared to wild carrot.

**Table 5:** Bulb mass and yield estimate considering the planting of 1,000 kg·ha$^{-1}$ of 'San Valentin' garlic bulbs treated with different combinations of herbicides during crop development. São Marcos, UCS, 2022.

| Treatment | Mass of bulbs (g) | Productivity (t·ha$^{-1}$) |
|---|---|---|
| T1 – Infested Control | 19 d | 4.50 d |
| T2 – Weeded Control | 50 a | 11.47 a |
| T3 – Pen[1], Pyr/Flu[2], Iox[3], Pen | 42 b | 9.74 b |
| T4 – Pyr/Flu, Pen, Iox, Pyr/Flu | 40 b | 9.16 b |
| T5 – Iox, Pen, Pyr/Flu, Iox | 32 c | 7.37 c |
| T6 – Pen, Iox, Pyr/Flu, Pen | 41 b | 9.42 b |
| T7 – Pyr/Flu, Iox, Pen, Pyr/Flu | 49 a | 11.42 a |
| T8 – Iox, Pyr/Flu, Pen, Iox | 42 b | 9.79 b |
| T9 – Pyr/Flu, Pyr/Flu, Iox, Pyr/Flu | 40 b | 9.20 b |
| T10 – Iox, Pyr/Flu, Pyr/Flu, Iox | 36 b | 8.44 b |
| Coefficient of variation (%) | 10.8 | 11.1 |

[1] – pendimethalin; [2] – pyroxasulfone/flumioxazin; [3] – ioxynil. The adopted doses were 4.0 l·ha$^{-1}$ for pendimethalin®, 0.15 l·ha$^{-1}$ for pyroxasulfone/flumioxazin, and 1.5 l·ha$^{-1}$ for ioxynil. DAA – days after application 4 - Considering the planting of 1,000 kg·ha-1 of bulbils. Means in column followed by the same letter do not differ statistically by the Scott-Knott test at a 5 % error probability. Source: Authors (2025).

Data on bulbs' classification according to the herbicide treatments used are compiled in Table 4.

Differences in bulb caliber were significant in class 7 (C7), highlighting the use of pyroxasulfone/flumioxazin 0.15 l·ha$^{-1}$ in the first application, followed by ioxynil 1.5 l·ha$^{-1}$, pendimethalin 4.0 l·ha$^{-1}$ and pyroxasulfone/flumioxazin in combination. In this treatment, 7 % of the total bulbs were classified as class 7, 50 % as class 6, and 32 % as class 5. Notably, weed control was important for greater uniformity and size of bulbs in the garlic crop.

The infested control (T1) showed statistical variation about the other treatments for bulbs smaller than 32 mm, with 37 % of the production as industry garlic, which indicates the negative effect of not controlling the weed species present during the development of the crop.

Data referring to the mass of bulbs and estimated productivity of the crop with the different herbicide application regimes are presented in Table 5.

According to Tables 4 and 5, the combination that stood out negatively from the others was ioxynil, pendimethalin, pyroxasulfone/flumioxazin, and ioxynil (T5), which had the smallest caliber of bulbs, being inferior to the other treatments due to the low control of weeds.

Among the evaluated treatments (Table 5), the combination of pyroxasulfone/flumioxazin herbicides at 7 DAP, ioxynil at 58 DAP, pendimethalin at 97 DAP, and pyroxasulfone/flumioxazin in its last application (T7), which had an average mass of 49 g per bulb stood out. The estimated productivity of 11.42 t·ha$^{-1}$ was very close to the weeded control (T2 – 11.47 t·ha$^{-1}$).

The residual effect of the product pyroxasulfone/flumioxazin at 0.15 l·ha$^{-1}$ was notorious when applied at 7 DAP, presenting an excellent residual effect and controlling the emergence of weeds, which kept the crop free of competition. The combination that had the slightest effect on yield was the one started with ioxynil, pendimethalin, pyroxasulfone/flumioxazin, and ioxynil (T5), with an estimated yield of 7.37 t·ha$^{-1}$, considering 1,000 kg·ha$^{-1}$ of bulbils planted.

Wilkinson et al. (2015) pointed out that the symptoms of poisoning by photosystem II inhibitors (FSII), such as ioxynil, appear quickly and are characterized by interveinal chlorosis and leaf edge chlorosis in seagrass. These symptoms develop rapidly after herbicide application, as seen within seven days after application. Therefore, the combination of herbicides that stood out, showing less phytotoxicity at 58 DAP, was pendimethalin, ioxynil, pyroxasulfone and flumioxazin, and pendimethalin (T6).

As highlighted by Siddhu et al. (2018), in addition to favorable climatic conditions, the emergence of diseases is associated with injuries or stress caused by applying chemical pesticides, mainly post-emergence herbicides.

Furthermore, Mudge and Haller (2009) stated that herbicides can cause crop toxicity, affecting development, growth, and productivity, as verified by 7 DAA of pyroxasulfone and flumioxazin when applied in corn, cotton, soybean, and wheat, begonia, impatiens, and snapdragon. Therefore, the application of pyroxasulfone and flumioxazin principles in this period is not indicated. It is believed that this phenomenon occurred due to the size of the crop being more prominent and having a larger leaf area in this period. In this way, the contact of the formulations with the soil and weeds is reduced.

The herbicide pendimethalin inhibits microtubule arrangement in mitosis, efficiently controlling annual grasses and specific broad leaves in pre-emergence. However, this principle does not control established plants before application (Chen et al., 2021).

It was also possible to observe the control efficiency of pyroxasulfone/flumioxazin at 7 DAA, followed by ioxynil at 58 DAA, on wild carrot and speedwell regarding the other combinations tested. After application, the areas remained free of these weeds. According to the *Sociedade Brasileira da Ciência das Plantas Daninhas* (1995), a product must have a minimum control threshold of 80 % to be recommended for weed control of invasive species in that respective culture.

Uzo and Currah (1990) pointed out that weeds can cause great productivity losses as they compete with the crop for space, nutrients, water, and light. In addition, invasive species can host diseases and release toxins/allelopathics that can inhibit or reduce crop development, thus decreasing final productivity.

The increased competition pressure between the weeds and the culture may have caused a reduced bulb caliber. Luz et al. (2022) commented that when efficient weed control is not carried out, this can result in important yield losses for garlic crops.

Most of the tested combinations showed satisfactory results for weed control, with emphasis on the pyroxasulfone/flumioxazin treatment at a dose of 0.15 $l \cdot ha^{-1}$ at 7 DAP (T7), which showed excellent control of weeds when applied to the garlic crop, not causing significant injuries due to phytotoxicity when compared to applications at other stages of the crop.

It was also noticed that, at 97 DAP where pyroxasulfone/flumioxazin was used, highlighting the combination ioxynil, pyroxasulfone/flumioxazin, pyroxasulfone/flumioxazin, and ioxynil (T10), the culture showed reduced growth due to phytotoxicity concerning the controls (T1 and T2). Therefore, this product is not indicated in this period but in earlier periods, in which the leaf area of the crop is smaller.

When using the pendimethalin herbicide in post-emergence of the crop, less phytotoxicity was observed in garlic in different application periods when compared to the pyroxasulfone/flumioxazin product, in addition to achieving excellent control of potato weed, lesser swinecress, and speedwell. On the other hand, this product did not effectively manage wild carrot. The use of ioxynil at 7 DAP did not have reasonable control and productivity results, making the ioxynil, pendimethalin, pyroxasulfone/flumioxazin, and ioxynil combination less effective than the other combinations assessed. Thus, the importance of applying pre-emergence herbicides at earlier crop stages is suggested to reduce the toxic effects on the garlic plants.

## 4 CONCLUSIONS

The sequential application of pyroxasulfone/flumioxazin at 7 DAP, ioxynil at 58 DAP, pendimethalin at 97 DAP, and pyroxasulfone/flumioxazin at 128 DAP (T7) yielded the best results regarding the relationship between phytotoxicity to the crop, weed control, and bulb classifications, presenting itself as an alternative to garlic producers, considering the current options for chemical control of weeds in the crop.

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# Effect of high residual sodium carbonat (RSC) water and amendments on soil properties under rice-mustard (*Brassica juncea* 'Khanpur Raya') rotation

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**Effect of high residual sodium carbonat (RSC) water and amendments on soil properties under rice-mustard (*Brassica juncea* 'Khanpur Raya') rotation**

**Abstract:** A trial was designed to study the deleterious effect of high RSC water on soil properties under rice-mustard crop rotation. Treatments included were; $T_1$: high RSC water, $T_2$: gypsum on the basis of RSC of water, $T_3$: $H_2SO_4$ on the basis of RSC of water, $T_4$: green manuring with guar, $T_5$: farm yard manure (FYM) at the rate of 10 t. ha$^{-1}$. For irrigation tube well water having (electrical conductivity (EC) 1.37 dS m$^{-1}$, sodium adsorption ratio (SAR) 8.40 (mmol l$^{-1}$)$^{1/2}$ and RSC 7.85 me l$^{-1}$) was used. The results revealed that lowest paddy (2.22 t ha$^{-1}$) and grain yield (1.00 t ha$^{-1}$) of rice and mustard were recorded when irrigated with high RSC water. In case of soil analysis, long-term use of high RSC water induces secondary salinity by increasing pH (1.92 %), EC (5.73 %) and SAR (35.71 %) over their initial values. Harmful effects of high RSC water were thwarted by all the treatments; however, positive effects of gypsum were more visible that increased crop growth and grain yield of rice-mustard crops by promoting soil properties. Gypsum recorded the highest paddy and grain yield (3.66 t ha$^{-1}$, 1.70 t ha$^{-1}$) of rice and mustard crop and decreased soil pH$_s$ (4.98 %), EC$_e$ (29.93 %) and SAR (54.54 %) over their initial values.

Key words: residual sodium carbonate, rice, mustard, salinity, sodic water, gypsum, sulfuric acid, guar

**Učinek velikih vsebnosti rezidualnega natrijevega karbonata (RSC) v vodi in dodatkov na lastnosti tal v kolobarju riža in gorjušice**

**Izvleček:** Poskus je bil izveden z namenom preučiti škodljive učinke velikih vsebnosti natrijeva karbonata na lastnosti tal v kolobarju riža in gorjušice. Obravnavanja so bila; $T_1$: velika vsebnost RSC v vodi, $T_2$: dodatek sadre vodi z veliko vsebnostjo RSC, $T_3$: dodatek $H_2SO_4$ vodi z veliko vsebnostjo RSC , $T_4$: zeleno gnojenje z guarom, $T_5$: dodatek hlevskega gnoja (FYM) v odmerku 10 t ha$^{-1}$. Za namakanje je bila uporabljena voda iz vodnjakov naslednjih lastnosti: električna prevodnost (EC 1.37 dS m$^{-1}$), razmerje adsorbiranega natrija (SAR; 8.40 mmol l$^{-1}$)$^{1/2}$ in RSC 7.85 me l$^{-1}$). Rezultati so pokazali, da je bil najmanjši pridelek biomase (2,22 t ha$^{-1}$) in zrnja (1,00 t ha$^{-1}$) v kolobarju riža in gorjušice pri zalivanju z RSC vodo. Analize tal so pokazale, da je dolgotrajno namakanje z RSC vodo povzročilo sekundarno salinizacijo s povečanjem pH (1,92 %), EC (5,73 %) in SAR (35,71 %) glede na začetne vrednosti. Škodljivi učinki namakanja z vodo z veliko vsebnostjo rezidualnega natrijeva karbonata so bili oblaženi z vsemi obravnavanji vendar je bil največji pozitivni učinek opažen pri dodatku sadre zaradi izboljšanja lastnosti tal, kar je povečalo rast poljščin in pridelek zrnja riža v kolobarju riža z gorjušico. Dodatek sadre je dal največji pridelek biomase in zrnja riža (3,66 t ha$^{-1}$, 1,70 t ha$^{-1}$), zmanjšal pH (4,98 %), električno prevodnost (EC$_{e\,;}$ 29,93 %) in SAR (54,54 %) glede na njihove izhodiščne vrednosti.

**Ključne besede:** rezidualni natrijev karbonat, riž, gorjušica, slanost, slana voda, sadra, žveplena kislina, guar

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## 1 INTRODUCTION

Freshwater resources has long played a critical role in production and cultivation of agricultural crops throughout history, it is primarily used as 75 % in global agriculture (Assouline et al., 2015). However, increasing population, industrial productions and agricultural expansion has led the huge difference between supply and demand of freshwater resources and most of the countries of the world are facing shortage of fresh water resources (Gilbert, 2018). In recent years, global warming, inadequate rainfall, droughts and strong wind under climate change scenario further aggravated water scarcity and poses a great challenge to food safety and human health (Kummu et al., 2010). So, to solve the problem of global water deficit, new technical measures are being developed in the utilization of non-conventional water resources (Chen et al., 2016). Underground poor-quality water has emerged as potential alternative way source of irrigation that could relive the water pressure, almost 27 countries are utilizing underground brackish water for agricultural crops (Zhu et al., 2021). Irrigated agriculture is the primary source of Pakistan's economy and groundwater is a big source primarily used to meet the > 40 % of countries irrigational requirements but unfortunately 70-75 % of underground is of poor quality (Cheema et al., 2014). Brackish water irrigation changes the soil physical, chemicals and structural properties. It has been reported that irrigating with poor quality water reduces soil permeability (Li et al., 2018), increased soil electrical conductivity (Yang et al., 2020) and interfere with nutrient uptake (Chen et al., 2018). Sodic water may degrade the soil porosity from 8.8 to 12.5 % and 7.4–13.8 %, and bulk density from 7.4–13.8 % (Öztürk et al., 2023). Poor quality water also has a direct effect on the morphophysiological of plant (Viana et al., 2021). Water with high soluble salts and Na content may create drought conditions in rhizosphere, inhibiting water uptake, limited $CO_2$ assimilation, stomatal closure and disrupting photosynthetic activity (Leite et al., 2017; Sá et al., 2018). Yang et al. (2020) irrigated cotton crop with saline water ($1 g·l^{-1}$, $3 g·l^{-1}$, $6 g·L^{-1}$, $9 g·l^{-1}$ and $12 g l^{-1}$). They reported that saline water causes secondary soil salinization by increasing salts in rootzone, soil pH increases but the cotton yield decreases as salt content increased in saline water. Similarly, it is reported by Wiedenfeld (2008) that water having EC (3.4 dS $m^{-1}$) used to irrigate the sugarcane crop results in 17 % reduction in cane and sugar yield. Arora et al. (2018) studied the results of tap water having EC (0.7 dS $m^{-1}$), alkli water (RSC) of 2.5 and 10 me $l^{-1}$) and saline water (EC 5.0 and 10 dS $m^{-1}$) on growth and grain yield performance of rice-wheat crops. They reported that alkali water builds up soil sodicity by increasing soil pH and exchangeable sodium percentage. While maximum grain mortality in rice-wheat crop was recorded when irrigation with saline water of EC (10 dS $m^{-1}$) was done.

Brackish water is rich source of beneficial micronutrients and short-term irrigation did not obviously deteriorate the soil health and can improve the quality and yield of crops (Jin et al., 2016). However, long-term brackish water irrigation may cause non-salinized soil to transform into salinized soil due to accumulation of certain toxic ions in root zone that lead to serious crop yield reduction and huge changes in the physico-chemical properties of the soil (Lee et al., 2016; Cao et al., 2016; Tahtouh et al., 2019). Therefore, secondary soil salinization is mainly determined by amount and kind of dissolved salt content of poor-quality water and utilization of brackish water has become a new problem to solve. Therefore, long run utilization of low-quality water needs to be investigated and appropriate protective measures need to framed that inhibit soil salt accumulation in soil. The damaging effect of brackish water on soil and crops can be alleviated by suitable agronomic practices, exogenous hormone regulation, proper nutrient management and application of inorganic and organic amendments that accelerate leaching of toxic salts from root zone. Gypsum application is an inexpensive, easy to handle and effective strategy that alleviate salt stress on crops by adequately leaching toxic ion from rootzone brought by brackish water into the soil (Wang and Yang, 2017). Gypsum releases sufficient amount of soluble $Ca^{2+}$ to replace $Na^{+}$ from exchange site (Wang et al., 2017; Koralegedaraa et al., 2019). Five-year application of gypsum improves forage yield of corn, alfalfa and emergence ratios of mignonettes (Wang et al., 2017). Qadir et al. (2019) evaluated the effect of gypsum (100 % on the basis of RSC of water), $H_2SO_4$ (50 % on the basis of RSC of water) poultry manure (10 t $ha^{-1}$) and press mud (10 t $ha^{-1}$) to counteract the detrimental effect of brackish water {EC = 1.17 dS $m^{-1}$, SAR = 6.75 and RSC = 5.30 me $l^{-1}$} on growth and yield of cotton and wheat crops. They reported that gypsum was most effective amendments to prevent secondary salinization by improving $EC_e$, $pH_s$ and SAR and create favorable soil condition for wheat and cotton crops. Rashmi et al. (2024) reported that gypsum application at the rate of 2.5 t $ha^{-1}$ + FYM at rate of10 t $ha^{-1}$ resulted a reduction of 45–48 % in exchangeable sodium percentage and 3–6 % in bulk density of sodic soil leading to higher crop yield in soybean (1.21 Mg $ha^{-1}$) and mustard (1.39 Mg $ha^{-1}$).

Green manuring with leguminous crops is very careful and effective sustainable means to prevent soil salinization. Short-lived plants with low C:N ratio decomposed easily and demonstrate positive impacts on soil health by enhancing soil fertility status, improve water holding capacity, aeration and inhibit soil salt accumulation (Shah et al., 2011). Incorporation of sesbania in salt affected soils during rice-wheat crop rotation improved soil salinity and sodicity indices and grain yield of both crops (Rizwan et al., 2018). Green manuring with guar and sesbania had positive effect on soil health by up-lifting nitrogen, phosphorus and organic carbon contents, improved water holding capacity, aeration, porosity, and microbial activities (Ibrahim et al., 2000; Shindo and Nishio, 2005).

Reduction in fresh water resources of country force farming community to explore poor-quality underground water, therefore in our study we used high RSC water to explore its effect on physicochemical properties of the soil and to develop a feasible preventive measure to control secondary salinization and ensure crop growth.

## 2 MATERIALS AND METHODS

### 2.1 SITE DESCRIPTION

The present study was conducted for four years (2016-20), following rice-mustard crop rotation, at SSRI, Pindi Bhattian, Punjab, Pakistan located at latitude 31.8950° N and longitude 73.2706° E). A field (Table 1) {($pH_{s\,=}$ 8.82, $EC_e$ = 4.71 dS $m^{-1}$, sodium adsorption ratio = 26.82 (mmol $l^{-1})^{1/2}$, hydraulic conductivity = 0.67 cm $hr^{-1}$ and bulk density = 1.37 Mg $m^{-3}$} was selected and prepared. An experiment was laid out in RCBD arrangement with four replications using plot size 6 x 4 $m^2$. During the period of (2016-20), the mean weather conditions were: sunshine hours ranging from 14h and 8 min to 7 h and 36 min, relative humidity ranges from 36.2 ± 2.8 % to 73.2 ± 4.8 % and temperature 14.5 ± 2.5 °C minimum temperature and 42.6 ± 3.5 °C maximum temperature.

### 2.2 TREATMENTS AND CROP ROTATION

The treatments applied were: $T_1$ = high RSC tube well water (No amendement), $T_2$ = gypsum application on the basis of RSC of tube well water, $T_3$ = $H_2SO_4$ application on the basis of RSC of tube well water, $T_4$ = green manuring with guar, $T_5$ = FYM @ 10 t $ha^{-1}$. Cluster bean or locally known as guar (*Cyamopsis tetragonoloba* (L.) Taub.) seed @ 50 kg $ha^{-1}$ was sown in 2nd week of May and incorporated after 45 days of sowing in respective treatment plots. Gypsum (30 mesh size, 80 % pure, 1 kg/plot/ year) and FYM (Total N (%) 0.96, Total $P_2O_5$ (%) 0.27, Total K (%) 0.75 and pH 7.88, 24 Kg/plot/ year) were applied one-month prior rice transplanting followed by irrigation and $H_2SO_4$ (96 % pure, 0.3 l/plot) was applied with every irrigation. Amendments were applied once in a year before transplanting of rice. Poor-quality ground water with high residual sodium carbonate pumped from tub well located at study site was used for all treatments (Table 2). On average 16 irrigations for rice and 4 irrigations for mustard crop at the rate of 7.5 cm were applied in each season.

During Kharif season, rice ('Shaheen Basmati') nursery at the age of 30 days was shifted in the field during second week of July and during Rabi season, mustard crop (*Brassica Juncea* ?Khanpur Raya') was sown during November. Fertilizers @150-85-60 and 70-70-60 NPK (kg $ha^{-1}$) was broadcasted to rice and mustard crops. Phosphatic and potassium fertilizers were applied as basal dose and nitrogen was broadcasted in three installments. Other cultural and plant protection measures were taken equally when required. Data regarding crop growth and yield was recorded and crops harvested at maturity.

### 2.3 SOIL AND WATER ANALYSIS

Composite soil samples were collected. Samples were air dried, ground, sieved and stored in bottles after the harvest of every crop and analyzed for pH, $EC_e$, SAR, hydraulic conductivity and bulk density using U.S. Salinity Laboratory Staff (1954). Soil pH of saturated paste was measured through pH meter (Microcomputer pH-vision cole parmer model 05669-20). Conductivity meter (WTW conduktometer LF 191) was used to measure the EC of water and soil extract. $Na^+$ contents were measured using flame photometer (digiflame code DV 710) and $Ca^{2+}$, $Mg^{2+}$ were measured through titration. Sodium adsorption ratio (SAR) was calculated as follows where ionic concentration of the saturation extracts is given in mmole $l^{-1}$. SAR = $Na^+$ / $[(Ca^{2+}+ Mg^{2+})/2]^{1/2}$. Hydrometer method (Bedaiwy, 2012) was used for textural class determination. $CO_3^{2-}$ and $HCO_3^-$ were determined via titration with standard $H_2SO_4$. Residual sodium carbonate (RSC) was calculated by (Eaton, 1950) as follows:

$$RSC= (CO_3^{2-} \text{ and } HCO_3^-)- (Ca^{2+}+ Mg^{2+}).$$

### 2.4 STATISTICAL ANALYSES

Recorded crop data was subjected to analysis of variance. Treatment means comparison was calculated by LSD Test at 5 % probability level (Steel et al., 1997) using STATISTIX 8.1 package software.

**Table 1:** Initial soil analysis

| Parameter | Value | Units |
|---|---|---|
| Electrical conductivity of soil extract ($EC_e$) | 4.71 | ($dS\ m^{-1}$) |
| pH of soil saturated paste ($pH_s$) | 8.82 | - |
| Sodium absorption ratio (SAR) | 26.82 | ($mmol\ l^{-1})^{1/2}$ |
| Hydraulic conductivity (HC) | 0.67 | ($cm\ hr^{-1}$) |
| Bulk density (BD) | 1.37 | ($Mg\ m^{-3}$) |
| Available phosphorus | 8.50 | $mg\ kg^{-1}$ |
| Organic matter | 0.62 | % |
| Available potassium | 105 | $mg\ kg^{-1}$ |
| Textural class | sandy loam | - |

**Table 2:** Water Analysis

| Parameters | Value | Units |
|---|---|---|
| Electrical conductivity of irrigation water ($EC_{iw}$ ) | 1.37 | ($dS\ m^{-1}$) |
| Sodium adsorption ratio (SAR) | 8.4 | ($mmol_e\ l^{-1})^{1/2}$ |
| Residual sodium carbonate (RSC) | 7.85 | ($me\ l^{-1}$) |
| Total dissolved solids (TDS) | 877 | ($mg\ l^{-1}$) |
| $CO_3^{2-}$ | Nill | ($me\ l^{-1}$) |
| $HCO^{3-}$ | 11.25 | ($me\ l^{-1}$) |
| $Ca^{2+} + Mg^{2+}$ | 3.40 | ($me\ l^{-1}$) |
| $Na^+$ | 10.93 | ($me\ l^{-1}$) |
| $Cl^-$ | 4.0 | ($me\ l^{-1}$) |

## 3 RESULTS

### 3.1 EFFECTS OF HIGH RSC WATER AND AMENDMENTS ON SOIL PROPERTIES

Soil analysis data revealed that long term use of high RSC water negatively affected the soil properties while all the remedial strategies used considerably improved the soil physico-chemical properties. Irrigation with high RSC water resulted a gradual increase in soil $pH_s$ value at the end of every season and after four years of experimentation an increase of 1.92 % over initial soil $pH_s$ value was observed with continuous use of high RSC water (Table 3). Meanwhile, all the remedial strategies thwarted the negative impact of high RSC water and significantly influenced soil $pH_s$. From the treatments maximum reduction of 5.44 % in $pH_s$ was recorded with $H_2SO_4$ followed by gypsum (4.98 %), whereas, FYM and green manuring reduces the $pH_s$ value by 3.17 % and 3.06 % respectively at the end of experiment. Effect of high RSC water and amendments on soil electric conductivity $EC_e$ is shown in (Table 4). Data indicated that soluble salts are brought into soil with continuous irrigation with high RSC water and salt accumulated in the upper soil surface, therefore an increase of 5.73 % in $EC_e$ with respect to initial value was recorded in control treatments at the end of study. However, at the same time amendments application can prevent the salt accumulation in top soil by accelerating the salt leaching process. Maximum improvement in soil salinity status was observed with gypsum application as a reduction of 54.54 % in $EC_e$ was noted in this treatment. While $H_2SO_4$, guar and FYM showed a reduction of 49.40 %, 41.05 %, 46.71 % respectively in $EC_e$ over initial value at the end of experiment. Similar trend was recorded in the case of soil SAR, irrigation with high RSC water (7.85 $me\ l^{-1}$) without any amendment elevated soil SAR by 35.71 % at the end of experimentation as compared to initial value (Table 5). Whereas, SAR was under safe range (≤ 15) in all other treatments receiving amendments. It was observed that maximum reduction in soil SAR (54.54 %) was for gypsum followed by $H_2SO_4$ (49.40 %), while Guar and FYM recorded a reduction

**Table 3:** Effect of high RSC water and amendments on soil pHs

| Treatments | 1st year | 2nd year | 3rd year | 4th year | + % increase or<br>-%decrease over initial value |
|---|---|---|---|---|---|
| High RSC water | 8.84 | 8.90 | 8.96 | 8.99 | (+1.92) |
| Gypsum @ RSC of water | 8.58 | 8.46 | 8.41 | 8.38 | (-4.98) |
| H2SO4 @ RSC of water | 8.57 | 8.46 | 8.39 | 8.34 | (-5.44) |
| Green manuring with guar | 8.72 | 8.68 | 8.66 | 8.55 | (-3.06) |
| FYM @ 10 t ha$^{-1}$ | 8.70 | 8.65 | 8.61 | 8.54 | (-3.17) |

**Table 4:** Effect of high RSC water and amendments on soil ECe

| Treatments | 1st year | 2nd year | 3rd year | 4th year | +% increase or<br>-% decrease over initial value |
|---|---|---|---|---|---|
| High RSC water | 4.75 | 4.81 | 4.85 | 4.98 | (+5.73) |
| Gypsum @ RSC of water | 3.48 | 3.37 | 3.32 | 3.30 | (-29.93) |
| H2SO4 @ RSC of water | 3.87 | 3.84 | 3.79 | 3.70 | (-21.44) |
| Green manuring with guar | 3.91 | 3.87 | 3.85 | 3.80 | (-19.32) |
| FYM @ 10 t ha$^{-1}$ | 3.92 | 3.86 | 3.81 | 3.76 | (-20.16) |

**Table 5:** Effect of high RSC water and amendments on soil SAR

| Treatments | 1st year | 2nd year | 3rd year | 4th year | +% increase or<br>-% decrease over initial value |
|---|---|---|---|---|---|
| High RSC water | 30.70 | 32.20 | 33.50 | 36.40 | (+35.71) |
| Gypsum @ RSC of water | 16.80 | 15.30 | 14.40 | 12.19 | (-54.54) |
| H2SO4 @ RSC of water | 17.65 | 16.40 | 15.30 | 13.57 | (-49.40) |
| Green manuring with guar | 19.40 | 17.80 | 17.50 | 15.81 | (-41.05) |
| FYM @ 10 t. ha$^{-1}$ | 18.20 | 17.30 | 16.80 | 14.29 | (-46.71) |

of 41.05 % and 46.71 % respectively compared to initial value of SAR.

Among physical properties, soil bulk density (BD) slightly increased (8.02 %) with continuous irrigation of high RSC water, whereas it decreased by all the applied amendments. BD decreased (about 15.32 %) with gypsum application, whereas, $H_2SO_4$, Guar and FYM showed a reduction of 14.59 %, 10.94 % and 11.67 % respectively (Table 6). On contrary, an opposite trend was observed in case of HC. Upon irrigation with high RSC water a reduction of 11.94 % was noted but at the same time gypsum and $H_2SO_4$ surpassed the hydraulic conductivity by an increase of 26.86 % and 25.37 % respectively over its initial value (Table 7).

## 3.2 EFFECT OF HIGH RSC WATER AND AMENDMENTS ON GROWTH AND PADDY YIELD OF RICE

Analysis of four years pooled data showed that use of high RSC water significantly ($p < 0.05$) affected the rice crop growth while simultaneously all the amendments thwarted the detrimental effects of high RSC water (Table 8). As far as plant height of rice crop was concerned

**Table 6:** Effect of high RSC water and amendments on soil bulk density

| Treatments | 1st year | 2nd year | 3rd year | 4th year | +% increase or<br>-% decrease over initial value |
|---|---|---|---|---|---|
| High RSC water | 1.37 | 1.41 | 1.46 | 1.48 | (+8.02) |
| Gypsum @ RSC of water | 1.28 | 1.22 | 1.19 | 1.16 | (-15.32) |
| H2SO4 @ RSC of water | 1.27 | 1.24 | 1.22 | 1.17 | (-14.59) |
| Green manuring with guar | 1.34 | 1.29 | 1.26 | 1.22 | (-10.94) |
| FYM @ 10 t ha$^{-1}$ | 1.33 | 1.29 | 1.25 | 1.21 | (-11.67) |

**Table 7:** Effect of high RSC water and amendments on soil hydraulic conductivity

| Treatments | 1st year | 2nd year | 3rd year | 4th year | +% increase or<br>-% decrease over initial value |
|---|---|---|---|---|---|
| High RSC water | 0.66 | 0.64 | 0.60 | 0.59 | (-11.94) |
| Gypsum @ RSC of water | 0.72 | 0.77 | 0.81 | 0.85 | (+26.86) |
| H2SO4 @ RSC of water | 0.72 | 0.76 | 0.79 | 0.84 | (+25.37) |
| Green manuring with guar | 0.71 | 0.73 | 0.75 | 0.76 | (+13.43) |
| FYM @ 10 t ha$^{-1}$ | 0.70 | 0.73 | 0.76 | 0.78 | (+16.41) |

maximum plant height (135.33 cm) was observed with application of gypsum application on RSC basis followed by sulphuric acid application. Farm yard manure @ 10 t ha$^{-1}$ and green manuring with guar were statistically ($p < 0.05$) non-significant. While, least plant height (124.00 cm) was recorded in treatment where field was irrigated with high RSC water without any amendment. Productive tiller data showed that maximum productive tiller/m$^2$ (235.33) was observed by $H_2SO_4$ application followed by gypsum on of RSC basis, however, both amendments were non-significant ($p < 0.05$) from each other (Table 8). High RSC water adversely affected productive tillers and lower numbers of productive tiller were recorded with use of high RSC water (220). Gypsum and $H_2SO_4$ produced the highest panicle length (16.66 cm) that was statistically ($p < 0.05$) non-significant with other treatments and minimum panicle length was noted in control (Table 8). Paddy yield was also significantly influenced by different treatments, data in Table 9 revealed that amendments had pronounced effect on yield attribute and all the organic and inorganic amendments exhibited an antistress effect against deleterious impact of high RSC water (Table 9). Gypsum application on RSC basis produces significantly ($p < 0.05$) more paddy yield (3.66 t ha$^{-1}$) which was similar with $H_2SO_4$ application on the basis of RSC of water (3.62 t ha$^{-1}$) followed by FYM @ 10 t ha$^{-1}$ (3.35 t ha$^{-1}$) and green manuring with guar (3.20 t ha$^{-1}$). High RSC water irrigation without any amendment resulted minimum paddy yield of 2.22 t ha$^{-1}$. Same trend was recorded for straw yield and 1000 grain mass (Table 9). Gypsum application recorded the highest mean value for straw (7.96 t ha$^{-1}$) and 1000 grain mass (28.33 g) however, difference was non-significant with $H_2SO_4$ application. On contrary lower mean value for straw (4.16 t ha$^{-1}$) and 1000 grain mass (19.66 g) was divulged in control plot irrigated with high RSC water.

### 3.3 EFFECT OF HIGH RSC WATER AND AMENDMENTS ON GROWTH AND YIELD OF MUSTARD

Information in Table 10 & 11 showed that use of amendment relieves the drastic effects of high RSC water on growth and yield of mustard crop. An obvious increased in plant height of mustard was observed with use of amendments (Table 10). Maximum plant height (155 cm) and number of branches/plant (26.66) were observed with gypsum application; however, values were at par ($p < 0.05$) with $H_2SO_4$ followed by green manuring with guar. While minimum plant height (119.33 cm) and number of branches/plant (17.33) were observed in con-

**Table 8:** Effect of high RSC water and amendments on rice growth (Average of four seasons)

| Treatments | Plant height (cm) | Number of productive tillers ($m^{-2}$) | Panicle length (cm) |
|---|---|---|---|
| High RSC water | 124.00 D | 220.00 C | 12.66 B |
| Gypsum @ RSC of water | 135.33 A | 232.67 A | 16.66 A |
| $H_2SO_4$ @ RSC of water | 133.00 B | 235.33 A | 16.66 A |
| Green manuring with guar | 128.33 C | 227.00 B | 15.33 A |
| FYM @ 10 t $ha^{-1}$ | 129.33 C | 225.00 B | 14.66 AB |

Different letters in the same column indicate significant differences by LSD at p ≤ 0.05

**Table 9:** Effect of high RSC water on and amendments rice growth (Average of four seasons)

| Treatments | Paddy yield (t $ha^{-1}$) | Straw yield (t $ha^{-1}$) | 1000-grain mass (g) |
|---|---|---|---|
| High RSC water | 2.22 C | 4.16 D | 19.66 D |
| Gypsum @ RSC of water | 3.66 A | 7.96 A | 28.33 A |
| H2SO4 @ RSC of water | 3.62 A | 7.70 AB | 27.00 AB |
| Green manuring with guar | 3.20 B | 6.93 C | 23.66 C |
| FYM @ 10 t $ha^{-1}$ | 3.35 B | 7.03 BC | 24.66 BC |

Different letters in the same column indicate significant differences by LSD at $p \leq 0.05$

trol. Similarly, the highest pods plant$^{-1}$ (258) and grains pods$^{-1}$ (13) were produced with gypsum application while, irrigation with high RSC water negatively affected these attributes and recorded minimum pods plant$^{-1}$ (227.33) and grains pods$^{-1}$ (7.33). Data showed (Table 11) that the highest grain yield (1.70 t ha$^{-1}$) and 1000-grain mass (8.81 g) was recorded with gypsum application on RSC basis which was statistically ($p < 0.05$) at par with $H_2SO_4$ application on RSC basis followed by FYM @ 10 t ha$^{-1}$, which was at par with green manuring with guar. While minimum grain yield (1.0 t ha$^{-1}$) and 1000-grain weight (6.06 g) were recorded in control (brackish water).

## 4 DISCUSSION

### 4.1 EFFECT OF HIGH RSC WATER ON SOIL PROPERTIES

Fresh water resources of the country are not enough to meet the agricultural requirements because of increased cropping intensity and drought condition. Therefore, use of unconventional water resources in addition to fresh water resources is necessary and ground water pumping is need of the time. However, underground water is generally inferior to canal water and may vary in quality depending upon type and quantity of dissolved salts. So, there is need for development and adoption of such technologies that can ensure the safe utilization of low-quality water and prevent the salt deposition. Therefore, an attempt has been made to study the harmful effects of high RSC water and efficiency of different amendments on physico-chemical properties of soil for rice-mustard rotation.

Continuous use of high RSC water without any amendment slightly increased the electrical conductivity, it was obvious effect of dissolved salts in high RSC water that accumulated in top soil due to lack of leaching and upward movement caused by soil evaporation and induces secondary salinization (Huo et al., 2017; Yang et al., 2019; Wang et al., 2019). A build of salt load in surface soil was also reported by Arora et al. (2018) in rice-wheat cropping system when irrigated with saline

**Table 10:** Effect of high RSC water and amendments on mustard growth (Average of four seasons)

| Treatments | Plant height (cm) | Number of branches plant$^{-1}$ | Number of pods plant$^{-1}$ |
|---|---|---|---|
| High RSC water | 119.33 C | 17.33 C | 227.33 D |
| Gypsum @ RSC of water | 155.00 A | 26.66 A | 258.00 A |
| H2SO4 @ RSC of water | 153.33 A | 24.33 AB | 250.00 AB |
| Green manuring with guar | 137.67 B | 21.33 BC | 240.67 C |
| FYM @ 10 t ha$^{-1}$ | 135.00 B | 20.66 BC | 248.33 BC |

Different letters in the same column indicate significant differences by LSD at $p \leq 0.05$

**Table 11:** Effect of high RSC water and amendments on mustard growth (Average of four seasons)

| Treatments | Number of grains pods$^{-1}$ | Grain yield (t ha$^{-1}$) | 1000-grain mass (g) |
|---|---|---|---|
| High RSC water | 7.33 D | 1.00 C | 6.06 B |
| Gypsum @ RSC of water | 13.00 A | 1.70 A | 8.81 A |
| H2SO4 @ RSC of water | 11.33 B | 1.68 A | 8.79 A |
| Green manuring with guar | 9.00 C | 1.35 B | 7.01 B |
| FYM @ 10 t ha$^{-1}$ | 9.66 C | 1.38 B | 7.49 AB |

Different letters in the same column indicate significant differences by LSD at $p \leq 0.05$

and alkali water. A rise in pH was also noted in this treatment, a possible reason for this increment was presence of $HCO_3^-$ and $Na^+$ in applied irrigation water which led to increase in pH (Jalali and Ranjbar 2009; He et al., 2018). Similar results were recorded earlier by Choudhary et al. (2011) that long-term use of high RSC alkali water (12.5 me l$^{-1}$) increased soil pH by 2.2 units under rice-wheat rotation in Punjab. Similarly (Yang et al., 2020) reported an increase of 0.37 unit in soil pH with irrigation of 12 g l$^{-1}$ brackish water treatment. Nearly similar trend was observed in case of SAR, that increased 35.71 % from its initial value. These results were supported by earlier findings that application of sodic and saline water without any preventive measures increased the sodicity of soil and deteriorate soil physical properties (Bajwa and Choudhary, 2014; Mwubahaman et al., 2024). High residual alkalinityof irrigation water had adverse effect of sodification due to high exchangeable sodium contents as compared to exchangeable calcium and magnesium (Choudhary et al., 2011; Arora et al., 2018). similar results were recorded by Liu et al. (2018) that irrigation with saline water (3 g l$^{-1}$) increased soil pH value, ESP, and SAR. Adverse effects of low-quality water on chemical properties were directly translated into soil physical properties. Soil bulk density was negatively influenced by continuous use of high RSC water and increased by 8.02 % from its initial value. This increase in bulk density may be attributed to, Na replaces Ca from exchange sites which causes soil dispersion (Qadir and Schubert, 2002; Kahlon et al., 2012; Qadir et al., 2001). HC was negatively related with water sodicity, high $Na^+$ contents in irrigation water deteriorate soil structure, cause dispersion of soil aggregates and block soil pores thus reducing hydraulic conductivity (Wu and Wang, 2010; Kang et al., 2014).

## 4.2 EFFECT OF AMENDMENTS ON SOIL PROPERTIES

It is clear from results that addition of amendments counteracted the detrimental effects induced by long term use of low-quality water and prevent the accumulation of salts in upper surface layers. All the amendments substantially improved physico-chemicals properties of soil; however, beneficial effects of gypsum were more visible than all others treatments. Salinity indices pH, EC, and SAR were significantly improved by gypsum application as compared to their initial values. These positives effects of gypsum could be due to that high amount of $Ca^{2+}$ replaces the exchangeable $Na^+$ on the exchange site and leaching it out from root zone (Sharma and Minhas, 2005). Sufficient leaching of $Na^+$ leads to lower values of pH, EC, and SAR, furthermore, replacement of hydrated monovalent $Na^+$ by divalent $Ca^{2+}$, increased soil aggregation and reorganized soil structure subsequently improved the soil bulk density and hydraulic conductivity. Similar results were reported by Amer and Hashem (2018) that application of only gypsum and along-with with other amendments improved soil physico-chemicals properties like infiltration rate, soil porosity, bulk density, EC and SAR. Gypsum application is a rapid, viable and effective approach for improving pH, EC, and ESP of sodic soils (Koralegedaraa et al., 2019; Yong-gan et al., 2021). Previous studies demonstrated that positive effects of gypsum on soil physico-chemicals properties may remained from 12 to 17 years (Tirado-Corbalá et al., 2019; Zhao et al., 2019). $H_2SO_4$ also significantly improved soil salinity indices (pH, EC, and SAR), BD and HC. $H_2SO_4$ is an indirect source of $Ca^{2+}$ in calcareous soils, it reacts with the native $CaCO_3$ and releases $Ca^{2+}$ in the soil solution that probably replaced the $Na^+$ from exchange sites (Abdelhamid et al.,2013). Consequently, soil phyico-chemical properties were improved after leaching of $Na^+$ and dissolved salts from the root zone. Comparable outcomes were observed by Qadir et al., (2019) that $H_2SO_4$ @ 50 % application on RSC basis counteracted negative effects of brackish (5.30 me $l^{-1}$) water and improved soil properties and cotton-wheat yield. Shaaban, et al. (2013) in a field study also concluded that $H_2SO_4$ was the best of the amendments for decreasing damaging effects of saline irrigation water and enhancing the productivity and quality of rice crop grown on saline soil. Use of organic amendments like FYM and green manuring is also an effective measure against sodicity build up in soil (Pang et al., 2010). Organic matter after decomposition release $CO_2$ and organic acids that decreased the precipitation of $Ca^{2+}$, displace $Na^+$ on the exchange site, accelerate leaching of soluble salts leading to a decline in pH, EC, and SAR (Liu et al., 2017; Ahmad et al., 2018; Ding et al., 2019). Leaching of larger $Na^+$ enhanced soil structure stability and improved soil bulk density and hydraulic conductivity (Hammer et al., 2015; Ding et al., 2019). Ding et al. (2019) stated that organic amendments could be successfully used to mitigate the soil salinity and to improve soil physico-chemical properties. Similar results were recorded by Yang et al. (2019) that straw incorporation decreased the BD by 1.6 %–4.7 %, in the 0-30 cm soil layer.

## 4.3 CROPS GROWTH AND YIELD

Parameters like growth and yield of rice and mustard crops were meaningfully influenced by all treatments. Irrigation with high RSC water significantly reduced plant height, grain yield and 1000-grain mass of rice and mustard crop as compared to other treatments. Reduction in growth and yield contributing parameters may be associated with accumulation of salts with constant use of low-quality water that deteriorate the physico-chemical properties of soil. Secondary salinization in root zone reduces absorption of water by crops, results ion toxicity, nutritional imbalance, affect stomatal conductance and photosynthetic activity (Rahm et al., 2018; Wang et al., 2019; Munns and Tester, 2008; Sá et al., 2019). Similar, results were observed by Praxedes et al. (2022) that saline water (4.5 dS $m^{-1}$) irrigation may reduce grain yield from 26 % to 54 % in cowpea. Alkali water (10.0 me $l^{-1}$) reduced grain yield of rice up to 87 % in saline soil (Arora et al., 2018). Gypsum application not only counteracted negative effects of high RSC water but also showed the highest value for most of the studied parameters of rice and mustard crops. Better crops growth performance may be corelated with ameliorating gypsum effects on soil properties. Gypsum application increased soil organic matter (Wang et al., 2017), improved nutritional status of soil and microbial activity (Zhao et al., 2019; Ekholm et al., 2024), these factors promote plant growth and subsequently final grain yield. Previous studies also demonstrated the improved yield in barley and rice (Amer and Hashem, 2018) and alfalfa (Yong-gan et al., 2021) with gypsum application in salt-affected soil. $H_2SO_4$ mobilizes the native $CaCO_3$ of soil and provides Ca in soil solution, which in turn alleviate the ill effect of high RSC water and improved physico-chemical properties. Consequently, crop took the advantages of improved soil conditions in this treatment and produced more yield over control. $H_2SO_4$ @ 50 % application on RSC basis counteracted negative effects of high RSC (5.30 me $l^{-1}$) water and improved soil properties and cotton-wheat yield (Qadir et al., 2019). Shaaban, et al. (2013) in a field study also concluded that $H_2SO_4$ was best of the amendments for decreasing damaging effects of saline irrigation water and enhancing the productivity and quality of rice crop grown on saline soil. Green manuring and FYM application also improved all the yield traits as compared to control. Addition of organic matter also improved microbial activities, soil organic matter and other properties (Urbaniak et al., 2017). Organic matter alleviated the adverse of high RSC water by increasing chelation of toxic Na, water holding capacity of soil and improved soil BD and HC (Liu et al., 2017). Rice and mustard crops were benefited by these positive effects of organic matter on soil environment leading to increased grain yield and other agronomic attributes. Ding et al. (2020) suggested that addition of organic matter is a successful management approach for improving the nutrient uptake and wheat productivity in salt-affected soil which reinforced our findings.

## 5 CONCLUSION

Findings of the current study highlighted that long-term use of high RSC water could deposit the salts and induce secondary salinity by increasing pH, EC and SAR which adversely affected the growth and yield of rice and mustard crops. Harmful effects of high RSC water were thwarted by all amendments and can be used as preventive measures against salinity-sodicity development. Among all amendments, positive effects of gypsum were more visible that increased growth and yield of rice-mustard crops by promoting soil properties. Efficiency of different amendments to alleviate adverse effects of high RSC water can be arranged as such gypsum> $H_2SO_4$ > FYM > green manuring with guar. Therefore, it is recommended that farmers should apply gypsum on the basis of RSC of water for safe use of poor-quality high RSC water. In present study we investigated the ameliorative role of each amendment individually; however, a long-term field trial about ameliorative role of different organic and inorganic amendments in combination should be explored in future.

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Original research article / izvirni znanstveni članek

# The role of volatile compounds and genes that involved in ester biosynthesisduring strawberry fruit (*Fragaria* × *ananassa* Duchesne) development

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**The role of volatile compounds and genes that involved in ester biosynthesisduring strawberry fruit *(Fragaria* × *ananassa* Duchesne) development**

**Abstract:** Strawberry (*Fragaria* × *ananassa* Duchesne) is one of the most crucial berry fruits because of its nutrients and pleasant taste. The present research is to identify volatile compounds, study the biosynthesis pathway during three developmental stages, and *insilico* analysis of lipoxygenase (LOX), alcohol dehydrogenase (ADH), and alcohol acyltransferase (AAT) genes in strawberries. The results indicated that 68 volatile compounds were identified in different developmental stages. The gas chromatography/mass spectrometry showed that the amounts of esters increased during the development of strawberry fruit, while aldehydes and alcohol components decreased during the red stage. The results showed LOX gene expression decreased during fruit development, while ADH and AAT gene expression increased in ripe fruit. It seems that alcohols have a minor contribution to producing the aroma of fruits due to early consumption. Furthermore, esters in the red stage play a significant role in the aroma of ripe fruit. The knowledge of the phytochemical profile of strawberries in the growing stages could be used in different applications of these materials in various fields, including food, medical, and pharmaceutical industries, and production of food essences and natural flavorings, as well as fragrance design.

**Key words:** alcohols, aldehydes, esters, lipoxygenase pathway, strawberry fruit.

**Vloga hlapnih spojin in genov vključenih v biosintezo estrov pri razvoju plodov jagodnjaka (*Fragaria* × *ananassa* Duchesne)**

**Izvleček:** Žlahtni jagodnjak (*Fragaria* × *ananassa* Duchesne) je ena izmed najpomembnejših vrst jagodičevja zaradi vsebnosti hranil v plodovih in dobrega okusa. Namen raziskave je bil *insilico* določiti hlapne spojine in njihovo biosintezo med tremi obdobji razvoja plodov in sicer delovanje genov za lipoksigenazo (LOX), alkohol dehidrogenazp (ADH) in alkohol aciltransferazo (AAT). V različnih razvojnih stopnjah je bilo identificiranih 68 hlapnih spojin. Analiza s plinsko kromatografijo in masno spektrometrijo je pokazala, da se med razvojem plodov jagodnjaka povečuje količina estrov medtem, ko se količina aldehidov in alkoholov zmanjšuje v rdečem obdobju razvoja plodov. Rezultati so pokazali, da se je delovanje genov za LOX zmanjševalo med razvojem plodov, medtem, ko se je delovanje genov za za ADH in AAT povečalo v zrelih plodovih. Izgleda, da imajo alkoholi manjši delež pri tvorbi arome plodov zaradi njihove hitre porabe, imajo pa estri v rdečem stadiju razvoja pommebmo vlogo pri aromi zrelih plodov. Vedenje o fitokemkičnem profile plodov jagodnjaka v rastnih obdobjih bi lahko bilo uporabljeno za različne namene in področja kot so prehrana, medicinska in farmacevtska uporaba, pri pripravi dodatkov hrani, naravnih barvilih kot pri načrtovanju vonjav.

**Ključne besede:** alkoholi, aldehidi, estri, cikel lipoksigenaze, plod jagodnjaka.

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## 1 INTRODUCTION

Strawberry is one of the most popular fruit crops cultivated worldwide, valued for its economic importance and consumer demand. In recent years, its production has significantly contributed to national economies, including over three billion dollars annually in the United States (Lu et al., 2020). Plants produce a wide range of volatile compounds (secondary metabolites), including alcohols, aldehydes, esters, ketones, lactones, terpenoids, and apocartenoids, which are not always essential for plant reproduction and survival. Moreover, volatile compounds play a role in protecting the plant against environmental stresses (Effah et al., 2019). Volatile compounds are an indicator of fruit ripening, which affect the aroma of the fruit and play a vital role in the acceptability and choice of fruit by consumers (Christensen et al., 2023). Esters are the most abundant volatile compounds of strawberry fruit (Yan et al., 2018).

The most significant step in the biosynthesis of volatile compounds is the availability of their precursor substrate, the amount and composition of which are strictly controlled during fruit development (Song et al., 2003). The activity of lipoxygenase enzyme (LOX) is one of the fundamental processes during fruit ripening, and its products have essential functions in the biosynthesis of volatile compounds (Li et al., 2014). Esters, alcohols, acids, and carbonyls in fruits are produced from the oxidative reduction of linolenic acid and linoleic acid by the LOX pathway, which mainly forms C6 volatile compounds (Ei Hadi et al., 2013). C6 volatiles are normally produced after chewing herbivore attack. In continuing the lipoxygenase pathway, aldehyde compounds can either be converted into their isomers by isomerases or reduced to alcohol by the alcohol dehydrogenase (ADH) enzyme. ADH can use aldehydes as a substrate, which quickly converts the C6 aldehydes of the ripening fruit into alcohol. ADH enzyme is dependent on NAD and NADP and is responsible for providing precursors that determine the production of the type of ester in strawberries (Yan et al., 2018). Alcohols produced through the lipoxygenase pathway are used as substrates for the enzyme alcohol acyltransferase (AAT) to produce esters (Cumplido-Laso et al., 2012). Furthermore, alcohols act as signaling molecules in stress conditions (biotic and abiotic) and induce the expression of defense genes (Weihua et al., 2020). AAT enzyme catalyzes the biosynthesis of esters, and in fruits with more aroma, AAT enzyme is more active (Beekwilder et al., 2004). AAT enzyme catalyzes the biosynthesis of esters and is responsible for the final stage of ester production, which shows a multifold increase in the gene expression of the Rosaceae family in the middle stages until the ripe fruit (Song et al., 2008).

The biosynthesis of volatile compounds is an important part of the fruit development, and their production during fruit ripening affects its final quality and taste (Li et al., 2021). The economic value of strawberry fruit and the role of its volatile compounds make it indispensable as a valuable fruit in various industries such as food, pharmaceuticals, and cosmetics. This study aims to identify the volatile compounds present at three key developmental stages of strawberry fruit—green, white, and red—using GC-MS. Additionally, it investigates the expression patterns of three key genes involved in volatile biosynthesis, namely LOX, ADH, and AAT, through RT-PCR analysis. Bioinformatic analyses were also conducted to predict the subcellular localization and functional properties of these proteins. We hypothesize that both the composition of volatile compounds and the expression levels of LOX, ADH, and AAT genes vary significantly across fruit developmental stages, correlating with changes in aroma profiles during ripening.

## 2 MATERIALS AND METHOD

### 2.1 PLANTS COLLECTION

Strawberry plants (Albion cultivar) were cultivated in a greenhouse (60-75 % humidity) under light and temperature conditions (16 hours of light and 8 hours of darkness at 25-27 °C). Water and nutrient solutions were provided directly to the plant and growth was carried out under controlled conditions (without stress). Then the fruits were harvested in different stages of development (green, white, and red stages). After collection, fruits were frozen at -80 °C for molecular studies.

### 2.2 ANALYSIS OF VOLATILE COMPOUNDS DURING STRAWBERRY FRUIT DEVELOPMENT

Volatile compounds from strawberry fruits at different developmental stages were analyzed using the Headspace Solid Phase Micro-Extraction (HS-SPME) method, following the protocol described by Kafkaz et al. (2005) with modifications. For each stage (green, white, and red), 10 g of fresh fruit were collected, immediately ground to a fine consistency, and placed into a sealed 20 ml glass vial. A silica fiber coated with polydimethylsiloxane/divinylbenzene (PDMS/DVB) was then inserted into the vial's headspace and exposed to the sample volatiles for 30 minutes at 65 °C to allow adsorption of compounds onto the fiber. After extraction, the fiber was immediately transferred to the injection port of the Gas Chromatography-Mass Spectrometry (GC-MS) system for thermal desorption.

GC-MS analysis was performed using an HP-5MS capillary column (30 m length × 0.25 mm inner diameter). Helium was used as the carrier gas at a constant flow rate of 1 ml min$^{-1}$. The oven temperature program began at 50 °C (held for 1 minute), then ramped to 200 °C at 4 °C per minute, followed by a 2-minute hold. The injector and detector temperatures were set at 280 °C. Mass spectra were recorded with an HP 5989A detector, scanning from m/z 40 to 400. Volatile compounds were identified by comparing their mass spectra and retention times with those in the NIST library database.

### 2.3 THE EXPRESSION OF AAT, ADH, AND LOX GENES DURING STRAWBERRY FRUIT DEVELOPMENT

For molecular investigations, the sequence of AAT KX450225.1, ADH X15588.1, and LOX AJ578035.1 genes in strawberry plants was extracted and then the primer design was done via Oligo Analyzer software (Table 1). Elongation factor 1-alpha gene (EF1) (DAA80492.1) was considered as the reference gene and the stage of receptacle formation was regarded as the control. Extraction of total RNA and reaction of cDNA synthesis of samples were performed using the YektaTajhiz Azma kit. Then the PCR reaction with temperature program 95 °C (10 min), 95 °C (15 S), 60 °C (1min), and 72 °C (15 S) in 40 cycles was performed via CFX96™ Real-Time System Bio-Rad (USA). The ΔΔCt method was used for the statistical analysis of the gene expression obtained in the present research.

### 2.4 BIOINFORMATICS ANALYSIS OF AAT, ADH, AND LOX PROTEINS OF STRAWBERRY FRUIT

To identify LOX (CAE17327.1), ADH (CAA33613.1), and AAT (AAG13130.1) enzymes in strawberry fruit, protein sequences were extracted from the NCBI database. Sequence alignment was performed by Mega7 software. Protein characteristics and possible location of proteins were predicted by ProtParam and LOCtree3 software. Moreover, the ligand binding site, and second and three-dimensional structures of proteins were predicted using COACH, Phyre2, and I-TASSER software, respectively (Faghani et al., 2022).

## 3 RESULTS AND DISCUSSION

### 3.1 IDENTIFICATION OF VOLATILE COMPOUNDS DURING STRAWBERRY FRUIT DEVELOPMENT

GC-MS results showed that 68 volatile compounds were identified in different stages of strawberry development. The number of identified compounds gradually increased during fruit development, which was more in the red stage than in other stages. The 13 compounds identified in the green stage included esters (1 compound), aldehydes (3 compounds), alcohols (3 compounds), terpenoids (2 compounds), alkanes (2 compounds), and other compounds (2 compounds). The most significant compounds in the green stage included trans, 2-hexenal (18.27 %), and myrtenol (1.64 %), which in this stage 68 % of the compounds belonging to aldehydes (Figure 1a). The number of volatile compounds detected in the white stage (Figure 1b) was significantly increased compared to the green stage (30 compounds). Volatile compounds

**Table 1:** The sequence of primers in Real-Time PCR evaluation.

| Target Gene | Forward Sequence (5’-3’) | Reverse Sequence (5'-3') |
|---|---|---|
| LOX | AGTGTGCTTCACCCGATACA | GTCTTCTCAAGTACCCCACCA |
| ADH | AGGAGGGATTGTGGAGAGTG | TTATCCTGAGCAGGTCACACA |
| AAT | ATGAGCGTTACCCCTTGCTT | GCACCCCAGGACTTGAGAAA |
| EF1 | TGAGATGCACCACGAAGCTC | CCAACATTGTCACCAGGAAGT |

in the white stage contained esters (6 compounds), aldehydes (6 compounds), alcohols (6 compounds), terpenoids (4 compounds), alkanes (4 compounds), and other compounds (4 compounds) (Supplementary table 1) (70 % alcohols). The most crucial compounds included 3-hexen-1-ol (15.07 %), linalool (4.04 %), myrtenol (9.21 %), and methyl salicylate (2.63 %).

The most important red phase compounds included ethyl hexanoate (24.98 %), gamma-decalactone (12.8 %), linalool (9.10 %), gammado-decalactone (5.28 %), hexanoic acid, hexyl ester (3.33 %) and trans-2-hexenal (2.89 %) (Supplementary table 1). At this stage, 79 % of the identified compounds belonged to esters (Figure 1c). It should be noted that numerous and diverse volatile compounds were observed in different developmental stages of strawberry fruit. Aldehydes, alcohols, and esters were the most abundant volatile compounds in the green, white, and red stages, respectively.

Volatile compounds result from several chemical changes, including hydroxylation, methylation, oxidation/reduction, and acetylation, which are produced through various biological pathways (El Hadi et al., 2013). It has been reported that the volatile compounds of strawberry fruit contain about 350 compounds (Yan et al., 2018), but the HS-SPME GC-MS results of the present study identified 68 compounds, which gradually increased during fruit development (13 compounds in the green stage, 30 compounds in the white stage and 37 compounds in the red stage). The investigation showed that gamma-decalactone and ethyl hexanoate are the dominant volatile compounds in the ripe fruit of strawberry. Esters often play a role in fruit maturation and ripening, and their presence is difficult to detect in the initial stages of fruit development, but the amount of these compounds increases in ripe fruit, which can be different depending on the species and cultivars (Padilla-Jimenez et al., 2019).

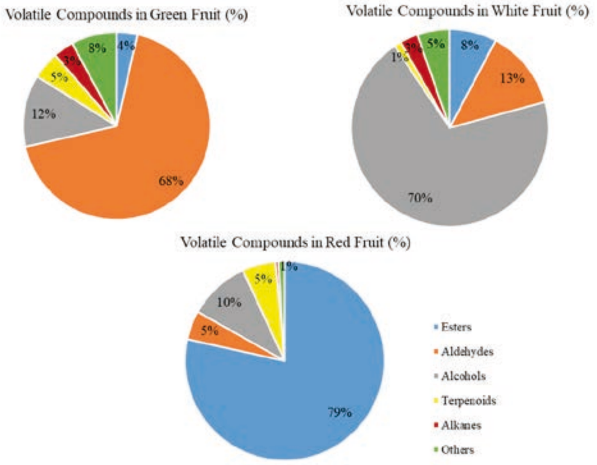

**Figure 1:** Abundant of volatile compounds identified in three stages of strawberry fruit development. a green stage, b white stage, c red stage

In this study, we observed a clear increase in the number and diversity of volatile compounds as strawberry fruit progressed from the green to the red (ripe) stage, with 13, 30, and 37 compounds identified in the green, white, and red stages, respectively. This trend indicates that fruit ripening is associated with enhanced metabolic activity, particularly in pathways responsible for aroma compound biosynthesis. One of the most notable findings was the significant increase in ester production during ripening, with the highest levels detected in the red stage. This is consistent with previous reports that identified esters such as ethyl butanoate, ethyl hexanoate, and 2-methyl butanoate as major contributors to strawberry aroma, accounting for 20 % to 90 % of total volatiles (Yan et al., 2018). Our results also support the findings of Forney et al. (2000), who emphasized the importance of methyl and ethyl esters in defining the characteristic strawberry scent. Interestingly, we found that aldehyde levels were the highest in the green stage, while alcohol content peaked during the white stage. This pattern may reflect the sequential activation of the LOX and ADH pathways during early fruit development.

Carboxylate esters, such as ethyl acetate, are lipophilic molecules that usually have a low odor threshold. They are widely present in many beverages and food products that provide a pleasant aroma and affect its desirable properties. Ethyl esters, including ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, are different in terms of sensory properties and have been identified in apples and pears (Saerens et al., 2010).

The present research showed that aldehydes and alcohols are the second most volatile compounds in strawberry fruit. Aldehydes compounds such as hexanal, decanal, benzaldehyde, benzeneacetaldehyde, nonanal and beta-cyclocitral, trans-2-hexanal were identified in different stages of strawberry development, and the concentration of aldehydes gradually decreased during fruit ripening. Hexenal, trans-2-hexenal, and cis-3-hexenal-1-ol are important volatile compounds in green stages and unripe fruits, which produces a unique aroma (Xu et al., 2017). Investigations showed that E, Z-2,6-nonadienal and E-2-nonanal are considerable volatile compounds in cucumber, which contributing to the taste of cucumber along with ketones and esters (Chen et al., 2015). Moreover, hexanal and trans-2-hexanal aldehydes are the main components of kiwifruit in the unripe stage (Carcia et al., 2013). The amount of aldehydes depends on the cultivar and the degree of immaturity of the fruit, which gradually decreases during the fruit ripening (Kafkaz et al., 2017). The produced aldehydes are converted into alcohols to improve the stability of compounds, and finally, alcohols are consumed as substrates for the production of esters (Lu et al., 2021). Therefore, there is a specific coordination between metabolic pathways during fruit development, so the product of one metabolic reaction can be used as a substrate in another pathway (Preeti et al., 2019).

The alcohols produced in strawberry fruit are benzene methanol, myrtenol, methyl chavicol, 3-hexen-1-ol, benzyl alcohol, linalool, and eugenol. Furthermore, alcohol compounds act as signaling molecules in stress conditions (Aguero et al., 2015). Studies showed that the amount of alcohol was constant during the development of the strawberry-Portola cultivar, while the amount of alcohol decreased significantly during fruit ripening in the Cigaline cultivar (Lu et al., 2020). Moreover, the investigations of blackberry (*Rubus ulmifolius* Schitt) showed that aliphatic alcohols increased during fruit development. Thus, the most abundant volatile compounds in ripe fruit include aldehydes, alcohols, ketones, and terpenoids, which indicates the activation of the biosynthetic pathway of these compounds in the final stages of fruit development (Castro et al., 2023). The current research showed different terpenoid compounds such as 1, 8-cineole, neophytadienen, delta-cadiene, hexadecane epoxide, pulegone, and limonene in different stages of strawberry fruit development. 8 alkanes, 3 ketones, 2 lactones, 1 furan, and 1 phenol were observed in this study, which showed different concentrations in different stages of fruit development. These compounds have a significant effect on characteristics, such as the aroma of strawberries (Kafkas et al., 2017).

### 3.2 THE EXPRESSION OF LOX, ADH AND AAT GENES DURING STRAWBERRY FRUIT DEVELOPMENT

RT-PCR results showed different relative expression patterns for LOX, ADH, and AAT genes in different developmental stages of strawberries (Figure 2). The results indicated that the LOX gene had the highest expression in the green fruit, while the LOX gene expression in the red stage had a significant decrease compared to the previous stages. It appears that as the fruit gets closer to the final stages of its development, the expression of the LOX gene decreases ($p \leq 0.05$) (Figure 2d). ADH and AAT genes showed a significant increase in the relative expression level from the green to the red stage. In other words, the expression levels of ADH and AAT genes increased in the white stage compared to the green stage. The results showed that the expression of ADH and AAT genes gradually increased during fruit development and reached the highest level in the red stage ($p \leq 0.05$) (Figure 2. e, f).

Evaluation of LOX pathway gene expression in different developmental stages of strawberry showed that the LOX gene expression level in the green stage was significantly higher than the other two genes, but expression of LOX gene decreased during fruit development. The findings revealed that the ADH gene had a very low expression level in the green stage, while it reached the highest expression level in the red fruit. Moreover, AAT gene expression increased significantly during strawberry fruit development.

The study of LOX gene expression in apples (Schiller et al., 2015), peaches (Zhang et al., 2010), and kiwifruit (Zhang et al., 2006) showed that the expression of the LOX gene decreased during fruit development, so that the highest expression was observed in the unripe fruit. The analysis of LOX gene expression in pears showed that the expression level was low in the early stages of development, then the expression increased in the later stages. At the stage of fruit ripening, the expression of LOX reached the lowest level according to the changes in aldehydes (Li et al., 2014). Studies have shown that LOX gene suppression completely blocks the biosynthesis pathway in transgenic *Zea ma*ys (Christensen et al., 2023). In addition, LOX gene expression is regulated according to tissue type, developmental stage, phytohormones such as abscisic acid, jasminic acid, salicylic acid, and nitric oxide, and environmental stimuli (injury, water deficit, and pathogen attack) (Chen et al., 2015).

The results demonstrated that the expression of LOX, ADH, and AAT genes varied significantly across strawberry fruit developmental stages, showing a dynamic correlation with the profile of volatile compounds detected by GC-MS. Notably, LOX expression was the highest in the green stage and decreased sharply toward the red stage. This trend aligns with the observed accumulation of aldehydes in the green fruit, suggesting that LOX is actively involved in the early generation of aldehyde volatiles during the initial stages of fruit development. Similar LOX expression patterns have been reported in other fruits, supporting its role in the lipoxygenase pathway for aldehyde biosynthesis (Lu et al., 2018, Iaria et al., 2012). In contrast, ADH and AAT gene expression levels increased progressively during Strawberry fruit development, peaking at the red stage. This pattern closely mirrors the increase in alcohols in the white stage and esters in the red stage, as identified by GC-MS. ADH catalyzes the reduction of aldehydes into alcohols, which serves as a critical step toward ester formation. AAT, which uses alcohol and acyl-CoA substrates to form esters, showed a strong upregulation in the red stage—corresponding

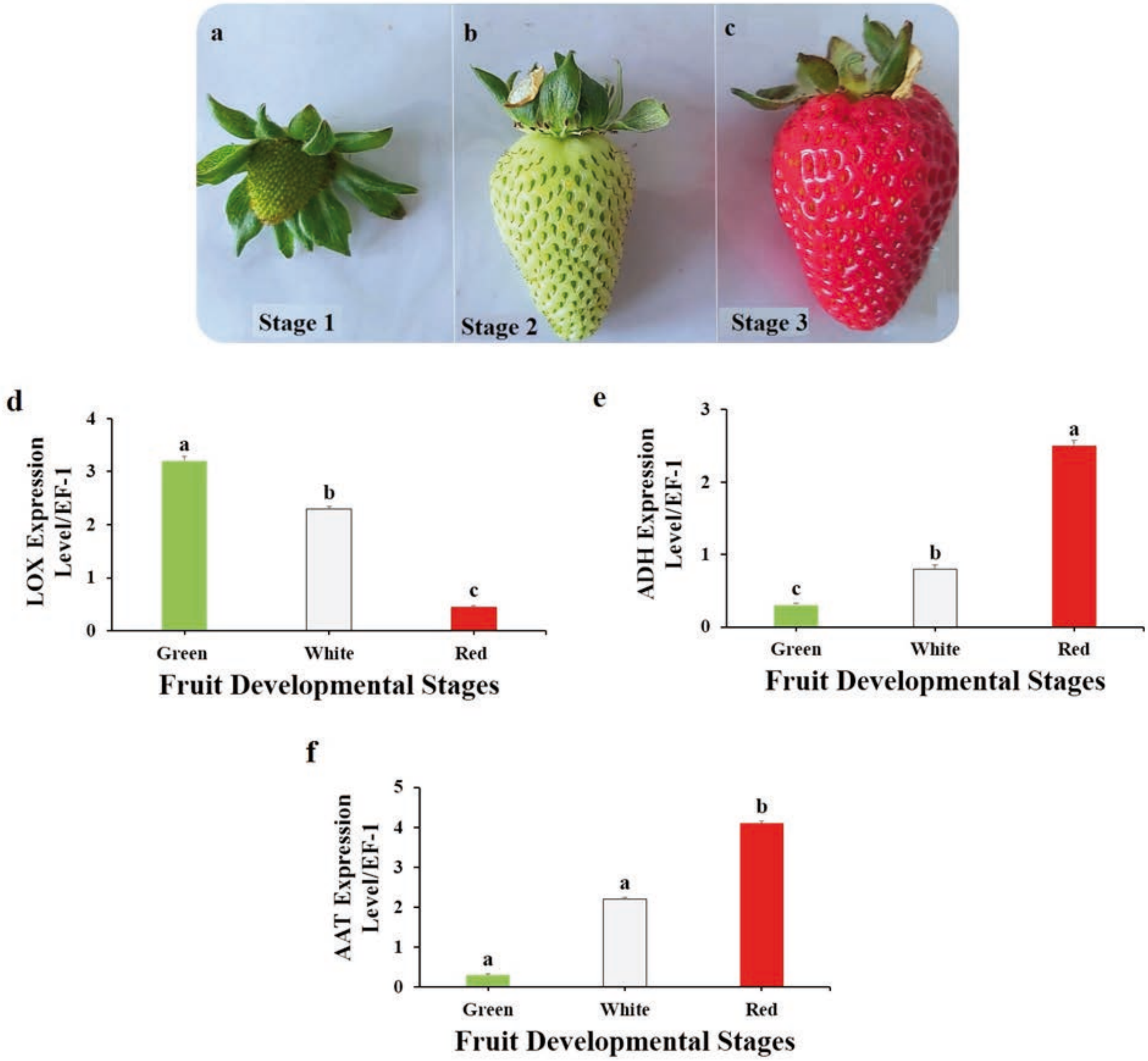

**Figure 2:** The relative expression pattern of genes involved in the biosynthesis of volatile compounds in three developmental stages of strawberry fruit (a green stage, b white stage, and c red stage). d, e, f is the expression of LOX, ADH, and AAT genes, respectively. Different letters in each column indicate significant differences at the $p \leq 0.05$ level

with the highest ester accumulation at fruit ripening. The dependence of ADH expression pattern on developmental stages has also been observed in many fruits, including apricot (Gonzalez-Aguero et al., 2009), pears (Gai-hua et al., 2017), and melons (Jin et al., 2016). The increased accumulation of ADH expression can probably be due to changes in cytoplasmic pH and cytoplasmic ion concentration caused by membrane leakage (Speirs et al., 1998). Investigations indicated that overexpression and silencing of the ADH gene in tomatoes cause a significant change in the content of alcohols (especially hexanol and 3-z-hexanol), respectively (Manriquez et al., 2006). ADH gene expression is induced by the abscisic acid hormone and various environmental stresses such as low temperature, drought, salinity, and mechanical damage (Davik et al., 2013). The transcript level of this gene in corn increased rapidly under oxygen deficiency conditions and the production of alcohols occurred through fermentation and then decreased under anaerobic conditions (Zeng et al., 2020).

The metabolism of esters is controlled by environmental factors (light and temperature), transcription factors, and AAT gene expression during fruit development (Zhou et al., 2021). Studies in bananas (Beekwilder et al., 2004), apples (Li et al., 2006), apricot (Gonzalez-Aguero et al., 2009), papaya (Balboltin et al., 2010), peach (Zhang et al., al., 2010) and pear (Chen et al., 2020) showed that the expression of AAT gene in these fruits starts from the initial stages of fruit development (low expression) and the maximum expression of this gene is observed in the final developmental stages of fruit. Therefore, it can be concluded that the lack of esters production in the initial stages is due to the lack of enzyme activity (Chen et al., 2020). Fruits produce various types of esters, the variety of which depends on the substrate specificity of the relevant enzymes (Liu et al., 2019). AAT enzymes use a variety of alcohol and acyl substrates available to form esters (Perez et al., 2002). Overexpression of AAT in transgenic tobacco plants leads to a significant increase in methyl benzoate concentration, which indicates that methanol and benzoyl-CoA were used as substrates (Li et al., 2008).

The observed increase in AAT gene expression during fruit ripening, particularly in the red stage, suggests that its regulation is tightly linked to developmental and hormonal signals. This upregulation coincides with the highest levels of ester production detected by GC-MS, highlighting AAT's central role in determining the final aroma profile of ripe strawberry fruit. Our findings are consistent with previous reports in apricot and other fruits, where suppression of AAT expression significantly reduced ester biosynthesis (Zhou et al., 2021). The accumulation of esters in the ripening stages may also be influenced by physiological processes such as cell wall degradation, which releases methanol and other alcohols serving as substrates for AAT activity (Beekwilder et al., 2004). This aligns with our observation that alcohol content increases before esters, suggesting a stepwise activation of the volatile biosynthetic pathway.

In addition to substrate availability, the regulation of AAT expression itself appears to be controlled by hormonal signaling and transcription factors. Ethylene and abscisic acid—both known to increase during ripening—are likely contributors to the induction of AAT in the red stage (Ortiz et al., 2010; Cumplido-Laso et al., 2012). Transcription factors such as ERFs and MYBs have also been shown to regulate AAT and other aroma-related genes (Wang et al., 2023). The activation of AAT by ERF overexpression in apples (Li et al., 2020) and the influence of MYBs on aldehyde biosynthesis (Lu et al., 2020) suggest that transcriptional regulation is a critical mechanism behind the coordinated rise of volatiles during ripening. Furthermore, recent evidence suggests that small RNAs such as miRNAs also participate in post-transcriptional control of aroma biosynthetic genes (Singh et al., 2021). While not directly assessed in our work, this layer of regulation may contribute to the fine-tuning of gene expression during fruit development.

### 3.3 BIOINFORMATICS INVESTIGATION OF GENES INVOLVED IN THE BIOSYNTHESIS OF STRAWBERRY FRUIT VOLATILE COMPOUNDS

The coding region of the LOX sequence in the strawberry plant encodes 844 amino acids with a molecular weight of 100,477 Da. The alignment of the LOX protein sequence in the Rosaceae family indicates the similarity and high conservation of the amino acids. The Pfam software has predicted the sequence of this protein as belonging to the Lipoxygenase family, which consists of two domains (PLAT/LH2 and Lipoxygenase). The second structure of strawberry LOX protein consists of 27 % alpha helix and 29 % beta sheets (98 % accuracy). The results of COACH software revealed that LOX protein can bind to fatty acids. The protein binding site with higher C-Score includes amino acids at position 293, 294, 297, 394, 532, 535, 536, 540, 541, 545, 579, 582, 587, 594, 598, 738, 742, 748, 795 and it is 844 (Table 2). The prediction of the intracellular location of LOX protein showed that this protein is located in the chloroplast.

Alignment of the ADH protein sequence in the Rosacea family indicated that this protein is highly conserved among co-family species. This protein belongs to the Zinc-binding dehydrogenase family. The secondary structure of ADH protein consists of 26 % alpha helix, 27 % beta sheets, and 4 % Tm helix. The results have determined that ADH protein can bind to NADH. The protein binding site with a higher C-Score includes amino acids at positions 48, 49, 50, 178, 182, 203, 204, 205, 206, 207, 227, 228, 232, 272, 273, 275, 278, 296, 297, 298, 321, 322, 323 and 373 (Table 2). ADH protein is mainly located in the cytosol and the non-secretory pathway of the cell.

AAT sequence alignment indicated low similarity and conservation in the Rosacea family. The sequence of this protein is predicted from the Transferase family. The secondary structure of the AAT is composed of 31 % alpha helix, 23 % beta sheets, and 4 % TM helix. The 3D structure (Figure 3) is modeled on the most likely organism related to *Sorghum bicolor* (L.) Moench and the transferase family. Moreover, the results showed that AAT interacts with NADH. The binding site with a higher C-Score includes amino acids at positions 36, 37, 38, 157, 305, 376, 402, 406, 407, 408, and 410 (Table 2). Prediction of the intracellular location showed that AAT is located in the cytosol and the non-secretory pathway.

**Figure 3:** Three-dimensional protein structure predicted by I-TASSER software, a LOX protein, b ADH protein, c AAT protein.

Bioinformatics analysis showed that the LOX enzyme belongs to the family of nonheme iron-containing dioxygenases and are found in plants, animals, and fungi (Viswanath et al., 2020). Plant LOX sequence has a highly conserved catalytic site, lipoxygenase domain (C-terminal), and PLAT/LH2 motif (N-terminal), which cooperating with the lipid bilayer. The catalytic site region has several conserved histidine amino acids that play a role in ligand binding (Wang et al., 2019). The studies of different plants showed that the cellular location of LOX was observed in the chloroplast and cytoplasm, probably due to the chromosomal duplication and evolution of this gene (Guo et al., 2017). Several MYC and MYB motifs were identified in the LOX of plants, and these regulatory elements of the promoter region play an essential role in regulating gene expression in stress conditions (Liu et al., 2020). Investigations presented that LOX is coded by multiple gene families and is active in different cell organelles (Hou et al., 2015). Based on the primary structure and sequence similarity, plant LOX13 is classified into two subfamilies (type I and type II) where type I lacks a temporary peptide (Kang et al., 2021). Exon and intron studies indicated that LOX has 6-9 introns and different exon lengths. This property can probably be due to the loss or gain of introns during evolution, which has created a specific functional role for LOX (Liu et al., 2020).

The ADH enzyme belongs to the large family of dehydrogenases/reductases and plays a crucial role in converting aldehydes to alcohols during fruit ripening. It is a glycoprotein with diverse physiological roles, and

**Table 2:** Protein characteristics that predicted by I-TASSER and COACH software

| protein | PDB Hit | Organism | Ligand | C-score | Z-score | Lig binding site |
|---|---|---|---|---|---|---|
| LOX | 1HSS | *Triticum aestivum* L. alpha-amylase | POL | 0.9 | 2.9 | 31,71,111,153,187,189,190,192,193,214,216,217,233,295,296,303 |
| | 3WN6 | *Oryza sativa* L. alpha-amylase | POL | 0.9 | 2.6 | 71, 154,187,189,190,193,214,217,233, 296 |
| | 1HSS | *Triticum aestivum* alpha-amylase | POL | 0.8 | 2.5 | 111,154, 189192,193,214,216,217,233,295 |
| ADH | 1YP4 | *Solanum lycopersicum* L. | GLC | 0.9 | 2.6 | 71,111,154,187,189,190,192,193,214,216,217,233,295,296 |
| | 1YP4 | *Solanum lycopersicum* | GLC | 0.8 | 2.5 | 71,111,154,187,189,190,192,193,214,216,217,233,295,296 |
| | 1YP3 | *Solanum lycopersicum* | GLC | 0.8 | 2.5 | 71,111,154,157,189,190,214,216 |
| AAT | 1YP4 | *Solanum lycopersicum* | GLC | 0.9 | 3.0 | 93,94,95,96,110,111,174,184,185,186,187,190,210,211,212,213,248,249,264,265,296,323,345 |
| | 1YP4 | *Solanum lycopersicum* | GLC | 0.9 | 2. 9 | 93,94,95,96,110,111,174,184,185,186,187,190,210,211,212,213 |
| | 1YP3 | *Solanum lycopersicum* | GLC | 0.8 | 2.8 | 93,94,95,96,110,111,174,184,185,186,187,210,211,212 |

it is classified into three major classes—Class I, II, and III—based on differences in structure, function, and cofactor specificity (such as NAD$^+$ or NADP$^+$) (Jornvall et al., 1995). Class I ADHs are the most common in plants and are primarily involved in fermentative pathways and aroma-related metabolism. Class II and III ADHs are typically associated with detoxification processes or more specialized metabolic functions. Structurally, ADH proteins are composed of four conserved subdomains or cores—A, B, C, and D—which together form the active enzyme. Core A typically contains binding sites for cofactors like NAD$^+$/NADP$^+$. Core B includes the catalytic zinc-binding site. Core C and D contribute to substrate binding and overall protein stability.

All the amino acids participating in the coreA were placed in the catalytic domain (50 % neutral amino acids and 33 % hydrophobic). Core C is a significant functional unit surrounded by four cysteine amino acids (Goihberg et al., 2007). Studies indicated that this enzyme contains zinc-binding (206-340 amino acids), NADPH-binding, and GroES-like (163-36 amino acids) domains in plants (Hayward 2004). Phylogenetic studies determined that zinc-binding domains in the ADH protein of each plant family have about 80 % similarities, which are known as GHE(X)2G(X)5G(X)2V pattern (Elleuche et al., 2014). ADH enzymes have 8-10 exons and 7-9 introns, which were changed during evolution to adapt to environmental changes. Furthermore, the cellular location of this protein was identified in the cytoplasm of different plants (Borras et al., 2014).

AAT enzyme belongs to acyltransferases family, commonly known as BAHD (Bontpart et al., 2015). The proteins belonging to this family have several common motifs, such as the HXXXD motif, which is highly conserved in higher plants and yeasts and can play a role in the catalytic mechanism (D'Auria, 2006; Molina and Kosma, 2015). The conserved sequence DFGWG is located near the C-terminal and maintains the structural integrity of the enzyme (El-Sharkawy et al., 2005). LXXYYPLAGR is the third conserved motif (less conservation compared to other motifs) which is located at the N-terminal of the sequence and is used in acyltransferases involved in the synthesis of fruits esters (Balbontin et al., 2010). The phylogenetic analysis determined that the AAT enzyme is classified into five clades where species with common motifs, such as the HXXXD domain and DFGWG, are placed in one clade (Tuominen et al., 2011).

## 4 CONCLUSIONS

This study analyzed volatile compound profiles and gene expression patterns during three developmental stages of strawberry fruit (green, white, and red). GC-MS results revealed a progressive increase in the number of volatile compounds, with esters peaking in the red stage. Aldehyde levels were the highest in green fruit, while alcohols peaked in the white stage. Gene expression analysis showed that **LOX** was highly expressed in the green stage, while **ADH** and **AAT** were significantly upregulated in the red stage. These expression patterns corresponded with changes in volatile compound composition. Bioinformatic predictions suggested that LOX, ADH, and AAT proteins localize to the chloroplast, cytosol, and non-secretory pathways, respectively. Our integrated analysis of gene expression and metabolite profiling provides insight into the temporal coordination of key enzymes involved in flavor development in strawberry. These data not only confirm findings from other fruit systems but also highlight the potential of manipulating LOX, ADH, and AAT expression to enhance fruit aroma quality in breeding programs. This study enhances understanding of aroma biosynthesis during strawberry ripening and provides insights valuable for improving fruit flavor and potential applications in food, pharmaceutical, and fragrance industries.

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# Confirmation of top cross hybrids in guava using morpho-molecular markers

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**Confirmation of top cross hybrids in guava using morpho-molecular markers**

**Abstract:** The study was conducted to confirm the genetic diversity and hybridity of seventeen guava progenies developed from top-crossing between genetically distinct green and purple guava varieties. Morphological, biochemical, and molecular markers effectively identified hybrids exhibiting phenotypes from both parents. Moreover, remarkable genetic diversity was revealed among these segregants. Biplot analysis demonstrated a strong positive relationship between: (1) chlorophyll and anthocyanin content, (2) leaf length-to-width ratio, (3) leaf area, and (4) petiole length, identifying G15 and G16 genotypes as superior top-cross hybrids. A set of 10 simple sequence repeat (SSR) markers identified 36 alleles with a mean of 3.6 alleles per primer. The polymorphism percentage was 80.83 %, with pairwise dissimilarity ranging from 0.071 to 0.357. Four SSR primers (mPgCIR03, mPgCIR08, mPgCIR11, and mPgCIR19) specifically confirmed the top-cross hybrid status of G6, G8, G9, G10, G15, and G16 genotypes. These diverse genetic resources will be maintained for homozygous plant production through selfing and subsequent guava improvement programs.

**Key Words:** genetic diversity, top cross, polymorphism, dissimilarity index, molecular marker, segregates

**Določitev najboljših križancev gvajave z morfološkimi in molekularnimi markerji**

**Izvleček:** Namen raziskave je bil potrditi genetsko raznolikost in hibridnost sedemnajstih potomcev gvajave pridobljenih s križanjem genetsko različnih zelenih in škrlatnih sort. Morfološki, biokemični in molekularni markerji so potrdili, da izražajo križanci fenotipe obeh staršev, pri čemer je bila med njimi ugotovljena opazna genetska raznolikost. Biplotna analiza je pokazala močne pozitivne povezave med lastnostmi kot so: (1) vsebnost klorofila in antocianov, (2) razmerje med dolžino in širino listov, (3) v listni površini in (4) dolžini listnih pecljev. Pri tem sta bila genotipa G15 in G16 prepoznana kot najboljša križanca. Z naborom 10 markerjev enostavnih ponavljajočih se zaporedij (SSR) je bilo določenih 36 alelov, s poprečjem 3,6 alela na marker. Odstotek polimorfizma je bil 80,83 %, parna različnost je bila med 0,071 in 0,357. Štirje SSR primerji (mPgCIR03, mPgCIR08, mPgCIR11 in mPgCIR19) so še posebej potrdili najboljše križance med genotipi kot so G6, G8, G9, G10, G15 in G16. Ta raznolik genetskih vir bo vzdrževan za vzgojo homozigotnih rastlin preko samoopraševanja v bodočih programih žlatnenja gvave.

**Ključne besede:** genetska raznolikost, vrhunski križanci, polimorfizem, indeks različnosti, molekularni marker, segregacija

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## 1 INTRODUCTION

Guava (*Psidium guajava* L., 2n = 22), a member of the Myrtaceae family, is widely cultivated in tropical and subtropical regions worldwide for its fleshy fruits (Grattapaglia et al., 2012; Morton, 1987). The genus *Psidium* comprises approximately 150 species, of which only 20 produce edible fruits (Jitendra et al., 2017). Due to its wide adaptability, nutritional value, and medicinal importance, guava has gained global popularity as a profitable crop (Medina & Herrero, 2016).

Although guava is well grown in almost all parts of Bangladesh, little attention has been paid to varietal improvement. Only four released varieties are available, while different obsolete varieties like Swarupkathi, Kanchannagar, Mukundapuri, Alahabad, Strawberry guava are still under cultivation at the farmers' level. Therefore, we assumed that a new guava variety could be developed with the introgression among the widespread varieties with desirable traits. Because of its vegetative propagation means facilitates the genetic purity of the successive generations. Guava is an allogamous fruit crop and self-pollination has been recorded to the extent of about 80 %. Singh (2007) reported that self-pollination in guava varies between 35–60 %, depending on the variety. For instance, 'Allahabad Safeda' shows a 50–60 % fruit set through self-pollination, while other varieties like 'Red Flesh' can achieve higher success rates (up to 70–80 %) under optimal conditions (Pommer & Murakami, 2009). However, 35 % natural cross-pollination occurs that creates the opportunity to develop a heterozygous population with an adequate genetic variation for selecting desirable commercial improved variety (Purseglove, 1968).

The determination of genetic diversity and hybridity among breeding materials using morphological markers represents a traditional approach that has been successfully employed for decades. However, this method presents significant constraints for breeding strategies, particularly in perennial crops (Chandra et al., 2005). Consequently, molecular markers have emerged as a superior alternative for varietal improvement programs, offering applications at multiple stages: (1) germplasm evaluation at variety or species level (Valdés-Infante et al., 2003; Rodríguez et al., 2004), (2) hybridity estimation (Barbour et al., 2010), (3) trait-specific association mapping (Feria-Romero et al., 2009), and (4) linkage mapping, quantitative trait locus (QTL) identification, and marker-assisted selection (Ritter, 2012).

Among various PCR-based techniques used in horticultural crop improvement, simple sequence repeats (SSRs) have proven particularly reliable for hybrid assessment. Compared to morphological markers, SSRs enable accurate hybrid identification at the seedling stage with greater efficiency, requiring smaller population sizes and shorter evaluation periods to select promising genotypes (Risterucci et al., 2010). In the present study, we employed two phenotypically distinct guava genotypes as parents for top-cross hybridization: a superior white-pulp cultivar (IPSA guava) and a local pink-pulp variety. Subsequent evaluation of phenotypic and genetic variability incorporated both traditional and advanced breeding techniques to identify effective molecular markers for precise genetic diversity assessment and top-cross hybrid confirmation.

## 2 MATERIALS AND METHODS

### 2.1 PLANT MATERIALS

The experiment was conducted using two cultivated varieties of guava and fifteen offspring developed from the top-crossing of these cultivated varieties. The study was carried out in the nursery and experimental field of the Department of Horticulture at Bangabandhu Sheikh Mujibur Rahman Agricultural University between 2019 and 2022. The experimental materials consisted of a top-cross population, which inherited traits from the parent plants and exhibited significant morphological variation. The parental lines included: G1: A purple guava (open-pollinated female) and G2: IPSA guava (purebred male). Additionally, 15 segregants (G3 to G17) were derived from hybridization. The plant materials are described in detail in Table 1.

### 2.2 EXPERIMENT DESIGN

The experiment was laid out in the Randomized Complete Block Design (RCBD) with three replications where 17 genotypes were allocated randomly in each experimental unit as the independent variables. For morphological parameters determination, nine leaves from each genotype were used in one replication and repeated three times. Different morphological markers were used following the guidelines of the International Union for the Protection of New Varieties of Plants (UPOV, 1987) and Alam et al. (2019).

### 2.3 QUALITATIVE CHARACTERS

Fully developed leaves of the fifth and sixth position from the apex of a shoot were selected for the evalua-

**Table 1:** Characteristics feature with the accession number of the parents and the derived segregates exploited in the study

| Sl. No. | Genotypes | Salient feature of the genotypes | Variety /Accession |
|---|---|---|---|
| 1 | G1 (Female) | Purple colored plant having purple colored fruit (both skin and flesh), fruit medium in size with hard seeds and astringency taste | Purple peyara (inferior) |
| 2 | G2 (Male) | Green colored plant having less seeded, sweet and medium size fruit | IPSA peyara (superior) |
| 3 | G3 | Purple plant | F1 Segregates of the crossing between G1 and G2 |
| 4 | G4 | Green plant | |
| 5 | G5 | Purple plant | |
| 6 | G6 | Green plant | |
| 7 | G7 | Green plant | |
| 8 | G8 | Purple plant | |
| 9 | G9 | Green plant | |
| 10 | G10 | Purple plant | |
| 11 | G11 | Green plant | |
| 12 | G12 | Green plant | |
| 13 | G13 | Green plant | |
| 14 | G14 | Green plant | |
| 15 | G15 | Purple plant | |
| 16 | G16 | Purple plant | |
| 17 | G17 | Green plant | |

tion of qualitative phenotypic characters based on the leaf base and apex shape (Alam et al. 2019; UPOV 1987), the color of the leaf, twigs and vein (IBPGR 1993), leaf surface nature (curvature or twisting type) (Methela et al., 2019; Nagar et al., 2018a) and plant habitus (erect or spreading type) features (Patel 2006; Sharma et al. 2010; Nagar et al., 2018b). All the characters were observed critically in the eye estimation and expressed in descriptive traits.

## 2.4 QUANTITATIVE CHARACTERS

The quantitative data of each plant of the parents and F1 generations were recorded based on the leaf length, leaf width and petiole length with the help of digital slide calipers and expressed as centimeters (Shiva et al., 2017). The leaf area (cm$^2$) was measured in leaf length and width, and average data was used to compare the studied accessions. The leaf blade length to width was calculated by the average length of the leaf blade divided by the average width of the respective leaf blade for randomly selected four leaves of each genotype.

## 2.5 BIOCHEMICAL ASSESSMENT

### 2.5.1 Total chlorophyll

Chlorophyll content was estimated by the SPAD chlorophyll fluorescence and acetone extraction procedure. A portable chlorophyll meter (SPAD-502 Plus, Minolta Corporation, Ltd., Osaka, Japan) was used to measure the leaf chlorophyll concentration as a rational unit. Measurements were made at a central point on the leaflet between the midrib and the leaf margin of 5th and 6th leaf from the top (Colla et al. 2013). Six random measurements per plant were taken and averaged to a single SPAD value for each treatment. Chlorophyll was extracted from 200 mg of leaf samples in 10 ml of acetone (80 % acetone), and the supernatant was made up to the final volume of 25 ml and preserved in dark condition for 24 hours. The absorbance was recorded at 663 and 645 nm using a UV visible spectrophotometer. Total chlorophyll was estimated using the following formula and expressed as mg/g FW (Khan et al. 2017).

$$Chl\ a = [12.7\ (A_{663}) - 2.69\ (A_{645})]\ [V/(1000 \times M)]$$
$$Chl\ b = [22.9\ (A_{645}) - 4.68\ (A_{663})]\ [V/(1000 \times M)]$$

$TChl\ (mg/g\ FM) = Chla + Chlb$

Where, Chl= Chlorophyll, V= Volume, M= Mass and TChl= Total chlorophyll

### 2.5.2 Anthocyanin

Fresh leaf (100 mg) was used for anthocyanin extraction following Chu et al. (2013) procedure with some modifications. The leaf sample was homogenized in 3 ml of acidic ethanol (1 % HCl w/v) on an ice base and the extracted sample was incubated at 4 °C for 1 hour on the shaker with moderate shaking mode. The suspension was centrifuged with 14,000 rpm at 4 °C for 5 minutes for clarified suspension and this suspension was used for further absorbance analysis. The absorption was measured with a UV- visible spectrophotometer at 530 nm and 657 nm wavelength.

Quantification of anthocyanin was performed using the following equation:

$$Q_{Anthocyanin} = (A_{530} - 0.25 \times A_{657}) \times M^{-1}$$

Here, $Q_{\text{Anthocyanin}}$ indicated the amount of anthocyanin,

$A_{530}$ and $A_{657}$ were the absorptions at the indicated wavelengths and M was the mass of the plant material used for extraction (g).

## 2.6 MOLECULAR CHARACTERIZATION

### 2.6.1 Materials for molecular characterization

For molecular characterization, we analyzed 12 distinct guava genotypes selected from an initial pool of 17, excluding five F1 progenies that exhibited close morphological resemblance to their parental lines. The genetic diversity assessment employed ten carefully selected SSR markers that demonstrated precise amplification across 10 F1 progenies and two parent plants. These markers were chosen based on their proven reproducibility, clear scorable banding patterns (150-320 bp), and prior validation in guava (*Psidium guajava*) as reported by Rodríguez et al. (2007) and Kareem et al. (2018). The selected primers generated distinct polymorphic profiles suitable for genetic differentiation, showed optimal amplification efficiency, and specifically targeted known guava loci.

### 2.6.2 Isolation of genomic DNA

The genomic DNA from the actively growing young, fresh and healthy leaves of the selected 12 genotypes was extracted following the modified CTAB (Cetyl Trimethylammonium Bromide) method (Chakrabarti et al. 2006). 150 mg of leaf materials were cut into small pieces and kept inside the mortar. Then some sand and 700 µl of DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2 % CTAB solution (w/v), 0.2 % (v/v) 2-Mercaptoethanol) was added and crushed with pestle. Crushed materials were transferred to a 1.5 ml microcentrifuge tube followed by adding 700 µl of DNA extraction buffer. This sample was incubated at 65 ℃ for one hour in a shaker with gentle shaking after thoroughly mixing by vortex mixture for 30 sec afterward, centrifuged for 15 minutes at 15000 rpm. The supernatant of 700 µl was transferred to the microcentrifuge tube, and then added 4 µl of RNase and kept 45 minutes at 37 °C. Then 700 µl of Chloroform: Isoamyl alcohol (24: 1, v/v) was added to the sample and mixed thoroughly in gentle mode. Spinning this sample at 12000 rpm for 10 minutes with the addition of and a 2/3$^{rd}$ volume isopropanol for spinning down the DNA pellet and supernatant was discarded carefully. The pellets were washed with 70 % ethanol and again spinned out at 10000 rpm for 10 minutes. After drying the pellets were dissolved in 100 µl of sterile water and stored at -20℃ until the PCR analysis.

### 2.6.3 PCR analysis for genotypes selection

Ten SSR primers were used to amplify the DNA sample of 12 genotypes. PCR was conducted in 25 of reaction volume for each reaction and total 12 reactions were done for each 10 SSR primers. The PCR reaction mixture contained template DNA (20 ng), 1 µM forward and 1 µM reverse primers, 200 µM of dNTPs, and 10X PCR buffer, 0.1 U Taq DNA polymerase and $MgCl_2$ solution (1.5 mM). The optimization of conditions was made separately for each marker. PCR was carried out in the thermal cycler with an initial denaturation at 94 °C for 5 min; denaturation at 94 °C for 30 sec; primer annealing at 55 °C for 45 sec; extension at 72 °C for 2 min and final extension at 72 °C for 4 min.

All amplifications were confirmed after running PCR product (10 µl) on agarose gels (1.5%). An 8 µl ladder (100 bp) with 2 µl loading dye was used for comparison. After staining with ethidium bromide (EtBr) gel was visualized with the gel documentation system.

### 2.6.4 Data analysis

Principal component analysis (PCA) was done using the R-statistical program to distinguish $F_1$ segregates with respective parents according to their morphological features. Marker (SSR) based data were analyzed, and Roger's genetic distance matrices were calculated between each pair of lines using DARwin software 6.0 (Perrier and Jacquemoud-Collet 2016).

## 3 RESULTS

### 3.1 MORPHOLOGICAL CHARACTERIZATION

#### 3.1.1 Qualitative characters

Morphological qualitative traits exhibited distinct visual differences between parents and their F1 segregants (Table 2, Figure 1). The female parent (G1) displayed oblanceolate leaf shapes, while the male parent (G2) showed elliptical leaves. Among the 15 segregants, we observed various leaf shapes including oblong, elliptical, lanceolate, oblong-to-elliptical, and ovate forms. While both parents shared an obtuse leaf base shape, their segregants exhibited deviations including cordate and rounded bases. Similar variation occurred in leaf apex shapes, with F1 progenies showing apiculate, rounded, and acute forms compared to the parents' obtuse apices. The male parent G2 exhibited leaf twisting and midrib curvature - traits absent in female parent G1 - with intermediate variations appearing among their segregants. Leaf surface texture varied from smooth to rough (Table 2). Ventral surface analysis (Figure 1a) revealed three texture types: smooth, moderately smooth, and rough. Dorsal surfaces (Figure 1b) showed moderately smooth textures only in G2 and G11, with rough textures in all other genotypes. Branching pattern attitudes varied between parents and segregating progenies (Table 2). Spreading growth habits characterized genotypes G1, G4-G5, G7-G8, and G11-G14, while the remaining genotypes exhibited erect growth forms.

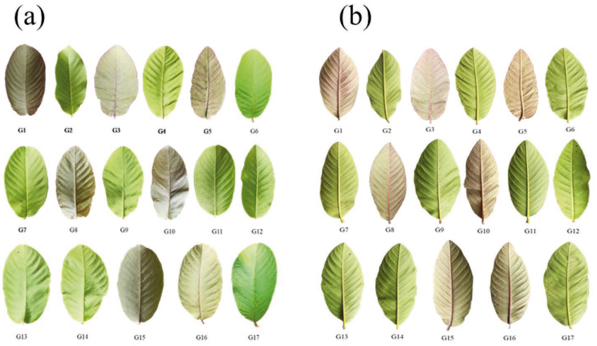

**Figure 1:** Shape and color of fully developed leaf (a) ventral surface (b) dorsal surface in different guava genotypes.

Though the female (G1) had greyed dark purple and male (G2) had light green color fully matured leaves, their pogenies showed different leaf colors viz. yellowish green, light green, green, maroon dark purple and greenish-purple. Similarly, G1 had dark red and G2 had light green twigs color while dark red, brownish red, yellowish-green, light green, light green with red streak, green with red streaks and reddish-green were observed among the segregates. Considerable variation for leaf vein color viz. red, dark red, reddish green and green was also noticed among the segregates, whereas G1 had red color leaf vein and G2 had green color leaf vein. Stem color variation viz. reddish brown, greenish brown, brown was found among the segregating progenies through their two parents such as G1 had dark reddish brown and G2 had greenish brown stem.

#### 3.1.2 Quantitative characters

Morphological data on five quantitative traits were showed significant variation among two parent guava lines and their 15 segregates (Table 3). Though, both the parents viz. G1 (9.98 cm) and G2 (9.8 cm) had almost similar fully developed leaf lengths, the segregates showed a slight variation. Among the progenies, the highest leaf length was found in G14 (11.80 cm) and the lowest in G8 (7.85 cm). The highest leaf width was found in G14 (6.70 cm) and the lowest was in G2 (3.93 cm). Although the parents G1 and G2 had identical petiole length (0.75 cm) but remarkable variations were observed among the progenies where G14 (0.88 cm) had the highest and G8 (0.38 cm) had the lowest petiole length. Similarly, the maximum leaf area was observed in G14 (79.57 cm$^2$) and the minimum in G8 (35.27 cm$^2$). The highest leaf length width ratio was identified in G2 (4.37) but the lowest was in G6 (1.63)

### 3.2 BIOCHEMICAL ANALYSIS

#### 3.2.1 Chlorophyll content

The total chlorophyll content estimated by the SPAD meter was statistically identical in both the parents and their progenies (Table 4). However, the highest chlorophyll content (%) was measured in G10 (45.20) and the lowest in G11 (35.45). On the other hand, Chla is almost similar in two parents, viz. G1

**Table 2:** Morphological characteristics of guava

| Genotype | Fully developed leaf shape | Leaf base shape | Leaf apex shape | Fully developed leaf twisting | Curvature in midrib | Leaf surface nature | | Tree habit | Fully developed leaf color | Twig color | Leaf vein color | Stem color |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Ventral surface | Dorsal surface | | | | | |
| G1 | Oblanceolate | Obtuse | Obtuse | Absent | Absent | Smooth | Rough | Spreading | Greyed dark purple | Dark red | Red | Dark reddish brown |
| G2 | Elliptical | Obtuse | Obtuse | Present | Present | Smooth | Moderately smooth | Erect | Light green | Light green | Green | Greenish brown |
| G3 | Ovate | Cordate | Obtuse | Absent | Absent | Smooth | Rough | Erect | Maroon purple | Dark red | Dark red | Reddish brown |
| G4 | Elliptical | Rounded | Apiculate | Absent | Present | Moderately smooth | Rough | Spreading | Yellowish green | Green with red streaks | Reddish green | Greenish brown |
| G5 | Lanceolate | Cordate | Acute | Present | Present | Moderately smooth | Rough | Spreading | Maroon purple | Dark red | Dark red | Reddish brown |
| G6 | Oblong | Cordate | Obtuse | Absent | Absent | Smooth | Rough | Erect | Yellowish green | Light green with red streak | Green | Greenish brown |
| G7 | Oblong | Rounded | Rounded | Present | Present | Smooth | Rough | Spreading | Light green | Light green | Green | Reddish brown |
| G8 | Oblong | Cordate | Acute | Present | Absent | Smooth | Rough | Spreading | Greenish purple | Brownish red | Red | Reddish brown |
| G9 | Elliptical | Obtuse | Rounded | Present | Absent | Smooth | Rough | Erect | Yellowish green | Light green with red streak | Reddish green | brown |
| G10 | Oblong | Cordate | Obtuse | Present | Absent | Rough | Rough | Erect | Greenish purple | Brownish red | Dark red | Reddish brown |
| G11 | Ovate | Rounded | Apiculate | Absent | Absent | Smooth | Moderately smooth | Spreading | Green | Light green with red streak | Green | Brown |
| G12 | Lanceolate | Cordate | Acute | Present | Absent | Smooth | Rough | Spreading | Green | Light green with red streak | Green | Greenish brown |
| G13 | Elliptical | Obtuse | Rounded | Absent | Present | Smooth | Rough | Spreading | Light green | Light green with red streak | Reddish Green | Greenish brown |
| G14 | Oblong to elliptical | Obtuse | Obtuse | Present | Absent | Moderately smooth | Rough | Spreading | Yellowish green | Light green with red streak | Green | Greenish brown |
| G15 | Oblong | Rounded | Acute | Present | Absent | Smooth | Rough | Erect | Maroon purple | Reddish green | Dark red | Reddish brown |
| G16 | Oblong | Rounded | Acute | Present | Absent | Smooth | Rough | Erect | Dark purple | Dark red | Red | Reddish brown |
| G17 | Ovate | Rounded | Obtuse | Present | Present | Smooth | Rough | Spreading | Light green | Yellowish green | Green | Greenish brown |

(1.10 mg $g^{-1}$ FM) and G2 (1.14 mg $g^{-1}$ FM) but remarkable variations were found among the segregates (Table 4). The highest Chla was determined in G16 (1.46 mg $g^{-1}$ FM); which was at par with G10 and G15and the lowest in G13 (0.86 mg $g^{-1}$ FM). Meanwhile, both the parent showed differences in Chlb content denoted as G1 (0.56 mg $g^{-1}$ FM) and G2 (0.45 mg $g^{-1}$ FM). Consequently, wide variation was observed among the segregates Depicted as G16 (0.74 mg $g^{-1}$ FM) had maximum and G13 (0.37 mg $g^{-1}$FM) had minimum Chlb content. Similar trends of result in the TChl content were observed in the parents G1 (1.65 mg $g^{-1}$ FM) and G2 (1.59 mg $g^{-1}$ FM) and the progenies of G16 (1.65 mg $g^{-1}$ FM) had maximum and G13 (1.24 mg $g^{-1}$ FM) had minimum TChl content.

### 3.2.2 Anthocyanin content

The results depict that anthocyanin content was varied significantly between the parents where purple parent G1 and the green parent G2 were showed about 14.03 mg $g^{-1}$ FM and 1.86 mg $g^{-1}$ FM, respectively (Figure 2a). So a large variation was found among the segregates where G3 (17.89 mg $g^{-1}$ FM) had maximum anthocyanin, which was at par with G16, G1, G15, G8, G5, and all these genotypes had a different shade of purple leaves. On the contrary, G6 (0.54 mg $g^{-1}$ FM) had minimum anthocyanin, which was statistically similar with the genotypes G4, G11, G14, G17, G13, G2, G9, G7, G12, and all of those had a different shade of green leaves (Figure 2b).

Morpho-biochemical characteristics variation visualize in Biplot of PCA analysis - The biplot (Figure 3) displayed 68.4 % of the total variation observed (PC1 in Dim1 = 46.5 % and PC2 in Dim2 = 21.9 %) in the standardized data of the 17 genotypes for the studied eight morpho-biochemical traits. This biplot was visualized from two perspectives (Yan and Reid, 2008), showing a strong positive correlation among Chla, TChl, Chlb, ChlSPAD and Anth traits due to having an acute angle and covered 46.5% of the variation (PC1). On the other hand, Anth and LWR; LA and PL also had a strong positive correlation that covered 21.9 % of the variation (PC2).

In addition, biplot analysis showed the genotypes' trait profiles, especially those positioned far away from the origin and correlation among the traits. Therefore, the scatter plot helped select genotypes for the yield contributing traits or traits that helped in better qualitative performance. In the present biplot visualization after loading variations by PC1 and PC2, it was evident that genotypes G10 and G12 had better performance for a higher percentage of SPAD value;

**Table 3:** Variation in leaf length, leaf width, petiole length, leaf area and leaf length width ratio among the guava genotypes

| Genotype | Leaf length (cm) | Leaf width (cm) | Petiole length (cm) | Leaf area (cm2) | Leaf length width ratio |
|---|---|---|---|---|---|
| G1 | 9.98 ± 0.40ab | 5.18 ± 0.29abcd | 0.75 ± 0.10ab | 51.71 ± 4.88ab | 1.93 ± 0.04a |
| G2 | 9.8 ± 0.71ab | 3.93 ± 2.02d | 0.75 ± 0.06ab | 37.83 ± 19.38b | 4.37 ± 4.86a |
| G3 | 8.72 ± 2.46ab | 4.53 ± 1.65abcd | 0.53 ± 0.10def | 42.52 ± 25.50ab | 1.98 ± 0.18a |
| G4 | 9.33 ± 1.03ab | 4.90 ± 0.35abcd | 0.65 ± 0.06bcd | 45.91 ± 7.65ab | 1.90 ± 0.13a |
| G5 | 8.80 ± 2.11ab | 4.48 ± 1.01bcd | 0.40 ± 0.00ef | 40.93 ± 17.93b | 1.96 ± 0.11a |
| G6 | 10.73 ± 1.21ab | 6.60 ± 0.81ab | 0.70 ± 0.08bc | 71.49 ± 16.50ab | 1.63 ± 0.06a |
| G7 | 10.60 ± 0.84ab | 5.68 ± 0.64abcd | 0.50 ± 0.00def | 60.41 ± 10.86ab | 1.88 ± 0.17a |
| G8 | 7.85 ± 2.84b | 4.30 ± 0.81cd | 0.38 ± 0.05f | 35.27 ± 18.05b | 1.79 ± 0.44a |
| G9 | 10.80 ± 0.84ab | 5.92 ± 0.25abcd | 0.55 ± 0.10cde | 64.12 ± 7.18ab | 1.82 ± 0.10a |
| G10 | 9.98 ± 1.97ab | 4.95 ± 1.01abcd | 0.53 ± 0.05def | 50.74 ± 20.31ab | 2.02 ± 0.17a |
| G11 | 9.98 ± 1.97ab | 4.95 ± 1.01abcd | 0.53 ± 0.05def | 50.74 ± 20.31ab | 2.02 ± 0.17a |
| G12 | 8.90 ± 0.28ab | 4.88 ± 0.68abcd | 0.40 ± 0.00ef | 43.48 ± 7.10ab | 1.85 ± 0.24a |
| G13 | 10.73 ± 1.74ab | 5.68 ± 0.88abcd | 0.63 ± 0.05bcd | 61.96 ± 18.57ab | 1.89 ± 0.10a |
| G14 | 11.80 ± 1.06 a | 6.70 ± 0.65a | 0.88 ± 0.05a | 79.57 ± 14.67a | 1.76 ± 0.03a |
| G15 | 9.23 ± 0.40ab | 5.65 ± 0.48abcd | 0.55 ± 0.06cde | 52.19 ± 5.99ab | 1.64 ± 0.13a |
| G16 | 10.85 ± 2.71ab | 5.45 ± 1.86abcd | 0.53 ± 0.05def | 62.86 ± 34.62ab | 2.04 ± 0.22a |
| G17 | 10.70 ± 2.08ab | 6.30 ± 0.76abc | 0.75 ± 0.06ab | 68.57 ± 21.15ab | 1.69 ± 0.14a |
| LSD0.05 | 3.70 | 2.19 | 0.16 | 37.93 | 3.08 |

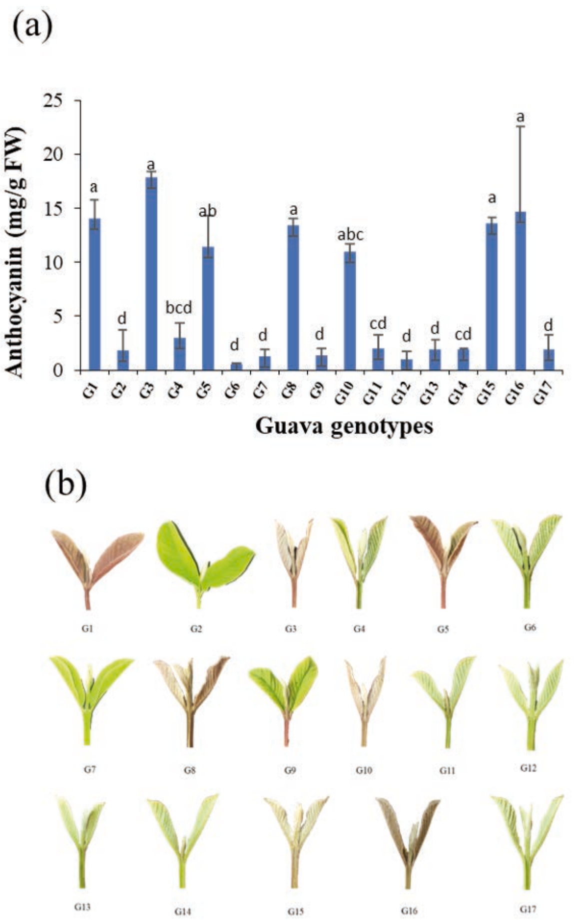

**Figure 2:** Anthocyanin pigment content (a) and variation in twig color due to having different level of anthocyanin (b) among 17 guava genotypes.

G16 could be a better choice for the maximum TChl; G16, G15, G10 were superior for a higher amount of Chla and Chlb content; G3 could be selected for the highest Anth content.

### 3.2.3 Analysis of correlation matrix

The correlation matrix analysis among the different morphological traits (Figure 4a and 4b) revealed that a strong positive correlation was observed between Tchl and Chla (0.99) followed by Tchl and Chlb (0.97). Meanwhile, Chla has a significant correlation with Chlb (0.94). Almost similar correlation matrix was noticed between ChlSPAD and Chla (0.65); ChlSPAD and TChl (0.64) whereas it was 0.59 between ChlSPAD and Chlb (0.59). Furthermore, PL was found correlated with LA (0.58) and Chlb with anthocyanin (0.53).

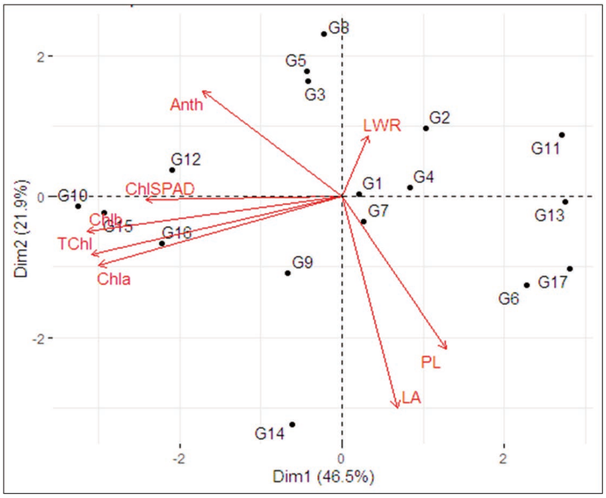

**Figure 3:** Biplot analysis of guava genotypes for morphological and biochemical character association. (LA = Leaf area ($cm^2$); PL = Petiole length (cm); LWR = Leaf length width ratio; Chl a = Chlorophyll a (mg $g^{-1}$ FM); Chl b = chlorophyll b (mg $g^{-1}$ FM); TChl = Total chlorophyll (mg $g^{-1}$ FM); ChlSPAD = SPAD value of chlorophyll (%); Anth =Anthocyanin (mg $g^{-1}$ FM).

## 3.3 MOLECULAR CHARACTERIZATION

### 3.3.1 Level of polymorphism

Different polymorphism levels were observed among all 12 studied guava genotypes using a set of ten SSR primers (Table 5). A total of 36 alleles were generated by SSR markers, with a mean of 3.6 alleles per primer. Among the ten primers, six (mPgCIR02, mPgCIR05, mPgCIR15, mPgCIR17, mPgCIR21, and mPgCIR25) produced both polymorphic and monomorphic bands, while the remaining four primers exhibited exclusively polymorphic banding patterns. All primers were selected from previously characterized guava loci (Rodríguez et al., 2007; Kareem et al., 2018).

In this study, mPgCIR02, mPgCIR03, mPgCIR08, mPgCIR11, and mPgCIR19 showed clear polymorphism, while the remaining primers displayed lower polymorphism levels. Among the SSR markers, mPgCIR03, mPgCIR08, and mPgCIR11 each produced 4 polymorphic bands, and mPgCIR19 yielded 3 polymorphic bands, with no monomorphic alleles detected. These four markers demonstrated 100 % polymorphism, making them strong candidates for varietal improvement programs.

The highest number of alleles was observed with mPgCIR02, which showed 83.33% polymorphism, while the lowest polymorphism (50%) was recorded for mPgCIR17.

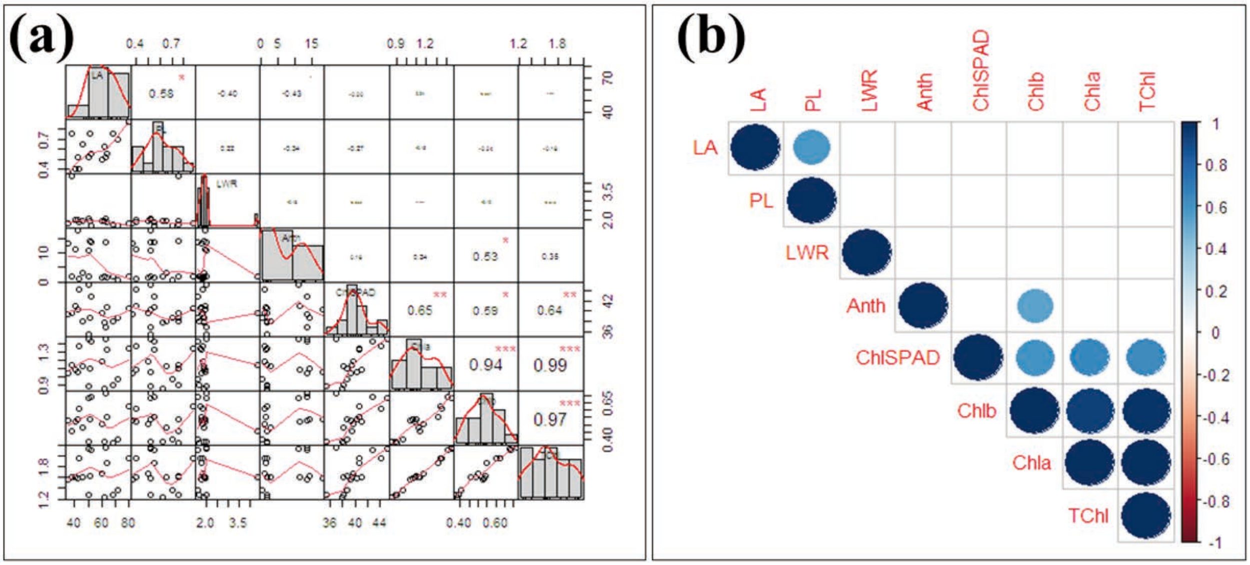

**Figure 4:** Correlation and visualization of guava genotypes. Correlation matrix with significant value among the different variables of 17 guava genotypes (a). Visualization of correlation matrix among the different variables of guava genotypes. (Blank space indicates insignificant correlation. Cyan to blue and cyan to red colors show significant ($p<0.05$) positive and negative correlation between traits respectively) (b). (LA = Leaf area ($cm^2$); PL = Petiole length (cm); LWR = Leaf length width ratio; Chla = Chlorophyll a (mg $g^{-1}$ FM); Chlb = chlorophyll b (mg $g^{-1}$ FM); TChl = Total chlorophyll (mg $g^{-1}$ FM); ChlSPAD = SPAD value of chlorophyll (%); Anth =Anthocyanin (mg $g^{-1}$ FM).

**Table 4:** Variation in Chlorophyll content (SPAD units), Chla, Chlb and TChl in different guava genotypes

| Genotype | Chlorophyll (SPAD units) | Chla (mg $g^{-1}$ FM) | Chlb (mg $g^{-1}$ FM) | TChl (mg $g^{-1}$ FM) |
|---|---|---|---|---|
| G1 | 38.75 ± 1.06 | 1.10 ± 0.004de | 0.56 ± 0.02bcde | 1.65 ± 0.02de |
| G2 | 39.60 ± 2.12 | 1.14 ± 0.002cd | 0.45 ± 0.01ef | 1.59 ± 0.02de |
| G3 | 40.30 ± 1.56 | 1.06 ± 0.004e | 0.53 ± 0.01cde | 1.58 ± 0.02de |
| G4 | 38.10 ± 0.57 | 1.15 ± 0.028cd | 0.47 ± 0.01def | 1.62 ± 0.01de |
| G5 | 41.05 ± 1.34 | 1.08 ± 0.001de | 0.55 ± 0.04bcde | 1.63 ± 0.04de |
| G6 | 38.75 ± 0.35 | 0.95 ± 0.021f | 0.42 ± 0.02f | 1.36 ± 0.00f |
| G7 | 39.70 ± 2.83 | 1.17 ± 0.001c | 0.42 ± 0.02cde | 1.71 ± 0.04d |
| G8 | 39.30 ± 1.56 | 1.04 ± 0.002e | 0.54 ± 0.01cde | 1.57 ± 0.01e |
| G9 | 40.70 ± 1.41 | 1.33 ± 0.021b | 0.56 ± 0.02bcd | 1.90 ± 0.04c |
| G10 | 45.20 ± 4.67 | 1.45 ± 0.014a | 0.65 ± 0.04ab | 2.10 ± 0.05ab |
| G11 | 35.45 ± 4.17 | 0.88 ± 0.007g | 0.37 ± 0.02f | 1.25 ± 0.03fg |
| G12 | 44.25 ± 4.88 | 1.37 ± 0.014b | 0.62 ± 0.02bc | 1.99 ± 0.04bc |
| G13 | 37.90 ± 1.84 | 0.86 ± 0.021g | 0.37 ± 0.01f | 1.24 ± 0.01g |
| G14 | 40.65 ± 1.91 | 1.35 ± 0.014b | 0.65 ± 0.04ab | 1.99 ± 0.05bc |
| G15 | 42.95 ± 5.16 | 1.44 ± 0.028a | 0.65 ± 0.04ab | 2.09 ± 0.02ab |
| G16 | 36.95 ± 10.54 | 1.46 ± 0.021a | 0.74 ± 0.03a | 2.20 ± 0.05a |
| G17 | 38.05 ± 0.64 | 0.87 ± 0.028g | 0.39 ± 0.01f | 1.26 ± 0.01fg |
| LSD0.05 | 14.79 | 0.07 | 0.11 | 0.13 |

### 3.3.2 Polymorphic information

Polymorphic information observed by SSR primers revealed that thirty alleles showed 83.33 % polymorphism among the total alleles and six alleles showed monomorphism of 16.67 %. The overall percentage of polymorphic alleles was 80.83 %. All primers produced specific, effective, and measurable alleles. The amplified alleles ranged from 220-1250 bp (Figure 5; Table 5). A representative image of mPgCIR08 primer showed the allelic difference between the parents and segregates (Figure 5). The average polymorphic information content (PIC) was found 0.576 among the genotypes. The primer mPgCIR03 showed highest (0.693) polymorphic information followed by mPgCIR08 and mPgCIR11. Thus, the primer mPgCIR03, mPgCIR11, and mPgCIR19 were shown effective for the selection of top cross hybrids and genetic diversity study.

Determination of genetic relatedness with dissimilarity matrix - A dissimilarity matrix using ten SSR markers was used to estimate the genetic relatedness of analyzed accessions of guava species. The dissimilarity matrix (Figure 6, Table 5) represented the pair-wise dissimilarity value ranged from 0.071 to 0.357. The lowest value was observed between the G8 and G14 (0.071); thus, these are the closest genotypes. Similarly, a lower value (0.097) was found between the genotypes G5 and G16; 0.103 was found between G6 and G8; 0.111 was found for three pairs of G2 and G9; G4 and G9; G6 and G14. So, it can be said that G5 and G16; G6 and G8; G2 and G9; G4 and G9; G6 and G14 were closer genotype pairs. Contrary, the highest dissimilarity matrix value (0.357) was found among G1 and G15; G6 and G15 genotype pairs indicated these genotypes were not closely

**Table 5:** Polymorphic information of ten SSR markers with their sequences

| Sl No. | Name of primer | Sequences (5'-3') | Observed size (bp) | NA | NPA | PIC | QMA | %PA |
|---|---|---|---|---|---|---|---|---|
| 1 | mPgCIR02 | F: AGTGAACGACTGAAGACC<br>R: ATTACACATTCAGCCACTT | 220-1250 | 6 | 5 | 0.569 | 1 | 83.33 |
| 2 | mPgCIR03 | F: TTGTGGCTTGATTTCC<br>R: TCGTTTAGAGGACATTTCT | 220-800 | 4 | 4 | 0.693 | 0 | 100 |
| 3 | mPgCIR05 | F: GCCTTTGAACCACATC<br>R: TCAATACGAGAGGCAATA | 220-800 | 3 | 2 | 0.567 | 1 | 66.67 |
| 4 | mPgCIR08 | F: ACTTTCGGTCTCAACAAG<br>R: AGGCTTCCTACAAAAGTG | 220-800 | 4 | 4 | 0.676 | 0 | 100 |
| 5 | mPgCIR11 | F: TGAAAGACAACAAACGAG<br>R: TTACACCCACCTAAATAAGA | 220-800 | 4 | 4 | 0.650 | 0 | 100 |
| 6 | mPgCIR15 | F: TCTAATCCCCTGAGTTTC<br>R: CCGATCATCTCTTTCTTT | 240-780 | 3 | 2 | 0.576 | 1 | 66.67 |
| 7 | mPgCIR17 | F: CCTTTCGTCATATTCACTT<br>R: CATTGGATGGTTGACAT | 300-700 | 2 | 1 | 0.393 | 1 | 50 |
| 8 | mPgCIR19 | F: AAAATCCTGAAGACGAAC<br>R: TATCAGAGGCTTGCATTA | 220-800 | 3 | 3 | 0.671 | 0 | 100 |
| 9 | mPgCIR21 | F: TGCCCTTCTAAGTATAACAG<br>R: AGCTACAAACCTTCCTAAA | 300-1250 | 4 | 3 | 0.476 | 1 | 75 |
| 10 | mPgCIR25 | F: GACAATCCAATCTCACTTT<br>R: TGTGTCAAGCATACCTTC | 200-780 | 3 | 2 | 0.546 | 1 | 66.67 |
| Total |  | ------ | ----- | 36 | 30 |  | 06 | 808.34 |
| Percentage/Average* |  | 3.6* | 83.3 | 0.575 | 16.67 | 80.83* |  |  |

NA number of alleles, NPA number of polymorphic alleles, PIC polymorphism information content, QMA quantity of monomorphic alleles, PPA percentage of polymorphic alleles, SSR simple sequence repeat and *indicates the average values of QAA and PPA produced by each primer

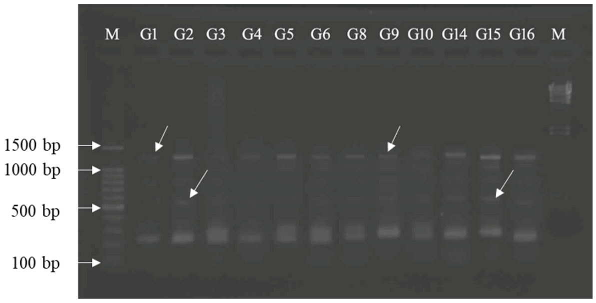

**Figure 5:** Polymorphic profile of primer mPgCIR08 for 12 guava genotypes. Hybridity testing of guava hybrid using the mPgCIR08 SSR marker. M= 100 bp ladder, Lane 2 and 3 indicated the two parent genotypes and lane 4-13 indicated their offspring genotypes viz. lane 3= G3, Lane 4= G4, lane 5= G5, lane 6= G6, lane 7= G8, lane 8= G9, lane 9= G10, lane 10= G14, lane 11= G14, lane 12= G15 and lane 13= G16. Lane 6, 8 and 12 (arrow) represents top cross hybrids (G6, G9 and G15) guava.

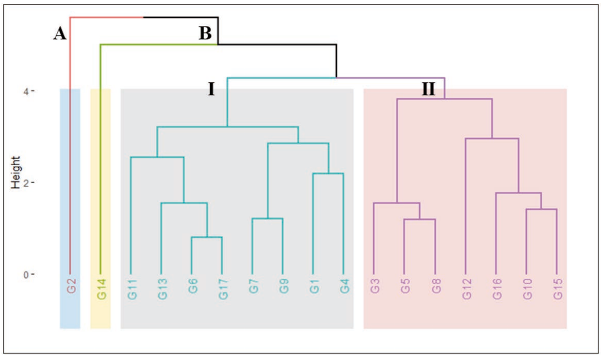

**Figure 6:** Dendrogram showing genetic relationship among 10 segregating guava progenies and their two parent genotypes based on SSR marker analysis.

related. Furthermore, a higher level of dissimilarity was also observed in several genotypes such as G15 and G14 (0.333); G2 and G15 (0.313); G16 and G15 (0.310).

## 4 DISCUSSIONS

The effectiveness of SSR markers for early-stage selection and screening of plants has been well established for assessing genetic diversity and identifying pure hybrids (Maravilla et al., 2017; Dawson et al., 2013; Tuler et al., 2015). In this study, we evaluated twelve guava genotypes using ten SSR primer pairs, among which four primers (mPgCIR03, mPgCIR08, mPgCIR11, and mPgCIR19) demonstrated 100 % polymorphism. These results align with previous findings by Ma et al. (2019), Dinesh et al. (2017), Campos-Rivero et al. (2017), and Urquía et al. (2019), who reported 90-97 % polymorphism using SSR markers for genetic diversity analysis and hybrid confirmation. Notably, Kanupriya et al. (2011) identified 23 microsatellite markers that successfully discriminated among nine guava varieties.

Besides molecular markers, morpho-biochemical markers are helpful for variety identification and are reliable in establishing the genetic relationships across more extensive and diverged accessions of guava (Padilla-Ramirez and Gonzalez-Gaona 2008). In this study morphological traits viz. fully developed leaf shape, leaf base and apex shape, leaf twisting, midrib curvature, leaf surface nature, tree habit, the color of fully developed leaf, twig, leaf vein and stem showed remarkable variations. The variations of leaf characteristics in guava were also reported in some recent studies (Alam et al. 2019; Methela et al. 2019; Nagar et al. 2018a; Nagar et al. 2018b). In an experiment, Dubey et al. (2016) found leaf length ranged from 10.75 cm to 13.95 cm, leaf width from 4.36 cm to 7.08 cm, and leaf area from 65.1 cm$^2$ to 95.71 cm$^2$. The observed leaf width value of this study was well supported by the findings of El-Sisy (2013) who found that leaf width was varied from 4.0 cm to 6.9 cm. El-Sharkawy and Othman (2009) stated that the leaf petiole length of five guava genotypes ranged from 0.84 cm to 0.55 cm. El-Sisy (2013) also reported that leaf area ranged from 30.67 to 88.33 cm$^2$ which were similar with the findings of this study.

Chlorophyll and anthocyanin are the two most essential pigments in leaves (Croft and Chen 2017). Chlorophyll, commonly responsible for green color, is an essential pigment for converting light into chemical energy and the increased synthesis of anthocyanins is the main reason leading to purple coloration (Croft et al. 2017). In this study chlorophyll SPAD value among the parent and their segregates were well supported by the previous study done by Afifi et al. (2019), who found about 35.47 % to 47.47 % chlorophyll content variation among the guava genotypes. In all the case, the Chla content in leaf was found higher than the Chlb. The possible reason could be that Chla is the primary pigment while others, including Chlb are accessory pigments (Srichaikul et al. 2011). Anthocyanin is responsible for the colors (red, purple, and blue) of leaves, stems, roots, flowers and fruits (Khoo et al. 2017) that reflect the color variation among the segregates. It might happen because the parents used in the hybridization process possess different colors with the significant anthocyanin variation that strongly influenced the pigmentation variation of their segregates. Again, biplot analysis of morpho-biochemical traits is considered an efficient way of suitable genotype selection and magnitude of the relationship among the agronomic traits (Farshadfar et al. 2013). Sau et al. (2017)

conducted biplot analysis to identify the principal yield attributes and considerable variations were observed in yield and yield contributing characters. From the biplot and correlation matrix analysis, a strong positive correlation was observed between Tchl and Chla (0.99) followed by Tchl and Chlb (0.97) in the present study that is supported by the findings of Santos et al. (2017).

## 5 CONCLUSIONS

Genetic diversity assessment and top-cross hybrid selection were conducted using morphological, physio-chemical, and molecular markers. Among the SSR primers tested, mPgCIR03, mPgCIR08, mPgCIR11, and mPgCIR19 effectively identified top-cross hybrids derived from the G1 × G2 hybridization scheme. Results revealed that progenies G5, G6, G8, G9, G10, G15, and G16 showed the highest segregation, exhibiting morphological characteristics from both parents. The study demonstrated that morphological variation and anthocyanin pigmentation serve as valuable selection criteria when combined with molecular markers for identifying superior hybrid progenies. These findings provide significant insights for hybridization programs and progeny selection in tropical guava, particularly when based on phenotypic characterization. Furthermore, the developed segregating progenies represent valuable genetic resources that can serve as foundation material for future guava improvement programs targeting desirable traits.

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## 7 CONFLICT OF INTEREST

The authors declare no conflict of interest.

## 8 AUTHOR CONTRIBUTIONS

The funding for the work was received through Md. Sanaullah Biswas. Md. Sanaullah Biswas, Mohammad Sharif Raihan and M. Mizanur Rahman conceptualized the initial work and the planned activities of this work. Masuma Zahan Akhi and Md. Sanaullah Biswas carried out the field experiment and performed the laboratory experiments. Masuma Zahan Akhi and Md. Sanaullah Biswas collection of the data. Masuma Zahan Akhi and Jahidul Hassan organized, analyzed and interpreted the data. Masuma Zahan Akhi and Md. Sanaullah Biswas drafted the initial manuscript. Jahidul Hassan and Mohammad Sharif Raihan carried out the review of the manuscript. Md. Sanaullah Biswas finally reviewed and shaped the manuscript. All the authors read and approved the final draft of the manuscript.

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# Uporaba termografije za spremljanje učinkov manualne terapije v obliki masaže na temperaturo površine nekaterih telesnih regij pri konjih: preliminarna raziskava

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**Uporaba termografije za spremljanje učinkov manualne terapije v obliki masaže na temperaturo površine nekaterih telesnih regij pri konjih: preliminarna raziskava**

**Izvleček:** Namen pilotne raziskave je bil ugotoviti, kako manualna terapija v obliki masaže (v nadaljevanju masaža) vpliva na površinsko temperaturo telesnih regij konja. S tem namenom je bil izveden poskus v treh dnevih, v katerem smo s posnetki termografske kamere Optris PI 400i izmerili temperaturo šestih različnih masiranih telesnih regij. V poskus je bilo vključenih šest toplokrvnih konj, ki so bili nameščeni v individualnih boksih. Meritve znotraj istega dne so bile izvedene: tik pred masažo (0) ter 1, 15, 30 in 60 minut po izvedeni masaži. Najvišjo povprečno temperaturo vseh merjenj smo zabeležili v zadnjem dnevu (31,2 °C), in se je statistično značilno razlikovala od predhodnih dni. Pri analizi posameznih telesnih področij konja je bila zabeležena najnižja povprečna temperatura (29,2 °C) v predelu križa in najvišja (31,7 °C) na področju prsi konja. Tik pred masažo je bila povprečna površinska temperatura značilno najnižja (30,5 °C) in se je značilno razlikovala od temperature po masaži. Trideset minut po masaži je bila površinska temperatura najvišja, potem je začela padati. Potrjene razlike v površinski temperaturi nakazujejo na pomemben fiziološki odziv, ki bi lahko pomagal razložiti poročila o terapevtskih koristih masaže.

**Ključne besede:** konji, manualna terapija, masaža, termografija, površinska telesna temperatura

**The use of thermography for monitoring the effects of manual therapy in the form of massage on the surface temperature of certain body regions in horses: a preliminary study**

**Abstract:** The aim of the pilot study was to determine the effect of manual therapy in the form of massage on the surface temperature of the horse's body areas. An experiment was carried out in three days, in which we measured the temperature in six different massaged body areas using the Optris PI 400i thermographic camera. Six warmblooded horses housed in individual stalls were included. The measurements were taken within the same day in the following time intervals: immediately before the massage (0) and 1, 15, 30 and 60 minutes after the massage. The highest average temperature of all measurements was recorded in the last observed day (31.2 °C) and was statistically significantly different from the two previous days. When analyzing the individual areas of the horse's body, the lowest average temperature (29.2 °C) was recorded in the back (lumbar spine) area and the highest (31.7 °C) in the chest. When analysing the time of measurement, the significantly lowest average temperature was detected before the massage (30.5 °C). Thirty minutes after the massage, the measured temperatures were the highest. The confirmed differences in surface temperature indicate an important physiological response that could help explain reports of therapeutic benefits of massage therapy.

**Key words:** horses, manual therapy, massage, thermography, surface body temperature

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## 1 UVOD

Manualna ali manipulativna terapija se nanaša na prakso v mišično-skeletni fizioterapiji, kjer terapevt uporablja pasivne ali aktivne tehnike manipulacije telesa. Poznamo več vrst manualne terapije, kot so kiropraktika, osteopatija, fizioterapija in masaža (O'Grady in sod., 2024). V naši raziskavi smo se osredotočili na manualno terapijo v obliki masaže. Masaže vključujejo uporabo rok na različnih delih telesa z diagnostičnim ali terapevtskim namenom in se v diagnostične namene uporablja predvsem za ocenjevanje stanja mišic, sklepov in tkiv ter za odkrivanje morebitnih nepravilnosti v telesu (Eddy, 2001). S pomočjo masaže lahko terapevt zazna spremembe v tkivih, kot so napetosti, zatrdline, vozli ali občutljivost, ki lahko nakazujejo na težave, kot so poškodbe, vnetja ali druge motnje. Terapevtski namen masaže je vplivati na procese znotraj živčnega, mišičnega in skeletnega sistema. Učinek različnih oblik masaž je generalno gledano lahko viden na celotnem telesu kot boljše splošno počutje, bolj lokalno pa kot vpliv na percepcijo bolečine, živčno-mišično kontrolo ali na prekrvljenost specifičnih tkiv (Haussler, 2009, Birt in sod., 2015). Na telo masaža vpliva tako na fiziološkem nivoju kot na nivoju obnašanja (Birt in sod., 2015).

Tudi fizioterapevti, specializirani za fizioterapijo živali, pri svojem delu uporabljajo različne terapije in tretmaje, s katerimi predvsem izboljšujejo delovanje živalskega mišično-skeletnega sistema. Osnovne fizioterapevtske obravnave vključujejo masažo, fizioterapijo in kinezioterapijo. Masaža je pogosto uporabljena oblika manualne terapije pri živalih, posebej razširjena je uporaba masaže za zmanjšanje mišične bolečine in spodbujanje mišičnega razvoja (Soroko-Dubrovina in Davies Morel, 2023). Pri konjih se masaža promovira za zmanjšanje anomalij v obnašanju in izboljšanje dobrobiti živali ter za izboljšanje odnosa med konjem in jahačem (McBride in sod., 2004). Masažo pogosto uporabljajo pri dirkalnih in športnih konjih predvsem za spodbujanje okrevanja in regeneracije po vadbi, za izboljšanje prožnosti ter za spodbujanje popolnega okrevanja mišično-skeletnega sistema po poškodbah. Redna masaža konj se priporoča pri živalih, ki imajo stare poškodbe, boleče sklepe ali njihov trening ni izveden pravilno in je preveč obremenjujoč (McBride in sod., 2004; Roberto in Souza, 2020).

Učinke masaže lahko spremljamo z različnimi tehnikami, med katerimi ima velik potencial termografija – neinvazivna slikovna metoda, ki temelji na merjenju površinske temperature in oddane toplote na daljavo (Kruljc, 2023a). Omogoča vizualizacijo porazdelitve toplotnega sevanja na telesni površini, saj beleži infrardeče (IR) sevanje v spektru valovnih dolžin od 0,7 do 1000 µm. Telesna površina oddaja infrardeče sevanje, ki ga termografske kamere zaznavajo in pretvorijo v termogram – barvno sliko temperaturnega polja, kjer različni odtenki ponazarjajo temperaturne razlike (Soroko in sod., 2015). Termografija se uporablja kot diagnostično orodje v veterini in zootehniki, saj omogoča primerjavo površinske temperature telesnih regij in s tem prispeva k diagnostiki poškodb, bolezni (Eddy in sod., 2001; Song in sod., 2007) ter prepoznavanju stresa (Herborn in sod., 2015; Stewart in sod., 2007). Poleg tega se raziskuje njena uporaba pri ocenjevanju čustvenih stanj pri živalih – pri psih so denimo zaznali spremembe temperature kot odziv na različne čustvene dražljaje (Travain in sod., 2015). Čeprav je metodologija ocenjevanja afektivnih stanj s termografijo še vedno v razvoju, je njena uporaba za namene diagnostike v veterini že dobro uveljavljena, zlasti v kombinaciji z drugimi kazalniki bolezni (Pichová in sod., 2023).

Izmenjava toplote med površino konjevega telesa in okoljem igra pomembno vlogo pri toplotnem ravnovesju živali, živalska koža in dlaka pa imata pomembno vlogo pri izmenjavi toplote med telesom živali in okoljem (Scott in Swenson, 2009). Organizem konja večino toplote izgubi skozi kožo s kondukcijo (prevajanje toplote), konvekcijo (prenos toplote), radiacijo (sevanje toplote), izhlapevanjem ter prevajanjem preko dihanja. Telesno temperaturo uravnava centralni regulacijski sistem v hipotalamusu v osrednjem živčevju. Ta center sprejema sporočila toplotnih receptorjev, ki so povsod po telesu, z namenom, da ohranijo telesno temperaturo ali zmanjšajo izgubo toplote. Telesna temperatura pri konjih je običajno uravnavana v ozkih mejah, namreč dnevne in sezonske spremembe telesne temperature ne presegajo ± 1,5 °C. Kljub temu, da se organizem prilagodi na spremembe v temperaturi okolja, lahko količina toplote, ki jo proizvaja, niha v širokih mejah (Boulant, 2000). Minimalne količine toplote se sproščajo pri nujnih, fizioloških procesih, ki ohranjajo življenje, največje količine toplote pa pri dolgotrajnem težkem fizičnem naporu (Hinchcliff in sod., 2008). Številni avtorji poročajo tudi o povišani površinski temperaturi po masaži (Cabak in sod., 2013; Miyaji in sod., 2018). Pri masaži je povišana površinska temperatura lahko posledica direktnega mehanskega pritiska (gnetenja) na kožo, kite, mišice, ligamente, tetive in sklepe. Mehanski pritisk pri masaži lahko povzroča povečanje krvnega pretoka preko povečanja tlaka v arteriolah in tako povzroči povišanje temperature v koži, mišičnem in vezivnem tkivu telesnega predela (Scott in Swenson, 2009; Sefton in sod., 2010). Weerapong in sod. (2005) predvidevajo, da bi lahko povečanje pretoka krvi pospešilo dostavo kisika v mišice in tako povečalo temperaturo mišice, kar potem vodi v boljšo zmogljivost mišice.

Površinska temperatura telesa, izmerjena s termovizijsko kamero, odraža vpliv notranje tvorbe telesne toplote, debeline kože, dolžine dlake ter okoljskih dejavnikov, kot so temperatura zraka, hitrost vetra in zračna vlažnost (Turner in sod., 2001; Soroko in sod., 2014; Westermann in sod., 2013; Kruljc, 2023a). Razumevanje normalnih variacij toplotnih vzorcev kopitarjev je zato ključnega pomena za razumevanje in interpretacijo termogramov (Soroko-Dubrovina in sod., 2024). Termografske kamere lahko zaznajo spremembe temperature, ki se začnejo pri 0,05 °C (Roberto in Souza, 2020), kar je nadvse natančno. S pomočjo omenjene opreme lahko te spremembe tudi zaznamo, kar nam omogoča hitro diagnostiko posameznih sprememb. Študija Soroko in sod. (2017) je preverila vpliv temperature okolja na površinsko temperaturo pri konjih z uporabo termografije. Ugotovili so, da obstaja močna korelacija med izmerjeno temperaturo okolja in absolutno temperaturo sklepov. Še natančneje so podobno tematiko s termografijo preverjali Roy in sod. (2020), in sicer pri meritvah površinske temperature konj zunaj in znotraj hleva. Zaznali so statistično značilne razlike pri meritvah, izvajanih na odprtem (temperaturno območje 19 do 22 °C; razpon vlažnosti 40 do 53 %). Sprememba temperature okolja se je odražala na spremembi izmerjene površinske temperature živali. Pri nižjih okoljskih temperaturah so torej izmerili nižje temperature telesnih območij živali, kar nakazuje na vpliv temperature okolja pri izvajanju tovrstnih meritev zunaj hleva. Ravno nasprotno, pri izvajanju meritev znotraj hleva (temperaturno območje med 20 do 24 °C; razpon vlažnosti 20 do 58 %), niso zaznali vpliva okoljske temperature na površinsko temperaturo. Izmerjene razlike zunaj hleva pojasnjujejo predvsem z načinom zajetja termografske slike, predvsem hladnega ozadja (okoliško ozadje), ki lahko vpliva na termografsko zaznavo, in drugih nastavitev kamere, kar nakazuje v prid meritvam, izvajanim v kontroliranih pogojih znotraj hleva. Prav tako na točnost meritev vpliva tehnika zajemanja meritev, ki jo uporablja operater, vključno z razdaljo do ciljne točke meritve, kotom zajetja posnetka ter gibanjem živali. Žival je redko pri miru. Na kvalitativne ali kvantitativne podatke, ki jih želimo zajeti s termografsko kamero, lahko vplivajo tudi dejavniki, povezani s samo živaljo in njenim individualnim odzivom na okolico, v kateri se nahaja. Ti vključujejo stopnjo aktivnosti pred zajetjem posnetka, fiziološko stanje živali (npr. brejost), dolžino in gostoto dlake ali poškodbo in bolezensko stanje (Roy in sod., 2020). Pravilna uporaba termografije za ovrednotenje površinskih toplotnih vzorcev zahteva torej nadzorovano okolje izvajanja meritev, pri čemer je treba upoštevati temperaturo okolja, čas izvajanja meritve in fiziološko stanje konja, s čimer se zmanjša možnost napak pri interpretaciji rezultatov (Purohit, 2008; Michelotto in sod., 2016).

V naši raziskavi smo uporabili termografijo kot metodo za analiziranje sprememb površinske temperature. S tem smo želeli poglobiti znanje o temeljnih fizioloških mehanizmih, ki se sprožijo med terapijo pri konjih. Spremembe smo spremljali z meritvami površinske temperature na šestih izbranih telesnih regijah (prsni koš spredaj – podprsje, levo pleče, desno pleče, križ, levo in desno stegno) pred in po masaži. Poleg tega smo preverili tehnične zmogljivosti uporabljene opreme za natančno termografsko analizo pri konjih.

## 2 MATERIALI IN METODE

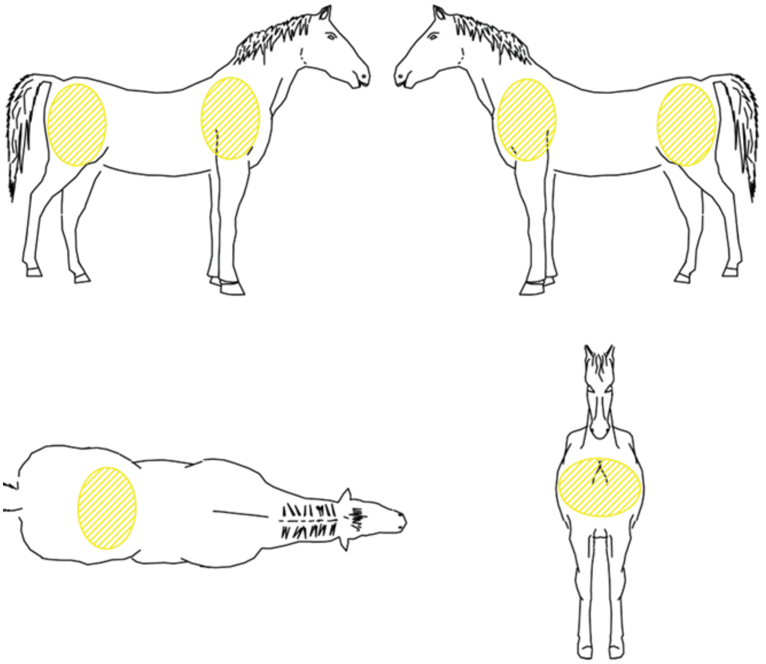
### 2.1 ŽIVALI V POSKUSU

Masažno terapijo smo izvajali na šestih konjih na lokaciji konjeniškega kluba Kasco Svečina. Vzorec je sestavljalo pet kastratov in ena kobila toplokrvnih pasem konj (belgijska, nizozemska in slovenska toplokrvna pasma). Konji so bili stari med 8 in 16 let. Vsi konji so bili del programa šole jahanja, kjer so redno sodelovali pri izvajanju jahalnih vaj. Vsakodnevno so bili vključeni v aktivnosti, tako dan pred kot dan po izvajanju masaže. Na dan masaže niso opravljali fizičnih aktivnosti, s čimer je bil zagotovljen nevtralni vpliv masaže na njihovo fiziološko stanje. Konji so imeli na voljo vodo in seno po volji. Imeli so dostop do pašnika, razen na dneve meritev, ko so bili ves čas trajanja poskusa nastanjeni v svojih boksih.

### 2.2 IZVEDBA POSKUSA

Masaža, ki jo je izvajala izkušena terapevtka, ki je bila konjem nepoznana oseba, je bila izvedena na šestih telesnih področjih konja: levo pleče, prsi spredaj (podprsje), desno pleče, križ, levo in desno stegno (slika 1). Masaža posameznega področja je trajala 4,5 minute, z izjemo podprsja in hrbta, kjer je trajala 9 minut (2 strani × 4,5 min). Takšen časovni okvir masaže smo izbrali, ker sta dela pleče in stegno na levi in desni strani, medtem ko sta regiji podprsje in križ enotni.

Vključitev omenjenih telesnih področij je temeljila na predhodnem posvetovanju s terapevtko, ki ima večletne izkušnje na tem področju, in na obstoječi literaturi (Haussler, 2009; Denoix, 2021). Stegno, podprsje, pleča in hrbet smo izbrali zato, ker gre za glavne gibalne mišice, ki jih konj izdatno uporablja pri delu pod sedlom. Gre za mišice, ki so odgovorne za kompleksne



**Slika 1**: Shematski prikaz masiranih telesnih področij: desno pleče in desno stegno; levo pleče in levo stegno; križ; podprsje (prsi spredaj)
**Figure 1**: Schematic representation of the massaged body areas: right shoulder and right thigh; left shoulder and left thigh; lower back; underbust (chest in front)

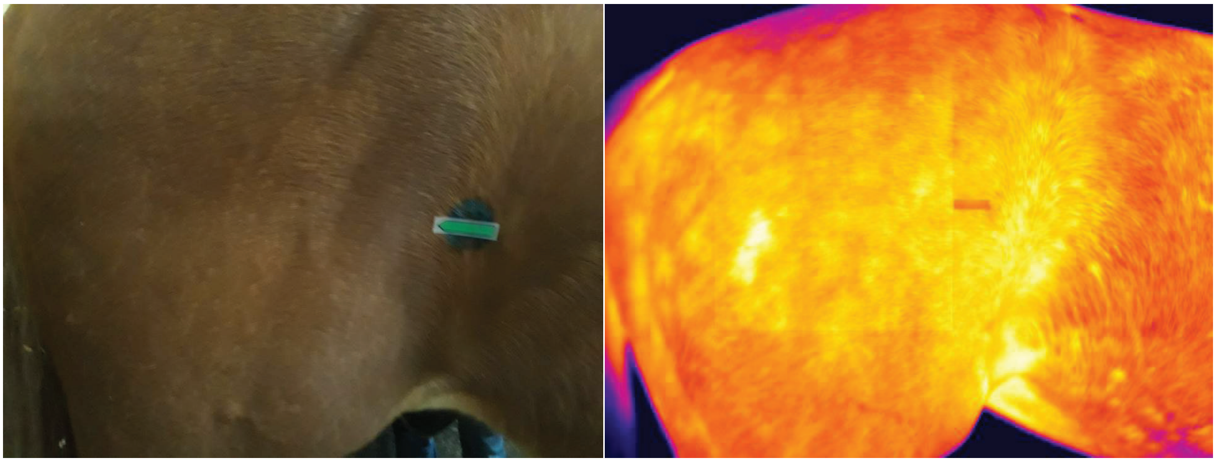
gibalne vzorce – izbrano gibanje, preskakovanje zaprek, menjave galopa v skok itd. (Denoix, 2021).

Zaporedje masaže tudi ni bilo naključno in je bilo izbrano glede na predhodne izkušnje maserke in glede na pridobljene informacije iz znanstvene literature. Za masažo se priporoča, da se izvaja (masira) proti srcu in da se masaža začne pri večjih mišicah in nadaljuje proti manjšim (Kleven in sod., 2019). Pri raziskavi smo se posledično odločili za naslednje zaporedje: levo pleče, podprsje, desno pleče, hrbet, levo stegno, desno stegno. Drug razlog predstavljenega zaporedja je ta, da so konji manj občutljivi na področju pleč kot na primer po hrbtu ali stegnu. Masažo torej začnemo na delu, kjer je konj najmanj občutljiv, s čimer zmanjšamo možnosti za negativno reakcijo (upiranje, grizenje, umikanje …). Najprej torej zmasiramo sprednji del, posledično pa je konj manj občutljiv na področju hrbta in stegen. Sproščanje napetosti na prednjem delu namreč pozitivno vpliva na zadnji del – mišice se med seboj povezujejo v funkcionalne verige. Sproščanje napetosti v enem delu verige posredno sprosti tudi mišice drugje v verigi (Denoix, 2021). Dodaten razlog je praktične narave. Želeli smo, da se maserka čim manjkrat premika iz ene strani konja na drugo – prepogosti premiki lahko namreč motijo konja in razbijejo naravni tok masaže.

Na vseh masiranih delih smo naredili posnetke s kamero tik pred masažo (0) ter 1, 15, 30 in 60 minut po končani terapiji. Postopek masaže smo ponovili v treh dneh (26. 3., 29. 3. in 5. 4. 2019). Na posamezni dan se je masaža začela v jutranjem času ob 8.00 uri in končala ob 14.00 uri. Konji so bili vsak dan obravnavani v enakem zaporedju, pri čemer smo v času terapije zajeli vse potrebne termografske posnetke. Posnetki posameznega konja so bili narejeni v njegovem individualnem boksu. V času izvajanja masaže smo okna v boksih prekrili in tako preprečili padec sončnih žarkov na konjevo telo tekom izvajanja meritev. Okna in sončni žarki bi lahko vplivali na izmerjeno površinsko temperaturo.

Masažo prvega konja smo začeli ob 8:00, pri čemer smo najprej zajeli termografske posnetke vseh obravnavanih telesnih področij. Nato je terapevtka pričela z masažo prvega izbranega področja. Takoj po zaključku masaže smo opravili termografski posnetek obdelanega območja, nato pa je terapevtka nadaljevala z masažo naslednjega telesnega področja. Ta postopek smo ponovili pri vseh obravnavanih telesnih regijah, pri vseh konjih in na vsak dan, pri čemer smo posnetke zajemali v vnaprej določenih časovnih intervalih (1, 15, 30 in 60 minut po masaži).

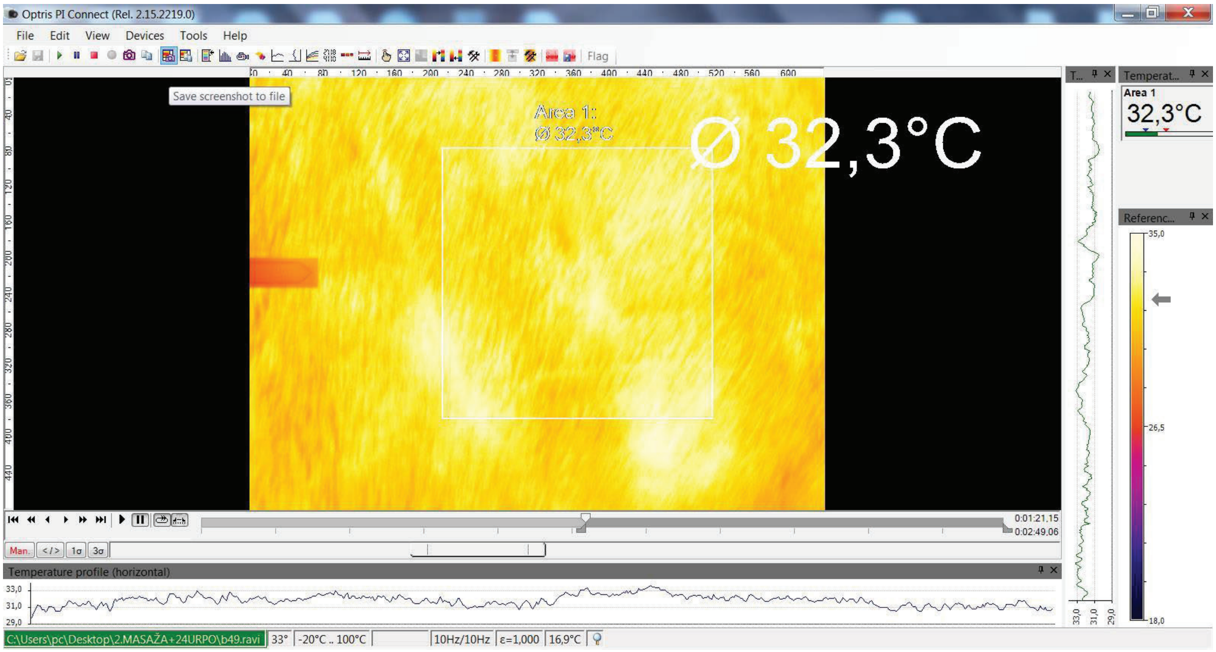
Za natančen zajem termografskih posnetkov in



**Slika 2**: Prikaz snemanja z infrardečo kamero Optris PI 400i. Leva slika prikazuje posnetek z običajno kamero, desna pa posnetek s termokamero. Samolepilna puščica je služila za orientacijo pri zajemanju posnetka (foto: M. Galun)
**Figure 2**: Image acquisition with the Optris PI 400i infrared camera. The left image shows a regular photographic recording and the right image shows a thermal camera recording. The adhesive arrow served as an orientation aid during the recording (photo: M. Galun)

dosledno upoštevanje časovnega protokola so skrbeli trije sodelujoči. Prva oseba je bila usposobljena za rokovanje s termografsko kamero, druga je s pomočjo vnaprej pripravljenega časovnega protokola in štoparice zagotavljala pravilno časovno izvedbo posnetkov, tretja pa je skrbela za ravnanje s konjem, da bi preprečila morebitno vznemirjenost ali premikanje med zajemanjem posnetkov.

Ob posamezni meritvi smo zabeležili tudi podatke o okoljski temperaturi hleva na višini konjeve glave. Povprečne temperature hlevskega zraka so bile naslednje: prvi dan masaže (13,9 °C ± 0,2), drugi dan masaže (13,8 °C ± 0,2) in tretji dan masaže (13,8 °C ± 0,4). Ker razlike v okoljski temperaturi med posameznimi dnevi niso bile dovolj velike, da bi pomembno vplivale na spremembe izmerjene površinske temperature (kot je



C:\Users\pc\Desktop\2.MASAŽA+24URPO\b49.ravi | 33° | -20°C .. 100°C | 10Hz/10Hz | ε=1,000 | 16,9°C

**Slika 3**: Prikaz analize posnetkov v programu *Optris PI Connect®* (foto: M. Galun)
**Figure 3**: Representation of the image analysis in the *Optris PI Connect®* software (photo: M. Galun)

opisano v uvodu; Roy in sod., 2020), korekcije temperature nismo vključili v statistično analizo podatkov.

## 2.3 MERITVE S TERMOKAMERO

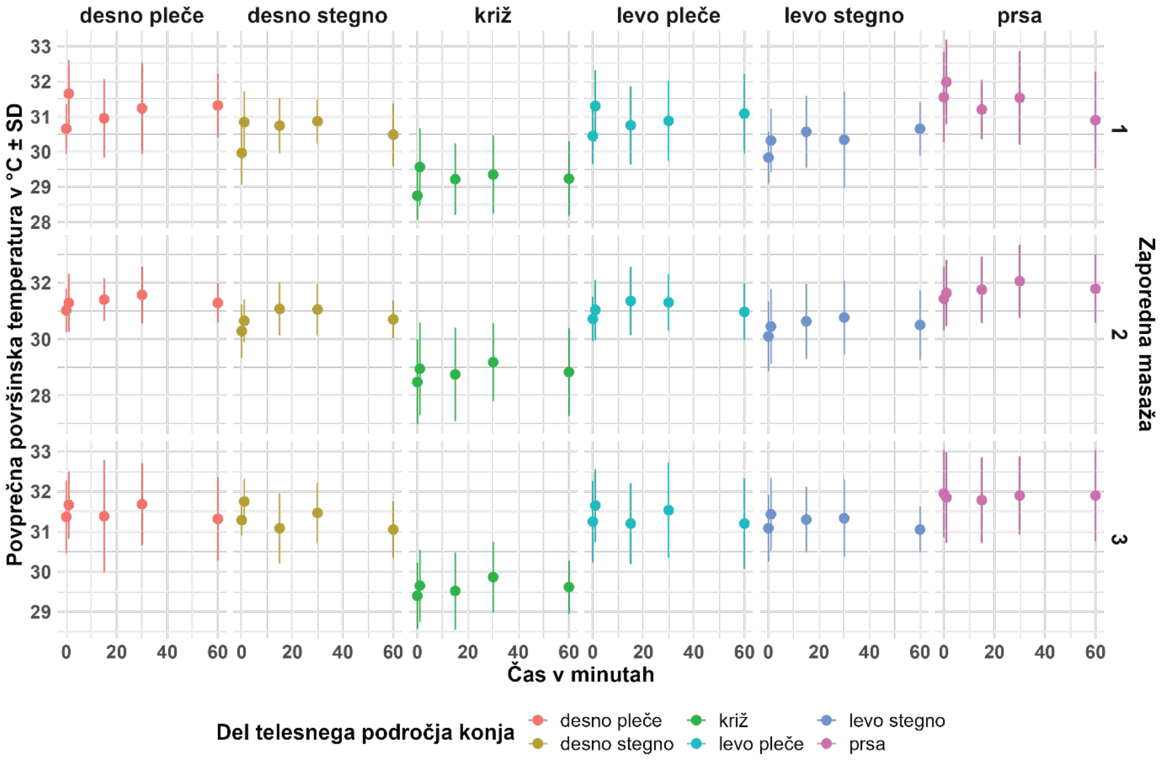
Za izvedbo meritev smo uporabljali infrardečo kamero Optris PI 400i z optično resolucijo 382 × 288 pikslov ter hitrostjo okvirjev 80 Hz, kar nam je omogočilo izvajanje meritev v realnem času in zagotavljalo jasno in natančno sliko. Med opravljanjem meritev (posnetkov) je bila termokamera priključena na prenosni računalnik in postavljena na stativ. Posnetki kamere so se shranili v formatu .tiff. Za analizo termografskih posnetkov smo uporabili program *Optris PI Connect*®.

Posnetke telesnih delov (slika 2) smo nato analizirali s pomočjo originalne programske opreme proizvajalca kamere *(Optris PI Connect*®*)*. Program nam je prikazal maksimalno, minimalno in povprečno temperaturo masiranega dela. Posnetki so bili pridobljeni z razdalje 40 centimetrov in pod kotom 90°. Velikost zajetega polja, v katerem je program izračunaval povprečno temperaturo, je bila 30 × 30 cm (slika 3).

## 2.4 STATISTIČNA ANALIZA

Statistična analiza je bila narejena s programom R, verzija 4.2.3. Za ugotavljanje vpliva raziskovanih dejavnikov (dan – zaporedna masaža, področje telesa in čas merjenja) na odvisno spremenljivko je bil uporabljen mešani linearni model. V model so bili vključeni trije ponovljeni sistemski dejavniki (*repeated measures*) in vse možne njihove interakcije, medtem ko je bil ID konja obravnavan kot naključni učinek (*random effect*). Analiza je bila narejena s paketom lmerTest (Kuznetsova in sod., 2017) z upoštevano Satterthwaitovo aproksimacijo. Za test mnogoterih primerjav smo izbrali Tukeyev test (α = 0,05), ki je bil narejen v paketu emmeans (Lenth in sod., 2018). V mešanem linearnem modelu ni bilo zaznanih statistično značilnih interakcij med dejavniki, kar pomeni, da kombinacije različnih nivojev teh dejavnikov niso imele pomembnega vpliva na odvisno spremenljivko. Zato smo v nadaljevanju interpretirali učinke vsakega dejavnika posebej na merjeno spremenljivko.

Grafični prikaz vključuje povprečne vrednosti (x) skupaj s standardnimi odkloni (sd) za vsa možna



**Slika 4**: Gibanje povprečne površinske temperature posameznega telesnega področja konjev, ločeno po času od izvedbe (v minutah, kjer 0 pomeni merjenje neposredno pred začetkom) in zaporedju masaže

**Figure 4**: The average surface temperature movement of individual body areas of horses, separated by execution time (in minutes, where 0 means the measurement immediately before the start) and massage sequence

obravnavanja (skupaj 90), kar omogoča podrobno vizualizacijo variabilnosti podatkov. Za raziskovane dejavnike (dan, področje telesa in čas merjenja) smo ločeno za vsak nivo dejavnika izračunali mere opisne statistike.

## 3 REZULTATI IN DISKUSIJA

Slika 1 prikazuje rezultate sprememb povprečne temperature šestih konj glede na čas, zaporedno masažo in mesto merjenja. Opaziti je, da se je temperatura telesnih regij minuto po masaži povečala v primerjavi z izhodiščnim stanjem, kar je verjetno posledica povečanega pretoka krvi, stimulacije živčnega sistema in trenja (Scott in Swenson, 2009). V večini primerov sta bili najvišji povprečni vrednosti doseženi minuto ali 30 minut po masaži, medtem ko je bila temperatura 15 minut po masaži nekoliko nižja. Najnižjo povprečno temperaturo, 28,5 °C, smo zabeležili pred drugo zaporedno masažo na križu, najvišjo pa 30 minut po drugi masaži na prsnem predelu, kjer je dosegla 33,8 °C. Vse izmerjene vrednosti so se gibale okoli 30 °C, kar je skladno z rezultati drugih študij (Jodkowska in sod., 2011; Soroko in sod., 2019; Čebulj-Kadunc in sod., 2019).

Nadalje smo z linearnim mešanim modelom ugotovili, da imajo vsi preučevani dejavniki statistično značilen vpliv na površinsko temperaturo konja. Vsi dejavniki so delovali neodvisno drug od drugega, kar pomeni, da med njimi ni bilo značilnih interakcij (preglednica 1).

Da bi preverili vpliv zaporedne masaže na povprečno temperaturo, smo primerjali vse tri zaporedne masaže med seboj. Značilno najvišjo povprečno temperaturo vseh telesnih področij smo zabeležili zadnji dan masiranja (31,2 °C), kjer opazimo postopen dvig povprečne temperature med zaporednimi masažami (preglednica 2). Teh dognanj pri konjih ni mogoče primerjati z literaturo, saj glede na vedenje avtorjev ne obstaja, so pa skladna z ugotovitvami pri ljudeh, kjer so Cabak in sod. (2013) ugotovili, da masaža poviša povprečno površinsko temperaturo, ki je ob vsaki ponovitvi masaže višja. Prav tako Miyaji in sod. (2018) poročajo, da je dolgotrajna ponavljajoča se masaža povzročila višje povprečne temperature masiranih telesnih področij. Omenjena študija domneva, da dolgotrajna masaža poveča vazodilatacijske odzive na masažno stimulacijo, masaža ne samo poveča pretok krvi, ampak tudi izboljša delovanje žil.

Pri analizi časovnega poteka sprememb povprečne temperature masiranih področij je bila temperatura neposredno pred začetkom masaže najnižja in je znašala 30,5 °C (preglednica 2). Najvišjo povprečno temperaturo, 31,0 °C, smo zabeležili 30 minut po masaži, nato pa se je v eni uri znižala na 30,8 °C. Statistična analiza vpliva časa na povprečne temperature posameznih telesnih področij ločeno po dnevu merjenja ni pokazala statistično značilnih razlik in ni vključena v preglednico 2. Razlog za nesignifikantnost je lahko relativno majhen vzorec – vključeval je šest konjev in tri zaporedne masaže. Poleg tega so nihanja površinskih temperatur pri masaži manj izrazita kot pri večjih fizičnih obremenitvah, kot so treningi, kjer literatura poroča o večjih temperaturnih spremembah pred in po aktivnosti (Čebulj-Kadunc in sod., 2022). Salter in sod. (2011) so, podobno kot naša študija, ugotavljali vpliv masaže na površinsko temperaturo treh različnih telesnih področij (regija 1: vrat in prsa, regija 2: ramenski del in hrbet, regija 3: stegna) in času pred in po masaži (5 in 60 minut). Tudi ta študija podaja rezultate povprečnih temperatur vseh masiranih delov skupaj. Ugotavljajo, da se je temperatura po masaži ves čas dvigovala in bila 60 minut po masaži značilno višja v primerjavi s temperaturo pred masažo. Takojšnji dvig temperature po masaži se pripisuje povečanemu pretoku krvi v te predele. Podoben prvotni dvig temperature pol ure po masaži in padec na izhodiščno temperaturo uro po masaži so

**Preglednica 1**: Vpliv zaporedne masaže, masiranega področja konja ter časa izvedene masaže na povprečno površinsko temperaturo
**Table 1**: The effect of sequential massage, the massaged area of the horse, and the time of massage on the average surface temperature

| Analiza variance | Dejavnik | F-statistika | P-vrednost |
|---|---|---|---|
| Glavni učinki dejavnikov | Zaporedna masaža (ZM) | 25,46 | *** |
| | Področje telesa konja (P) | 111,28 | *** |
| | Čas (Č) | 6,57 | *** |
| Interakcije med dejavniki | ZM*Č | 1,23 | ns |
| | P*Č | 0,26 | ns |
| | ZM*P | 1,64 | ns |
| | ZM*P*Č | 0,29 | ns |

ns – ni statistično značilno ($p > 0,05$), *** statistično značilen vpliv ($p \leq 0,001$)

**Preglednica 2**: Površinska temperatura konjev v °C ločeno po zaporedni masaži, področju telesa in času od izvedene masaže
**Table 2**: Surface temperature of horses in °C, separated by successive massages, body area and time after the massage

| Zaporedna masaža | povprečje | SD | min | maks | $q_1$ | mediana | $q_3$ |
|---|---|---|---|---|---|---|---|
| Prva masaža | 30,59$^{a}$ | 1,22 | 27,50 | 33,80 | 29,80 | 30,65 | 31,50 |
| Druga masaža | 30,70$^{a}$ | 1,42 | 27,00 | 33,60 | 29,90 | 30,85 | 31,60 |
| Tretja masaža | 31,16$^{b}$ | 1,12 | 27,90 | 33,80 | 30,40 | 31,20 | 31,90 |
| Področje telesa (P) | povprečje | SD | min | maks | $q_1$ | mediana | $q_3$ |
| prsa | 31,69$^{e}$ | 1,11 | 29,40 | 33,80 | 30,70 | 31,80 | 32,50 |
| križ | 29,22$^{a}$ | 1,14 | 27,00 | 31,70 | 28,20 | 29,40 | 30,10 |
| desno stegno | 30,89$^{bc}$ | 0,84 | 28,80 | 32,70 | 30,38 | 30,90 | 31,50 |
| levo stegno | 30,69$^{b}$ | 1,05 | 28,00 | 32,60 | 30,00 | 30,70 | 31,40 |
| desno pleče | 31,33$^{d}$ | 0,93 | 29,00 | 33,40 | 30,80 | 31,35 | 32,00 |
| levo pleče | 31,11$^{cd}$ | 1,00 | 28,90 | 33,50 | 30,60 | 31,20 | 31,80 |
| Čas v min (Č) | povprečje | SD | min | maks | $q_1$ | mediana | $q_3$ |
| 0 | 30,53$^{a}$ | 1,28 | 27,00 | 33,30 | 29,75 | 30,65 | 31,40 |
| 1 | 30,98$^{b}$ | 1,28 | 27,30 | 33,80 | 30,20 | 31,20 | 31,85 |
| 15 | 30,82$^{b}$ | 1,29 | 27,30 | 33,30 | 30,10 | 30,90 | 31,70 |
| 30 | 31,00$^{b}$ | 1,29 | 27,90 | 33,60 | 30,30 | 30,95 | 31,90 |
| 60 | 30,77$^{ab}$ | 1,22 | 27,40 | 33,80 | 30,00 | 30,90 | 31,65 |

a–e povprečja (± SD) označena z različnimi črkami se med seboj statistično značilno razlikujejo (Tukey, α = 0,05), SD=standardni odklon, min=minimalna vrednost, maks=maksimalna vrednost, $q_1$=prvi kvartil, $q_3$=tretji kvartil

ugotovili tudi v raziskavi Sefton in sod. (2010), in sicer pri ljudeh. Ugotovili so, da je masaža povzročila signifikantne spremembe temperature masiranih telesnih regij. Holey in sod. (2011) so raziskovali učinek masaže hrbta in nog na temperaturo kože pri ljudeh. Na obeh telesnih delih se je temperatura takoj po masaži najprej dvignila in bila signifikantno višja kot pred masažo. Po prvotnemu dvigu se temperatura kože po masaži (15, 30, 45, 60 minut) ni spreminjala, vse naštete meritve pa so bile značilno višje od meritev pred začetkom masaže. Avtorji trdijo, da je kljub majhnemu dvigu površinske temperature dvig klinično pomemben.

Statistično značilno najnižjo povprečno temperaturo 29,2 °C smo izmerili na križu, najvišjo, 31,7 °C, pa na prsnem predelu. Razlike med telesnimi regijami so posledica variacij v debelini kože, velikosti mišic in podkožnem ožilju, pri čemer so višje temperature značilne za predele, kjer so krvne žile bližje površini kože (Head in Dyson, 2001). Podobne ugotovitve navajajo tudi druge študije, ki najvišje temperature poročajo na vratu, prsih in stegnih, najnižje pa v distalnih delih okončin, kjer prevladujejo kite (Simon in sod., 2006; Čebulj-Kadunc in sod., 2020; Stamberov in sod., 2023; Jodkowska in Dudek, 2000). Višja temperatura v bolj omišičenih telesnih regijah je posledica intenzivnejše presnovne aktivnosti in večje proizvodnje toplote v teh mišicah (Soroko-Dubrovina in Morel, 2023). Tudi Stamberov in sod. (2023) so najvišje temperature zabeležili na vratu, prsih in stegnih, najnižje pa na križu in distalnih delih okončin, kar potrjuje naše rezultate. Mišice prsi, plečet ter stegen so močno obremenjene pri jahalnih konjih. Zaradi obremenitev so zato te mišice pogosto zakrčene, kar na dolgi rok poslabšuje konjevo atletsko sposobnost. Masaža teh mišic odpravlja napetost in izboljšuje sposobnost konjevega gibalnega aparata. Ker te mišice spadajo med največje v konjevem telesu, pospeševanje prekrvavitve v teh mišicah pozitivno vpliva na celoten mišičen sistem (Haussler, 2009).

Naši rezultati kažejo na simetrijo temperature med obema stranema telesa (stegno, pleča), kar je skladno z ugotovitvami prejšnjih študij. Le te so ugotovile, da porazdelitev temperature med simetričnimi telesnimi regijami kaže visoko stopnjo podobnosti (Selfe in sod., 2008; Soroko in sod., 2017, Čebulj-Kadunc in sod., 2019, Čebulj-Kadunc in sod., 2022, Stamberov in sod., 2023).

## 4 SKLEP

V naši raziskavi smo na vzorcu šestih konj proučevali vpliv manualne terapije na površinsko temperaturo šestih masiranih telesnih področij konja (podprsje, levo in desno pleče, hrbet, levo in desno stegno). Rezultati kažejo, da je najhladnejše masirano območje križ, najtoplejše pa podprsje, pri čemer na površinsko temperaturo vplivata tako čas po masaži kot tudi zaporedna masaža. Statistično potrjene razlike v temperaturi kažejo na pomemben fiziološki odziv, ki podpira dosedanja poročila o terapevtskih koristih masaže.

V študiji smo se soočili z nekaterimi omejitvami. Vzorec živali je bil majhen, konji pa so bili različnih pasem, kar lahko vpliva na izmerjene površinske temperature zaradi razlik v dolžini in gostoti dlake. V prihodnjih raziskavah bi bilo smiselno povečati število preiskovanih živali in standardizirati pasemsko sestavo. Prav tako bi bilo koristno vključiti dodatne parametre, kot so meritve srčnega utripa pred in po masaži, kar bi omogočilo celovitejšo analizo fiziološkega odziva. Kljub tem omejitvam je naša preliminarna raziskava pokazala možne vplive masaže na površinsko temperaturo ter opozorila na dejavnike, ki jih je treba upoštevati pri nadaljnjih študijah. Naše ugotovitve potrjujejo uporabnost termografije kot dragocenega orodja za ocenjevanje učinkov masaže. Termografija ima velik potencial kot zanesljiva, varna in uporabniku prijazna metoda za hitro ter natančno kvantitativno analizo sprememb površinske temperature pri konjih po masaži.

## 5 ZAHVALA

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# Hranilna in senzorična ustreznost celodnevnih obrokov v domu starejših občanov

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**Hranilna in senzorična ustreznost celodnevnih obrokov v domu starejših občanov**

**Izvleček:** V Sloveniji se kot starejši odrasli smatrajo osebe stare 65 let in več. Študije so pokazale, da je staranje povezano s številnimi fiziološkimi spremembami, vključno z zmanjšanjem energijskega in hranilnega vnosa, zaradi česar je ustrezna prehrana starejših odraslih bistvena pri preprečevanju podhranjenosti. Namen raziskave je bil ovrednotiti energijsko in hranilno vrednost ponujenih in zaužitih obrokov v domu za starejše občane z orodjem za vrednotenje prehrane na osnovi referenčnih podatkov o sestavi živil in s kemijskimi analizami ter preveriti skladnost sestave obrokov z aktualnimi priporočili in potrebami udeležencev. Glede na celokupne srednje vrednosti, je večina sodelujočih v raziskavi s celodnevnimi zaužitimi obroki dosegala priporočene dnevne energijske vnose, določene glede na spol, starost in ocenjeno raven telesne dejavnosti. Rezultati so pokazali, da je odstotek energije iz ogljikovih hidratov v celodnevnih zaužitih obrokih premajhen, odstotek energije iz maščob pa prevelik. Odstotek energije iz beljakovin je bil sicer skladen s priporočili, vendar pri večini udeležencev premajhen glede na njihovo telesno maso. Pri udeležencih smo preverili tudi všečnost ponujenih kosil in večerij ter njihove ocene primerjali z ocenami strokovnega panela. Tako vključeni starejši odrasli kot člani strokovnega panela so na 5-stopenjskih lestvicah dodelili visoke povprečne ocene za senzorično sprejemljivost obrokov.

**Ključne besede:** prehrana starejših odraslih, dom starejših občanov, energijska vrednost, hranilna vrednost, senzorična sprejemljivost

**Nutritional and sensory adequacy of all-day meals in a nursing home**

**Abstract:** In Slovenia, older adults are defined as individuals aged 65 and over. Studies have shown that aging is associated with several physiological changes, including a decrease in energy and nutrient intake, making adequate nutrition crucial to prevent malnutrition. This study aimed to evaluate the energy and nutritional value of meals offered and consumed in a nursing home using the reference food composition data tool for dietary assessment and chemical analyses. Additionally, the study assessed the alignment of meal composition with current dietary recommendations and the specific needs of the participants. Based on overall mean values, most participants met the recommended daily energy intake according to their gender, age, and estimated physical activity level. However, results indicated that the proportion of energy from carbohydrates in the consumed meals was too low, while the proportion from fats was too high. Protein intake met general recommendations but was insufficient relative to body mass for most participants. The study also assessed the acceptability of lunches and dinners, comparing participants' ratings with those of an expert panel. Both residents and experts assigned high average scores on a 5-point scale for sensory acceptability.

**Key words:** nutrition for older adults, nursing home, energy value, nutritional value, sensory acceptability

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## 1 UVOD

Staranje prebivalstva je proces, v katerem se spreminja starostna sestava prebivalstva na način, da se povečuje delež starejših odraslih, kamor v Sloveniji uvrščamo osebe stare 65 let in več (Eurostat, 2019; Filipovič Hrast in Hlebec, 2015). Po najnovejših ocenah portala Statista (2024) je v Evropi delež starejših od 65 let (20 %) že presegel delež mlajših od 15 let (16 %), kar predstavlja velik javnozdravstveni izziv, saj staranje prebivalstva vodi v večjo prevalenco kroničnih bolezni, kognitivni upad, krhkost, anoreksijo ter težave z žvečenjem in požiranjem, kar lahko vpliva na nezadosten energijski in hranilni vnos (Cristea in sod., 2020).

Podhranjenost definiramo kot stanje neuravnoteženega energijskega in/ali hranilnega vnosa glede na potrebe, ki jih ima posameznik, kar vpliva na poslabšanje telesne sestave in indeks telesne mase (ITM) (Cederholm in sod., 2017). Tveganje za podhranjenost se povečuje s starostjo in prisotnostjo kroničnih ali akutnih bolezenskih stanj (Van Wymelbeke in sod., 2020). V primeru, da podhranjenost ni odkrita pravočasno, lahko vodi v šibkost, utrujenost, izgubo telesne mase, slabokrvnost, večjo tveganje za padce in zlome, funkcionalno oviranost, oslabitev imunskega sistema, povečano dovzetnost za bolezni in okužbe, podaljšan čas okrevanja po bolezni in/ali operaciji, zmanjšano kognitivno delovanje, slabše kakovosti življenja in celo smrt (Volkert in sod., 2019b). S starostjo se pojavljajo fiziološke, patofiziološke in socialno-ekonomske spremembe, ki povečujejo tveganje za izgubo telesne mase in podhranjenost pri starejših odraslih (Kaur in sod., 2019; Landi in sod., 2016). S staranjem se zmanjšuje delež funkcionalne mase (mišične in kostne mase) ter kopiči delež maščobnega tkiva, kar povečuje tveganje za prehransko stanje debelosti in razvoj sarkopenije (Tyrovolas in sod., 2015; Pilgrim in sod., 2015; Cederholm in sod., 2017; Lavriša in sod., 2024). Eden glavnih dejavnikov, ki pri starejših odraslih bistveno poveča tveganje za nezadosten energijski vnos in podhranjenost je zmanjšan apetit. Etiologija zmanjšanja apetita pri starejših odraslih je kompleksna in večfaktorska. Vključuje fiziološke vidike, kot so hormonske in presnovne spremembe, upad senzorične zaznave hrane, težav z zobovjem in žvečenjem, upočasnjene peristaltike, ter disfagije. Zajema tudi dejavnike, povezane z zdravstvenim stanjem, kot so kronične bolezni ali akutne okužbe, depresijo, demenco in uporaba zdravil. S starostjo se zmanjšuje tudi občutek žeje, kar povečuje tveganje za dehidracijo in kognitivni upad (Malisova in sod., 2018; Volkert in sod., 2019a). Poleg fizioloških in patofizioloških dejavnikov na prehrano vplivajo tudi psihosocialni dejavniki. Osamljenost zmanjšuje motivacijo za pripravo in uživanje hrane, socialna izolacija ter ekonomske omejitve pa lahko vodijo v neustrezno prehrano (Pilgrim in sod., 2015; Landi in sod., 2016). Omenjeni dejavniki pa dodatno otežujejo tudi vsakodnevna opravila, kot so nakupovanje, kuhanje in hranjenje (Aigbogun in sod., 2017).

Zaradi neenotne uporabe orodij za ugotavljanje prehranske ogroženosti v različnih raziskavah je razširjenost podhranjenosti med starejšimi težko opredeliti, vendar je več študij izpostavilo, da je pojavnost podhranjenosti večja pri starejših odraslih, ki živijo v domovih za starejše občane (DSO) (20-68 %), v primerjavi s tistimi, ki živijo doma (4-10 %) (Kaur in sod., 2019; Landi in sod., 2016; Van Wymelbeke in sod., 2020).

Prvi korak pri preprečevanju podhranjenosti v DSO je redno ugotavljanje prehranske ogroženosti tudi pri prekomerno hranjenih in debelih starejših (NIJZ, 2020b). Priporočila za prehransko obravnavo bolnikov v bolnišnicah in starostnikov v domovih za starejše občane (Cerović in sod., 2008) zato navajajo, da je potrebna redna, enkrat tedenska ocena prehranskega stanja starejših odraslih v DSO s pomočjo presejalnih orodij. Za diagnosticiranje podhranjenosti na podlagi mednarodno sprejetega soglasja se trenutno uporablja vprašalnik GLIM, kjer je potrebna prisotnost vsaj enega fenotipskega kriterija: nenamerna izguba telesne mase, nizek ITM ali zmanjšana mišična masa in vsaj enega etiološkega kriterija: zmanjšan vnos hrane, malabsorpcija ali huda bolezen s prisotnim vnetjem (Jensen in Cederholm, 2018). Tveganje za podhranjenost se poveča, če je energijski vnos izrazito zmanjšan (npr. pod 50 % glede na dnevne potrebe več kot tri dni) ali če so prisotni dejavniki tveganja, ki posledično zmanjšajo vnos hrane ali povečajo energijske in hranilne potrebe (npr. akutne bolezni, nevropsihološke težave, nepokretnost, težave z žvečenjem, težave s požiranjem) (Volkert in sod., 2019a). V primeru pozitivnega rezultata presejalnega testa sledi nadaljnja prehranska obravnava, z željo prepoznati tveganja za nastanek podhranjenosti ali že prisotno podhranjenost, kateri nato sledi posamezniku prilagojen prehranski načrt (Kaur in sod., 2019; Volkert in sod., 2019a).

Pri preprečevanju podhranjenosti pri institucioniranih starejših odraslih je bistvena ustrezna energijska in hranilna sestava ter senzorična ustreznost ponujenih obrokov. Selitev v DSO je pomemben preobrat v življenju starejših odraslih, saj spremeni njihovo fizično in družbeno okolje ter vsakodnevne dejavnosti, vključno s prehranjevalnimi navadami. Stanovalci DSO so le redko vključeni v odločitve v zvezi s hrano in obroki, ker so te običajno vezane na prehranske smernice in organizacijske omejitve, zato jedi v DSO težko izpolnijo pričakovanja in želje vseh stanovalcev. Van Wymelbeke in sod. (2020) so preverjali kako prilagajanje senzoričnih lastnosti in raznolikost jedi vpliva na energijski vnos stanovalcev DSO in ugotovili, da se s temi ukrepi dnevni energijski vnos stanovalcev poveča za 5-7 %. Po ugotovitvah magistrske naloge (Marković, 2017), ki je v raziskavo vključevala 20 domov za starejše v Sloveniji in preučevala splošno zadovoljstvo življenja v DSO, so stanovalci pri intervjujih največkrat izrazili nezadovoljstvo s prehrano. Izpostavili so predvsem nezadovoljstvo z okusom, ponavljanje jedi in okrnjeno izbiro obrokov. Do podobnih ugotovitev so prišli v DSO Sežana (Skupina Fabrika, 2023), kjer so stanovalci izrazili zmerno zadovoljstvo s prehrano in poudarili, da si želijo bolj okusno hrano, več raznolikosti in izbire, več sadja in zelenjave ter manj mesa.

Cilj raziskave je bil ovrednotiti energijsko in hranilno vrednost celodnevnih ponujenih in zaužitih obrokov stanovalcev v DSO v Ljubljani in preveriti skladnost z obstoječimi Priporočili za prehransko obravnavo bolnikov v bolnišnicah in starostnikov v domu starejših občanov (Cerović in sod., 2008), PANGeA prehranskimi priporočili (Rotovnik Kozjek in sod., 2014), Smernicami za izvajanje prehranske oskrbe v domovih za starejše (NIJZ, 2020b) in Referenčnimi vrednostmi za energijski vnos ter vnos hranil (referenčnimi vrednostmi) (NIJZ, 2020a) glede na spol, starost in raven telesne dejavnosti, ki so povzeta po Referenčnih vrednostih Nemškega prehranskega društva (DGE, 2019). Priporočila smo primerjali še s priporočili ESPEN (Volkert in sod., 2019a) in priporočili Evropske agencije za varnost hrane (EFSA) (EFSA, 2017). Poleg tega smo z raziskavo želeli ugotoviti, kakšna je senzorična sprejemljivost obrokov med stanovalci DSO ter senzorična kakovost po oceni strokovnega panela Biotehniške fakultete Univerze v Ljubljani.

## 2 MATERIAL IN METODE

### 2.1 VZOREC

V raziskavi, ki je potekala od 20. maja do 2. junija 2019, je prostovoljno sodelovalo 15 stanovalk in 6 stanovalcev DSO starih med 72 in 94 let. K raziskavi so bili povabljeni starejši odrasli, ki so bili funkcionalno neodvisni in so bili na »navadni« prehrani, ne dietni. Raziskava je bila odobrena s strani Komisije za študij 1. in 2. stopnje Oddelka za živilstvo Biotehniške fakultete UL. Sodelujoči v raziskavi so bili seznanjeni s potekom raziskave in s tem, da lahko od nje kadarkoli odstopijo. Pred pričetkom izvedbe so podpisali Soglasje za sodelovanje v raziskavi.

Antropometrične meritve (telesna masa, telesna višina) in oceno stopnje telesne dejavnosti so izvedli delavci zdravstvene nege DSO. Raziskovalci smo imeli dostop samo do informacij, ki so bistvene za določanje dnevne potrebe po energiji in hranilih - spol, starost, telesna višina, telesna masa in stopnja telesne dejavnosti.

### 2.2 METODE

V prvem delu raziskave smo ovrednotili energijsko in hranilno vrednost celodnevnih ponujenih in zaužitih obrokov stanovalcev DSO. V ta namen smo sodelujočim v raziskavi, v 14-dnevnem obdobju pred postrežbo vsakega ponujenega obroka (zajtrk, kosilo, popoldanska malica in večerja), s kuhinjsko tehtnico stehtali posamezne jedi oziroma komponente v obroku. Po zaključenem obroku smo stehtali še morebitne ostanke vsake komponente posebej in tako določili dejanske količine zaužite hrane in pijače. Tekom raziskave nismo spremljali in beležili dodatno zaužite hrane in pijače sodelujočih v raziskavi, t. j. hrano in pijačo, ki jo, poleg ponujenih obrokov v domu, zaužijejo tekom dneva (živila, ki jih prinesejo svojci, živila, ki jih kupijo sami v trgovini itd). Z odprto platformo za klinično prehrano (OPKP), ki predstavlja spletno orodje za vrednotenje prehrane na osnovi referenčnih podatkov o sestavi živil, smo nato na podlagi povprečnih ponujenih in zaužitih količin hrane in pijače sodelujočih v raziskavi določili povprečno energijsko in hranilno vrednost celodnevnih ponujenih obrokov in zaužitih obrokov. Všečnost toplih obrokov (kosil in večerij), ponujenih tekom tedna, so sodelujoči v raziskavi ocenili s 5-točkovno hedonsko lestvico in komentirali, kaj jih je najbolj motilo. Prav tako je senzorične lastnosti (videz, vonj, okus, aromo, teksturo) obrokov ocenil tudi strokovni panel Biotehniške fakultete Univerze v Ljubljani.

V drugem delu raziskave smo s kemijsko analizo določili energijsko in hranilno vrednost celodnevnih ponujenih obrokov. Po dve porciji vsakega ponujenega obroka, smo stehtali, homogenizirali in shranili za nadaljnje kemijske analize na Biotehniški fakulteti.

#### 2.2.1 Antropometrične meritve in ocena ravni telesne dejavnosti sodelujočih v raziskavi

Delavci zdravstvene nege DSO so pri sodelujočih v raziskavi izvedli antropometrične meritve. Sodelujočim v raziskavi so izmerili telesno maso z elektromehansko medicinsko tehtnico SECA 799 (seca GmbH & Co. KG., Nemčija) na 0,5 kg natančno. Tehtnica SECA 799 ima tudi merilno letev s katero so sodelujočim izmerili višino. Telesna višina je bila izmerjena kot razdalja med najvišjo točko na glavi in vodoravno podlago. Pri tem je merjenec bos stal zravnano in z rokami spuščenimi ob telesu. Meritve telesne višine so delavci zdravstvene nege izmerili na 0,5 cm natančno. Meritve telesne mase in višine sodelujočih v raziskavi so omogočile izračun ITM. Vrednosti ITM sodelujočih v raziskavi smo nato primerjali s priporočili SZO (WHO, 2019).

Sodelujoči v raziskavi so delavcem zdravstvene nege DSO poročali, koliko so telesno dejavni (oblikovali so vprašalnik o pogostosti in trajanju telesnih dejavnosti). Na podlagi intenzivnosti poročanih telesnih dejavnosti smo s pomočjo seznama Centra za nadzor in preprečevanje bolezni (CDC, 2020) sodelujočim v raziskavi določili raven telesne dejavnosti. V skupino z »nizko« ravnjo telesne dejavnosti so bili uvrščeni tisti sodelujoči v raziskavi, ki so bili na invalidskem vozičku ali tisti, ki so za hojo potrebovali posebne pripomočke, npr. hodulje, rolatorje. Takšnih je bilo 80,9 % sodelujočih. »Zmerno« telesno dejavni so bili le 4 sodelujoči (19,1 %), ti so se več ur dnevno sprehajali in/ali vozili s kolesom. Nihče izmed sodelujočih ni bil »visoko« telesno dejaven. Izvajalci raziskave nismo imeli neposrednega stika s sodelujočimi v raziskavi.

### 2.2.2 Vnos živil in receptov v spletno orodje OPKP

Z vnosom povprečnih ponujenih in zaužitih količin živil in jedi, v spletno orodje OPKP, smo dobili podatke o povprečni energijski in hranilni vrednosti ponujenih in zaužitih obrokov. Da so bili podatki, vneseni v OPKP, čim bolj natančni, nam je vodja prehrane in strežbe pripravil recepte jedi, po katerih so pripravljeni obroki v DSO.

V ponujenih in zaužitih obrokih smo spremljali vsebnost makrohranil (skupni ogljikovi hidrati, skupne beljakovine in maščobe), vsebnost mikrohranil (kalcij, vitamina D in B12) ter energijsko vrednost.

### 2.2.3 Kemijske analize celodnevnih ponujenih obrokov

V 14-dnevnem obdobju smo hkrati izvajali tudi vzorčenje ponujenih obrokov. Vse tekom dneva ponujene obroke (zajtrk, kosilo, popoldanska malica in večerja) smo najprej fotografirali. Nato smo vsako jed oziroma komponento v obroku in napitek stehtali in homogenizirali v dveh ponovitvah ter sproti zamrzovali na -20 °C.

V sklopu AOAC kemijskih analiz smo v homogeniziranih vzorcih določali vsebnost vode z gravimetrično metodo sušenja pri 105 °C, vsebnost pepela s suhim sežigom pri temperaturi 550 °C do konstantne mase, vsebnost beljakovin s Kjeldahlovo metodo in vsebnost maščob z Weibull-Stoldtovo metodo ter izračunali vsebnost skupnih ogljikovih hidratov in energijsko vrednost. Vsebnosti prehranske vlaknine nismo določali. Naštete analize so bile opravljene po postopkih, ki so opisani v Korošec in sod. (2019) ter KTMVŽ in FOSS (2019).

### 2.2.4 Primerjava rezultatov energijske in hranilne vrednosti celodnevnih ponujenih obrokov, določenih s spletnim orodjem OPKP in kemijsko analizo

Rezultate o povprečni energijski in hranilni vrednosti celodnevnih ponujenih obrokov, pridobljene s spletnim orodjem OPKP, smo primerjali z rezultati kemijske analize. V primerjavo smo vključili vsebnost makrohranil (skupni ogljikovi hidrati, beljakovine in maščobe) ter energijsko vrednost na 100 g vzorca (t. j. celodnevnih ponujenih obrokov) in maso celotnega vzorca.

Vse rezultate, ki smo jih dobili s spletnim orodjem OPKP in kemijsko analizo, smo uredili in statistično obdelali z uporabo računalniškega programa Microsoft Excel. Uporabili smo naslednje statistične parametre: povprečna vrednost (X), standardna deviacija (SD), minimalna vrednost (min) in maksimalna vrednost (max).

### 2.2.5 Skladnost energijske in hranilne vrednosti celodnevnih ponujenih obrokov s priporočili

Rezultate o povprečni energijski in hranilni vrednosti celodnevnih ponujenih obrokov, pridobljenih s spletnim orodjem OPKP in kemijsko analizo, smo primerjali s Smernicami za izvajanje prehranske oskrbe v domovih za starejše (NIJZ, 2020b), Priporočili za prehransko obravnavo bolnikov v bolnišnicah in starostnikov v domovih za starejše občane (Cerović in sod., 2008), PANGeA prehranskimi priporočili (Rotovnik Kozjek in sod., 2014) in referenčnimi vrednostmi (NIJZ, 2020a), ki so povzeta po Referenčnih vrednostih Nemškega prehranskega društva (DGE, 2019). V primerjavo smo vključili tudi mednarodna priporočila ESPEN (Volkert in sod., 2019a) in EFSA (2017).

### 2.2.6 Primerjava energijske in makrohranilne vrednosti celodnevnih zaužitih obrokov s priporočili

Sodelujoči v raziskavi so imeli stalen sedežni red. Po hranjenju so pladenj z ostanki hrane pustili na mizi, kar nam je omogočilo, da smo na podlagi sedežnega reda beležili količine zaužite hrane in pijače. Tako kot pri celodnevnih ponujenih obrokih, smo tudi v tem koraku s kuhinjsko tehtnico stehtali vsako komponento obroka posebej in kot razliko mase ponujena obroka in morebitnih ostankov določili maso zaužitega obroka. S spletnim orodjem OPKP smo tako določili povprečne energijske in hranilne vnose s celodnevnimi zaužitimi obroki vseh sodelujočih v raziskavi.

### 2.2.7 Senzorično ocenjevanje vzorcev kosil in večerij

Tople obroke (kosila in večerje) sta ocenjevala dva panela preskuševalcev. Panel potrošnikov so predstavljali sodelujoči v raziskavi, ki so ocenjevali všečnost zaužitih toplih obrokov, ponujenih tekom tedna. Za ocenjevanje so uporabili 5-točkovno hedonsko lestvico (1 = nezadostno, 5 = odlično).

Po dva vzorca kosila in večerje smo vsak dan shranili v termo posode in takoj prepeljali iz DSO na Biotehniško fakulteto na senzorično analizo. Vsak dan smo posneli tudi fotografijo obroka, serviranega na krožniku, za dodatno informacijo strokovnemu panelu pri ocenjevanju videza. Štiričlanski strokovni panel Oddelka za živilstvo Biotehniške fakultete je ocenjeval senzorično kakovost posameznih toplih obrokov z vrednotenjem senzoričnih lastnosti. Za oceno senzorične kakovosti videza, vonja, okusa, arome in teksture so uporabljali 5-točkovno lestvico (1 = nesprejemljiva, 5 = odlična). Ker senzorično ocenjevanje ni potekalo neposredno ob serviranju obrokov, je strokovni panel ob senzorični oceni upošteval morebitni vpliv neustrezne temperature na senzorične lastnostni obrokov.

## 3 REZULTATI IN DISKUSIJA

### 3.1 INDEKS TELESNE MASE

V raziskavo je bilo vključenih 21 stanovalk in stanovalcev DSO, katerih povprečna starost je znašala 85,7 ± 5,5 let. Na podlagi telesne meritve mase in višine smo izračunali ITM sodelujočih ter jih primerjali s priporočili SZO (WHO, 2019).

V preglednici 1 so zbrani antropometrični podatki sodelujočih v raziskavi.

**Preglednica 1:** Število sodelujočih v raziskavi ter povprečna starost (leta), telesna masa (kg) in višina (m) ter ITM* (kg m$^{-2}$) sodelujočih v raziskavi

| | Ženske | Moški | Skupaj |
|---|---|---|---|
| Število sodelujočih v raziskavi | 15 (76 %) | 6 (24 %) | 21 (100 %) |
| Povprečna starost ± SD (let) | 85,9 ± 5,6 | 85,2 ± 5,1 | 85,7 ± 5,5 |
| Povprečna telesna masa ± SD (kg) | 69,2 ± 10,3 | 68,4 ± 8,8 | 69,0 ± 9,9 |
| Povprečna telesna višina ± SD (m) | 1,54 ± 0,05 | 1,67 ± 0,04 | 1,57 ± 0,08 |
| Povprečen ITM* ± SD (kg m$^{-2}$) | 29,5 ± 5,0 | 24,7 ± 3,2 | 28,1 ± 5,0 |

*ITM (Indeks telesne mase)

Povprečen ITM sodelujočih v raziskavi je znašal 28,1, kar se po SZO klasifikaciji uvršča v razred čezmerne hranjenosti (WHO, 2019). Nasprotno Porter Starr in Bales (2015) za starejše z ITM 25,0-29,9 priporočata ohranjanje telesne mase z uživanjem hranilno goste hrane ter telesno dejavnostjo. Prav tako so tudi Winter in sod. (2014) v območju ITM med 24,0 in 30,9 (kamor sodi tudi povprečen ITM sodelujočih v raziskavi) ugotovili najšibkejšo povezavo med ITM in smrtnostjo starejših odraslih.

Iz preglednice 1 je razvidno, da so bile stanovalke, vključene v raziskavo, po SZO klasifikaciji (WHO, 2019), v povprečju čezmerno hranjene (ITM 25,0-29,9). Tri stanovalke (20 %) so bile uvrščene v razred normalne hranjenosti, šest stanovalk (40 %) v razred čezmerne hranjenosti in šest stanovalk (40 %) v razred debelosti. Največji ITM stanovalke je znašal 40,8, kar predstavlja debelost III. stopnje, najmanjši pa 21,6, kar predstavlja normalno hranjenost. Nobena od stanovalk, vključenih v raziskavo, ni bila uvrščena v razred podhranjenosti (ITM manj kot 18,5).

Moški stanovalci, vključeni v raziskavo, so imeli manjši povprečni ITM kot stanovalke. Povprečni ITM stanovalcev je znašal je 24,7, kar jih je uvrščalo v razred normalne hranjenosti (ITM 18,5-24,9). Največji ITM stanovalca znašal 27,6, kar predstavlja čezmerno hranjenost, najmanjši pa 18,8, kar predstavlja normalno hranjenost. Enako kot med stanovalkami, tudi med stanovalci ni bilo zaznane podhranjenosti (ITM pod 18,5). Dva stanovalca (33 %) sta se uvrščala v razred normalne hranjenosti, štirje stanovalci (67 %) pa v razred čezmerne hranjenosti. V nasprotju s stanovalkami se med stanovalci glede na vrednost ITM nihče ni uvrščal v razred debelosti (WHO, 2019).

### 3.2 OCENA RAVNI TELESNE DEJAVNOSTI SODELUJOČIH V RAZISKAVI

Od skupno 21 sodelujočih v raziskavi smo jih 17 (81,0 %), med njimi 14 stanovalk in tri stanovalce, uvrstili med slabo telesno dejavne, t. j. brez ali z malo naporne telesne dejavnosti (NIJZ, 2020a). Pretežno so bili to tisti sodelujoči v raziskavi, ki so bili na invalidskem vozičku ali tisti, ki so za hojo potrebovali posebne pripomočke, npr. hodulje, rolatorje. Štiri (19,1 %) sodelujoče v raziskavi (3 stanovalci in 1 stanovalka) smo uvrstili med zmerno telesno dejavne (tisti, ki so se več ur dnevno sprehajali oziroma planinarili in/ali kolesarili). Med visoko telesne dejavne nismo uvrstili nobenega izmed sodelujočih v raziskavi.

## 3.3 ENERGIJSKA IN HRANILNA VREDNOST CELODNEVNIH PONUJENIH IN ZAUŽITIH OBROKOV

### 3.3.1 Primerjava energijske vrednosti celodnevnih ponujenih obrokov, ovrednotene s spletnim orodjem OPKP in določene kemijsko analizo ter vnos energije s celodnevnimi zaužitimi obroki, ovrednoten s spletnim orodjem OPKP in skladnost s priporočili

Glede na spol (ženski, moški), starost (> 65 let) in določeno raven telesne dejavnosti (nizka in zmerna) smo za vsakega sodelujočega v raziskavi iz referenčnih vrednosti (NIJZ, 2020a) določili njegov priporočen dnevni energijski vnos. Za žensko z nizko ravnjo telesne dejavnosti znaša 7113 kJ (1700 kcal), za moškega pa 8786 kJ (2100 kcal). Za žensko z zmerno ravnjo telesne dejavnosti znaša 7950 kJ (1900 kcal) ter za moškega 10460 kJ (2500 kcal) (NIJZ, 2020a).

Energijska vrednost celodnevnih ponujenih obrokov v prvem tednu se je gibala med 6433 kJ (1537 kcal) in 9234 kJ (2206 kcal), v povprečju 8157 ± 919 kJ (1949 ± 219 kcal). Največja razlika med z OPKP ovrednotenimi in s kemijsko analizo določenimi energijski vrednosti je znašala 1619 kJ (386 kcal) (9. dan), najmanjša pa 78 kJ (18 kcal) (10. dan). S pomočjo Microsoft Excel smo izvedli parni t-test, ki je pokazal statistično značilno razliko med energijsko vrednostjo celodnevnih ponujenih obrokov, ki smo jo ocenili z OPKP in določili s kemijsko analizo (p = 0,001), iz česar lahko sklepamo, da OPKP ni povsem zanesljiva metoda vrednotenja in ne izraža dejanske energijske vrednosti obrokov.

Glede na ostanke po hranjenju sodelujočih smo določili povprečno energijsko vrednost zaužitih obrokov, ki smo jih primerjali s dnevnimi energijskimi potrebami vsakega sodelujočih v raziskavi. Energijska vrednost celodnevnih zaužitih obrokov sodelujočih v raziskavi je v prvem tednu v znašala 7545 ± 860 kJ (1803 ± 205 kcal), v drugem tednu pa 7133 ±1049 kJ (1704 ± 250 kcal). To pomeni, da so sodelujoči v raziskavi v prvem tednu povprečno zaužili 92,5 ± 0,7 % celodnevnih ponujenih obrokov, v drugem tednu pa 93,1 ± 1,4 % celodnevnih ponujenih obrokov. Priporočen energijski vnos (NIJZ, 2020a) glede na spol, starost in ocenjeno raven telesne dejavnosti, je doseglo 14 od 21 sodelujočih.

O podobnih ugotovitvah so poročali tudi Jyväkorpi in sod. (2015), ki so pri 374 institucionaliziranih starejših odraslih na Finskem beležili povprečen dnevni energijski vnos 7370 kJ (1760 kcal). O nekoliko manjših povprečnih energijskih vnosih in sicer 6760 kJ (1615 kcal) so poročali Rakıcıoğlu in sod. (2016), ki so s 24-urnim priklicem jedilnika prejšnjega dne spremljali prehranjevalne navade 102 institucionaliziranih starejših odraslih v Turčiji. Lavriša in Pravst (2024) so v pilotni študiji ugotavljala kakšni so prehranski izzivi v treh DSO po Sloveniji in ugotovili, da stanovalci niso zadostili dnevnim potrebam po energiji. Moški so dnevno v povprečju zaužili le 6845 kJ (1637 kcal), ženske pa 5673 kJ (1356 kcal), kar je bistveno pod priporočenim energijskim vnosom 8786 kJ (2100 kcal) in 7113 kJ (1700 kcal). Prav tako so Lavriša in sod. (2024) isto leto objavili raziskavo v katero je bilo vključenih 317 stanovalcev iz 20 DSO po Sloveniji. Tokrat so bili rezultati energijskega vnosa bolj ustrezni glede na nacionalna priporočila (NIJZ, 2020a). Stanovalci so v povprečju zaužili 9572 kJ (2288 kcal), stanovalke pa 8740 kJ (2089 kcal).

### 3.3.2 Primerjava vsebnosti makrohranil v celodnevnih ponujenih obrokih, ovrednotenih s spletnim orodjem OPKP in kemijsko analizo, ter vnos makrohranil s celodnevnimi zaužitimi obroki, ovrednoten s spletnim orodjem OPKP in skladnost s priporočili

#### 3.3.2.1 Ogljikovi hidrati

Vsebnosti ogljikovih hidratov v vzorcih celodnevnih ponujenih obrokov, ovrednotene s spletnim orodjem OPKP, so se gibale med 149 in 260 g, določene s kemijsko analizo pa med 126 in 258 g. Pridobljene vrednosti smo primerjali s parnim t-testom in ugotovili, da je razlika statistično značilna ($p = 0,027$). Zanimivo je bilo opaziti, da razlika ni bila statistično značilna ($p > 0,05$), kadar smo preračunali odstotek ogljikovih hidratov glede na energijsko vrednost ponujenih obrokov.

Referenčne vrednosti (NIJZ, 2020a) navajajo, da naj ogljikovi hidrati predstavljajo več kot 50 % dnevnega energijskega vnosa, kar v povprečju niso dosegali niti celodnevni ponujeni obroki, niti zaužiti obroki prvega tedna (44,5 ± 3,7 %) in drugega tedna (47,5 ± 7,2 %) (Preglednica 2). Dejstvo, da je vnos OH manjši kot 50 % dnevnega energijskega vnosa, je zaskrbljujoče, saj ti odstotki predstavljajo tako izkoristljive OH kot prehransko vlaknino, kar pomeni, da je izračunana energija na račun izkoristljivih OH še nekoliko precenjena. Rakıcıoğlu in sod. (2016) so v raziskavi prehrane starejših odraslih, ki bivajo v domu starejših občanov v Turčiji, beležili primerljive odstotke ogljikovih hidratov, saj so povprečno predstavljali 48,4 % dnevnega energijskega vnosa. Nekoliko večji odstotek, povprečno 51,3 % dnevnega energijskega vnosa, so ogljikovi hidrati predstavljali v celodnevni prehrani pri institucionaliziranih starejših odraslih na Finskem (Jyväkorpi in sod., 2015).

#### 3.3.2.2 Beljakovine

Vsebnost beljakovin v ponujenih obrokih, določena z OPKP in kemijsko analizo, je v povprečju dveh tednov znašala 79 in 76 g/dan. Rezultati OPKP in kemijskih analiz se niso statistično razlikovali ($p$ > 0,05), rezultati statistične analize pa so predstavljeni v preglednici 2. Vnos beljakovin z zaužitimi obroki je prvi teden znašal 71 ± 10 g oziroma 16,3 ± 2,6 % dnevnega energijskega vnosa, drugi teden pa 71 ± 13 g oziroma 17,0 ± 2,8 % dnevnega energijskega vnosa, kar je skladno s priporočili, ki navajajo, da naj beljakovine predstavljajo 15-20 % dnevnega vnosa energije (Hursti in Becker, 2012). Rezultati so se razlikovali od Rakıcıoğlu in sod. (2016), ki so v svoji raziskavi ugotovili, da so pri starejših odraslih, ki so bivali v DSO v Turčiji, beljakovine povprečno predstavljale nekoliko manjši odstotek dnevnega energijskega vnosa, 14,9 %. Ugotovili so namreč, da najbolj pogost nezadosten vnos pri starejših odraslih predstavljajo ravno beljakovine ter nekateri vitamini in minerali (kalcij, železo, cink, magnezij, folat in vitamin C). Podobno navajajo tudi Jyväkorpi in sod. (2015) za institucionalizirane starejše na Finskem, kjer so beljakovine v celodnevni prehrani povprečno predstavljale 14,8 % dnevnega energijskega vnosa.

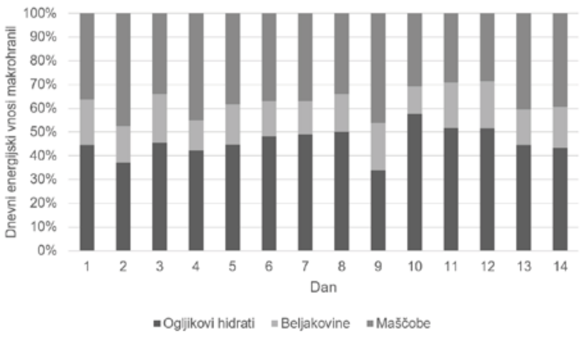
Priporočila za prehransko obravnavo bolnikov v bolnišnicah in starostnikov v domovih za starejše občane (Cerović in sod., 2008) navajajo, da naj bi starejši dnevno zaužili vsaj 0,8 g beljakovin/kg TM. Ob upoštevanju slednjih priporočil lahko glede na izmerjeno telesno maso sodelujočih trdimo, da je s celodnevnimi zaužitimi obroki prvega in drugega tedna večina sodelujočih v raziskavi (20 od 21) dosegala (presegala) priporočene dnevne vnose beljakovin. V novejših smernicah za izvajanje prehranske oskrbe v domovih za starejše (NIJZ, 2020b) pa je navedeno, da naj bi starejši dnevno zaužili vsaj 1,0 g beljakovin/kg telesne mase (TM). Enake vrednosti navajajo tudi Bauer in sod. (2013), referenčne vrednosti (NIJZ, 2020a), priporočila ESPEN (Volkert in sod., 2019a) ter PANGeA priporočila (Rotovnik Kozjek in sod., 2014). Ob upoštevanju slednjih priporočil lahko glede na izmerjeno telesno maso sodelujočih trdimo, da je s celodnevnimi zaužitimi obroki prvega in drugega tedna 67 % sodelujočih v raziskavi (14 od 21) dosegalo priporočene dnevne vnose beljakovin. Naši rezultati so pokazali večji vnos beljakovin kot raziskava Lavriša in sod. (2024), kjer so ugotovili, da priporočen vnos beljakovin (1 g $kg^{-1}$ TM) doseže samo 40 % stanovalcev in 35 % stanovalk DSO.

#### 3.3.2.3 Maščobe

Maščobe so glede na bazo podatkov OPKP in kemijske analize predstavljale 36 % in 35 % dnevnega energijskega vnosa ob upoštevanju, da stanovalke in stanovalci DSO-ja niso poleg ponujenih obrokov v tistih dneh zaužili ničesar več. Razlika med vrednostmi ni bila statistično značilna ($p$ > 0,05).

Povprečen odstotek energije iz maščob v celodnevnih zaužitih obrokih v prvem (39,3 ± 4,6 %) in drugem tednu (35,5 ± 6,2 %) je presegal priporočila, ki navajajo, da naj bi maščobe predstavljale 30 % dnevnega energijskega vnosa (Cerović in sod., 2008; NIJZ, 2020a, NIJZ, 2020b). Tako povprečen odstotek energije iz maščob prvega tedna kot tudi energijski odstotki maščob v posameznih celodnevnih zaužitih obrokih vseh dni prvega tedna (34,0-47,5 % dnevnega energijskega vnosa) so bili glede na priporočila preveliki. V drugem tednu pa so bili energijski odstotki maščob preveliki v celodnevnih zaužitih obrokih petih dni, saj so maščobe manj kot 30 % dnevnega energijskega vnosa predstavljale samo 11. in 12. dan raziskave, kar je razvidno s slike 1. Tudi Lavriša in sod., (2024) so poročali, da je 37 % celokupnega energijskega vnosa stanovalcev v DSO izviral iz maščob. Ustrezen dnevni vnos maščob (< 30 % dnevnega energijskega vnosa) je imelo manj kot 10 % stanovalcev DSO. Rakıcıoğlu in sod. (2016) so v svoji raziskavi predstavili primerljive rezultate. Pri starejših odraslih, ki so bivali v DSO v Turčiji, so maščobe povprečno predstavljale 36,7 % dnevnega energijskega vnosa. Manjše odstotke energije, povprečno 31,0 %, so maščobe predstavljale v raziskavi Jyväkorpi in sod. (2015) pri institucionaliziranih starejših odraslih na Finskem.

Na sliki 1 so grafično predstavljen dnevni energijski vnosi (%) posameznih makrohranil, določeni z OPKP.



**Slika 1:** Dnevni energijski vnosi (%) makrohranil v celodnevnih zaužitih obrokih 1. teden (1.-7. dan) in 2. teden (8-14. dan)

**Preglednica 2:** Minimalne, maksimalne in povprečne vrednosti energije (kJ) in makrohranil (g) v celodnevnih ponujenih obrokih, določene z OPKP ter kemijsko analizo.

| | OPKP | | | Kemijska analiza | | | |
|---|---|---|---|---|---|---|---|
| | Minimalna vrednost | Maksimalna vrednost | Povprečna vrednost | Minimalna vrednost | Maksimalna vrednost | Povprečna vrednost | *p*-vrednost |
| Energija (kJ) | 6433 | 9736 | 7913 | 5603 | 8749 | 7291 | 0,001* |
| Ogljikovi hidrati (g) | 149 | 260 | 210 | 126 | 258 | 196 | 0,027* |
| % dnevnega energijskega vnosa | 34 | 57 | 45 | 38 | 56 | 46 | 0,864 |
| Beljakovine (g) | 46 | 104 | 79 | 48 | 110 | 76 | 0,446 |
| % dnevnega energijskega vnosa | 12 | 23 | 17 | 12 | 27 | 18 | 0,144 |
| Maščobe (g) | 49 | 111 | 77 | 49 | 93 | 68 | 0,018* |
| % dnevnega energijskega vnosa | 28 | 45 | 36 | 26 | 43 | 35 | 0,472 |

### 3.4 SENZORIČNO OCENJEVANJE KOSIL IN VEČERIJ

V preglednici 3 so zbrane najnižje in najvišje povprečne hedonske ocene kosil, ki so jih dodelili sodelujoči v raziskavi in strokovne ocene kosil strokovnega panela.

Pri najslabše ocenjenem kosilu (kosilo, 9. dan) so bili sodelujoči v raziskavi mnenja, da je bila omaka zažgana, meso pa trdo. S kuharskim osebjem smo prišli do zaključka, da je dodeljena ocena verjetno zaradi nepoznavanja jedi, mongolskega mesa, ki vključuje sojino omako zelo temne, skoraj črne barve. Strokovni panel je to kosilo ocenil s 4,2 ± 0,5. Prežganko so ocenili kot preslano, pretemne barve z vonjem po zažganem ter da daje masten pookus. Pri mongolskem mesu jih je zmotilo predvsem, da v vonju in aromi izstopa sojina omaka. Za paradižnikovo solato pa so bili mnenja, da je bil paradižnik kašast in slabo očiščen zaradi prisotnega stržena ter narezan na neenakomerno velike koščke. Prav tako so menili, da je bila solata pretirano poprana in okisana.Po oceni strokovnega panela je bilo najslabše senzorične kakovosti kosilo 4. dne (3,8 ± 0,4). Člani panela so menili, da je bila bučkina kremna juha premalo kremna, prazna, preslana ter da ni imela okusa po bučkah temveč po krompirju. Riž za zelenjavno-mesno rižoto je bil razkuhan, zaradi česar je bila aroma rižote premalo intenzivna. Prav tako jih je zmotila zelenjava, ki ni bila sveža ampak zamrznjena. Solato so prav tako ocenili kot preveč oljnato. Stanovalci DSO so omenjenemu kosilu dodelili višjo oceno, 4,1 ± 0,9. Enega od stanovalcev je motilo, da je bilo dodano preveč mesa, drugega pa, da je bila hrana hladna, vsi ostali pa so bili z obrokom zadovoljni, še posebej s solato.

Podobno nizko oceno za senzorično kakovost, 3,8 ± 0,3, je strokovni panel dodelil tudi kosilu 12. dne. Podali so komentarje, da je bila pri porovi juhi aroma pora neizrazita, saj je bilo zaznati smetano, krompir in začimbe, predvsem pa slan okus in aromo po peteršilju. File osliča je bil presušen zaradi tankih rezin in močno soljen, prisotne

**Preglednica 3:** Najnižje in najvišje povprečne ocene ± SD za kosila, dodeljene s strani sodelujočih v raziskavi in strokovnega panela

| | Hedonska ocena (1-5) | Meni | Strokovna ocena (1-5) | Meni |
|---|---|---|---|---|
| Najnižja ocena kosila | 3,3 ± 1,2 | Prežganka, mongolsko meso, dušen riž in paradižnikova solata (kosilo, 9. dan) | 3,8 ± 0,4 | Bučkina kremna juha, zelenjavno-mesna rižota in solata (zelena solata, radič, zelje) (kosilo, 4. dan) |
| | | | 3,8 ± 0,3 | Porova juha, file osliča po tržaško, blitva s krompirjem, zelena solata z lečo in cviček (kosilo, 12. dan) |
| Najvišja ocena kosila | 4,3 ± 0,8 | Fižolova juha, file osliča po dunajsko, krompirjeva solata in cviček (kosilo, 5. dan) | 4,3 ± 0,4 | Lečina juha, mesni kanelon in krompirjeva solata (kosilo, 10. dan) |

so bile tudi kosti. Hkrati je bila preveč intenzivna aroma olja in česna v dodani tržaški omaki. Blitva s krompirjem je bila razkuhana, aroma jedi je bila nekoliko neharmonična. Zelena solata je imela rjave robove, leča pa je bila razkuhana in zmečkana. Cviček je imel neharmonično aromo (kisel okus, vonj po alkoholu). Še nižjo oceno, 3,5 ± 1,2, so temu kosilu dodelili sodelujoči v raziskavi. Enega od vprašanih je motilo, da juha ni bila precejena in so bila prisotna vlakna ter drugega, da sta bila riba in krompir hladna. Sicer pa so bili na kosilo različni komentarji: da je kosilo okusno in vitaminsko, da je kosilo prav dobro ter vse do komentarja, da kosilo ni vredno ocene.

Pri kosilu z najvišjo hedonsko oceno s strani sodelujočih v raziskavi (kosilo, 5. dan), je dva sodelujoča sicer zmotilo bučno olje in enega preveč soljena krompirjeva solata, na splošno pa so kosilo pohvalili, da je odlično. Strokovni panel je omenjenemu kosilu dodelil oceno 4,2 ± 0,6. Bili so mnenja, da je fižolova juha primerno slana, a so jih zmotile luščine in zakrita aroma fižola zaradi prevelike količine peteršilja. Pri osliču jih je zmotila panada, ki je imela preveč arome po moki. Tudi krompirjeva solata je bila po njihovem mnenju preveč slana in preveč poprana, zato je aroma popra zakrivala aromo po krompirju. Prav tako so bili kosi krompirja neenakomerno veliki. Cviček so ocenili kot neharmoničen (preveč kisel okus in z intenzivnim vonjem po alkoholu).

Pri kosilu z najvišjo oceno za senzorično kakovost (kosilo, 10. dan) je bil strokovni panel sicer mnenja, da je lečina juha preslana, moteče so jim bile tudi luščine in vonj po prežganju. Mesni kanelon je bil nekoliko neenakomerno zapečen. Krompirjeva solata je bila po njihovi presoji preslana ter s preveliko količino popra in čebule. Prav tako so bili kosi krompirja različno veliki in različno čvrsti. Sodelujoči v raziskavi so omenjenemu kosilu dodelili nekoliko nižjo oceno, 4,0 ± 0,7 točk. Dvema stanovalcema ni bilo všeč bučno olje na krompirjevi solati, dva pa so zmotile luščine v juhi. Sicer pa so kosilo ocenili kot super ter brez pripomb.

V preglednici 5 so zbrane najnižje in najvišje povprečne hedonske ocene večerij, ki so jih dodelili sodelujoči v raziskavi in strokovne ocene večerij strokovnega panela.

Za najslabše ocenjeno večerjo s strani sodelujočih v raziskavi (večerja, 1. dan), z vprašalniki nismo dobili veliko konkretnih odgovorov, kaj jih je motilo. Eden od sodelujočih v raziskavi je menil, da je zelenjava sicer zdrava, vendar jo je bilo preveč, drugega pa je motilo, da je bila mineštra hladna. Strokovni panel je omenjeni večerji namenil višjo senzorično oceno, in sicer 4,1 ± 0,3, vendar pa so bili vseeno mnenja, da je bila mineštra preslana, z razkuhano zelenjavo in neenakomerno velikostjo koščkov zelenjave, zaradi česar je bila mineštra neenotne teksture.Pri večerji z najnižjo oceno senzorične kakovosti (večerja, 11. dan) je strokovni panel motila predvsem preslana mineštra z razkuhano zelenjavo, med katero so prevladovale križnice (ohrovt, zelje). Sodelujoči v raziskavi so omejeno večerjo ocenili s podobno povprečno oceno 3,7 ± 1,0. Konkretnih odgovorov, kaj jih moti, pa z vprašalniki nismo dobili.

Vsi, razen enega sodelujočega v raziskavi, ki je menil, da je potica preveč suha, so večerjo z najvišjo hedonsko oceno (večerja, 4. dan) pohvalili, da je bila zelo dobra/super/odlična in da jih nič ni motilo. Strokovni panel je tej večerji za senzorično kakovost dodelil 3,9 ± 0,9 točk. Ocenili so, da potica glede na ime ni ocvirkova, saj namesto ocvirkov v nadevu vsebuje prekajeno meso ter skuto oziroma smetano. Prav tako so bili mnenja, da je domač metin čaj brez okusa in arome po meti in preveč svetle barve.

Strokovni panel je večerjo z najvišjo oceno za senzorično kakovost (večerja, 12. dan) označil za eno izmed najboljših od vseh jedi, ki so jih ocenjevali v 14 dneh, predvsem zaradi ustrezne uporabe začimb, mehkega mesa in primerno kuhanega krompirja. Sodelujoči v raziskavi so omenjeno večerjo ocenili z nekoliko nižjo povprečno oceno, vendar še vedno visoko na 5-točkovni hedonski lestvici, 4,4 ± 0,6. Prav vsi stanovalci, ki so izpolnili anketo po zaužitem obroku so bili mnenja, da je bila večerja zelo dobra in okusna/odlična.

Strokovni panel je opozoril, da na splošno v hrani pogrešajo pestrost sestavin, barv in arom, saj se pogosto ponavlja uporaba zamrznjene zelenjave v obliki juh (zelenjavna mineštra, telečja obara, štajerska kisla juha). Prav tako bi zamrznjeno zelenjavo lahko zamenjali s

**Preglednica 5:** Najnižje in najvišje povprečne ocene ± SD za večerje, dodeljene s strani sodelujočih v raziskavi in strokovnega panela

| | Hedonska ocena (1-5) | Meni | Strokovna ocena (1-5) | Meni |
|---|---|---|---|---|
| Najnižja ocena kosila | 3,6 ± 0,9 | Zelenjavna mineštra z žličniki in bel kruh (večerja, 1. dan) | 3,8 ± 0,3 | Zelenjavna mineštra z žličniki (večerja, 11. dan) |
| Najvišja ocena kosila | 4,5 ± 0,7 | Ocvirkova potica in domač metin čaj (večerja, 4. dan) | 4,7 ± 0,2 | Krompirjev golaž in bel kruh (večerja, 12. dan) |

svežo sezonsko zelenjavo. Pogosto je bila hrana razkuhana (riž, ribana kaša v goveji juhi, zelenjava v mineštri). V lečini in fižolovi juhi so bile moteče luščine. Solate so bile pogosto preveč oljnate, z neenakomernimi kosi uvele solate/paradižnika/krompirja. Strokovni panel priporoča uporabo začimb (poleg soli in popra), saj so bili obroki pogosto preveč slani. Prav tako bi bilo potrebno paziti na kombinacije v obroku (npr. mlečni zdrob in kompot iz višenj). Poleg tega je ponujeno vino ob jedeh močno kislo ter prazne arome in neharmoničnega okusa.

Sodelujoči v raziskavi z vprašalniki pogosto niso dajali konkretnih odgovorov, kaj jih je pri zaužitem obroku najbolj motilo (pogosto so napisali, da jih ne moti nič, da pošiljajo pozdrave kuhinjskemu osebju ali pa so polje za odgovor pustili prazno), prav tako so pogosto zaužitemu obroku zgolj dodelili oceno. V prehrani si na splošno želijo zamenjavo bučnega olja z drugimi olji. Prav tako so večkrat opozorili, da je meso pretrdo (kuhana govedina, mongolsko meso). V lečini juhi so jih, tako kot strokovni panel, motile luščine.

V Sloveniji je do sedaj nastala le peščica raziskav, s katerimi smo primerjali naše rezultate senzoričnega ocenjevanja in senzorične sprejemljivosti obrokov v DSO. Ena izmed teh je magistrska naloga (Marković, 2017), kjer so preučevali splošno zadovoljstvo življenja v 20 DSO po Sloveniji. Stanovalci so kot eno izmed najslabših vidikov življenja v DSO ocenili prav prehrano. Izpostavili so predvsem nezadovoljstvo z okusom, ponavljanje jedi in okrnjeno izbiro obrokov. Do podobnih ugotovitev so prišli tudi v DSO Sežana (Skupina Fabrika, 2023), kjer so stanovalci izrazili zmerno zadovoljstvo s prehrano in poudarili, da si želijo bolj okusno hrano, več raznolikosti in izbire, več sadja in zelenjave ter manj mesa. Kljub temu, da so bile hedonske ocene obrokov v naši raziskavi relativno visoke, ostaja prostor z izboljšave, ki bi ga morda zapolnili že z dodatno prilogo pri kosilu (Van Wymelbeke in sod., 2020).

S t-testom smo potrdili, da med hedonskimi ocenami in ocenami za senzorično kakovost kosil in večerij obstajajo statistično značilne razlike ($p \leq 0{,}05$), kar je pričakovano, saj visoka senzorična kakovost ne pomeni nujno, da je živilo tudi všečno potrošniku in obratno.

### 3.5 OMEJITVE RAZISKAVE

Tekom naše raziskave smo določali povprečne energijske in hranilne vrednosti ponujenih in zaužitih obrokov. Te povprečne vrednosti smo nato primerjali v potrebami sodelujočih stanovalcev glede na spol, starost in raven telesne dejavnosti, zato podatki o ustreznosti energijske in hranilne vrednosti ponujenih in zaužitih obrokov niso bile povsem individualne. Da bi dobili bolj natančne podatke, bi bilo potrebno za vsakega sodelujočega v raziskavi na podlagi količin ponujenih in zaužitih jedi v obrokih s pomočjo OPKP določiti energijsko in hranilno vrednost celodnevnih ponujenih in zaužitih obrokov. Nato bi podatke primerjali s priporočenim dnevnim energijskim vnosom sodelujočega v raziskavi, določenim glede na spol, starost in določeno raven telesne dejavnosti. To bi omogočilo, da bi za vsakega sodelujočega v raziskavi določili, v kolikšni meri so bili njegovi dejanski energijski vnosi v skladu z zanj priporočenimi energijskimi vnosi. Ena izmed omejitev raziskave je tudi, da pri sodelujočih nismo spremljali hrane in pijače, ki so jo dodatno morebiti zaužili npr. živila, ki so jih prinesli svojci in živila, ki so si jih lahko kupili v trgovini. Tovrstno spremljanje bi zahtevalo celodnevno opazovanje sodelujočih v raziskavi, kar bi lahko vplivalo na njihov način in ritem prehranjevanja. Lahko bi sicer sodelujoče zaprosili za informacije glede dodatno zaužitih živil, ampak bi te lahko bile izkrivljene, saj bi se morali zanašati na njihov spomin. Prav tako okviru raziskave nismo določili vsebnosti prehranske vlaknine, zaradi česar sta lahko precenjena odstotek energije ogljikovih hidratov v obrokih in energijska vrednost obrokov.

## 4 SKELPI

Staranje prebivalstva je pojav, do katerega v razvitih državah, vključno s Slovenijo, prihaja zaradi zmanjševanja rodnosti in posledično zmanjševanja števila otrok, hkrati pa se, zaradi izboljšanih zdravstveno-higienskih in socialnih razmer, podaljšuje življenjska doba ljudi. Še dodatno k staranju prebivalstva prispeva odseljevanje mladih (SiSTAT, 2023). Iz leta v leto tako vedno bolj aktualno postaja vprašanje, kako v vedno večji in predvsem heterogeni skupini starejših odraslih zagotoviti čim daljše obdobje zdravih in aktivnih ter funkcionalno neodvisnih let (Filipovič Hrast in Hlebec, 2015).

Tekom vseživljenjskega procesa staranja se starejši odrasli poleg fizioloških sprememb in pogosto pridruženih akutnih in/ali kroničnih bolezni ter običajno jemanja zdravil pogosto soočajo z več socialnimi, psihološkimi ter socialno-ekonomskimi spremembami (Cerović in sod., 2008; Deutz in sod., 2014). Skupek vseh omenjenih sprememb pogosto vpliva na zmanjšan vnos hrane in/ali apetit starejših odraslih, kar lahko vodi v izgubo tako TM. To povzroča povečano tveganje za nastanek sarkopenije, osteoporoze in krhkosti, posledično pa prihaja do večje nagnjenosti za padce in zlome, okužbe in splošno povečano tveganje za obolevnost in umrljivost (Clegg in Williams, 2018; Cruz-Jentoft in Sayer, 2019).

Rezultati naše raziskave, kjer smo ovrednotili energijsko in hranilno vrednost celodnevnih ponujenih obrokov stanovalcev DSO Ljubljana s pomočjo spletnega orodja OPKP in kemijske analize, kažejo, da večina stanovalcev dosega priporočene energijske vnose. Kljub temu prehrana ni bila optimalno uravnotežena, saj je bil odstotek energije iz ogljikovih hidratov v celodnevnih zaužitih obrokih premajhen, enako velja za beljakovine ob upoštevanju smernic za izvajanje prehranske oskrbe v domovih za starejše (2020), ki navajajo, da mora biti dnevni vnos beljakovin pri starejših odraslih najmanj 1 g kg$^{-1}$ TM. Odstotek energije iz maščob je presegel priporočene vnose (NIJZ, 2020a; NIJZ, 2020b). Za doseganje ustreznih dnevnih vnosov makrohranil bi bilo priporočeno v obroke v večjem obsegu vključevati žita in izdelke iz žit, med njimi tudi polnozrnate, saj so poleg ogljikovih hidratov tudi bogat vir prehranske vlaknine ter vitaminov in mineralov. Zmanjšanje vnosa maščob bi lahko dosegli z ustreznejšim izborom mesa in mesnin ter večjim vnosom rib (Rotovnik Kozjek in sod., 2014). Še naprej je v obrokih potrebno zagotavljati zadostne količine kakovostnih beljakovin za ohranjanje mišične mase in zaviranje sarkopenije, ki predstavlja glavni razlog za krhkost in padce starejših odraslih (Deutz in sod., 2014).

Senzorična ocena obrokov, opravljena tako s strani stanovalcev kot tudi strokovnega panela, je pokazala zmerno zadovoljstvo. To nakazujejo povprečne hedonske ocene sodelujočih v raziskavi (3,8 ± 0,9 od skupno 5 točk) in strokovne ocene strokovnega panela (4,0 ± 0,5 od skupno 5 točk). Komentarji so se največkrat nanašali na neprimerno teksturo jedi (npr. razkuhan krompir, riž), prekomerne slanosti obrokov, ponavljajoče se uporabe zamrznjene zelenjave, ki bi jo lahko zamenjali s svežo sezonsko zelenjavo. Rezultati naše raziskave so primerljivi z ugotovitvami Marković in sod. (2017) ter Fabrika (2020), iz česar lahko sklepamo, da je senzorična kakovost obrokov v večini DSO področje, kjer bi bilo smiselno uvesti spremembe s ciljem izboljšanja senzorične kakovosti obrokov in posledično preprečevanja nezadostnega energijskega vnosa stanovalcev v DSO.

Naša raziskava dopolnjuje znanstveno področje s podrobnim ovrednotenjem energijske in hranilne vrednosti obrokov v DSO, pri čemer smo kombinirali spletno orodje OPKP in kemijske analize ter hkrati vključili senzorično oceno obrokov, kar omogoča celovitejše razumevanje povezave med prehransko ustreznostjo in sprejemljivostjo obrokov pri starejših odraslih. Glede na naše rezultate lahko zaključimo, da je na področju zagotavljanja uravnotežene in senzorično ustrezne prehrane v DSO, tudi v prihodnje, potrebno izvajati še več, tudi strateških aktivnosti.

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Hranilna in senzorična ustreznost celodnevnih obrokov v domu starejših občanov

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# Dissecting the phenotypic diversity of Ethiopian barley (*Hordeum vulgare* L.) genotypes through variance components and multivariate analysis

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**Dissecting the phenotypic diversity of Ethiopian barley (*Hordeum vulgare* L.) genotypes through variance components and multivariate analysis**

**Abstract:** Exploring the genetic diversity of barley germplasm conserved in the gene bank is critical for climate-resilient breeding. This study explored the phenotypic diversity and associations of 10 quantitative agro-morphological traits among 229 Ethiopian barley genotypes to identify desirable traits and promising accessions. The experiment was conducted at Holeta Agricultural Research Center in 2023 using an augmented design. The result of the analysis of variance revealed significant variation among the studied genotypes. A significant variability and wide range of mean performance were observed for the studied traits. Key traits like heading and maturity, grain filling period, 1000-kernel mass, kernel number per spike, and productive tillers were the most discriminating traits contributing to the highest variability (46.9 %). Cluster analysis identified three major trait-specific clusters. Grain yield exhibits a moderate correlation with plant height and kernel number per spike. High phenotypic and genotypic coefficients of variation were obtained for grain yield, productive tillers, kernel number per spike, and spikelet per spike. In addition, high heritability and genetic advance were observed for days to heading, productive tillers, spike length, kernel number and spikelet per spike, and grain yield suggesting selective breeding for these traits is likely to be effective for crop improvement programs.

**Key words:** barley, characterization, genetic variability, conservation, multivariate, augmented RCBD

**Preučevanje fenoptipske raznolikosti etiopskih genotipov ječmena (*Hordeum vulgare* L.) na osnovi variance in multivariatne analize**

**Izvleček:** Preučevanje genetske raznolikosti ječmena shranjenega v genski banki je nujno potrebno za podnebno odporno žlahtnjenje. V raziskavi je bila preučevana fenotipska raznolikost in z njo povezane lastnosti desetih kvantitativnih agro-morfoloških lastnosti med 229 etiopskimi genotipi ječmena z namenom določiti željene lastnosti in obetavne akcesije. Izsledki analize variance so pokazali značilne raznolikosti med preučevanimi genotipi. Med preučevanimi lastnostmi je bila ugotovljena značilna variabilnost z širokim razponom poprečij. Ključne lastnosti kot so čas do klasenja, zrelost, obdobje polnjenja zrnja, masa 1000-zrn, število zrn na klas, in število fertilnih poganjkov so se najbolj razlikovale in so največ prispevale (46.9 %). S klastersko analizo so bili prepoznani trije specifični grozdi, glede na glavne lastnosti. Pridelek zrnja je pokazal zmerno korelacijo z višino rastlin in številom zrn na klas. Doseženi so bili veliki fenotipični in genotipični koeficienti raznolikosti za pridelek zrnja, plodne poganjke, število zrn na klas in število klaskov na klas. Dodatno je bila opažena velika dednost in genetska prednost v znakih kot so število dni do klasenja, v številu plodnih poganjkov, v dolžini klasa, v številu zrn in klaskov na klas ter v pridelku zrnja, kar nakazuje, da bi bilo selektivno žlahtnjenje za te lastnosti verjetno učinkovito v programih izboljšanja te poljščine.

**Ključne besede:** ječmen, ovrednotenje, genetska spremenljivost, ohranjevanje, multivaritna analiza, izboljšani bločni poskus

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**ABBREVIATIONS**

CSA- Central Statistical Authority; DAP-Diammonium phosphate; EBI- Ethiopian Biodiversity Institute; GCV-Genotypic coefficient of variance; $h^2$- Broad sense heritability; GAM- Genetic advance over mean; HARC- Holeta Agricultural Research Center; ICARDA- Intenational Center for Agricultural Research for the Dry Areas; IPGRI-International Plant Genetic Resource Institute; m. a. s. l- meter above sea level; PCV- Phenotypic coefficient of variance; PCs- Principal components

## 1 INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the earliest domesticated crops that was originating from its wild progenitor H. spontaneum (K. Koch) Thell. in the Fertile Crescent ~10,000 years ago (Badr et al., 2000; Harlan and Zohary, 1966). It has been cultivated in a wide range of agroecologies from high altitudes to regions with low rainfall. In Ethiopia, barley was domesticated for more than 5000 years (Harlan, 1968) and is one of the fifth most important traditional food crops next to teff, wheat, maize, and sorghum in terms of area acreage and production (CSA, 2021). Barley cultivation covers more than one million hectares in Ethiopia and contributing to 8 % of the nation's total cereal production (Abtew, 2019; Kaso, 2015). Due to its short maturity duration relative to other crops, it is hugely consumed by small-scale farmers in the highlands of Ethiopia for poverty reduction and hunger alleviation during the lean period (Rashid et al., 2019). More than 80 % of barley grown by Ethiopian farmers is for food as compared to malt barley (Kaso, 2015). Besides, the straw is used for animal feed during the dry season and for thatching of roofs (Kaso, 2015). Cognizant of this, it is named as "*Gebis ye ehil nigus*" in highlanders of Ethiopia to mean "barley the king of all crops" (Mohammed et al., 2016).

Ethiopia is a secondary center of genetic diversity for its native two-rowed deficient and irregular barley types (Harlan, 1969; Vavilov, 1951).The Ethiopian barley landraces are diverse in morphology (two and six rows and irregular types) and color (black, white, and pink) (Asfaw, 1988) and also preferred by foreign breeders because of quality traits such as disease resistance, drought tolerance, high lysine, and protein content (Bjørnstad et al., 1997; Engels, 1991; Milner et al., 2019). It is also reported that the diverse in altitude, soil types, climates, topography, geographical isolation for long periods together with the farming system and wide socio-cultural diversity are the main driving forces for the diversity of barley in Ethiopia (Harlan, 1969; Teklemariam et al., 2022). Like other cereals, barley production is heavily influenced by spatial and temporal variations in the environment (Dido et al., 2021) and thus exploring and harnessing the genetic diversity available in barley germplasm conserved in the gene bank is crucial for the improvement of the crop.

According to ICARDA (2025), around 400,000 barley accessions are conserved worldwide in gene banks, breeders, and research collections. In Ethiopia, barley has been conserved in-situ in community seed banks located in different agroecologies of the country and ex-situ in gene bank. For instance, Ethiopian Biodiversity Institute (EBI) has maintained the largest barley farmer varieties (> 17,000 (~4.25 %)) in its cold storage facilities as compared with other crops (https://ebi.gov.et/biodiversity/conservation/genetic-material-holdings/). In Ethiopia, farmer varieties or landraces constitute 90 % of the total land devoted to barley production (Hadado et al., 2009). In fact, landraces or farmer varieties exhibited both within and between variation as compared to the genetic uniformity of modern cultivars (Zhu et al., 2000). Thus, maintaining the genetic diversity in crops is required to sustain genetic improvement for polygenic traits, such as yield. Previous studies showed that landraces are the major constituent of useful genes for adaptation to biotic and abiotic stresses (Asfaw, 1988; Gegnaw & Hadado, 2014). Most specifically, recent studies by Megersa et al. (2015), Monteagudo et al. (2019), and Wosene et al. (2015) revealed that barley landraces have shown higher yield stability and comparable yield with improved varieties. As such, characterization, and evaluation of genetic variability between and within barley landraces is an essential component for sustainable conservation and improvement. Owing to these, numerous genetic diversity studies were carried out on Ethiopian barley in relation to altitude, eco-geographical distribution of isozyme and allozyme, and association of hordein and morpho-agronomic traits (Asfaw, 1988, 1989; Assefa et al., 2016; Bedasa et al., 2015; Engels, 1994; Fantahun et al., 2023b; Gadissa et al., 2021)

Multivariate analysis techniques such as principal component analysis (PCA), clustering, and correlation were commonly employed by researchers (Abebe et al., 2010; Angassa & Tesfaye, 2019; Derbew, 2020; Enyew et al., 2019; Fantahun et al., 2023a) to investigate the genetic relationship between genotypes and association of traits. Besides, genetic parameters such as genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV); heritability, and genetic advance were used by researchers (Addisu & Shumet, 2015; Kaur et al., 2022; Shiferaw et al., 2020; Shtaya et al., 2015) to explore genetic relationships between genotypes and the association of traits. Despite the enormous efforts made by national and international programs to conserve barley diversity, there is, however, little information is yet available as compared with the existing huge genetic resources in Ethiopia which remained underutilization of germplasm accessions preserved in the gene bank. Therefore, a rigorous characterization and evaluation are utmost important to have sufficient characterized accessions to researchers and breeders. In this context, the present study was conducted to explore the genetic variability and association of yield and yield-related components among 229 barley genotypes and identify superior traits and accessions that can contribute to future barley grain yield improvement program and conservation.

## 2 MATERIAL AND METHODS

### 2.1 PLANT MATERIALS AND EXPERIMENTAL SITE

A total of 232 barley genotypes comprised of 229 accessions obtained from the Ethiopian Biodiversity Institute and three improved check cultivars containing six-rowed food barley (HB-1307 and HB-1966) and two-rowed malt barley (Suba) (Figure 1B; Additional File 1, Sheet 1) released by Holeta agricultural research center (HARC) were characterized. The accessions were requested from the gene bank owing to the major barley growing regions, altitudinal class (1940-3546 m. a. s. l) and previous characterization history in the gene bank. The study was conducted in 2022/23 main growing season at the research field of HARC located in the Oromia regional state of West Shewa zone, Walmera woreda (Figure 1C) (9$^{o}$00’00” N, 38$^{o}$30’00” E, elevation 2400 meter above sea level. The temperature varied from 6-22 $^{0}$C with an average annual rainfall of 1144 mm and light soil (Nitosols and Vertosols (http://www.eiar.gov.et/holetta/).

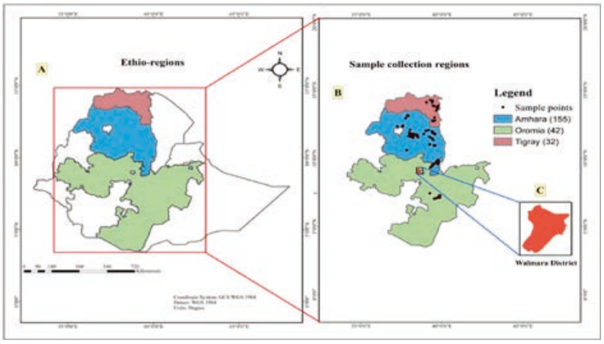

**Figure 1:** Map of Ethiopia showing the administrative regions (A), sample points where barley study materials were collected (B) and experimental site (C). Map was generated by ArcMap 10.2.2 (Esri, 2014). (All boundaries were prepared in accordance with Ethiopia Geoportal (Last updated on May 2020; https://ethiopia.africageoportal.com/)

The experiment was laid out by an Augmented block design where the check cultivars were replicated and appeared once in each block but test treatments appeared once in the design. The design assumes checks as fixed effects whereas entries as random effects. The experimental area contained a total of seven blocks. Each block had a total of 36 entries comprised of 33 accessions and three checks. To scrutinize the bias among treatments, randomization were done by Agricolae package of R-software (Mendiburu, 2020). Each accession was drilled by hand in 4 rows of 2 m$^2$ having with 2.5 m row length with 0.2 m row spaced at 85 kg ha$^{-1}$ seed rate (17 g plot$^{-1}$). The distance between each plot & block was 0.4 m & 1 m respectively. All the recommended agronomic practices were applied to each treatment equally before and after sowing according to the standard operational procedure of characterization (Ethiopian Biodiversity Institute, 2021). Artificial fertilizer was applied equally to each treatment before sowing at the rate of 36.4 g NPS and 11.2 g UREA split to 7.5 g (during sowing) and 3.7 g (during tillering after first weeding).

### 2.2 DATA COLLECTION

All data were electronically recorded through tablet using the FieldScorer Android App (http://www.katmandoo.org/Help/Fieldscorer4Android/index.html) after developing a standardized comma delimited (*csv*) trial and trait files (Tamirat Bejiga and Amare Seyoum, 2018). The traits were prepared in accordance with the barley descriptor list developed by Bioversity International (IPGRI, 1994) with minor modifications. Since three accessions were missed due to heavy water stress, data were exclusively collected for 229 genotypes (226 accessions and three checks). Quantitative data were collected on a plant and plot basis after randomly selecting and tagging 20 representative plants from each plot and the average of these samples was used for the analysis. Three phenological and seven agro-morphological traits were recorded in this study. Days to heading (DTH) were counted as the number of days from sowing to 50 % of plants fully emerged (Z55 stage (Zadoks et al., 1974)); days to maturity (DTM) was counted as the number of days from sowing to 75 % of the plants physiologically matured); grain filling period (GFP = DTM - DTH) was measured as the number of days from the time that half of the plants have started flowering to 75 % of the plants fully matured; plant height (PH, cm) was measured at full maturity from the ground level to the top of the spike excluding awns; productive tillers (PT) was counted on the number of effective tillers arising from the main plant; spike length (SL, cm) was taken from the neck to the base of the lemma awns of the uppermost grain; spikelet per spike (SPS) was counted as the number of spikelet raised from each node; kernel number per spike (KNS) was counted as the number of kernels obtained after threshing of spikes; thousand kernel mass (TKM, g) were measured after weighing and converting of 250 seeds and grain yield (GY, kg ha$^{-1}$) were obtained from the field plot (g m$^{-2}$) after extrapolating the area of 2 m$^2$ to hectare basis. TKM and GY were determined after adjusting to 12.5 % seed moisture content.

## 2.3 STATISTICAL DATA ANALYSIS

### 2.3.1 Estimates of analysis of variance and genetic variability

The quantitative data obtained from augmented design were subjected to analysis of variance (ANOVA), and genetic variability analysis using augmented RCBD bulk function of the augmented RCBD package (Aravind et al., 2021) in R software (Team, 2024). The phenotypic (), genotypic (and environmental variance ( were estimated from the ANOVA tables according to the expected value of the mean square described by Federer and Searle (1976) as follows:

$$\text{Genotypic variance } (\sigma^2 g) = \frac{\text{MSg-MSe}}{r} \tag{1}$$

$$\text{Phenotypic variance } (\sigma^2 p) = \sigma^2 g + \sigma^2 e \tag{2}$$

Where MSg-mean square due to the genotype, MSe-error mean square, r-number of replications for the check (control) genotypes, -Phenotypic variance, $\sigma^2$g -Genotypic variance, $\sigma^2$e - Environmental variance = eror mean square (MSe). The phenotypic and genotypic coefficients of variation (PCV & GCV) were estimated according to (Burton, 1951) and categorized as low (< 10), medium (10-20) and high (≥ 20) according to (Sivasubramaniam S, 1973).

$$\text{PCV} = \frac{\sigma^2 p}{\sqrt{\bar{x}}} \text{x}100 \tag{3}$$

$$\text{GCV} = \frac{\sigma^2 g}{\sqrt{\bar{x}}} \text{x}100 \tag{4}$$

Where $\sigma^2$p = Phenotypic variance, $\sigma^2$g = Genotypic variance and $\bar{x}$ = the grand mean of the trait under consideration.

Heritability (h$^2$) in the broad sense was estimated according to the method of (Lush, 1940) and categorized according to (Robinson, 1966) suggestion as low (< 30), medium (30-60) and high (≥ 60).

$$h^2 = \frac{\sigma^2 g}{\sigma^2 p} \text{x}100 \tag{5}$$

Genetic advance (GA) and genetic advance as a percent of the mean (GAM) were calculated by assuming the selection of more than 5 % of the genotypes estimated per the methods illustrated by (Johnson et al., 1955).

where GA - expected genetic advance, $\sigma_p$- phenotypic standard deviation on the mean basis, h$^2$ - Heritability in a broad sense, K = selection differential (where k = 2.06 at 5 % selection intensity).

$$\text{GA} = k \times \sigma_p \times \frac{h^2}{100} \tag{6}$$

The genetic advance (as a percent of the mean) (GAM) was computed to compare the extent of the predicted genetic advance of different traits under selection and categorized as low (< 10 %), moderate (10 %-20 %), or high ( > 20%) according to (Johnson et al., 1955)

$$\text{GAM} = \frac{\text{GA}}{\bar{x}} \text{x}100 \tag{7}$$

Where GAM - genetic advance as a percentage of the mean,  - Grand mean of the quantitative character

### 2.3.2 Multivariate analysis

The adjusted mean values generated from the ANOVA analysis were subsequently used for the computation of principal component analysis, pairwise correlations, and clustering analysis. Prior to analysis, the adjusted mean values were standardized to means of zero and variances of unity to avoid variations in the scales used during data collection (Manly, 1986; Sneath and Sokal, 1973) using the scales function in R software (Wickham & Seidel, 2020). The principal component and biplot analysis was computed using FactoMineR (Lê et al., 2008), and ggbiplot (Vu, 2011). A Spearman rank correlation was generated by GGally (Schloerke et al., 2024) and ggplot2 (Wickham et al., 2016) packages. A phylogenetic tree-based cluster analysis was performed after determining the cut-off point using the NbClust package (Charrad et al., 2014). The cluster analysis was hierarchically categorized by the Wards linkage clustering method. Besides, the genetic distance among genotypes and divergence among clusters was performed by using NbClust package (Charrad et al., 2014) and clv package (Nieweglowski & Nieweglowski, 2015) respectively.

## 3 RESULTS AND DISCUSSION

### 3.1 ANALYSIS OF VARIANCE (ANOVA)

The results of ANOVA presented in Table 1 showed a significant difference ($p \leq 0.05$) to highly significant ($p \leq 0.01$) difference among tests (accessions) and genotypes (entries) for PT, DTH, DTM, PH, SL, SPS, KNS, TGW, and GY. In contrast, a non-significant difference was observed for GFP. These notable significant differences among accessions revealed that substantial genetic variability exists which can be harnessed through selection. In agreement with our study, Zewodu et al. (2025) reported highly significant differences ($p \leq 0.01$) for DTH, DTM, PH, SL, SPS, KNS, TKM, and GY. Similarly, (Derbew, 2020; Dido et al., 2020) also reported a non-significant difference for GFP and a highly significant difference for other parameters. The two-food barley and one-malt barley improved varieties used for standard checks showed significant ($p \leq 0.05$) variation for GFP, PH, and PT to highly significant variation ($p \leq 0.01$) for the rest of the parameters except DTH which showed non-significant variation. A similar study on 102 Ethiopian food barley landraces and five checks (Bedasa et al., 2015) reported highly significant variation for PH, SL, KNS, and TKM. In addition, the mean square due to test (accessions) v/s checks were highly significant ($p \leq 0.01$) for DTM, DTH, GFP, PH, and KNS traits indicating the presence of significant differences between landraces and checks in these traits.

**Table 1:** The mean square of ANOVA for 10 morpho-agronomic traits of studied barley genotypes (n = 229) resulted from augmented design

| Sources | Mean squares† | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | DTH | DTM | GFP | PH | PT | SL | SPS | KNS | TKM | GY |
| Blocks (6) | 4.98$^{ns}$ | 14.43$^{ns}$ | 11.94$^{ns}$ | 140.52** | 0.64* | 1.02* | 3.25* | 5.62$^{ns}$ | 5.91* | 1304592.5** |
| Among genotypes (228) | 95.73** | 158.17** | 24.09$^{ns}$ | 108.73** | 0.49* | 1.35** | 36.77** | 108.9** | 26.6** | 690112.7** |
| Among check (2) | 11.48$^{ns}$ | 147.57** | 76.76* | 136.92* | 2.59** | 6.53** | 185** | 951.9** | 64.3** | 951074.6** |
| Among accessions (225) | 92.43** | 143.71** | 20.52$^{ns}$ | 102.22** | 0.47* | 1.31** | 35.6** | 100.3** | 26.4** | 688720.7** |
| Accessions vs. Check (1) | 1006.8** | 3432.7** | 721.4** | 1517.7** | 0.3$^{ns}$ | 0.54$^{ns}$ | 3.53$^{ns}$ | 369.3** | 4.77$^{ns}$ | 481399.7$^{ns}$ |
| Residuals (12) | 4.37 | 17.9 | 12.65 | 21.8 | 0.16 | 0.33 | 0.96 | 3.56 | 1.91 | 136411.1 |
| CV (%) | 2.85 | 3.69 | 8.58 | 5.5 | 22.4 | 7.02 | 4.75 | 5.52 | 2.81 | 17.6 |
| | Standard Errors | | | | | | | | | |
| A Test Treatment and a Control Treatment | 2.58 | 5.22 | 4.39 | 5.76 | 0.49 | 0.71 | 1.21 | 2.33 | 1.7 | 455.92 |
| Control Treatment Means | 1.12 | 2.26 | 1.9 | 2.5 | 0.21 | 0.31 | 0.52 | 1.01 | 0.74 | 197.42 |
| Two Test Treatments (Different Blocks) | 3.41 | 6.91 | 5.81 | 7.62 | 0.65 | 0.94 | 1.6 | 3.08 | 2.25 | 603.13 |
| Two Test Treatments (Same Block) | 2.95 | 5.98 | 5.03 | 6.6 | 0.56 | 0.82 | 1.39 | 2.67 | 1.95 | 522.32 |

† DTH-Days to 50 % heading, DTM-days to 75 % maturity, GFP-grain filling period, PH-plant height (cm), PT-productive tiller, SL-spike length (cm), SPS-spikelet per spike, TKM-thousand kernel mass (g) and GY-grain yield (Kg ha$^{-1}$); genotypes-accession + checks. ns p > 0.05, * p ≤ 0.05, ** p ≤ 0.01; CV- coefficient of variation; Numbers in parenthesis represented degree of freedom.

### 3.2 AGRO-MORPHOLOGICAL TRAIT DIVERSITY AND MEAN PERFORMANCE OF GENOTYPES

The mean performance of the studied barley genotypes for 10 morpho-agronomic traits is presented in Additional File 1, Sheet 2. In general, barley genotypes exhibited a high level of variation in yield and related yield components (Figure 2; Additional File 1, Sheet 2). Meanwhile, the mean value of DTH was 72.7 days and varied from 54 days to 99 days. On the other hand, the number of days to attain 75 % physiological maturity (DTM) had a mean of 113.7 days with a range of 89 days to 146 days. In line with our work, a combined two and six-row barley study by (Gadissa & Gudeta, 2023) and (Angassa & Tesfaye, 2019) showed a comparable DTH (56-97 & 56-93 days) and DTM (99-138 & 89-140 days) respectively. In contrast, a wide range of DTH (91-116 days) and DTM (117-174 days) were also reported on Ethiopian six-row barley genotypes by Alemayehu & Parlevliet (1997) and Megersa et al. (2015). In the present study, two-rowed barley accession-243314 had taken earlier heading (53 days) and maturing time (89 days) as compared to six-rowed barley accession-243209 (98 days and 146 days respectively) which is consistent with (Fantahun et al., 2023a; Kandić et al., 2018; Setotaw et al., 2010; Zewodu et al., 2025) studies. These results, however, disagree with Kaur et al. (2022) study who reported as two-rowed varieties took many days to mature as compared to six-rowed varieties. GFP on the other hand is found to be a crucial determinant of the genetic variation in rice (Yang et al., 2008) which ranged from 31 days to 59 days with a mean of 41 days and CV of 8.58. In a similar study, a wide range of GFP (23.88-34.22) with a mean of 30.11 was reported by (Megersa et al., 2015). In general, the difference in the number of days (DTH, DTM, and GFP) observed in our study is mainly attributed from the genetic background of genotypes (two-rowed, six-rowed, irregular, and hulless) and the mixture of materials used. This is from the fact that the barley genetic materials preserved in the gene bank are in the population form.

The regional diversity of agronomic traits (Table 2) also revealed that accessions collected from the Amhara region showed minimum values of DTH (54 days), DTM (89 days), and GFP (31 days) as compared to the maximum values of DTH (99 days) and DTM (146 days) observed for accessions collected from Oromia and local improved varieties. In fact, earliness is one of the key adaptive traits as demonstrated in wheat (Hyles et al., 2020; Mondal et al., 2013) that enables crops to escape terminal moisture stress and ensuring more reliable yields under unpredictable and re-

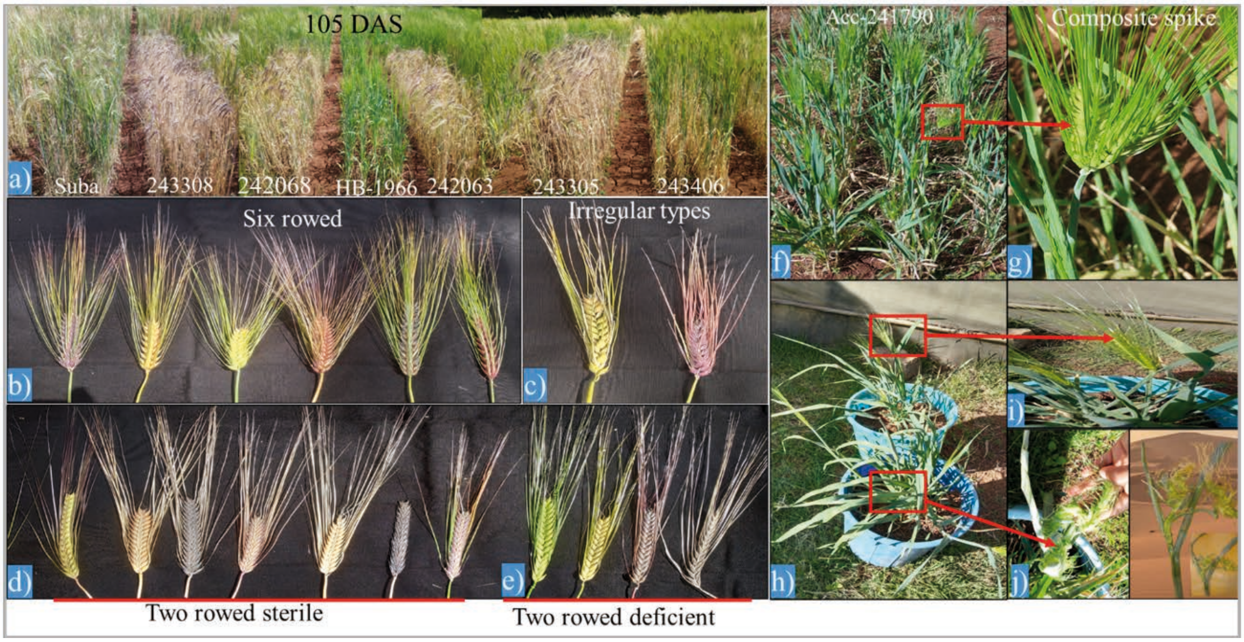

**Figure 2:** Diversity of some of barley genotypes based on maturity and grain filling period at 105 days after sowing (DAS) (a); row types i.e. six row (b) irregular types (c) two rowed sterile, long awn and awnless (d) two rowed deficient (e), hulless and composite barley (Acc-241790) (f &g) found in the field (top right corner) and a pot containing the same composite barley seeds planted in the Lathouse (h) and emerged two different spike forms i.e. a single spike (i) and triple-spikes (j)

source-limited conditions (Kandić et al., 2018; Megersa et al., 2015; Yadav et al., 2018) and hunger alleviation during the lean period (Amri et al., 2005; Rashid et al., 2019). Therefore, the wide range of variation present in the studied genotypes across regions for phenological traits allows breeders to improve grain yield for short and extended growing seasons accordingly.

Plant height (PH) is another important agronomic trait highly valued by farmers (for animal fodder, thatching roofs) and breeders (lodging tolerance) (Kaso, 2015; Kaur et al., 2022). In this study, the highest (108.4 cm) PH was found for accession 243296 and the lowest (57.8 cm) being for accession 241790 with a mean of 85.6 cm and CV of 5.5. Interestingly, accession 241790 (Figure 2f) is found to be six-rowed hulless and merely has composite spike forms with dwarf plant height (57.8 cm) and also a minimum grain filling period (32 days). To further elucidate the mode of inheritance, we planted the seed obtained from composite barley spike (Figure 2g) in two pots in Lathouse (Figure 2h) and it was found that > 99 % of the spikes that emerged were single spikes while only a single spike showed triple-spike form (Figure 2j). This stipulated that accession 241790 could have a potential poly-row-branch-spike (*prbs*) gene (Poursarebani et al., 2015, 2020; Terzi et al., 2017) which needs further investigation at the multi-location and genomic level.

Grain yield (GY) is the consequence of multiplicative interaction of intricate traits such as SL, SPS, KNS, PT, TKM, and rarely on PH (Dziurdziak et al., 2020; Kaur et al., 2022). In this study, a wide range of variation was observed for SL (5.2-11.5 cm), SPS (12.5-33.9), KNS (14.3-53.9), and TKM (31.9-62.4 g). Whereas, a narrow range of PT (0.23-3.7) with a mean value of 1.78 was observed for accession 243274 and 243307 respectively (Table 1). Previous studies (Angassa & Tesfaye, 2019; Dziurdziak et al., 2020, 2021) also reported a closer range and mean value of PH, SPS, KNS, SL and TKM A comprehensive phenotypic characterization and genetic diversity analysis of 6,778 barley germplasm (Kaur et al., 2022) also revealed a wide variation in PH (45.96-171.32cm), SL (3.44-13.73 cm), KNS (10.48-82.35) and TKM (12-68.6 g). Besides, the lowest GY (292.3 kg ha$^{-1}$) was recorded for two-rowed barley accession-243212 and the highest (3599.9 kg ha$^{-1}$) being for six-rowed accession-243313 with an average of 2118.3 kg ha$^{-1}$ (Additional File 1, Sheet 2). Derbew et al., (2013) reported a wide range of variation in GY (436-3752.5 kg ha$^{-1}$). It is evident from (Verma et al., 2021) study that two-rowed barley generally have a reduced number of grains per spike which is significantly affecting the final grain yield as compared to six-rowed ones. The regional diversity of morpho-agronomic parameters (Table 2) also indicated that accessions collected from Amhara have high SPS (50.5), TKM (62.9 g), and GY (3599.9 kg ha$^{-1}$) as compared to SPS (26.2), TKM (51.6) and GY (2225.8 kg ha$^{-1}$) of standard checks exploited in the study. In general, out of 229 barley genotypes, 119 (52 %) genotypes have given higher GY than the grand mean (2118.3 kg ha$^{-1}$) (Additional File 1, Sheet 2). Despite the poor attitude towards landraces on their low yield potential (Azeez et al., 2018), the average GY obtained in this study, however, is significantly higher than the improved varieties HB-1307 (2102.9 kg ha$^{-1}$), HB-1966 (2225.8 kg ha$^{-1}$), and Suba (1534.8 kg ha$^{-1}$). In agreement with our results, a multi-environment field study (Wosene et al., 2015) also indicated that Ethiopian barley landraces or farmer varieties show higher yield stability and comparable yield to improved varieties. A similar study conducted in Ethiopia (Lakew et al., 1997), Syria (Ceccarelli, 1996) and Spain (Yahiaoui et al., 2014) also supported our study and concluded that landraces outperformed improved varieties grown in favorable to modern stress conditions.

## 3.3 PAIRWISE CORRELATION ANALYSIS

Results of the pairwise Spearman rank correlation coefficient ($r_s$) among 10 morpho-agronomic traits are displayed in Figure 3. The estimates of trait association revealed that a strong positive association exists among pheno-agronomic traits such as DTH-DTM ($r_s = 0.9$), DTM-GFP ($r_s = 0.67$), and GY-PH ($r_s = 0.5$). In contrast, a strong negative correlation was observed between KNS and TKM ($r = -0.62$). However, most of the trait associations showed weak correlations

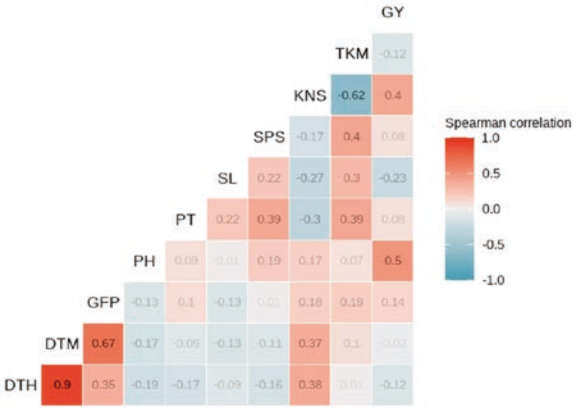

**Figure 3:** Spearman correlations of 10 morpho-agronomic traits of barley germplasm accessions. Keys: no correlation (−0.1 ≤ rs ≤ 0.1), weak correlation (−0.3 ≤ rs < −0.1 or 0.1 < rs ≤ 0.3), moderate correlation (-0.3 < rs < -0.5 or 0.3 < rs < 0.5) and strong correlation (rs < −0.5 or rs > 0.5) where rs is Spearman correlation. Trait abbreviations are given in Table 1 footnote.

**Table 2:** Mean, minimum, and maximum values of agro-morphological parameters of Ethiopian barley genotype and local released varieties based on regional diversity

| Region | Morpho- agronomic traits† | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DTH | DTM | GFP | PH | PT | SL | SPS | KNS | TKM | GY |
| Amhara | Mean | 72.9 | 113.7 | 40.8 | 86.7 | 1.7 | 8.2 | 20.5 | 35.0 | 48.7 | 2109.5 |
| (n = 153) | Min | 54 | 89 | 31 | 57.8 | 0.2 | 5.2 | 14.1 | 14.3 | 31.9 | 513.0 |
| | Max | 97 | 141 | 59 | 108.4 | 3.7 | 11.2 | 50.5 | 52.2 | 62.4 | 3599.9 |
| Oromia | Mean | 73.4 | 114.8 | 41.4 | 85.1 | 1.8 | 8.2 | 19.9 | 35.0 | 48.2 | 2190.7 |
| (n = 41) | Min | 59 | 96 | 31 | 67.6 | 0.6 | 5.4 | 16.0 | 18.8 | 31.9 | 292.3 |
| | Max | 99 | 146 | 55 | 99.7 | 3.0 | 11.5 | 39.9 | 49.0 | 56.3 | 3511.9 |
| Tigray | Mean | 70.0 | 111.0 | 41.0 | 82.1 | 2.1 | 8.1 | 21.5 | 26.6 | 52.4 | 2082.7 |
| (n = 32) | Min | 58 | 101 | 31 | 69.2 | 0.8 | 5.9 | 16.8 | 18.2 | 45.6 | 787.2 |
| | Max | 82 | 126 | 47 | 100.6 | 3.5 | 10.4 | 32.0 | 53.9 | 60.4 | 3055.3 |
| Local checks | Mean | 79.8 | 127 | 47 | 76.8 | 1.9 | 8.4 | 20.3 | 38.2 | 49.6 | 1954.5 |
| (n = 3) | Min | 79 | 124 | 45 | 73.6 | 1.4 | 7.8 | 16.8 | 24.9 | 46.1 | 1534.8 |
| | Max | 81 | 132 | 51 | 81.8 | 2.6 | 9.5 | 26.2 | 46.6 | 51.6 | 2225.8 |
| Accessions | Mean | 72.6 | 113.5 | 40.9 | 85.7 | 1.8 | 8.2 | 20.6 | 33.8 | 49.1 | 2120.5 |
| (n = 226) | Min | 54 | 89 | 31 | 57.8 | 0.2 | 5.2 | 14.1 | 14.3 | 31.9 | 292.3 |
| | Max | 99 | 146 | 59 | 108.4 | 3.7 | 11.5 | 50.5 | 53.9 | 62.4 | 3599.9 |
| Genotypes | Mean | 72.7 | 113.7 | 41.0 | 85.6 | 1.8 | 8.2 | 20.5 | 33.9 | 49.1 | 2118.3 |
| (n = 229) | Min | 54 | 89 | 31 | 57.8 | 0.2 | 5.2 | 14.1 | 14.3 | 31.9 | 292.3 |
| | Max | 99 | 146 | 59 | 108.4 | 3.7 | 11.5 | 50.5 | 53.9 | 62.4 | 3599.9 |

Min-minimum, Max-maximum; †Trait abbreviations are given in Table 1 footnote

(44 %) followed by no correlations (29 %), moderate correlations (18 %) and strong correlations (9 %). For instance, moderate correlation was found for KNS-GY ($r_s$ = 0.4), TKM-SPS ($r_s$ = 0.4), TKM-SL ($r_s$ = 0.3), TKM-PT ($r_s$ = 0.39), DTM-KNS ($r_s$=0.37), DTH-KNS ($r_s$ = 0.38), DTH-GFP ($r_s$ = 0.35) traits. Similarly, GY showed a weak correlation with all phenological traits. The better the correlations among traits the more likely it is that breeders can indirectly select one trait based on other traits with high heritability. From the breeders point of view, the strong correlation between GY and PH ($r_s$ = 0.5) is found to be problematic with respect to lodging and yield penalty. Therefore, reducing plant height without incurring grain yield is highly recommended to escape lodging as also reported by Monteagudo et al. (2019) study that found a positive correlation between PH and GY.

### 3.4 PRINCIPAL COMPONENT ANALYSIS

Principal component analysis (PCA) was estimated to elucidate the spatial distribution and diversity of genotypes and eventually identify the key traits that contributed utmost to the observed variations. In this study, the principal component analysis based on the correlation between 10 measured traits was used to discern the pattern of variation for the studied 229 barley genotypes (Table 3). The first four principal components (PCs) having Eigenvalue ≥ 1 and explained 74.3 % of the total cumulative variance present in the studied barley germplasm were retained (Table 3, Figure S1 a & b). Likewise, Yadav et al., (2018)reported Eigenvalue ≥1 that explained a total variability of 79.5 %. In addition, (Bedasa et al., 2015) and (Demissie & Bjørnstad, 1996) reported four principal components explaining a total variance of 72 % and 63 % in the studied 102 and 49 barley accessions respectively.

**Table 3:** Eigen values, Eigen vectors, variance, cumulative variance and contribution of variables to four dimensions obtained from the considered 10 morpho-agronomic traits

| Traits† | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| DTH | **-0.50** (24.99) | 0.19 (3.80) | 0.15 (2.15) | -0.26 (6.81) |
| DTM | **-0.55** (29.74) | 0.28 (8.06) | 0.05 (0.22) | -0.14 (1.92) |
| GFP | **-0.37** (13.36) | 0.31 (9.80) | -0.16 (2.68) | 0.16 (2.54) |
| PH | 0.09 (0.86) | -0.12 (1.35) | **-0.57** (32.69) | **-0.39** (15.58) |
| PT | 0.20 (4.12) | **0.38** (14.4) | -0.25 (6.36) | 0.09 (0.85) |
| SL | 0.22 (5.05) | 0.28 (7.89) | 0.09 (0.88) | **-0.66** (43.64) |
| SPS | -0.10 (1.08) | 0.23 (5.36) | **-0.35** (12.2) | **0.48** (23.52) |
| KNS | **-0.42** (17.65) | **-0.37** (13.7) | -0.16 (2.52) | -0.19 (3.43) |
| TKM | 0.16 (2.52) | **0.55** (30.58) | -0.18 (3.41) | -0.10 (0.99) |
| GY | -0.08 (0.64) | -0.22 (5.05) | **-0.61** (36.88) | -0.09 (0.73) |
| Eigenvalue | 2.7 | 2.0 | 1.8 | 1.0 |
| Variance (%) | 27.0 | 19.8 | 17.7 | 9.8 |
| Cumulative variance (%) | 27.0 | 46.9 | 64.6 | 74.3 |

Values in bold indicate the highest absolute values among all four PCs for each trait which represents relative contribution and values in parentheses indicate factor loadings with > 10 % positive contribution to the variation. †Trait abbreviations are given in Table 1 footnote.

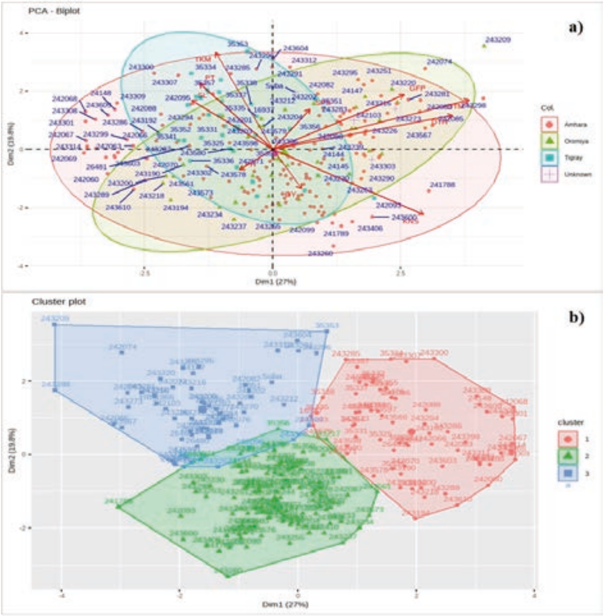

**Figure 4:** A PCA biplot analysis based on the mean value of 10 morpho-agronomic traits depicting the scatter of 226 barley accessions and three checks along with their measured traits and entire collection sites (a) and a Principal Component Analysis (PCA) biplot where the samples are colored by cluster (b). Trait abbreviations are given in Table 1. The vector indicates the traits and unknown represents improved varieties (Suba, HB-1307, and HB-1966). The more the deviation from the center towards the direction of the vector, the higher the value and vice versa. In other words, an angle of 90° or less between any two vectors (traits) indicates a positive correlation and vice versa.

Factor loadings were considered with > 10 % positive contribution (Figure S1c & d, Table 3) and indicated that DTH (24.99), DTM (29.74), KNS (17.65), and GFP (13.36) were mostly contributed for PC1 in the negative direction. The major traits that contributed to PC2 were TKM (30.58) in the positive direction and PT (14.4) and KNS (13.7) in the negative direction. Similarly, GY (36.88), PH (32.69), and SPS (12.2) were contributed for PC3 in the negative direction while SL (43.64) and PH (15.58) attributed for PC4 in the negative direction and SPS (23.52) in the positive direction. In line with our study (Dido et al., 2020) reported that DTH was heavily loaded in the negative direction for PC1. In contrast, the findings of (Fantahun et al., 2023a) indicated that PC1 was mostly affected by phenological traits in the positive direction while DTM was highly loaded.

To visualize the associations of genotypes and measured traits, a PCA scatter biplot was generated based on the first two PCs (Figure 4a). The first and the second PCA biplot accounted for 46.8 % of the total variability among the genotypes, indicating that DTH, DTM, GFP, TKM, KNS, and PT were considered as the most discriminating traits contributing to the highest variability (Figure 4a, Table 3). The genotypes positioned on the top right quadrant were characterized by late heading and maturity whereas, the genotypes clustered on the bottom right quadrant were associated with the highest GY and KNS; the genotypes positioned on the top left quadrant are associated with high TKM, PT and SL whereas, the genotypes assembled at the bottom left quadrant is characterized by high PH, early heading and maturity. On the other hand, the genotypes concentrated around the origin had similar genetic characteristics, while the genotypes that were found far from the origin are considered as unrelated genotypes (Figure 4a). Therefore, the distribution of the genotype over the four quadrants indicates the presence of significant genetic diversity among the studied barley genotypes. On the other hand, a PCA cluster biplot (Figure 4b) indicated that 229 barley genotypes were grouped in three major clusters.

## 3.5 CLUSTERING ANALYSIS

Clustering is grouping of individuals according to their similarities. Cluster analysis (Figure 5) based on 10 quantitative traits for 229 barley genotypes were grouped into three major clusters, each with a trait-specific pattern. The mean values of the three clusters for the 10 quantitative traits of the tested barley genotypes (Table 4) showed that a high cluster mean was recorded in cluster (I) for PH, KNS, and GY and the lowest for PT and TKM. These findings suggest that the genotypes in cluster I could be preferable for improving grain yield while maintaining high plant height, making them suitable for both animal fodder and human consumption. cluster (II) exhibited the lowest mean value for all phenological traits studied and the highest value for PH, PT, SL, and TKM. This indicates that the genotypes in cluster II could serve as a valuable genetic resource for developing early maturing and high-yielding barley cultivars, particularly suited for short rainy seasons. In contrast, the genotypes in cluster III were characterized by late heading and maturity, a high number of spikes per plant (SPS), and shorter plant height compared to the other clusters. These traits make cluster III genotypes a potential source to cross-hybridize and develop dwarf barley varieties suitable for extended rainy seasons.

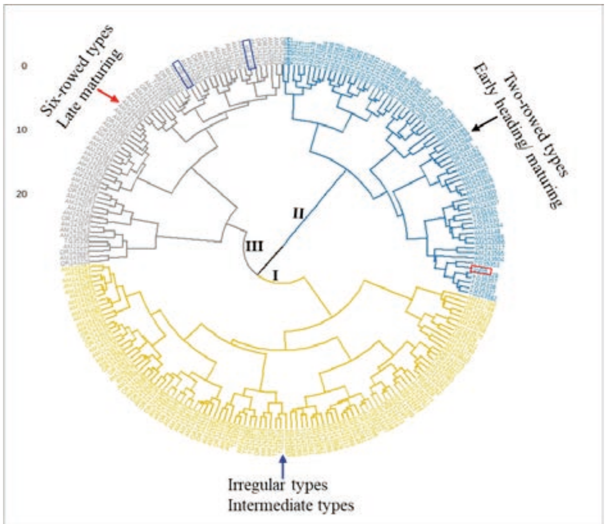

**Figure 5:** A circular dendrogram-based phylogenetic tree of 229 barley genotypes obtained from 10 quantitative traits. Genotypes highlighted in the blue box (cluster III) indicate six-rowed improved barley genotypes and red box (cluster II) for two-rowed ones. The prefix presented before each accession number indicates their region of collection (e.g. AM-Amhara, OR-Oromia, TG-Tigray).

Among the 229 genotypes studied, 97 (42.4 %) were grouped in Cluster I, which exhibited intermediate phenological characteristics. Meanwhile, 71 genotypes (31 %) were classified as early-maturing (cluster II), and 61 genotypes (26.6 %) were late-maturing (cluster III) (Table 4). Statistical tests further confirmed that the observed differences among clusters were significant for DTH, DTM, GFP, PH, SL, SPS, KNS, and GY. However, no significant differences were found for TKM and PT (Table 4). Overall, the genotypes were clustered based on morphological similarity rather than their collection region. This is consistent with previous studies (Bedasa et al., 2015; Benlioglu et al., 2025; Kaur et al., 2022), which found that cluster analysis groups genotypes according to morphological traits and less on their geographic location. Figure 5 further illustrates that two-

**Table 4:** Cluster means and size among three clusters generated from 10 morpho-agronomic traits

| Clusters | $^{\dagger}$Traits | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | DTH | DTM | GFP | PH | PT | SL | SPS | KNS | TKM | GY | Cluster size |
| I | 72.4 | 112.0 | 39.5 | 85.6 | 1.51 | 7.9 | 19.9 | 41.3 | 45.3 | 2398.0 | 97 (42.4%) |
| II | 64.1 | 102.8 | 38.7 | 86.9 | 2.19 | 8.6 | 21.0 | 22.6 | 52.5 | 1893.5 | 71 (31%) |
| III | 83.0 | 129.1 | 46.1 | 83.6 | 1.74 | 8.2 | 21.7 | 35.1 | 51.4 | 1929.3 | 61 (26.6%) |
| *p*-values | 0.027 | 0.008 | 0.049 | 0.000 | 0.211 | 0.036 | 0.007 | 0.000 | 0.632 | 0.000 | |

The means/medians of the clusters are significantly different for the traits if $p < 0.01$ (highly significant), $p < 0.05$ (significant), and $p > 0.05$ (non-significant); †Trait abbreviations are found in Table 1 footnote

**Table 5:** Intra (diagonal and bold) and inter (above diagonal) cluster distance (D2) among three clusters generated from 229 barley genotypes

| Clusters | I | II | III |
|---|---|---|---|
| I | **3.83** | 4.51 | 4.57 |
| II | | **4.08** | 5.06 |
| III | | | **3.69** |

rowed barley accessions were predominantly grouped in cluster II, six-rowed ones in cluster III, and a mix of irregular and six-rowed barley spikes in cluster I. Similar findings were reported by (Fantahun et al., 2023a).

The Euclidean distance analysis revealed the highest genetic distance (10.02) between accession numbers AM-242060 (early heading/maturing) and OR-243209 (late heading/maturing). In contrast, the smallest genetic distance (0.68) was observed between accessions TG-25357 and TG-35329, both collected from the Tigray region, indicating their similar genetic backgrounds (Additional File 1, Sheet 3). These results align with the findings of Angassa and Mohammed (2021), who reported a genetic distance range of 0.61 to 8.06 among 138 barley genotypes. The inter-cluster distances based on the Euclidean dissimilarity matrix (Table 5) showed the highest distance (5.06) between clusters II and III, suggesting that these clusters are genetically distinct and makes them ideal candidates for crossing to create high-yielding, early-maturing, or dwarf barley varieties with diverse traits. In contrast, the lowest (4.51) inter-cluster distance between clusters I and II revealed that the clusters are genetically similar or less divergence in traits. On the other hand, the highest intra-cluster distance (4.08) observed in cluster II suggests greater genetic diversity or variability among the genotypes within the cluster. In contrast, the low intra-cluster distance (3.69) in cluster III indicates that the genotypes within a cluster are genetically similar or closely related. Overall, the highest and lowest values of intra-cluster and inter-cluster distances provide important insights into the genetic diversity and relationships among the clusters and genotypes.

## 3.6 ESTIMATES OF VARIANCE COMPONENTS, HERITABILITY, AND GENETIC ADVANCE

The genetic variability (Table 6) was computed to estimate the level of genetic variation for environmental and genetic factors. High magnitude of phenotypic variance ($\sigma^2p$) and genotypic variance ($\sigma^2g$) were observed in GY, DTM, PH, KNS and DTH and low magnitude in SL, PT, SPS, TKM and GFP traits. Because of variations in mean and measurement units, the value of phenotypic and genotypic variants cannot be directly compared across traits (Gadissa et al., 2021). Therefore, coefficient of variation at phenotype and genotype levels was used to compare variability among traits (Table 6). The coefficient of variation study indicated that the highest PCV and GCV were noted on GY (39.18 and 35.08), PT (38.41 and 31.19), KNS (29.56 and 29.03) and SPS (28.83 and 28.44) indicating high degree of genetic variability and

**Table 6:** Genetic variability analysis of 10 yield and yield-related traits of 229 barley genotypes

| Trait | Mean ± SE | Range | $\sigma^2p$ | $\sigma^2g$ | $\sigma^2e$ | GCV (%) | PCV (%) | ECV | $h^2b$ (%) | GA | GAM (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DTH | 72.70 ± 0.62 | 54-99 | 92.43 | 88.07 | 4.37 | 12.92 | 13.23 | 2.88 | 95.28 | 18.90 | 26.01 |
| DTM | 113.7 ± 0.79 | 89-146 | 143.71 | 125.80 | 17.90 | 9.87 | 10.55 | 3.72 | 87.54 | 21.65 | 19.04 |
| GFP | 41.0 ± 0.33 | 31-59 | 20.52 | 7.87 | 12.65 | 6.84 | 11.04 | 8.67 | 38.34 | 3.58 | 8.73 |
| PH | 85.6 ± 0.56 | 57.8-108.4 | 102.22 | 80.42 | 21.80 | 10.47 | 11.81 | 5.45 | 78.67 | 16.41 | 19.16 |
| PT | 1.78 ± 0.04 | 0.2-3.7 | 0.47 | 0.31 | 0.16 | 31.19 | 38.41 | 22.42 | 65.95 | 0.93 | 52.26 |
| SL | 8.2 ± 0.07 | 5.2-11.5 | 1.31 | 0.98 | 0.33 | 12.07 | 13.97 | 7.03 | 74.68 | 1.76 | 21.52 |
| SPS | 20.7 ± 0.4 | 12.5-33.9 | 35.60 | 34.63 | 0.96 | 28.44 | 28.83 | 4.75 | 97.29 | 11.98 | 57.87 |
| KNS | 33.9 ± 0.66 | 14.3-53.9 | 100.27 | 96.71 | 3.56 | 29.03 | 29.56 | 5.57 | 96.45 | 19.92 | 58.82 |
| TKM | 49.1 ± 0.34 | 31.9-62.4 | 26.39 | 24.48 | 1.91 | 10.07 | 10.45 | 2.81 | 92.78 | 9.83 | 20.01 |
| GY | 2118.3 ± 43.3 | 292.3-3599.9 | 688720.7 | 552309.5 | 136411.1 | 35.08 | 39.18 | 17.44 | 80.19 | 1372.97 | 64.82 |

Keys: $\sigma^2$ p-phenotypic variance, $\sigma^2$ g-genotypic variance, $\sigma^2$ e-environmental variance, GCV-genotypic coefficient of variance, PCV-phenotypic coefficient of variance, ECV-environmental coefficient of variance, h2-heritability in the broad sense, GA-genetic advance, GAM-genetic advance over mean. PCV, GCV and GAM values < 10 % (low), 10-20 % (medium), > 20 % (high); h2 values < 30 (low), 30-60 (medium), and ≥ 60 (high). Trait abbreviations are given in Table 1 footnote.

close relationship with yield attributing traits and thus could be a useful trait for screening yield traits. Whereas medium PCV and GCV values were observed on the remaining traits except for DTM and GFP which showed low GCV. The magnitude of PCV value was generally greater than GCV for all studied traits as expected and revealed that the apparent variation was due to the environmental factors up to some extent. However, the magnitude of the difference was low for all studied traits suggesting the observed variation was due to genetic factors and less influenced by environmental factors. Moreover, the low GCV estimates for DTM and GFP suggest a direct selection on these traits for improvement depending on phenotype expression. In line with our findings, high coefficient of variation (PCV and GCV) were reported by (Addisu & Shumet, 2015; Angassa, 2021; Zewodu et al., 2025) in barley for SPS, KNS and GY traits.

Broad sense heritability ($h^2$) of a trait represents the extent at which the genotypes are affected by environment and environmental errors (You et al., 2017). High heritability was obtained on SPS (97.29 %) followed by KNS (96.45 %), DTH (95.28 %), TKM (92.78 %), DTM (87.54 %), GY (80.19 %), PH (78.67 %), SL (74.68 %) and PT (65.95 %) while low for GFP (38.34 %) (Table 6). Similar results have been reported by Addisu and Shumet (2015) and Zewodu et al. (2025). Estimation of heritability coupled with genetic advance offers the most suitable condition for selection (Singh and Chaudhary, 1985). High heritability and genetic advance were observed for GY, KNS, SPS, PT, DTH, SL and TKM traits (Table 6) which revealed the presence of additive genetic variance in the trait (Johnson et al., 1955) and suggested that reliable crop improvement could be effective through phenotypic selection. Similar studies in barley (Angassa, 2021; Kaur et al., 2022; Kumar & Shekhawat, 2013; Shrimali et al., 2017) and flax (Abeje & Zewodu, 2024; You et al., 2017) also indicated that estimates of heritability with genetic advance are more reliable than individual consideration of the parameters for selection.

## 4 CONCLUSION

Understanding the extent and magnitude of phenotypic diversity and trait associations is essential for the maximum utilization of gene bank materials in breeding programs and conservation. This study examined the phenotypic diversity of 226 Ethiopian barley landraces and three improved varieties using variance components and multivariate analysis. Significant genetic variability was found in most traits, except for GFP, which had non-significance differences and medium heritability. Key traits such as grain yield (GY), days to maturity (DTM), and plant height (PH) showed notable variability, aiding breeders in selection efforts. PCA biplot analysis identified early (Acc-243314, 242070 and 242060) and late heading/maturing types (Acc-243210, 243209,243273,243298 and 243074). In agreement with PCA Biplot results, cluster analysis grouped genotypes into three major clusters according to their morphological similarity and rarely on their geographical location. Notably, Accession 241790 collected from the Amhara region exhibited low GFP, low PH, and high GY (above the grand mean of 2118.3 kg $ha^{-1}$) and rarely have composite spike forms. The study suggests further multi-location trials to explore genes linked to composite spikes and other valuable traits. In conclusion, the superior grain yield of landraces compared to improved varieties highlights the potential of Ethiopian farmer varieties for breeding programs aimed at enhancing food security.

## 5 ACKNOWLEDGMENTS

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## 6 CONFLICT OF INTEREST

The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## 7 SUPPLEMENTARY MATERIALS

The following list of supplementary tables and figures are attached with this manuscript.

**Additional File 1** (Xlsx): Geospatial data of the studied barley accessions (sheet 1; Passport), Mean performance of 229 barley genotypes based on 10 morpho-agronomic traits (Sheet 2; Mean performance) and Euclidean distance between the studied barley genotypes (Sheet 3; Genetic distance)

**Figure S1:** A scree plot of four dimensions/PCs (a&b) and contribution of variables (c&d)retained from 10 morpho-agronomic traits

## 8 FUNDING

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## 9 CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

**Gizie Abeje:** Conceptualization, Visualization, Validation, Methodology, Investigation, Data curation, Formal analysis, Resources, Project administration, Supervision, Funding acquisition, Writing–original draft, Writing-review, and editing.

**Alemayehu Zewodu**: Resources, Visualization, Supervision, Investigation, Data curation, Validation, Writing-review, and editing.

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# Identifying gene actions to rice yield and its components traits under both normal and drought conditions

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**Identifying gene actions to rice yield and its components traits under both normal and drought conditions**

**Abstract:** Eight rice genotypes were selected to conduct all crosses in diallel Method 2 Model 1 design to generate genotypes tolerance to drought stress with high yield potential. The parents Giza179 and Sakha 108 and the crosses UYR 3472 × GZ10739 and GZ10487 × Giza179 reported the highest mean values of the grain yield plant$^{-1}$ under drought conditions. Two and twelve hybrids revealed desirable highly and highly significant positive heterotic effects as a deviation over mid parent, one hybrid obtained significant positive heterotic over better-parent under natural condition. Regarding the grain yield plant$^{-1}$, four and six parents showed desirable significant and highly significant positive general combing ability effects under either natural and drought conditions, respectively. Five and thirteen hybrids showed desirable significant and highly significant positive specific combining ability effects under both natural and drought conditions, respectively regarding grain yield plant$^{-1}$. The high significant and positive associated among grain yield and days to heading, relative water content, number of panicles plant$^{-1}$, thousand grain mass and water use efficiency for both natural and drought environments. The crosses GZ10739 × WAB 638-1, GZ10739 × IRAT112 and WAB 638-1 × IRAT 112 had superior in most agronomic traits at drought condition.

**Key words:** combing ability, heterosis, heatmap, correlation, drought

**Določanje delovanja genov, ki vplivajo na pridelek riža in njegove komponente v normalnih in sušnih razmerah**

**Izvleček:** Izbranih je bilo osem genotipov riža za izvedbo dialelnih križanj (Metoda 2 Model 1) za vzgojo genotipov s toleranco na sušo in velikim potencialom pridelka. Križanci starševskih genotipov Giza179 in Sakha 108 in križanci UYR 3472 × GZ10739 ter GZ10487 × Giza179 so imeli največji poprečni pridelek zrnja na rastlino v razmerah suše. Dva in dvanajst križancev je imelo zaželjen velik, značilen pozitivni heterotični učinek kot odklon od poprečja staršev, eden od križancev je v naravnih razmerah dosegel značilno pozitiven heterotični učinek glede na boljšega starša. Glede na pridelek zrnja na rastlino so štiri in šest staršev pokazali zaželjene značilne, pozitivne, splošne kombinacijske učinke v naravnih razmerah kot v razmerah suše. Pet in trinajst križancev je v naravnih in sušnih razmerah pokazalo zaželjene in visoko značilne pozitivne specifične kombinacijske učinke glede na pridelek zrnja na rastlino. Ugotovljena je bila zelo značilna in pozitivna povezava med pridelkom zrnja, dnevi do klasenja, relativno vsebnostjo vode, številom latov na rastlino, maso tisočih zrn in učinkovitostjo izrabe vode v normalnem in sušnem okolju. Križanja GZ10739 × WAB 638-1, GZ10739 × IRAT112 in WAB 638-1 × IRAT 112 so bila superiorna v vseh agronomskih lastnostih v razmerah suše.

**Ključne besede:** sposobnost kombinacije, heteroza, dvodimezionalna barvna predstavitev podatkov, korelacija, suša

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## 1 INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important agriculture food crops for more than half of the world population. Moreover, it is a very important cereal crop in Egypt for both consumption and export (Zheng et al., 2020)breeders manually perform extensive testing of hybridizations between restorer and sterile lines, which is a laborious and time-consuming process. Here, we report that a modified flowering stimulant containing methyl jasmonate (MeJA. Egypt is facing two major challenges, food security and water shortage, particularly in the terminal areas. Water shortage is one of the most limiting factors in more than 30 % of paddy fields in Egypt, where the developed varieties cannot perform well under water shortage. Therefore, the development of water stress tolerant genotypes that maintain good yield under drought condition is a priority area of rice research for sustainable rice production (Abdallah et al., 2013). Drought affects rice at morphological, physiological, and molecular levels where it reduces plant height, tiller numbers plant$^{-1}$, panicle length, leaf area, increase sterility, induce leaf rolling, reduce water potential, stomatal closure, dry matter accumulation and decrease photosynthetic capacity. Moreover, high temperature is often accompanied with low water supply, so the primary aim of rice breeding program is developing promising varieties tolerant to both types of stresses (Abdel-Hafez et al., 2017). To increase the yield potential and to reduce the yield gap, improving varietal adaptability, yield potential and grain quality under stress are needed. Conventional plant breeding played important role in developing rice varieties tolerant to abiotic stresses, which is very complex due to the intricate interactions between stress factors that affect plant growth and development. Rice breeders follow all breeding methods to improve characters of the newly developed varieties to cover the continuous changes in breeding objectives. The information about the used varieties is essential to plan a successful breeding program. The diallel analysis provides information on the nature and amount of genetic parameters and general and specific combining abilities of parents and their crosses. Combining ability analysis is a useful method to assess the potential ability of varieties. This will be helpful in choosing parents in hybridization programs to get desirable segregates (Gaballah et al., 2021a). The information about gene effects including additive and dominance gene effects, non-allelic gene interaction i.e. additive × additive, additive × dominance, and dominance × dominance are very important and essential to rice breeders for improving new rice varieties under both natural as well as adverse conditions (Hassan et al., 2023). Today yield safety has gained more significance because of the forecasted climate changes. It can only be improved if future breeding attempts are based on the valuable new knowledge acquired on yield (Barnabas et al., 2008). The major objectives of the study to identify some rice genotypes tolerant to drought stress conditions as donors for rice breeding program, to study the variation among the studied genotypes and their characters in terms of drought tolerance in all traits and determine the general and specific combining abilities, genetically analysis in parents and the possible crosses of rice to identify the most important traits and genotypes for drought tolerance in rice.

## 2 MATERIALS AND METHODS

The present study was carried out at the Experimental Farm of the Rice Research and Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt, during rice growing seasons, 2022 and 2023. Eight rice genotypes namely UYR 2184, UYR 3472, GZ10487, GZ10739, WAB 638-1, IRAT 112, Giza 179 and Sakha 108 representing a wide range of diversity for several agronomic characters and drought resistance measurements were selected for this study. The names, pedigree and origin of these genotypes are presented in Table (1).

### 2.1 EXPERIMENTAL WORK

In 2022 season, seeds of the parental genotypes were sown at three planting dates (May 1st, May 10th and May 20th) in order to overcome the differences in time of flowering. Seedling of each parent were individuals transplanted in the permanent field in five rows. Each row was five meters long and contained 25 hills. At flowering the eight parents were diallel crossed in all possible

**Table 1:** Origin and type of the eight rice genotypes used as parents in the studied half diallel cross.

| Genotype | Origin | Type | drought tolerance |
|---|---|---|---|
| UYR 2184 | Ukraine | Japonica | Sensitive |
| UYR 3472 | Ukraine | Japonica | Sensitive |
| GZ10487 | Egypt | Japonica | Moderate |
| GZ10739 | Egypt | Japonica | Tolerant |
| WAB 638-1 | Côte d'Ivoire | Indica | Tolerant |
| IRAT 112 | Côte d'Ivoire | Indica | Tolerant |
| Giza 179 | Egypt | Indica/Japonica | Moderate |
| Sakha 108 | Egypt | Japonica | Sensitive |

combinations without reciprocal giving a total of twenty-eight crosses according to Griffing's, Method 2, Model 1 includes both parental lines and their $F_1$ hybrids, assumes genotypes are fixed (not randomly sampled) and ignores maternal/paternal effects (reciprocal differences). The observed phenotypic value of a hybrid ($Y_{ij}$) is modeled

$$Y_{ij} = \mu + g_i + g_j + s_{ij} + e_{ij}$$

Where: μ: grand mean. $g_i$, $g_j$: GCA effects of parents i and j. $s_{ij}$: SCA effect for the cross between i and j and $e_{ij}$: random error term. In 2023 season, all hybrid combination seeds and their parental were sown in May 10th in the nursery and after 30 days the seedlings of the parents and their $F_1$ crosses were individually transplanted in randomized complete blocks design with three replications. The rows were five meters long with 20 cm between rows and comprised 25 hills each of a single plant. The genotypes were grown under both natural and drought conditions. Drought stress was imposed by applying irrigation every 12 days without remaining water. The amount of irrigation water applied was measured by flowmeter. Weeds were chemically controlled by applying 2 liters of Saturn/ feddan after transplanting by five days. Nitrogen fertilizer was applied according to both recommended rate and application time. The physical and chemical analysis of soil properties of the experimental field in both 2022 and 2023 seasons are shown in Table 2, according to USDA soil survey manual (2017).

## 2.2 STUDIED CHARACTERS

Growth characters, plant samples were collected randomly from each treatment at tillering, panicle initiation, booting and heading stages to estimate the following characters. Days to 50 % heading (day): It was counted as number of days starting from date of sowing till the date of main panicle appearance. Plant height (cm): It was measured from the soil surface to the tip of the tallest panicle of each plant at maturity. Number of tillers plant$^{-1}$: It was determined by counting the number of tillers per hill, when the panicles were at the full ripe stage.

**Table 2:** Physical and chemical analysis of the experimental soil (0-30 cm) depth.

| Characters | 2022 season | 2023 season |
|---|---|---|
| Mechanical analysis: | | |
| Sand % | 16.20 | 15.95 |
| Clay % | 55.05 | 55.60 |
| Silt % | 28.75 | 28.45 |
| Chemical analysis: | | |
| 1-pH | 7.3- 7.8 | 7.9- 8.3 |
| 2-Organic matter % | 1.45 | 1.67 |
| 3-E.C (ml mhose) | 1.95 | 2.04 |
| 4- ($Ca^{++}$) | 2.00 | 1.97 |
| 5- ($Mg^{++}$) | 20.00 | 23.00 |
| 6-($Na^+$) mg 100 $g^{-1}$ | 93.54 | 99.00 |
| 7-($K^+$) mg 100 $g^{-1}$ | 42.00 | 39.40 |
| 8-P mg 100 $g^{-1}$ | 2.06 | 1.90 |
| 9-Mg mg 100 $g^{-1}$ | 85.00 | 90.00 |
| Fe ppm | 8.08 | 7.13 |
| Mn ppm | 3.08 | 4.55 |
| Zn ppm | 0.93 | 0.85 |
| Cu ppm | 1.00 | 1.83 |

Panicle length (cm): The main panicle was measured from panicle base to the upper most of the panicle (IRRI, 1996). Flag leaf area (cm$^2$): It was measured at the maximum tillering stage according to the formula reported by Yoshida et al. (1976) as follows:

$$\textit{Leaf area } (cm^2) = K \times \textit{leaf length } (cm) \times \textit{leaf width } (cm)$$

K, is the correction factor which can be used for all growth stages, except the seedling and maturity stages. It approximately equals 0.75. Chlorophyll content (SPAD): Total chlorophyll content was determined by using chlorophyll analytical apparatus; chlorophyll meter SPAD-502 Minolta Camera Co. Ltd., Japan. Five flag leaves were measured from the widest part of the leaf of the main culm for each entry in all replications. Relative water content (RWC): It was determined by the method of Barrs and Weatherley, (1962). Leaf rolling: It was recorded based on methods proposed by De Datta et al., (1988); Loresto and Chang (1994) as shown in Table (3).

Grain yield and its components: The plants were individually harvested and threshed separately to determine the grain yield and yield components. Number of panicles plant$^{-1}$: It was determined by counting the number of panicles plant$^{-1}$ at the ripening stage. 1000-grain

**Table 3:** Scores and symptoms for leaf rolling and drought resistance at vegetative stage.

| Scores | Reaction | Leaf rolling | Leaf firing |
|---|---|---|---|
| 0 | Highly resistant | No symptoms of stress | No symptoms |
| 1 | Resistant | No rolling | Slight leaf tip drying |
| 3 | Moderately resistant | Partially rolled, unrolled in evening | Leaf tip drying extende to 1/4 in top three leave |
| 5 | Intermediate | Partially; unrolling at late evening and early morning. | Half of younger leaf blades dried, all lower leaf dried. |
| 7 | Susceptible | Complete, unrolling in morning | 3/4 of younger leaf blade dried. |
| 9 | Highly susceptible | Like tube; no unrolling in morning | All leaves dried |

mass (g): It was recorded by the mass of 1000 random chosen filled grains plant$^{-1}$ at ripening stage. Sterility percentage (%): The unfilled grains of the main panicle were separated and counted. Then, sterility percentage was calculated as follows:

*Sterility (%) = (number of unfilled grains/panicle)/ Number of total spikelets) * 100*

Grain yield plant$^{-1}$ (g): It was recorded as the mass of grain yield of each individual plant and adjusted to 14 % moisture content. Water use efficiency (g ml$^{-1}$): It was measured by the following formula

*WUE = Dry matter production (g)/Water loss (ml)*

Water use efficiency was expressed in $\text{gDM-ml}^{-1}\text{H}_2\text{O}$

## 2.3 STATISTICAL ANALYSIS

The data were analyzed by using the ordinary analysis of variance to test the significant differences among the genotypes studied. Data of each experiment was analyzed using procedure of Griffing (1956) (method II, model I) to estimate the general and specific combining ability.

## 2.4 ESTIMATION OF HETEROSIS

Heterosis as proposed by Mather and Jinkes (1982) was determined as the deviation of the $F_1$ means from mid-parents (MP) and better parent (Bp) means and expressed as percentage. Average heterosis value for each trait was computed as parents vs. $F_1$ hybrids as follows: Mid-parents Heterosis (MP):

$$HMP\ \% = \frac{\bar{F}_1 - M\bar{P}}{M\bar{P}} \times 100$$

To test the significance of heterosis for the above case, LSD. values were estimated according to the following formula suggested by Wynne et al. (1970).

$$LSD\ for\ HMP = t^{0.05}_{0.01}\frac{\sqrt{3MSe}}{r}$$

Better -parent heterosis (BP):

$$HBP\ \% = \frac{\bar{F}_1 - B.\bar{P}}{B\bar{P}} \times 100$$

The better parent for any character is that having the higher mean value, except for heading date and plant height, where the better parent which has the lower mean value. Appropriate LSD values were calculated to test the significance of the heterotic effects for better parent, according to the following formula, suggested by Wynne et al. (1970).

$$LSD\ for\ HBP = t^{0.05}_{0.01}\ \frac{\sqrt{2MSe}}{r}$$

Where: : Mean value of the first generation, MP: Mid-parents value, BP: Value of the better parent, t: Tabulated (t) value at certain probability level and given degrees of freedom, (df), MSe: Error mean squares from the analysis of variance, r: Number of replications.

## 2.5 ESTIMATION OF COMBINING ABILITY

The statistical analysis was done using method 2, model 1 of Griffing (1956). This a fixed and most appropriate model as its requirements can be met experimentally. Data were analyzed according to Cochran and Cox (1967) to test the significance of the different genotypes. The analysis of variance was calculated for each character. Then, the differences between genotypes were further partitioned to GCA and SCA, follow Griffing (1956) (Method2, Model1) as a fixed model. Variances due to general and specific combining abilities were estimated. The mathematical model for a single cross value ($X_{ij}$) as given by Griffing (1956).

### 2.6 PHENOTYPIC CORRELATION COEFFICIENT AND HEATMAP

Phenotypic correlations between all possible pairs of the studied characters were estimated by the application of the formula used by Dewey and Lu (1959), as follows: Phenotypic correlation

$$(R_{ph}) = \{(Cov.\ XY)/ \sqrt{}\,(Var.\ X * Var.Y)\}$$

Where, Cov. XY = Phenotypic covariance between X and Y characters, Var. X and Var. Y = Variance of characters X and Y. The "R" values were tested for significance against the minimum significant "R" values calculated for different degrees of freedom listed by Snedecor (1956). All calculated were done by Genes Package Software Version 2019. A heatmap with clustering was generated using the heatmap package implemented in R software (R Core Team 2021).

## 3 RESULTS

### 3.1 ANALYSIS OF VARIANCE

The mean square values for days to heading, plant height, number of tillers plant$^{-1}$, panicle length, flag leaf area, leaf rolling, chlorophyll content and relative water content were given in Table 4, 5 and 6. Genotypes, parents, crosses and parent vs. crosses mean squares were found to be highly significant for all the traits studied in both natural and drought condition, except leaf rolling under natural conditions. Both general and specific combining ability variance were found to be highly significant for all characters studied at the two environments except leaf rolling and water use efficiency. The GCA: SCA ratios were found to be higher than unity under all environments for most traits, except leaf rolling under natural condition was less than unity.

### 3.2 MEAN PERFORMANCE

The mean performance for genotypes of all characters studied in both environments are given in supplementary Table 7, 8 and 9. For days to heading, the parents, UYR 2184 and UYR 3472 and the crosses UYR 2184 × UYR 3472, and UYR 2184 × Giza179 gave the earliness mean values under both natural and drought condition. With respect to plant height, the most desirable mean values towards dwarfing were obtained from the parents UYR 2184 and UYR 3472 under both natu-

**Table 4:** Mean of squares estimates of the ordinary and combining ability analysis for growth characters under both natural and drought conditions.

| Source of variance | df | Days to heading (day) | | Plant height (cm) | | Number of tillers plant$^{-1}$ | | Panicle length (cm) | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | D | N | D | N | D | N | D |
| Replication | 2 | 1.03 | $8.90^{**}$ | $36.21^{**}$ | $4.69^{*}$ | 0.65 | 0.57 | 0.12 | 0.32 |
| Genotypes | 35 | $322.56^{**}$ | $264.35^{**}$ | $367.75^{**}$ | $378.21^{**}$ | $45.54^{**}$ | $37.33^{**}$ | $45.09^{**}$ | $27.09^{**}$ |
| Parents (P) | 7 | $770.33^{**}$ | $581.99^{**}$ | $656.87^{**}$ | $496.36^{**}$ | $63.27^{**}$ | $68.03^{**}$ | $72.22^{**}$ | $48.98^{**}$ |
| Crosses (C) | 27 | $214.87^{**}$ | $190.65^{**}$ | $305.54^{**}$ | $359.80^{**}$ | $41.72^{**}$ | $29.67^{**}$ | $38.37^{**}$ | $22.38^{**}$ |
| P vs. Cross | 1 | $96.01^{**}$ | $30.57^{**}$ | $23.35^{**}$ | $48.21^{**}$ | $24.51^{**}$ | $29.19^{**}$ | $36.56^{**}$ | 0.94 |
| Error | 70 | 1.57 | 1.67 | 4.51 | 2.93 | 2.21 | 1.60 | 1.84 | 1.49 |
| GCA | 7 | $382.17^{**}$ | $263.99^{**}$ | $371.10^{**}$ | $403.76^{**}$ | $44.57^{**}$ | $34.26^{**}$ | $42.63^{**}$ | $28.56^{**}$ |
| SCA | 28 | $38.86^{**}$ | $44.15^{**}$ | $60.45^{**}$ | $56.65^{**}$ | $7.83^{**}$ | $6.99^{**}$ | $8.13^{**}$ | $4.15^{**}$ |
| Error | 70 | 0.52 | 0.56 | 1.50 | 0.98 | 0.74 | 0.53 | 0.61 | 0.50 |
| GCA:SCA | --- | 9.83 | 5.98 | 6.14 | 7.13 | 5.69 | 4.90 | 5.24 | 6.89 |

N: natural condition, D: drought condition, $^{*}$ and $^{**}$ significant at 0.05 and 0.01 levels of probability, respectively.

**Table 5:** Mean square estimates of the ordinary and combining ability analysis for growth characters under both natural and drought conditions.

| Source or variance | df | Chlorophyll content (SPAD) | | Flag leaf area (cm$^2$) | | Leaf rolling | | Relative water content | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | D | N | D | N | D | N | D |
| Replication | 2 | $5.42^{**}$ | 1.87 | $5.08^{**}$ | 1.64 | 0.53 | $13.21^{**}$ | 0.74 | $30.98^{**}$ |
| Genotypes | 35 | $74.76^{**}$ | $69.08^{**}$ | $90.83^{**}$ | $69.29^{**}$ | 0.83 | $3.37^{**}$ | $47.97^{**}$ | $77.36^{**}$ |
| Parents (P) | 7 | $32.67^{**}$ | $38.47^{**}$ | $122.60^{**}$ | $87.94^{**}$ | 0.80 | $12.67^{**}$ | $53.31^{**}$ | $85.25^{**}$ |
| Crosses (C) | 27 | $82.05^{**}$ | $78.72^{**}$ | $77.62^{**}$ | $64.24^{**}$ | 0.85 | 0.94 | $44.54^{**}$ | $77.77^{**}$ |
| P vs. cross | 1 | $172.63^{**}$ | $22.93^{**}$ | $225.26^{**}$ | $75.20^{**}$ | 0.29 | 3.82 | $103.03^{**}$ | $11.18^{**}$ |
| Error | 70 | 3.18 | 2.54 | 1.82 | 8.21 | 0.42 | 0.39 | 2.95 | 3.85 |
| GCA | 7 | $26.11^{**}$ | $35.15^{**}$ | $84.84^{**}$ | $47.55^{**}$ | 0.25 | 1.25 | $53.94^{**}$ | $66.48^{**}$ |
| SCA | 28 | $24.62^{**}$ | $19.99^{**}$ | $16.64^{**}$ | $16.98^{**}$ | 0.28 | 1.09 | $6.50^{**}$ | $15.61^{**}$ |
| Error | 70 | 1.06 | 0.85 | 0.61 | 2.74 | 0.14 | 0.13 | 0.98 | 1.28 |
| GCA:SCA | --- | 1.06 | 1.76 | 5.10 | 2.80 | 0.87 | 1.14 | 8.30 | 4.26 |

N: natural condition, D: drought condition, $^{*}$ and $^{**}$ significant at 0.05 and 0.01 levels of probability, respectively.

**Table 6:** Mean squares estimate of the ordinary and combining ability analysis for grain yield and its components characters under both natural and drought conditions.

| Source or variance | df | Number of panicles plant$^{-1}$ | | 1000-grain mass (g) | | Sterility percentage | | Grain yield plant$^{-1}$ (g) | | Water use efficiency (g ml$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | D | N | D | N | D | N | D | N | D |
| Replication (Rep) | 2 | 1.16 | $3.68^{*}$ | 0.79 | 1.81 | 0.33 | $6.02^{**}$ | $5.22^{**}$ | $25.48^{**}$ | $0.00^{**}$ | $0.02^{**}$ |
| Genotypes (G) | 35 | $48.91^{**}$ | $37.10^{**}$ | $39.06^{**}$ | $24.82^{**}$ | $12.10^{**}$ | $265.39^{**}$ | $112.17^{**}$ | $153.23^{**}$ | $0.04^{**}$ | $0.14^{**}$ |
| Parents (P) | 7 | $70.13^{**}$ | $68.84^{**}$ | $26.13^{**}$ | $21.38^{**}$ | $12.14^{**}$ | $423.90^{**}$ | $128.93^{**}$ | $123.08^{**}$ | $0.05^{**}$ | $0.11^{**}$ |
| Crosses (C) | 27 | $44.05^{**}$ | $28.58^{**}$ | $40.87^{**}$ | $23.86^{**}$ | $8.63^{**}$ | $210.91^{**}$ | $104.43^{**}$ | $165.96^{**}$ | $0.04^{**}$ | $0.15^{**}$ |
| P vs. F1 | 1 | $31.49^{**}$ | $44.92^{**}$ | $80.79^{**}$ | $74.67^{**}$ | $105.24^{**}$ | $626.92^{**}$ | $203.87^{**}$ | $20.58^{**}$ | $0.07^{**}$ | $0.02^{**}$ |
| Error | 70 | 1.47 | 1.00 | 1.37 | 1.69 | 1.61 | 2.43 | 1.00 | 0.52 | 0.00 | 0.00 |
| GCA | 7 | $49.19^{**}$ | $34.61^{**}$ | $19.07^{**}$ | $9.06^{**}$ | $6.46^{**}$ | $94.55^{**}$ | $153.34^{**}$ | $210.43^{**}$ | 0.06 | 0.19 |
| SCA | 28 | $8.08^{**}$ | $6.80^{**}$ | $11.51^{**}$ | $8.08^{**}$ | $3.42^{**}$ | $86.94^{**}$ | $8.40^{**}$ | $11.24^{**}$ | 0.00 | 0.01 |
| Error | 70 | 0.49 | 0.33 | 0.46 | 0.56 | 0.54 | 0.81 | 0.33 | 0.17 | 0.00 | 0.00 |
| GCA: SCA | --- | 6.09 | 5.09 | 1.66 | 1.12 | 1.89 | 1.09 | 18.25 | 18.73 | 18.25 | 18.73 |

N: natural condition, D: drought condition, $^{*}$ and $^{**}$ significant at 0.05 and 0.01 levels of probability, respectively.

ral and drought condition, and the dwarfing values were obtained from the crosses UYR 2184 × UYR 3472 and UYR 2184 × Sakha 108 under natural conditions, in addition to the crosses GZ10487 × Sakha108 and GZ10739 × Sakha108 under drought condition. Concerning the number of tillers plant$^{-1}$, the parents Giza179 and Sakha 108 got the highest mean values under natural condition and the parents GZ10487 and WAB 638-1 showed the highest mean values under drought condition and the crosses UYR 3472 × IRAT 112 and UYR 3472 × Giza179 gave the highest mean values under natural and drought condition. While, the parents UYR 2184 and UYR 3472 and the crosses UYR 2184 × UYR 3472 and UYR 2184 × Giza 179 showed the lowest mean values under natural and drought condition. For panicle length, the parents IRAT 112 and Sakha 108 illustrated the highest mean values under natural condition therefor the parents WAB 638-1 and Sakha 108 gave the highest mean values under drought condition, However the crosses GZ10739 × WAB 638-1 and WAB 638-1 × IRAT 112 gave the highest mean values under natural condition in addition to the crosses GZ10739 × WAB 638-1 and WAB 638-1 × Sakha108 gave the highest mean values under drought condition. While, the parents UYR 2184 and UYR 3472 and the crosses UYR 2184 × UYR 3472 and UYR 2184 × IRAT 112 obtained the lowest mean values under natural and drought condition. With respect to chlorophyll content, the parents WAB 638-1 and Giza 179 showed the highest mean values under either natural and drought condition, and the crosses UYR 3472 × GZ10739 and WAB 638-1 × Sakha108 gave the highest mean values under natural condition, and the crosses UYR 2184 × GZ10739 and WAB 638-1 × Sakha108 displayed the highest mean values under drought condition. Concerning the flag leaf area, the parents WAB 638-1 and GZ10739 showed the highest mean values of the flag leaf area under either natural and drought condition, and the crosses GZ10739 × WAB 638-1 and GZ10739 × IRAT112 gave the highest mean values of the flag leaf area under natural condition, and the crosses GZ10739 × IRAT112 and WAB 638-1 × IRAT 112 provided the highest mean values of the flag leaf area under drought condition. While, the parents UYR 3472 and GZ10487 and the crosses UYR 2184 × Giza 179 and UYR 2184 × Sakha 108 got the lowest mean values under both natural and drought conditions. For leaf rolling, the parents UYR 2184 and GZ10487 gave the highest mean values under natural condition, and the parents UYR 2184 and Sakha 108 gave the highest mean values under drought condition, and the crosses UYR 2184 × UYR 3472 and IRAT 112 × Sakha108 provided the highest mean values under natural condition, and the crosses UYR 2184 × UYR 3472 and Giza179 × Sakha108 obtained the highest mean values under drought condition. While, the parents UYR 3472 and Giza 179 and the crosses GZ10487 × GZ10739, GZ10739 × WAB 638-1 and WAB 638-1 × IRAT 112 got the lowest mean values of the leaf rolling under both natural and drought conditions. concern relative water content, the parents Sakha 108 and IRAT 112 showed the highest mean values under both natural and drought conditions, the crosses UYR 3472 × Giza179 and UYR 3472 × IRAT 112 got the highest mean values under both natural and drought conditions, respectively. On the other hand, the parent UYR 3472 gave the lowest mean values under natural condition, and the parent GZ10487 illustrated the lowest mean values under drought condition, and the cross UYR 2184 × GZ10739 gave the lowest mean values under both natural and drought conditions. Concerning the number of panicles plant$^{-1}$, the parents GZ10487 and WAB 638-1 gave the highest mean values under natural and drought condition, and the crosses UYR 3472 × Giza179 and IRAT 112 × Giza179 obtained the highest mean values under natural condition. and the crosses UYR 3472 × GZ10487 and UYR 3472 × Giza179 got the highest mean values under drought condition. While, the parents Sakha 108 and UYR 2184 gave the lowest mean values under natural and drought condition, respectively. and the crosses UYR 2184 × UYR 3472 and UYR 2184 × GZ10739 provided the lowest mean values under natural and drought condition, respectively. For 1000-grain mass, the parents UYR 2184 and IRAT 112 and the crosses UYR 3472 × IRAT 112 and UYR 3472 × Giza179 showed the highest mean values of the 1000-grain weight under natural conditions. And the parents GZ10739 and IRAT 112 and the crosses UYR 3472 × Giza179 and WAB 638-1 × IRAT112 provided the highest mean values under drought condition. on the other hand, the parent GZ10487 and the cross UYR2184 × GZ10487 gave the lowest mean values under natural and drought condition, respectively. Concerning the sterility percentage, the parents GZ10739 and Sakha 108 and the crosses IRAT 112 × Giza179 and Giza179 × Sakha108 displayed the lowest mean values of the sterility percentage under natural conditions, and the parents IRAT112 and Giza179 and the crosses GZ10739 × WAB638-1 and WAB 638-1 × IRAT 112 gave the lowest mean values of the sterility percentage under drought conditions. While, the parent IRAT 112 and the cross UYR 2184 × Giza 179 showed the highest mean values under natural condition, and the parent UYR 3472 and the cross GZ10487 × Sakha108 provided the highest mean values under drought condition. Concerning the grain yield plant$^{-1}$, the parents GZ10739 and Sakha 108 and the crosses GZ10487 × Sakha108 and IRAT 112 × Giza179, showed the highest mean values of the grain yield plant$^{-1}$ under natural conditions, and the parents Giza179 and Sakha 108 and the crosses UYR 3472 × GZ10739 and GZ10487 × Giza179 got the highest mean values of the grain yield plant$^{-1}$ under drought conditions. While, the parents WAB 638-1 and IRAT112 and the crosses UYR 2184 × WAB 638-1 and UYR 2184 × IRAT 112 gave the lowest mean values under either natural and drought condition, respectively. For water use efficiency, the parents GZ10739 and Sakha 108 and the crosses GZ10487 × Sakha108 and IRAT 112 × Sakha108 got the highest mean values under natural condition, and the parents Giza179 and Sakha 108 and the crosses UYR 3472 × GZ10739 and GZ10487 × GZ10739 showed the highest mean values of the water use efficiency under drought conditions. While, the parents WAB 638-1 and IRAT112 and the crosses UYR 2184 × WAB 638-1 and UYR 2184 × IRAT 112 provided the lowest mean values under either natural and drought condition, respectively.

### 3.3 HETEROSIS:

Useful heterosis, expressed as the percentage deviation of $F_1$ mean performance from mid and better parents for all studied traits in each natural and drought conditions. Concerning days to heading, data in Table 7, 8, 9 and 10 showed that, eight and fifteen hybrids revealed desirable significant and highly significant negative heterotic effects as a deviation from the mid parent, 17 and 21 combinations were obtained significant and highly significant negative heterotic over better parent under both natural and drought condition, respectively. on the other hand, undesirable highly and highly significant positive heterotic effects were recorded for 11 and 10 hybrids over mid parent and 6 and 6 combinations were obtained significant and highly significant positive heterotic over better parent at the natural and drought environments, respectively. With respect to plant height, 10 and 12 hybrids revealed desirable highly and highly significant negative heterotic effects as a deviation over mid parent, 18 and 23 combinations were showed significant and highly significant negative heterotic over better parent under both natural and drought condition, respectively. on the other hand, undesirable highly and highly significant positive heterotic effects were recorded for 10 and 9 hybrids over mid parent and 2 hybrids under natural condition and there are no crosses under drought condition significant and highly significant positive heterotic over better parent at the natural and drought environments, respectively. For number of tillers plant$^{-1}$, seven and five hybrids showed significant and highly significant positive heterosis over mid-parent, two and one crosses obtained significant and highly significant positive heterotic over better-parent under both natural and drought condition, respectively. Otherwise, the negative heterotic over mid-parent was found with eleven crosses over mid-parent, and 17 and 26 crosses over better parent under natural and drought condition, respectively. Regarding panicle length, five hybrids showed significant and highly significant positive heterosis over mid-parent, one cross obtained significant and highly significant positive heterotic over better-parent under both natural and drought condition, respectively. Otherwise, the negative heterotic over mid-parent was found with eleven and seven crosses over mid-parent, and 19 and 23 crosses over better parent under natural and drought condition, respectively. For to chlorophyll content showed that, 18 and 13 hybrids showed significant and highly significant positive heterosis over mid-parent, 8 and 1 crosses obtained significant and highly significant positive heterotic over better-parent under both natural and drought condition, respectively. However, the negative heterotic over mid-parent was found with 3 and 10 crosses over mid-parent, and 7 and 22 crosses over better parent under natural and drought condition, respectively. With respect to flag leaf area, three and two hybrids revealed desirable highly and highly significant positive heterotic effects as a deviation from the mid parent, one hybrid obtained significant positive heterotic over better parent under natural condition, but there is no hybrid under drought condition, respectively. on the other hand, undesirable highly and highly significant negative heterotic effects were recorded for 18 and 10 hybrids over mid parent and 21 and 24 over better parent at the natural and drought environments, respectively. These findings indicated that these crosses would be very important in drought breeding program for selecting high flag leaf area plants. With regard to leaf rolling, one and six hybrids showed significant and highly significant positive heterosis over mid-parent, there are no crosses under natural condition, but there are six hybrids obtained significant and highly significant positive heterotic over better-parent under drought condition, respectively. There for, the negative heterotic over mid-parent was found with two and twelve crosses over mid-parent, and 1 and 8 crosses over better parent under natural and drought condition, respectively. Regarding relative water content, two and six hybrids revealed desirable significant and highly significant positive heterotic effects as a deviation from the mid parent, one GZ10487 × Giza179 was significant positive heterotic over better parent under natural condition but, there is no hybrid under drought condition, respectively. on the other hand, undesirable highly and highly significant negative heterotic effects were recorded for 18 and 11 hybrids over mid parent and 23 and 26 over better parent at the natural and drought environments, respectively. Concerning the number of panicles plant$^{-1}$, six and five hybrids revealed desirable highly and highly significant positive heterotic effects as a deviation over mid parent, two hybrids obtained significant positive heterotic over better-parent under natural condition and there is no hybrid under drought condition, respectively. There for, undesirable highly and highly significant negative heterotic effects were recorded for 13 and 15 hybrids over mid parent and 17 and 26 from over better parent at the natural and drought environments. Regarding 1000-grain weight three and four hybrids revealed desirable highly and highly significant positive heterotic effects as a deviation over mid parent, three hybrids obtained significant positive heterotic over better-parent under natural condition and there is no hybrid under drought condition, respectively. On the other hand, undesirable highly and highly significant negative heterotic effects were recorded for 16 and 17 hybrids over mid parent and 20 and 28 from over better parent at the natural and drought environments. With respect to sterility percentage, there

**Table 7:** Percentage of heterosis over mid and better parents for growth characters under natural and drought conditions

| Crosses | Days to heading (day) | | | | Plant height (cm) | | | | Number of tillers plant-1 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MP | | BP | | MP | | BP | | MP | | BP | |
| | N | D | N | D | N | D | N | D | N | D | N | D |
| UYR 2184 × UYR 3472 | -2.62 | -12.1** | -0.53 | -9.14** | 5.11** | 0.20 | 4.66 * | -7.45** | 4.64 | 25.38** | 3.268 | -18.7** |
| UYR 2184 × GZ10487 | 11.25** | 0.43 | 36.92** | 18.46** | -2.45 | -1.71 | -8.65** | -8.15** | 9.97 | -1.51 | -11.141 | -29.3** |
| UYR 2184 × GZ10739 | 8.90 ** | -0.87** | 34.87** | 17.44** | 3.03 | 3.04* | -3.52* | -3.55 | -14.2** | -16.3** | -29.7** | -38.7** |
| UYR 2184 × WAB 638-1 | 12.35** | 12.67** | 40.00** | 27.69** | -12.8** | -9.15** | -25.39* | -20.1** | 31.73** | 51.52** | 26.45** | 8.2** |
| UYR 2184 × IRAT 112 | 9.64** | 6.96** | 40.00** | 26.15** | 11.65** | 13.66** | -4.06** | -3.161* | -0.64** | -0.28 | -14.01* | -21.5** |
| UYR 2184 × Giza 179 | 9.32** | 5.73** | 35.38** | 23.08** | 3.49* | 5.88** | 0.000 | -4.15* | -24.59 | -18.7** | -39.5** | -40.8** |
| UYR 2184 × Sakha 108 | 14.97** | 11.45** | 47.69** | 32.31** | 2.45 | 2.86 | 0.000 | -7.45** | -35.31 | -18.11 | -47.9** | -35.0** |
| UYR 3472 × GZ10487 | 19.49** | 12.50** | -1.05 | -7.69** | 3.09* | 2.22 | -3.84* | -2.12 | 12.42* | 16.75** | -10.06* | -13.4** |
| UYR 3472 × GZ10739 | 12.42** | 12.00** | -7.29** | -8.36** | 3.44* | -0.19** | -3.52* | -4.27** | -15.6** | -27.3** | -31.5** | -44.9** |
| UYR 3472 × WAB 638-1 | 15.48** | 16.28** | -5.15** | -1.96 | 17.48** | 17.38** | 0.26 | 5.57** | 7.82 | -3.93 | 2.204 | -26.1** |
| UYR 3472 × IRAT 112 | 15.10** | 11.16** | -6.93** | -8.79** | 11.86** | 10.89** | -4.22** | -3.44** | -5.12 | -4.38 | -18.7** | -19.4** |
| UYR 3472 × Giza179 | -15.3** | -21.2** | -30.2** | -34.8** | 6.05** | 4.74** | 2.055 | -1.85 | -20.5** | -29.1** | -36.9** | -46.6** |
| UYR 3472 × Sakha 108 | 10.75** | 11.31** | -10.7** | -9.06** | 1.44 | -0.99 | -1.399 | -7.77** | 20.00** | 28.63** | -4.382 | 6.250 |
| GZ10487 × GZ10739 | -0.52 | -3.65** | -1.04 | -7.37** | -5.77** | -4.44** | -5.76** | -13.7** | -1.92 | -35.3** | -3.75 | -45.5** |
| GZ10487 × WAB 638-1 | -5.21** | -7.20** | -6.19** | -14.0** | 3.75** | 6.12** | -5.75** | -0.61 | 10.13* | -11.55 | -8.054 | -39.3** |
| GZ10487 × IRAT112 | -5.10** | -7.69** | -7.92** | -11.5** | -12.3** | -14.2** | -20.0** | -22.4** | 13.62** | 15.81** | 4.69 | -15.4** |
| GZ10487 × Giza179 | -3.32** | -6.67** | -3.82** | -11.5** | 2.32 | -7.17** | -0.962 | -21.1** | 12.88** | 4.18 | 11.69* | -11.4** |
| GZ10487 × Sakha108 | -1.52 | -4.92** | -4.90** | -8.42** | -2.3** | -2.47 | -6.41** | -17.6** | -3.87 | -4.26 | -4.382 | -27.5** |
| GZ10739 × WAB 638-1 | -4.32** | -8.30** | -4.81** | -15.6** | -10.6** | -8.94** | -18.8** | -14.8 | 14.47** | -12.74* | -2.9 | -40.3** |
| GZ10739 × IRAT112 | -6.94** | -9.12** | -9.24** | -13.5** | -9.12** | -11.2** | -17.1** | -19.8** | -7.81 | -8.12 | -13.5** | -32.9** |
| GZ10739 × Giza179 | 0.00 | -3.69 | 0.00 | -9.38** | 6.95** | -1.32 | 3.5* | -16.34** | -11.64** | -19.74** | -14.19** | -31.38** |
| GZ10739 × Sakha108 | 1.68 | -1.63 | -1.31 | -5.90** | -2.68 | -12.55** | -6.73** | -26.28** | -14.29** | 1.38 | -16.33** | -23.15** |
| WAB 638-1 × IRAT 112 | -1.01 | 1.14** | -2.97** | -8.25** | -0.66 | 5.51** | -1.047 | -7.33** | -9.49 | -4.18 | -18.7** | -21.8** |
| WAB 638-1 × Giza179 | -5.70 | -6.13** | -6.19** | -15.81** | -9.20** | -4.38** | -19.89** | -28.53** | -19.05** | -21.45** | -32.98** | -36.99** |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WAB 638-1 × Sakha108 | -1.51** | -9.60** | -3.92** | -17.53** | -12.57** | -18.31** | -23.56** | -39.26** | -14.06** | -6.09 | -28.55** | -19.63** |
| IRAT 112 × Giza179 | -0.51 | -7.78** | -2.97** | -17.82** | -9.09** | -9.40** | -19.52** | -28.76** | -28.01** | -33.71** | -34.29** | -44.10** |
| IRAT 112 × Sakha108 | -2.46** | -12.20** | -2.94** | -20.46** | -14.29** | -15.01** | -24.80** | -33.50** | -26.14** | -28.26** | -32.27** | -47.45** |
| Giza179 × Sakha108 | -2.62 | -9.39** | -2.94** | -14.58** | -0.35 | 3.45* | -1.370 | -12.67** | -19.95** | -17.20** | -20.36** | -40.21** |

MP: Mid-Parent, BP: Better Parent, N: natural condition, D: drought condition, * and ** significant at 0.05 and 0.01 levels of probability, respectively.

**Table 8:** Percentage of heterosis over mid and better parents for growth characters under natural and drought conditions

| Crosses | Panicle length (cm) | | | | Chlorophyll content (SPAD) | | | | Flag leaf area (cm$^2$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MP | | BP | | MP | | BP | | MP | | BP | |
| | N | D | N | D | N | D | N | D | N | D | N | D |
| UYR 2184 × UYR 3472 | -3.28 | -17.20* | -8.88 | -39.57** | -2.63 | -5.15 | -2.70 | -16.38** | -4.83 | 16.94 | -6.05 | -18.56* |
| UYR 2184 × GZ10487 | -8.66 | 1.37 | -22.40** | -20.13** | 8.11** | 13.18** | 2.13 | -7.44** | -10.00** | -6.96 | -17.04** | -21.84** |
| UYR 2184 × GZ10739 | -9.72 | 12.90* | -18.60** | -12.162* | 7.07* | 7.29* | 3.62 | -8.72** | -7.78** | 25.28** | -19.30** | 8.91 |
| UYR 2184 × WAB 638-1 | -7.89 | 8.35 | -25.88** | -17.42** | 13.67** | 27.72** | 6.17* | 0.50 | -4.42 | 5.88 | -19.98** | -8.51 |
| UYR 2184 × IRAT 112 | 13.83** | 17.83** | -9.195* | -8.73 | 9.37** | 12.00** | -1.06 | -11.98** | -11.14** | -15.51* | -24.51** | -29.71** |
| UYR 2184 × Giza 179 | -11.66* | -5.15 | -23.14** | -27.13** | 12.56** | 9.89** | 3.62 | -7.37** | 1.98 | 3.56 | -1.25 | -19.38** |
| UYR 2184 × Sakha 108 | -1.01 | 1.46 | -16.99** | -20.49** | -4.73 | -8.75** | -10.78** | -21.27** | -5.68* | -18.50* | -9.74** | -35.17** |
| UYR 3472 × GZ10487 | 17.60** | 26.21** | -4.86 | 6.16 | 7.59** | 14.88** | 1.71 | -5.68* | 8.55** | 8.11 | -1.13 | -14.42 |
| UYR 3472 × GZ10739 | 6.98 | 25.29** | -8.53 | 18.478** | 6.78* | 13.01** | 3.41 | -3.48 | 4.80 | -11.59 | -9.30** | -29.01** |
| UYR 3472 × WAB 638-1 | -11.31* | -14.95** | -31.76** | -31.06** | 8.28** | 1.12 | 1.21 | -20.09** | -17.78** | 13.22 | -31.88** | -7.57 |
| UYR 3472 × IRAT 112 | -30.72** | -18.27** | -47.12** | -32.54** | 19.89** | 9.45** | 8.523** | -13.63** | -12.07** | -24.19** | -26.09** | -41.45** |
| UYR 3472 × Giza179 | -6.39 | -13.74** | -22.57** | -29.45** | -5.55 | -7.43* | -12.99** | -21.66** | -20.44** | -11.56 | -23.91** | -35.57** |
| UYR 3472 × Sakha 108 | 28.37** | 17.65** | 2.61 | -1.64 | 12.39** | -7.09* | 5.33 | -19.53** | -31.68** | -15.60 | -35.42** | -37.05** |
| GZ10487 × GZ10739 | -16.31** | -1.04 | -21.72** | -29.15** | 23.82** | 13.53** | 20.77** | 0.72 | -13.68** | -3.89 | -18.46** | -20.93** |
| GZ10487 × WAB 638-1 | -8.74* | -20.70** | -14.58** | -32.51** | -2.99 | -3.77 | -4.15 | -21.53** | -19.98** | -26.93** | -28.00** | -42.12** |
| GZ10487 × IRAT112 | 0.56 | -2.52 | -6.90 | -19.02** | 2.21 | -2.64 | -2.39 | -20.73** | 10.54** | -36.71** | 1.08 | -44.81** |
| GZ10487 × Giza179 | 16.59** | 2.73 | 13.36** | -13.63** | 14.75** | 3.68 | 11.64** | -8.93** | 12.52** | 14.18 | 6.92* | -17.82** |
| GZ10487 × Sakha108 | -5.71 | 4.05 | -7.19 | -14.98** | -10.66** | -6.95* | -11.48** | -16.18** | -4.18 | -20.09* | -7.87** | -41.34** |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GZ10739 WAB 638-1 | ×-23.75$^{**}$ | -8.93 | -32.94$^{**}$ | -22.72$^{**}$ | 8.61$^{**}$ | 10.29$^{**}$ | 4.70 | -10.61$^{**}$ | -13.84$^{**}$ | -4.05 | -18.18$^{**}$ | -28.07$^{**}$ |
| GZ10739 IRAT112 | ×-17.49$^{**}$ | 1.47 | -28.16$^{**}$ | -14.26$^{**}$ | -0.57 | -8.76$^{**}$ | -7.27$^{*}$ | -26.15$^{**}$ | -13.91$^{**}$ | -19.21$^{**}$ | -16.84$^{**}$ | -33.69$^{**}$ |
| GZ10739 Giza179 | ×-6.32 | -2.26 | -10.00$^{*}$ | -16.27$^{**}$ | 12.81$^{**}$ | -8.89$^{**}$ | 7.12$^{*}$ | -20.69$^{**}$ | -20.55$^{**}$ | -19.19$^{**}$ | -28.46$^{**}$ | -44.61$^{**}$ |
| GZ10739 Sakha108 | ×2.13 | 6.54 | -5.88 | -11.62$^{*}$ | -4.63 | -7.31$^{*}$ | -7.80$^{*}$ | -17.36$^{**}$ | -16.35$^{**}$ | -20.66$^{**}$ | -23.84$^{**}$ | -44.61$^{**}$ |
| WAB 638-1 Giza179 | ×-21.81$^{**}$ | -17.24$^{**}$ | -28.70$^{**}$ | -36.47$^{**}$ | 15.44$^{**}$ | -14.37$^{**}$ | 13.64$^{**}$ | -25.98$^{**}$ | -20.39$^{**}$ | -7.66 | -31.53$^{**}$ | -45.36$^{**}$ |
| WAB 638-1 Sakha108 | ×-13.93$^{**}$ | -23.62$^{**}$ | -18.23$^{**}$ | -42.94$^{**}$ | 17.23$^{**}$ | 7.92$^{*}$ | 16.897$^{**}$ | -4.25 | -9.03$^{**}$ | 9.75 | -20.95$^{**}$ | -33.81$^{**}$ |
| IRAT 112 Giza179 | ×-19.75$^{**}$ | -5.10 | -27.58$^{**}$ | -30.46$^{**}$ | -8.64$^{**}$ | -12.64$^{**}$ | -10.37$^{**}$ | -19.10$^{**}$ | -16.26$^{**}$ | -16.67$^{*}$ | -26.88$^{**}$ | -43.36$^{**}$ |
| IRAT 112 Sakha108 | ×-2.14 | 8.06 | -8.04$^{*}$ | -22.98$^{**}$ | 45.62$^{**}$ | 37.57$^{**}$ | 40.29$^{**}$ | 25.06$^{**}$ | -13.57$^{**}$ | -11.35 | -23.72$^{**}$ | -38.71$^{**}$ |
| Giza179 Sakha108 | ×13.31$^{**}$ | 5.18 | 8.50 | -5.71 | -11.71$^{**}$ | -21.80$^{**}$ | -13.32$^{**}$ | -23.63$^{**}$ | 3.04 | 1.33 | 1.78 | -23.24$^{**}$ |

MP: Mid-Parent, BP: Better Parent, N: natural condition, D: drought condition, * and ** significant at 0.05 and 0.01 levels of probability, respectively.

**Table 9:** Percentage of heterosis over mid and better parents for growth characters under natural and drought conditions

| Crosses | Leaf rolling | | | | Relative water content | | | | Number of panicles plant$^{-1}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MP | | BP | | MP | | BP | | MP | | BP | |
| | N | D | N | D | N | D | N | D | N | D | N | D |
| UYR 2184 × UYR 3472 | -37.50$^{*}$ | -55.56$^{**}$ | -37.5 | -55.5$^{**}$ | -3.59$^{*}$ | 4.66$^{*}$ | -5.147$^{**}$ | -14.61$^{**}$ | 9.09 | 19.93$^{**}$ | 8.585 | -20.41$^{**}$ |
| UYR 2184 × GZ10487 | 23.08 | -38.46$^{**}$ | 0 | 0 | -3.77$^{*}$ | -10.25$^{**}$ | -4.460$^{*}$ | -23.45$^{**}$ | 7.31 | -3.79 | -14.7$^{**}$ | -31.32$^{**}$ |
| UYR 2184 × GZ10739 | -23.08 | -33.33$^{**}$ | -37.5 | 0 | -3.26$^{*}$ | 0.91 | -6.199$^{**}$ | -12.12$^{**}$ | -17.60$^{**}$ | -21.69$^{**}$ | -34.03$^{**}$ | -43.49$^{**}$ |
| UYR 2184 × WAB 638-1 | -23.08 | -25.00$^{*}$ | -37.5 | 12.5 | -6.23$^{**}$ | -10.60$^{**}$ | -10.49$^{**}$ | -19.30$^{**}$ | 30.54$^{**}$ | 42.15$^{**}$ | 21.643$^{**}$ | 6.03 |
| UYR 2184 × IRAT 112 | -16.67 | -21.74 | -37.5 | 12.5 | -4.87$^{**}$ | -0.32 | -9.668$^{**}$ | -10.21$^{**}$ | -0.68 | -5.68 | -14.78$^{**}$ | -23.67$^{**}$ |
| UYR 2184 × Giza 179 | -33.33 | -38.46$^{**}$ | -37.5 | 0 | -7.11$^{**}$ | -2.61 | -10.93$^{**}$ | -14.94$^{**}$ | -28.02$^{**}$ | -25.00$^{**}$ | -43.62$^{**}$ | -46.20$^{**}$ |
| UYR 2184 × Sakha 108 | -33.33 | -35.14$^{**}$ | -37.5 | -36.8$^{**}$ | -1.54 | 12.33$^{**}$ | -5.015$^{**}$ | -6.80$^{**}$ | -38.85$^{**}$ | -17.15$^{**}$ | -51.92$^{**}$ | -34.34$^{**}$ |
| UYR 3472 × GZ10487 | 23.08 | -38.46$^{**}$ | 0 | 0 | -1.86 | -5.53$^{**}$ | -4.133$^{*}$ | -17.93$^{**}$ | 8.71 | 9.66$^{*}$ | -13.93$^{**}$ | -18.76$^{**}$ |
| UYR 3472 × GZ10739 | 23.08 | -16.67 | 0 | 25 | -7.07$^{**}$ | -8.58$^{**}$ | -11.30$^{**}$ | -18.87$^{**}$ | -19.41$^{**}$ | -30.99$^{**}$ | -35.70$^{**}$ | -48.27$^{**}$ |
| UYR 3472 × WAB 638-1 | -7.69 | -16.67 | -25 | 25 | -3.55$^{*}$ | -4.01$^{*}$ | -9.358$^{**}$ | -12.53$^{**}$ | 5.83 | -5.47 | -1.80 | -25.05$^{**}$ |
| UYR 3472 × IRAT 112 | 0 | 4.35 | -25 | 50.000$^{*}$ | -0.72 | -6.05$^{**}$ | -7.167$^{**}$ | -14.24$^{**}$ | -6.71 | -7.86 | -20.26$^{**}$ | -21.83$^{**}$ |
| UYR 3472 × Giza179 | -20.00$^{**}$ | -30.77$^{**}$ | -25 | 12.5 | -4.04$^{**}$ | 1.23 | -9.410$^{**}$ | -9.91$^{**}$ | -20.54$^{**}$ | -30.59$^{**}$ | -37.97$^{**}$ | -48.30$^{**}$ |
| UYR 3472 × Sakha 108 | -46.67 | -62.16$^{**}$ | -50.00$^{**}$ | -63.15$^{**}$ | -2.99$^{*}$ | 4.57$^{*}$ | -7.870$^{**}$ | -11.66$^{**}$ | 20.34$^{**}$ | 31.59$^{**}$ | -5.71 | 9.13 |
| GZ10487 × GZ10739 | 0 | 42.86$^{*}$ | 0 | 66.667$^{*}$ | -3.09$^{*}$ | -4.80$^{*}$ | -5.380$^{**}$ | -15.95$^{**}$ | -3.52 | -40.78$^{**}$ | -4.508 | -50.00$^{**}$ |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GZ10487 × WAB 638-1 | 60 | 71.43$^{**}$ | 60 | 100.0$^{**}$ | -1.14 | -4.07$^{*}$ | -4.981$^{**}$ | -12.31$^{**}$ | 9.67$^{*}$ | -14.06$^{**}$ | -7.787 | -41.12$^{**}$ |
| GZ10487 × IRAT112 | -11.11 | 84.62$^{**}$ | -20 | 140.0$^{**}$ | 5.41$^{**}$ | 7.65$^{**}$ | 0.784 | -1.8 | 13.94$^{**}$ | 12.78$^{**}$ | 3.825 | -18.03$^{**}$ |
| GZ10487 × Giza179 | -33.33 | 50.00$^{**}$ | -42.85 | 50.00$^{*}$ | 7.71$^{**}$ | 12.78$^{**}$ | 4.000$^{*}$ | -0.16 | 12.53$^{**}$ | 0.96 | 10.381$^{**}$ | -13.93$^{**}$ |
| GZ10487 × Sakha108 | -33.33 | -33.33$^{**}$ | -42.85 | -52.63$^{**}$ | -4.37$^{**}$ | -6.66$^{**}$ | -7.099$^{**}$ | -21.39$^{**}$ | -3.03 | -0.83 | -4.382 | -26.23$^{**}$ |
| GZ10739 × WAB 638-1 | 0 | 33.33 | 0 | 33.33 | -4.74$^{**}$ | -9.42$^{**}$ | -6.264$^{**}$ | -19.57$^{**}$ | 14.47$^{**}$ | -12.74$^{**}$ | -2.929 | -40.30$^{**}$ |
| GZ10739 × IRAT112 | -11.11 | 45.45 | -20 | 60 | -5.03$^{**}$ | -9.24$^{**}$ | -7.055$^{**}$ | -19.57$^{**}$ | -9.48$^{*}$ | -11.90$^{**}$ | -16.73$^{**}$ | -35.98$^{**}$ |
| GZ10739 × Giza179 | 0 | 71.43$^{*}$ | -14.286 | 50.000$^{*}$ | -4.82$^{**}$ | -3.99$^{*}$ | -5.905$^{**}$ | -17.38$^{**}$ | -11.64$^{**}$ | -19.74$^{**}$ | -14.19$^{**}$ | -31.38$^{**}$ |
| GZ10739 × Sakha108 | -33.33 | -4 | -42.857 | -36.84$^{**}$ | -2.04 | 2.17 | -2.546 | -16.29$^{**}$ | -14.29$^{**}$ | 3.28 | -16.33$^{**}$ | -23.15$^{**}$ |
| WAB 638-1 × IRAT112 | 11.11 | 45.45 | 0 | 60 | -3.85$^{**}$ | -2.19 | -4.367$^{**}$ | -13.28$^{**}$ | -12.26$^{*}$ | -11.00$^{*}$ | -19.76$^{**}$ | -27.85$^{**}$ |
| WAB 638-1 × Giza179 | 0 | 28.57 | -14.286 | 12.5 | -0.7 | 2.87 | -1.17 | -11.35$^{**}$ | -19.05$^{**}$ | -21.45$^{**}$ | -32.98$^{**}$ | -36.99$^{**}$ |
| WAB 638-1 × Sakha108 | -16.67 | -28.00$^{**}$ | -28.571 | -52.63$^{**}$ | -5.00$^{**}$ | 3.31 | -6.038$^{**}$ | -15.09$^{**}$ | -14.06$^{**}$ | -3.84 | -28.55$^{**}$ | -19.63$^{**}$ |
| IRAT 112 × Giza179 | 45.45 | 84.62$^{**}$ | 14.286 | 50.00$^{*}$ | -3.17$^{*}$ | 0.66 | -4.143$^{**}$ | -14.37$^{**}$ | -26.63$^{**}$ | -33.40$^{**}$ | -34.29$^{**}$ | -43.29$^{**}$ |
| IRAT 112 × Sakha108 | 27.27 | -16.67 | 0 | -47.36$^{**}$ | -1.65 | 0.21 | -3.247$^{*}$ | -18.70$^{**}$ | -24.72$^{**}$ | -26.26$^{**}$ | -32.27 | -45.18$^{**}$ |
| Giza179 × Sakha108 | 42.86$^{*}$ | -33.33$^{**}$ | 42.857 | -52.63$^{**}$ | -0.94 | 6.24$^{**}$ | -1.562 | -14.70$^{**}$ | -19.95$^{**}$ | -15.66$^{**}$ | -20.36$^{**}$ | -40.21$^{**}$ |

MP: Mid-Parent, BP: Better Parent, N: natural condition, D: drought condition, * and ** significant at 0.05 and 0.01 levels of probability, respectively.

**Table 10:** Percentage of heterosis over mid and better parents for growth characters under natural and drought conditions

| Crosses | 1000-grain mass (g) | | | | Sterility percentage | | | | Grain yield plant$^{-1}$ (g) | | | | Water use efficiency (g ml) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MP | | BP | | MP | | BP | | MP | | BP | | MP | | BP | |
| | N | D | N | D | N | D | N | D | N | D | N | D | N | D | N | D |
| UYR 2184 × UYR3472 | -4.9 | -5.5 | -7.2$^{*}$ | -25.2$^{**}$ | 6.3 | 21.9$^{**}$ | 7.7 | 275.0$^{**}$ | -0.1 | -12.2$^{**}$ | -0.3 | -46.8$^{**}$ | -0.1 | -12.2$^{**}$ | -0.3 | -16.4$^{**}$ |
| UYR 2184 × GZ10487 | -28.1$^{**}$ | -13.1$^{**}$ | -32.7$^{**}$ | -31.5$^{**}$ | 20.3$^{*}$ | 24.6$^{**}$ | 47.6 | 237.5$^{**}$ | -13.6$^{**}$ | -28.2$^{**}$ | -25.5$^{**}$ | -44.0$^{**}$ | -13.6$^{**}$ | -28.2$^{**}$ | -25.5$^{**}$ | -44.0$^{**}$ |
| UYR 2184 × GZ10739 | -4.5 | 11.86$^{**}$ | -5.4 | -7.447$^{*}$ | -15.6 | -9.01$^{**}$ | -3.54$^{**}$ | 147.5$^{**}$ | -12.2$^{**}$ | -26.9$^{**}$ | -24.8$^{**}$ | -44.1$^{**}$ | -12.2$^{**}$ | -26.9$^{**}$ | -24.8$^{**}$ | -44.1$^{**}$ |
| UYR 2184 × WAB 638-1 | -7.20$^{**}$ | -13.0$^{**}$ | -13.2$^{**}$ | -23.4$^{**}$ | 19.41$^{*}$ | -23.2$^{**}$ | 46.6 | 90.36$^{**}$ | -8.0 | -26.8$^{**}$ | -13.0$^{**}$ | -47.1$^{**}$ | -7.98$^{**}$ | -26.8$^{**}$ | -13.0$^{**}$ | -38.2$^{**}$ |
| UYR 2184 × IRAT 112 | -0.1 | -6.84$^{*}$ | -6.042$^{*}$ | -17.1$^{**}$ | 21.3$^{**}$ | -43.8$^{**}$ | 26.63$^{*}$ | 50.0 | -10.8$^{**}$ | -33.2$^{**}$ | -15.3$^{**}$ | -49.7$^{**}$ | -10.8$^{**}$ | -33.2$^{**}$ | -15.3$^{**}$ | -45.1$^{**}$ |
| UYR 2184 × Giza 179 | -3.8 | 5.8 | -10.5$^{**}$ | -18.4$^{**}$ | 7.3 | -34.4$^{**}$ | 32.2 | 80.00$^{*}$ | -15.7$^{**}$ | -7.02$^{*}$ | -28.7$^{**}$ | -29.1$^{**}$ | -15.7$^{**}$ | -7.0$^{**}$ | -28.7$^{**}$ | -24.1$^{**}$ |
| UYR 2184 × Sakha 108 | -3.1 | -13.5$^{**}$ | -3.4 | -25.4$^{**}$ | 3.3 | -31.8$^{**}$ | 35.92$^{*}$ | 102.5 | -11.1$^{**}$ | -5.68$^{**}$ | -23.4$^{**}$ | -34.5$^{**}$ | -11.1$^{**}$ | -5.6$^{**}$ | -23.4$^{**}$ | -17.9$^{**}$ |
| UYR 3472 × GZ10487 | -30.7$^{**}$ | -21.1$^{**}$ | -36.6$^{**}$ | -34.3$^{**}$ | 28.4$^{**}$ | 43.4$^{**}$ | 9.5 | 78.2$^{**}$ | -13.4$^{**}$ | -19.2$^{**}$ | -25.5$^{**}$ | -35.8$^{**}$ | -13.4$^{**}$ | -19.2$^{**}$ | -25.5$^{**}$ | -35.8$^{**}$ |
| UYR 3472 × GZ10739 | -15.1$^{**}$ | 0.6 | -18.0$^{**}$ | -11.9$^{**}$ | -5.0 | 26.2$^{**}$ | -14.6 | 55.3$^{**}$ | -21.2$^{**}$ | -32.0$^{**}$ | -32.7$^{**}$ | -47.0$^{**}$ | -21.2$^{**}$ | -32.0$^{**}$ | -32.7$^{**}$ | -47.0$^{**}$ |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UYR 3472 × WAB 638-1 | -14.0** | -18.6** | -21.4** | -26.1** | 27.5** | 106.0** | 8.7 | 238.3** | -16.9** | -24.2** | -21.6** | -43.7** | -16.9** | -24.2** | -21.6** | -34.5** |
| UYR 3472 × IRAT 112 | -17.4** | -25.4** | -24.1** | -32.8** | 13.7 | -5.8 | 10.5 | 20.62** | -13.3** | -27.0** | -18.0** | -43.6** | -13.3** | -27.0** | -18.0** | -38.7** |
| UYR 3472 × Giza179 | -10.5** | -13.2** | -14.8** | -29.2** | 38.4** | 38.5** | 17.6* | 67.4** | -15.5** | -4.82* | -28.6** | -25.5** | -15.5** | -4.82** | -28.6** | -20.7** |
| UYR 3472 × Sakha 108 | -7.9* | -20.9** | -9.8** | -27.8** | 7.7 | -26.5** | -12.3 | -22.7** | -15.8** | -0.7 | -27.6** | -29.1** | -15.8** | -0.7 | -27.6** | -11.6** |
| GZ10487 × GZ10739 | 0.7 | 12.4** | -4.9 | -18.7** | 5.1 | 10.3** | -1.0 | 9.4 | -2.9 | 2.8* | -3.7* | -19.2** | -2.9* | 2.8* | -3.7* | 0.2 |
| GZ10487 × WAB 638-1 | -18.4** | -9.7** | -18.4** | -30.5** | 21.8* | 51.2** | 21.8 | 91.8** | -8.6** | 1.7 | -17.1** | -29.5** | -8.6** | 1.7 | -17.1** | 10.6** |
| IRAT 112 × Giza179 | -3.5** | -18.5** | -15.2** | -37.1** | 40.4** | 91.9** | 22.2** | 83.1** | -7.4** | 11.7** | -18.0** | -7.1** | -7.4** | 11.7** | -18.0** | 10.6** |
| IRAT 112 × Sakha108 | 1.9 | -10.7** | -4.5 | -23.9** | 56.6** | 16.8** | 30.4** | -0.3 | -10.4** | 14.3** | -19.2** | -12.0** | -10.4** | 14.3** | -19.2** | 22.8** |
| Giza179 × Sakha108 | 16.0** | 1.7 | 8.3* | -8.1* | 64.4** | 7.2 | 56.04** | -4.7 | 6.1** | 13.2** | 4.04* | -32.0** | 6.1** | 13.2** | 4.0** | 6.7** |
| GZ10487 × IRAT112 | 12.8** | 4.0 | 12.2** | -19.2** | 12.0 | 33.5** | -2.2 | 36.9** | -9.8** | -3.5* | -18.6** | -31.1** | -9.8** | -3.5** | -18.6** | 3.9** |
| GZ10487 × Giza179 | 30.6** | 30.8** | 14.3** | -11.8** | 40.8** | 85.5** | 40.3** | 81.2** | 0.2 | 6.8** | -2.0 | -22.9** | 0.2 | 6.8** | -2.0 | 13.8** |
| GZ10487 × Sakha108 | -13.4** | -15.3** | -19.2** | -36.2** | 40.0** | 27.7** | 32.3** | 11.27* | -1.6 | 17.8** | -1.7 | -20.9** | -1.6 | 17.8** | -1.7 | 24.2** |
| GZ10739 × WAB 638-1 | -17.5** | -24.6** | -22.1** | -31.8** | 37.2** | 50.3** | 29.2** | 92.6** | -4.78** | 4.8** | -14.3** | -26.4** | -4.7** | 4.8** | -14.3** | 15.5** |
| GZ10739 × IRAT112 | -13.9** | -26.9** | -18.3** | -33.3** | 33.1** | 51.2** | 22.8** | 56.3** | -11.0** | 3.9** | -20.2** | -24.9** | -11.0** | 3.9** | -20.2** | 15.2** |
| GZ10739 × Giza179 | -4.2 | 8.07* | -11.7** | -13.9** | 29.1** | 6.6 | 21.2* | 5.0 | -1.4 | 8.8** | -2.7 | -20.6** | -1.4 | 8.8** | -2.6* | 19.2** |
| GZ10739 × Sakha108 | -0.7 | 2.6 | -2.0 | -9.03** | 20.50* | 105.5** | 7.7 | 80.39** | 3.49* | 10.85** | 2.8 | -24.6** | 3.4* | 10.8** | 2.7* | 18.4** |
| WAB 638-1 × IRAT 112 | -18.2** | -20.2** | -18.8** | -31.7** | 8.6 | 95.67** | -5.2 | 64.64** | 0.0 | 0.7 | -0.3 | -20.5** | 0.0 | 0.7 | -0.3 | -2.6 |
| WAB 638-1 × Giza179 | -12.8** | -6.6 | -23.8** | -29.7** | 48.5** | 97.4** | 48.0** | 60.0** | -5.9** | 9.19** | -16.4** | -12.9** | -5.99** | 9.19** | -16.4** | 4.5** |
| WAB 638-1 × Sakha108 | -6.3* | -11.8** | -12.7** | -26.6** | 30.0** | 57.5** | 22.9* | 16.7** | -6.3** | 14.4** | -15.2** | -15.6** | -6.3** | 14.4** | -15.2** | 18.6** |

MP: Mid-Parent, BP: Better Parent, N: natural condition, D: drought condition, * and ** significant at 0.05 and 0.01 levels of probability, respectively.

is no hybrid desirable significant and highly significant negative heterotic effects as a deviation from the mid parent under natural condition, but there are six hybrids under drought condition, one and one combinations were obtained significant and highly significant negative heterotic over better parent under both natural and drought condition, respectively. However, undesirable highly and highly significant positive heterotic effects were recorded for 18 and 19 hybrids over mid parent and 13 and 21 combinations were obtained significant and highly significant positive heterotic over better parent at the natural and drought environments, respectively. Concerning the grain yield plant$^{-1}$, two and twelve hybrids revealed desirable highly and highly significant positive heterotic effects as a deviation over mid parent, one hybrid obtained significant positive heterotic over better-parent under natural condition and there is no hybrid under drought condition, respectively. Otherwise, undesirable highly and highly significant negative heterotic effects were recorded for 19 and 13 hybrids over mid parent and 21 and 28 from over better parent at the natural and drought environments. For water use efficiency, two and twelve hybrids showed significant and highly significant positive heterosis over mid-parent, 2 and 13 crosses obtained significant and highly significant positive heterotic over better-parent under both natural and drought condition, respectively. Otherwise, the negative heterotic over mid-parent was found with 21 and 13 crosses over mid-parent, and 22 and 13 crosses over better parent under natural and drought condition, respectively.

## 3.4 GENERAL COMBINING ABILITY EFFECTS (GCA)

Variation in general combining ability effects was estimated among the eight parental genotypes for studied traits to identify the best combiner parents for subsequent hybrid development program (Table 11, 12 and 13). For number of days to heading six and four parents showed desirable significant and highly significant in negative direction effects under both natural and drought conditions, respectively. Regarding plant height, six and four parents showed desirable significant and highly significant in negative direction effects under both natural and drought conditions, respectively. For number of tillers plant$^{-1}$, four and three parents showed desirable significant and highly significant positive GCA effects under both natural and drought conditions, respectively. Concerning panicle length out of four and four parents showed desirable significant and highly significant positive general combining ability effects under either natural and drought conditions, respectively. For chlorophyll content, three parents showed desirable significant and highly significant positive GCA effects under both natural and drought conditions, respectively. With respect to flag leaf area, four and three parents showed desirable significant and highly significant positive general combining ability effects under either natural and drought conditions, respectively. Otherwise, four and three parents provided negative significant and highly significant in general combining ability under both natural and drought condition, respectively. For leaf rolling there are no parents desirable significant and highly significant positive general combining ability effects under natural condition, but there are three parents desirable significant and highly significant positive general combining ability effects under drought condition, respectively. Regarding the relative water content, four and three parents showed desirable significant and highly significant positive general combining ability effects under either natural and drought conditions, respectively. However, two and four parents obtained negative significant and highly significant in general combining ability under both natural and drought condition, respectively. Regarding number of panicles plant$^{-1}$, four parents showed desirable significant and highly significant positive general combining ability effects under either natural and drought conditions, respectively. On the other hand, four parents provided negative significant and highly significant in general combining ability under

**Table 11:** Estimates of general combining ability effects of the parental genotypes for the studied growth characters under natural and drought conditions.

| Parents | Days to heading (day) | | Plant height (cm) | | Number of tillers plant$^{-1}$ | | Panicle length (cm) | |
|---|---|---|---|---|---|---|---|---|
| | N | D | N | D | N | D | N | D |
| UYR 2184 | -5.09$^{**}$ | -3.17$^{**}$ | -5.09$^{**}$ | -3.17$^{**}$ | -2.71$^{**}$ | -2.23$^{**}$ | -2.49$^{**}$ | -2.82$^{**}$ |
| UYR 3472 | -1.43$^{**}$ | 0.97$^{**}$ | -1.43$^{**}$ | 0.97$^{**}$ | -2.15$^{**}$ | -1.82$^{**}$ | -2.99$^{**}$ | -2.03$^{**}$ |
| GZ10487 | -0.93$^{*}$ | -0.43 | -0.93$^{*}$ | -0.43 | 3.93$^{**}$ | 3.44$^{**}$ | 1.61$^{**}$ | 1.18$^{**}$ |
| GZ10739 | -1.23$^{**}$ | -1.97$^{**}$ | -1.23$^{**}$ | -1.97$^{**}$ | 1.17$^{**}$ | 1.02$^{**}$ | -1.39$^{**}$ | -0.81$^{**}$ |
| WAB 638-1 | 9.43$^{**}$ | 7.50$^{**}$ | 9.43$^{**}$ | 7.50$^{**}$ | -1.06$^{**}$ | -1.12$^{**}$ | 0.67$^{**}$ | 0.35 |
| IRAT 112 | 9.15$^{**}$ | 10.63$^{**}$ | 9.15$^{**}$ | 10.63$^{**}$ | -0.56$^{*}$ | -0.63$^{**}$ | 1.83$^{**}$ | 1.46$^{**}$ |
| Giza 179 | -3.00$^{**}$ | -4.93$^{**}$ | -3.00$^{**}$ | -4.93$^{**}$ | 0.62$^{*}$ | 1.04$^{**}$ | 0.23 | 1.13$^{**}$ |
| Sakha 108 | -6.90$^{**}$ | -8.60$^{**}$ | -6.90$^{**}$ | -8.60$^{**}$ | 0.76$^{**}$ | 0.29 | 2.53$^{**}$ | 1.53$^{**}$ |
| L.S.D.05 (gi) | 0.72 | 0.58 | 0.72 | 0.58 | 0.50 | 0.43 | 0.46 | 0.42 |
| L.S.D.01 (gi) | 0.96 | 0.77 | 0.96 | 0.77 | 0.67 | 0.57 | 0.61 | 0.55 |
| L.S.D .05(gi-gj) | 1.09 | 0.88 | 1.09 | 0.88 | 0.76 | 0.65 | 0.70 | 0.63 |
| L.S.D .01(gi-gj) | 1.45 | 1.17 | 1.45 | 1.17 | 1.01 | 0.86 | 0.92 | 0.83 |

N: natural condition, D: drought condition, * and ** significant at 0.05 and 0.01 levels of probability, respectively.

**Table 12:** Estimates of general combining ability effects of the parental genotypes for some growth characters studied under natural and drought conditions.

| Parents | Chlorophyll content (SPAD) | | Flag leaf area (cm$^2$) | | Leaf rolling | | Relative water content | |
|---|---|---|---|---|---|---|---|---|
| | N | D | N | D | N | D | N | D |
| UYR 2184 | 2.07$^{**}$ | 3.24$^{**}$ | -2.31$^{**}$ | -0.31 | 0.05 | 0.28$^{**}$ | -3.01$^{**}$ | -2.44$^{**}$ |
| UYR 3472 | 2.18$^{**}$ | 1.94$^{**}$ | -3.77$^{**}$ | -3.49$^{**}$ | 0.22 | 0.35$^{**}$ | -3.73$^{**}$ | -3.94$^{**}$ |
| GZ10487 | -0.58 | -0.51 | 1.53$^{**}$ | -0.44 | -0.05 | -0.08 | 0.04 | -0.67 |
| GZ10739 | 0.99$^{**}$ | 0.54$^{*}$ | 1.28$^{**}$ | 2.16$^{**}$ | -0.18 | -0.32$^{**}$ | -0.43 | -0.96$^{**}$ |
| WAB 638-1 | -0.07 | -1.44$^{**}$ | 3.46$^{**}$ | 2.71$^{**}$ | -0.08 | -0.42$^{**}$ | 1.36$^{**}$ | 2.24$^{**}$ |
| IRAT 112 | -1.21$^{**}$ | -1.27$^{**}$ | 3.92$^{**}$ | 2.18$^{**}$ | -0.18 | -0.28$^{**}$ | 2.79$^{**}$ | 3.18$^{**}$ |
| Giza 179 | -2.30$^{**}$ | -2.47$^{**}$ | -2.01$^{**}$ | -1.17$^{*}$ | 0.22 | -0.08 | 1.99$^{**}$ | 2.91$^{**}$ |
| Sakha 108 | -1.07$^{**}$ | -0.03$^{**}$ | -2.11$^{**}$ | -1.64$^{**}$ | 0.02 | 0.55$^{**}$ | 0.98$^{**}$ | -0.33$^{**}$ |
| L.S.D.05 (gi) | 0.61 | 0.54 | 0.46 | 0.97 | 0.22 | 0.21 | 0.58 | 0.67 |
| L.S.D.01 (gi) | 0.80 | 0.72 | 0.61 | 1.29 | 0.29 | 0.28 | 0.77 | 0.89 |
| L.S.D .05(gi-gj) | 0.92 | 0.82 | 0.69 | 1.47 | 0.33 | 0.32 | 0.88 | 1.01 |
| L.S.D .01(gi-gj) | 1.21 | 1.09 | 0.92 | 1.95 | 0.44 | 0.43 | 1.17 | 1.34 |

N: natural condition, D: drought condition, * and ** significant at 0.05 and 0.01 levels of probability, respectively.

**Table 13:** Estimates of general combining ability effects of the parental genotypes for grain yield and its component characters under natural and drought conditions.

| Parents | Number of panicles plant$^{-1}$ | | 1000-grain mass (g) | | Sterility percentage | | Grain yield plant$^{-1}$ (g) | | Water use efficiency (g ml) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | D | N | D | N | D | N | D | N | D |
| UYR 2184 | -3.09$^{**}$ | -2.51$^{**}$ | -0.52$^{*}$ | -0.52$^{*}$ | 0.45$^{*}$ | 5.85$^{**}$ | -4.50$^{**}$ | -7.58$^{**}$ | -0.09$^{**}$ | -0.23$^{**}$ |
| UYR 3472 | -2.33$^{**}$ | -1.92$^{**}$ | -2.57$^{**}$ | -2.05$^{**}$ | 0.88$^{**}$ | 3.25$^{**}$ | -5.54$^{**}$ | -6.58$^{**}$ | -0.11$^{**}$ | -0.20$^{**}$ |
| GZ10487 | 3.83$^{**}$ | 3.12$^{**}$ | 1.63$^{**}$ | 0.05 | -0.72$^{**}$ | -0.54$^{*}$ | 2.79$^{**}$ | 3.58$^{**}$ | 0.05$^{**}$ | 0.11$^{**}$ |
| GZ10739 | 1.26$^{**}$ | 1.12$^{**}$ | -0.33 | 0.97$^{**}$ | -0.75$^{**}$ | -1.44$^{**}$ | 3.20$^{**}$ | 4.40$^{**}$ | 0.06$^{**}$ | 0.13$^{**}$ |
| WAB 638-1 | -0.89$^{**}$ | -0.99$^{**}$ | 0.21 | 0.27 | -0.48$^{*}$ | -2.60$^{**}$ | -1.56$^{**}$ | 0.31$^{*}$ | -0.03$^{**}$ | 0.01$^{*}$ |
| IRAT 112 | -0.73$^{**}$ | -0.63$^{**}$ | 1.75$^{**}$ | 0.50$^{*}$ | 1.24$^{**}$ | -3.38$^{**}$ | -2.23$^{**}$ | 0.93$^{**}$ | -0.04$^{**}$ | 0.03$^{**}$ |
| Giza 179 | 0.89$^{**}$ | 1.26$^{**}$ | -0.62$^{**}$ | 0.02 | 0.20 | -0.85$^{**}$ | 4.31$^{**}$ | 3.28$^{**}$ | 0.08$^{**}$ | 0.10$^{**}$ |
| Sakha 108 | 1.06$^{**}$ | 0.56$^{**}$ | 0.46 | 0.76$^{**}$ | -0.82$^{**}$ | -0.29$^{**}$ | 3.53$^{**}$ | 1.65$^{**}$ | 0.07$^{**}$ | 0.05$^{**}$ |
| L.S.D.05 (gi) | 0.41 | 0.34 | 0.40 | 0.44 | 0.43 | 0.53 | 0.34 | 0.25 | 0.01 | 0.01 |
| L.S.D.01 (gi) | 0.55 | 0.45 | 0.53 | 0.59 | 0.57 | 0.70 | 0.45 | 0.33 | 0.01 | 0.01 |
| L.S.D .05 (gi-gj) | 0.62 | 0.51 | 0.60 | 0.67 | 0.65 | 0.80 | 0.51 | 0.37 | 0.01 | 0.01 |
| L.S.D .01 (gi-gj) | 0.83 | 0.68 | 0.80 | 0.89 | 0.86 | 1.06 | 0.68 | 0.49 | 0.01 | 0.01 |

N: natural condition, D: drought condition, * and ** significant at 0.05 and 0.01 levels of probability, respectively.

either natural and drought condition, respectively. Concerning 1000-grain mass, two and three parents showed desirable significant and highly significant positive general combining ability effects under either natural and drought conditions, respectively. However, three and two parents got negative significant and highly significant in general combining ability under either natural and drought condition, respectively. With respect to sterility percentage, four and six parents showed desirable significant and highly significant negative general combining ability effects under both natural and drought conditions, respectively.

On the other hand, three and two parents displayed positive significant and highly significant in general combining ability under both natural and drought condition, respectively. Regarding the grain yield plant$^{-1}$, four and six parents showed desirable significant and highly significant positive general combining ability effects under either natural and drought conditions, respectively. Otherwise, four and two parents

**Table 14:** Estimates of specific combining ability effects for the studied growth characters under natural and drought conditions.

| Crosses | Days to heading (day) | | Plant height (cm) | | Number of tillers plant$^{-1}$ | | Panicle length (cm) | |
|---|---|---|---|---|---|---|---|---|
| | N | D | N | D | N | D | N | D |
| UYR 2184 × UYR 3472 | -1.99 | -5.47$^{**}$ | -1.99 | -5.47$^{**}$ | 0.68 | 1.78$^{**}$ | -0.82 | -2.72$^{**}$ |
| UYR 2184 × GZ10487 | -2.49$^{*}$ | -1.73 | -2.49$^{*}$ | -1.73 | 0.87 | -0.65 | -1.98$^{**}$ | -0.37 |
| UYR 2184 × GZ10739 | 3.14$^{**}$ | 3.80$^{**}$ | 3.14$^{**}$ | 3.80$^{**}$ | -1.64$^{*}$ | -1.10 | -0.65 | 0.79 |
| UYR 2184 × WAB 638-1 | -12.86$^{**}$ | -10.00$^{**}$ | -12.86$^{**}$ | -10.00$^{**}$ | 4.82$^{**}$ | 5.21$^{**}$ | 0.79 | 2.63$^{**}$ |
| UYR 2184 × IRAT 112 | 13.62$^{**}$ | 13.20$^{**}$ | 13.62$^{**}$ | 13.20$^{**}$ | 1.29 | 0.15 | 4.97$^{**}$ | 2.52$^{**}$ |
| UYR 2184 × Giza 179 | 1.91 | 3.43$^{**}$ | 1.91 | 3.43$^{**}$ | -2.56$^{**}$ | -1.31 | -1.84$^{*}$ | -0.65 |
| UYR 2184 × Sakha 108 | 3.81$^{**}$ | 4.10$^{**}$ | 3.81$^{**}$ | 4.10$^{**}$ | -4.96$^{**}$ | -2.37$^{**}$ | -0.90 | -0.55 |
| UYR 3472 × GZ10487 | -1.15 | -0.20 | -1.15 | -0.20 | 0.57 | 2.38$^{**}$ | 2.86$^{**}$ | 4.11$^{**}$ |
| UYR 3472 × GZ10739 | -0.52 | -1.00 | -0.52 | -1.00 | -2.63$^{**}$ | -2.77$^{**}$ | 2.02$^{**}$ | 3.00$^{**}$ |
| UYR 3472 × WAB 638-1 | 16.15$^{**}$ | 13.53$^{**}$ | 16.15$^{**}$ | 13.53$^{**}$ | 0.23 | -0.77 | -0.37 | -1.15 |
| UYR 3472 × IRAT 112 | 9.76$^{**}$ | 8.73$^{**}$ | 9.76$^{**}$ | 8.73$^{**}$ | -0.27 | -0.25 | -5.53$^{**}$ | -3.26$^{**}$ |
| UYR 3472 × Giza179 | 0.25 | 0.63 | 0.25 | 0.63 | -2.45$^{**}$ | -2.92$^{**}$ | -1.20 | -1.94$^{**}$ |
| UYR 3472 × Sakha 108 | -1.19 | -1.03 | -1.19 | -1.03 | 5.41$^{**}$ | 3.82$^{**}$ | 4.60$^{**}$ | 2.50$^{**}$ |
| GZ10487 × GZ10739 | -3.35$^{**}$ | 0.40 | -3.35$^{**}$ | 0.40 | -1.18 | -5.64$^{**}$ | -2.91$^{**}$ | -0.88 |
| GZ10487 × WAB 638-1 | 7.98$^{**}$ | 8.27$^{**}$ | 7.98$^{**}$ | 8.27$^{**}$ | -0.02 | -1.96 | -0.10 | -2.87$^{**}$ |
| GZ10487 × IRAT112 | -10.74$^{**}$ | -11.87$^{**}$ | -10.74$^{**}$ | -11.87$^{**}$ | 2.65$^{**}$ | 3.48$^{*}$ | 1.54$^{*}$ | -0.64 |
| GZ10487 × Giza179 | 3.41$^{**}$ | -4.30$^{**}$ | 3.41$^{**}$ | -4.30$^{**}$ | 3.80$^{**}$ | 2.82$^{**}$ | 4.14$^{**}$ | 1.02 |
| GZ10487 × Sakha108 | 1.65 | 3.03$^{**}$ | 1.65 | 3.03$^{**}$ | -0.67 | -0.44 | -2.49$^{**}$ | 0.28 |
| GZ10739 × WAB 638-1 | -8.39$^{**}$ | -5.53$^{**}$ | -8.39$^{**}$ | -5.53$^{**}$ | 3.11$^{**}$ | -0.35 | -2.30$^{**}$ | -0.54 |
| GZ10739 × IRAT112 | -6.77$^{**}$ | -7.33$^{**}$ | -6.77$^{**}$ | -7.33$^{**}$ | 0.08 | 0.93 | -1.63$^{*}$ | -0.22 |
| GZ10739 × Giza179 | 8.38$^{**}$ | 2.23$^{*}$ | 8.38$^{**}$ | 2.23$^{*}$ | 0.00 | -0.37 | 0.13 | -0.33 |
| GZ10739 × Sakha108 | 1.61 | -4.43$^{**}$ | 1.61 | -4.43$^{**}$ | -0.91 | 2.34 | 0.84 | 0.27 |
| WAB 638-1 × IRAT 112 | 3.89$^{**}$ | 8.20$^{**}$ | 3.89$^{**}$ | 8.20$^{**}$ | -1.36 | 0.04 | -3.19$^{**}$ | 0.52 |
| WAB 638-1 × Giza179 | -7.95$^{**}$ | -3.23$^{**}$ | -7.95$^{**}$ | -3.23$^{**}$ | -2.55$^{**}$ | -1.63$^{**}$ | -2.72$^{**}$ | -1.48$^{*}$ |
| WAB 638-1 × Sakha108 | -8.72$^{**}$ | -13.23$^{**}$ | -8.72$^{**}$ | -13.23$^{**}$ | -1.75$^{*}$ | -0.52 | -2.05$^{**}$ | -3.72$^{**}$ |
| IRAT 112 × Giza179 | -8.01$^{**}$ | -7.37$^{**}$ | -8.01$^{**}$ | -7.37$^{**}$ | -3.37$^{**}$ | -3.41$^{**}$ | -3.08$^{**}$ | -0.43 |
| IRAT 112 × Sakha108 | -10.77 | -9.70$^{**}$ | -10.77$^{**}$ | -9.70$^{**}$ | -3.18$^{**}$ | -3.37$^{**}$ | 0.29 | 1.34$^{*}$ |
| Giza179 × Sakha108 | 2.38$^{*}$ | 6.87$^{**}$ | 2.38$^{*}$ | 6.87$^{**}$ | -1.16 | -0.87 | 2.88$^{**}$ | 1.33$^{*}$ |
| L.S.D 0.05 (sij) | 2.21 | 1.78 | 2.21 | 1.78 | 1.55 | 1.32 | 1.41 | 1.27 |
| L.S.D 0.01 (sij) | 2.94 | 2.37 | 2.94 | 2.37 | 2.05 | 1.75 | 1.88 | 1.69 |
| L.S.D 0.05 (sij-sik) | 3.27 | 2.64 | 3.27 | 2.64 | 2.29 | 1.95 | 2.09 | 1.88 |
| L.S.D 0.01 (sij-sik) | 4.34 | 3.50 | 4.34 | 3.50 | 3.04 | 2.59 | 2.77 | 2.50 |
| L.S.D 0.05 (sij-skl) | 3.09 | 2.49 | 3.09 | 2.49 | 2.16 | 1.84 | 1.97 | 1.78 |
| L.S.D 0.01 (sij-skl) | 4.10 | 3.30 | 4.10 | 3.30 | 2.86 | 2.44 | 2.62 | 2.36 |

N: natural condition, D: drought condition. * and ** significant at 0.05 and 0.01 levels of probability, respective illustrated negative significant and highly significant in general combining ability under either natural and drought condition, respectively. Concerning water use efficiency four and six parents showed desirable significant and highly significant positive general combining ability effects under both natural and drought

**Table 15:** Estimates of specific combining ability effects for growth characters under natural and drought conditions.

| Crosses | Chlorophyll content (SPAD) | | Flag leaf area (cm$^2$) | | Leaf rolling | | Relative water content | |
|---|---|---|---|---|---|---|---|---|
| | N | D | N | D | N | D | N | D |
| UYR 2184 × UYR 3472 | -3.27$^{**}$ | -4.22$^{**}$ | 0.44 | 3.08$^{*}$ | -0.54 | -1.28$^{**}$ | 0.17 | 4.05$^{**}$ |
| UYR 2184 × GZ10487 | 1.76 | 2.43$^{**}$ | -3.43$^{**}$ | -1.04 | 0.72$^{*}$ | -0.85$^{*}$ | -1.93$^{*}$ | -6.32$^{**}$ |
| UYR 2184 × GZ10739 | 0.89 | 0.77 | -0.18 | 7.63$^{**}$ | -0.14 | -0.61 | 0.91 | 3.07$^{**}$ |
| UYR 2184 × WAB 638-1 | 3.15$^{**}$ | 7.09$^{**}$ | 1.24 | 0.14 | -0.24 | -0.18 | -2.02$^{*}$ | -5.89$^{**}$ |
| UYR 2184 × IRAT 112 | 0.89 | 1.05 | -2.69$^{**}$ | -2.73 | -0.14 | -0.31 | -1.86$^{*}$ | 0.47 |
| UYR 2184 × Giza 179 | 4.18$^{**}$ | 4.42$^{**}$ | 2.41$^{**}$ | 0.49 | -0.54 | -0.85$^{*}$ | -3.79$^{**}$ | -3.06$^{**}$ |
| UYR 2184 × Sakha 108 | -3.82$^{**}$ | -4.56$^{**}$ | 0.34 | -4.17$^{**}$ | -0.34 | -0.15 | 1.35 | 6.71$^{**}$ |
| UYR 3472 × GZ10487 | 1.38 | 4.50$^{**}$ | 4.17$^{**}$ | 3.84$^{*}$ | 0.56 | -0.91$^{**}$ | -0.94 | -2.53$^{*}$ |
| UYR 3472 × GZ10739 | 0.61 | 4.47$^{**}$ | 5.62$^{**}$ | -1.96 | 0.69$^{*}$ | -0.01 | -2.74$^{**}$ | -2.97$^{**}$ |
| UYR 3472 × WAB 638-1 | 0.64 | -1.35 | -3.03$^{**}$ | 2.86 | -0.08 | 0.09 | -0.3 | -0.57 |
| UYR 3472 × IRAT 112 | 5.21$^{**}$ | 1.52 | -1.95$^{**}$ | -3.95$^{*}$ | 0.02 | 0.62 | 1.1 | -3.11 |
| UYR 3472 × Giza179 | -3.79$^{**}$ | -1.04 | -3.99$^{**}$ | -2.13 | -0.38 | -0.58 | -1.74 | 0.13 |
| UYR 3472 × Sakha 108 | 3.57$^{**}$ | -2.49$^{**}$ | -7.32$^{**}$ | -2.13 | -0.84$^{*}$ | -1.88$^{**}$ | -0.39 | 2 |
| GZ10487 × GZ10739 | 7.94$^{**}$ | 3.73$^{**}$ | -3.65$^{**}$ | 1.56 | -0.04 | 0.42 | -1.44 | -0.81 |
| GZ10487 × WAB 638-1 | -4.03$^{**}$ | -3.59$^{**}$ | -6.47$^{**}$ | -7.16$^{**}$ | 0.86$^{*}$ | 1.19$^{**}$ | -0.2 | -1.04 |
| GZ10487 × IRAT112 | -2.16$^{*}$ | -3.43$^{**}$ | 5.38$^{**}$ | -7.66$^{**}$ | -0.38 | 1.05$^{**}$ | 4.43$^{**}$ | 6.59$^{**}$ |
| GZ10487 × Giza179 | 4.80$^{**}$ | 2.71$^{**}$ | 5.51$^{**}$ | 6.09$^{**}$ | -0.78$^{*}$ | 0.85$^{*}$ | 6.23$^{**}$ | 8.19$^{**}$ |
| GZ10487 × Sakha108 | -6.10$^{**}$ | -2.77$^{**}$ | -0.09 | -2.51 | -0.58 | -0.78$^{*}$ | -3.49$^{**}$ | -5.87$^{**}$ |
| GZ10739 × WAB 638-1 | 0.36 | 1.85$^{*}$ | -1.48$^{*}$ | -0.89 | -0.01 | 0.09 | -0.86 | -3.41$^{**}$ |
| GZ10739 × IRAT112 | -3.77$^{**}$ | -5.15$^{**}$ | -2.71$^{**}$ | -2.8 | -0.24 | -0.05 | -2.10$^{*}$ | -4.36$^{**}$ |
| GZ10739 × Giza179 | 3.66$^{**}$ | -1.55 | -4.44$^{**}$ | -4.18$^{**}$ | 0.02 | 1.09 | -1.96$^{*}$ | -2.22$^{**}$ |
| GZ10739 × Sakha108 | -4.14$^{**}$ | -2.53$^{**}$ | -2.34$^{**}$ | -3.71$^{*}$ | -0.44 | 0.45 | 0.91 | 1.96 |
| WAB 638-1 × IRAT 112 | -0.87 | 3.73$^{**}$ | 3.61$^{**}$ | 6.42$^{**}$ | -0.01 | 0.05 | -1.49 | 0.28 |
| WAB 638-1 × Giza179 | 3.99$^{**}$ | -4.23$^{**}$ | -4.63$^{**}$ | -2.4 | -0.08 | 0.19 | 1.21 | 2.25$^{*}$ |
| WAB 638-1 × Sakha108 | 4.36$^{**}$ | 2.19$^{*}$ | 0.57 | 3.64$^{*}$ | -0.21 | -0.45 | -2.08$^{*}$ | 2.2 |
| IRAT 112 × Giza179 | -5.80$^{**}$ | -3.84$^{**}$ | -4.08$^{**}$ | -1.87 | 0.69$^{*}$ | 1.05$^{**}$ | -1.93$^{*}$ | -0.52 |
| IRAT 112 × Sakha108 | 15.10$^{**}$ | 12.68$^{**}$ | -2.52$^{**}$ | 0.77 | 0.56 | -0.25 | -0.12 | -1.15 |
| Giza179 × Sakha108 | -5.81$^{**}$ | -5.48$^{**}$ | 4.15$^{**}$ | 2.25 | 1.16$^{**}$ | -0.78$^{*}$ | 0.42 | 1.16 |
| L.S.D 0.05 (sij) | 1.86 | 1.66 | 1.41 | 2.99 | 0.68 | 0.65 | 1.79 | 2.05 |
| L.S.D 0.01 (sij) | 2.46 | 2.2 | 1.87 | 3.96 | 0.9 | 0.86 | 2.38 | 2.71 |
| L.S.D 0.05 (sij-sik) | 2.75 | 2.46 | 2.08 | 4.42 | 1 | 0.96 | 2.65 | 3.03 |
| L.S.D 0.01 (sij-sik) | 3.64 | 3.26 | 2.76 | 5.86 | 1.33 | 1.28 | 3.51 | 4.01 |
| L.S.D 0.05 (sij-skl) | 2.59 | 2.32 | 1.96 | 4.17 | 0.95 | 0.91 | 2.5 | 2.85 |
| L.S.D 0.01 (sij-skl) | 3.44 | 3.07 | 2.6 | 5.53 | 1.25 | 1.21 | 3.31 | 3.78 |

N: natural condition, D: drought condition. * and ** significant at 0.05 and 0.01 levels of probability, respective

**Table 16:** Estimates of specific combining ability effects for grain yield and its component characters under natural and drought conditions

| Crosses | Number of panicles plant$^{-1}$ | | 1000-grain mass (g) | | Sterility percentage | | Grain yield plant$^{-1}$ (g) | | Water use efficiency (g ml) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | D | N | D | N | D | N | D | N | D |
| UYR 2184 × UYR 3472 | 1.52$^{*}$ | 1.67$^{**}$ | 1.77$^{**}$ | 0.86 | 0.25 | 10.96$^{**}$ | 4.60$^{**}$ | 3.70$^{**}$ | 0.09$^{**}$ | 0.11$^{**}$ |
| UYR 2184 × GZ10487 | 0.57 | -0.41 | -6.83$^{**}$ | -3.01$^{**}$ | 1.38$^{*}$ | 9.75$^{**}$ | -2.67$^{**}$ | -4.42$^{**}$ | -0.05$^{**}$ | -0.13$^{**}$ |
| UYR 2184 × GZ10739 | -1.90$^{**}$ | -1.37$^{*}$ | 0.57 | 2.87$^{**}$ | -2.13$^{**}$ | -1.36 | -2.14$^{**}$ | -4.18$^{**}$ | -0.04$^{**}$ | -0.13$^{**}$ |
| UYR 2184 × WAB 638-1 | 4.72$^{**}$ | 4.54$^{**}$ | 0.96 | -0.93 | 1.04 | -8.20$^{**}$ | -0.08 | -3.28$^{**}$ | 0.00 | -0.10$^{**}$ |
| UYR 2184 × IRAT 112 | 1.42$^{*}$ | 0.01 | 1.35$^{*}$ | 0.61 | 1.42$^{*}$ | -12.41$^{**}$ | -0.57 | -4.81$^{**}$ | -0.01 | -0.14$^{**}$ |
| UYR 2184 × Giza 179 | -3.00$^{**}$ | -1.84$^{**}$ | -1.11 | 0.73 | -1.04 | -10.94$^{**}$ | -4.15$^{**}$ | -0.09 | -0.08$^{**}$ | 0.00 |
| UYR 2184 × Sakha 108 | -5.40$^{**}$ | -2.18$^{**}$ | -0.20 | -1.99$^{**}$ | -0.96 | -8.50$^{**}$ | -2.31$^{**}$ | -0.32 | -0.04$^{**}$ | -0.01 |
| UYR 3472 × GZ10487 | 0.01 | 1.64$^{**}$ | -6.04$^{**}$ | -3.17$^{**}$ | 1.66$^{*}$ | 3.35$^{**}$ | -1.63$^{**}$ | -2.45$^{**}$ | -0.03$^{**}$ | -0.07$^{**}$ |
| UYR 3472 × GZ10739 | -3.06$^{**}$ | -2.92$^{**}$ | -1.01 | 1.91$^{**}$ | -1.45$^{*}$ | 0.15 | -4.86$^{**}$ | -6.27$^{**}$ | -0.09$^{**}$ | -0.19$^{**}$ |
| UYR 3472 × WAB 638-1 | 0.06 | -0.62 | 0.35 | -0.69 | 1.32 | 13.84$^{**}$ | -2.38$^{**}$ | -3.21$^{**}$ | -0.05$^{**}$ | -0.10$^{**}$ |
| UYR 3472 × IRAT 112 | -0.44 | -0.31 | -2.39$^{**}$ | -2.43$^{**}$ | -0.17 | -6.61$^{**}$ | -0.53 | -3.81$^{**}$ | -0.01 | -0.11$^{**}$ |
| UYR 3472 × Giza179 | -2.32$^{**}$ | -2.86$^{**}$ | -1.46$^{*}$ | -1.77$^{*}$ | 1.81$^{**}$ | 3.10$^{**}$ | -3.07$^{**}$ | 0.01 | -0.06$^{**}$ | 0.00 |
| UYR 3472 × Sakha 108 | 5.44$^{**}$ | 3.90$^{**}$ | -0.14 | -2.15$^{**}$ | -1.08 | -11.90$^{**}$ | -3.27$^{**}$ | 0.42 | -0.06$^{**}$ | 0.01 |
| GZ10487 × GZ10739 | -1.28$^{*}$ | -6.23$^{**}$ | 1.99$^{**}$ | 2.50$^{**}$ | -0.68 | -5.49$^{**}$ | 0.74 | 1.57$^{**}$ | 0.01 | 0.05$^{**}$ |
| GZ10487 × WAB 638-1 | 0.07 | -1.96$^{**}$ | -2.89$^{**}$ | -0.63 | -0.05 | -1.60 | -1.64$^{**}$ | 0.76$^{*}$ | -0.03$^{**}$ | 0.02$^{*}$ |
| GZ10487 × IRAT112 | 2.74$^{**}$ | 3.32$^{**}$ | 5.47$^{**}$ | 2.80$^{**}$ | -0.93 | 0.32 | -1.63$^{**}$ | -0.60 | -0.03$^{**}$ | -0.02 |
| GZ10487 × Giza179 | 3.79$^{**}$ | 2.43$^{**}$ | 8.51$^{**}$ | 5.69$^{**}$ | 0.97 | 9.82$^{**}$ | 1.80$^{**}$ | 0.92$^{*}$ | 0.03$^{**}$ | 0.03$^{*}$ |
| GZ10487 × Sakha108 | -0.38 | 0.12 | -3.44$^{**}$ | -2.96$^{**}$ | 1.25 | 1.16 | 0.74 | 3.52$^{**}$ | 0.01 | 0.11$^{**}$ |
| GZ10739 × WAB 638-1 | 3.33$^{**}$ | -0.05 | -2.13$^{**}$ | -4.42$^{**}$ | 2.21$^{**}$ | -0.61 | -0.01 | 2.01$^{**}$ | 0.00 | 0.06$^{**}$ |
| GZ10739 × IRAT112 | -0.13 | 0.62 | -2.76$^{**}$ | -5.08$^{**}$ | 2.16$^{**}$ | 4.95$^{**}$ | -2.17$^{**}$ | 2.11$^{**}$ | -0.04$^{**}$ | 0.06$^{**}$ |
| GZ10739 × Giza179 | 0.12 | -0.17 | -1.13 | 1.00 | 0.70 | -5.42$^{**}$ | 1.03 | 1.83$^{**}$ | 0.02 | 0.06$^{**}$ |
| GZ10739 × Sakha108 | -0.82 | 2.49$^{**}$ | 0.59 | 1.66$^{*}$ | 0.31 | 20.85$^{**}$ | 3.13$^{**}$ | 1.57$^{**}$ | 0.06$^{**}$ | 0.05$^{**}$ |
| WAB 638-1 × IRAT 112 | -1.78$^{**}$ | -0.57 | -3.11$^{**}$ | -1.42$^{*}$ | -1.54$^{*}$ | 7.71$^{**}$ | 2.52$^{**}$ | 0.64 | 0.05$^{**}$ | 0.02 |
| WAB 638-1 × Giza179 | -2.50$^{**}$ | -1.46$^{**}$ | -2.37$^{**}$ | -0.26 | 1.43$^{*}$ | 7.38$^{**}$ | -1.02 | 1.23$^{**}$ | -0.02 | 0.04$^{**}$ |
| WAB 638-1 × Sakha108 | -1.73$^{**}$ | -0.40 | 0.14 | -0.01 | 0.15 | 4.71$^{**}$ | -1.35$^{*}$ | 1.80$^{**}$ | -0.03$^{*}$ | 0.05$^{**}$ |
| IRAT 112 × Giza179 | -3.00$^{**}$ | -3.12$^{**}$ | -1.48$^{*}$ | -3.20$^{**}$ | 1.15 | 13.06$^{**}$ | -1.11$^{*}$ | 2.53$^{**}$ | -0.02$^{*}$ | 0.08$^{**}$ |
| IRAT 112 × Sakha108 | -2.83$^{**}$ | -3.12$^{**}$ | 0.87 | 0.29 | 3.16$^{**}$ | 0.87 | -2.57$^{**}$ | 2.30$^{**}$ | -0.05$^{**}$ | 0.07$^{**}$ |
| Giza179 × Sakha108 | -1.25 | -0.85 | 3.01$^{**}$ | 0.78 | 2.40$^{**}$ | -2.86$^{**}$ | 4.02$^{**}$ | -0.01 | 0.08$^{**}$ | 0.00 |
| L.S.D 0.05 (sij) | 1.26 | 1.04 | 1.22 | 1.35 | 1.32 | 1.63 | 1.04 | 0.75 | 0.02 | 0.02 |
| L.S.D 0.01 (sij) | 1.68 | 1.38 | 1.62 | 1.80 | 1.75 | 2.16 | 1.38 | 1.00 | 0.03 | 0.03 |
| L.S.D 0.05 (sij-sik) | 1.87 | 1.54 | 1.80 | 2.00 | 1.96 | 2.40 | 1.54 | 1.11 | 0.03 | 0.03 |
| L.S.D 0.01 (sij-sik) | 2.48 | 2.04 | 2.39 | 2.66 | 2.59 | 3.19 | 2.05 | 1.48 | 0.04 | 0.04 |
| L.S.D 0.05 (sij-skl) | 1.76 | 1.45 | 1.70 | 1.89 | 1.84 | 2.27 | 1.46 | 1.05 | 0.03 | 0.03 |
| L.S.D 0.01 (sij-skl) | 2.34 | 1.93 | 2.25 | 2.50 | 2.45 | 3.01 | 1.93 | 1.39 | 0.04 | 0.04 |

N: natural condition, D: drought condition. * and ** significant at 0.05 and 0.01 levels of probability, respective

conditions, respectively. On the other hand, four and two parents obtained negative significant and highly significant in general combining ability under both natural and drought condition, respectively

## 3.5 SPECIFIC COMBINING ABILITY EFFECTS (SCA):

Estimates of SCA effects of all crosses for each trait under natural and drought conditions are presented in Table 14, 15 and 16. Regarding days to heading nine and eleven hybrids showed desirable significant and highly significant negative SCA effects under either natural and drought conditions, respectively. For plant height, ten and eleven hybrids showed desirable significant and highly significant negative SCA effects under both natural and drought conditions, respectively. However, ten and eleven hybrids got positive significant and highly significant in SCA under both natural and drought condition, respectively. For number of tillers plant$^{-1}$, five and seven hybrids showed desirable significant and highly significant positive SCA effects under either natural and drought conditions, respectively. Seven hybrids showed desirable significant and highly significant positive SCA under both natural and drought conditions, respectively with respect to panicle length. For chlorophyll content ten and eleven hybrids showed desirable significant and highly significant positive SCA effects under either natural and drought conditions, respectively. Seven and six hybrids showed desirable significant and highly significant positive SCA effects under either natural and drought conditions for flag leaf area, respectively. Regarding leaf rolling, five and three hybrids showed desirable significant and highly significant negative SCA effects under both natural and drought conditions, respectively. Two and six hybrids showed desirable significant and highly significant positive SCA effects under either natural and drought conditions regarding relative water content, respectively. For number of panicles plant$^{-1}$, seven hybrids showed desirable significant and highly significant positive SCA effects under either natural and drought conditions, respectively. Six hybrids showed desirable significant and highly significant positive SCA effects under both environments concerning 1000-grain mass, respectively. Regarding sterility percentage, three and nine hybrids showed desirable significant and highly significant negative SCA effects under either natural and drought conditions, respectively. Regarding grain yield plant$^{-1}$, 5 and 13 hybrids showed desirable significant and highly significant positive SCA effects under both environments, respectively. For water use efficiency, out of 5 and 13 hybrids showed desirable significant and highly significant positive SCA effects under either natural and drought environments, respectively.

## 3.6 PHENOTYPIC CORRELATION COEFFICIENT:

Observation values were recorded on yield contributing and drought tolerance characters. Data in Fig.1 The correlation coefficient at Fig.1 revealed the high significant and positive associated among grain yield and days to heading, number of tillers plant$^{-1}$, panicle length, relative water content, number of panicles plant$^{-1}$, thousand grain mass and water use effi-

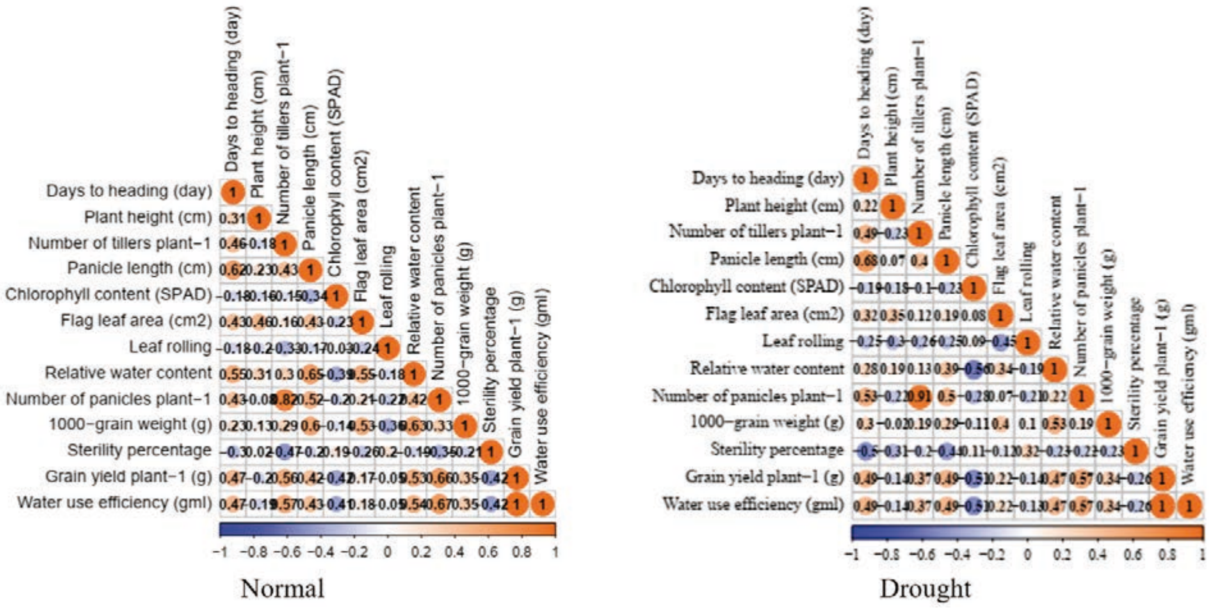

**Fig. 1:** Correlation coefficient of characteristic study under both natural and drought conditions.

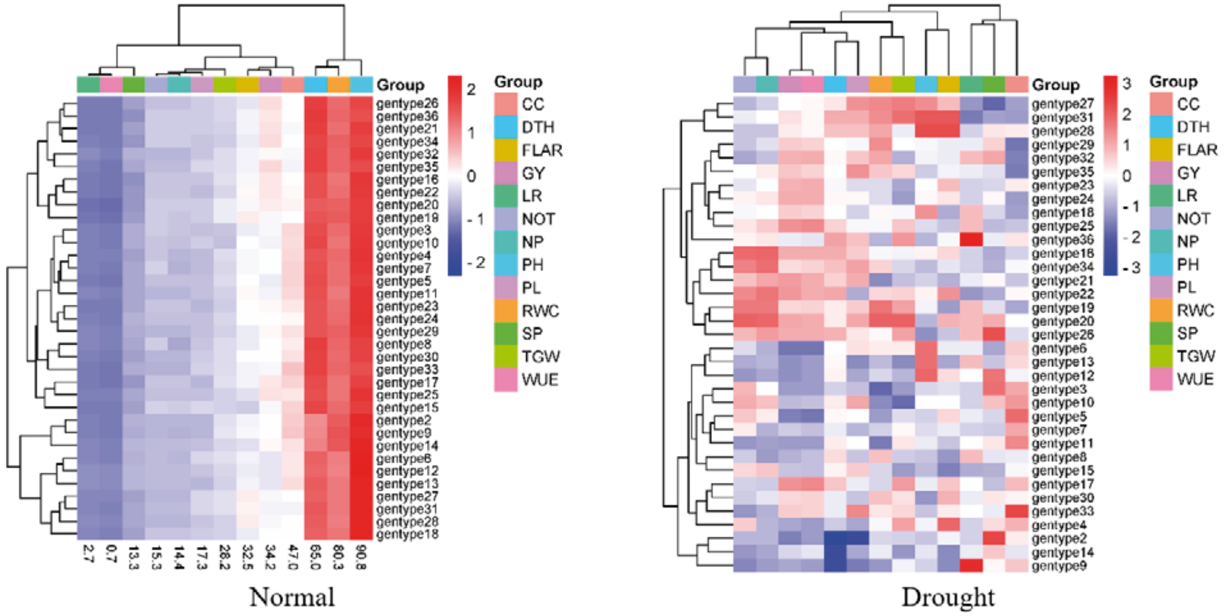

**Fig. 2:** Heatmap and hierarchical clustering divided the genotypes into different clusters based on the evaluated characteristics for both environments natural and drought. Red and blue colors reveal high and low values for the corresponding characteristics, respectively.

ciency for both natural and drought environments. For days to heading, significant and highly significant positive correlation was obtained with plant height, flag leaf area, chlorophyll content, relative water content, root length and grain yield plant$^{-1}$. Also, highly significant negative correlation was observed with leaf rolling and sterility percentage. Plant height was significant and highly significant positive correlation was obtained with flag leaf area, chlorophyll content, relative water content and root length. Also, highly significant negative correlation was observed with leaf rolling and sterility percentage. Chlorophyll content was significant and highly significant positive correlation was found with flag leaf area, relative water content, root length, with 100-grainmass and grain yield plant$^{-1}$. Also, significant and highly significant negative correlation was observed with leaf rolling and sterility percentage. Flag leaf area significant and highly significant positive correlation with relative water content, root length, 100-grain and grain yield plant$^{-1}$. Highly significant negative correlation was observed with leaf rolling and sterility percentage. The relative water content showed significant and highly significant positive correlation with number of rootsplant$^{-1}$, root length and grain yield plant$^{-1}$. On the other hand, highly significant negative correlation was observed with leaf rolling sterility percentage. Leaf rolling highly significant positive correlation was detected with sterility percentage. On the other hand, significant and highly significant negative correlation with root length and grain yield plant$^{-1}$. Concerning number of roots plant$^{-1}$ had significant positive correlation with grain yield plant$^{-1}$ and significant negative correlation with sterility percentage. With respect to root volume, significant and highly significant positive correlation was obtained with number of panicles plant$^{-1}$, harvest index and grain yield plant$^{-1}$. For root length, highly significant positive correlation was detected with grain yield plant$^{-1}$ and highly significant negative correlation with sterility percentage. For sterility percentage, significantly negative correlation with grain yield plant$^{-1}$ and harvest index.

## 3.7 CLUSTERING OF GENOTYPES

The heatmap and hierarchical clustering based on agronomic characteristics divided the evaluated 36 genotypes (parents and their offsprings into different clusters (Figure 2). The genotype 26, 36, 21 and 34 possessed the highest values for most of the agronomic factors (depicted in red) under natural condition while the genotypes 27,31 and 28 had superior in most agronomic traits at drought condition. On the contrary, the parental cultivars 27,31,28 and 18 had the lowest values (depicted in blue) at natural irrigation otherwise the genotypes 2, 14 and 9 at drought condition.

## 4 DISCUSSION

Drought stress is a serious limiting factor for rice production and yield stability (Asma et al., 2021). Therefore, the achievement of modern Egyptian rice varieties with stable yield under water stress conditions would be great importance. Significant differences were observed among genotypes (parents, crosses, and parents vs. crosses) under drought and natural conditions for all the studied traits, implying the appreciable amount of genetic variability of the parents, crosses, and parents vs. crosses used. Thus, the evaluated genotypes could be selected for further genetic improvement on the basis of grain yield and other physiological traits under drought and natural conditions, the selected genotypes represent a scientifically robust pool for genetic improvement because they address yield gap in both stress and non-stress environments, exhibit physiological resilience (WUE, sterility control) critical for climate adaptation and enable trait-based breeding to develop varieties with balanced performance. Highly significant genetic variability is present among lines, testers, and line × tester interactions for flag leaf area, panicle density, harvest index, biological yield per plant, and yield per plant (Saleem et al., 2010). Tiwari et al. (2011) found that parents, crosses, and parents vs. crosses are highly significant for various traits, *i.e.,* days to 50 % flowering, effective tillers plant$^{-1}$, panicle length, number of spikelets panicle$^{-1}$, number of fertile spikelets, spikelet fertility percent, grain yield plant$^{-1}$, Hundred grain mass, biological yield, and harvest index. Significant parents vs. crosses populations were studied for useful heterosis, which can be used to identify improved genotypes for all the traits under water stress conditions. Previous researchers have emphasized the importance of genetic variation in the breeding of new improved rice varieties (Wang et al., 2018). General combining ability effects (GCA) varied from the parent to another, giving negative or positive values. The significant values of GCA refer to genetic variance (additive) and (additive × additive) play a major role in the positive direction of the desired character in all the crosses in which the genotype is involved (Gaballah et al., 2021b). The negative and significant ones indicated that the flexible portion of genetic variance response well in the positive direction of desired character in some crosses and negative direction in other crosses that having the genotype in question as a constant parent. So, this response would be clearly shown in the performance of the specific crosses. The non-significant (GCA) indicated that the genotypes have no important effects in the crosses in which they will be involved, but in some cases, they may show important response in specific cross which could be estimated by the (SCA). Combining ability analysis revealed significant GCA and SCA variances for all the traits under natural and drought conditions, suggesting the importance of additive and non-additive gene actions in the expression of these traits. GCA can be applied to identify superior parental genotypes, whereas SCA helps in the identification of promising hybrids that may ultimately lead to the development of hybrid populations (Saleem et al., 2010). The GCA: SCA ratio is used to identify the nature of existing gene action. The GCA: SCA ratio was less than unity for all studied traits, except for leaf rolling under natural condition indicating that the non-additive type of gene action was of greater importance in the inheritance of this traits, so, the selection for most traits were delay to late generation. Therefore, selection based on the accumulation of non-additive effects would be more effective and successful in improving these traits in later generations. Malemba et al. (2017) revealed that in rice, the GCA:SCA for spikelet fertility, grain yield, thousand-grain mass, and panicles plant$^{-1}$ are controlled by non-additive genes under drought conditions. Hybridization and then intensive selection in later generations are recommended for the improvement of traits that are governed by non-additive gene actions. The relatively high level of GCA variances indicated the main role of additive gene action for all traits, except leaf rolling under natural conditions. Selection and pedigree breeding methods are feasible for the improvement of rice traits governed by additive gene action (Devi et al., 2018). The significant and positive GCA effects of the parental genotypes for the studied traits are a good indication of enhancement in tolerance to drought stress conditions. The estimated GCA effects help in identifying the parental genotypes with the best genetic potential to produce individuals with desirable traits after consequent selections (Gramaje et al., 2020). The genotypes GZ10487, GZ10739, Giza 179 and Sakha 108 were good general combiners for improving most traits studied under both natural and water deficit conditions. The $F_1$ hybrids UYR 2184 × UYR 3472, GZ10487 × GZ10739, GZ10487 × Sakha108, GZ10739 × Sakha108 and WAB 638-1 × IRAT 112 were pranking populations for natural and drought tolerance and yield components. A good combination of crosses with high SCA values are ideal for heterosis breeding. The promising crosses shared one of the good general combiner with drought tolerance, signifying that these crosses will eventually yield desirable transgressive segregants (Collard et al., 2017). Positive significant heterosis over the mid and better parents was expressed by $F_1$ hybrids *viz*., GZ10739 × Sakha108 and Giza179 × Sakha108 for the majority of the traits, indicating that these hybrids were found to be best suited to natural and aerobic conditions and provide desirable direction for the further improvement of tolerance to drought stress conditions. The production of rice, being the staple food in most Asian countries, has to be increased through the exploitation of heterosis breeding to meet the food security challenges of the 21st century (Prahalada et al., 2021). In the study, the association has been done to identify the nature of association among important yield contributing traits of drought tolerance with grain yield in parent and $F_1$ generation of rice. High yield at the drought conditions has been attributed to the drought tolerance nature of the genotypes correlation analysis reveals the relationship between the various independent yield contributing characters with the dependent variable of interest the grain yield (Acevedo-siaca et al., 2020). Also, yield improvement in drought condition is difficult without understanding the association of secondary and putative traits of drought tolerance with grain yield. correlations are the magnitude of the level of association between traits (Gaballah et al., 2021a). Grain yield showed highly significant and positive correlations with the majority of the traits. The selection for one trait resulted in progress for all other traits that are positively correlated.

## 5 CONCLUSION

The governance of yield components, including grain yield, fertility percentage, hundred grain mass, and productive tillers plant$^{-1}$, by non-additive genes suggested that hybridization followed by intensive selection in later generations might be effective for the further improvement of these traits under natural and drought conditions. Four parents, *i.e.,* GZ10487, GZ10739, Giza 179 and Sakha 108, were found to be good general combiners for yield components under natural and water stress conditions and hence could be utilized in future hybridization programs for the introgression of drought tolerance into elite rice lines. The $F_1$ hybrids, *viz*., GZ10739 × Sakha108 and Giza179 × Sakha108, showed the best performance for the studied traits with desirable heterosis over the better parent. These promising populations could be used in future breeding program to develop natural and drought tolerant and high yielding rice genotypes.

## 6 DECLARATIONS

**Ethics approval and consent to participate**

**Consent for publication:** The article contains no such material that may be unlawful, defamatory, or which would, if published, in any way whatsoever, violate the terms and conditions as laid down in the agreement.

**Availability of data and material:** Not applicable.

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# Pathogenicity assessment of *Fusarium clavum* associated with wheat head blight in Algeria

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**Pathogenicity assessment of *Fusarium clavum* associated with wheat head blight in Algeria**

**Abstract:** Durum wheat (*Triticum durum* Desf) is one of the most important cereal crops in Algeria. During the agricultural season 2021-2022, typical symptoms of *Fusarium* Head Blight were observed in wheat fields in the Setif region. One of the causal agents was identified on the basis of morphological characters and DNA sequences as *Fusarium clavum* J.W. Xia, L. Lombard, Sand.-Den., X.G. Zhang & Crous, a member of the *Fusarium incarnatum-equiseti* species complex. It was isolated from symptomatic wheat glumes. In vitro and in vivo pathogenicity tests were conducted on three Algerian durum wheat varieties to assess the effect of this isolate on the seedling and the mass of durum wheat kernels. The results showed that *Fusarium clavum* caused a significant reduction in the coleoptile (38.9 %) and root length (42 %) and decreased kernels mass by 20.8 %. This study further confirms the presence of *Fusarium clavum* as an agent causing *Fusarium* Head Blight on wheat in Algeria.

**Key words:** durum wheat, *Fusarium clavum*, pathogenicity, Algeria.

**Ocena patogenosti glive *Fusarium clavum*, kot povzročiteljice pšeničnega ožiga v Alžiriji**

**Izvleček:** Trda pšenica (*Triticum durum* Desf) je eno izmed najpomembnejših žit v Alžiriji. V pridelovalnih sezonah 2021-2022 so bili na pšeničnih poljih na območju Setifa opaženi značilni simptomi pšeničnega ožiga, ki ga povzroča gliva iz rodu *Fusarium*. Kot povzročitelj je bila na osnovi morfoloških znakov in DNK zaporedij prepoznana gliva *Fusarium clavum* J.W. Xia, L. Lombard, Sand.-Den., X.G. Zhang & Crous, predstavnica iz kompleksa vrst *Fusarium incarnatum-equiseti*. Izolirana je bila iz simptomatičnih pčeničnih plev. In vitro in in vivo testi patogenosti so bili narejeni na treh alžirskih sortah trde pšenice za oceno učinka tega izolata na sejanke in maso pšeničnih zrn. Rezultati so pokazali, da je gliva *Fusarium clavum* povzročila značilno zmanjšanje dolžine koleoptile (38,9 %) in dolžine korenin (42 %) ter zmanjšala maso zrn za 20,8 %. Raziskava potrjuje prisotnost glive *Fusarium clavum* kot fuzarijskega povzročitelja pšeničnega ožiga v Alžiriji.

**Ključne besede:** trda pšenica, *Fusarium clavum*, patogenost, Alžirija

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## 1 INTRODUCTION

Wheat (*Triticum aestivum* L.) is a key cereal crop and a major global source of food for people. Due to its strategic importance for human and animal nutrition, wheat occupies a privileged place in Algerian agriculture. For instance, it is used to make bread and other Algerian food as couscous, which is the country's most popular dish (Kezih et al., 2014). Numerous phytopathogenic *Fusarium* species are found around the world, infecting a variety of crop plants, including cereals such as wheat, maize, oats, and barley (Boutigny et al., 2011). *Fusarium* drastically reduces grain quality and output. More significantly, several species of the *Fusarium* genus produce mycotoxins, which prevent the synthesis of proteins and as a result, exposure to these mycotoxins can lead to several health issues in humans and animals (Pestka, 2010). The wheat head, grain, and occasionally the peduncle are the only areas where *Fusarium* head blight (FHB) symptoms can occur. Usually, when healthy heads are still green, the first discernible indication is the bleaching of a part of or all the spikelets. Spikelets above and below the initial spot of infection may also become bleached as the fungus spreads across the rachis. When examining infected spikelets, pink to orange masses of spores may be seen. It is during rainy, humid conditions that these spore masses are generated. Infected kernels, sometimes known as tombstones, have a shriveled, discolored appearance and are low in mass (Freije & Wise, 2015). There is currently not enough information available in Algeria regarding the causative agent of *Fusarium* head blight of durum wheat (Laraba et al., 2017); besides, *Fusarium clavum* was discovered for the first time in Algeria in a study by Belabed et al. (2025), as a cause of this disease in durum wheat. This study was conducted to isolate and identify *Fusarium clavum* species from infected wheat in the Setif region of Algeria and to evaluate their pathogenicity towards durum wheat varieties. Through morphological characterization, molecular identification, phylogenetic study, and pathogenicity tests conducted in vitro and in vivo, we aim to report *Fusarium clavum* as a head blight agent on durum wheat in Algeria.

## 2 MATERIALS AND METHODS

### 2.1 FUNGAL MATERIAL

### 2.1.1 Isolation of *Fusarium clavum*

In this study, symptomatic infected wheat samples were collected randomly during the agricultural season 2021/2022 from several farms located in the Setif region, Algeria.

Diseased samples (seeds, glumes, and crowns) were disinfected in 2 % sodium hypochlorite (NaClO) (commercial bleach, Bref, Henkel, Algeria) for 5 min and rinsed three successive times with sterile distilled water (Benhamou & Chet, 1996). They were then dried between two sheets of sterile paper towels, plated on potato dextrose agar (PDA) medium in sterile Petri dishes, and incubated at 25 °C in the dark for seven days. Resultant colonies resembling *Fusarium* spp. were further sub-cultured on PDA for purification.

### 2.1.2 Macroscopic and microscopic characterization

A mycelial disc subculture from strain F15B was placed on the PDA for evaluating macroscopic characteristics (Leslie & Summerell, 2006 and Xia et al., 2019). After a 21 - day incubation period in the dark at 25 °C, the morphological characterization was conducted based on the colony's growth, appearance, and texture, and the pigmentation of the Petri dish's face and reverse was examined macroscopically (Leslie & Summerell, 2006 and Xia et al., 2019). Synthetic Nurtrient-poor Agar medium (SNA) (Nirenberg, 1976) was used for the diagnosis of micromorphological characteristics. The strain was observed under a light microscope to describe microscopic characters. The presence or absence of microconidia and macroconidia, their shapes and sizes, and the presence or absence of chlamydospores are diagnostic characteristics used for the identification of *Fusarium* species (Leslie & Summerell, 2006 and Xia et al., 2019).

### 2.1.3 Molecular identification and phylogenetic analysis

Molecular analysis was performed to validate identification of strain F15B. The mycelium was harvested, and DNA was extracted using Nucleo Spin Plant II kit (Macherey-Nagel Germany). The internal transcribed spacers of ribosomal DNA (ITS) and the transcription elongation factor 1 alpha (TEF1) were amplified using two primers ITS1/ITS4 (CTTGGTCATTTAGAGGAAGTAA/ TCCTCCGCTTATTGATATGC) (White et al., 1990), and EF-728F/EF-2 (CATYGAGAAGTTCGAGAAGG/ GGARGTACCAGT SATCATGTT) (O'Donnell et al., 1998; Carbone & Kohn, 1999). The PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel (Germany). Amplicons were sequenced with the Sanger technique

(Sanger et al., 1977). The sequences were edited using MEGA 11 software and compared with sequences in databases by using Blastn (National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). MEGA 11 was used to phylogenetically place our sample. The tree is based on TEF1 sequences and calculated by using neighbor-joining clustering and bootstrap analyses with 1,000 replications.

## 2.2 PATHOGENICITY TESTS

### 2.2.1 Pathogenicity test of F15B isolate towards wheat seedling

A pathogenicity assay was performed following the protocol of Belabed et al. (2023) with some modifications, using strain F15B to determine its pathogenic potential. Durum wheat seeds from each Algerian variety (Guemgoum Rkham, Djnah Khotaifa, and Oued Znati) were surface disinfected for 5 min in 2 % NaClO (commercial bleach, Bref, Henkel, Algeria), rinsed three times in sterile distilled water, and dried. Five healthy wheat seeds from the three varieties were each inoculated with a 5mm diameter fungal disc taken from a 21 day old PDA culture and a sterile PDA disc as a control. Three replicates were set up for all combinations of F15B isolate and wheat variety. The inoculated seeds were placed on sterile double-layer filter paper soaked with Potato Dextrose Broth (PDB) in Petri dishes. Petri dishes were sealed with parafilm and incubated in the dark at 25 °C for five days. The length of the root system, coleoptile, and germination rate were measured to assess the pathogenicity. The reduction rate of coleoptile length (CLr %) and the reduction rate of root length (RLr %) were calculated using the following equation:

$$\text{CLr (\%) or RLr (\%)} = \frac{\text{Length of coleoptile or root (control)} - \text{Length of coleoptile or root (Fusarium-treated)}}{\text{Length of coleoptile or root (control)}} \times 100$$

### 2.2.2 Pathogenicity test of F15B isolate on wheat heads

For the pathogenicity test on Algerian durum wheat heads (Guemgoum Rkham, Djnah Khotaifa, and Oued Znati varieties), the experimental spray inoculation protocol of Mesterhazy (1995) was used. For each variety, three durum wheat seeds were sowed in plastic pots with four replicates distributed randomly. A spore suspension of $4 \times 10^5$ spores ml$^{-1}$, obtained from F15B cultures was prepared using a Malassez hemocytometer. 3 spikes from each replicate were inoculated with spores during the full flowering stage, using glass sprayers, while the control spikes were sprayed with sterile distilled water. The inoculated spikes were covered with a damp polyethylene bag to retain humidity for 24 hours. For comparison purposes, the same experiment was conducted using a known *Fusarium culmorum* isolate (PV123206), which is recognized as pathogenic to wheat heads. This allowed assessing the potential impact of F15B isolate on wheat heads. After harvesting, the ears of each statistical unit were placed in a paper bag. Treated and untreated wheat spikes were harvested and threshed, and the kernels mass was measured. The mass of a thousand kernels (TKM) was then estimated. Subsequently, the reduction rate in the thousand-grain mass (TKMr %) of the *Fusarium culmorum* and *Fusarium clavum*-inoculated spikes was calculated using the following equation:

$$\text{TKMr (\%)} = \frac{\text{Mass of 1000 grains (control)} - \text{Mass of 1000 grains (}\textit{Fusarium}\text{ inoculated)}}{\text{Mass of 1000 grains (control)}} \times 100$$

## 2.3 STATISTICAL ANALYSIS

Data was subjected to analyses of variance ANOVA using SPSS software (IBM SPSS Statistics version 26) at a 5 % level with a 95 % confidence interval.

# 3 RESULTS AND DISCUSSION

## 3.1 MACROSCOPIC AND MICROSCOPIC CHARACTERIZATION

Fungal colonies that resembled *Fusarium* spp. were obtained. Only a single *Fusarium clavum*-like isolate was obtained from durum wheat glumes and assigned as F15B. The morphological characters observed on SNA and PDA (not shown) agreed with the phylogenetic analyses.

## 3.2 MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS

The obtained sequences of F15B isolate were deposited in GeneBank under accession numbers OR900216 (ITS) and PP035912 (TEF). The blastn search of ITS showed 98-99 % identity with several sequences of *Fusarium clavum* (e.g: OR582979, OR123379), while the

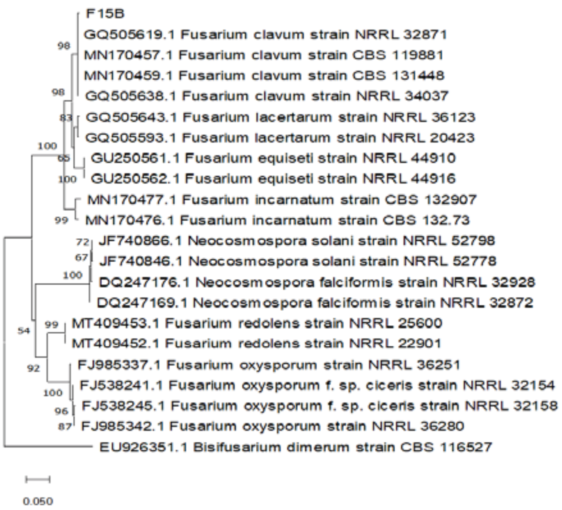

**Figure 1:** Phylogenetic tree generated from translation elongation factor 1-alpha (TEF1) gene sequences of *Fusarium* spp. Bootstrap values (based on 1,000 replications) are indicated next to the branches. Based on the Neighbor-joining method, isolate F15B clusters with *F. clavum* isolates. The tree is rooted with *Bisifusarium dimerum* (Penz.) L. Lombard & Crous strain CBS 116527

TEF sequence showed 99-100 % with *Fusarium clavum* isolates (e.g: GQ505672, MN170457).

## 3.3 PATHOGENICITY TESTS

### 3.3.1 Pathogenicity test of F15B isolate towards wheat seedling

In vitro, the isolate F15B was responsible for 29,9 %, 60,4 %, and 26,3 % of coleoptile length reduction on Guemgoum Rkham, Djnah Khotaifa, and Oued Znati varieties. Furthermore, it induced 25,7 %, 64,3 %, and 35,9 % of root length reduction on Guemgoum Rkham, Djnah Khotaifa, and Oued Znati varieties (Tab. 1).

After 5 days of incubation, it appears that 'Djnah Khotaifa' was more sensitive to *F. clavum* as coleoptile length and root length reduction were highest compared to vars. Guemgoum Rkham and Oued Znati. Also, it appears that var. Djnah khotaifa was most susceptible to attacks of F15B isolate with a germination rate of 93 % compared to vars. Guemgoum Rkham (100 % germination rate) and Oued Znati (95,3 %) (Tab. 1).

There was no statistically significant difference between coleoptile length reduction rate ($p = 0.33 > 0.05$), root length reduction rate ($p = 0.31 > 0.05$), and seed germination rate ($p = 0.39 > 0.05$) of the controls of the tested durum wheat varieties. On the contrary, the results showed that there was a significant difference ($p < 0.05$) between the infected plants and the non-infected control plants. This result shows that *Fusarium clavum* F15B isolate was pathogenic and significantly affected coleoptile and root lengths (Tab.1).

### 3.3.2 Pathogenicity test of F15B isolate on wheat heads

Twenty-six days after inoculation, typical *Fusarium* head blight symptoms appeared on the inoculated spikes, while the control spikes remained free of symptoms. After harvest, inoculated grains exhibited deformities compared to the negative controls. Re-isolation from the infected grains and glumes was conducted to satisfy Koch's postulates. The results of TKM after means comparing (Tab. 1) showed that *Fusarium clavum* reduced TKM from 55,9 g to 44,8 g in 'Guemgoum Rkham', and from 45,3 g to 26,5 g in 'Djnah Khotaifa', compared to the negative controls. On the other hand, no TKM reduction was observed in 'Oued Znati'. Concerning the rate of TKM reduction (TKMr %) by the isolate F15B, the results were 21,1 % for 'Guemgoum Rkham' and -7,1 % for 'Oued Znati'. However, it appears that 'Djnah Khotaifa' was the most susceptible to attacks of the isolate with a TKMr % of 41,3 %. The pathogenic isolate *Fusarium culmorum* (PV123206) exhibited a higher pathogenic potential, causing severe disease symptoms in the tested wheat varieties. This was reflected in the TKM, recorded as 29,9 g for var. Goumgoum Rkham, 16,5 g for var. Djenah Khotifa, and 29,1 g for var. Oued Zenati. Compared to the non-infected plants, this represents a TKMr % of 44,7 % for var. Goumgoum Rkham, 63 % for var. Djenah Khotifa, and 36,3 % for var. Oued Zenati. These reductions were higher than those caused by *Fusarium clavum* isolate.

Statistical analysis showed a significant difference ($p = 0.01 < 0.05$) between the inoculated and uninoculated control spikes. The reduction in TKM caused by the F15B isolate is directly associated with yield and quality loss, confirming its pathogenic impact on wheat heads.

The most serious diseases affecting root, stem, and spike of wheat and at all stages of growth are caused by *Fusarium* species. In Algeria, some studies have been carried out on *F. culmorum* the main *Fusarium* Head Blight agent focusing on its occurrence, pathogenicity, and diversity. Other pathogenic *Fusarium* species have not yet been studied well in Algeria (Touati-Hattab et al., 2016; Laraba et al., 2017; Abdallah-Nekache et al., 2019).

*Fusarium clavum* is a species of the *Fusarium incarnatum-equiseti* species complex (FIESC) which is a

**Table 1:** Observed effects of pathogenicity tests and thousand kernels mass reduction

| Varieties | Treatment | Coleoptile length (cm) | CLr (%) | Germination (%) | Root length (cm) | RLr (%) | TKM (g) | TKMr (%) |
|---|---|---|---|---|---|---|---|---|
| Guemgoum Rkham | Control | 5,03 | 0 | 100 | 5,5 | 0 | 55,87 | 0 |
| | F15B | 3,36 | 29,93 | 100 | 4,03 | 25,72 | 44,79 | 21,12 |
| Djnah Khotaifa | Control | 5,3 | 0 | 100 | 2,53 | 0 | 45,26 | 0 |
| | F15B | 2 | 60,38 | 93 | 0,7 | 64,25 | 26,48 | 41,28 |
| Oued Znati | Control | 3,86 | 0 | 100 | 4,76 | 0 | 45,84 | 0 |
| | F15B | 2,86 | 26,29 | 95,33 | 3,03 | 35,86 | 49,08 | -7,06 |

*Each number in the table represents the average of the replicates in each parameter (F15B: inoculated by F15B isolate)

phylogenetically species-rich complex that includes over 30 recognized phylogenetic species (Xia et al., 2019). *Fusarium clavum* was reported for the first time in Algeria in the study of Belabed et al. (2025) as a causal agent of wheat head blight. The F15B isolate was morphologically identified as *F. clavum*. It was isolated from durum wheat glumes in Algeria's Setif area in the North. The results of macroscopic and microscopic characteristics were similar to those described by Manganiello et al. (2021) and Belabed et al. (2025). The micro-morphological and plate-culturing characteristics of F15B isolate were also similar to those of *Fusarium clavum* (Wang et al., 2019; Xia et al., 2019). The phylogenetic tree, constructed using the Neighbor-Joining (NJ) method based on partial TEF1-α sequences, showed that the F15B isolate clustered with *Fusarium clavum*, indicating its genetic relatedness to this species and confirming its classification within it. This study reports the presence of an *F. clavum* isolate in Algerian durum wheat fields. In contrast, Belabed et al. (2025) identified multiple isolates of this species, further supporting its occurrence in the region.

*Fusarium clavum* is a plant pathogen occurring worldwide as it can infect a wide range of plant hosts, such as *Cucumis melo* L. (Meshram et al., 2023), *Solanum lycopersicum* L. (Gilardi et al., 2021), *Beta vulgaris* L. (Khan et al., 2024), *Rosa* spp (Manganiello et al., 2021) and *Phoenix dactylifera* L. (Rabaaoui et al., 2021).

Few research was conducted on the pathogenicity of *F. clavum* on wheat. *Fusarium clavum* has been identified as a pathogenic species responsible for *Fusarium* head blight in wheat in Mexico (Leyva-Mir et al., 2022). Azil et al. (2021) investigated the *Fusarium incarnatum-equiseti* species complex associated with tuber dry rot and wilt of potatoes but did not report *F. clavum*. This highlights the need for further studies on the occurrence of *F. clavum* in different crops.

The obtained results showed that strain F15B had a moderate aggressiveness toward wheat coleoptile length, root length, and seed germination, which concord with the finding of Belabed et al. (2023). They concluded that members of the *F. incarnatum-equiseti* species complex are moderate or weak pathogens. The F15B isolate had a moderate effect on wheat TKM (20.8 % TKM reduction), especially if compared with *F. culmorum* (Wm.G.Sm.) Sacc. one of the major FHB agents of wheat plants (48 % TKM reduction) (Abdallah-Nekache et al., 2019). The significantly greater virulence of *F. culmorum* aligns with its well-documented ability to induce severe disease symptoms in wheat, leading to substantial yield losses. This strong impact can be attributed to its aggressive infection mechanisms, including rapid colonization, toxin production, and disruption of host physiological processes. Additionally, the reduction rate of TKM caused by *F. culmorum* and *F. clavum* strains may be linked to the susceptibility of the wheat genotypes used in the experiment, highlighting differences in host responses to fungal infection. This parameter plays a very important role and impacts the results. In this study, var. Djnah Khotaifa was the most sensible, comparing to vars. Guemgoum Rkham and Oued Znati. This is an important information benefiting breeding programs that aim at reducing susceptibilies of cultivars against *Fusarium* head blight agents.

## 4 CONCLUSIONS

In Algeria, *Fusarium clavum* has been identified on wheat, and its pathogenic effects have been confirmed across three durum wheat varieties. Observed impacts included reduced seed germination, decreased coleoptile and root lengths, and reduced thousand kernel mass. Our findings highlight the importance of expanding studies focusing on determining the distribution, prevalence, and toxigenic potential of *Fusarium* species associated with wheat diseases in Algeria.

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# The effect of the preharvest treatment of sour cherry fruit (*Prunus cerasus* L.) with chitosan and mixture of chitosan and salicylic acid on fruit quality

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**The effect of the preharvest treatment of sour cherry fruit (*Prunus cerasus* L.) with chitosan and mixture of chitosan and salicylic acid on fruit quality**

**Abstract:** The effect of the pre-harvest treatment of sour cherry fruit with chitosan and salicylic acid on product quality as well as mass losses after storage is shown in the paper. The research was carried out with sour cherry cultivars 'Alfa' and 'Pamiat Artemenko' at the experimental pomology station named after L.P. Symyrenko of the institute of horticulture of NAAS. The pre-harvest treatment of sour cherry fruit with mixture of 1 %-chitosan with 100 mg $l^{-1}$ of salicylic acid appeared to be efficient; this makes it possible to extend the storage period up to 30 days, to increase the output of marketable produce by 7.8–8.6 %, to have mass losses which do not exceed 3 %. The pre-storage treatment of sour cherry fruit with the solution of salicylic acid in combination with chitosan facilitates the decrease of the number of mesophylic aerobic and facultative anaerobic microorganisms by 5.2 times, that of yeast and mold – 6.3 times. Along with this, taste properties of the produce are maintained at the level of fresh fruit, also the product has an excellent tasting evaluation – 5 points.

**Key words:** product evaluation, mass loss, sour cherry fruit, chitosan, salicylic acid.

**Učinek obravnavanja plodov višnje (*Prunus cerasus* L.) pred obiranjem s hitozanom in mešanico hitozana in salicilne kisline na njihovo kakovost**

Izvleček: V prispevku je prikazan učinek tretiranja plodov višnje pred obiranjem s hitozanom in mešanico hitozana in salicilne kisline na njihovo kakovost in izgubo mase po spravilu. Raziskava je bila izvedena na sortah višnje 'Alfa' in 'Pamiat Artemenko' na poskusni postaji za sadjarstvo imenovani po L.P. Symyrenku, inštituta za hortikulturo NAAS. Obravnavanje plodov višnje pred obiranjem z mešanico 1 %-hitozana z 100 mg $l^{-1}$ salicilne kisline se je izkazalo za učinkovito, kar je omogočilo čas shrambe do 30 dni in povečalo prodajno vrednost pridelka za 7,8–8,6 % ter zmanjšalo izgubo mase pod 3 %. Obravnavanje plodov višnje pred shrambo z raztopino salicilne kisline v kombinaciji s hitozanom je omogočilo zmanjšanje števila mezofilnih aerobnih in fakultativno anaerobnih mikroorganizmov za 5,2 krat, kvasovk in plesni za 6,3 krat. Hkrati so lastnosti okusa ostale na isti ravni kot pri svežih plodovih saj je bila ocean okusa odlična in je znašala 5 točk.

**Ključne besede:** ovrednotenje pridelka, izguba mase, plodovi višnje, hitozan, salicilna kislina

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## 1 INTRODUCTION

Fruits with a short shelf life, including sour cherries, have considerable respiration intensity with a high level of transpiration, they are susceptible to physiological diseases; so they spoil and are lost at the first stages of harvesting on the way from producers to consumers.

Cultivar, harvesting time, handling, and packaging greatly affect the quality and shelf life of sweet and sour cherry. However, its high perishability after harvest is mainly due to its high respiration rate, physical-chemical changes (such as mass loss, softening, darkening, mechanical damage etc.), and the development of microorganisms spoilage. Among them, primarily diverse species of filamentous fungi cause relatively faster spoilage after harvesting. The most important spoilage fungi of sweet cherries are *Botrytis cinerea* Pers., *Penicillium expansum* Link., and *Monilinia* spp. Therefore, strict storage conditions must be applied, alone or in a combination with different postharvest strategies, to delay spoilage and maintain quality (Cabañas et al., 2023).

At present new storage technologies in combination with existing ones are worked out – the effect of a low temperature, radiation, the use of the substances with anti-microbial effect/ aloe vera coating, 1-metylcyclopropen (Jianglian & Shaoying, 2013; Wani et al., 2014). However, the application of these substances for the extension of storage duration of cherry fruit has not been studied thoroughly, and preparation 1-MCP is very cost consuming to be used.

For chitosan to be effective in use, it is combined with other substances: essential oils, salicylic and metylsalicylic acids, etc. (Jianglian & Shaoying, 2013; Pasquariello et al., 2015).

Chitosan is a linear polysaccharide obtained by deacetylation of chitin, which, after cellulose, is the most abundant natural biopolymer in nature (Pereira, 2018). It has great potential for active packaging due to its biocompatibility, antimicrobial activity, non-toxicity and physical properties. Chitosan is active against pathogens and microorganisms, including bacteria (Thabet, 2019).

The chitosan coating has been approved by the (US-FDA) as a GRAS substance and its use is safe for the consumer and the environment. The use of chitosan in horticulture, and especially on highly perishable products (including sour cherry fruit), is based on the following properties such as cost, availability, functional attributes, mechanical properties (flexibility, tensile strength), optical properties (brightness and opacity), barrier effect against gas flow, structural resistance to water and microorganisms, and sensitivity (Romanazzi et al., 2017).

Salicylic acid and acetylsalicylic acid are plant hormones which play the main role in a wide spectrum of physiological processes. The prior salicylate treatment of fruit prevents spoilage, reduces damages and improves appearance and their density (Razavi et al., 2018). Salicylic acid is a natural and safe phenolic compound, it has a high potential as to the control over losses after harvesting. The use of salicylic acid is efficient to prevent damage of peach, kiwi, sweet cherry, apricot, pomegranate, plum (Youzuo et al., 2015).

Besides, the prior treatment of fruit with salicylic acid helps extend a storage period and preserve their quality. Peach fruit, treated with salicylic acid, were stored at 1 °C for 28 days. The content of phenols, flavonoids, ascorbic acid was higher as compared with the untreated fruit (Razavi et al., 2018).

Thus, the postharvest treatment with salicylic acid can be a safe, ecological technique aimed at preserving fruit quality (Zapata, 2017).

According to the research done by A. A. Lo'ay, A. Mohamed, M. A. Taher (2018), salicylic acid used in combination with chitosan enhances the resistance to damage of guava fruit during 15 days at temperature 27 °C. The effect of chitosan and salicylic acid on the storage of grapefruit is shown in the research (Shi et al., 2019). The pretreatment prevented a green mold formation, inhibited the effect of enzymes and contributed to the firmness of apricot, grapefruit, sweet cherry (Ghaouth et al., 1997; Gimenez et al., 2014; Shi et al., 2019; Cui et al., 2020).

The treatment of citrus fruit with oligochitosan, salicylic acid and Pichia membranaefaciens (E.C. Hansen) E.C. Hansen contributed to a significantly lower morbidity and damages during storage (Pereira, 2017; Xoca-Orozco et al., 2018).

However, not enough research was conducted concerning the effect of the combined use of salicylic acid and chitosan for the pretreatment of stone fruit, in particular sour cherries, on their quality (Vasylyshyna, 2018; Vasylyshyna & Chernega, 2022; Vasylyshyna, 2023). To improve the quality of fruit-berry output, the fruit treatment with salicylic acid in combination with chitosan and other substances is widely used (Zheng et al., 2007; Youzuo et al., 2015).

The aim of the study was to investigate the effect of pretreatment with a solution of chitosan and salicylic acid on the marketability, organoleptic and microbiological parameters of sour cherry fruits during storage.

## 2 MATERIAL AND METHODS

### 2.1 PRE-AND POST-HARVEST TREATMENT

The research was conducted with sour cherry cultivars 'Alfa' and 'Pamiat Artemenko' at the experimental pomology station named after L.P. Symyrenko of the institute of horticulture of NAAS in the years of 2016–2019. For the trial, the day before harvesting, 15 trees of each cultivar were sprayed with the solution of salicylic acid (100 mg $l^{-1}$); 1% chitosan mixture with salicylic acid (100 mg $l^{-1}$). Untreated sour cherry fruit were taken as the control. A three-time replication of the trial was applied. The fruit of a certain cultivar and a certain treatment technique were harvested at a consumer stage from four different places in the crown of each tree; they were placed for storage in boxes № 5 (5 kg each) at temperature 1 ± 0.5 °C and relative humidity 95 ± 1 % in an industrial refrigeration chamber KH with a capacity of 10.8 cubic meters. The control fruits were stored for 15 days, and the experimental ones – for 30 days.

### 2.2 ANALYTICAL METHODS

To determine product quality, sour cherry fruit of the first grade were chosen after storage. As to their appearance, they had a shape and coloring, typical for a given sour cherry cultivar. Sour cherry fruit were similar by the degree of ripeness, no overripe ones were there. The number of fruit without peduncle and with scarred injuries was not higher than 4 %. Fruit were of the same size with an average diameter not less than 16 mm (15.51 ± 0.05 mm), red color, they did not have injuries and they met the requirements of fresh sour cherries. Technical conditions (GSTU 01.1-37-167:2004). Fruits with mechanical damage exceeding 3 % and browning (5 % and higher) were considered to be of technical defects.

### 2.3 MASS LOSSES

At the end of storage, natural mass losses were determined by means of weighing on the scales (TWE) with an accuracy of 0.01 g. Mass loss was expressed in percents as the difference of two weighings before and after storage. The criterion of the end of storage was mass loss which would not exceed 6 % (Naichenko, 2001).

The determination of the number of mesophylic aerobic and facultative anaerobic microorganisms (MAFAnM) was done with help of cup method according to food products. Methods for determining the number of mesophilic aerobic and facultative anaerobic microorganisms (DSTU 8446:2015). By determining the number of mesophilic aerobic and facultative anaerobic microorganisms by sowing into solid product nutrient media, incubating the cultures, counting all visible colonies that have grown. For the determination, the following was used: (MPA) with contents (in %) – meat water, peptone – 1 %, agar–agar – 2 %. Endo's Medium (for defining of coliform bacteria) with contents (g $dm^{-3}$) – peptone – 5.0; triptone – 5.0; lactose – 10.0.

### 2.4 ORGANOLEPTIC EVALUATION

Organoleptic evaluation of sour cherry fruit (by sensory analysis) as to overall, sweetness, color, consistence, scent, taste, appearance, the consumers evaluated by 5-point scale (Naichenko, 2001). Scale range (0 to 5) with expression of perception (poor 0– 2, fair–3, good–4, excellent–5).

### 2.5 STATISTIC ANALYSIS

All statistic analyses were made at probability level 5 % ($p \leq 0.05$) with help of STATISTICA 10 (factorial ANOVA). The data is presented as average values with standard deviations of three measurements.

## 3 RESULTS AND DISCUSSION

As the research results showed, pretreatment of sour cherry fruit with chitosan with salicylic acid facilitated the extension of the storage duration up to 30 days, as compared with 15 days in the control.

The output of marketable produce (Fig.1) of sour cherry fruit 'Alfa' and 'Pamiat Artemenko' was at the same level after 15 days of storage – 85.2 and 83.6 %, respectively. The maximal output of marketable produce was recorded for the fruit pretreated with 1 % chitosan with salicylic acid – by 7.8–8.6 % higher as compared with the control, absolute waste was 2.3 times (3.1–3.6 %).

Mass losses of fruit result from respiration and moisture transpiration. During storage of sour cherry fruit 'Alfa' and 'Pamiat Artmenko', mass losses in the control were at levels 5.4–5.7 % (Fig. 2). The pretreatment of sour cherry fruit with salicylic acid resulted in the decrease of mass losses up to 3.4–3.5 %, and the treatment with chitosan in combination with salicylic acid – up to 2.7–3.0 % during 30-day storage; it occurred due to semi-permeable coating formed on fruit surface which prevented moisture losses and reduced respiration intensity; this was proved by researchers Z.Youzuo, Z. Meiling.,Y. Huqing (2015).

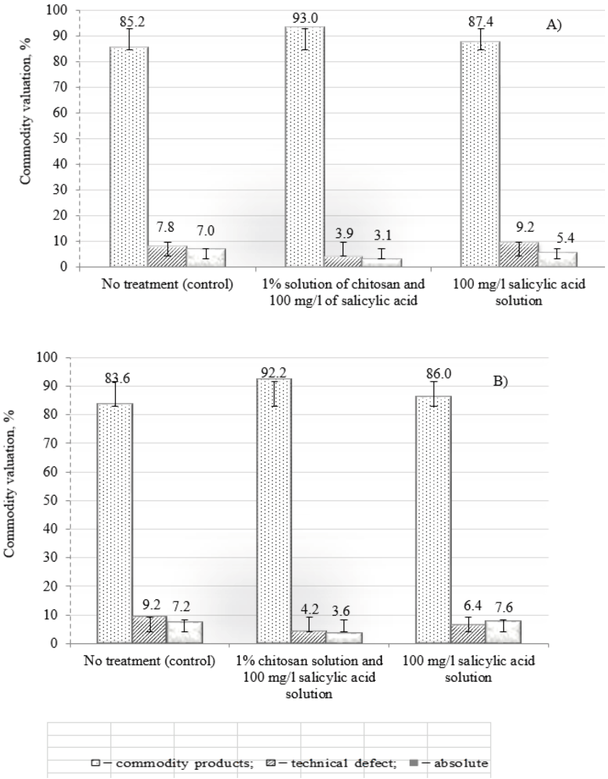

**Figure 1:** Output of marketable produce of sour cherry fruit 'Alfa' (A) and 'Pamiat Artemenko' (B) after storage ($LSD_{05}$ of commodity produce = 2.6; $LSD_{05}$ of technical defect = 0.2; $LSD_{05}$ of absolute waste = 0.2).

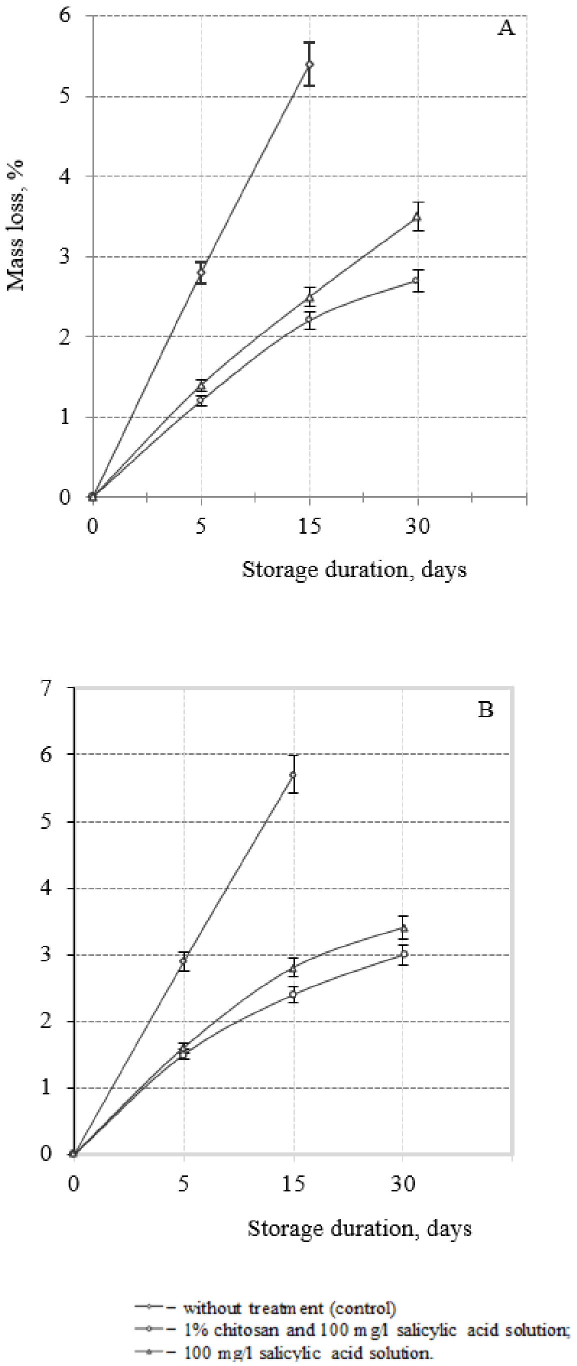

**Figure 2:** Mass losses of sour cherry fruit 'Alfa' (A) and 'Pamiat Artemenko' (B) during storage ($LSD_{05}$ = 0.4).

Hence, the pre-storage treatment of sour cherry fruit with 1 % chitosan and 100 mg $l^{-1}$ of salicylic acid increases the duration of their storage to 30 days, enhances the output of marketable produce by 7.8–8.6 %, reduces the level of technical defect by 3.9–5.0 %, and absolute waste – by 2.3 times as compared with mass losses which do not exceed 3 %.

The results of tasting evaluation of the sour cherry fruit, treated with polysaccharide compositions after storage, are shown in Fig. 3. After storage sour cherry fruit were evaluated by the following indicators: appearance, consistence, taste, scent, color.

By appearance, treated fruit were much better than the control, and they had excellent indicators. By scent, such differences were not recorded, except for the fruit treated with salicylic acid; they had a much lower tasting estimate – 4.5 points. The fruit treated with chitosan in combination with salicylic acid had an excellent scent.

Fruit color after harvesting was natural for sour cherry fruit. Significant differences from the control indicators and an excellent tasting evaluation was recorded on the sour cherry fruit treated with 1 % chitosan with 100 mg $l^{-1}$ of salicylic acid. Contrary to this, the sour cherry fruit treated with salicylic acid alone almost did not differ from the control – 4.5 points.

Along with color, another important indicator of product quality is consistence which plays a decisive role in choosing fruit for consumers. Softening occurred in the untreated sour cherry fruit (the control) after stor-

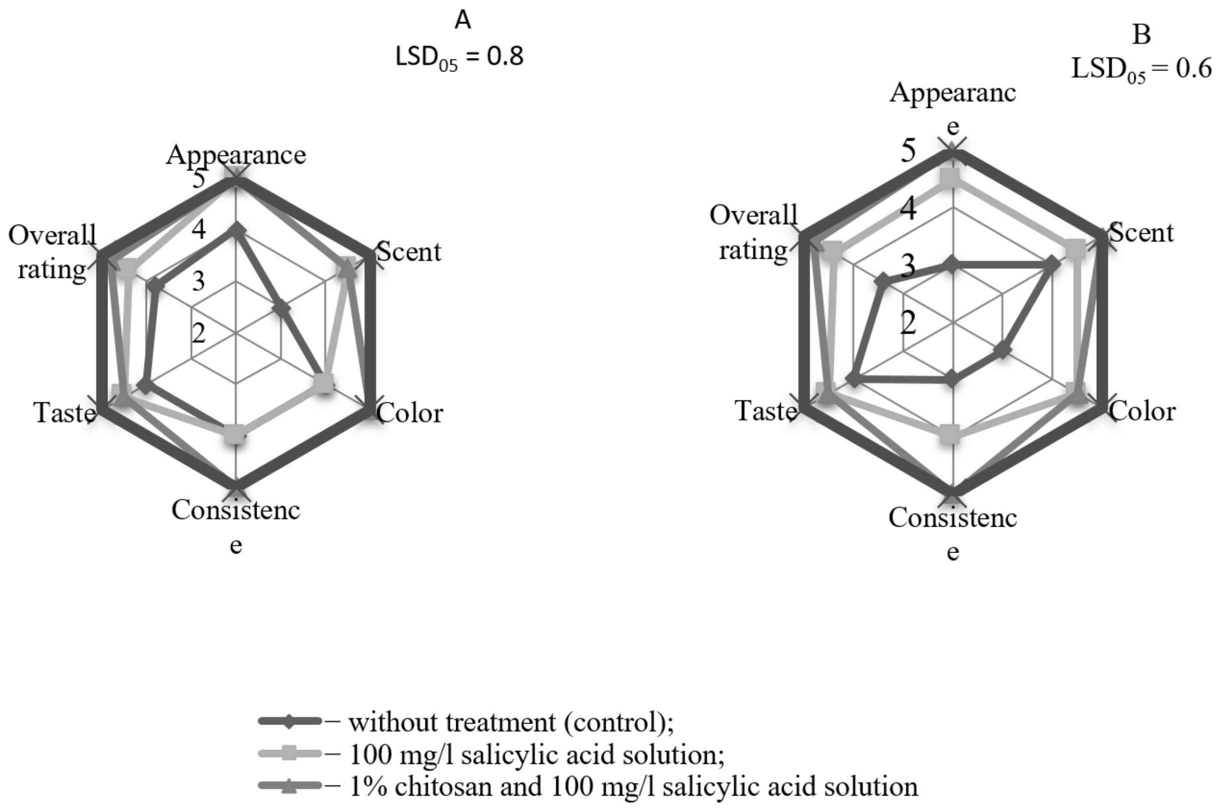

**Figure 3:** Organoleptice valuation of the sour cherry fruit 'Alfa' (A) and 'Pamiat Artemenko' (B) pretreated with polysaccharide compositions at the end of storage, 2016–2019.

age; this was caused by the worsening of a cell wall composition, due to pectin hydrolysis where such enzymes as polygalacturonase and pectinesterase were present. Similar findings were recorded in strawberry fruit during storage (Tomadoni et al., 2019).

For the sour cherry fruit which were pretreated with the combination of chitosan and salicylic acid, fruit consistence differed significantly from the control and it was evaluated in 5 points. Whereas the fruit treated with the solution of salicylic acid had a good tasting estimate (4 points).

The most important indicator of product quality is its taste. After storage, untreated sour cherry fruit had good taste properties (4 points). The evaluation grade of the fruit treated with the combination of chitosan and salicylic acid differed significantly. Namely, the sour cherry fruit treated with this solution had an excellent tasting estimate. It can be explained by the fact that chitosan can most likely add some taste to cherry fruit, which in turn influences their tasting estimate (Tomadoni et al., 2019).

Thus, the pre-storage treatment of sour cherry fruit with a polysaccharide composition of chitosan with salicylic acid appears to be the most efficient one due to the preservation of organoleptic properties of the produce at the level of fresh fruit and an excellent tasting estimate.

The complication in the organization of juicy fruit protection during their storage is defined by not only their mechanical structure, chemical composition and organoleptic indicators but also by the lack of active protective responses from plants themselves.

Microscopic fungi are the reason why diseases occur during juicy fruit storage. They can be classified into two groups – parasitic (phyto-pathogenic) and saprotrophic. The examples of such fungi are monilios carriers (*Monilinia fructigena* Honey and *Monilinia laxa* (Aderh. & Ruhland) Honey), grey mold (*Botrytis cinerea*), leaf browning (*Schizothyrium pomi* (Mont. & Fr.) Arx,), alternarios (*Alternaria* spp.).

Saprotrophic fungi cannot infect live healthy plant and they usually inhabit fruit after harvesting (during their transportation and storage). The example of such fungi are molds which are caused by some kinds of species *Aspergillus*, *Penicillium*, *Trichothecium*, *Mucor*, *Rhizopus* and others.

The most harmful disease is a bacterial disease caused by *Pseudomonas syringae* Van Hall. Fungus damage caused such kinds of species as *Penicillium, Botrytis* and M*onilia* leads to blue rot and grey mold. The occurrence of these rots and their effect on fruit, cherry fruit in particular, depend on varietal features and the ripening stage of the yields (Akulov, 2012).

Sweet and sour cherry fruit are damaged by various causative agents of species monilinia (*M. laxa, M. frutigena and M. fructicola* (G.Winter) Honey), blue mold, caused by *Pencillium expansum*, *Alternaria* and *Cladosporium* molds, caused by *Alternaria alternata* (Fr.) Keissl and *Cladosporium* sp. *Penicillium expansum;* they all result in yield losses and worse fruit quality (Wani et al., 2014).

Caused by fungus, *Pseudomonas syringae (*species *Monilia)* leads to brown rot. The occurrence of rot and its impact on sour cherry fruit depend on a cultivar and fruit ripeness degree (Wani et al., 2014).

To limit the development of phytopthogenic fungi on fruit, it is important to organize the control over them in the period of plant vegetation. The more efficient the control over them "in the orchard", the fewer of them will be during the fruit storage. To limit the development of saprotrophic fungi, it is important to minimize fruit damage. Besides, the fruit planned for a longer storage period must not be damaged by pests.

To improve the appearance of fruit and to slow down their spoilage, the procedure of putting an artificial wax-like layer on their surface is often done after harvesting the yield. This layer blocks gas exchange between the fruit and the atmosphere, and it also prevents water evaporation. Various fungicides are frequently applied right before or even during the smearing of this protective layer.

Such substances as salicylic acid, chitosan, and alginate are the inducers of the acquired system resistance of the plants to diseases, or they are used directly to protect cherry fruit from diseases during their storage (Akulov, 2012).

Chitosan is a safe alternative to synthetic fungicides against *B. cinerea* in strawberry (Petriccione et al., 2015). Disease *Botrytis* (grey mold) and strawberry rotting begin on the 6th day of their cold storage both on untreated fruit and those treated with 1 % chitosan covering. The strawberry fruit, covered with 1.5 and 2 % solution of chitosan were affected by microbiological spoilage only on the 9th day (Nasrin et al., 2017).

It was established (Tokatlı & Demirdove, 2020) that the total quantity of mesophylic aerobic bacteria in sweet cherry fruit, covered with chitosan, was smaller than expected, whereas it was equal to 2.74 log CFU $g^{-1}$ at 4 °C in the untreated control group.

The research of the treatment effect with the solutions of polysaccharide compositions on micro-flora of sour cherry fruit after storage showed that the average amount of epiphytic micro-flora in fresh sour cherry fruit was at the level $1.1{\cdot}10^3$ CFU $g^{-1}$ (Fig. 4).

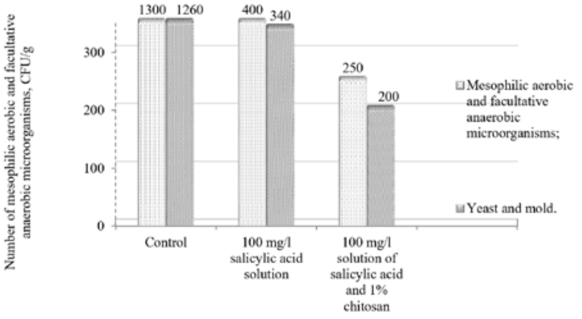

**Figure 4:** Effect of the treatment with the solutions of a polysaccharide combination on a quantitative and qualitative composition of epiphytic microflora on the surface of sour cherry fruit at the end of storage ($LSD_{05MAFAnM} = 17.5$; $LSD_{05\ yeast} = 36$)

After storage its amount increased up to $1,3{\cdot}10^3$ CFU $g^{-1}$. The treatment of sour cherry fruit with chitosan and salicylic acid decreased significantly the quantity of MAFAnM – by 5.2 times, as compared with the control. The decrease in the number of MAFAnM on sour cherry fruit, treated with the composition of chitosan and salicylic acid, can be explained by the availability of chitosan in it; and chitosan shows anti-bacterial properties. Positively charged amino-groups on chitosan bind to negatively charged carboxyl groups on a membrane of a bacterial cell, changing the distribution of a charge on a cell surface which causes the disturbance of membrane stability (Tokatlı &Demirdove, 2020).

The pretreatment of sour cherry fruit before storage led to the growth suspension of yeast and mold. In the control, they were at the level of $1.26{\cdot}10^3$ CFU $g^{-1}$; when fruit were treated with the solutions of polysaccharide compositions it decreased by 3.7–6.3 times. And the smallest amount of epiphytic micro-flora remained in sour cherry fruit treated with the solution of chitosan and salicylic acid.

Similarly, the decrease of the amount of yeast and mold on the cherry fruit, covered with aloe-vera, was recorded by D. Martinez-Romero et al. (2006). M. Rassa, M. Ghasemnezhad et al. (2013) stated that the treatment with 1% solution of chitosan reduces the growth of fungi, as compared with the control (Nasrin et al., 2017). After storage, monilios disease was mostly detected (it was caused by fungal flora of *Monilia* species).

Thus, the pre-storage treatment of sour cherry fruit with salicylic acid in combination with chitosan, ensures the decrease in the number of MAFAnM by 5.2 times, yeast and mold – by 6.3 times, as compared with the untreated fruit.

## 4 CONCLUSIONS

The pre-storage treatment of sour cherry fruit with 1 % solution of chitosan with 100 mg $l^{-1}$ of salicylic acid extends the duration of their storage up to 30 days, enhances the output of marketable produce by 7.8–8.6 %, decreases the level of technical defect by 3.9–5.0 %, and absolute waste – by 2.3 times as compared with mass losses which does exceed 3 %.

The pretreatment of sour cherry fruit with polysaccharide composition of chitosan with salicylic acid before storage appears to be the most efficient technique due to the preservation of organoleptic properties of the produce at the level of fresh fruit and an excellent tasting estimate.

The treatment of sour cherry fruit with salicylic acid in combination with chitosan before storage results in the decrease of the quantity of MAFAnM by 5.2 times, yeast and mold – by 6.3 times, as compared with the untreated fruit.

Taking into consideration a high percent of the fruit spoilage after harvesting, during transportation and storage, it is believed to be promising to continue further research aimed at studying the effect of postharvest treatment with food films on their quality during storage.

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# Effect of low-temperature stress on antioxidant defense in *Malus* spp.

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**Effect of low-temperature stress on antioxidant defense in *Malus* spp.**

**Abstract:** The antioxidant defence system of plants consists of various antioxidant compounds and enzymes that play a key role in reducing oxidative damage and regulating the decay of reactive oxygen species, which are vital for metabolic processes. To evaluate the relationship between antioxidant components and stress resistance of apple plants, we studied the level of malondialdehyde, ascorbic acid, anthocyanins and chalcones in the bark of annual shoots. The results show that at sub-zero temperatures, the level of malondialdehyde in the apple plants studied increased by 1.6 % compared to positive temperatures. Based on the normal probability distribution, the correlation coefficient was r = 0.91 for anthocyanins, r = 0.97 for chalcones, r = 0.98 for vitamin C and r = 0.99 for malondialdehyde. The correlation value between anthocyanins and chalcones was r = 0.1 ($p > 0.62$), between anthocyanins and vitamin C r = -0.4 ($p > 0.29$), and between anthocyanins and malondialdehyde r = 0.5 ($p > 0.05$). The correlation analysis shows that among the secondary metabolites, chalcones (r = 0.7) have a greater ability, while anthocyanins (r = 0.5) have a lower ability, to inhibit excessive accumulation of malondialdehyde and protect lipid membranes from severe degradation of apple cells.

**Key words:** *Malus*, malondialdehyde, ascorbic acid, anthocyanins, chalcones

**Učinek mraznega stresa na antioksidativno obrambo pri jablani (*Malus* spp.)**

**Izvleček:** Antioksidativni obrambni sistem rastlin sestavljajo različne antioksidativne spojine in encimi, ki imajo ključno vlogo pri zmanjševanju oksidativnih poškodb in uravnavanju razpada reaktivnih kisikovih vrst, ki so bistvene za presnovne procese. Da bi ocenili povezavo med antioksidativnimi sestavinami in odpornostjo jablan na stres, smo v skorji enoletnih poganjkov preučevali raven malondialdehida, askorbinske kisline, antocianinov in halkonov. Rezultati kažejo, da se je pri temperaturah pod ničlo raven malondialdehida v preučevanih jablanah v primerjavi s pozitivnimi temperaturami povečala za 1,6 %. Na podlagi normalne verjetnostne porazdelitve je bil korelacijski koeficient r = 0,91 za antocianine, r = 0,97 za halkone, r = 0,98 za vitamin C in r = 0,99 za malondialdehid. Vrednost korelacije med antocianini in halkoni je bila r = 0,1 ($p > 0,62$), med antocianini in vitaminom C r = -0,4 ($p > 0,29$) ter med antocianini in malondialdehidom r = 0,5 ($p > 0,05$). Korelacijska analiza je pokazala, da imajo med sekundarnimi metaboliti halkoni (r = 0,7) večjo, antocianini (r = 0,5) pa manjšo sposobnost zaviranja prekomernega kopičenja malondialdehida in zaščite lipidnih membran pred hudo degradacijo jabolčnih celic.

**Ključne besede:** *Malus,* malondialdehid, askorbinska kislina, antocianini, halkoni

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## 1 INTRODUCTION

Recent studies indicate that several fruit tree cultivars exhibit insufficient adaptive resistance to cope with climate change, particularly when facing sudden temperature fluctuations (Cascia et al., 2013; Stevens et al., 2017; Suekawa et al., 2017). As a result, the development of new cultivars with greater ecological flexibility and improved adaptive potential remains a critical goal. In this regard, physiological and biochemical research plays a key role in identifying primary indicators of winter hardiness in plants, with a particular emphasis on apple trees (Ishikawa et al., 2018).

It is well documented that plants have evolved strategies to mitigate stress and can modify their physiological processes in response to environmental changes (Zechmann, 2018). Under stress conditions, plants increase the production of reactive oxygen species (ROS) – such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals – which can lead to oxidative stress (Bi et al., 2014). This phenomenon represents a delicate balance between pro-oxidant and antioxidant mechanisms, significantly influencing plant adaptation and acclimation (Maheshwari et al., 2009). Under favorable conditions, the antioxidant defense system efficiently neutralizes ROS and free radicals (Gao et al., 2008); however, both natural and anthropogenic stressors can trigger excessive production of reactive oxygen derivatives, leading to an intensified antioxidant response (Fernandez-Lorenzo et al., 1999).

As essential components of cell membranes, lipids are primary targets for ROS-induced damage. The process of lipid peroxidation (LPO), initiated by ROS, compromises membrane integrity, thereby reducing its permeability to ions and organic compounds (Pereir et al., 2016). Consequently, the extent of LPO serves as a key indicator of a plant's physiological status and stress response. This process is typically assessed by measuring the accumulation of hydroperoxides and malondialdehyde (MDA) (Cheeseman et al., 2007; Mullineaux et al., 2010), with increased MDA levels under abiotic stress signaling oxidative degradation of membrane lipids and decreased tolerance to low temperatures (Choudhury et al., 2016).

In response to biotic and abiotic stressors, plants activate a complex, multi-tiered antioxidant system comprising both high- and low-molecular-weight compounds to mitigate lipid peroxidation (Farmer et al., 2003; Weber et al., 2004). This protective mechanism includes enzymes such as peroxidase, antioxidants like ascorbic acid, and various secondary metabolites that collectively neutralize ROS and their oxidized derivatives, preventing harmful chain reactions within cells (Raza et al., 2019; Esterbauer et al., 1991; Vollenweider et al., 2000). The effectiveness of this antioxidant defense, along with the ability to rapidly enhance enzyme activity in response to stress, is a crucial determinant of plant resilience. More resistant species generally display heightened antioxidant enzyme activity and elevated concentrations of ascorbic acid, anthocyanins, and chalcones (Gill et al., 2010).

Previous research (Alscher, 1997; Fecht-Christoffers, 2003) has shown that malondialdehyde, ascorbic acid, anthocyanins, and chalcones in the bark of annual shoots of small-fruited apple trees play an essential role in their defense mechanism against low-temperature stress. Malondialdehyde (MDA) is widely recognized as an indicator of oxidative lipid damage, with its levels increasing in response to both biotic and abiotic stress factors. Since the 1950s, the thiobarbituric acid reactive substances (TBARS) assay has been extensively used to measure lipid peroxidation in biological membranes and systems (Sinnhuber et al., 1958; Heath et al., 1968; Pelle et al., 1990; Du et al., 1992; DeLong et al., 1997).

Among the most important plant antioxidants is ascorbic acid, which is abundantly distributed in chloroplasts, the cytosol, vacuoles, and the apoplastic space of both vegetative and reproductive cells (Foyer et al., 1991). It plays a key role in the ascorbate-glutathione cycle, responsible for detoxifying and breaking down hydrogen peroxide (Noctor et al., 1998), and serves as a redox buffer in the apoplast, regulating plant growth and defense mechanisms under stress (Pignocchi, 2003). The redox state of vitamin C, often expressed as the AsA/DHA ratio, is considered a sensitive indicator of oxidative stress and is crucial for plant development (Suekawa et al., 2017; Zechmann et al., 2018). A higher AsA/DHA ratio signifies stronger antioxidant defense and better protection against oxidative damage (Fecht-Christoffers et al., 2003; Maheshwari et al., 2009), whereas lower levels and an imbalanced vitamin C redox state are often linked to premature aging (Pastori et al., 2003; Lin et al., 2011; Conklin et al., 2004).

Anthocyanins, secondary metabolites synthesized in both vegetative and reproductive tissues (Ficco et al., 2014; Levon et al., 2017; Goncharovska et al., 2022), contribute to ROS scavenging. Typically colorless or light blue in the cytoplasm, they adopt a red hue once stored in vacuoles (Goncharovska et al., 2018). Numerous studies highlight their role in modulating signal transduction pathways in response to physiological stress. Due to their association with various stress factors, anthocyanins are considered both stress markers and active participants in stress mitigation (Levon et al., 2017), with their accumulation influenced by environmental factors such as temperature, light exposure, water availability, and mechanical damage (Goncharovska et al., 2021).

Chalcones, a group of specialized plant metabolites consisting of two phenolic rings (A and B) connected by an α, β-unsaturated ketone bridge, have gained attention in medicinal chemistry due to their simple synthesis and broad bioactivity. Like other secondary metabolites, chalcones accumulate in response to biotic and abiotic stress and act as potent antioxidants (Heim et al., 2002; Ishikawa et al., 2018).

Building on these insights, the present study seeks to expand upon previous research by assessing physiological and biochemical parameters in apple cultivars. The primary objective is to identify key metabolites and evaluate their responses to low-temperature exposure. The findings are expected to support the selection of the most promising small-fruited apple cultivars that exhibit either high frost resistance or enhanced winter hardiness, thereby contributing to future breeding efforts. Notably, this study represents the first investigation of selected *Malus* spp. and cultivars within the M.M. Gryshko National Botanical Garden.

## 2 MATERIALS AND METHODS

### 2.1 PLANT MATERIALS

Objects of study 6 species and 9 cultivars of *Malus* spp., in particular *Malus niedzwetzkyana* Dieck, *(*cultivars Era*,* King Beauty), *M. halliana* Koehne, *M. coronaria* (L.) Mill., *M. fusca* (Raf.) C. K. Schneid, *M. baccata* (L). Borkh, *(*cultivars Pendula, Evereste), *Malus* × *denticulata, M.* × *gloriosa* (cultivar Lemoine), *M.* × *zumi* (Matsum.) Rehder (cultivar Golden Hornet), *M.* × *purpurea* (E. Barbier) Rehder, *(*cultivars Ola, Royalty).

### 2.2 EXPERIMENTAL DESIGN

The annual sprouts were selected at the Form Garden site of the M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine.

To study the bark, samples with lengths of 15-20 mm were cut from the sprouts of each apple tree (in the middle part), cut longitudinally into 2 sections and the bark was separated from the wood. The study was conducted in 3-fold biological replication, each replication consisted of 10 samples. Samples were collected on 13.01.2023 at an average daily temperature of -4.8 $^0$C and on 14.02.2024 at an average daily temperature of +6.5 $^0$C.

Physiological and biochemical methods were used, namely, the total content of malondialdehyde, ascorbic acid, anthocyanins, and chalcones.

### 2.3 MALONDIALDEHYDE DETERMINATION

The applied method is based on the reaction between malondialdehyde (MDA) and thiobarbituric acid, leading to the formation of a colored trimethine complex under high-temperature conditions in an acidic medium. This complex exhibits a maximum absorption at 532 nm (Ohkawa et al., 1979). To determine the MDA content, freshly harvested plant leaves were homogenized with 5 % trichloroacetic acid and then centrifuged at 12,000 g for 10 minutes at 27 °C. Equal volumes of the resulting supernatant and 0.5 % thiobarbituric acid (TBA) were mixed with 20 % trichloroacetic acid, incubated at 96 °C for 30 minutes, and rapidly cooled in an ice bath. Following centrifugation at 12,000 g for 10 minutes, the optical density of the supernatant was measured at 532 and 600 nm. The MDA concentration was determined using the following formula:

$$C_{MDA} = (D1–D2)/155*m$$

where:

$C_{MDA}$ – TBA-reactive products, mM g$^{-1}$ of fresh mass;
D1 – optical density at 532 nm
D2 – optical density at 600 nm
155 – TBA extinction coefficient, mM g$^{-1}$
m – mass of plant material, g

### 2.4 THE TOTAL CONTENT OF ASCORBIC ACID

The ascorbic acid content was determined using a titrimetric method based on the addition of an alkaline solution of 2,6-dichlorophenolindophenol (Tilman's reagent) to an acidified solution containing ascorbic acid. For the analysis, 2 g of fresh mass was mixed with an acidic solution (hydrochloric acid and oxalic acid in a 1:4 ratio) and kept in darkness for 20 minutes. After this, the obtained solution was filtered and titrated with Tilman's reagent. In the presence of ascorbic acid, the deep blue color of 2, 6-dichlorophenolindophenol gradually fades as it is reduced to its leuco form. Once all the ascorbic acid has been oxidized to dehydroascorbic acid, the solution turns red, indicating the oxidized form of 2,6-dichlorophenolindophenol in an acidic medium. The obtained results are expressed in mg·100 g$^{−1}$ of dry matter (DM) (Hrytsajenko et al., 2003).

The mass fraction of ascorbic acid in the analyzed material was calculated using the following formula:

$$C_{AA} = (T*V1*V2)/(m*V3)$$

where:

T – titer of 0.001 n solution of 2,6-dichlorophenolindophenol by ascorbic acid, 0.088 mg ml$^{-1}$;

$V_1$ – volume of 0.001 n solution of 2,6-dichlorophenolindophenol used for titration of the extract, ml;
$V_2$ – the total volume of the extract, 100 ml;
$V_3$ – the volume of the extract taken for titration;
m – the mass of the sample of the test material, g;
100 – conversion factor per 100 g.

### 2.5 ANTHOCYANIN DETERMINATION.

The anthocyanin content was determined using a spectrophotometric method at a wavelength of 530 nm. The analysis included alcohol extraction from a homogenized plant material that had been acidified with 3.5 % hydrochloric acid (Nurlinda et al., 2021).

The anthocyanin concentration, expressed as cyanidin-3-glucoside, was calculated using the following formula:

$$C_{\text{ан}} = (D*V*R*K)/(l*m)$$

where:
D – the optical density of the solution;
V – the volume of the extract, ml;
R – dilution ratio of a solution of 3.5 % hydrochloric acid in ethanol;
l – working length of the cuvette, cm;
m – weighed quantity, g;
K – is the conversion factor, based on a calibrated graph for cyanidine glycosides in acidified ethanol K = 5.

### 2.6 CHALCONES DETERMINATION.

The chalcone content was determined using a spectrophotometric method based on Udovenko's approach (1988) with slight modifications. For the analysis, a 0.2 g sample was ground and mixed with a 0.1 N hydrochloric acid solution, then allowed to infuse for 2 hours with periodic shaking. The resulting mixture was transferred to a dry test tube and centrifuged at 2,000 rpm for 2–3 minutes.

The absorbance of the solution was measured at a wavelength of 364 nm. Due to high absorbance levels, the initial solution was diluted fivefold with 0.1 N hydrochloric acid before measurement.

The calculation was performed using the following formula:

$$X = (E*V*A)/(p*l)$$

where:
E – optical density at 364 nm;
A – dilution index (how many times the initial solution is diluted);
P – sample mass, g;
V – volume of extract, ml;
l – thickness of cuvette, cm.

### 2.7 STATISTICAL ANALYSES

The study results were analyzed using mathematical statistics methods with the aid of EXCEL and STATISTICA 6.0 software. Hierarchical cluster analyses were conducted to assess genotype similarity based on the Bray-Curtis similarity index. Descriptive statistical analysis was performed, and the results were expressed as the mean of n replicates with standard deviation, utilizing Statgraphics 5 Plus software. Statistically significant differences were determined through analysis of variance (ANOVA).

## 3 RESULTS

In the course of the study, the intensity of lipid peroxidation (LPO) in the bark of annual sprouts of apple trees was determined by the accumulation of malondialdehyde (MDA), which is one of the main end products of LPO.

In the genotypes of the study, the average value for 2 years of the study, the content of malondialdehyde in shoots ranged from 1.23 to 1.87 % (Fig. 1).

A comparative analysis of the obtained data strongly supports previous findings that malondialdehyde (MDA) accumulates in the bark of apple shoots under abiotic stress. In our study, this was particularly evident under subzero temperatures, specifically at –4.8 °C. On average, the difference in MDA accumulation between positive and negative temperatures was 1.6 %. The highest MDA content was recorded in *M. niedzwetzkyana* 'Era' at 0.442 mg·100 $g^{-1}$ of fresh matter, while the lowest was observed in *M. coronaria* at 0.202 mg·100 $g^{-1}$ of dry matter (Fig. 2).

A review of the existing literature in this research area suggests that low-molecular-weight organic antioxidants, such as ascorbic acid, anthocyanins, and chalcones, play a crucial role in protecting metabolic processes from the harmful effects of reactive oxygen species (ROS). Ascorbic acid, in particular, is a well-known antioxidant due to its ability to participate in redox reactions, effectively neutralizing superoxide and hydroxyl radicals and thereby reducing their intracellular concentrations. Based on this, we can conclude that ascorbic acid is essential for enhancing plant resistance to low temperatures, with winter-hardy apple cultivars accumulating

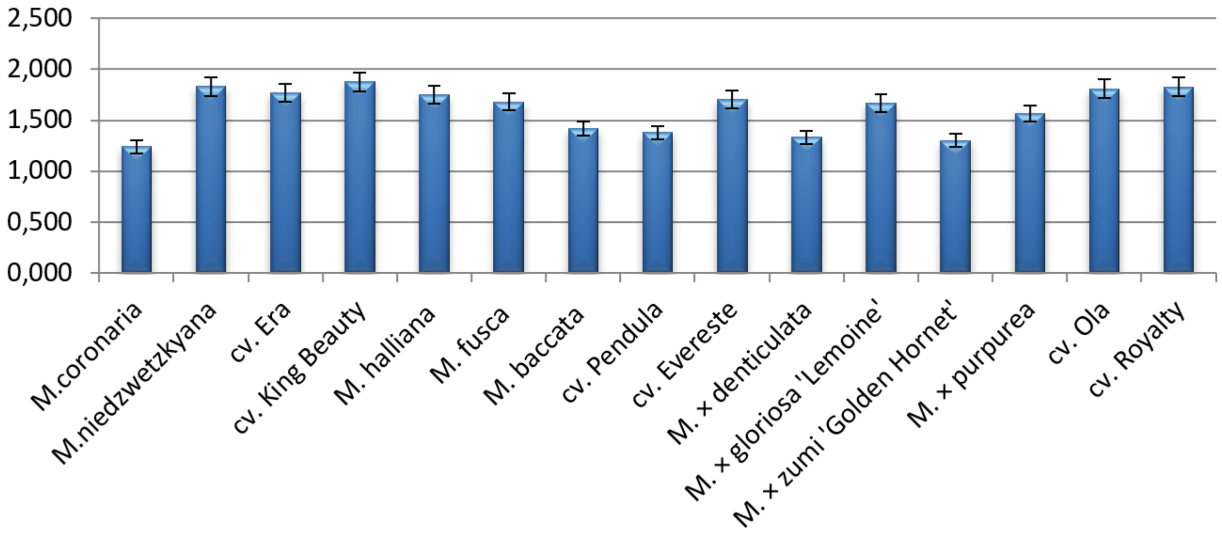


**Figure 1:** Difference in malondialdehyde accumulation in shoot bark in % for January-February 2023-2024, %

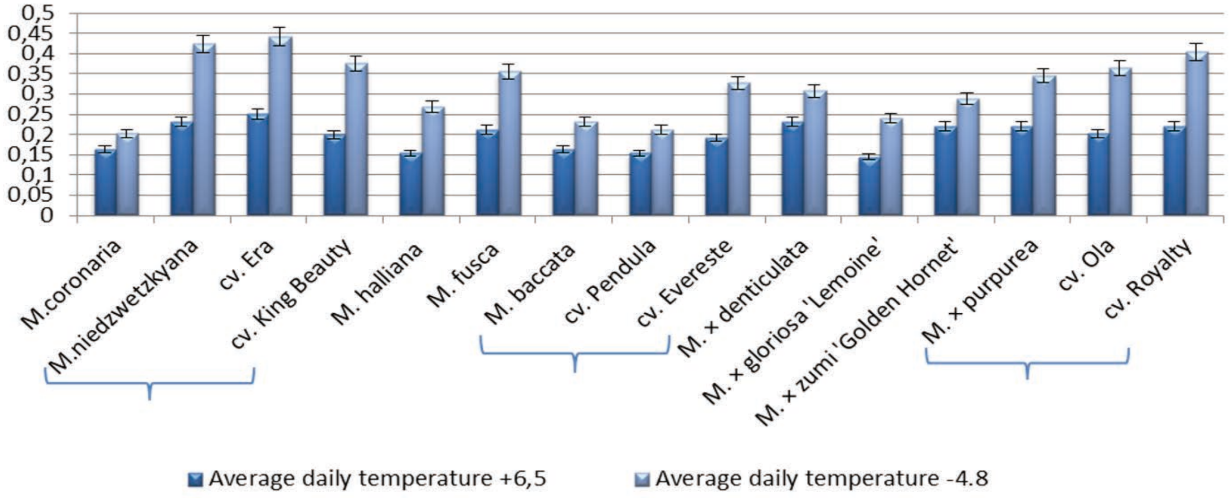


**Figure 2:** Content of malondialdehyde in the bark of annual sprouts of representatives of the genus *Malus* spp., mg.100 $g^{-1}$

higher levels of this compound compared to those with moderate cold tolerance.

Analyzing the data from the normal probability graph (Fig. 3), it is evident that at a positive average daily winter temperature of +6.5 °C, the correlation coefficient for malondialdehyde accumulation in the bark of annual apple shoots was r = 0.96, while at a lower temperature of −4.8 °C, it increased to r = 0.98. This indicates that malondialdehyde actively accumulates under stressful conditions, particularly during subzero temperatures.

Furthermore, multivariate linear regression analysis revealed a correlation coefficient of r = 0.83, with a significance level of $p > 0.0001$ (Fig. 4).

Upon examining Fig. 5, a clear distinction in the accumulation of ascorbic acid in the bark of annual apple tree shoots was observed, indicating varying plant responses to sudden temperature changes. The highest vitamin C concentration was found in the apple cultivar *M. baccata* ‘Pendula’ at 41.067 mg.100 $g^{-1}$, while the low-

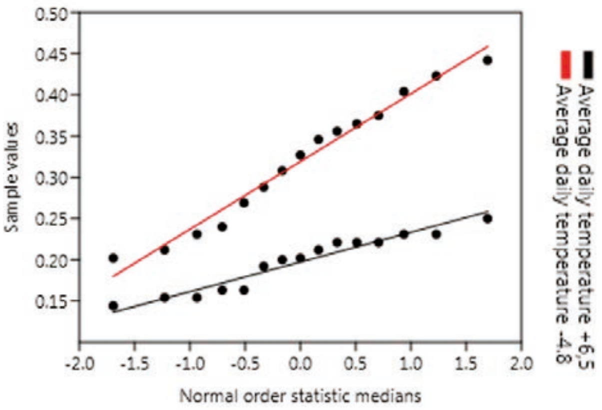


**Figure 3:** Normal probability plot

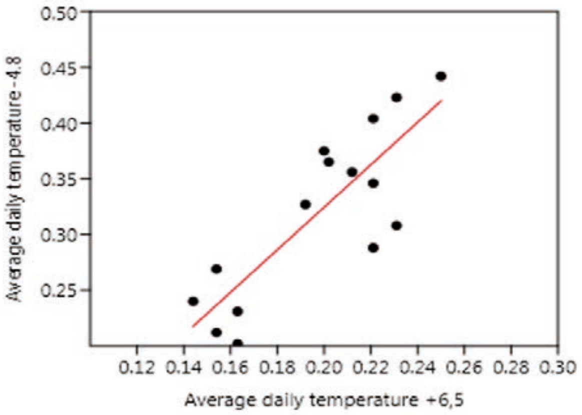


**Figure 4:** Multivariate linear regression

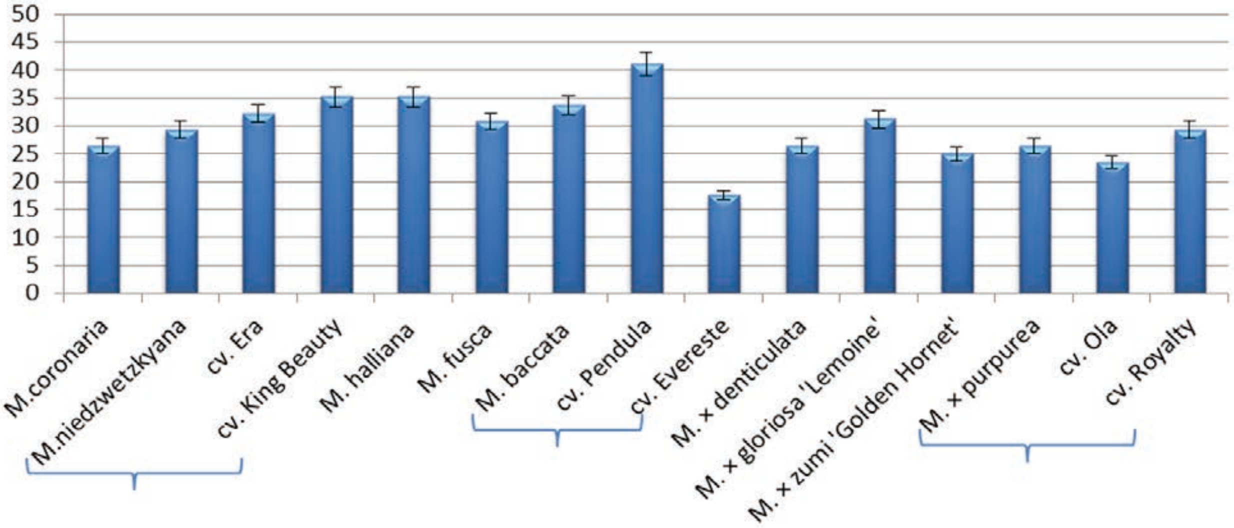


**Figure 5:** Ascorbic acid content in the bark of annual sprouts of representatives of the genus *Malus* spp., mg.100 g$^{-1}$ (average value for 2023–2024)

est was observed in *M. baccata* 'Evereste' at 17.6 mg.100 g$^{-1}$ of dry matter.

The next phase of our research involved quantifying the content of anthocyanins and chalcones, which are potent antioxidants that enhance the resilience of fruit plants to stress. Typically, anthocyanins and chalcones (anthochlorines) are localized in the bark. In our study, the highest anthocyanin concentration was found in *M. baccata* 'Evereste' at 587.5 mg.100 g$^{-1}$, and the lowest in M. *coronaria* at 132.9 mg.100 g$^{-1}$ of dry matter. Regarding chalcones, the highest content was detected in *M.* × *purpurea* 'Royalty' at 98.3 mg.100 g$^{-1}$, while the lowest was in *M.* × *gloryosa* 'Lemoine' at 52.9 mg.100 g$^{-1}$ of dry matter (Fig. 6).

**Figure 6:** Content of anthocyanins and chalcones in the bark of annual sprouts of representatives of the genus *Malus* spp. (average value for 2023–2024)

By comparing the levels of anthocyanins, chalcones, ascorbic acid, and malondialdehyde based on the normal probability graph (Fig. 7), the following correlation coefficients were obtained: anthocyanins r = 0.91, chalcones r = 0.97, vitamin C r = 0.98, and malondialdehyde r = 0.99.

Subsequent analysis sought to examine the density of accumulation of anthocyanins and chalcones, revealing a correlation of r = 0.1; $p > 0.62$ between anthocyanins and chalcones, r = -0.4; $p > 0.29$ between anthocyanins and vitamin C, and r = 0.5; $p > 0.05$ between anthocyanins and malondialdehyde. These results indicate an inverse relationship between the accumulation of anthocyanins and ascorbic acid, as shown by the negative

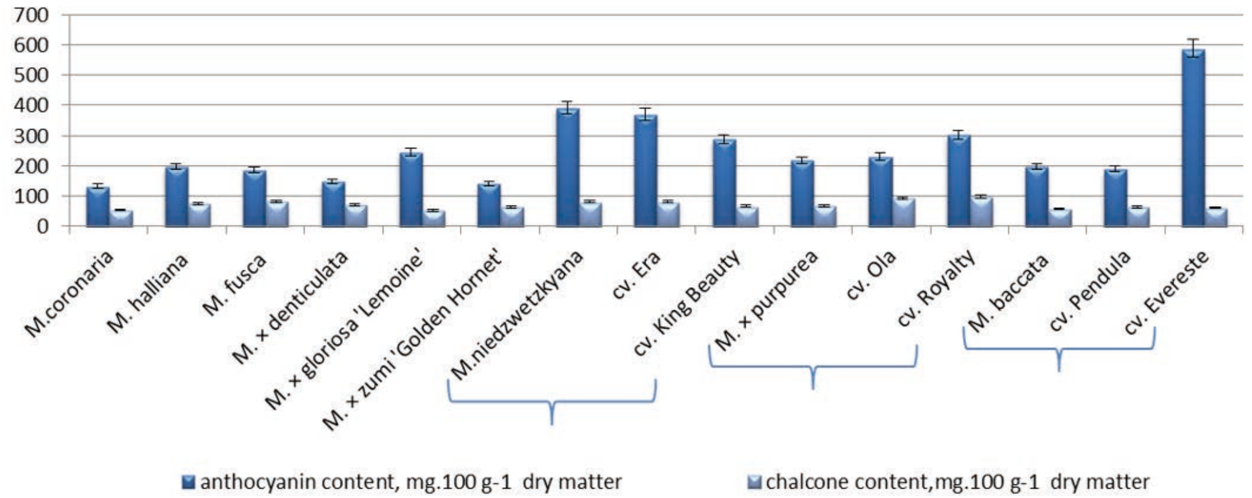


**Figure 6:** Content of anthocyanins and chalcones in the bark of annual sprouts of representatives of the genus *Malus* spp. (average value for 2023–2024)

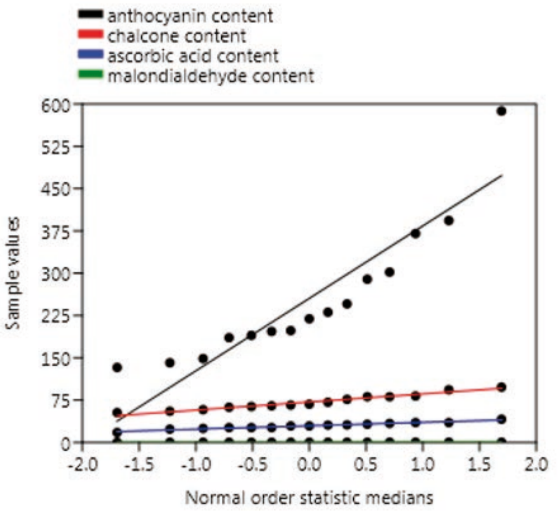


**Figure 7:** Normal probability plot between content malondialdehyde, vitamin C, anthocyanins, and chalcones in the bark of annual sprouts of the genus *Malus* spp.

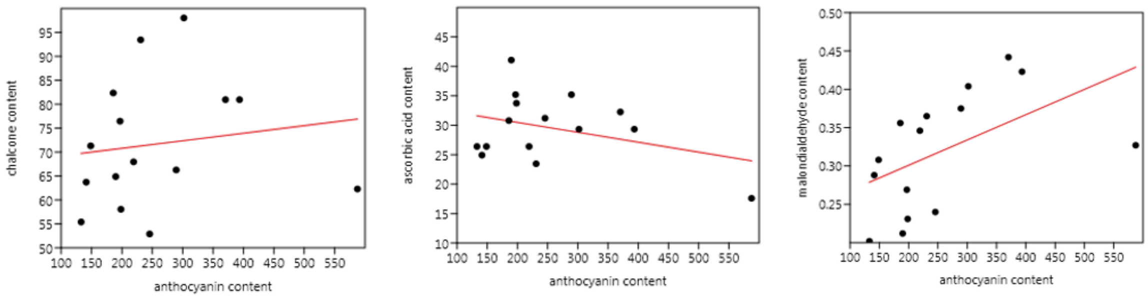
correlation coefficient, and a direct relationship between anthocyanins and malondialdehyde (Fig. 8).

The correlation between the levels of the studied secondary metabolites and organic acids was analyzed (Fig. 9). A weak positive relationship was observed between malondialdehyde accumulation and anthocyanins ($r = 0.5$), as well as between malondialdehyde and chalcones ($r = 0.7$), with $p > 0.05$. These findings suggest that malondialdehyde levels decrease primarily due to the accumulation of chalcones and anthocyanins in plants. Consequently, these two indicators serve as key markers of winter hardiness in apple plants.

Analyzing the dendrogram from the cluster analysis of the studied indicators, we observe a distinct Cluster I, which includes *M.* × *zumi* 'Golden Hornet' – characterized by the highest anthocyanin content (Fig. 10). The similarity between clusters, particularly among *M. baccata* 'Evereste' and *M.* × *purpurea* 'Ola'; *M. purpurea*; as well as *M. niedzwetzkyana* 'Era' and *M. niedzwetzkyana*; and *M. niedzwetzkyana* 'King Beauty' and *M.* × *purpurea* 'Royalty', can be attributed to their genetic origin and the anthocyanin pigmentation of both generative and vegetative organs. All of these cultivars exhibit red to burgundy-colored flowers, fruits, leaves, and shoots, reflecting their high anthocyanin concentration.

A separate cluster, which includes *M.* × *denticulata*, is distinguished by yellow-hued fruits, bright green leaves, white flowers, and brown shoot bark. These visual characteristics align with laboratory tests on natural antioxidant content, reinforcing the well-documented correlation between high anthocyanin levels and burgundy pigmentation. This pigmentation, in turn, serves as an indicator of increased anthocyanin accumulation.

The Gower similarity index further highlights genotype clustering based on anthocyanin pigmentation in both vegetative and generative organs. Genotypes with burgundy coloration exhibit higher network density, indicating a stronger relationship among anthocyanin-rich cultivars (Fig. 10).



Plot between anthocyanins and chalcones
r = 0.1; $p > 0.62$

Plot between anthocyanins and vitamin C
r = -0.4; $p > 0.29$

Plot between anthocyanins and malondialdehyde
r =0.5; $p > 0.05$

**Figure 8:** Multivariate linear regression

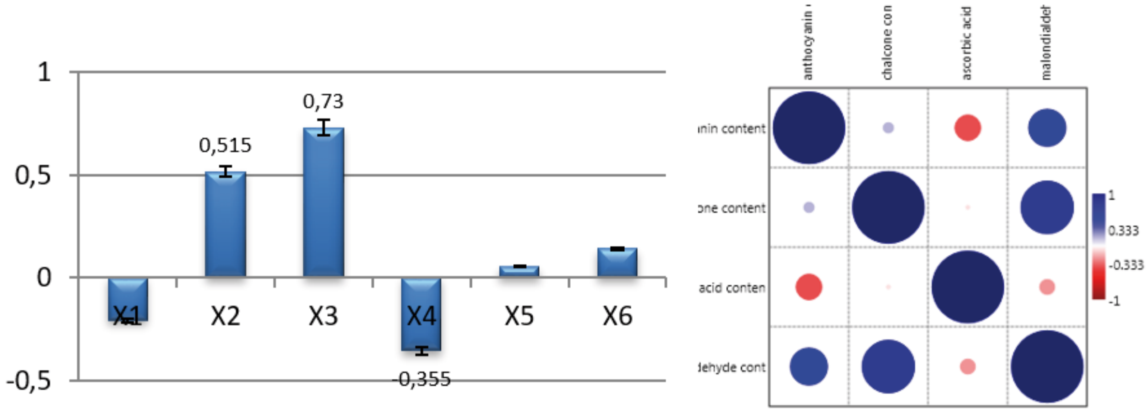


**Figure 9**: Pearson correlation coefficient: X1 – dependence of malondialdehyde on ascorbic acid content; X2 – dependence of malondialdehyde on anthocyanin content; X3 – dependence of malondialdehyde on chalcone content; X4 – dependence of ascorbic acid on anthocyanin content; X5 – dependence of ascorbic acid on chalcone content; X6 – dependence of anthocyanins on chalcone content

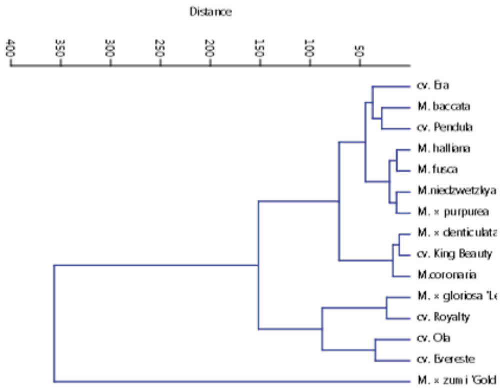


**Figure 10**: Dendrograms of the cluster analysis of the content of malondialdehyde, ascorbic acid, anthocyanins and chalcones in the bark of annual sprouts of representatives of the genus *Malus* spp.

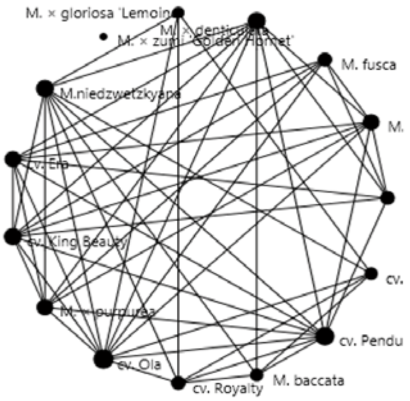


**Figure 11**: Network section similarity index Gower of the content of malondialdehyde, ascorbic acid, anthocyanins and chalcones in the bark of annual sprouts of representatives of the genus *Malus* spp.

## 4 DISCUSSION

Members of the *Malus* spp. genus endure various challenging winter conditions, including early frosts, extreme cold, and periodic thaws, all of which contribute to potential stress and damage. As a result, breeding for enhanced resistance remains a key research priority (Davey et al., 2000).

Currently, there are approximately 55 known *Malus* spp. cultivars worldwide (Goncharovska et al., 2020). Through long-term natural selection, many of these cultivars have developed unique traits tailored to specific environmental conditions. For example, *Malus xiaojinensis* Cheng et Jiang, *M. toringoides* (Rehd.) Hughes, and *M. kansuensis* (Batal.) Schneid. exhibit strong drought tolerance; *M. hupehensis* (Pamp.) Rehd. and *M. toringoides* (Rehd.) Hughes. are resistant to excessive moisture; *M. baccata* and *M. sieversii* withstand extremely low temperatures; *M. robusta* (Carr.) Rehd., *M. sieversii*, and *M. sikkimensis* (Wenzig.) Koehne can survive in saline soils, while *M. zumi* can tolerate soil salinity levels of up to 0.60 % (Gu, 1996; Wang et al., 2002).

Previous studies (Foyer et al., 1991; Juszczuk et al., 2001; Heim et al., 2002) indicate that free radicals and other reactive oxygen species (ROS) are naturally produced during biological redox reactions. A comparative assessment of small-fruited apple varieties for cold tolerance revealed significant fluctuations in malondialdehyde (MDA) levels in the bark of one-year-old shoots, as well as variations in ascorbic acid, anthocyanin, and chalcone content.

Research on MDA accumulation under salt stress (Wang et al., 2022) and during leaf chilling (Mark et al., 1999) confirms its formation in plants subjected to environmental stress. Antioxidant concentrations in shoot tissues varied among samples, suggesting that ROS detoxification is facilitated by different antioxidant defense mechanisms (Esterbauer et al., 1991; Alscher et al., 1997). Numerous studies support the idea that MDA serves as a key marker of oxidative lipid damage, with its levels changing in response to biotic and abiotic stressors (Deighton et al., 1999). It is hypothesized that, in addition to MDA, other endogenous compounds significantly contribute to the adaptive capacity of plants. However, findings confirm that anthocyanins, chalcones, and vitamin C play crucial roles in counteracting abiotic stress, forming an integral part of the antioxidant defense system responsible for neutralizing ROS.

In selecting samples for this study, temperature variations were considered to assess MDA accumulation under stress conditions. The results demonstrated that MDA levels were 1.6 % lower at positive temperatures than at subzero temperatures. Notably, *M. niedzwetzkyana* and *M. niedzwetzkyana* ‘Era’ showed the highest MDA accumulation in cell membranes. However, due to the presence of antioxidant systems – particularly anthocyanins and chalcones – MDA levels remained regulated. The deep red pigmentation of leaves and shoots in these apple varieties is likely attributed to high anthocyanin concentrations, which not only provide coloration but also play a protective role against oxidative lipid damage. Anthocyanin content in the studied genotypes ranged from 139.9 to 587.5 mg.100 $g^{-1}$ dry matter, with the highest values recorded in *M. baccata* ‘Evereste’, *M. niedzwetzkyana* ‘Era’, and *M. niedzwetzkyana*. Based on these results and previous research (Christie et al., 1994; Gould et al., 2000; Goncharovska et al., 2018; Goncharovska et al., 2021), anthocyanins function as protective signaling pigments in plants throughout the seasons. Therefore, apple cultivars with higher anthocyanin levels exhibit enhanced resistance to both biotic and abiotic stressors.

Chalcones, belonging to the largest group of secondary metabolites, serve as precursors to flavonoids and isoflavonoids (Panche et al., 2016). These compounds protect cell membranes by mitigating oxidative damage caused by ROS. Chalcone concentrations in the studied samples ranged from 52.9 to 68.03 mg.100 $g^{-1}$ dry matter, with the highest levels observed in *M.* × *purpurea* ‘Royalty’. Due to its high chalcone content, this cultivar is a strong candidate for future breeding programs aimed at enhancing apple cold resistance.

Extensive studies (Davey et al., 2000; Agius et al., 2003; Ang et al., 2018) emphasize the critical role of vitamin C as a plant antioxidant that strengthens resistance to environmental stressors. Among the analyzed samples, vitamin C content ranged from 17.6 to 47.06 mg.100 $g^{-1}$ dry matter, playing a significant role in suppressing free oxygen radicals and protecting cell membranes from oxidative damage. The highest vitamin C concentration was detected in *M. baccata* Pendula’, making this hybrid a promising candidate for breeding highly stress-resilient apple cultivars.

Pearson correlation analysis confirmed that MDA accumulation in apple plants occurs as a stress response, with chalcones playing a direct role in the antioxidant defense mechanism ($r = 0.73$). A dendrogram derived from cluster analysis further reinforced the biochemical similarities among apple cultivars based on their secondary metabolite accumulation patterns, providing valuable information for selecting genotypes for breeding programs targeting resistance to abiotic stress.

In conclusion, the apple cultivars demonstrating the highest cold resistance at the physiological level were *M. niedzwetzkyana* (including cultivars Era and King Beauty) and *M. baccata* (including cultivars Pendula and Evereste).

The results of this study highlight the essential physiological roles of malondialdehyde, ascorbic acid, anthocyanins, and chalcones in the adaptation of apple plants to low temperatures. These biochemical markers should be utilized as diagnostic tools for selecting small-fruited apple varieties and cultivars best suited to specific climatic conditions.

## 5 CONCLUSIONS

Our findings suggest that biochemical markers can serve as valuable indicators of cellular processes in *Malus* spp. Since the biosynthesis of various polyphenols is regulated at the molecular level, their concentrations fluctuate in response to abiotic factors. This variability provides insight into the oxidative processes that underlie plant stress responses and adaptation mechanisms.

Based on the total content of the analyzed compounds, the most winter-hardy small-fruited apple species were identified as *M. niedzwetzkyana* and the cultivars *M. baccata* 'Evereste', *M. niedzwetzkyana* 'Era', and *M.* × *purpurea* 'Royalty'. In contrast, *M. coronaria*, *M.* × *zumi* 'Golden Hornet', and *M.* × *denticulata* exhibited the lowest tolerance to low temperatures.

These findings are valuable for breeding programs, offering a diagnostic tool for assessing plant resistance to severe mid-winter frosts. Additionally, they provide a foundation for further research into the dynamics of these antioxidants.

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# Combining mulching and drip irrigation for water conservation in tomato crops: case of Mitidja plain (Algeria)

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**Combining mulching and drip irrigation for water conservation in tomato crops: case of Mitidja plain (Algeria)**

**Abstract:** Water scarcity in agriculture necessitates innovative practices for sustainable water management. This study evaluated the effects of organic mulching, specifically a mixture (composed of wood chips, sawdust, and wheat straw in equal proportions) and recycled crop waste (RCW), combined with drip irrigation, on water conservation in tomato crops cultivated in the Mitidja Plain, Algeria. Field experiments were conducted from July to October 2023, comparing mulched and non-mulched plots under surface and subsurface drip irrigation (SSDI). Results showed that the mixture mulch with SSDI saved 29.6 % of water, while RCW saved 22.2 %, compared to surface irrigation. Mulching regulated soil moisture, temperature, and electrical conductivity, though water application efficiency slightly decreased (96.1 % for mixture, 96.4 % for RCW vs. 97.1 % for control). However, the slight soil acidification observed in mulched plots (pH 6.20–6.52) warrants attention, as it may influence nutrient availability over time. Despite this, soil organic matter (OM) remained stable (1.09 %–1.70 %), confirming that mulching enhances soil fertility without compromising its structure. These findings highlight the potential of combining organic mulching with drip irrigation for sustainable water management in tomato cultivation, offering practical insights for farmers in arid regions.

**Key words:** water scarcity, soil protection, sustainability, water application efficiency (EA), irrigation; mulch, Mitidja plain

**Vpliv prekrivk in kapljičnega namakanja na ohranjanje vode v nasadu paradižnika: vzorčen primer iz planote Mitidja (Alžirija)**

**Izvleček:** Pomanjkanje vode v kmetijstvu zahteva inovativne prakse za trajnostno upravljanje z vodo. Ta študija je ovrednotila učinke organskih prekrivk (mešanica iz enakih deležev lesnih sekancev, žagovine in pšenične slame) ter recikliranih kmetijskih ostankov (RCW), kombiniranih s kapljičnim namakanjem, na varčevanje z vodo pri pridelavi paradižnika na planoti Mitidja v Alžiriji. Terenski poskusi so potekali od julija do oktobra 2023, pri čemer so primerjali prekrite in neprekrite parcele pri površinskem in podpovršinskem kapljičnem namakanju (SSDI). Rezultati so pokazali, da je mešanica z SSDI prihranila 29,6 % vode, RCW pa 22,2 % v primerjavi s površinskim namakanjem. Prekrivka je uravnavala vlažnost, temperaturo in električno prevodnost tal, čeprav se je učinkovitost namakanja rahlo zmanjšala (96,1 % za mešanico, 96,4 % za RCW v primerjavi s 97,1 % pri kontrolni skupini). Kljub temu je rahlo zakisanje tal v prekritih parcelah (pH 6,20–6,52) potrebno spremljati, saj lahko sčasoma vpliva na dostopnost hranil. Organska snov v tleh (OM) je ostala stabilna (1,09 %–1,70 %), kar potrjuje, da prekrivka izboljšuje rodovitnost tal brez ogrožanja njihove strukture. Te ugotovitve poudarjajo potencial kombinacije organske prekrivke s kapljičnim namakanjem za trajnostno upravljanje z vodo pri pridelavi paradižnika, kar ponuja praktične vpoglede za kmete v sušnih območjih.

**Ključne besede:** pomanjkanje vode, zaščita tal, trajnostnost, učinkovitost namakanja (EA), namakanje, prekrivka, planota Mitidja

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## 1 INTRODUCTION

Water scarcity is one of the most pressing challenges of the 21st century, with agriculture accounting for nearly 70 % of global freshwater withdrawals (FAO 2017). In arid and semi-arid regions, such as Al geria's Mitidja Plain, the situation is exacerbated by climate change, prolonged droughts, and increasing water demand (Meddi et al., 2015). This region, known as the "breadbasket of Algeria," plays a critical role in the nation's food security, particularly for tomato production: a crop that not only sustains local livelihoods but also fuels the country's agri-food industries (Bessaoud et al., 2019). However, the sustainability of this vital sector is under threat due to inefficient water management practices and the overexploitation of limited water resources (Karambiri et al., 2021; Rastogi et al., 2024).

In response, conservation agriculture has emerged as a transformative approach, integrating innovative practices such as drip irrigation, mulching, and soil cover to optimize water use, enhance soil health, and reduce environmental impacts (Yang et al., 2023; Du Preez et al., 2024). Among these practices, the combination of organic mulching and subsurface drip irrigation (SSDI) has shown exceptional promise. While drip irrigation minimizes water loss by delivering water directly to the root zone (Ayars et al., 2015), organic mulching acts as a protective barrier, reducing evaporation, regulating soil temperature, and improving moisture retention (Simsek et al., 2017; Telkar et al., 2017). Together, these techniques offer a (synergistic solution to the dual challenges of water scarcity and sustainable crop production (Sharma et al., 2023; Wang et al., 2024).

Despite their individual benefits, the combined effects of organic mulching and SSDI remain underexplored, particularly in the context of tomato cultivation in water-stressed regions like the Mitidja Plain. Previous studies have highlighted the advantages of mulching in conserving soil moisture (Simsek et al., 2017; Telkar et al., 2017) and the efficiency of SSDI in reducing water use (Ayars et al., 2015; Yang et al., 2023). Recent research by-Wang et al. (2024) has demonstrated that mulched drip irrigation significantly enhances water use efficiency and crop yields in arid regions, particularly for high-value crops like tomatoes. Similarly, (Tankeuoo et al., 2023) have shown that organic mulches improve soil moisture retention and water infiltration in tomato crops, underscoring their potential for sustainable agriculture in water-scarce environments. However, there is a critical gap in understanding how these practices interact to enhance water conservation, improve crop yields, and support long-term agricultural sustainability (Admasu & Tamiru, 2019).

This study addresses this gap by evaluating the performance of different organic mulches in combination with surface and subsurface drip irrigation systems. Conducted in the Mitidja Plain, the research aims to identify the most effective strategies for water conservation in tomato cultivation. By providing evidence-based insights, this study seeks to empower farmers, researchers, and policymakers with practical solutions to enhance water efficiency, ensure food security, and promote sustainable agricultural practices in Algeria and beyond (Iqbal et al., 2020; Gouda et al., 2023).

## 2 MATERIALS AND METHODS

### 2.1 DESCRIPTION OF THE EXPERIMENTAL SITE

The experiments were conducted on the Mitidja Plain, a fertile agricultural region in northern Algeria, specifically at the experimental site of the Higher National School of Hydraulics (ENSH) in Blida. Geographically located at 36°30'31" N and 2°53'15" E , with an altitude of 116 m above sea level, the site lies within the sub-humid Mediterranean bioclimatic zone, characterized by mild winters and hot, dry summers (Laribi et al., 2023). The Mitidja Plain spans four wilayas (Blida, Tipaza, Boumerdès, and Algiers) and is a vital agricultural hub, contributing significantly to Algeria's food production, particularly for high-value crops like tomatoes. The study was carried out over four months, from July to October 2023, during the peak growing season for tomatoes. Climatic data collected by an on-site agro-meteorological station revealed an average air temperature (Tmean) of 26.39 °C, with extremes ranging from 26.31 °C (Tmin) to 41.30 °C (Tmax). The average relative humidity (Hmean) was 62.40 %, fluctuating between 19.54 % and 86.48 %, while solar radiation (Rs) averaged 227.04 W $m^{-2}$. Wind speed (Ws) was moderate, averaging 1.97 km $h^{-1}$, and precipitation (Pi) was scarce, with a sum of 22.4 mm over the study period. The daily reference evapotranspiration (ETR) averaged 3.17 mm $day^{-1}$, reflecting the high evaporative demand typical of the region. Soil analysis conducted by (Tankeuoo et al., 2023) confirmed that the experimental site features a fine loam soil.

### 2.2 SITE DESIGN AND TREATMENTS

The experimental site was prepared through light plowing and harrowing to ensure optimal soil conditions for seeding. A total of six plots were established, each measuring 2 m × 2 m (4 m²), resulting in a total experimental area of 24 m² (Fig. 1). The design included two replications, with each replication comprising two plots: one equipped with a surface drip irrigation system (SURDI) and the other with a subsurface drip irrigation system (SSDI) installed at a depth of 10 cm. This setup allowed for a direct comparison of the two irrigation methods under identical environmental conditions. Each plot contained 18 tomato plants of the hybrid variety Badja F1, known for its high yield and adaptability to Mediterranean climates. The plants were spaced at 50 cm intervals, while the drippers were positioned 33 cm apart, ensuring uniform water distribution to the root zone. The irrigation system operated at a constant flow rate of 2.3 l h$^{-1}$, with irrigation cycles scheduled every 2 hours between 4 a.m. and 7 a.m. to minimize evaporation losses during the cooler morning hours. Three distinct treatments were applied: P1 (mixture mulch of wood chips, sawdust and wheat straw in equal proportions); P2 (bare soil without mulch) and P3 (RCW mulch composed of wood chips and dead leaves) and control The choice of mulching materials was guided by their local availability, cost-effectiveness, and proven efficacy in previous studies (Chachoua, 2015; Telkar et al., 2017). The experimental design followed a Latin square pattern, ensuring balanced spatial distribution of treatments and minimizing the impact of soil variability. Irrigation scheduling was optimized based on the field capacity (FC) and wilting point (WP) of the soil, which ranged from 32-38 % and 14-17 %, respectively. These parameters were determined using a calibration sheet provided by the TDR 150 manufacturer, ensuring precise irrigation control and efficient water use.

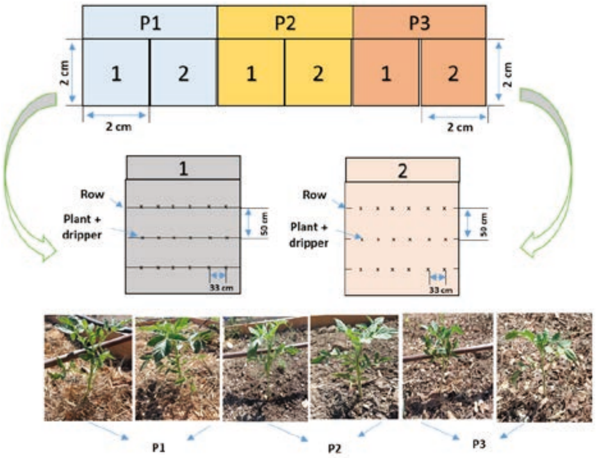


**Figure 1:** Design of the experimental area. P1 = Mixture plots (same ratio of wood chips + sawdust + wheat straw); P2 = Control plots (bare soil); P3 = RCW plots (woody debris and dead leaves). 1 = Surface drip irrigation system; 2= Subsurface drip irrigation system (10 cm depth)

## 2.3 SOIL PHYSICAL AND CHEMICAL ANALYSIS

To assess the suitability of the soil for tomato cultivation and to understand its impact on crop performance, physical and chemical properties were analyzed for each experimental plot. Specifically, soil pH and soil organic matter (OM) were measured, as these parameters are critical for nutrient availability, microbial activity, and overall soil health.

Soil pH was determined using a calibrated pH meter, which provided accurate and reliable measurements of soil acidity or alkalinity. This parameter is essential for understanding nutrient solubility and uptake by plants, as well as for identifying potential soil amendments to optimize growing conditions.

Soil organic matter (OM) was quantified using the loss on ignition (LOI) method, a widely recognized technique for measuring organic content. This method involves heating soil samples to high temperatures to combust organic materials, with the mass loss corresponding to the organic matter content. OM is a key indicator of soil fertility, as it influences water retention, nutrient cycling, and soil structure.

## 2.4 SOIL MOISTURE, TEMPERATURE, AND ELECTRICAL CONDUCTIVITY DATA COLLECTION

Soil water levels were monitored continuously from the sowing of the tomato plants until the end of the growing period, spanning July to October 2023. The TDR 150 probe was selected for this study due to its affordability, reliability, accuracy, and ease of use. This advanced instrument is capable of measuring soil moisture, electrical conductivity, and temperature simultaneously at the same depth and time scale, providing comprehensive data essential for effective irrigation and nutrient management. In the field, the TDR 150's rods were inserted at four depths: 5 cm, 10 cm, 15 cm, and 20 cm, adjacent to selected tomato plants in each plot. These depths were chosen to capture variations in soil water dynamics across the root zone, ensuring a detailed understanding of water distribution and uptake by the plants. Measurements were conducted daily, with three replicates per plot, between 7 a.m. and 9 a.m. to minimize variability caused by diurnal fluctuations in temperature and evaporation. All data were meticulously recorded on standardized data sheets for subsequent analysis.

The recorded data were analyzed using XLSTAT software, employing advanced statistical tools to ensure robust and reliable results. A correlation matrix was first generated to assess the relationships between key variables, including soil moisture, temperature, and electrical conductivity parameters. This step provided preliminary insights into the interactions between the studied factors. Subsequently, an Analysis of Variance (ANOVA) was performed to evaluate the significance of differences among treatments. The F-test was applied at a significance level of $p \leq 0.05$ to determine whether the observed variations were statistically significant. For parameters significantly affected by the studied factors, treatment means were compared using the Critical Difference (CD) method.

### 2.5 COLLECTION OF PLANT HEIGHT DATA ON CROPS, IRRIGATION, AND ACTUAL EVAPOTRANSPIRATION (ETA).

Plant height was measured at each stage (stage 1 = plant growth; stage 2 = plant flowering; stage 3 = plant fruiting; stage 4 = plant maturation + harvest) of development to monitor growth patterns and assess the impact of different treatments.

Irrigation data were meticulously recorded, with water volumes measured during each irrigation event. These volumes were then summed for each growth stage and treatment, allowing for a detailed analysis of water use efficiency. At the end of the growing season, the total water volumes applied to each plot were converted to m³ per hectare to facilitate comparisons and evaluate water savings across the different irrigation systems. Additionally, irrigation frequencies were tracked and summed for each growth stage.

Actual evapotranspiration (ETA) was calculated using the FAO Penman-Monteith formula, a widely recognized method for estimating crop water requirements. This formula incorporates weather station data (such as temperature, humidity, wind speed, and solar radiation) and crop coefficients (K) specific to each growth stage. The equation used is as follows:

$$ETA = ETR \times K \qquad (2.1)$$

Where: *ETA* = Actual evapotranspiration (mm/day); *ETR* = Daily reference evapotranspiration (mm day$^{-1}$), calculated using the Penman-Monteith method and *K* = Crop coefficient, which varies according to the growth stage of the tomato plants

### 2.6 IRRIGATION APPLICATION EFFICIENCY (EA)

Water application efficiency (EA) is a critical metric in irrigation management, expressed as a percentage that indicates how effectively irrigation water is delivered to the crop root zone. Specifically, EA measures the proportion of applied water that is stored in the root zone and made available for plant use, minimizing losses due to evaporation, runoff, or deep percolation. According to (Gouda et al. 2023), the equation for calculating EA is as follows:

$$EA = \frac{e \times q_{min} \times T}{V} \times 100 \qquad (2.2)$$

Where: *EA* = Application efficiency (%); *e* = Total number of drippers; *qmin* = Minimum flow rate of drippers (l h$^{-1}$); *T* = Total irrigation time (h); *V* = Total volume of water applied (l)

## 3 RESULTS

### 3.1 SOIL PHYSICAL AND CHEMICAL ANALYSIS

The Table 1 compares two irrigation systems (SURDI and SSDI), by analyzing OM and soil pH across six plots (P1-P6). For OM, values range from 1.09 % to 1.70 %, with no significant differences between SURDI and SSDI (Pr > F = 0.87), indicating that the irrigation system does not significantly affect OM. However, for

**Table 1:** Statistical analysis of soil organic matter (OM) and soil pH across plots under two irrigation types (SURDI and SSDI)

| IR. Type | Plot unit | OM % | Soil pH - |
|---|---|---|---|
| SURDI | P1 | 1.54a | 7.04a |
| | P2 | 1.50a | 6.90a |
| | P3 | 1.70a | 7.06a |
| SSDI | P1 | 1.24a | 6.20b |
| | P2 | 1.54a | 6.30b |
| | P3 | 1.09a | 6.52b |
| | SEm± | 0.09 | 0.16 |
| | Pr > F | 0.87 | 0.91 |
| | CD (P=0.05) | 6.08 | 6.08 |

The treatment details are provided in the Materials and Methods section soil pH, SURDI plots show higher pH values (6.90–7.06) compared to SSDI plots, which have lower pH values (6.20–6.52), marked with “b” to indicate significant differences (Pr > F = 0.91). This suggests that SSDI may lead to slight soil acidification, possibly due to reduced surface evaporation and altered nutrient dynamics. Despite this, both systems perform similarly in terms of OM content, highlighting that SSDI remains a viable option for water conservation without significantly compromising soil organic matter.

## 3.2 LENGTH AND SIZE OF EACH GROWING SEASON

Figure 2 shows plant duration and height at each stage in mulched and bare plots. During growth (P3), plants are the largest and take the longest time. Flowering phase (P2) lasts longer under both irrigation systems, and P1 is larger with SURDI. Fruiting sees P1 as the tallest and longest. Mulched plots, especially P1, outperform others. RCW mulch excels with SSDI, mixture mulch with SSDI.

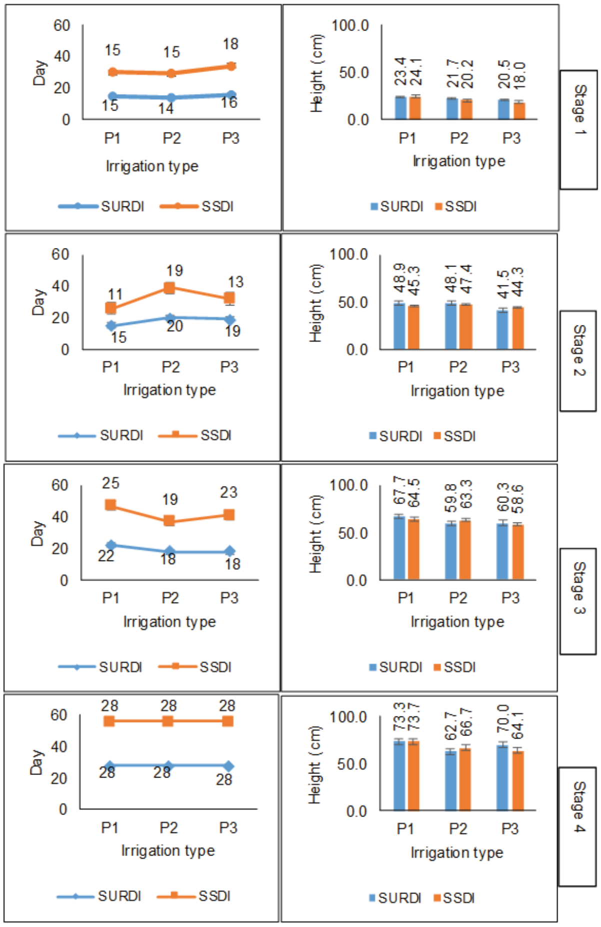


**Figure 2:** Average duration and height of plants per growing stage according to irrigation system and treatment applied to tomato crop. The bars indicate the standard errors. Details of the treatment are provided in the Materials and Methods section.

## 3.3 CHARACTERIZATION OF SOIL MOISTURE, TEMPERATURE AND ELECTRICAL CONDUCTIVITY OF EACH GROWING SEASON

### 3.3.1 Growth stage (stage 1)

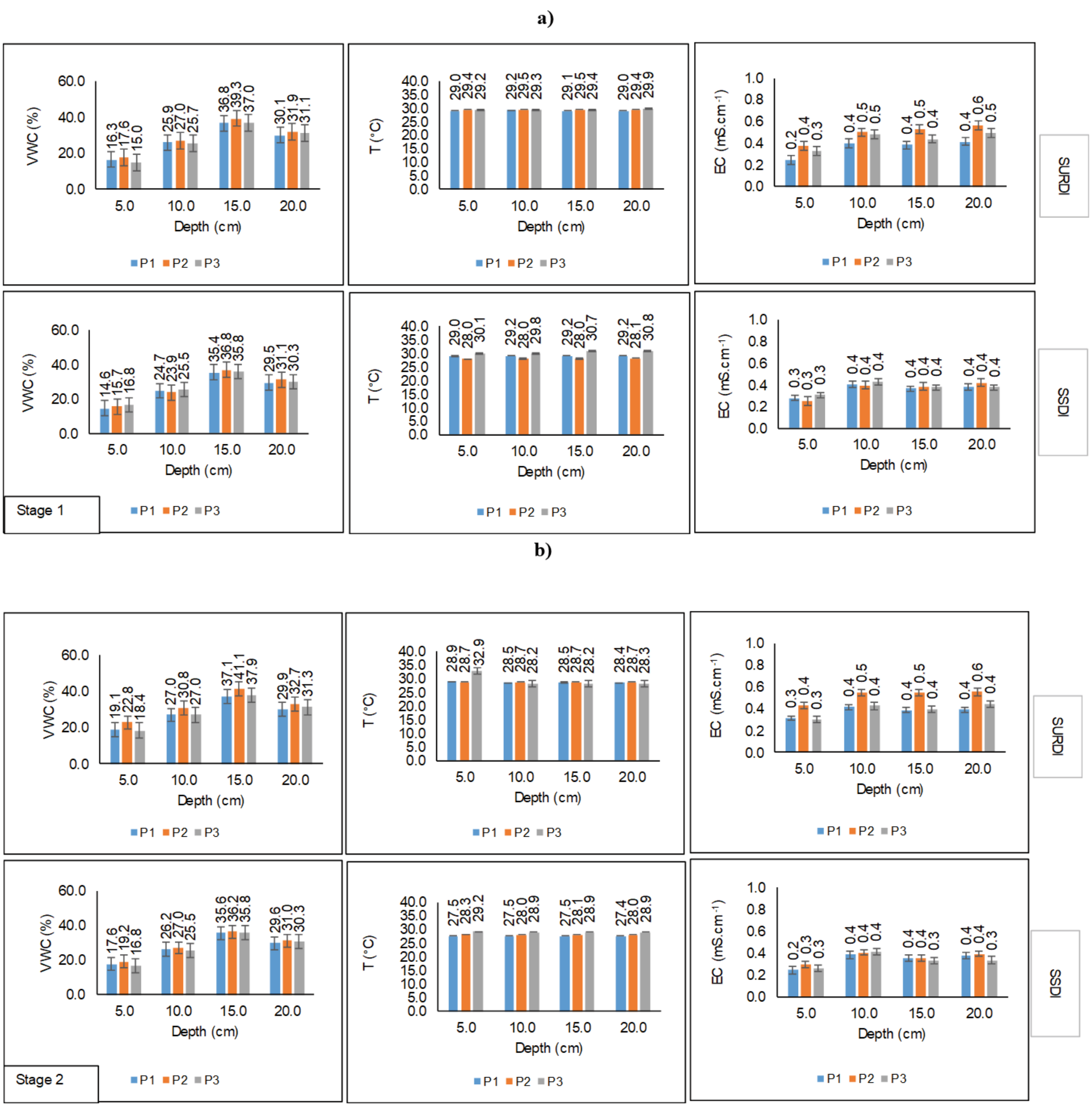
Figure 3a shows the impact on soil under SURDI and SSDI irrigation systems. With SURDI, mulch reduces soil moisture at 15-20 cm depth, especially in P1, but increases it at 5-10 cm in P3. Mulch lowers soil temperature, with P1 being the coolest, and decreases electrical conductivity, with P1 the lowest. Under SSDI, mulch acts as a regulator, increasing moisture at 5 cm in P3 and decreasing it in P1, while fully increasing at 10 cm and decreasing at 15-20 cm in both P1 and P3. Mulch raises temperature, notably in P3, and affects electrical conductivity, increasing it at 5-10 cm and decreasing at 15-20 cm.

### 3.3.2 Flowering stage (stage 2)

Figure 3b illustrates that in the SURDI scenario, mulched plots exhibit lower soil moisture levels compared to un-mulched plots across different depths, with P3 showing the lowest values. Soil temperature in mulched plots is also lower than the control, with P1 recording the lowest temperature. Additionally, soil electrical conductivity is lower in mulched plots compared to the control, with P3 being lower than P1 at 5 cm depth. In the SSDI scenario, mulched plots display lower soil moisture levels compared to the control, with P3 having lower values than P1. Soil temperature varies, with P1 being the lowest, and electrical conductivity is generally lower in mulched plots compared to the control, although variations exist at different depths.

### 3.3.3 Fruiting stage (stage 3)

In Figure 3 c’s analysis, within the SURDI context, mulched-covered plots consistently show lower soil moisture levels compared to the control across various depths, with P3 surpassing P1. Similarly, soil temperature is lower in mulched plots than the control, with P1 recording the lowest temperatures. In



terms of soil electrical conductivity, mulched plots consistently demonstrate lower values than the control. In the SSDI scenario, mulched plots exhibit lower soil moisture levels than the control at different depths, with P3 surpassing P1 from 10 cm upwards but the reverse at 5 cm. Soil temperature in mulched plots varies, both increasing (P3) and decreasing (P1) compared to the control at different depths. Electrical conductivity varies, with some depths showing lower (P1) and higher (P3) values compared to the control.

### 3.3.4 Maturation stage + harvest (stage 4)

In Figure 3d, SURDI shows that mulched plots have lower soil moisture compared to un-mulched ones, with P3 higher than P1. Soil temperature varies, with both lower (P1) and higher (P3) values compared to control. Soil electrical conductivity is lower in mulched plots, with P3 higher than P1. In SSDI, mulched plots have lower soil moisture, with P1 lower than P3 at certain depths. Soil temperature increases at some depths in mulched plots compared to control, with P3 showing the highest increase. Soil electrical conductivity varies, with P3 higher than P1 at certain depths.

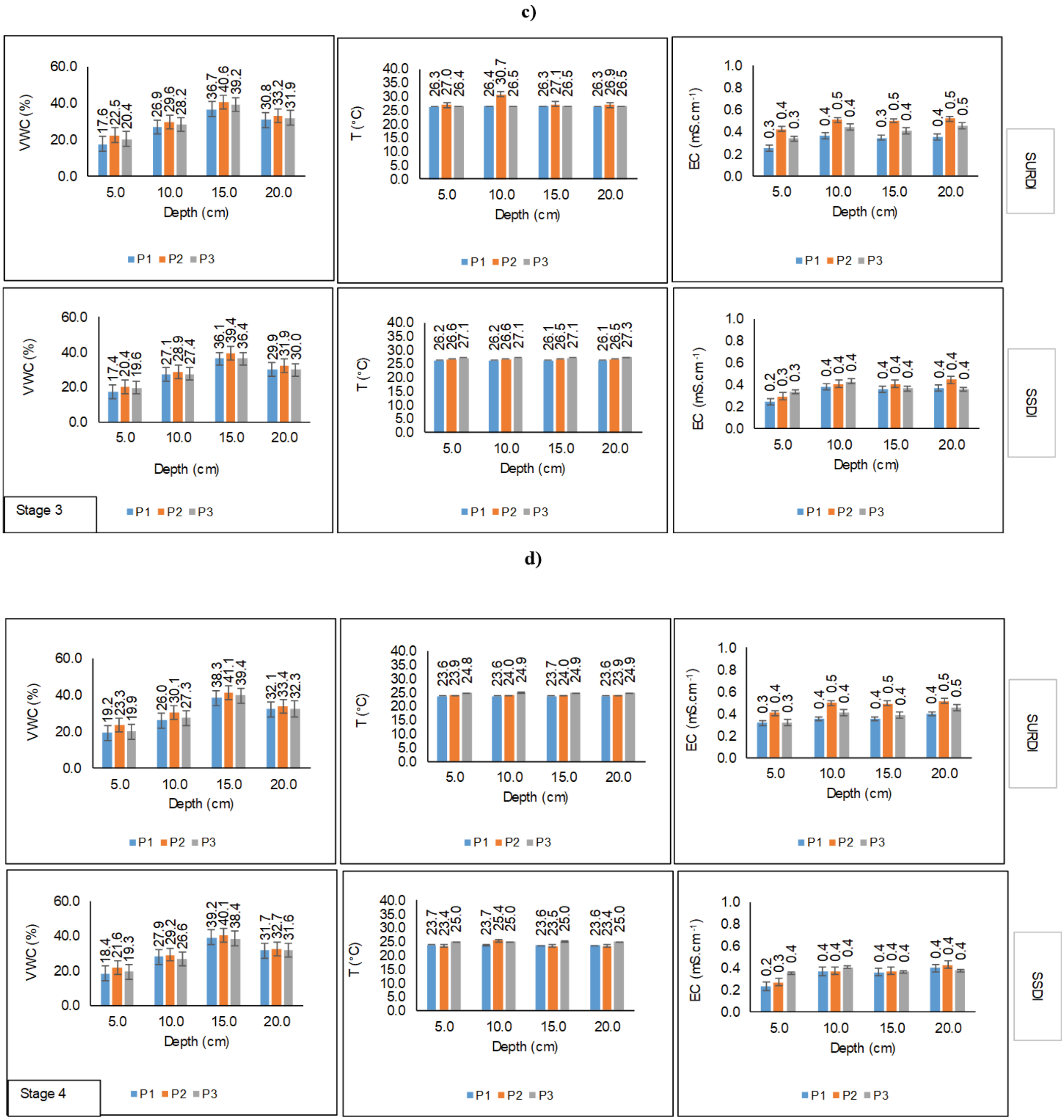


**Figure 3:** Evaluation of soil moisture (VWC), temperature (T), and electrical conductivity (EC) at depths of 5 cm, 10 cm, 15 cm, and 20 cm across different growth stages: (a) Stage 1, (b) Stage 2, (c) Stage 3, and (d) Stage 4. Error bars represent standard errors. Treatment details are provided in the Materials and Methods section.

## 3.4 STATISTICAL ANALYSIS FOR SOIL MOISTURE, TEMPERATURE AND ELECTRICAL CONDUCTIVITY PARAMETERS

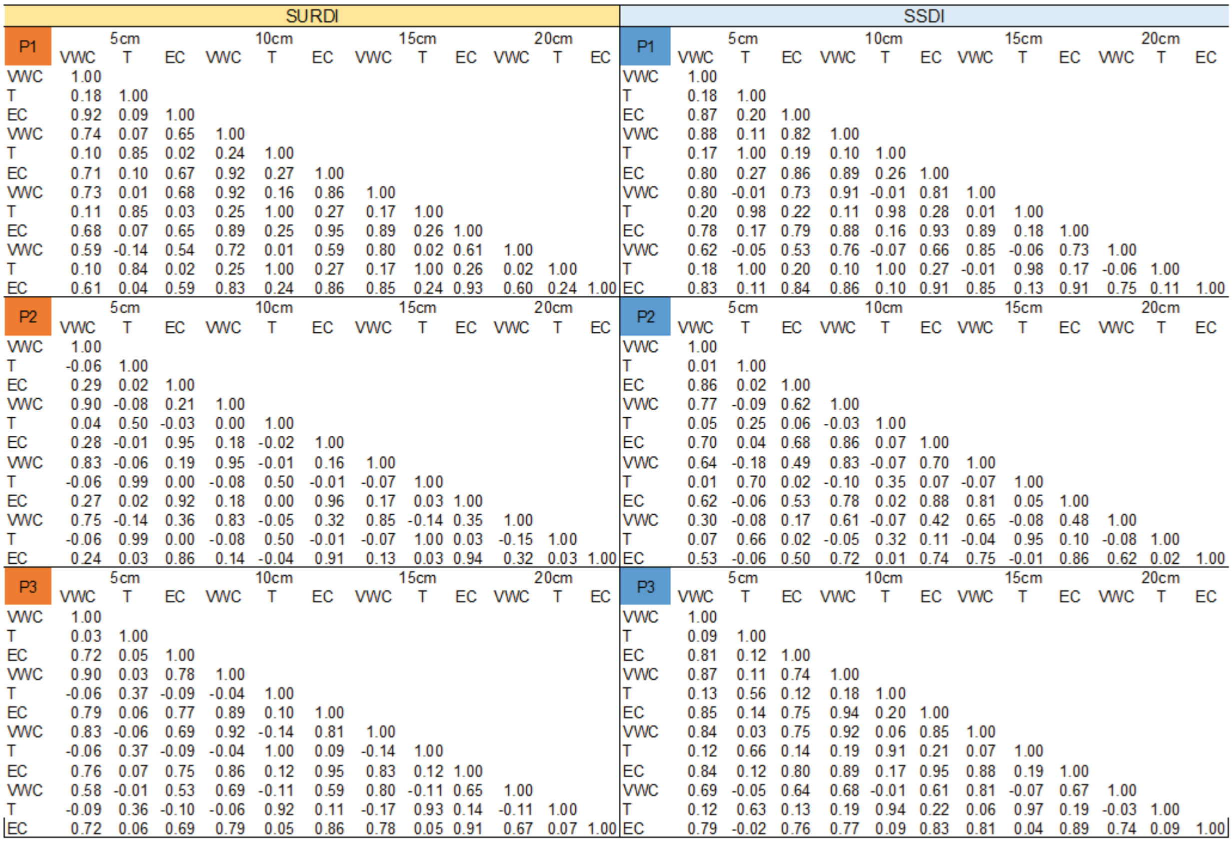
### 3.4.1 Correlation matrix between soil moisture, soil temperature and soil conductivity during plant development

The correlation matrices (Table 2) analyze the relationships between Volumetric Water Content (VWC), Temperature (T), and Electrical Conductivity (EC) across different depths (5 cm, 10 cm, 15 cm, and 20 cm) for three plots (P1, P2, and P3) under two irrigation systems (SURDI and SSDI). Generally, VWC and EC show strong positive correlations (ranging from 0.53 to 0.95), reflecting the role of water content in enhancing electrical conductivity through improved ion mobility. Temperature, however, exhibits minimal correlation with VWC and EC, indicating its limited influence on these variables. Specifically, in P1, the VWC-EC relationship is robust (0.68–0.95) across depths, with SSDI slightly strengthening this correlation compared to SURDI, likely due to the mulch's ability to retain moisture and improve water distribution under SSDI. In P2, the VWC-EC correlation is weaker under SURDI (0.19–0.36) but improves under SSDI (0.42–0.86), suggesting that SSDI enhances water-conductivity dynamics even in the absence of mulch. P3 shows strong VWC-EC correlations (0.53–0.89), similar to P1, with SSDI further enhancing this relationship, indicating that RCW mulch, like the mixture mulch, effectively retains moisture and optimizes water distribution under SSDI. Across all plots, VWC-EC correlations tend to decrease with depth, particularly in P2, likely due to reduced water movement or soil heterogeneity at deeper layers. Temperature remains stable and independent across depths, reflecting uniform thermal behavior. Overall, the data highlight the critical role of mulch types and irrigation systems in shaping soil water-conductivity dynamics: mixture and RCW mulches enhance water retention and conductivity, particularly under SSDI, while the control plot shows weaker relationships unless SSDI is applied. These findings underscore the importance of mulch and irrigation strategies in optimizing soil water management and conductivity.

### 3.4.2 Statistical analysis of VWC, T, and EC under SURDI and SSDI during plant development

The Table 3 presents data on soil parameters (VWC), T, and EC) measured at four depths (5 cm, 10 cm, 15 cm, 20 cm) under two irrigation systems (SURDI, SSDI). The results show that SSDI significantly improves water distribution in deeper soil layers (15 cm, 20 cm), with lower VWC values at 5 cm but higher efficiency in retaining moisture at greater depths compared to SURDI. Additionally, SSDI effec-

**Table 2:** Correlation matrices of VWC, T, and EC across different depths (5 cm, 10 cm, 15 cm, 20 cm) and mulch types (P1, P2, P3) under SURDI and SSDI irrigation systems.



| | SURDI | | | | | | | | | | | | SSDI | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P1 | 5cm | | | 10cm | | | 15cm | | | 20cm | | | P1 | 5cm | | | 10cm | | | 15cm | | | 20cm | | |
| | VWC | T | EC | VWC | T | EC | VWC | T | EC | VWC | T | EC | | VWC | T | EC | VWC | T | EC | VWC | T | EC | VWC | T | EC |
| VWC | 1.00 | | | | | | | | | | | | VWC | 1.00 | | | | | | | | | | | |
| T | 0.18 | 1.00 | | | | | | | | | | | T | 0.18 | 1.00 | | | | | | | | | | |
| EC | 0.92 | 0.09 | 1.00 | | | | | | | | | | EC | 0.87 | 0.20 | 1.00 | | | | | | | | | |
| VWC | 0.74 | 0.07 | 0.65 | 1.00 | | | | | | | | | VWC | 0.88 | 0.11 | 0.82 | 1.00 | | | | | | | | |
| T | 0.10 | 0.85 | 0.02 | 0.24 | 1.00 | | | | | | | | T | 0.17 | 1.00 | 0.19 | 0.10 | 1.00 | | | | | | | |
| EC | 0.71 | 0.10 | 0.67 | 0.92 | 0.27 | 1.00 | | | | | | | EC | 0.80 | 0.27 | 0.86 | 0.89 | 0.26 | 1.00 | | | | | | |
| VWC | 0.73 | 0.01 | 0.68 | 0.92 | 0.16 | 0.86 | 1.00 | | | | | | VWC | 0.80 | -0.01 | 0.73 | 0.91 | -0.01 | 0.81 | 1.00 | | | | | |
| T | 0.11 | 0.85 | 0.03 | 0.25 | 1.00 | 0.27 | 0.17 | 1.00 | | | | | T | 0.20 | 0.98 | 0.22 | 0.11 | 0.98 | 0.28 | 0.01 | 1.00 | | | | |
| EC | 0.68 | 0.07 | 0.65 | 0.89 | 0.25 | 0.95 | 0.89 | 0.26 | 1.00 | | | | EC | 0.78 | 0.17 | 0.79 | 0.88 | 0.16 | 0.93 | 0.89 | 0.18 | 1.00 | | | |
| VWC | 0.59 | -0.14 | 0.54 | 0.72 | 0.01 | 0.59 | 0.80 | 0.02 | 0.61 | 1.00 | | | VWC | 0.62 | -0.05 | 0.53 | 0.76 | -0.07 | 0.66 | 0.85 | -0.06 | 0.73 | 1.00 | | |
| T | 0.10 | 0.84 | 0.02 | 0.25 | 1.00 | 0.27 | 0.17 | 1.00 | 0.26 | 0.02 | 1.00 | | T | 0.18 | 1.00 | 0.20 | 0.10 | 1.00 | 0.27 | -0.01 | 0.98 | 0.17 | -0.06 | 1.00 | |
| EC | 0.61 | 0.04 | 0.59 | 0.83 | 0.24 | 0.86 | 0.85 | 0.24 | 0.93 | 0.60 | 0.24 | 1.00 | EC | 0.83 | 0.11 | 0.84 | 0.86 | 0.10 | 0.91 | 0.85 | 0.13 | 0.91 | 0.75 | 0.11 | 1.00 |
| P2 | 5cm | | | 10cm | | | 15cm | | | 20cm | | | P2 | 5cm | | | 10cm | | | 15cm | | | 20cm | | |
| | VWC | T | EC | VWC | T | EC | VWC | T | EC | VWC | T | EC | | VWC | T | EC | VWC | T | EC | VWC | T | EC | VWC | T | EC |
| VWC | 1.00 | | | | | | | | | | | | VWC | 1.00 | | | | | | | | | | | |
| T | -0.06 | 1.00 | | | | | | | | | | | T | 0.01 | 1.00 | | | | | | | | | | |
| EC | 0.29 | 0.02 | 1.00 | | | | | | | | | | EC | 0.86 | 0.02 | 1.00 | | | | | | | | | |
| VWC | 0.90 | -0.08 | 0.21 | 1.00 | | | | | | | | | VWC | 0.77 | -0.09 | 0.62 | 1.00 | | | | | | | | |
| T | 0.04 | 0.50 | -0.03 | 0.00 | 1.00 | | | | | | | | T | 0.05 | 0.25 | 0.06 | -0.03 | 1.00 | | | | | | | |
| EC | 0.28 | -0.01 | 0.95 | 0.18 | -0.02 | 1.00 | | | | | | | EC | 0.70 | 0.04 | 0.68 | 0.86 | 0.07 | 1.00 | | | | | | |
| VWC | 0.83 | -0.06 | 0.19 | 0.95 | -0.01 | 0.16 | 1.00 | | | | | | VWC | 0.64 | -0.18 | 0.49 | 0.83 | -0.07 | 0.70 | 1.00 | | | | | |
| T | -0.06 | 0.99 | 0.00 | -0.08 | 0.50 | -0.01 | -0.07 | 1.00 | | | | | T | 0.01 | 0.70 | 0.02 | -0.10 | 0.35 | 0.07 | -0.07 | 1.00 | | | | |
| EC | 0.27 | 0.02 | 0.92 | 0.18 | 0.00 | 0.96 | 0.17 | 0.03 | 1.00 | | | | EC | 0.62 | -0.06 | 0.53 | 0.78 | 0.02 | 0.88 | 0.81 | 0.05 | 1.00 | | | |
| VWC | 0.75 | -0.14 | 0.36 | 0.83 | -0.05 | 0.32 | 0.85 | -0.14 | 0.35 | 1.00 | | | VWC | 0.30 | -0.08 | 0.17 | 0.61 | -0.07 | 0.42 | 0.65 | -0.08 | 0.48 | 1.00 | | |
| T | -0.06 | 0.99 | 0.00 | -0.08 | 0.50 | -0.01 | -0.07 | 1.00 | 0.03 | -0.15 | 1.00 | | T | 0.07 | 0.66 | 0.02 | -0.05 | 0.32 | 0.11 | -0.04 | 0.95 | 0.10 | -0.08 | 1.00 | |
| EC | 0.24 | 0.03 | 0.86 | 0.14 | -0.04 | 0.91 | 0.13 | 0.03 | 0.94 | 0.32 | 0.03 | 1.00 | EC | 0.53 | -0.06 | 0.50 | 0.72 | 0.01 | 0.74 | 0.75 | -0.01 | 0.86 | 0.62 | 0.02 | 1.00 |
| P3 | 5cm | | | 10cm | | | 15cm | | | 20cm | | | P3 | 5cm | | | 10cm | | | 15cm | | | 20cm | | |
| | VWC | T | EC | VWC | T | EC | VWC | T | EC | VWC | T | EC | | VWC | T | EC | VWC | T | EC | VWC | T | EC | VWC | T | EC |
| VWC | 1.00 | | | | | | | | | | | | VWC | 1.00 | | | | | | | | | | | |
| T | 0.03 | 1.00 | | | | | | | | | | | T | 0.09 | 1.00 | | | | | | | | | | |
| EC | 0.72 | 0.05 | 1.00 | | | | | | | | | | EC | 0.81 | 0.12 | 1.00 | | | | | | | | | |
| VWC | 0.90 | 0.03 | 0.78 | 1.00 | | | | | | | | | VWC | 0.87 | 0.11 | 0.74 | 1.00 | | | | | | | | |
| T | -0.06 | 0.37 | -0.09 | -0.04 | 1.00 | | | | | | | | T | 0.13 | 0.56 | 0.12 | 0.18 | 1.00 | | | | | | | |
| EC | 0.79 | 0.06 | 0.77 | 0.89 | 0.10 | 1.00 | | | | | | | EC | 0.85 | 0.14 | 0.75 | 0.94 | 0.20 | 1.00 | | | | | | |
| VWC | 0.83 | -0.06 | 0.69 | 0.92 | -0.14 | 0.81 | 1.00 | | | | | | VWC | 0.84 | 0.03 | 0.75 | 0.92 | 0.06 | 0.85 | 1.00 | | | | | |
| T | -0.06 | 0.37 | -0.09 | -0.04 | 1.00 | 0.09 | -0.14 | 1.00 | | | | | T | 0.12 | 0.66 | 0.14 | 0.19 | 0.91 | 0.21 | 0.07 | 1.00 | | | | |
| EC | 0.76 | 0.07 | 0.75 | 0.86 | 0.12 | 0.95 | 0.83 | 0.12 | 1.00 | | | | EC | 0.84 | 0.12 | 0.80 | 0.89 | 0.17 | 0.95 | 0.88 | 0.19 | 1.00 | | | |
| VWC | 0.58 | -0.01 | 0.53 | 0.69 | -0.11 | 0.59 | 0.80 | -0.11 | 0.65 | 1.00 | | | VWC | 0.69 | -0.05 | 0.64 | 0.68 | -0.01 | 0.61 | 0.81 | -0.07 | 0.67 | 1.00 | | |
| T | -0.09 | 0.36 | -0.10 | -0.06 | 0.92 | 0.11 | -0.17 | 0.93 | 0.14 | -0.11 | 1.00 | | T | 0.12 | 0.63 | 0.13 | 0.19 | 0.94 | 0.22 | 0.06 | 0.97 | 0.19 | -0.03 | 1.00 | |
| EC | 0.72 | 0.06 | 0.69 | 0.79 | 0.05 | 0.86 | 0.78 | 0.05 | 0.91 | 0.67 | 0.07 | 1.00 | EC | 0.79 | -0.02 | 0.76 | 0.77 | 0.09 | 0.83 | 0.81 | 0.04 | 0.89 | 0.74 | 0.09 | 1.00 |

Treatment details are provided in the Materials and Methods section.

**Table 3:** Statistical analysis of soil moisture, temperature and electrical conductivity parameters in plots at 5, 10, 15 and 20 cm depth until two types of irrigation (SURDI, SSDI)

|  | Depth |  | 5 cm |  |  | 10 cm |  |  | 15 cm |  |  | 20 cm |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IR. Type | Plot | VWC | T | EC | VWC | T | EC | VWC | T | EC | VWC | T | EC |
| SURDI | P1 | 18.23a | 26.28a | 0.28a | 26.40a | 26.29a | 0.38a | 37.37a | 26.26a | 0.36a | 30.99a | 26.21a | 0.39a |
|  | P2 | 22.05a | 26.80a | 0.41a | 29.68a | 27.63a | 0.51a | 40.67a | 26.75a | 0.51a | 32.93a | 26.72a | 0.53a |
|  | P3 | 18.71a | 27.86a | 0.32a | 27.11a | 26.84a | 0.43a | 38.56a | 26.87a | 0.40a | 31.75a | 26.96a | 0.46a |
| SSDI | P1 | 17.12a | 25.94a | 0.25b | 26.78a | 25.96a | 0.38b | 37.03b | 25.93a | 0.36b | 30.44b | 25.89b | 0.38b |
|  | P2 | 19.67a | 26.06a | 0.28b | 27.64a | 26.75a | 0.39b | 38.47b | 26.03a | 0.38b | 31.84b | 26.01b | 0.42b |
|  | P3 | 18.13a | 27.27a | 0.33b | 26.47a | 27.19a | 0.42b | 36.75b | 27.39a | 0.36b | 30.47b | 27.44b | 0.37b |
|  | SEm± | 0.70 | 0.31 | 0.02 | 0.50 | 0.25 | 0.02 | 0.59 | 0.23 | 0.02 | 0.39 | 0.25 | 0.03 |
|  | Pr > F | 0.03 | 0.32 | 0.00 | 0.03 | 0.69 | 0.00 | 0.00 | 0.74 | < 0.0001 | < 0.0001 | 0.67 | < 0.0001 |
|  | CD (P=0.05) | 2.82 | 2.82 | 2.82 | 2.82 | 2.82 | 2.82 | 2.82 | 2.82 | 2.82 | 2.82 | 2.82 | 2.82 |

Treatment details are provided in the Materials and Methods section.

tively reduces soil salinity, as indicated by significantly lower ECs values across all depths ($p < 0.0001$). Temperature remains stable and unaffected by the irrigation system, suggesting that neither SURDI nor SSDI significantly impacts soil thermal properties. Statistical indicators (SEm±, Pr > F, CD) confirm the reliability of the data, with significant differences ($p < 0.05$) in VWC and EC between the two systems. These findings highlight the advantages of SSDI in optimizing water use and controlling soil salinity, making it a promising solution for sustainable agriculture in arid and semi-arid regions. Farmers and researchers are encouraged to adopt SSDI to enhance irrigation efficiency and mitigate salinity-related challenges.

## 3.5 CHARACTERIZING ACTUAL EVAPOTRANSPIRATION (ETA)

### 3.5.1 Evaluation of eta during plant development stages

Observations at stage 1 (Fig. 4) reveal similar behavior in both irrigation systems initially, with slight differences emerging towards the cycle's end. Mulched plots (P1 and P3) exhibit higher ETA compared to non-mulched ones (P2) in both systems. Stage 2 (Fig.4) shows slight variations within P3 in both SURDI and SSDI. ETA is generally lower in SSDI compared to SURDI, with P3 displaying the lowest rates. Stage 3 (Fig.4) demonstrates differing behavior, with constant ETA in SURDI, higher in mulched plots, while overall, SSDI exhibits higher ETA. Stage 4 (Fig.4) displays consistent behavior across irrigation systems and plots. Key findings include higher ETA in mulched plots during phases 1 and 3, higher ETA under drip irrigation in general, but lower values in phase 2, and consistent ETA across plots in both systems during phase 4.

### 3.5.2 Relationship between actual evapotranspiration (ETA) and plant height

Figure 5 illustrates that mulched plot P1 displays the highest ETA and the largest plant size, consistent across

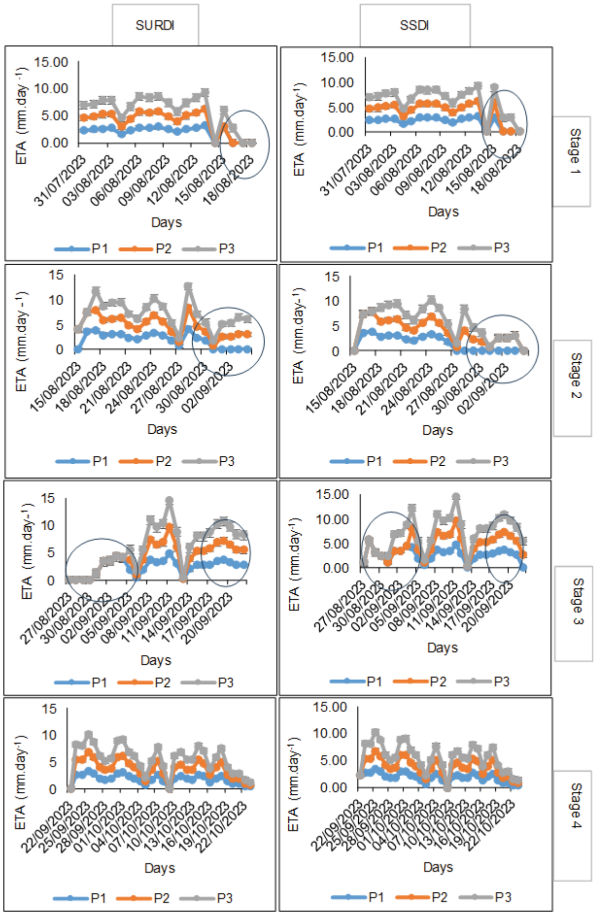


**Figure 4:** Evaluation of actual evapotranspiration (ETA) in different plots and growth stages under various irrigation systems. The bars indicate the standard errors. The treatment details are provided in the Materials and Methods section

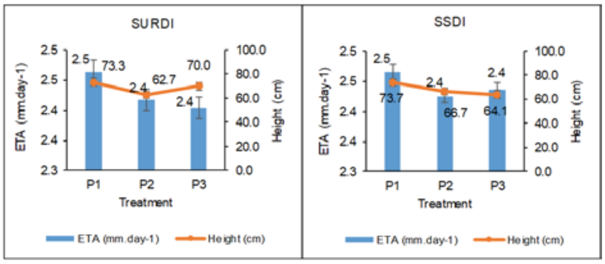


**Figure 5:** Relationship between actual evapotranspiration (ETA) and plant size in different treatments (mulch and control). The bars indicate the standard errors. The treatment details are provided in the Materials and Methods section

both surface and subsurface drip irrigation systems. The irrigation method doesn't alter this relationship. Mulching enhances plant transpiration over soil evaporation, maintaining stable soil moisture. Consequently, P1 benefits from consistent water access, fostering increased evapotranspiration and plant growth by reducing water stress. Comparing P2 and P3 under subsurface drip, despite equal ETA, plant sizes vary, with the control plot having the largest plants. This suggests deeper root systems seeking water, resulting in comparable or slightly higher transpiration rates despite smaller visible plant size.

## 3.6 IRRIGATION PARAMETERS

### 3.6.1 Irrigation frequency and total volume irrigation

Figure 6 presents the analysis of both irrigation frequency and total volume of irrigation across different plots and systems. In terms of irrigation frequency, P5 received the least surface irrigation (13 cycles), with stage 3 receiving the least water. On the other hand, P1 received the least subsurface irrigation (11 cycles), with stage 2 receiving the least water. Overall, P1 received the least irrigation across both systems. Subsurface plots generally required less water compared to surface plots, and subsurface drip irrigation demonstrated more favorable outcomes in terms of irrigation cycles. The mulch mixture was found to be more effective when used with subsurface drip, while RCW mulch performed better with surface irrigation.

Regarding the total volume of irrigation, P1 received the lowest total underground water (786.6 l), with the initial phase receiving the least amount (124.2 l). In contrast, P2 had the highest total water volume above ground (1117.8 l), with the flowering and ripening + harvesting phases recording the highest rates (414 l). This comprehensive analysis highlights the differences in water usage and efficiency between surface and subsurface drip irrigation systems.

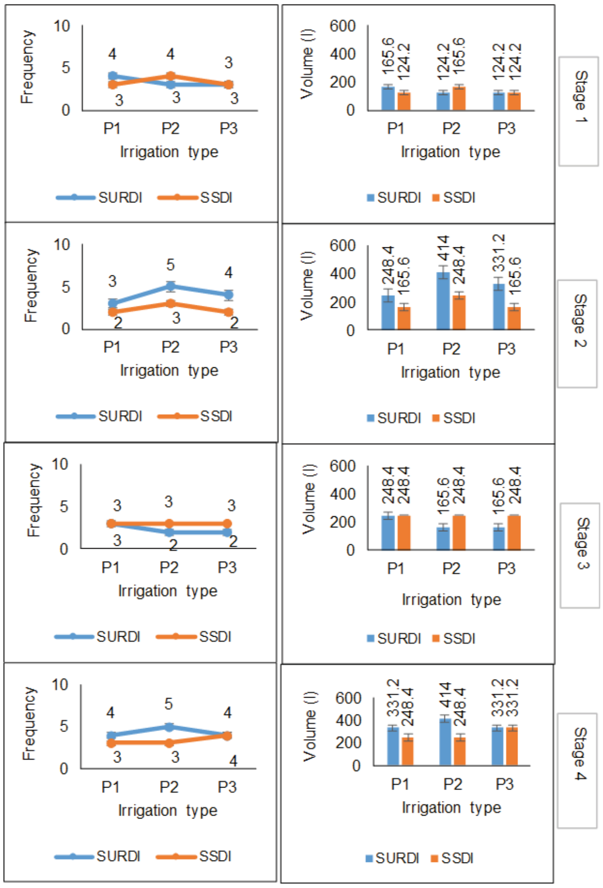


**Figure 6:** Irrigation frequency and total volume across treatments, crop development stages, and irrigation systems. The bars represent standard errors. Treatment details are provided in the materials and methods section

### 3.6.2 Water savings and efficient irrigation application (EA) in different plots following various treatments

Figure 7a demonstrates the water savings achieved in each plot under surface and subsurface drip irrigation compared to control conditions. For example, P1 achieved significant water savings of 414 $m^3$ (14.8 %) under SURDI, while P2 saved 310.5 $m^3$ (11.1 %) under SSDI. Furthermore, when compared to surface drip irrigation alone, P1 saved nearly 828 $m^3$ (29.6 %), and P3 saved 621 $m^3$ (22.2 %).

Figure 7b illustrates the efficiency of irrigation application (EA) under different systems. Under SURDI, P2 recorded the highest EA at 97.1 %, slightly outperforming

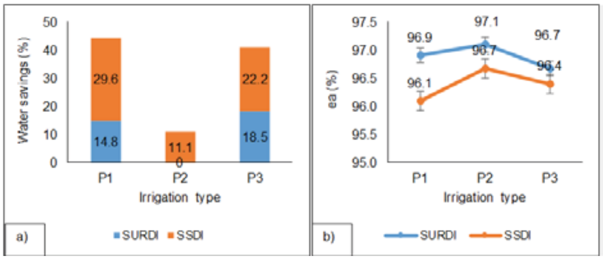


**Figure 7:** Overview of (a) water savings ($m^3$) compared to the surface control plot under surface and subsurface drip irrigation systems, and (b) water application efficiency (EA) in each plot

P1 (96.9 %) and P3 (96.7 %), with P1 showing a marginal advantage over P3. Similarly, under SSDI, P2 maintained the highest EA at 96.7 %, followed by P3 (96.4 %) and P1 (96.1 %). These findings underscore the effectiveness of specific treatments and systems in enhancing irrigation efficiency, with P2 consistently demonstrating superior performance across both systems.

## 4 DISCUSSION

The findings of this study highlight the significant potential of combining organic mulching with subsurface drip irrigation (SSDI) to enhance water conservation and improve soil conditions in tomato cultivation. These results align with previous research, which has shown that mulching and SSDI individually contribute to water savings and improved crop performance (Simsek et al., 2017; Yang et al., 2023). However, this study provides novel insights into their combined effects, particularly in the context of the Mitidja Plain, a region facing severe water scarcity.

The analysis of soil physico-chemical properties revealed significant differences between mulched and non-mulched plots. Mulched plots exhibited a slight soil acidification, with lower pH values (6.20–6.52) under SSDI compared to non-mulched plots (6.90–7.06). This acidification could be attributed to the decomposition of organic mulches, which release organic acids into the soil. While this acidification is moderate, it may influence nutrient availability and requires long-term monitoring. On the other hand, soil organic matter (OM) content remained stable, with values ranging from 1.09 % to 1.70 %, showing no significant differences between irrigation systems. This confirms that organic mulching improves soil fertility without altering its organic structure, which is crucial for the sustainability of agricultural systems (Kumar & Lal, 2012).

Mulching effectively regulated soil moisture, temperature, and electrical conductivity across all growth stages. The mixture mulch, in particular, showed superior performance in maintaining soil moisture levels, especially under SSDI. These findings are consistent with those of (Telkar et al., 2017), who reported that organic mulches reduce evaporation and improve water retention. The slight reduction in soil temperature in mulched plots further supports the role of mulch as a thermal regulator, as noted by (Gan et al., 2013). Additionally, the lower electrical conductivity in mulched plots suggests that mulching mitigates soil salinization, a critical issue in arid regions (Kumar & Lal, 2012).

Mulched plots exhibited higher plant growth and evapotranspiration (ETA) rates, particularly during the growth and fruiting stages. This is consistent with the findings of (Liasu & Achakzai, 2007), who reported that mulching enhances plant transpiration by reducing soil evaporation. The higher ETA in mulched plots under SSDI further highlights the synergistic effects of these practices in promoting crop growth and water use efficiency.

The combination of mulching and SSDI resulted in significant water savings, with the mixture mulch saving 29.6 % and RCW saving 22.2 % compared to surface irrigation. These findings are consistent with studies by (Yang et al., 2023) and (Sharma et al., 2023), who highlighted the water-saving potential of mulched drip irrigation systems. However, the slight decrease in water application efficiency (EA) in mulched plots (96.1 % for mixture, 96.4 % for RCW vs. 97.1 % for control) suggests that while mulching improves water retention, it may also create channels that lead to uneven water distribution. This underscores the need for further optimization of mulching materials and irrigation strategies.

The results of this study have important implications for sustainable agriculture in water-scarce regions. By reducing irrigation frequency and water consumption, the combination of mulching and SSDI offers a practical solution for farmers in the Mitidja Plain and similar regions.

## 5 CONCLUSION

This study demonstrates that combining organic mulching with subsurface drip irrigation (SSDI) is an effective strategy for water conservation and sustainable tomato cultivation in the Mitidja Plain. The mixture mulch, in particular, saved up to 29.6 % of water compared to surface irrigation, while also regulating soil moisture, temperature, and electrical conductivity. However, the slight soil acidification observed in mulched plots (pH 6.20–6.52) warrants attention, as it may influence nutrient availability over time. Despite this, soil organic matter (OM) remained stable (1.09 %–1.70 .%), confirming that mulching enhances soil fertility without compromising its structure. Farmers in arid regions are encouraged to adopt these practices to improve water efficiency and crop yields, but should monitor soil pH and consider amendments if necessary. Policymakers should support the adoption of these practices through subsidies, training programs, and infrastructure development, while also promoting research and innovation in conservation agriculture. Scientific researchers should focus on long-term studies to evaluate the effects of mulching and SSDI on soil health, crop productivity, and environmental sustainability, as well as explore the economic feasibility and scalability of these practices for smallholder farmers. A key limitation of this study is its short-term scope, which restricts the ability to assess long-term impacts on soil health and crop productivity. Future research should also investigate the broader applicability of these practices to other crops and regions, as well as their potential to mitigate climate change impacts through improved water and soil management. By addressing these gaps, mulching and SSDI can be further optimized to support sustainable agriculture in water-scarce environments.

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### DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

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# Water quality effects on germination of okra seed (*Abelmoschus esculentus* L.)

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**Water quality effects on germination of okra seed** (*Abelmoschus esculentus* **L.**)

**Abstract:** This study, conducted at Bakrajo Technical Institute in 2023, assessed the water quality of 24 resources using the Irrigation Water Quality Index (IWQI). Results revealed two categories: "Excellent" (19 resources, IWQI 91.4-96.5) and "Good" (5 resources, IWQI 70-90). Water in the "Excellent" category was highly suitable for irrigation, while the "Good" category was of lower quality but still acceptable. Electrical conductivity (EC) was identified as a key factor influencing the IWQI, with higher EC correlating with lower water quality. Principal Component Analysis (PCA) and Agglomerative Hierarchical Clustering (AHC) were used to classify resources based on cation, anion, and heavy metal content. A negative correlation between EC and IWQI emphasized the importance of monitoring EC for irrigation purposes. The study also found weak, non-significant correlations between pH, EC, and germination ratio, but noted that higher IWQI values and lower EC levels generally promoted better seed germination. The findings highlight the value of advanced models in water quality classification, offering essential insights for agricultural water management.

**Key words:** water quality, germination ratio, okra, irrigation water, pH, EC

**Kakovost vode vpliva na kalitev semen jedilnega osleza** (*Abelmoschus esculentus* **L.**)

**Izvleček:** V raziskavi, izvedeni na Bakrajo Technical Institute, v letu 2023 je bila ocenjena kakovost 24 vodnih virov z indeksom kakovosti vode (IWQI). Rezultati so odkrili dve kategoriji, "odlična" (19 virov, IWQI 91.4-96.5) in "dobra" (5 virov, IWQI 70-90). Voda iz virov "odlična" je bila zelo primerna za namakanje, med tem, ko je bila voda iz kategorije "dobra" slabše kakovosti vendar še sprejemljiva. Električna prevodnost (EC) je bila prepoznana kot ključni dejavnik, ki vpliva na indeks kakovosti vode (IWQI), pri čemer so večje vrednosti EC korelirale s slabšo kakovostjo vode. Analiza glavnih komponent (PCA) in hierarhično aglomerativno grozdenje (AHC) sta bila uporabljena za klasifikacijo vodnih virov glede na vsebnost kationov, anionov in težkih kovin. Ugotovljena je bila negativna korelacija med EC in IWQI, kar poudarja pomen monitoringa EC za namene namakanja. Raziskava je tudi odkrila šibko, neznačilno povezavo med pH, EC in deležem kalitve, pri čemer so večje vrednosti IWQI in manjše vrednosti EC navadno pospeševale boljšo kalitev. Ta odkritja pojasnjujejo vrednost naprednejših modelov pri klasifikaciji kakovosti vod in ponujajo bistven vpogled v kmetijsko upravljanje z vodo.

**Ključne besede:** kakovost vode, kalitev, jedilni oslez, voda za namakanje, pH, EC

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## 1 INTRODUCTION

Water quality plays a crucial role in determining both agricultural productivity and the overall health of ecosystems. It directly impacts plant growth, germination, and the subsequent development of crops, influencing both yield and quality (Yuan et al., 2024: Wakchaure et al., 2023). The importance of water in agriculture is underscored by its effect on various physiological processes within plants, such as nutrient uptake, photosynthesis, and transpiration. For crops like okra (*Abelmoschus esculentus* L.), which is widely cultivated in tropical regions, water quality can significantly alter germination rates and early vegetative growth, ultimately affecting overall crop performance. Okra is a staple vegetable in many countries, with a global cultivation area of approximately 2.5 million hectares, yielding 10.5 million tons annually (Ibrahim, 2024): Food and Agriculture Organization of the United Nations, 2018).

Seed priming, a pre-sowing treatment of seeds using water or other solutions, is an important technique to enhance germination and improve early seedling growth. When combined with high-quality irrigation water, seed priming has been shown to accelerate seedling emergence, leading to more robust and healthy plants. Water quality, defined by parameters such as salinity, pH, dissolved oxygen, and presence of contaminants, directly affects seed priming outcomes. Numerous studies have demonstrated that water with high salinity or other undesirable characteristics can hinder seedling emergence and reduce crop yields. Okra is not only valuable for its role in agriculture but also an important source of nutrition, providing essential vitamins, minerals, and dietary fiber. Given the crop's significance, understanding how different water qualities impact okra germination and seedling development is vital for optimizing agricultural practices, particularly in regions where water resources may be limited or of varying quality. Previous studies have highlighted the influence of water quality on seed germination and vegetative growth across different regions (Rima, 2021).

The aim of the present research is to assess the water quality from 25 different water resources, classifying the irrigation water based on the Sulaimani Irrigation Water Quality Index (SIWI) proposed by Marif and Esmael (2023), as well as the classification system by Todd (1966). Additionally, this study utilizes advanced statistical techniques such as Principal Component Analysis (PCA) and Cluster Analysis to classify the water resources further and investigate their impact on okra seed germination. This research will provide a comprehensive understanding of how various water qualities influence the early

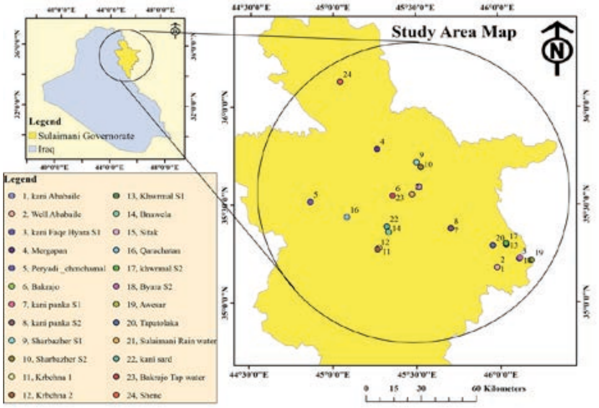

**Figure 1:** Study Area Map

**Tabel 1:** Study area description and GPS Coordination's

| No. | Location | GPS Coordinate | | Altitude |
|---|---|---|---|---|
| 1 | Kani Ababaile | 35.18235 | 45.98377 | 915 |
| 2 | Well Ababaile | 35.18235 | 45.98377 | 910 |
| 3 | Kani Faqe Byara S1 | 35.2250909 | 46.11599645 | 1087 |
| 4 | Mergapan | 35.78892 | 45.26581 | 1226.85 |
| 5 | Peryadi _chmchamal | 35.51728 | 44.86407 | 697.58 |
| 6 | Bakrajo | 35.550052 | 45.358326 | 743.36 |
| 7 | Kani panka S1 | 35.3816374 | 45.7082884 | 549 |
| 8 | Kani panka S2 | 35.3816374 | 45.7082884 | 549 |
| 9 | Sharbazher S1 | 35.720167 | 45.502614 | 827.28 |
| 10 | Sharbazher S2 | 35.695679 | 45.528932 | 846.55 |
| 11 | Krbchna 1 | 35.283103 | 45.276888 | 989 |
| 12 | Krbchna 2 | 35.27582 | 45.26 786 | 933 |
| 13 | Khwrmal S1 | 35.2971744 | 46.03963419 | 567 |
| 14 | Bnawela | 35.3647.1 | 453351.2 | 1130 |
| 15 | Sitak | 35.593677 | 45.517543 | 1126 |
| 16 | Qarachatan | 354410.3 | 450842.2 | 807 |
| 17 | Khwrmal S2 | 35.30378 | 46.03852 | 567 |
| 18 | Byara S2 | 35.23021 | 46.1212 | 1117 |
| 19 | Awesar | 35.21647 | 46.18916 | 1671 |
| 20 | Tapatolaka | 35.29402 | 45.95852 | 508 |
| 21 | Sulaimani Rain water | 35.557215 | 45.47464 | 1002 |
| 22 | Kani sard | 35.3932.4 | 453229 | 896 |
| 23 | Bakrajo Tap water | 35.550052 | 45.358326 | 743.36 |
| 24 | Shene | 36.1327 | 450425 | 570 |

stages of okra cultivation and offer insights into improving irrigation practices for better crop performance.

## 2 MATERIALS AND METHODS

### 2.1 STUDY AREA

The research was conducted at the Bakrajo Technical Institute Field, located in the Sulaimani Governorate of the Kurdistan Region, Iraq. This field site is situated in Sulaimani City, which lies at an elevation of 888 meters above sea level. The geographical coordinates of the location are approximately 35.39705° N latitude and 45.28260° E longitude, as shown in Figure 1 and detailed in Table 1. The specific location's altitude and geographic position are important factors in determining the climate, soil conditions, and overall environmental characteristics of the study area, all of which play significant roles in influencing the results and interpretation of the research. Sulaimani's climate is influenced by its semi-

**Table 2:** Water cations and anions analysis

| | | | EC | TDS | Na | Ca $^{2+}$ | $Mg^{2+}$ | $Na^+$ | $K^+$ | $CO_3^-$ | $HCO_3^-$ | $SO_4^{--}$ | $Cl^-$ | $NO_3^-$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Location | PH | dS $m^{-1}$ | mS.$cm^{-1}$ | % | mg $l^{-1}$ | | | | | | | | |
| 1 | Kani Ababaile | 7.3 | 0.58 | 368.36 | 8.0 | 1.14 | 1.083 | 0.172 | 0.021 | 0 | 1.967 | 0.219 | 0.014 | 0.013 |
| 2 | Well, ababaile | 7.1 | 0.57 | 362.83 | 9.1 | 1.28 | 1.156 | 0.234 | 0.010 | 0 | 1.901 | 0.474 | 0.199 | 0.018 |
| 3 | Kani Faqe Byara S1 | 7.1 | 0.54 | 346.58 | 8.8 | 1.17 | 1.142 | 0.179 | 0.044 | 0 | 1.820 | 0.215 | 0.243 | 0.075 |
| 4 | Mergapan | 7.1 | 1.01 | 645.67 | 9.1 | 2.20 | 1.190 | 0.335 | 0.006 | 0 | 1.059 | 1.310 | 1.029 | 0.171 |
| 5 | Shwan _chmchamal | 7.2 | 0.83 | 533.85 | 14.5 | 1.48 | 1.124 | 0.397 | 0.044 | 0 | 1.803 | 0.077 | 1.086 | 0.065 |
| 6 | Bakrajo | 7.3 | 1.02 | 649.64 | 12.8 | 1.20 | 1.125 | 0.190 | 0.151 | 0 | 1.984 | 0.216 | 0.329 | 0.021 |
| 7 | Kani panka S1 | 7.4 | 0.54 | 344.13 | 3.5 | 1.65 | 1.250 | 0.101 | 0.005 | 0 | 1.984 | 0.500 | 0.429 | 0.013 |
| 8 | Kani panka S2 | 7.3 | 0.60 | 386.82 | 4.5 | 1.30 | 1.100 | 0.108 | 0.006 | 0 | 1.746 | 0.328 | 0.191 | 0.013 |
| 9 | Shakhasur | 7.1 | 0.45 | 288.45 | 6.5 | 1.24 | 1.138 | 0.158 | 0.008 | 0 | 1.910 | 0.191 | 0.286 | 0.022 |
| 10 | Sharbazher tagaran | 7.2 | 0.44 | 284.50 | 6.6 | 1.30 | 1.243 | 0.165 | 0.014 | 0 | 1.929 | 0.654 | 0.029 | 0.032 |
| 11 | Krbchna 1 | 7.3 | 1.48 | 946.58 | 16.7 | 1.95 | 1.821 | 0.219 | 0.536 | 0 | 1.115 | 1.890 | 1.186 | 0.129 |
| 12 | Krbchna 2 | 7.2 | 0.70 | 450.67 | 3.5 | 1.35 | 1.123 | 0.087 | 0.003 | 0 | 1.931 | 0.431 | 0.091 | 0.023 |
| 13 | Khwrmal S1 | 7.1 | 1.99 | 1273.61 | 6.8 | 2.10 | 2.103 | 0.248 | 0.059 | 0 | 1.459 | 1.550 | 1.229 | 0.177 |
| 14 | Bnawela | 7.2 | 0.69 | 443.46 | 5.8 | 1.30 | 1.271 | 0.148 | 0.010 | 0 | 1.820 | 0.595 | 0.029 | 0.022 |
| 15 | Sitak | 7.1 | 0.74 | 475.24 | 8.9 | 1.12 | 1.283 | 0.220 | 0.015 | 0 | 1.900 | 0.088 | 0.629 | 0.013 |
| 16 | Qarachatan | 7.3 | 0.43 | 275.03 | 4.0 | 1.31 | 1.212 | 0.078 | 0.026 | 0 | 1.787 | 0.398 | 0.243 | 0.083 |
| 17 | Khwrmal S2 | 7.2 | 0.50 | 318.08 | 19.0 | 1.81 | 1.159 | 0.626 | 0.070 | 0 | 1.838 | 0.763 | 0.571 | 0.099 |
| 18 | Byara S2 | 7.1 | 0.52 | 329.94 | 4.7 | 1.23 | 1.010 | 0.104 | 0.006 | 0 | 1.484 | 0.475 | 0.243 | 0.025 |
| 19 | Awsar | 7.1 | 0.37 | 237.74 | 8.1 | 1.05 | 0.890 | 0.143 | 0.028 | 0 | 1.203 | 0.805 | 0.029 | 0.043 |
| 20 | Tapatolaka | 7.2 | 0.58 | 372.74 | 8.8 | 1.393 | 0.910 | 0.200 | 0.023 | 0 | 1.216 | 0.683 | 0.322 | 0.324 |
| 21 | Rain water | 7.1 | 0.34 | 218.88 | 2.2 | 1.1 | 0.920 | 0.030 | 0.014 | 0 | 1.323 | 0.353 | 0.280 | 0.028 |
| 22 | Kani sard | 7.2 | 0.32 | 204.80 | 6.0 | 0.7 | 0.430 | 0.059 | 0.013 | 0 | 0.436 | 0.446 | 0.186 | 0.048 |
| 23 | Bakrajo Tap water | 7.3 | 0.25 | 162.56 | 10.3 | 0.411 | 0.410 | 0.082 | 0.012 | 0 | 0.492 | 0.165 | 0.169 | 0.063 |
| 24 | Shene | 7.1 | 0.321 | 205.44 | 8.5 | 0.332 | 0.367 | 0.054 | 0.011 | 0 | 0.321 | 0.210 | 0.154 | 0.043 |

**Table 3:** Water heavy metals analysis

| | | Fe | Mn | Cu | Co | Ni | Zn | Cd | Cr |
|---|---|---|---|---|---|---|---|---|---|
| No | Location | mg l$^{-1}$ | | | | | | | |
| 1 | Kani Ababaile | 0.011 | 0.036 | 0.02383 | 0.00150 | 0.007 | 0.016 | 0.00090 | 0.00156 |
| 2 | Well ababaile | 0.014 | 0.020 | 0.01257 | 0.00105 | 0.004 | 0.012 | 0.00326 | 0.00152 |
| 3 | Kani Faqe Byara S1 | 0.019 | 0.033 | 0.02368 | 0.00201 | 0.005 | 0.002 | 0.00427 | 0.00300 |
| 4 | Mergapan | 0.031 | 0.046 | 0.02214 | 0.00213 | 0.005 | 0.012 | 0.00105 | 0.00190 |
| 5 | Shwan _chmchamal | 0.022 | 0.029 | 0.02334 | 0.00195 | 0.006 | 0.003 | 0.00166 | 0.00115 |
| 6 | Bakrajo | 0.025 | 0.004 | 0.03474 | 0.00184 | 0.007 | 0.006 | 0.00179 | 0.00210 |
| 7 | Kani panka S1 | 0.057 | 0.014 | 0.01360 | 0.00415 | 0.001 | 0.046 | 0.00215 | 0.00331 |
| 8 | Kani panka S2 | 0.016 | 0.032 | 0.02377 | 0.00120 | 0.004 | 0.001 | 0.00158 | 0.00393 |
| 9 | Shakhasur | 0.017 | 0.026 | 0.02286 | 0.00117 | 0.004 | 0.001 | 0.00275 | 0.00104 |
| 10 | Sharbazher tagaran | 0.014 | 0.026 | 0.01256 | 0.00111 | 0.004 | 0.03110 | 0.00569 | 0.00211 |
| 11 | Krbchna 1 | 0.045 | 0.037 | 0.02890 | 0.00166 | 0.001 | 0.00164 | 0.00244 | 0.00664 |
| 12 | Krbchna 2 | 0.023 | 0.016 | 0.03490 | 0.00156 | 0.005 | 0.00532 | 0.00530 | 0.00332 |
| 13 | Khwrmal S1 | 0.033 | 0.039 | 0.02225 | 0.00174 | 0.003 | 0.00131 | 0.00353 | 0.00091 |
| 14 | Bnawela | 0.002 | 0.023 | 0.01124 | 0.00190 | 0.002 | 0.00210 | 0.00231 | 0.00100 |
| 15 | Sitak | 0.013 | 0.039 | 0.01560 | 0.00138 | 0.037 | 0.00313 | 0.00174 | 0.00125 |
| 16 | Qarachatan | 0.010 | 0.014 | 0.01856 | 0.00112 | 0.001 | 0.00210 | 0.00171 | 0.00210 |
| 17 | Khwrmal S2 | 0.013 | 0.038 | 0.01530 | 0.00110 | 0.001 | 0.00132 | 0.00217 | 0.00132 |
| 18 | Byara S2 | 0.007 | 0.002 | 0.01260 | 0.00200 | 0.002 | 0.00142 | 0.00132 | 0.00142 |
| 19 | Awsar | 0.024 | 0.023 | 0.01220 | 0.00416 | 0.004 | 0.00673 | 0.00388 | 0.00273 |
| 20 | Tapatolaka | 0.029 | 0.034 | 0.01490 | 0.00125 | 0.006 | 0.00598 | 0.00129 | 0.00218 |
| 21 | Rain water | 0.022 | 0.033 | 0.02230 | 0.00118 | 0.003 | 0.00031 | 0.00140 | 0.00031 |
| 22 | Kani sard | 0.019 | 0.035 | 0.01410 | 0.00108 | 0.003 | 0.00104 | 0.00138 | 0.00144 |
| 23 | Bakrajo Tap water | 0.003 | 0.039 | 0.01530 | 0.00196 | 0.002 | 0.00091 | 0.00126 | 0.00091 |
| 24 | Shene | 0.016 | 0.034 | 0.01250 | 0.00110 | 0.002 | 0.00560 | 0.00135 | 0.05600 |

arid conditions, with distinct seasonal variations that can impact agricultural practices and ecological studies. These geographic and climatic features, combined with the region's unique environmental factors, provide valuable insights into the scope of the research, highlighting the importance of this field site in understanding local agricultural systems, climate adaptation, and ecological sustainability in the context of the Kurdistan Region.

## 2.2 WATER SAMPLING COLLECTING

The water sampling process for evaluating the water quality index in this research followed these steps:

First step: Water samples were collected from 24 different locations or wells (labeled 1 to 24) in the study area. These samples were analyzed and compared based on their electrical conductivity (EC) and pH values.

Second step: A total of 24 water samples were selected for the study.

Third step: The water from the 24 selected wells or water resources was tested for physicochemical properties and some heavy metals. Additionally, 24 of these samples were used for germination experiment with okra seeds. The samples were classified according to various methods outlined in Tables 2 and 3. Each water sample was collected in a 1.5-liter container for physicochemical analysis and was also used for germinating okra seeds in the laboratory at Bakrajo Technical Institute, maintained at a temperature of 25 °C.

## 2.3 WATER ANALYSIS AND COMPUTING THE IRRIGATION WATER QUALITY INDEX (IWQI).

The water analysis was conducted as follows:

### 2.3.1 pH and electrical conductivity (EC) measurement

A portable pH meter (Hanna pH H 98107) was used to measure the pH of the water sample, and an EC meter (HI981311) was employed to determine the electrical conductivity, following the standard methods described in the APHA (1998) guidelines for water quality analysis.

### 2.3.2 Cation and anion analysis

The concentrations of various cations calcium ($Ca^{2+}$), magnesium ($Mg^{2+}$), potassium ($K^{+}$), and sodium ($Na^{+}$)) and anions carbonate ($CO_3^{2-}$, bicarbonate ($HCO_3^{-}$), sulfate ($SO_4^{2-}$), nitrate ($NO_3^{-}$), and chloride ($Cl^{-}$)) were measured to assess the chemical composition of the water.

### 2.3.3 Heavy metal concentration analysis

The concentrations of heavy metals, including cobalt ($Co^{2+}$), copper ($Cu^{2+}$), iron ($Fe^{2+}$), manganese ($Mn^{2+}$), zinc ($Zn^{2+}$), chromium ($Cr^{2+}$), cadmium ($Cd^{2+}$), and nickel ($Ni^{2+}$), were determined using a Shimadzu ICP-9820 inductively coupled plasma atomic emission spectrometer (ICP-AES), made in Japan, which is capable of detecting trace amounts of these metals in the water. The results from these analyses are summarized in Table 3.

## 2.4 GERMINATION RATIO CALCULATIONS

A germination assessment is often the most reliable method to evaluate whether a seed is ready for planting. For this particular test, local varieties of okra seeds were used. Germination in these seeds typically begins after approximately 4 days, provided that the seeds are kept under optimal temperature and humidity conditions. The germination rate can be calculated using a specific equation, which helps to quantify the proportion of seeds that successfully sprout. The data collected during this experiment is summarized in Table 4, where the calculated germination ratio is recorded for each observation. This test is essential for determining the viability of the seeds before planting.

$$Germination\ ratio\% = \frac{Number\ of\ germination\ seeds}{Number\ of\ all\ seeds} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (1)$$

## 2.5 IRRIGATION WATER QUALITY CALCULATION

### 2.5.1 Irrigation water quality calculation according to modified SIWi 2023 (Marif and Esmael, 2023)

**Table 4:** Germination ratio of the study area

| No | Location | Germination Ratio % |
|---|---|---|
| 1 | Kani Ababaile | 88.3 |
| 2 | Well ababaile | 90 |
| 3 | Kani Faqe Byara S1 | 86.5 |
| 4 | Mergapan | 87.0 |
| 5 | Shwan _chmchamal | 85 |
| 6 | Bakrajo | 88 |
| 7 | Kani panka S1 | 81.7 |
| 8 | Kani panka S2 | 80 |
| 9 | Shakhasur | 83.0 |
| 10 | Sharbazher tagaran | 85.0 |
| 11 | Krbchna 1 | 86.67 |
| 12 | Krbchna 2 | 88.3 |
| 13 | Khwrmal S1 | 81.0 |
| 14 | Bnawela | 82.0 |
| 15 | Sitak | 80.0 |
| 16 | Qarachatan | 83.3 |
| 17 | Khwrmal S2 | 79 |
| 18 | Byara S2 | 83.0 |
| 19 | Awesar | 91.7 |
| 20 | Tapatolaka | 88.3 |
| 21 | Rain water | 92 |
| 22 | Kani sard | 83.3 |
| 23 | Bakrajo Tap water | 88.3 |
| 24 | Shene | 87.0 |

The irrigation water quality calculation, according to the modified SIWi 2023 as modified by (Marif and Esmael, 2023), involves a detailed analysis of several water quality parameters, including salinity, pH, sodium, chloride, and other essential factors that affect crop growth and soil health. This method incorporates updated thresholds and classifications to assess the suitability of water for irrigation, ensuring its compatibility with specific soil types and crop needs. In this context, the quality of irrigation water is categorized based on these criteria, which are then cross-referenced with the standards listed in Table 5 of the modified SIWi 2023. This table provides a classification system that ranks water quality into different categories, helping to determine whether the water is suitable for different agricultural purposes, these calculations and classifications are critical for managing

**Table 5** Irrigation water quality calculation according to modified SIWi 2023(Marif and Esmael, 2023)

| No | Location | IWQI | Classes |
|---|---|---|---|
| 1 | kani Ababaile | 92.1 | Excellent |
| 2 | Well Ababaile | 93.8 | Excellent |
| 3 | kani Faqe Byara S1 | 93.1 | Excellent |
| 4 | Mergapan | 90.9 | Good |
| 5 | Shwan _chmchamal | 91.4 | Excellent |
| 6 | Bakrajo | 89.7 | Good |
| 7 | kani panka S1 | 84.4 | Good |
| 8 | kani panka S2 | 92.4 | Excellent |
| 9 | Shakhasur | 94.0 | Excellent |
| 10 | sharbazher tagaran | 93.3 | Excellent |
| 11 | Krbchna 1 | 86.4 | Good |
| 12 | Krbchna 2 | 91.5 | Excellent |
| 13 | Khwrmal S1 | 85.5 | Good |
| 14 | Bnawela | 93.8 | Excellent |
| 15 | Sitak | 92.3 | Excellent |
| 16 | Qarachatan | 94.7 | Excellent |
| 17 | khwrmal S2 | 92.5 | Excellent |
| 18 | Byara S2 | 96.0 | Excellent |
| 19 | Awesar | 94.9 | Excellent |
| 20 | Tapatolaka | 92.5 | Excellent |
| 21 | Rain water | 95.0 | Excellent |
| 22 | kani sard | 96.5 | Excellent |
| 23 | Bakrajo Tap water | 96.3 | Excellent |
| 24 | Shene | 91.4 | Excellent |

**Table 6:** Irrigation water quality classification according (Todd,1966)

| No | Location | Classes |
|---|---|---|
| 1 | kani ababaile | Suitable for Irrigation |
| 2 | well ababaile | Suitable for Irrigation |
| 3 | kani faqe byara s1 | Suitable for Irrigation |
| 4 | Mergapan | Suitable for Irrigation |
| 5 | shwan _chmchamal | Suitable for Irrigation |
| 6 | Bakrajo | Suitable for Irrigation |
| 7 | kani panka s1 | Suitable for Irrigation |
| 8 | kani panka s2 | Suitable for Irrigation |
| 9 | Shakhasur | Suitable for Irrigation |
| 10 | sharbazher tagaran | Suitable for Irrigation |
| 11 | krbchna 1 | Suitable for Irrigation |
| 12 | krbchna 2 | Suitable for Irrigation |
| 13 | khwrmal s1 | Suitable for Irrigation |
| 14 | Bnawela | Suitable for Irrigation |
| 15 | Sitak | Suitable for Irrigation |
| 16 | Qarachatan | Suitable for Irrigation |
| 17 | khwrmal s2 | Suitable for Irrigation |
| 18 | byara s2 | Suitable for Irrigation |
| 19 | Awesar | Suitable for Irrigation |
| 20 | Tapatolaka | Suitable for Irrigation |
| 21 | rain water | Suitable for Irrigation |
| 22 | kani sard | Suitable for Irrigation |
| 23 | bakrajo tap water | Suitable for Irrigation |
| 24 | Shene | Suitable for Irrigation |

irrigation practices, preventing soil degradation, and maximizing crop yield.

### 2.6 DATA ANALYSIS

The data were analyzed using XLSTAT 2019.2.2.59614, a comprehensive statistical software tool. Principal Component Analysis (PCA) was applied to reduce the dimensionality of the data while retaining its most significant features, helping to uncover patterns and structure in complex datasets. Agglomerative Hierarchical Clustering (AHC) was also utilized to group similar data points based on their characteristics, allowing for the identification of distinct clusters or patterns within the data. Additionally, correlation analysis was performed to examine the relationships between various variables, helping to understand the strength and direction of their associations. Together, these analytical methods provided a thorough exploration of the data, generating valuable insights for further interpretation and decision-making.

## 3 RESULTS AND DISCUSSIONS

### 3.1 CLASSIFICATION OF WATER RESOURCES ACCORDING TO MODIFIED SIWI (MARIF & ESMAEL, 2023)

The study results classify the water resources into two distinct quality categories based on the Irrigation Water Quality Index (IWQI). Nineteen of the resources were categorized as "Excellent" for irrigation, demonstrating IWQI values above 90, with individual values ranging from 91.4 to 96.5, indicating that these water sources are highly suitable for agricultural use. These resources, spread across various locations (1, 2, 3, 5, 8, 9, 10, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, and 24), exhibit optimal quality for irrigation, as reflected in their consistently high IWQI scores. In contrast, five water sources (locations 4, 6, 7, 11, and 13) were classified as "Good," with IWQI values between 70 and 90, indicating that they are still suitable for irrigation but with slightly lower quality compared to the "Excellent" category. These lower IWQI scores correspond to higher electrical conductivity (EC) values, as seen in the data, with EC emerging as a key factor influencing water quality. Specifically, the study found that water resources with lower EC values tended to have higher IWQI scores, while those with higher EC values showed lower IWQI values. This pattern is consistent with findings from Dhaoui et al. (2023) and Benaafi et al. (2024), which also identified a strong correlation between EC levels and IWQI, underlining the critical role of EC in evaluating the suitability of water for irrigation purposes. This classification provides valuable insights into the variability of water quality across different locations and highlights the importance of monitoring EC as a predictor of irrigation water suitability.

### 3.2 CLASSIFICATION OF WATER RESOURCES ACCORDING TO ACCORDING (TODD,1966)

Water classification results indicated that all water resources (1, 2, 3, 4, ... to 24) were initially considered suitable for irrigation, with no variation observed across the resources. However, upon comparing the findings to the classification framework proposed by Todd (1966), it was evident that recent studies have led to significant changes in water classification due to the inclusion of additional parameters and updated models for calculating the Irrigation Water Quality Index (IWQI). These changes in classification can be attributed to the introduction of more comprehensive criteria for assessing water quality, which in turn influenced the classification of water resources. The results presented in Table 6 align with recent research conducted by Mahammad and Islam (2024), as well as Laaraj et al. (2024), which highlights the evolving nature of water classification models as they adapt to new data and methodologies. This shift underscores the importance of using more advanced and detailed models to accurately reflect the quality and suitability of water resources for irrigation purposes, as these models provide a more nuanced understanding of water quality and its potential impact on agricultural practices.

### 3.3 CLASSIFICATION OF WATER RESOURCES ACCORDING TO CATIONS AND ANIONS USING PRINCIPAL'S COMPONENT ANALYSIS PCA

The classification of 24 water resources based on their cation and anion content using Principal Component Analysis (PCA) reveals significant insights into water quality variations across different locations. As shown in Figure 2, the water resources are divided into seven distinct classes, each represented by a unique shape. Class 1, depicted by a left arrow shape, corresponds to water resource number 13. Class 2, represented by a north arrow shape, includes water resource number 11. Class 3, with a circle shape, includes water resources 4 and 17. Class 4, represented by a pentagon shape, is assigned to water resource number 20. Class 5, with a cylinder shape, corresponds to water location number 19. Class 6, represented by a triangle shape, groups water resources 22, 23, and 24. Finally, Class 7, depicted by a square shape, includes a broad range of water locations, including 1, 2, 3, 5, 6, 7, 8, 9, 10, 12, 14, 15, 16, 18, and 21. PCA analysis indicates that Factor 1 (F1) accounts for 48.17 % of the variation in water classification, while Factor 2 (F2) explains 14.53 %, together making up 62.70 % of the total variance. These findings are consistent with recent studies by Hammoumi et al. (2024), Arıman et al. (2024), and Ali et al. (2024), which similarly applied PCA to classify water resources, highlighting the effectiveness of this statistical approach in understanding water quality and variability across different regions. This analysis emphasizes the substantial influence of the first factor (F1), suggesting that cation-anion concentrations are the primary determinants in classifying water resources.

### 3.4 CLASSIFICATION OF WATER RESOURCES ACCORDING TO CATIONS AND ANIONS USING AGGLOMERATIVE HIERARCHICAL CLUSTERING (AHC)

The classification of water resources according to their cation and anion concentrations using Agglomerative Hierarchical Clustering (AHC) provides a structured approach to grouping water samples based on their chemical composition. As shown in Table 7 and Figure 3, the AHC analysis divided the water resources into five distinct classes. Class 1, which includes 16 water resources (locations 1, 2, 3, 7, 8, 9, 10, 16, 17, 18, 19, 20, 21, 22,

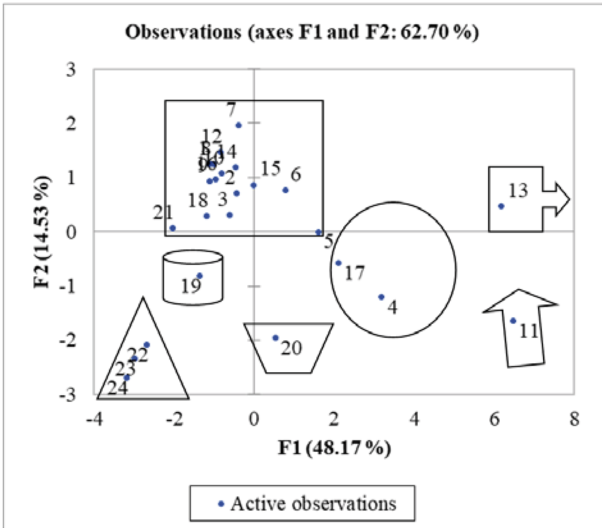

**Figure 2:** Classification of water resources according to cations and anions using principal's component analysis PCA

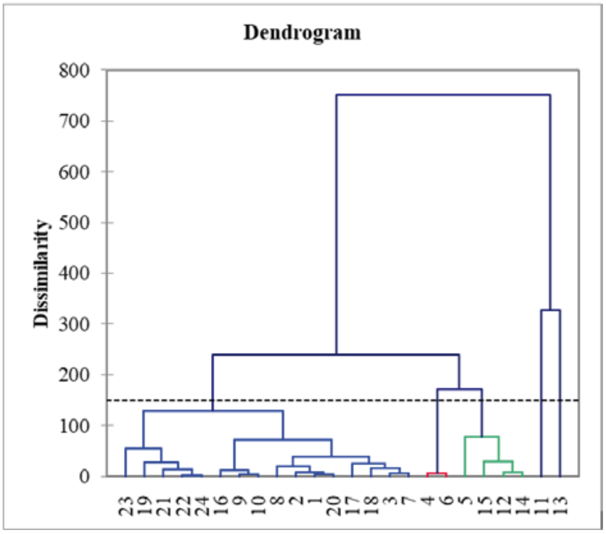

**Figure 3:** Classification of water resources according to cations and anions using agglomerative hierarchical clustering (AHC)

23, and 24), exhibited relatively low variation in terms of ion concentration. In contrast, Classes 2 and 3 each included fewer resources, with Class 2 comprising only locations 4 and 6, and Class 3 encompassing resources 5, 12, 14, and 15. Both Class 4 and Class 5 contained only one water resource each, located at positions 11 and 13, respectively. The lower variation observed in Classes 1 and 3 suggests a more homogeneous ionic composition, whereas the higher variation in Classes 4 and 5 can be attributed to the greater influence of electrical conductivity (EC), which likely caused more significant differentiation in the clustering. The impact of EC on water classification is well-documented in previous studies (Marif and Esmail, 2023; Mishra et al., 2023; Djaafri et al., 2024), supporting the findings of this research. This clustering technique thus underscores the complex interplay of cations, anions, and EC in determining the quality and characteristics of water resources.

**Table 7:** Classification of water resources according to cations and anions using agglomerative hierarchical clustering (AHC)

| Classes | Class 1 | Class 2 | Class 3 | Class 4 | Class 5 |
|---|---|---|---|---|---|
| Number of classes | 16 | 2 | 4 | 1 | 1 |
| Water Resources or Locations | 1, 2, 3, 7, 8, 9, 10, 16, 17, 18, 19, 20, 21, 22, 23 and 24 | 4 and 6 | 5, 12, 14, and 15 | 11 | 13 |

## 3.5 CLASSIFICATION OF WATER RESOURCES ACCORDING TO HEAVY METAL CONTENTS USING PRINCIPAL'S COMPONENT ANALYSIS PCA

The classification of water resources based on heavy metal content using Principal Component Analysis (PCA) offers a comprehensive approach to understanding the variability and quality of water bodies in relation to pollutants. In this study, 24 water resources were categorized into six distinct classes according to their heavy metal profiles, as demonstrated in Figure 4. These classes are represented by different geometric shapes, each corresponding to specific water resource locations. Class 1, marked by a circle, includes water resources at locations 6 and 12, while Class 2, represented by a north arrow, encompasses resources at locations 5, 3, 8, 9, 11, 13, 16, and 21. Class 3, shown with a rectangular shape, includes resources at locations 1, 2, 4, 14, 15, 17, 18, 20, 22, and 23. Class 4, marked by a pentagon, represents locations 10 and 19, while Class 5, identified by a triangle, corresponds to location 7. Finally, Class 6, represented by a cylinder shape, is composed of resource 24. The PCA results, as depicted in Figure 2, show that the first factor (F1) accounts for 26.68% of the total variability affecting water classification, while the second factor (F2) contributes 16.59%. Together, these factors explain 43.28 % of the variance in the water classification, highlighting the significant influence of heavy metal content on water quality. These findings align with previous research by Hammoumi et al. (2024) and Arıman et al. (2024), validating the effectiveness of PCA in classifying water

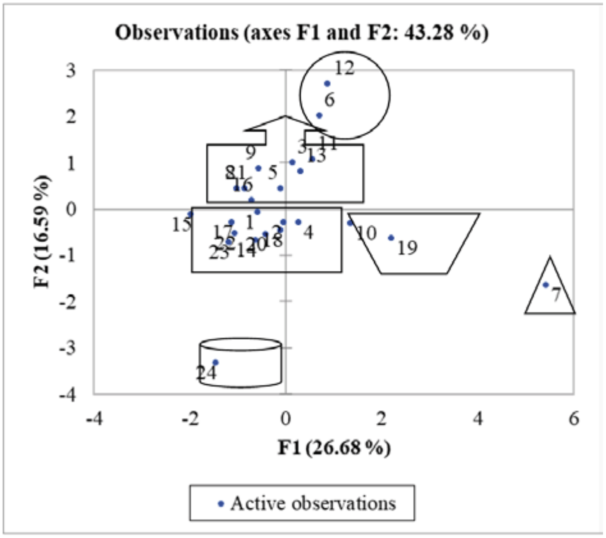

**Figure 4:** Classification of water resources according to heavy metal contents using principal's component analysis PCA

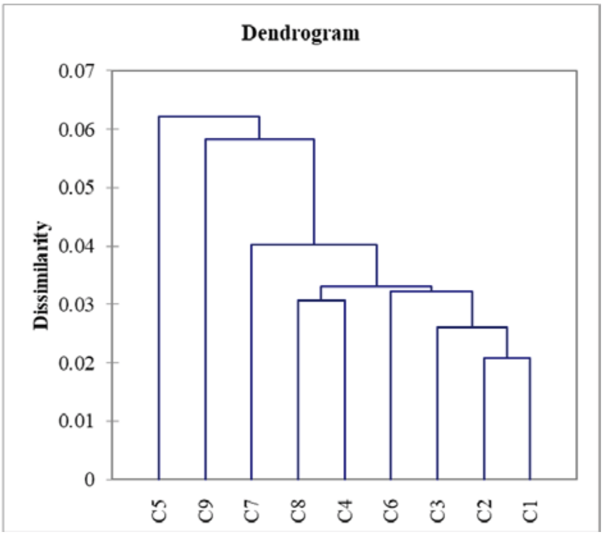

**Figure 5:** Classification of water resources according to heavy metal contents using agglomerative hierarchical clustering (AHC)

resources based on their contamination levels. This analysis underscores the importance of understanding the principal factors contributing to water quality variations and their implications for environmental monitoring and management.

## 3.6 CLASSIFICATION OF WATER RESOURCES ACCORDING TO HEAVY METAL CONTENTS USING AGGLOMERATIVE HIERARCHICAL CLUSTERING (AHC)

The classification of water resources based on heavy metal contents, utilizing Agglomerative Hierarchical Clustering (AHC), groups the water sources into nine distinct classes, as shown in Table 8 and Figure 5. Class 1, which includes water sources 1, 3, 5, 8, 9, 17, 21, 22, and 23, represents the largest cluster with nine water resources. Class 2, containing four locations (2, 14, 16, and 19), and Class 3, which includes locations 4, 11, 13, and 20, each contain four water resources. Class 4, consisting of only two locations (6 and 12), demonstrates a more limited variation in heavy metal content. In contrast, Classes 5, 6, 7, 8, and 9 are more distinct, each containing a single water resource—specifically, water sources 7, 10, 15, 18, and 24, respectively. The classification shows low variability in heavy metal concentrations for Class 1 and Class 3, which are likely influenced by similar environmental or anthropogenic factors, while a greater degree of variability is observed in Classes 5 through 9. This greater variation can be attributed to factors such as electrical conductivity (EC), which significantly impacts water quality classification by affecting the solubility and mobility of heavy metals in aquatic environments. These findings align with previous studies by Marif and Esmail (2023) and Mohsine et al. (2023), confirming that clustering based on heavy metal content provides a reliable method for assessing water quality, revealing both regional differences and the influence of chemical processes on water resources.

## 3.7 CORRELATION COEFFICIENT BETWEEN PH, EC, IWQI AND GERMINATION RATIO

The correlation analysis presented in Tables 4 and 9 reveals intriguing insights into the relationships between

**Table 8:** Classification of water resources according to cluster analysis of heavy metal contents

| Classes | Class 1 | Class 2 | Class 3 | Class 4 | Class 5 | Class 6 | Class 7 | Class 8 | Class 9 |
|---|---|---|---|---|---|---|---|---|---|
| Number of classes | 9 | 4 | 4 | 2 | 1 | 1 | 1 | 1 | 1 |
| Water resources or Locations | 1, 3, 5, 8, 9, 17, 21, 22, and 23 | 2, 14, 16, and 19 | 4, 11, 13, and 20 | 6 and 12 | 7 | 10 | 15 | 18 | 24 |

pH, electrical conductivity (EC), irrigation water quality index (IWQI), and germination ratio. Notably, the study found a positive but non-significant relationship between the germination ratio and IWQI, with a correlation coefficient (r) of 0.218, suggesting that although IWQI may have some influence on germination, its effect is weak and not statistically meaningful (Lal et al., 2024). Additionally, a similarly weak but negative correlation between pH and the germination ratio (-0.157), and EC and the germination ratio (-0.196), indicates that as pH and EC increase, there is a slight decrease in the germination ratio. However, these correlations are also non-significant, implying that other factors may be influencing the germination rate more strongly. A more robust and significant negative correlation was observed between EC and IWQI, with an r value of -0.727, which implies a strong inverse relationship. This suggests that as EC (a measure of salinity) increases, the overall quality of irrigation water as indicated by the IWQI declines. This negative significant relationship is critical because high EC typically denotes saline water, which can have detrimental effects on plant growth and germination. In summary, while the correlations between pH, EC, and germination ratio are weak and non-significant, the strong negative relationship between EC and IWQI underscores the importance of monitoring EC levels in maintaining irrigation water quality and optimizing germination success.

### 3.8 EFFECTS OF WATER QUALITY ON GERMINATION RATIO

The data presented in Tables 10 and 4 provides valuable insights into the relationship between water quality and the germination ratio of okra seeds. The maximum germination ratio recorded was 92 %, observed under excellent water quality conditions with an electrical conductivity (EC) of 0.34 dS m$^{-1}$ and a standard deviation of 3.72. In contrast, the minimum germination ratio of 79 % was observed at a slightly higher EC of 0.5 dS m$^{-1}$, accompanied by the same standard deviation, indicating that even small variations in EC can influence seed germination. This decline in germination is likely due to the combined effects of EC and pH on water quality, which have been shown to impact the osmotic potential and the uptake of water by seeds, ultimately affecting their ability to sprout. Higher EC levels can cause osmotic stress, making it more difficult for the seed to absorb sufficient water, which is crucial for the germination process. This aligns with the findings of Seymen et al. (2023), Singh et al. (2023), and Nautiyal et al. (2023), who demonstrated that water quality parameters such as EC and pH are critical factors in seed viability and germination. In this study, the mean values for pH, EC, and IWQI (Irrigation Water Quality Index) were 7.19, 0.66 dS m$^{-1}$, and 92.28, respectively, indicating that maintaining water quality within optimal ranges is essential for maximizing germination rates and ensuring successful crop establishment.

**Table 9:** Correlation between pH, EC, IWQI and germination ratio

| Variables | pH | EC dS m$^{-1}$ | IWQI | Germination Ratio |
|---|---|---|---|---|
| pH | 1 | | | |
| EC dS m$^{-1}$ | -0.012 | 1 | | |
| IWQI | -0.322 | -0.727 | 1 | |
| Germination Ratio | -0.157 | -0.196 | 0.218 | 1 |

**Table 10:** Summary of water quality effect on germination ratio

| Variable | Observations | Minimum | Maximum | Mean | Std. deviation |
|---|---|---|---|---|---|
| PH | 24 | 7.1 | 7.4 | 7.19 | 0.01 |
| EC | 24 | 0.26 | 1.2 | 0.66 | 0.40 |
| IWQI | 24 | 84.43 | 96.55 | 92.28 | 3.18 |
| Germination Ratio | 24 | 79 | 92 | 85.36 | 3.72 |

### 3.9 RELATION BETWEEN GERMINATION RATIO AND EC DS M$^{-1}$

Figure 6 illustrates the inverse relationship between the germination ratio and electrical conductivity (EC, measured in dS m$^{-1}$), showing a significant negative correlation. As EC increases, the germination ratio decreases dramatically, which can be attributed to the detrimental effects of high salinity on seed germination. Electrical conductivity in soil is a direct measure of its salinity, and when the EC is high, it indicates that the soil solution has a higher concentration of dissolved salts. These salts can create an osmotic pressure that reduces the availability of water to seeds, impairing their ability to absorb water and thus hindering the germination process. This negative relationship is supported by the correlation coefficient (r = -0.07), which highlights the weak yet consistent inverse trend between EC and germination. These findings align with those of Hossain et al. (2023) and Naher et al. (2024), who also observed similar impacts of salinity on seed germination. High salinity can induce

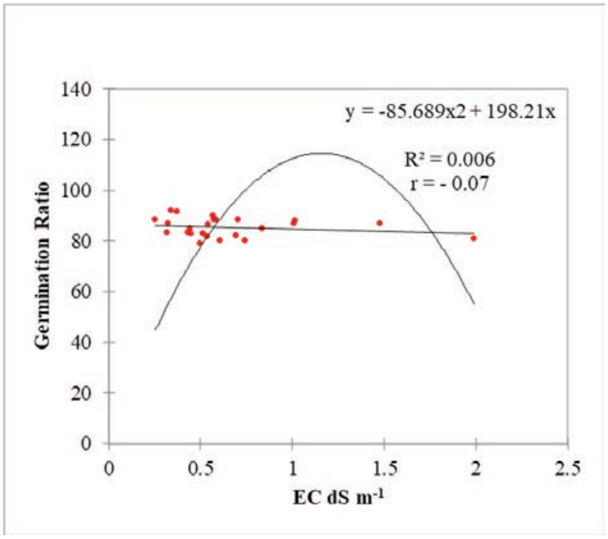

**Figure 6:** Relation between germination ratio and EC dS $m^{-1}$

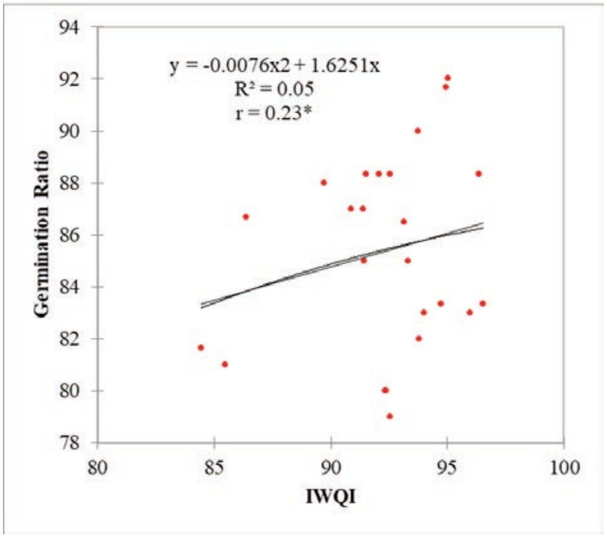

**Figure 7:** Relation between germination ratio and IWQI

physiological stress in seeds, affecting enzyme activity, cell membrane integrity, and nutrient uptake, all of which are essential for successful germination and early seedling development.

### 3.10 RELATION BETWEEN GERMINATION RATIO AND IRRIGATION WATER QUALITY INDEX (IWQI)

Figure 7 illustrates a positive correlation between germination ratio and the Irrigation Water Quality Index (IWQI), demonstrating that as the germination ratio increases, so does the IWQI, indicating better water quality. This relationship suggests that higher IWQI values, which reflect cleaner, more suitable water for irrigation, promote better seed germination and overall plant growth. Conversely, a decrease in IWQI corresponds to lower germination ratios, highlighting the detrimental effects of poor water quality on seedling establishment. This finding is consistent with previous studies, such as those by Marif and Esmail (2023) and Mezlini et al. (2024), which emphasize the significant role of water quality in agricultural productivity. Poor-quality irrigation water, characterized by high salinity, contamination, or imbalanced nutrient content, can impede seedling growth by creating osmotic stress, altering nutrient availability, or introducing toxic compounds, all of which negatively affect germination. Thus, maintaining a high IWQI is critical for ensuring successful crop establishment and maximizing agricultural yields.

## 4 CONCLUSIONS

The study of irrigation water quality (IWQ) from various locations or water sources reveals significant variations in water quality parameters, with conductivity being one of the most influential factors. Electrical conductivity (EC) serves as an indicator of the ion concentration in water, reflecting the level of dissolved salts or minerals. In agricultural practices, water with high EC values can lead to salinity stress, which adversely affects plant growth and seed germination. Conversely, water with lower EC values, indicating fewer dissolved salts, tends to be more favorable for seedling establishment and plant growth. The observed variations in water quality across different sources can thus be attributed to the local environmental conditions, such as soil composition and water sources, which influence the ion concentrations and overall quality of irrigation water.

Furthermore, the study demonstrates a positive correlation between irrigation water quality and the germination rate of okra seeds. As the IWQ index increases, particularly in relation to lower EC values, the germination ratio of okra seeds also increases. This suggests that water with lower salt concentrations provides a more conducive environment for seed sprouting, likely due to reduced osmotic stress and enhanced water uptake. High-quality irrigation water, characterized by low EC values, ensures that seeds receive the optimal conditions necessary for proper germination, leading to higher success rates in seedling emergence. This finding underscores the importance of maintaining high-quality water for irrigation to promote healthy crop development and improve agricultural productivity. Therefore, the results highlight that managing water salinity, by monitoring and controlling EC levels, is crucial for optimizing crop germination and growth.

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# *Brassica* oilseed crops in Japan: cultivation, consumption, and cultivars

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*Brassica* **oilseed crops in Japan: cultivation, consumption, and cultivars**

**Abstract:** *Brassica* oilseed crops are the third most important source of edible vegetative oils in the world. Among these crops, the cultivation history of *B. rapa* goes back to very ancient times in Japan. Its cultivation area expanded substantially in the 17th century and *Brassica* oil was used as fuel for lamps and cooking oil. *Brassica napus* L. ssp. *napus* was introduced into Japan mainly as an oil crop in the late 19th century, after which it gradually replaced the heirloom *B. rapa* L. ssp. *oleifera* (DC.) Metzg. cultivars used in the production of edible oil. The rapeseed (*B. rapa* and *B. napus*) cultivation area in Japan reached its peak in the 1950s and then decreased rapidly due to increase of imports of inexpensive oilseed crops. In recent years, however, domestic cultivation of *B. napus* has started to increase again. Japanese people consume rapeseed oil well and consider it to be a healthy oil with low levels of saturated fatty acids. This article aims to provide the information about the history, current state, problems, and prospects of rapeseed cultivation in Japan. The paper also describes an overview of agronomic characteristics of representative Japanese cultivars as well as cultural practices.

**Key words:** *Brassica napus*; *Brassica rapa*; breeding; cultivation history; cultural practices; vegetative oil

**Oljarice iz rodu** *Brassica* **na Japonskem: gojenje, uporaba in sorte**

**Izvleček:** Oljarice iz rodu *Brassica* so tretji najpomembnejši vir jedilnega rastlinskega olja v svetovnem merilu. Med njimi se je gojenje oljne repice (*B. rapa*) na Japponskem pričelo že v pradavnini. Površine njenega gojenja so se močno povečale v 17. stoletju, ko se je njeno olje uporabljalo kot gorivo za svetiljke in kuhanje. Oljna ogrščica (*Brassica napus* L. ssp. *napus*) je bila uvedena na Japonsko pretežno kot oljarica v poznem 19. stoletju in je nadomestila sorte oljne repice (*B. rapa* L.ssp. *oleifera* (DC.) Metzg.), ki so se uporabljale za pridelavo jedilnega olja. Površina gojenja obeh vrst je na Japonskem dosegla višek v petdesetih letih prejšnega stoletja in potem hitro upadla zaradi povečanega uvoza poceni jedilnega olja iz teh vrst. V zadnjih letih se domača pridelava oljne ogrščice spet povečuje, ker ga prebivalstvo rado uporablja kot zdravo jedilno olje z majhno vsebnostjo nasičenih maščobnih kislin. Namen članka je podati informacije o zgodovini, sedanjem stanju, problemih in bodočem gojenju oljne ogrščice na Japonskem. Članek daje tudi pregled agronomskih lastnosti japonskih sort kot tudi o načinih gojenja.

**Ključne besede:** *Brassica napus, Brassica rapa,* žlahtnjenje, zgodovina gojenja, tehnike gojenja, rastlinsko olje

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## 1 INTRODUCTION

*Brassica* crop species are of particular importance in agricultural production within the Brassicaceae family and have undergone extensive domestication (Gulden et al., 2008; OECD, 2016; McAlvay et al., 2021). Among these, *B. napus* L. ssp. *napus* (oilseed rape), *B. rapa* L. (syn. *B. campestris* L., turnip rape), *B. juncea* (L.) Czern. et Coss. (Indian mustard), and *B. carinata* Braun (Ethiopian mustard) form the oilseed group, and in Japan, rapeseed chiefly refers to *B. napus* and *B. rapa* (MRC Institute for Environment & Health, 1997; Zając et al., 2016; Kawasaki et al., 2022). As a side note, *B. juncea* is grown as an oilseed crop mainly in India as well as in some regions of China, and the cultivation of *B. carinata* is basically limited to Northeast Africa (Zając et al., 2016). In this paper, "rapeseed" is used to collectively designate *B. napus* and *B. rapa*.

*Brassica rapa* was one of the first *Brassica* species to be domesticated (Guo et al., 2014). Mizushima and Tsunoda (1969) hypothesized that *B. rapa* originated in cold uplands near Turkey from where it migrated into the flatlands of Europe and Siberia. However, the exact center of origin of *B. rapa* has been debated; proposed centers of origin include Europe (Zhao et al., 2005), Central Asia (Ignatov et al., 2008; Qi et al., 2017), and East Asia (Song et al., 1988; Zhao et al., 2005). *Brassica napus* ($2n = 38$) is an amphidiploid with an AACC genome, and evolved through the spontaneous hybridization between *B. rapa* L. ($2n = 20$) with an AA genome and *B. oleracea* L. (cabbage/kale; $2n = 18$) with a CC genome (OECD, 2016; Quezada-Martinez et al., 2021). It seems likely that the southwest European Mediterranean region, where the wild forms of the two parental species exist, is one of the places where this hybridization event occurred (Prakash and Hinata, 1980; Rahman, 2013).

Rapeseed oil, which is low in saturated fatty acids (e.g., less than 4 % palmitic acid), with relatively high levels of oleic acid (55-68 %) and linolenic acid (7-10 %), is thought to offer health benefits (MRC Institute for Environment & Health, 1997; Fujimura-Ito et al., 2011; Huang et al., 2015). Linolenic acid has valuable nutritional functions in humans (Nakui and Mikami, 2024), and oleic acid provides thermal stability, making rapeseed oil desirable for cooking oil. High oleic-acid oil also tastes better (Cartea et al., 2019). Nevertheless, *Brassica* seeds generally contain more than 40 % erucic acid in oil, and more than 100 µmoles of glucosinolates per gram of the oil-free meal (Rahman, 2013). Oil with high erucic acid content has anti-nutritional properties and is considered unsuitable as a source of food for humans (Rahman, 2013; Cartea et al., 2019). Additionally, glucosinolates are considered nutritionally undesirable since the presence of glucosinolates limits the use of this protein-rich meal in animal feed (Rahman, 2013). Intensive breeding efforts have been made to develop elite cultivars with low erucic acid, low glucosinolate content, and increased yields. The resulting improved crop is called "double-low" rapeseed or "canola" and has become one of the most important oilseed crops in the world (Quezada-Martinez et al., 2021). Recently, rapeseed oil has drawn Japanese consumers' attention because of its healthful properties (Japan Oilseed Processors Association, 2024). Among vegetable oils, the food-use consumption of rapeseed oil is the highest in Japan, followed by palm oil and soybean oil (Yagi et al., 2023).

In the present article, we review literature to provide an up-to-date summary in relation to the history, current state, problems, and perspectives of rapeseed cultivation in Japan. The paper also focuses on the agronomic characteristics of representative Japanese cultivars and cultural practices.

## 2 HISTORY OF RAPESEED CULTIVATION IN JAPAN

### 2.1 *Brassica rapa* L.

*Brassica rapa* exists in various forms such as oilseed, leafy (e.g., Chinese cabbage, mizuna) and root (e.g., turnip) type vegetables, and feed for livestock (e.g., fodder turnip) (Guo et al., 2014). The history of *B. rapa* cultivation goes back to very ancient times in Japan (Nishizawa et al., 2010). The initial use of the *B. rapa* crops introduced into Japan seems to have been as leafy vegetables rather than as an oilseed crop (Nishizawa et al., 2010). It remains unclear as to when oil production from *B. rapa* seeds began in Japan, but *Brassica* oil is believed to have been already utilized as lamp fuel in the end of the 16th century (Japan Oilseed Processors Association, 2015; Tokyo Oil Inquiry House Market, 2016). In those days, the oil market was monopolized by a group of merchants and the oil extracted from oil perilla (*Perilla frutescens* (L.) Britt. var. *frutescens*) was the major source of lamp fuel in the country (Japan Oilseed Processors Association, 2015; Nakui and Mikami, 2023). Perilla seed oil was also widely used as drying oil for waterproofing paper umbrellas, polishing or coating wood, and in the manufacture of lacquer wares (Nitta et al., 2003; Yamanaka Aburaten, 2023).

After the 17th century, rapeseed was recognized to have high economic value; *B. rapa* seeds were crushed more readily for oil in comparison with oil perilla seeds, and the light produced by burning rapeseed oil was brighter than that of perilla oil (Yamanaka Aburaten, 2023). Rapeseed oil production was officially encouraged and the cultivation areas of *B. rapa* then expanded substantially (Tokyo Oil Inquiry House Market, 2016). We would like to add that *B. rapa* was also grown for cooking oil alongside sesame, as western-influenced fried foods including tempura (deep-fried fish and vegetables) and hiryouzu (deep-fried tofu fritter) became popular from the 17th to the 19th centuries (Ohashi, 2007).

### 2.2 *Brassica napus* L. ssp. *napus*

Unlike *B. rapa*, the cultivation of *B. napus* in Japan is fairly recent, with a European cultivar being first introduced into the southwestern area of the country in 1878 (Nishizawa et al., 2010; Kawasaki et al., 2022). This cultivar was known as the name of 'Daichosen' and was characterized by late maturity and high plant height. Afterwards, 'Hamburg' was introduced from Germany into the northernmost island, Hokkaido in 1886 (Nishizawa et al., 2010).

'Hamburg' was a very late maturing cultivar that was well adapted to a cold climate (Nishizawa et al., 2010). At the beginning of the 20th century, two *Brassica* oilseed crops, viz., *B. napus* and heirloom *B. rapa* were cultivated throughout the country, but the acreage of *B. napus* cultivars gradually increased because these cultivars exhibited higher seed and oil yield as well as resistance to a major disease, *Sclerotinia* stem rot (Shiga, 1970). In a 5-year average from 1926 to 1930, rapeseed (*B. napus* and *B. rapa*) was harvested from ca. 72,000 ha with a total yield of ca. 73,000 metric tons annually in Japan (Fig. 1).

Planned rapeseed improvement by Japanese public research institutions commenced in the 1930s (Chen et al., 2017). The methods of breeding rapeseed were actually pure line selection and interspecific hybridization. The interspecific crossing was aimed at introducing early maturity from *B. rapa* into *B. napus* cultivars. Public breeding program also included the introgression of moisture resistance from *B. rapa* into *B. napus* (Shiga, 1970). This was because a number of farmers in southwestern Japan grew rapeseed after the rice harvest in the paddy fields with the purpose of raising farm incomes (Nishizawa et al., 2010). Breeding and intensive selection for desirable agronomic traits led to the generation of various *B. napus* cultivars, some of which gained wide acceptance commercially. Consequently, *B. napus* cultivars replaced conventional *B. rapa* (Nishizawa et al., 2010).

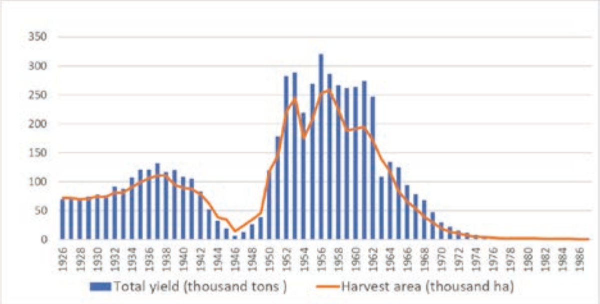

**Figure 1:** Production and cultivation area of rapeseed (*B. napus* and *B. rapa*) from 1926 to 1987 in Japan. Source: MAFF (1963, 2024a)

Domestic rapeseed production ranged between ca. 77,000 tons and 132,000 tons in the 1930s, but production decreased sharply during World War II (Fig. 1). Following the war, the rapeseed acreage increased again and the largest total production of 320,000 tons was achieved in 1956. Thereafter, the cultivation areas and production of rapeseed decreased rapidly (Fig. 1). There are several reasons for this, of which the most important one must be that the import volume of inexpensive oilseed crops including rapeseed and soybean jumped sharply due to the import liberalization (Kawasaki et al., 2021).

## 3 CURRENT STATE OF DOMESTIC OILSEED RAPE CULTIVATION

### 3.1 PRODUCTION

Japan's oilseed rape production nearly disappeared from the 1980s to the 2000s. In the 2000s, the average annual consumption of oilseed rape in Japan was ca. 2.2 million tons (Saiwai Shobo, 2011), and the domestic demand was almost entirely met by imports mostly from Canada and Australia (MAFF, 2024a). Apart from being used as edible oil, rapeseed oil has been utilized for several purposes in industry, including chemical manufacturing, paint, cosmetics, and pharmaceuticals (USDA, 2023). Almost all the rapeseed imported as edible oil material was of canola quality [low erucic acid (less than 2 % in the oil), low glucosinolate content (less than 30 μmoles per gram in the oil-free meal)] (CODEX, 2001; OECD, 2001).

In recent years, however, oilseed rape acreage and production in Japan have started to increase again. As shown in Table 1, average production per year from 2018 to 2023 inclusive was ca. 3,600 tons. The limited recovery in oilseed rape production is undoubtedly due to the subsidies granted by the Japanese government to farmers who grow oilseed rape and other crops (e.g., wheat, barley, soybean), with the aim of encouraging stable farm management (Kawasaki et al., 2022). Moreover, this ten-

**Table 1:** Production and import of oilseed rape from 2018 to 2023 in Japan

| | 2018 | 2019 | 2020 | 2021 | 2022 | 2023 |
|---|---|---|---|---|---|---|
| Harvest area (ha) | 1,920 | 1,900 | 1,830 | 1,640 | 1,740 | 1,740 |
| Total yield (t) | 3,120 | 4,130 | 3,580 | 3,230 | 3,680 | 3,680 |
| Import volume (t) | 2,337,350 | 2,359,212 | 2,252,378 | 2,342,162 | 2,100,818 | 2,021,557 |

Source: MAFF (2024a), Statistics Bureau of Japan (2024)

dency is partly owing to the choice of health-conscious people to use more domestic produces, in order to ensure safety of the foods they consume on a daily basis.

## 3.2 CULTIVAR DEVELOPMENT

Since the 1980s, oilseed-rape breeding programs in Japan have focused on the development of cultivars with low erucic acid, low glucosinolate content, and increased yields, because there were no domestic cultivars which did meet the canola standards (Kawasaki et al., 2022). Attempts have also been made to breed the genotypes with improved winter hardiness and high resistance to diseases, and a number of cultivars have been released. Agronomic characteristics of representative Japanese cultivars are described below.

### 3.2.1 'Kizakinonatane'

'Kizakinonatane' was produced by crossing 'Tohoku 72' as the seed parent with 'Rapora' as the pollen parent , and registered as an erucic acid-free cultivar in 1992 (Okuyama et al., 1994). This cross was made with the aim of combining the high yield potential of 'Tohoku 72' and the zero-erucic acid trait of 'Rapora', and 'Kizakinonatane' was developed using pedigree method. It is a high-yielding and medium-maturing cultivar with resistance to lodging and *Sclerotinia* stem rot. The cultivar also exhibits tolerance to cold and snow damage, and is recommended for northern Japan where the climate has long, cold winter.

### 3.2.2 'Nanashikibu'

This cultivar was derived from a cross of 'Morishi 148' x 'Oominatane', and registered as 'Nanashikibu' in 2002 (Kato et al., 2005). It is characterized by high seed-yield potential, zero erucic acid content, good lodging resistance, and moderate resistance to *Sclerotinia* stem rot. 'Nanashikibu' is a relatively early-flowering cultivar and its seeds can be harvested before the rainy season (commonly from beginning of June to mid-July in Japan with the exception of Hokkaido where there is no rainy season) begins; this cultivar has been predominantly grown in temperate central and southwestern Japan.

### 3.2.3 'Kirariboshi'

'Kirariboshi' originated from a cross between 'Morikei 188' and 'Karat', and in 2004, it was registered as the first Japanese high-yielding, double-low cultivar (Ishida et al., 2007). This cultivar is medium-maturing, and resistant to lodging and *Sclerotinia* stem rot. Tolerance to cold and snow damage is relatively high.

### 3.2.4 'Kirakiraginga'

'Kirakiraginga' is a high-yielding cultivar free of erucic acid and with low glucosinolate content (Honda et al., 2017). It resulted from hybridization of 'CASCADE' with 'Kirariboshi', and was released in 2014. The cultivar is useful both for the edible oil and livestock feed markets. 'Kirakiraginga' has resistance to lodging and is tolerant to cold and snow damage; it grows well in cold northern climates.

### 3.2.5 'CR Nanashikibu'

This clubroot resistant (CR) cultivar was produced by introducing two CR loci, *Crr1* and *Crr2*, from Chinese cabbage (*B. rapa*) into 'Nanashikibu' via DNA marker-assisted selection and backcrossing (Kawasaki et al., 2021, 2022). It was registered in 2022. 'CR Nanashikibu' has desirable characteristics similar to those of 'Nanashikibu', e.g., zero erucic acid content, lodging resistance, and moderate resistance to *Sclerotinia* stem rot.

### 3.2.6 'Penokanoshizuku'

The cultivar originated from a cross between 'OZ028-2' and 'Kizakinonatane' (Kawasaki et al., 2022). The release of 'Penokanoshizuku' in 2019 provided the industry and growers with high-yielding and double-low rapeseed. This cultivar exhibits resistance to *Sclerotinia* stem rot. It is also tolerant to cold and snow damage, and adapted to cold northern climates.

## 4 CULTURAL PRACTICES

Oilseed rape requires well-drained, loose fertile soil with a pH of 5.8-6.5 (Mori, 2009). In Japan, feral rapeseed populations are found throughout the country (Nishizawa et al., 2010; Chen et al., 2020). Oilseed rape can outcross to the feral rapeseed if they are in close proximity and there is synchrony of flowering. In order to ensure the seed purity, the commercial seeds should be multiplied not by growers themselves but by certified seed-production agencies. Time of seeding plays an important role for successful harvest and good yield of the crop. In northern Japan, the optimum time of sowing is between mid-August and mid-September, while in warmer central and southwestern Japan, sowing normally occurs from late September through early November (MAFF, 2024b). The seed sowing depth should be around 2-3 cm for best germination. Seeding rates vary by seeding methods, genotypes, seeding date and moisture status of the soil. Seeding is mostly done using drill seeder at 300-600 g per 10 a in the country. Oilseed rape competes poorly with weeds during the early stage of growth, making early weed control essential. An effective weed control program includes both cultural and herbicidal approaches. Post emergence herbicides can be used to control grassy weeds such as annual bluegrass, leading to minimizing yield decreases.

In Japan, oilseed rape is cultivated as an autumn-planted winter crop that needs vernalization (winter chilling) to flower. Flowering usually begins around late February (southwestern Japan) or late April (northern Japan) of the second growing season and continues for three to four weeks. Oilseed rape is ready to harvest when the plant is well dried and there are no more green pods (Mori, 2009). Harvesting too early results in too many immature seeds and low-quality seed oil, whereas late harvesting can cause excessive shattering and yield loss. Traditional mechanical pressing method is commonly applied to edible-oil extraction from rapeseed in Japan (MAFF, 2024b). Pressing oil retains the nutritional value, natural golden color, and inherent flavor of the oil. Additionally, mechanical pressing does not need solvents and chemicals, making it a simple and safe process. However, this method usually results in low oil yield.

For optimum crop yields and disease control, growers should not plant oilseed rape in the same field more often than once in three years. It is also important to avoid planting oilseed rape after cruciferous crops. In Japan, oilseed rape is commonly rotated with soybean, wheat, common buckwheat, and sugar beet (Mori, 2009).

## 5 DISEASE AND PEST PROBLEMS

*Sclerotinia* stem rot has been the most widespread and serious disease of rapeseed in Japan. It is caused by the soil-borne fungus *Sclerotinia sclerotiorum* (Libert) de Bary and has resulted in considerable yield losses. In the fields having a history of *Sclerotinia* infestations, crop rotation and selection of resistant cultivars (see preceding section) should be followed. Other major diseases that can cause crop losses include clubroot (caused by *Plasmodiophora brassicae* Woronin) and root knot [caused by *Meloidogyne arenaria* (Neal, 1889) Chitwood]. Clubroot has a wide host range and can attack almost all *Brassica* crops. The disease is difficult to manage by disease control practices such as crop rotation, increasing soil pH, and use of agricultural chemicals (Kawasaki et al., 2021). Thus, the most effective way to control this disease is by sowing resistant cultivars; as mentioned above, Japanese breeders released a promising clubroot-resistant cultivar ‘CR Nanashikibu’ carrying two resistant genes, *Crr1* and *Crr2* (Kawasaki et al., 2021).

Rapeseed insect pests include cabbage armyworm (*Mamestra brassicae* (L., 1758), small cabbage white (*Pieris rapae crucivora* Boisduval, 1836), and cabbage aphid *(Brevicoryne brassicae* (L., 1758)). It is important to protect the plants via insecticide application or other integrated pest management strategies. In addition, timely scouting is recommended for early detection and best management of insect pests.

## 6 CONCLUDING REMARKS

In the early 2010s, the Japanese government decided to promote the cultivation of oilseed rape by allocating subsidies, and then the rapeseed production area has increased gradually (Kawasaki et al., 2022). However, Japan still only produces ca. 0.2 % of what it consumes (see Table 1), and hence it is becoming essential to make the country self-sufficient even a little by increasing oilseed rape yields via the development of high-yielding cultivars. According to the statistics available (MAFF, 2024a; USDA, 2024), the rapeseed yield (2,110 kg ha$^{-1}$ in 2023) in Japan is considerably lower than that (3,310 kg ha$^{-1}$ in 2023) in the European Union where hybrid cultivars predominate. Hybrids seem likely to be the best method of enhancing yields (Ma et al., 2000; Gehringer et al., 2007). Lefort-Buson et al., (1987) previously reported that the

F1 hybrids between Japanese and European winter-type rapeseed cultivars exhibited high levels of yield heterosis.

Meanwhile, all Japanese rapeseed cultivars currently grown are derived from pedigree selection (Kawasaki et al., 2022). In the country, therefore, oilseed rape has yet to benefit from enhanced yield through hybrid breeding. Cytoplasmic male sterility (CMS) among cruciferous crops has been broadly investigated to implement it as a low-cost, efficient and reliable system for the production of F1 rapeseed hybrids (Kamiński, 2013; Yamagishi and Bhat, 2014). Several hybrid breeding systems are available at present, of which the Ogu-INRA CMS system is widely used in Europe and North America, and the Pol CMS system is preferentially utilized in China (Li et al., 2022). Dominant genic male sterility may be also usable for hybrid breeding in the crop (Li et al., 2022). Efforts are required to establish hybrid breeding program of oilseed rape using appropriate male sterility systems in Japan.

Moreover, winter damage remains a significant barrier for oilseed rape cultivation in the main producing area, Hokkaido where the crop is usually grown under thick snow cover from winter to spring. Thick snow cover acts as a heat insulator between the atmosphere and the soil, suppressing soil freezing; snow cover protects the plants from low temperature stress in winter (Shimoda et al., 2023). During recent years, oilseed rape has often suffered from a severe freezing injury in eastern Hokkaido, due to the late onset of snow cover and less snow fall. Because of that, some farmers are reducing rapeseed plantings and diverting more land under other crops which give them better return. There is an urgent need to identify cold-resistant germplasm and to develop oilseed rape cultivars with robust cold hardiness.

In Japan, more than 600 rapeseed germplasm accessions, including Japanese landraces and cultivars as well as overseas accessions, are maintained at the Genetic Resources Center of the National Agriculture and Food Research Organization (Chen et al., 2017). This collection is expected to harbor huge genetic variation and provide opportunities for trait improvement. Nowadays, genomics takes an important position in genetics and plant breeding. Through the use of whole-genome sequencing technology and diverse molecular markers, the genomics studies in *B. napus* have achieved great progress (Quezada-Martinez et al., 2021; Gu et al., 2024). The availability of various genomic resources allows the breeders to better understand agronomic traits at the genetic level, which will facilitate the generation of novel oilseed rape cultivars with improved traits.

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**Conflicts of interest**

The authors declare no conflict of interest.

**Data availability**

Original data are available from the corresponding author upon reasonable request.

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# Nove genomske tehnike za natančno žlahtnjenje rastlin

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**Nove genomske tehnike za natančno žlahtnjenje rastlin**

**Izvleček:** Podnebne spremembe, nove bolezni ter omejeni viri vplivajo na trajnostno pridelavo zadostne količine kakovostne hrane, kar zahteva stalno prilagajanje sort kmetijskih rastlin obstoječim in prihodnjim sistemom pridelave. Sorte kmetijskih rastlin morajo biti v prihodnosti odpornejše na biotske in abiotske dejavnike, prav tako pa morajo prejeto energijo in hranila učinkoviteje pretvoriti v kakovostne sestavine, pri čemer je veliko prostora za inovacije. Z razvojem rastlinske biotehnologije in genetike, uporabo znanj pridobljenih z visokozmogljivimi omskimi pristopi in naprednimi statističnimi analizami ter z metodami strojnega učenja se je močno pospešilo doseganje specifičnih žlahtniteljskih ciljev, ki lahko pomembno prispevajo k trajnostnemu kmetijstvu in varni preskrbi s hrano. S pregledom znanstvene literature pojasnjujemo, kako delujejo nove genomske tehnike, v čem se razlikujejo od splošno sprejetih metod žlahtnjenja in kakšne prednosti imajo v primerjavi s klasičnimi tehnikami žlahtnjenja rastlin. V nadaljevanju obravnavamo, katere rastline in njihove lastnosti so bile spremenjene z novimi tehnikami, kako je oblikovana ustrezna zakonodaja, tudi z vidika gensko spremenjenih organizmov, ter povzemamo zaključke javne razprave o njihovi potrebi in možnih tveganjih.

**Ključne besede:** preurejanje genoma, nove genomske tehnike, NGT, žlahtnjenje rastlin, agronomske lastnosti, GSO, zakonska ureditev

**New genomic techniques for precision plant breeding**

**Abstract:** Climate change, new diseases and resource constraints are affecting the sustainable production of sufficient and quality food, requiring the continuous adaptation of plant varieties to existing and future production systems. Crop varieties of the future must be more resilient to biotic and abiotic stresses, and able to convert the energy and nutrients they receive into food more efficiently, presenting significant opportunities for innovation. The development of plant biotechnology and genetics, the application of knowledge gained from high-throughput omics approaches, and advanced statistical analysis and machine learning methods have greatly accelerated the identification of specific breeding targets that can make an important contribution to sustainable agriculture and food security. We review the scientific literature to explain how new genomic techniques work, how they differ from commonly accepted breeding methods and what advantages they have over traditional techniques. We then discuss which plants, and their traits have been modified, the intricacies of relevant legislation, particularly from the perspective of GMOs, and summarise the conclusions drawn from the public debate concerning the necessity and potential risks of these techniques.

**Key words:** genome editing, new genomic techniques, NGT, plant breeding, agronomic traits, GMO, legislation

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## 1 OD UDOMAČITVE DO MOLEKULARNEGA ŽLAHTNJENJA RASTLIN

Žlahtnjenje rastlin je starodavna dejavnost, ki sovpada z začetki kmetijstva okrog 12.000 let pred našim štetjem. Nedavni genetski in arheobotanični dokazi kažejo na obstoj vsaj petih neodvisnih središč udomačitve gojenih rastlin: riža na Kitajskem, žit in stročnic na Bližnjem vzhodu, koruze, fižola in buč v Srednji Ameriki, krompirja in kvinoje v Andih ter koruze, manioke, buč, sladkega krompirja in maranta v jugozahodni Amazoniji (Iriarte in sod., 2020; G. Larson in sod., 2014). Ljudje so na svojih poljih pridelovali rastline in izbirali lastnosti kot so večji plodovi, semena in boljši okus. Semena najboljših rastlin so shranjevali za setev v naslednji rastni sezoni. Takšne poskusne selekcijske metode so bile predhodnice zgodnjih postopkov žlahtnjenja rastlin (Slika 1).

S pojavom Mendelove genetike v 19. stoletju ter boljšim razumevanjem principov dedovanja so se razvili sistematični pristopi križanja rastlin, ki so vključevali rodovnike in linijsko selekcijo ter se osredotočali na predvidljivo dedovanje lastnosti (Louwaars, 2018; Mittelsten Scheid, 2022). Pomemben mejnik v žlahtnjenju rastlin v začetku 20. stoletja je dosegel George Shull s poskusi križanja linij koruze in opisom pojava heteroze. Ugotovil je, da sta pridelek in vitalnost pri inbridiranih linijah koruze na splošno slabša, medtem ko je pridelek hibridov (njihovih potomcev) pogosto presegal pridelek inbridiranih starševskih linij. Poleg tega so imeli hibridi zelo zaželeno lastnost, bili so uniformni (Crow, 1998).Velik uspeh hibridov koruze na trgu je prispeval k hitremu prenosu tehnologije tudi na druge kmetijske rastline, predvsem tujeprašne poljščine.

Temelj sedanjemu molekularnemu žlahtnjenju rastlin zagotovo predstavlja odkritje strukture DNK (Watson in Crick, 1953), ki je omogočilo pripisovanje genotipa fenotipu ter oblikovanje centralne dogme molekularne biologije. Poleg osnovnih molekularnih tehnologij razvitih v osemdesetih in devetdesetih letih prejšnjega

**Slika 1:** Zgodovinski mejniki v žlahtnjenju rastlin
**Figure 1:** Historical milestones of plant breeding

stoletja, kot so kloniranje, določanje nukleotidnega zaporedja (sekvenciranje) in tehnologija rekombinantne DNK (Berg in Mertz, 2010; Duffin in Mach, 2022; Shendure in sod., 2017), je bil za sodobno žlahtnjenje rastlin ključen tudi napredek na področju rastlinske biotehnologije, ki je zagotovil gojenje rastlinskih celic in tkiv *in vitro* ter regeneracijo in klonsko razmnoževanje novih rastlin (Thorpe, 2007).

Osnovno načelo žlahtnjenja rastlin je ne glede na vrsto pridelka vedno enako: temelji na genetski raznolikosti, ki je nastala naravno z mutacijami in spolnim razmnoževanjem, ali pa jo je z različnimi metodami ustvaril človek. Hermann J. Muller je prvi opisal induciranje mutacij z rentgenskimi žarki pri vinskih mušicah (Muller, 1928). Njegove ugotovitve je kmalu zatem potrdil Lewis J. Stadler z obsevanjem semen ječmena z rentgenskimi žarki ali obdelavo z radijem (Stadler, 1928). Tretirane rastline so imele recesivne mutacije povezane s klorofilom, ki naj bi bile posledica kromosomske aberacije, ali uničenja gena. Povzročanje mutacij z ionizirajočim sevanjem ali kemijskimi mutageni je še vedno pomembno orodje za žlahtnjenje rastlin, kot tudi za rastlinsko genetiko in funkcijsko genomiko (Jankowicz-Cieslak in sod., 2016; Sikora in sod., 2012). Čeprav so z mutacijskim žlahtnjenjem v zadnjih osemdesetih letih pridobili že več kot 3.000 novih sort (Maluszynski, 2001), se na regulatorni ravni ne razlikuje med rastlinami, ki so nove lastnosti pridobile z mutacijskim žlahtnjenjem in rastlinami, ki so nove lastnosti pridobile s klasičnim žlahtnjenjem s križanji; potrebni niso nobeni postopki presoje tveganja in odobritve pred dajanjem na trg, prav tako zanje ni potrebno posebno označevanje.

Mutacije pogosto ne prinašajo nobenih koristi, vendar se občasno lahko pojavijo nove in dragocene lastnosti. S klasičnim genskim inženiringom je mogoče gene za želene lastnosti vnesti v genom rastlinskih celic. Naključno vgrajena zaporedja DNK so lahko natančna kopija zaporedij, ki so že prisotna v vrsti, lahko pa izvirajo iz druge vrste. Tako so leta 1983 v celice tobaka prvič vnesli bakterijska gena, ki sta se pod rastlinskim promotorjem funkcionalno izrazila in tobaku zagotovila odpornost na antibiotik kanamicin (Herrera-Estrella in sod., 1983). Produkt klasičnega genskega inženiringa so gensko spremenjeni organizmi (GSO) oziroma gensko spremenjene (GS) rastline ali transgene rastline. Sledili so prvi poljski poskusi s transgenimi rastlinami odpornimi na herbicide leta 1986, medtem ko je bila prva vrsta transgene rastline, ki je prišla na trg leta 1994, paradižnik z upočasnjenim zorenjem (Herrera-Estrella in sod., 2005). Po podatkih iz leta 2023 je bilo z GS rastlinami zasajenih 206,3 milijona hektarjev, kar predstavlja 13,4 % celotne svetovne kmetijske površine, pri čimer prevladujejo GS soja, koruza in bombaž z odpornostjo na herbicide in insekte (X. Cheng in sod., 2024).

Od objave prvega rastlinskega genoma navadnega repnjakovca (*Arabidopsis thaliana* (L.) Heynh) leta 2000 (Kaul in sod., 2000) je bilo javno razkritih več kot 700 genomov zelenih rastlin Viridiplantae (O'Leary in sod., 2024), pri čemer prevladujejo gojene rastline (Marks in sod., 2021). Dostopnost genomskih podatkov za asociacijske raziskave po celotnem genomu (angl. genome-wide association study, GWAS) in analize pangenoma ter razvoj novih tehnik sekvenciranja, kot je na primer genotipizacija s sekvenciranjem (angl. genotyping by sequencing, GBS), so privedli do odkritja velikega števila molekularnih označevalcev (angl. marker) ter lokusov kvantitativnih lastnosti (angl. quantitative trait loci, QTL), ki so povezani z različnimi agronomsko zaželenimi lastnostmi (He in sod., 2014; Nadeem in sod., 2018).

V zadnjem času se v žlahtniteljskih programih, poleg selekcije na podlagi genomov, uporabljajo visoko zmogljivi pristopi fenotipizacije, metabolomike, proteomike in transkriptomike, ki so v kombinaciji z naprednimi statističnimi orodji in metodami strojnega učenja močno pospešili realizacijo specifičnih žlahtniteljskih ciljev (Sun in sod., 2024). Z najnovejšim sklopom orodij za preurejanje genoma (angl. genome editing) je mogoče izvesti usmerjene spremembe na točno določenem mestu v genomu rastlinske vrste in v relativno kratkem času izboljšati izbrane lastnosti (Pixley in sod., 2022). Poleg teh orodij so v nadaljevanju predstavljene posamezne nove genomske tehnike, mehanizmi njihovega delovanja in predlagano zakonsko (ne)urejanje področja v EU.

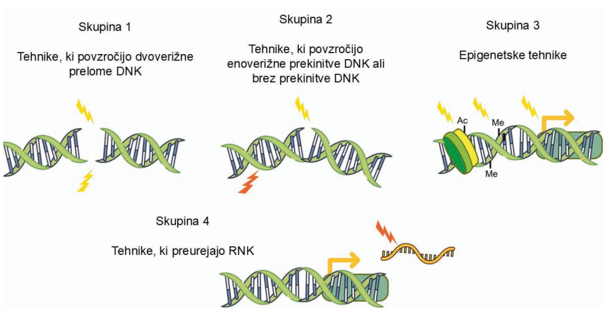
## 2 KAJ SO NOVE GENOMSKE TEHNIKE?

Nove genomske tehnike (NGT) so po definiciji „tehnike, ki lahko spremenijo genski material organizma in so bile razvite po objavi Direktive EU 2001/18/ES o namernem sproščanju gensko spremenjenih organizmov v okolje " (Direktiva - 2001/18 - EN - EUR-Lex, b. d.). Z uporabo nekaterih NGT lahko na ravni zaporedja DNK povzročijo komaj zaznavne spremembe v obliki variacij posameznih nukleotidov (zamenjave (substitucije), kratke vstavitve (insercije) ali izbrise (delecije)), s pomočjo drugih pa lahko uvedejo daljše vstavitve ali izbrise odsekov DNK. Te spremembe lahko ustvarijo celični popravljalni mehanizmi DNK, ki so nagnjeni k naključnim napakam, ali pa nastanejo namerno s kopiranjem iz donorske matrice DNK. Le-ta lahko vsebuje i) homologna zaporedja, ki so prisotna pri drugem posamezniku iste vrste (npr. divjem tipu rastline, ki je prednik gojene rastline) ali ii) heterologna zaporedja iz drugih vrst, ki se v naravi ne morejo križati (transgene rastline). Kopirana zaporedja se nato mestno specifično integrirajo v genom ciljnega organizma, pri čemer nastane produkt, podoben tistemu, ki ga dobimo s klasičnim genskim inženirstvom. Na ravni zaporedja RNK lahko z NGT uvedemo spremembe, kot so variacije posameznih nukleotidov, modulacije spajanja pre-mRNK in razgradnje RNK, vključno z nekodirajočimi RNK, ki imajo regulatorne učinke na druge gene (Broothaerts in sod., 2021).

NGT vključujejo naslednje štiri skupine tehnik (Slika 2), kot so jih razložili (Broothaerts in sod., 2021):

- tehnike, ki povzročijo dvoverižne prelome DNK, vključno z RNK usmerjanimi nukleazami (kot so na primer Cas9 in Cpf1) povezanimi z gručami enakomerno prekinjenih kratkih palindromskih ponovitev (angl. clustered regularly interspaced short palindromic repeats, CRISPR), nukleaze TAL efektorjev (angl. transcription activator-like effector nucleases, TALEN), nukleaze z motivi cinkovih prstov (angl, zinc finger nucleases, ZNF) ter ‘prenašalne’ endonukleaze (angl. homing nuclease);
- tehnike, ki povzročijo enoverižne prekinitve DNK ali ne vključujejo prekinitve DNK, kot so mutageneza posredovana z oligonukleotidi (angl. oligonucleotide-directed mutagenesis, ODM), urejevalci baz (angl. base editors, BE) ali sistem urejanja z vnosom (prime editing, PE);
- epigenetske tehnike, kot so z RNK usmerjana metilacija DNK (RNA-directed DNA methylation, RdDM) in interferenca CRISPR (CRISPRi);
- tehnike, ki preurejajo RNK.

Kot je razvidno iz Slike 2, lahko načrtno povzročimo specifične spremembe, ki so usmerjene v točno določeno regijo v genomu, vplivamo na epigenom ali na RNK, zato imajo NGT velik potencial v žlahtnjenju rastlin. Rezultati NGT so tudi bolj predvidljivi, čeprav obstaja majhna verjetnost za uvedbo nenačrtovane spremembe drugod v genomu (angl. ‘off-target’ effect) (Anderson in sod., 2015).



**Slika 2:** Delitev novih genomskih tehnik
**Figure 2:** Classification of new genomic techniques

Določene NGT so bile razvite, da se odpravi tveganja (npr. nevarnost za okolje in vpliv na zdravje), ki omejujejo uporabo transgenih rastlin. Cisgeneza tako vključuje gensko spreminjanje z uporabo popolne kopije naravnih genov, vključno z izvornimi regulatornimi zaporedji, ki pripadajo spolno združljivim vrstam, medtem ko se intrageneza nanaša na prenos edinstvenih kombinacij genov in regulatornih zaporedij znotraj iste vrste (Vasudevan in sod., 2023).

## 3 DOSTAVNI SISTEMI ZA VNOS MOLEKULARNIH ORODIJ NGT

Najpogosteje uporabljena metoda za vnos in stabilno integracijo transgenov v rastline je transformacija posredovana z bakterijo *Agrobacterium tumefaciens* (ATMT) (Altpeter in sod., 2016). Drug način vnosa genov je obstreljevanje s tako imenovano ‚gensko pištolo‘, pri čemer je DNK vezana na kovinske delce, ki se v celice izstrelijo z veliko hitrostjo. Čeprav na ta način lahko transformiramo širši nabor genotipov kot z ATMT, je velika pomanjkljivost te metode slabša regeneracija rastlin, kot tudi majhno število kopij in integracija na številna mesta v genomu (Wu in sod., 2015). Neposreden vnos rekombinantne DNK iz gojišča v protoplaste, s pomočjo elektroporacije, polietilenglikola (PEG) ali lipofekcije se zaradi zahtevne priprave protoplastov in njihove regeneracije uporablja le pri določenih rastlinah (Altpeter in sod., 2016). Virusni vektorji pripravljeni iz geminivirusov (krožnih enoverižnih DNK virusov s širokim naborom rastlinskih gostiteljev) imajo številne prednosti pri vnosu tuje DNK ali RNK v rastline: preprosta manipulacija, veliko število kopij na celico, sistemsko širjenje po rastlini in učinkovita ekspresija genov (Cody in Scholthof, 2019). Omejitve te tehnike so majhen genom virusov, prehodna ekspresija genov, sprememba se ne deduje, stranski učinki na gostitelja ter možnost prenosa na druge rastline. Trenutno je v ospredju razvoj naprednih dostavnih sistemov iz nanomaterialov, kot so karbonske nanocevke, polporozni silikatni nanodelci, nanokompoziti iz gline (angl. clay nanosheets), celice penetrirajoči peptidi (angl. cell penetrating peptides, CPP), nanostrukture DNK in kvantne točke (Demirer in sod., 2021; Li in sod., 2024). Poleg ciljne dostave, nadzorovanega izražanja in zaščite pred razgradnjo, nekateri nanodelci na podlagi fluorescence omogočajo sledenje dedni spremembi in izražanja v rastlini.

Na podlagi integrirane transgene DNK se v tarčnih celicah izrazijo aktivne komponente za NGT, ki povzročijo spremenjeno zaporedje DNK, epigenomski učinek

**Preglednica 1:** Primerjava novih genomskih tehnik (NGT) in uveljavljenih žlahtniteljskih metod
**Table 1:** Comparison of new (NGT) genomic techniques and conventional breeding methods

| Kriterij* | Klasično žlahtnjenje | Mutacijsko žlahtnjenje | Genski inženiring | Nove genomske tehnike |
|---|---|---|---|---|
| Način uvedbe genske spremembe | Križanje starševskih linij | Ionizirajoče sevanje ali mutagene kemikalije | Tehnologija rekombinantne DNK | Preurejanje genoma |
| Število genskih sprememb | Nekaj genov do celoten genom | Nekaj 100 do nekaj 1000 | 1-8 | 1 ali več |
| Rezultat žlahtnjenja | Zaželene spremembe genoma skupaj z drugim haploidnim genomom | Naključne spremembe genoma | Naključna integracija transgena | Načrtne mestno specifične spremembe |
| Povratno križanje | 5-7 generacij | Več generacij | Ni potrebno | Ni potrebno |
| Na trgu | 8-10 let | 8-10 let | 8-12 let | 2-5 let |
| Uredba | Varnostno testiranje ni zahtevano | Varnostno testiranje ni zahtevano | Potrebno varnostno testiranje, močno regulirano | Potrebno varnostno testiranje, mešana regulacija |

*Povzeto po (Chen in sod., 2019; Gao, 2021)

ali učinek na ravni RNK. Po opravljeni modifikaciji se ta integriran sistem lahko odstrani s križanjem v naslednji generaciji rastlin, ali z uporabo mestno specifične rekombinacije, kot je na primer sistem Cre-LoxP (Broothaerts in sod., 2021).

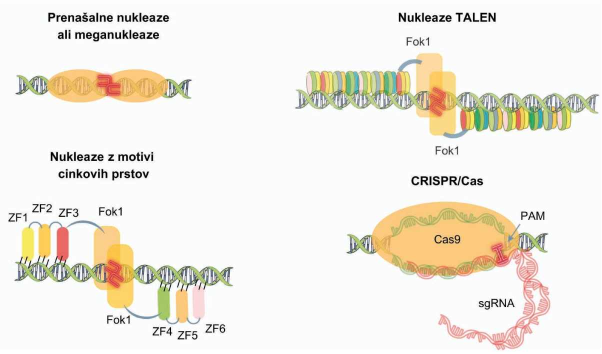
Alternativa integraciji transgenov je spreminjanje genoma ob prehodni ekspresiji aktivnih komponent za NGT (proteinov in/ ali RNK) iz rekombinantnih plazmidnih ali virusnih vektorjev, ki se po določenem času razgradijo z endogeno nukleazno aktivnostjo (Veillet in sod., 2019). Poleg tega so opisani tudi pristopi NGT, kjer se z biolistiko ali s transformacijo protoplastov s PEG v rastlinske celice vnesejo zgolj proteini, ribonukleoproteinski delci ali mRNK (Broothaerts in sod., 2021).

## 4 MOLEKULARNI MEHANIZMI UREJANJA GENOMA

### 4.1 SKUPINA 1 – NGT, KI POVZROČIJO DVOVERIŽNE PRELOME DNK

Nastanek dvoverižnih prelomov DNK je naravni fenomen, ki nastane pri podvojevanju DNK, rekombinaciji DNK, transpoziciji DNK, po delovanju produktov metabolizma (reaktivne kisikove zvrsti) ali eksogenih dejavnikov (UV, ionizirajoče sevanje, kemijski mutageni) (Mehta in Haber, 2014). Prelomi DNK so nevarni, saj lahko vodijo v celično smrt, če jih popravljalni mehanizmi ne odpravijo dovolj hitro.

Preurejanje genomov pri tej skupini tehnik poteka v dveh korakih in sicer se začne s prepoznavo tarčnega zaporedja in vezavo endonukleaze, ki povzroči dvoverižni prelom DNK, ter nadaljuje s popravilom preloma DNK s celičnimi popravljalnimi mehanizmi. Prva generacija nukleaz, ki se je uporabljala za mestno specifično preurejanje genomov, je temeljila na prepoznavi DNK po interakciji s proteini, kot so nukleaze TAL efektorjev (angl. transcription activator-like effector nucleases, TALEN), nukleaze z motivi cinkovih prstov (angl, zinc finger nucleases, ZNF) ter ‚prenašalne' endonukleaze (angl. homing endonuclease ali tudi meganuclease) (Slika 3). Nova generacija nukleaz, kot so sistemi CRISPR/Cas, pa tarčna mesta DNK prepozna po interakciji z RNK (Broothaerts in sod., 2021).



**Slika 3:** Splošen mehanizem delovanja različnih tipov mestno specifičnih nukleaz
**Figure 3:** General mode of action of different types of site-directed nucleases

### 4.1.1 'Prenašalne' endonukleaze

V naravi zapise za ‚prenašalne' endonukleaze najdemo ali v intronih ali v inteinih (Marcaida in sod., 2009). Funkcija ‚prenašalnih' endonukleaz je iniciacija rekombinacije z uvedbo dvoverižnih prelomov DNK, ki so asimetrični. Za razliko od običajnih restrikcijskih encimov so njihova prepoznavna mesta zelo dolga (12-40 bp) in izjemno redka. Poleg tega pri prepoznavi tarčnega zaporedja lahko tolerirajo manjše spremembe zaporedja nukleotidov.

Za preurejanje genomov so te encime priredili z mutagenezo in presejalnim testiranjem, da so domeno za prepoznavo DNK ločili od katalitičnega mesta in s kombiniranjem različnih ‚prenašalnih' nukleaz razširili nabor vezavnih mest (Arnould in sod., 2011). Ker je inženiring teh encimov neučinkovit, časovno potraten in zahteven, se jih danes le redko uporablja za preurejanje genomov.

### 4.1.2 Nukleaze z motivi cinkovih prstov (ZFN)

ZFN so hibridni encimi, ki so jih ustvarili z združevanjem endonukleaze, kot je bakterijski encim FokI, in proteinov s cinkovimi prsti, ki prepoznajo in se vežejo na različna zaporedja DNK (Osakabe in Osakabe, 2015). V domeni za vezavo DNK najdemo 3-4 cinkove prste, pri čemer je vsak sestavljen iz tripleta baz. Nukleazna domena FokI za cepitev DNK potrebuje dimerizacijo, zato se morata dve ZFN vezati na nasprotni verigi DNK. To pomeni, da je prepoznavno zaporedje dolgo 18 bp, kar je dovolj za določitev edinstvenega tarčnega mesta znotraj genoma.

Omejitve ZFN, kot so zahteven inženiring tarčnih mest, aktivnost ter občasna citotoksičnost (verjetno povezana z nenačrtovanim delovanjem na drugih tarčnih mestih), so prispevale k manjši uporabi ZFN za preurejanje genomov rastlin (Broothaerts in sod., 2021).

### 4.1.3 Nukleaze TAL efektorjev (TALEN)

Efektorji TAL so transkripcijski aktivatorji, ki so jih odkrili pri bakterijah rodu *Xanthomonas* (Mak in sod., 2012). Ko so jih po vzoru ZFN načrtno združili z endonukleazami, kot je encim *Fok*I, so ustvarili mestno specifične nukleaze TALEN (Becker in Boch, 2021). Domena za vezavo na DNK je sestavljena iz motivov tandemskih ponovitev 33-35 aminokislin. Vsak motiv prepozna posamezno bazo v zaporedju DNK, pri čemer sta pomembni predvsem aminokislini v motivu na mestih 12 in 13. Motive lahko med seboj poljubno kombiniramo, da zagotovimo bolj specifično prepoznavo tarčnih mest. Tako kot pri ZFN je tudi pri TALEN za cepitev DNK potrebna vezava nukleaze na nasprotnih verigah DNK in dimerizacija. Inženiring domene za vezavo DNK zahteva veliko truda in nekaj poskusnega testiranja, poleg tega je sama velikost konstruktov omejitev za številne dostavne sisteme (Broothaerts in sod., 2021).

### 4.1.4 Sistem CRISPR-Cas

Sistem CRISPR-Cas je vsestransko in najpogosteje uporabljeno orodje za preurejanje genoma. Tehnologija izvira iz prokariontskega imunskega sistema tipa II, ki je prilagodljiv obrambni sistem odvisen od RNK, ki bakterije in arheje ščiti pred fagnimi okužbami ter drugimi invazivnimi genskimi elementi (Barrangou in sod., 2007). Lokusi CRISPR-Cas vsebujejo nekaj sto kratkih odsekov DNK (30-40 bp), ostankov predhodnih okužb, tako imenovanih protovmesnikov, ki so ločeni s ponovitvami dolgimi 25-35 bp. Ti protovmesniki se prepišejo v CRISPR RNK (crRNA) in povežejo v kompleks s trans-aktivno CRISPR RNK (tracrRNA), na katerega se veže nukleaza Cas. Nastane aktiven ribonukleoproteinski kompleks, ki je namenjen razgradnji tujih nukleinskih kislin.

Z inženiringom so združili crRNK in tracrRNK v usmerjevalno RNK (angl. single-guide RNA, sgRNA) ter obdržali ključne lastnosti: 20 nukleotidov dolgo zaporedje na 5' koncu sgRNK, ki se veže na komplementarna mesta tarčne DNK, ter dvoverižno strukturo na 3' koncu, kamor se veže Cas (Jinek in sod., 2012). Tako je nastal preprost dvokomponentni sistem, v katerem lahko s spremembami 20 nukleotidov v sgRNK programiramo sistem CRISPR-Cas, da se usmeri na katerokoli zaporedje DKA, če le meji na PAM (angl. protospacer adjacent motif), 2-6 nukleotidov dolgo zaporedje takoj za tarčnim mestom. V primeru najpogosteje uporabljane nukleaze Cas9 iz bakterije Streptococcus pyogenes Rosenbach 1884 je to zaporedje 5'-NGG-3', pri čemer je prvi nukleotid poljuben, sledita pa mu dva zaporedna gvaninska nukleotida (Doudna in Charpentier, 2014). Od leta 2012 so odkrili številne ortologe Cas9 in jih razdelili v dva razreda ter 6 tipov glede na število podenot in njihovo arhitekturo (Makarova in sod., 2019). Sistemi CRISPR--Cas razreda 1 imajo efektorske module, sestavljene iz več proteinov Cas, ki tvorijo kompleks za vezavo crRNK in delujejo skupaj pri vezavi in procesiranju tarče. Sistemi razreda 2 imajo en sam večdomenski protein, ki veže crRNK in je funkcionalno podoben celotnemu efektorskemu kompleksu razreda 1.

Tehnologija CRISPR-Cas se še naprej razvija, vključno z izboljšavami ter novimi načini uporabe. Posebna pozornost je namenjena zmanjševanju vplivov na nenačrtovana tarčna mesta, predvsem z natančnim izborom tarčnih mest in načrtovanjem sgRNK, zmanjšanjem izražanja Cas9 z uporabo šibkih promotorjev, uvedbo Cas-mRNKK ali ribonukleoproteinskih kompleksov namesto DNK vektorjev ter uporabo delno ali popolnoma inaktivne različice Cas (nikaze ali dCas) (Broothaerts in sod., 2021).

### 4.1.5 Popravilo dvoverižnega preloma DNK

Drug korak pri urejanju genoma je popravilo dvoverižnih prelomov DNK. V evkariontskih celicah obstajata dva mehanizma: povezovanje nehomolognih koncev (angl. non-homologous end joining, NHEJ) in popravljanje na podlagi homologije (angl. homology-directed repair, HDR) (Osakabe in Osakabe, 2015). Popravilo z NHEJ je pogostejše in se običajno zgodi že po nekaj minutah po prelomu DNK z neposrednim zlepljanjem koncev DNK, pri katerem sodeluje kopica proteinov. Postopek NHEJ je podvržen napakam, predvsem nastanku majhnih insercij in delecij (indel), kar vodi do mutacij (zamikov bralnih okvirjev in nesmiselnih mutacij). Če so mesta preloma DNK blizu skupaj, lahko nastanejo delecije, inverzije ali duplikacije. S sistemom CRISPR-Cas lahko ustvarimo tudi daljše delecije, če uporabimo dve sgRNK, ki ciljata zaporedji okrog regije, ki jo želimo izbrisati. Tako lahko določen gen v celoti izbijemo. Mehanizem popravila s HDR je počasnejši, a natančen, in poleg številnih encimov zahteva določeno mero homologije z donorsko matrico, ki je lahko endogenega ali eksogenega izvora. V takem primeru lahko s CRISPR-Cas popravimo okvarjen gen ali ga zamenjamo. Poleg tega je možno multipleksiranje, oziroma ciljanje več genov hkrati (Broothaerts in sod., 2021).

## 4.2 SKUPINA 2 – NGT, KI POVZROČIJO ENOVERIŽNE PREKINITVE DNK ALI BREZ PREKINITVE DNK

Popravilo dvoverižnih prelomov DNK s celičnimi popravljalnimi mehanizmi, ki so podvrženi napakam, je težko nadzorovati in ima lahko neželene posledice. Zato so z mutagenezo mestno specifičnih nukleaz razvili manj škodljive encime za preurejanje genoma, tako imenovane nikaze (nCas), ki imajo inaktivirano eno katalitično domeno ter povzročijo enoverižne prekinitve DNK, ki se v celicah običajno popravijo na zelo zanesljiv način s popravljanjem z izrezom baze (angl. base excision repair) (Komor in sod., 2016).

### 4.2.1 Tarčna mutageneza z oligonukleotidi

Ta tehnika je bila razvita že v osemdesetih letih prejšnjega stoletja za spreminjanje kratkih zaporedij (nekaj baznih parov) v genomih bakterij in kvasovk, kasneje pa so jo izkoristili tudi za rastline in živali (Sauer in sod., 2016). Temelji na uporabi homolognih kratkih enoverižnih oligonukleotidov DNK, himernih RNK/DNK oligonukleotidov, ali dvoverižnih DNK molekul, ki se s tarčnim zaporedjem ne ujemajo na enem ali več mestih. Preurejanje genoma se začne s prileganjem oligonukleotida na tarčno zaporedje po principu komplementarnega parjenja baz. Zaradi neujemanja baznih parov, se aktivirajo celični popravljalni mehanizmi, ki vključujejo neposredno popravilo neujemanja (angl. mismatch repair), ali vključitev v verigo v prisotnosti ali odsotnosti aktivnosti povezane z replikacijo DNK (Broothaerts in sod., 2021).

### 4.2.2 Urejevalci baz

Urejanje baz (angl. base editing, BE) je novejši pristop k preurejanju genoma, ki omogoča uvedbo točkovnih mutacij brez prekinitve dvoverižne DNK. Urejevalci baz so sestavljeni iz katalitično neaktivne dCas (angl. dead Cas), ki je spojena s citozin ali adenin deaminazno domeno (Gaudelli in sod., 2017; Komor in sod., 2016). Urejevalca baze na tarčno mesto vodi usmerjevalna sgRNK. Ob vezavi nastane tako imenovana ‚R‘ zanka, ki vsebuje kratek enoverižen odsek DNK, na katerega deluje deaminaza, ki odstrani amino skupino iz citozina (angl. cytosine base editor, CBE). Nastane uracil, ki ga polimeraze DNK prepoznajo kot timin. V celicah je prisoten tudi encim uracil DNK glikozilaza, ki lahko par U:G zamenja nazaj v par C:G (Komor in sod., 2016). Zato so za povečanje učinkovitosti urejevalcem CBE na C- terminalni del dodali dve domeni inhibitorja uracil DNK glikozilaze. V primeru ABE (angl. adenine base editor) z deaminacijo adenina nastane inozin, ki ga polimeraze DNK prepoznajo kot gvanin. Z uporabo različic Cas, deaminaz in povezovalnih molekul so razvili urejevalce baz naslednje generacije, ki imajo boljšo učinkovitost in specifičnost uvedenih sprememb (Anzalone in sod., 2020).

### 4.2.3 Sistem urejanja z vnosom

Urejanje z vnosom (angl. prime editing, PE) je vsestranska in natančna metoda urejanja genoma, ki neposredno zapisuje novo genetsko informacijo na točno določeno mesto DNK. Pri tem uporablja nikazo (nCas) spojeno z reverzno transkriptazo ter pegRNK, ki vodi sistem PE do tarčnega mesta in nosi genetsko informacijo za želeno preurejanje (Anzalone in sod., 2019). Kjer pegRNK prepozna tarčno zaporedje DNK, nikaza uvede enoverižno prekinitev DNK s prostim 3‘ koncem, ki služi reverzni transkriptazi kot začetnik za prepisovanje informacije iz pegRNK v DNK. Nastane razvejan intermediat DNK, ki ga popravijo celični popravljalni mehanizmi tako, da odstranijo nespremenjeno (staro) zaporedje DNK, ki štrli na 5‘ koncu.

Urejanje z vnosom omogoča uvedbo substitucij posameznih nukleotidov, insercij in delecij do 100 baznih parov. Pomembna prednost te tehnike je, da ob urejanju ne nastanejo neželeni indeli, omejitev pa je vnos v celice zaradi same velikosti sistema (Broothaerts in sod., 2021).

### 4.3 SKUPINA 3 - NGT, KI TEMELJIJO NA SPREMINJANJU EPIGENOMA

Epigenom je skupek dednih modifikacij kromatina, ki uravnavajo izražanje genov v diferenciranih celicah brez spreminjanja nukleotidnega zaporedja v DNK. Epigenetske modifikacije so lahko neposredne, kot na primer vezava metilnih skupin na DNK ter vezava metilnih ali acetilnih skupin na lizin v histonih, ali posredne, ki vplivajo na regulacijo transkripcije z aktivatorji ali represorji (McCutcheon in sod., 2024).

#### 4.3.1 Neposredne modifikacije kromatina

Metilacija DNK poteka z encimi DNK metiltrasferazami, ki uvedejo metilno skupino na mesto C5 v citozinu. Pri rastlinah je večina citozinov v odsekih CpG, CpHpG ali CpHpH (pri čemer je H lahko A , C ali T), ki so del promotorskih regij ali zaporedij DNK, ki se aktivno prepisujejo, nemetiliranih (Gallego-Bartolomé, 2020). Medtem ko je metilacija teh mest povezana s kondenzacijo kromatina in represijo transkripcije. Poleg citozina lahko metilno skupino v nekaterih primerih najdemo tudi na adeninu na mestu N6.

Za uvedbo metilne skupine na tarčno mesto v DKA so katalitično domeno metiltransferaze spojili z mestno specifičnim proteinom za vezavo na DNK (npr. proteini s cinkovimi prsti, efektorji TAL, dCas). Za odstranitev metilne skupine iz tarčne DNK, pa so proteine za vezavo na DNK spojili z demetilazo (Kungulovski in Jeltsch, 2016).

Najpogostejši modifikaciji histonov pri rastlinah sta metilacija histona H3 na mestu lizina 4 ali 27 ter acetilacija histona H3 na mestu lizina 27. Metilacija histonov pri rastlinah je povezana s številnimi razvojnimi procesi (K. Cheng in sod., 2020), medtem ko je acetilacija histonov povezana s prilagajanjem rastlin na abiotski stres (Wang in sod., 2024).

Zaenkrat so mestno specifični modifikatorji kromatina slabše raziskani in v uporabi zgolj pri modelnih rastlinah (Ueda in Seki, 2020).

#### 4.3.2 Posredne epigenetske modifikacije

S številnimi transkripcijskimi faktorji, ki delujejo kot aktivatorji ali represorji lahko uravnavamo prepisovanje genov. Katalitično neaktivna nukleaza dCas se lahko specifično veže na mesta pred promotorjem ter onemogoči vezavo polimeraze RNK ali transkripcijskih faktorjev na promotor. S tem inhibira izražanje določenega gena brez spreminjanja genoma. To strategijo uravnavanja izražanja genov so poimenovali CRISPR interferenca (CRISPRi) (M. H. Larson in sod., 2013).

S kombinacijo usmerjevalne sgRNK, katalitično neaktivne dCas in efektorskih domen, ki lahko delujejo kot represorji (CRISPRi) ali aktivatorji (CRISPRa) izražanja genov, so dosegli tarčne spremembe kromatinskih oznak na izbranih mestih v genomu (Kungulovski in Jeltsch, 2016). Transkripcijski aktivatorji delujejo tako, da privabijo modulatorje kromatina, ki povzročijo dekondenzacijo kromatina, kopičenje histonskih oznak (npr. acetilacija histona H3 na mestu lizina 27 ali trimetilacija histona H3 na mestu lizina 4) ter vezavo polimeraze RNK II, da se začne prepisovanje mRNK. Transkripcijski represorji delujejo tako, da vežejo inhibitorne transkripcijske faktorje ali encime za modifikacijo histonov (npr. histonske metiltransferaze, ki povečajo trimetilacijo histona H3 na mestu lizina 9), kar povzroči lokalno kondenzacijo kromatina (Pei in sod., 2020).

### 4.4 SKUPINA 4 – NGT, KI PREUREJAJO RNK

#### 4.4.1 Od PAM neodvisna RNK-intereferenca posredovana s CRISPR-Cas

Čeprav so v zadnjih letih razvili številna orodja CRISPR, ki ciljajo in cepijo RNK, se pri rastlinah uporabljata zgolj CRISPR-Cas13a in CRISPR-Cas13d (Broothaerts in sod., 2021). Cas13a potrebuje CRISPR-RNK (crRNA) s sekundarno strukturo RNK v obliki lasnične zanke ter vmesnikom dolžine 22-28 nukleotidov za prepoznavo tarčne RNK (Abudayyeh in sod., 2017). Cepitev tarčne RNK je izven mesta vezave crRNK, pri tem pa imajo različne variante Cas13a različne preference za cepitev za določenimi nukleotidi.

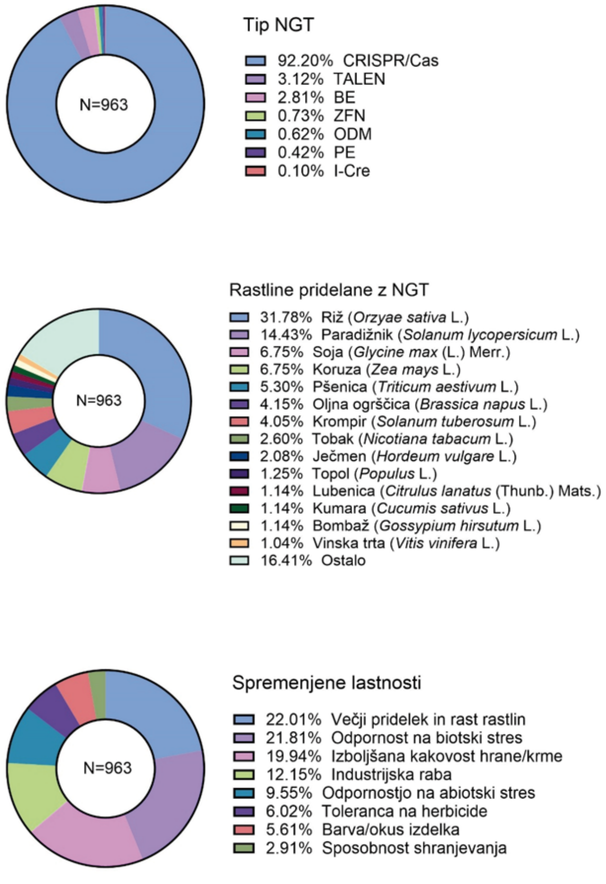
Cas13d je najmanjša in za preurejanje RNK najučinkovitejša ribonukleaza iz družine Cas13 (Zhang in sod., 2018). Tudi Cas13d je sestavljena iz dveh režnjev, prvi (angl. recognition, REC) je namenjen prepoznavi in vezavi crRNK, drugi (angl. nuclease, NUC) pa cepitvi RNK. Kompleks Cas13-crRNK je katalitično neaktiven, dokler ne veže tarčne enoverižne RNK (Gupta in sod., 2022). Poleg majhnosti je prednost tega sistema tudi to, da ne potrebuje motiva ob protovmesniku ter da ni opisanih neželenih učinkov izven tarčne RNK ali toksičnosti. Pri rastlinah se na primer uporablja za razvoj odpornosti proti RNK virusom (Zhan in sod., 2023).

## 5 Z NGT SPREMENJENE LASTNOSTI RAS-

## TLIN IN NJIHOVA UPORABA

EU-SAGE je mreža znanstvenikov iz 134 evropskih inštitutov in društev povezanih z rastlinami, ki so združili moči, da bi zagotovili ustrezne informacije o preurejanju genomov rastlin ter spodbujali razvoj evropskih politik in politik držav članic EU, ki omogočajo uporabo NGT za trajnostno kmetijstvo in pridelavo hrane (Dima in sod., 2020). Objavili so prosto dostopno interaktivno podatkovno zbirko o genomsko preurejenih rastlinah (Dima in sod., 2022).

Glede na razdelitev NGT v štiri skupine (slika 2 in (Broothaerts in sod., 2021)), pri preurejanju genomov rastlin prevladujejo NGT iz skupine 1 in 2. Ker je preurejanje genomov s sistemom CRISPR-Cas poceni, hitro in učinkovito, je tudi daleč najpogostejše in v uporabi pri kar 92 % rastlin (Slika 4, zgoraj). S približno 3 % ji sledita tehniki TALEN in BE. Epigenetske tehnike (skupina 3) in tehnike za preurejanje RNK (skupina 4) so bile do sedaj uporabljene le v začetnih fazah raziskav in razvoja (za dokazovanje koncepta) (Parisi in Rodriguez, 2021).



**Slika 4:** Podatkovna zbirka rastlin s preurejenim genomom EU-SAGE (Dima in sod., 2022), spremenjene lastnosti in uporabljene NGT
Podatki pridobljeni na: https://www.eu-sage.eu/genome-search (Februar 2025)
**Figure 4:** EU-SAGE Database of genome-edited crop plants (Dima in sod., 2022), modified traits and NGT used
Data retrieved from: https://www.eu-sage.eu/genome-search (February 2025)

NGT so bile uporabljene za preurejanje genomov pri 72 različnih rastlinskih vrstah (https://www.eu-sage.eu/genome-search, Februar 2025). Največ raziskav in razvoja NGT je bilo narejenih na rižu (*Oryza sativa* L.), paradižniku (*Solanum lycopersicum* L.), koruzi (*Zea mays* L.), soji (*Glycine max* (L.) Merr.) in pšenici (*Triticum aestivum* L.) (Slika 4, sredina). Izboljšave z NGT so uvedli tudi pri manj pomembnih poljščinah, sadju in širokem spektru zelenjadnic, kot tudi pri industrijsko pomembnih in okrasnih rastlinah (Parisi in Rodriguez, 2021). Večino rastlin s preurejenim genomom so požlahtnili na Kitajskem in v ZDA, čeprav tovrstne raziskave potekajo po vsem svetu (Dima in sod., 2022). V Evropi največ primerov uporabe NGT pri rastlinah prihaja iz Nemčije in Francije.

Leta 2021 je bilo za poljske poskuse odobrenih 117 primerov rastlin s preurejenim genomom, ki se lahko pojavijo na trgu do leta 2030 ter 292 primerov v zgodnji fazi razvoja (za dokazovanje koncepta) (Parisi in Rodriguez, 2021). Spekter uporabe NGT glede lastnosti in vrste rastline je zelo raznolik (Slika 4, spodaj). Najpogosteje so bile izboljšane: (i) agronomske pomembne lastnosti (22 %), kot so večji pridelek (boljša fotosintetska aktivnost, več cvetov, semen, plodov in/ali večja masa) in rast rastlin (izboljšana oblika -habitus, rastni vzorec), da se poveča produktivnost in izogne izgubam pred spravilom pridelka; (ii) odpornost na bolezni in škodljivce (22 %), da se zmanjša potreba po uporabi fitofarmacevtskih sredstev; (iii) kakovost rastlin za pridelavo hrane in krme (20 %) (na primer vsebnost vitaminov, toksičnih snovi, škroba, olja, proteinov, vlaknin, alergenov); (iv) ter lastnosti povezane z industrijsko rabo (12 %) (proizvodnja biogoriv, učinkovitejša raba dušika) (Dima in sod., 2022) Poleg tega se NGT uporabljajo tudi kot orodje za žlahtnjenje in sicer za spreminjanje reproduktivnih lastnosti (sterilnost, samoinkompatibilnost, apomiksis), zgodnejše cvetenje, povečanje ali zmanjšanje genetske rekombinacije, indukcijo haploidov in podvojenih haploidov (Parisi in Rodriguez, 2021). V preglednici 2 je seznam rastlin s preurejenim genomom, ki so že na trgu ali so prejele vse potrebne odobritve, vendar še niso na voljo potrošnikom, saj strategija trženja takih rastlin še ni izdelana (Polidoros in sod., 2024).

**Preglednica 2:** Seznam rastlin s preurejenim genomom odobrenih za prodajo
Podatki pridobljeni na: https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/united-states-crops-food (Februar 2025)
**Table 2:** The list of genome-edited crops approved for sale
Data retrieved from: https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/united-states-crops-food (February 2025)

| Lastnost | Opis (NGT) | Država (leto) | Podjetje |
|---|---|---|---|
| Voščena koruza | Koruza z veliko vsebnostjo škroba (CRISPR) | Japonska (2024) | Corteva Agriscience |
| Solata, ki ne porjavi | ‘GreenVenus’, romanska solata, ki ne porjavi | ZDA (2024) | Intrexon |
| Pšenica odporna na glive | Pšenica odporna na pepelasto plesen (*Blumeria graminins* Speer.) | Kitajska (2024) | Suzhou, Chinese Academy of Sciences |
| Zelena gorčica | ‘Conscious Greens’, blažja in manj grenka zelena gorčica (CRISPR-Cas12a) | ZDA (2023) dostopno na trgu | Pairwise |
| Banana, ki ne porjavi | Banana z upočasnjenim rjavenjem za podaljšanje roka uporabnosti (CRISPR) | Filipini (2023) | Tropic Biosciences |
| GABA paradižnik | ‘Sicilian Rouge’, paradižnik z večjo vsebnostjo GABA, znižuje krvni tlak (CRISPR) | Japonska (2021) dostopno na trgu | Sanantech Seed |
| Sojino olje z visoko vsebnostjo oleina | ‘Calyno’, Sojino olje z manj nasičenimi maščobami in brez trans maščob (TALEN) | ZDA (2019) dostopno na trgu | Calyxt |
| Jabolka, ki ne porjavijo | ‘ArticApple’, jabolka, ki ne porjavijo (sorte Golden, Granny, Fuji, Gala, Honey) (RNKi) | Kanada (2017) dostopno na trgu<br>ZDA (2015) dostopno na trgu | Okanagan Speciality Fruits |
| Krompir, ki ne porjavi | ‘White Russet Potato’, krompir, ki ne porjavi, odporen na plesni, z manj sladkorji in akrilamida (RNKi) | ZDA (2015) dostopno na trgu<br>Kanada (2015) dostopno na trgu | Simplot |
| Oljna ogrščica | Oljna ogrščica odporna na herbicide (ODM) | ZDA (2014)<br>Kanada (2013) | Cibus |

## 6 ZAKONODAJNA UREDITEV Z NGT PRIDELANIH RASTLIN IN NJIHOVIH PRODUKTOV V EU

Evropska unija ima vzpostavljen strog pravni okvir, ki zagotavlja, da razvoj moderne biotehnologije in zlasti GSO poteka pod varnimi pogoji. Namenjen je: (i) zaščiti zdravja ljudi in živali ter okolja z uvedbo ocene varnosti po najvišjih možnih standardih na ravni EU, preden se kateri koli GSO da na trg; (ii) uvedbi usklajenih postopkov za oceno tveganja in odobritev GSO, ki bodo učinkoviti, časovno omejeni in pregledni; (iii) zagotavljanju jasnega označevanja GSO, ki se dajejo na trg, da bi potrošnikom in strokovnjakom (npr. kmetom in upravljavcem prehranske verige) omogočili ozaveščeno izbiro; ter (iv) zagotavljanju sledljivost GSO, danih na trg. Trenutno veljavni pravni akti o GSO, ki uresničujejo te cilje so: (i) Direktiva EU o namernem sproščanju gensko spremenjenih organizmov v okolje (Directive 2001/18/EC); (ii) Uredba EU o gensko spremenjenih živilih in krmi (Regulation (EC) 1829/2003); (iii) Direktiva EU o spremembi Direktive 2001/18/ES glede možnosti držav članic, da omejijo ali prepovejo gojenje gensko spremenjenih organizmov (GSO) na svojem ozemlju (Directive (EU) 2015/412); (iv) Uredba EU o sledljivosti in označevanju gensko spremenjenih organizmov ter sledljivosti živil in krme, proizvedenih iz gensko spremenjenih organizmov (Regulation (EC) 1830/2003); (v) Direktiva EU o uporabi gensko spremenjenih mikroorganizmov v zaprtih sistemih (prenovitev) (Directive 2009/41/EC); ter (vi) Zakon o gensko spremenjenih organizmih in dajanju izdelkov na trg, ki ureja področje GSO v Sloveniji (in implementira Direktivo 2001/18).

Po Direktivi 2001/18/EC (Direktiva - 2001/18 - EN - EUR-Lex, b. d.) je GSO definiran kot “organizem, razen človeka, pri katerem je bil genski material spremenjen na način, ki ne nastane naravno s križanjem in/ali naravno rekombinacijo”. Ali se rastline pridelane z NGT obravnavajo kot GSO, je odvisno od interpretacije te definicije (Katsarova, 2024). Pri običajni razlagi te opredelitve kot procesa, torej zadostuje že sama uporaba tehnike genskega spreminjanja, da se dobljeni organizem šteje za GSO. Pri razlagi, ki temelji na produktu, pa mora nastali organizem vsebovati novo kombinacijo genskega materiala, da se šteje za GSO. Tretja možna razlaga združuje obe merili. V aneksih te direktive so opredeljene tehnike, ki povzročijo genske spremembe (Aneks IA, 1. del), tehnike, za katere se ne šteje, da lahko povzročijo genske spremembe (Aneks IA, 2. del) ter tehnike, ki povzročijo genske spremembe, vendar je nastali organizem izključen iz te direktive (Člen 3 in Aneks IB). Taki tehniki sta mutageneza in celična fuzija. Poleg tega je v direktivi zapisano (preambula 17), da se direktiva ne bi smela uporabljati za organizme, pridobljene z nekaterimi tehnikami genskega spreminjanja, ki se običajno uporabljajo v številnih aplikacijah in imajo dolgo zgodovino varnosti.

Leta 2018 je Sodišče EU razsodilo, da so organizmi, pridobljeni z usmerjeno mutagenezo, GSO - ker mutageneza spremeni genski material organizma na način, ki se ne pojavi v naravi - in zato za take organizme veljajo pravila EU o odobritvi, sledljivosti in označevanju (EUR-Lex - 62016CJ0528 - EN - EUR-Lex, b. d.). Glede vprašanja, ali se zakonodaja EU o GSO uporablja za organizme, pridobljene z NGT, je Sodišče EU menilo, da so tveganja, povezana z uporabo teh tehnik, podobna tveganjem pri transgenezi – vnosu tujega gena v organizem -, saj neposredna sprememba genskega materiala organizma z mutagenezo omogoča doseganje enakih učinkov. Pri tem je izvzelo sorte, ki so bile pridobljene s „tehnikami mutageneze, ki se običajno uporabljajo v številnih aplikacijah in imajo dolgo zgodovino varnosti“. Evropska komisija je nato leta 2021 objavila študijo o statusu novih genomskih tehnik v skladu s pravom EU, ki je ugotovila, da so organizmi, pridobljeni z NGT, zlasti z usmerjeno mutagenezo, cisgenezo in intragenezo, GSO (EC study…, 2025.)

Na podlagi opravljenih strokovnih študij in poročil Evropske agencije za varnost hrane (EFSA) (Mullins in sod., 2022) ter Skupnega raziskovalnega središča (JRC) (Broothaerts in sod., 2021) je Evropska komisija leta 2023 predložila predlog Uredbe o rastlinah, pridobljenih z nekaterimi novimi genomskimi tehnikami, ter hrani in krmi iz njih (COM (2023) 411) (Polfjärd, 2025), katere cilj je ohraniti visoko raven varovanja zdravja ljudi in hkrati spodbujati razvoj sort uspešnih v boju proti podnebnim spremembam in zmanjšujejo uporabo pesticidov. V predlogu uredbe in njenih dopolnitvah sta opredeljeni dve kategoriji rastlin (Kahrmann in Leggewie, 2024):

(i) rastline NGT kategorije 1 vključujejo rastline, ki so enakovredne rastlinam, ki jih je možno pridobiti s klasičnim žlahtnjenjem, pod pogojem, da se od starševskih rastlin ne razlikujejo za več kot 20 nukleotidnih sprememb, kot so tarčne substitucije in insercije nukleotidov, ali delecije poljubnega števila nukleotidov. Mutacije v intronih in regulatornih zaporedjih so izključene iz tega obsega. Med te spremembe sodijo tudi insercije, substitucije, inverzije ali translokacije zaporedja DNK, ne glede na velikost, če ta zaporedja že obstajajo v genskih virih žlahtniteljev in pod pogojem, da ne povzročijo prekinitve endogenih genov ter da ne nastane himeren protein. Za te rastline zakonodaja o GSO ne velja, zato postopek odobritve z oceno tveganja ni potreben. Toda namerno sproščanje in dajanje takih rastlin na trg je dovoljeno šele po uradni potrditvi statusa kategorije 1. V primeru namernega sproščanja je za to pristojen ustrezen organ članice EU, medtem ko dajanje na trg potrdi EFSA (angl. European Food Safety Authority). Zaradi zagotavljanja transparentnosti so uradno priznane rastline NGT kategorije 1 navedene v javno dostopni zbirki EU-SAGE, potrebno pa je tudi označevanje vseh izdelkov iz teh rastlin, vključno z razmnoževalnim materialom. Poleg tega se v ekološki pridelavi teh rastline ne sme uporabljati.

(ii) rastline NGT kategorije 2 so vse ostale rastline pridobljene z NGT, za katere velja zakonodaja o GSO. Zanje je potrebno izdelati oceno tveganja za zdravje ljudi, živali in okolje, pred dajanjem na trg je potrebno pozitivno mnenje EFSA, prav tako mora biti zagotovljeno ustrezno sledenje in označevanje kot GSO. To velja tudi za hrano ali krmo pridelano iz teh rastlin.

Predlog nove uredbe je 7. februarja 2024 v Evropskem parlamentu podprlo 307 poslancev, proti jih je bilo 263 in 41 se jih je vzdržalo (Katsarova, 2024) in je trenutno v javni razpravi.

## 7 KAKŠNA JE PERSPEKTIVA RASTLIN PRIDELANIH Z NGT?

Osnutek uredbe o NGT je sprožil veliko razprav (Kahrmann in Leggewie, 2024). Negativne kritike prihajajo predvsem iz okoljskih nevladnih organizacij in delov živilskega sektorja: Friends of the Earth v Evropi menijo, da je ogrožena narava, podobno tudi IFOAM Organics Europe meni, da je osnutek „korak nazaj za biološko varnost, svobodo izbire in obveščenost potrošnikov“, medtem ko Evropsko združenje industrije brez GSO poudarja tveganja za proizvodnjo brez GSO. Pesticide Action Network Europe meni, da predlog „krši previdnostno načelo“ in „ni v skladu z obljubami evropskega zelenega dogovora“, saj bodo nove tehnike „koristile le semenski industriji, kmetje, državljani in okolje pa bodo ostali nezaščiteni“.

Priznane znanstvene organizacije in semenarska podjetja imajo do osnutka uredbe zelo pozitiven odnos: Evropska organizacija za znanost o rastlinah pozdravlja osnutek predloga kot „uravnotežen kompromis“ in podpira večino njegove vsebine (*Feedback* …, 2025.). Podobno Euroseeds meni, da sta predlog in NGT priložnost za večjo odpornost in trajnost pri varni proizvodnji hrane (Planting …, 2025).

Evropski parlament je predlagal, da se v predlog uredbe uvede tudi člen, v skladu s katerim rastline NGT ne bodo patentirane (*New Genomic Techniques…*, 2024). Ta pristop naj bi bil nujen, saj bi v nasprotnem primeru multinacionalna semenarska podjetja lahko imela še večjo moč in monopol nad dostopom do semen. Z družbeno-ekonomskega vidika je predlog smiseln, vendar ga je potrebno obravnavati v okviru Evropske patentne konvencije in upoštevati, da patentiranje izdelka ni odvisno od tega, ali je njegova uporaba dovoljena. Po drugi strani patentni strokovnjaki opozarjajo, da brez ustreznega varstva podjetja morda ne bodo mogla vlagati v razvoj NGT (European Parliamant …, 2025).

## 8 ZAKLJUČEK

Prilagajanje rastlin zahtevam ljudi je nekaj, kar počnemo že od začetka kmetijstva. Še naprej se bodo razvijale nove tehnike žlahtnjenja in gojenja rastlin, zato je le vprašanje časa, kdaj bo tehnologija, s katero je rastlina pridobila določeno lastnost, postala drugotnega pomena glede na pomen same lastnosti, ki jo je rastlina pridobila (New report…, 2023.). Napredne NGT na eni strani prinašajo številne koristi za potrošnike, saj naslavljajo nekatere ključne izzive kmetijstva povezane s prilagajanjem na podnebne spremembe, z zdravjem in prehransko varnostjo, ter okoljsko trajnostjo in diverzifikacijo kmetijstva. Tveganja nove tehnologije so znanstveno dokazano primerljiva s tistimi, ki jih prinašajo že uveljavljene metode žlahtnjenja (Mullins in sod., 2024). Po drugi strani se potencialne koristi tehnologije zapravljajo zaradi neuspešnega obravnavanja regulativnega, pravnega in trgovinskega okvira ter večinoma negativnega sprejemanja družbe, verjetno predvsem na račun strahu in nerazumevanja.

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