  

 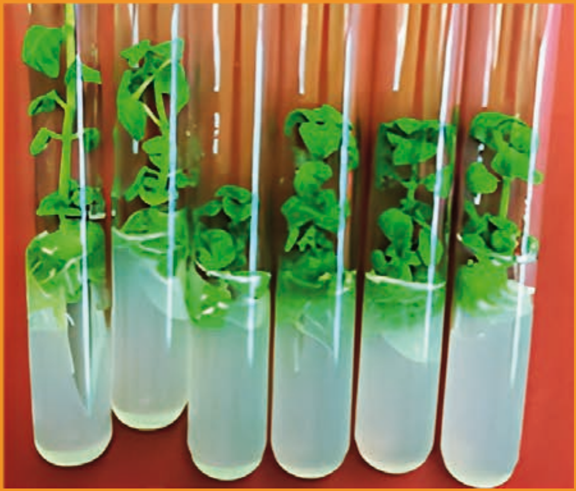

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Ovitek: Mikropropagacija vrste *Sideritis raeseri* Boiss. & Heldr: a) Kalitev semen v *in vitro* razmerah, b) in c) Diferenciacija poganjkov in korenin, d) Regeneracija poganjkov v podkulturi, e) Indukcija nastanka korenin (Foto: Valbona Sota, 1–15)
Cover: Sideritis raeseri *Boiss. & Heldr. micropropagation: a) Seed germination under* in vitro *conditions, b) and c) Shoot and root differentiation, d) Shoots regeneration during subculture, e) Rhizogenesis induction (Photo: Valbona Sota, 1–15)*

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Original Scientific Article / Izvirni znanstveni članek

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Investigating the effects of plant growth-promoting rhizobacteria isolates on germination and physiology status of durum wheat under salt stress

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Investigating the effects of plant growth-promoting rhizobacteria isolates on germination and physiology status of durum wheat under salt stress

Abstract: The aim of this work is to evaluate the seedling growth and physiology status of wheat seeds inoculated with a suspension of eight plant growth-promoting rhizobacteria (PGPR) isolates. For this purpose, rhizobacteria strains were isolated from the roots of native plants growing in the Algerian steppe, then evaluated for their plant growth promotion (PGP) features, and finally applied on wheat seeds. The obtained results showed that the majority of the tested strains displayed pertinent PGP features. In *in vitro* experiments, results showed that salinity affected negatively seed germination and impaired plant growth while the inoculation with BC3, BC6 and BC7 strains induced a good germination rate and improved significantly the root length. In greenhouse experience, data demonstrated that non-inoculated plants accumulated a significant amount of osmoregulators (proline and glycine betaine), and recorded a decrease of their chlorophyll content, compared to inoculated plants, where the salinity tolerance of this latter has been much better with a high seedling growth as well as high chlorophyll and low osmolyte contents. The results may be a useful extension of our knowledge of the interaction between plant and PGPR, in view of their possible applications as a biofertilizer to improve plant growth in salinity-impacted regions.

Key words: bacterial inoculation; biofertilization; PGPR; plant-microbe interactions; omoregulators; salt stress; durum wheat

Preučevanje učinka izolatov rizobakterij, ki pospešujejo rast rastlin na kalitev in fiziološke parametre trde pšenice pod slanostnim stresom

Izvleček: Namen raziskave je bil ovrednotiti rast sejank in fiziološko stanje trde pšenice, katere semena so bila inokulirana s suspenzijo devetih izolatov rizobakterij, ki pospešujejo rast rastlin (PGP). V ta namen so bili izolati rizobakterij izolirani iz korenin samoniklih rastlin, ki rastejo v alžirski stepi. Kasneje je bil ovrednoten njihov učinek na pospeševanje rasti rastlin, nakar so bili uporabljeni za inokulacijo semen pšenice. Dobljeni rezultati so pokazali, da je imela večina preiskušenih sevov pomembne PGP lastnosti. V *in vitro* poskusih so rezultati pokazali, da je slanost negativno vplivala na kalitev semen in zavrla rast rastlin med tem, ko je inokulacija z BC3, BC6 in BC7 sevi povečala kalitev in značilno izboljšala dolžino korenin. Poskusi v rastlinjaku so pokazali, da so ne inokulirane rastline značilno povečale količino osmoregulatorjev (prolina in glicin betaina), zabeležen je bil upad vsebnosti klorofila v primerjavi z inokuliranimi rastlinami. Toleranca inokuliranih rastlin na slanost je bila kasneje mnogo večja, kar se je pokazalo v boljši rasti, v večji vsebnosti klorofila in zmanjšanju osmotikov. Rezultati bi lahko bili koristni za izboljšanje razumevanja interakcij med rastlinami in PGPR, tudi z vidika njihove uporabe kot biognojil za izboljšanje rasti rastlin na zasoljenih območjih.

Ključne besede: bakterijska inokulacija; biognojenje; PGPR; interakcije rastline-mikrobi; omoregulatorji; solni stres; trda pšenica

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1 INTRODUCTION

Soil is the main support for most agricultural products. It also represents a heterogeneous environment that allows the development of many microorganisms, which are in continuous interaction with other species, under conditions of symbiosis, antagonism, mutualism, parasitism and saprophytes (Gouda et al., 2018; Bhat et al., 2019). For a long time, soil microbes were seen exclusively as pathogens, recent studies on plants and soil microbiome interactions have made this negative view obsolete (Lopes et al., 2021). The rhizosphere fungal and bacterial communities can harbor beneficial organisms. These organisms have the ability to colonize plant roots providing benefits to their hosts, by increasing the growth of the plant through the production of a variety of bioactive compounds such as phytohormones and several active enzymes and facilitating the nutrient uptake which enables plant growth in nutrient-poor soils (Khanna et al., 2019; Mülner et al., 2020), they can also contribute to protecting plants through direct biocontrol via the production of harmful compounds for neighboring phytopathological microorganisms (Viaene et al., 2016). Cultural practices such as plowing cause a decrease in the diversity of soil-borne microorganisms, a phenomenon amplified by the introduction of xenobiotic compounds (pesticides, chemical fertilizers) and soil salinization due to climate change (Marlet & Job, 2006), in this situation, it is unlikely that cultures will have a chance to naturally recreate an optimal microbial ecosystem.

Algeria produces more than 60 % of its cereals on agricultural land that is situated in salinity-prone areas. However, salinity is one of the primary factors limiting yield in these regions (Djermoun, 2009). According to FAO, land salinization should be considered a major risk likely to affect around 25 % of irrigated areas or 10 % of global food production. (FAO, 2015). Technical measures exist to control this phenomenon, but their application can take a long time and its implementation can reach considerable costs. To remedy this problem, the establishment of sustainable and environmental-friendly systems such as biofertilization can be a solution. For this purpose, present study was conducted to test the hypothesis that the isolated rhizobacteria have multiple PGP traits and that they can be used as biofertilizers to promote wheat (*Triticum durum* L.) growth under salinity stress.

2 MATERIALS AND METHODS

2.1 ISOLATION AND STRAIN PURIFICATION PROCEDURES

The bacterial strains used in this study were isolated from the rhizosphere of the halophyte plant Sparte (*Lygeum spartum* L.), which is found in steppe regions of Algeria. Location of the soil samples collection site: 34°35'51.8"N; 1°18'51.0"E. According to the Vincent, (1972) procedure, 10 g of rhizosphere soil was added to 90 ml of sterile distilled water, and the mixture was then incubated on a rotary shaker at 120 rpm for 10 min. Following this, 1 ml of the sample was serially diluted up to a concentration of 10^{-7}, and 0.1 ml of the diluted sample was then plated on sterile nutrient agar medium (containing 0.5 % peptone, 0.3 % beef extract, 1.5 % agar, 0.5 % NaCl, pH is adjusted to neutral 6.8) and incubated at 28 °C for three days. In order to obtain pure culture, single colonies were eventually picked up and streaked on sterile nutrient agar medium plates.

2.2 PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF ISOLATES

Colonies that had been carefully isolated had their morphology examined, and their Gram stain was checked. Standard biochemical and physiological tests were used to confirm the identification, and plant growth promotion (PGP) activity assays, such as inorganic phosphate solubilization, IAA production, fixation atmospheric nitrogen, and catalase enzyme production.

2.2.1 Catalase Test

The catalase was revealed by depositing of a bacterial colony on a clean glass slide, in the presence of H_2O_2. Positive reactions are evident by immediate effervescence (bubble formation) (Delarras, 2007), the catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2$).

2.2.2 Indole acetic acids (IAA) test

According to MacWilliams (2009), the bacterial isolates were examined for their ability to produce indole acetic acid (IAA). They were cultivated at 28 °C for 48 hours, in a liquid medium called "Tryptone Soya Broth" (TSB), and then 5 drops of Kovac's reagent were added by pouring them directly into the tube. The development of a pink to red colour in the reagent layer above the centre denotes a positive indole test.

2.2.3 Fixation of atmospheric nitrogen

According to the protocol of Rodge et al. (2016), bacteria were grown at 28 °C for 48 hours, on solid medium free of nitrogen «Ashby» or «Burk›s N-free» medium without mannitol. Any growth in this medium reveals the bacteria's capacity to fix atmospheric nitrogen.

2.2.4 Test for inorganic phosphate solubilization

The isolates were checked for phosphate solubilizing ability on the solid Pikovskaya (PVK) agar medium amended with 2 % tricalcium phosphate (TCP) (Kumar et al., 2001). Formation of a clear halo zone around the growth after 5 days of incubation indicates phosphate solubilizing ability.

2.3 RHIZOBACTERIA STRAINS EVALUATION ON WHEAT SEED GERMINATION UNDER SALT STRESS

Eight isolates (BC1…BC8) were selected to study their effect on the germination parameters (germination rate and root length) under different salinity levels (80 and 160 mM) developed with NaCl in sterilized distilled water. Seeds of the local Algerian durum wheat variety "Mohamed Ben Bachir" were used in this study. The experimental approach of our work takes place in several stages, which are summarized in the Figure 1.

The seeds were surface repeatedly washed with distilled water after being disinfected with sodium hypochlorite 2 % for 3 min and 75 % ethanol for 3 min. They were then immersed separately for 24 hours in each of the eight bacterial isolate solutions (10^8 CFU ml^{-1}). After being soaked with various NaCl solution concentrations, the treated seeds were incubated in Petri dishes at 25 °C in the dark. Only non-inoculated, unstressed seeds were used in subsequent tests as a control. The treatments of the experiment included the salinity at two levels: 80, and 160 mM, and seed inoculation with eight bacterial strains and three replicates per treatment for each strain (8 inocula × 2 treatments × 3 replicates). After 7 days, the emergence of the radicle from the seed was considered an index of germination (Johnson & Wax, 1978). The germination rate was calculated as follows: Germination (%) = (number of seeds germinated / total number of seeds sown) × 100.

By dividing the total length of the roots for each treatment by the total number of seeds (sprouted or not) the average root length of sprouted seeds is calculated (Darrah, 1993).

2.4 EVALUATION OF RHIZOBACTERIA STRAINS EFFECTS ON WHEAT CULTIVARS GROWTH UNDER SALT STRESS

Before installing cultures, the soil was autoclaved at 121 °C for 30 min to sterilize it, then it was divided into equal portions (1.2 kg each pot). Prior to planting, sterilized seeds were given 30 minutes to soak in a mix-

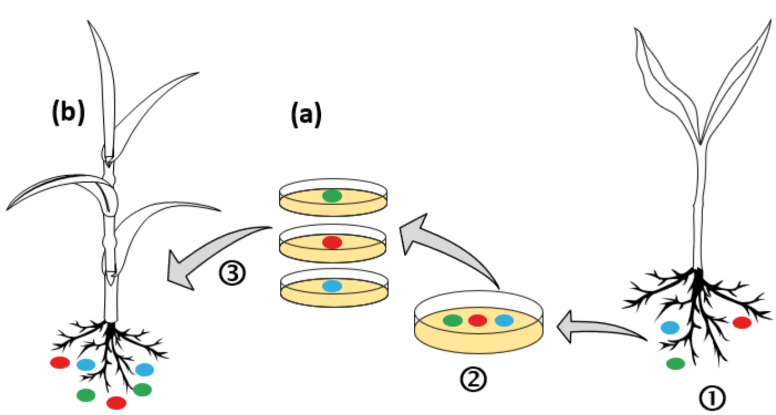

Figure 1: The scheme of the methodological steps of the study. 1: The isolation of bacteria from native plant roots growing in Algerian steppe areas, 2: The characterization and identification of strain isolates, 3: *in vitro* seed germination (a) and seedling development in greenhouse (b)

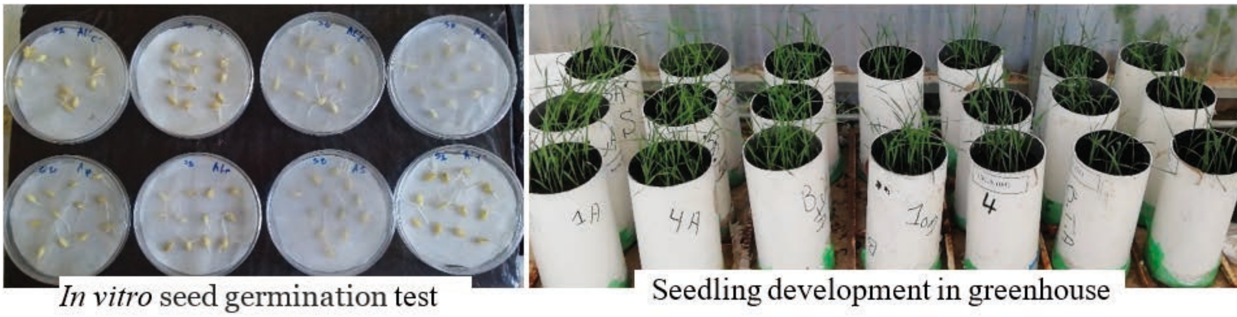

Figure 2: Effects of rhizobacterial strains on wheat cultivars growth under salt stress

ture of the eight bacterial suspensions. After that, the seeds were sowed in pots filled with sterilized soil and cultivated under salt stress conditions (80 and 160 mM) in the greenhouse at 25 °C, and 60 % relative humidity. Some sterilized seeds were sowed in pots without any salt treatment (unstressed control) (Fig. 2). Each treatment group comprised: 10 pots × 2 salt stress treatments × 3 replicates.

2.4.1 Leaf chlorophyll measurements

Plants stressed at 60 days after sowing were used for each salinity-restricted treatment, and the chlorophyll content of well-developed leaves was determined using a Chlorophyll Content Meter (SPAD 502 Plus). Measurements with the SPAD-502 meter produce relative SPAD meter values that are proportional to the amount of chlorophyll present in the leaf (Ling et al., 2011).

2.4.2 Determination of leaf proline content

Fresh leaves (100 mg) of each sample were chopped up and put in a test tube before being tested for proline using the Paquin & Lechasseur (1979) method. They were mixed thoroughlin 10 ml of 3 % sulfosalicylic acid aqueous solution ($C_7H_6O_6S$) and then filtered through Whatman filter paper. 2 ml of the filtrate was combined with 2 ml of ninhydrin ($C_9H_6O_4$) and 2 ml of acetic acid, glacial ($C_2H_4O_2$) in a 20 ml test tube. Samples were heated for 1 hour at 100 °C in a water bath. To stop the reaction, samples were put on ice, 4 ml of toluene was added to them. Then the whole mixture was vigorously stirred for 10 to 15 seconds. After standing for 20 min, a spectrophotometer was used to calculate the toluene portion's optical density at 520 nm. Finally, the standard range is established by pure proline.

2.4.3 Determination of leaf glycine betaine content

The amount of glycine betaine (GB) in each treatment was calculated using the technique described by Park et al. (2004). A sample of dry plant material (0.5 g) was combined with20 ml of distilled water. Sulfuric acid (NH_2SO_4) was used to dilute the resulting solution after it had been cultured for 48 hours at 25 °C. In cold water the resulting solution (0.5 ml) was chilled for 1 hour. After adding the reagent KI-I_2 (0.2 ml) the mixture was gently agitated using the vortex. Next, perform a 5-minute 14 000 rpm at 0 °C. After being aspirated, the supernatant is dissolved in 9 ml of 1,2-dichloroethane. First, it is washed and left with 0.5 ml for 5 minutes. After 2 hours, a UV-visible spectrophotometer was used to measure the absorbance at 365 nm. By using a standard curve created from a glycine betaine solution made in a sulfuric acid based on known concentrations, the glycine betaine concentration can be calculated.

2.5 STATISTICAL ANALYSIS

Data were analyzed for significant mean differences via two-way Analysis of Variance (ANOVA) using XL-STAT software (version 2014). The effects of the bacterial inocula, were assessed using Dunnett's test for multiple comparisons among class means.

3 RESULTS AND DISCUSSION

3.1 PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF ISOLATES

On the basis of phenotypic and functional characterization, the isolates were identified into 8 different strains, the latter were coded as: BC1, BC2, … BC8. Except for BC1, all of the isolates were Gram-negative, BC2, BC3, and BC6 were able to dissolve inorganic phosphate while BC4 was effective at fixing atmospheric nitrogen. BC7 outperformed the other seven isolates in terms of production of IAA, The promotion of plant growth (PGP) features were found in the majority of isolates. Table 1 and Figure 3 provide a phenotypic and functional characterization of isolates.

3.2 INOCULATION EFFECTS ON SEED GERMINATION UNDER SALT STRESS *IN VITRO*

The findings show that there were differences in how durum wheat seedlings responded to salt stress based on salinity levels and inoculation treatments. Under salt stress, most of the seeds undergo a reduction in their germination rate compared to the unstressed seeds,

Table 1: Morphological and biochemical characterization of rhizospheric isolates

	Rhizospheric isolates							
Characteristics	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC8
Form	Filamentous	Coccobacillus	Bacillus	Coccus	Coccobacillus	Coccobacillus	Coccus	Coccobacillus
Colour	Pale yellow	White	Orange	Pale yellow	Orange	Pink	White	Pale green
Gram Staining	+	-	-	-	-	-	-	-
Catalase test	-	+	+	+	-	+	-	+
Atmospheric nitrogen fixation	-	-	+	+++	+	+	++	+
Indole production	-	-	++	+	+	+	+++	+
Inorganic phosphate solubilization	-	+	+	-	-	+	-	+

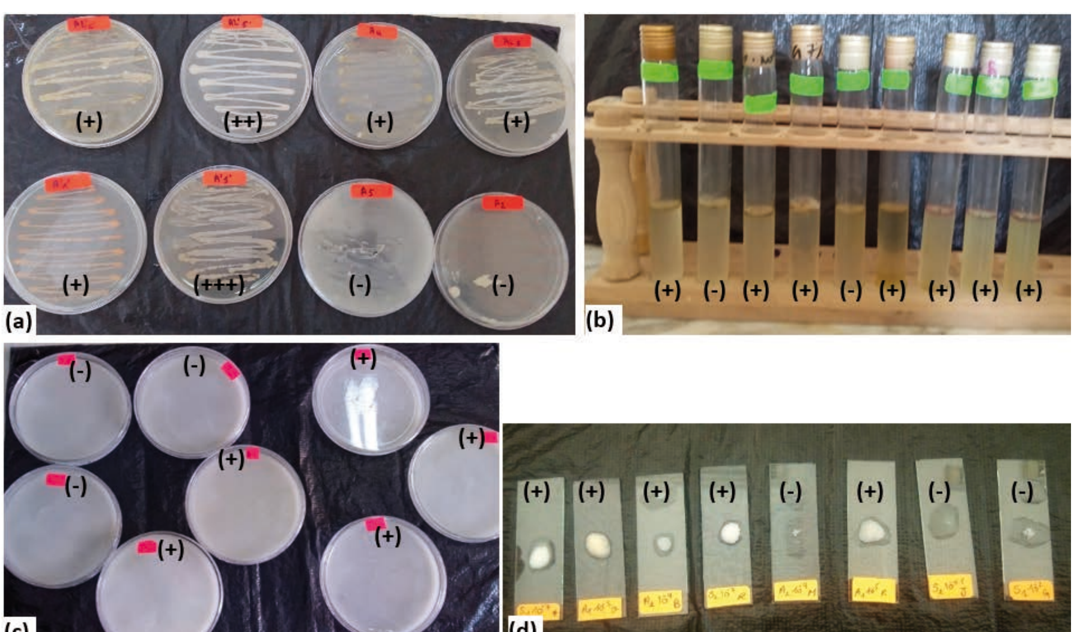

Figure 3: Screening of rhizospheric isolates for the plant growth promotion features. (a) Plates assay for screening of atmospheric N fixation, (b) Characterization of IAA producing isolates, (c) Plates assay for screening of inorganic phosphate solubilization, (d) Catalase test results, the absence of bubbling indicates a negative test. "+" indicates a positive test, whereas "-" indicates a negative test

however, the inoculated seeds were the exception, showing a much higher germination rate than non-inoculated. Among some isolates (BC3, BC6 and BC7) were able to keep their germination rates between 98 and 100 % slightly close to the witnesses (Fig. 4 A). Root length was also significantly improved with inoculated seeds under salt stress conditions. As may be seen below (Fig. 4 B), the highest root length was recorded with both BC3 and BC7 strains treated seeds.

According to the variables examined, the dendrogram providesgives the best depiction of the strains distribution into hierarchical clusters. The resulting tree's topology reveals two hierarchical clusters, one of which regrouped the three strains BC3, BC6 and BC7 that exercised a beneficial effect on the germination, seedling growth and give a clear capability to tolerate the salt stress as illustrated in Figure 5 A and B.

According to Johnson & Wax, (1978) the effect of inoculation is generally seen as an increase in germination rate and root length, two significant predictors frequently used to assess the success of the culture. Several publications have appeared in recent years (Egamberdieva et al., 2019; Rodge et al., 2016) documenting that, inoculating seeds with rhizobacteria strains significantly increases in germination rate and roots elongation. According to Egamberdieva et al. (2019) rhizobacteria might be chosen for their involvement in seed resistance to salt stress based on germination characteristics.

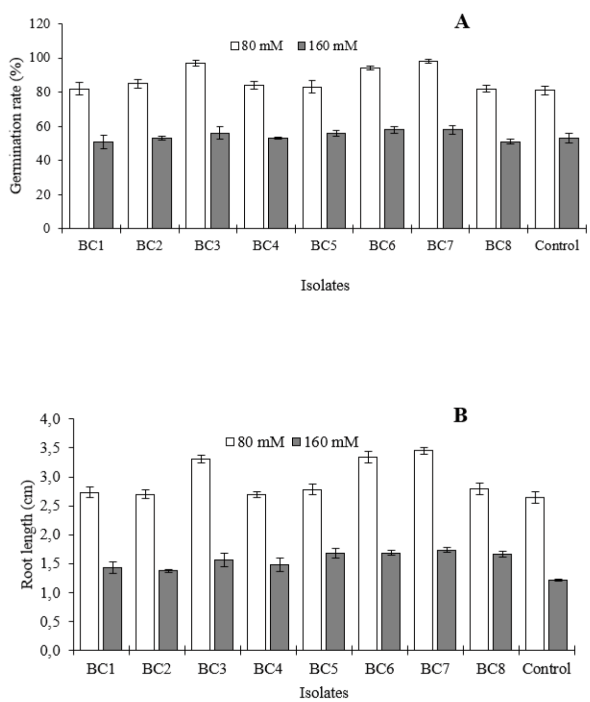

Figure 4: Inoculation effects on germination rates (A) and root length (B) under salt stress

3.3 INOCULATION EFFECTS ON WHEAT SEEDLING GROWTH UNDER SALT STRESS

3.3.1 Chlorophyll content

The results obtained for plant pigments after 60 days of stress showed that salinity leads to marked reductions in the total chlorophyll contents. In fact, the watering with the salt-water at different salinity levels induced a decrease of chlorophyll content in all treatments compared to unstressed control (Fig. 6). This reduction was less important in inoculated plants, where the total chlorophyll recorded was 28.31 and 21.13 significantly ($p < 0.05$) higher compared to 20.06 and 14.13 (in non-inoculated plants) respectively, for 80 and 160 mM.

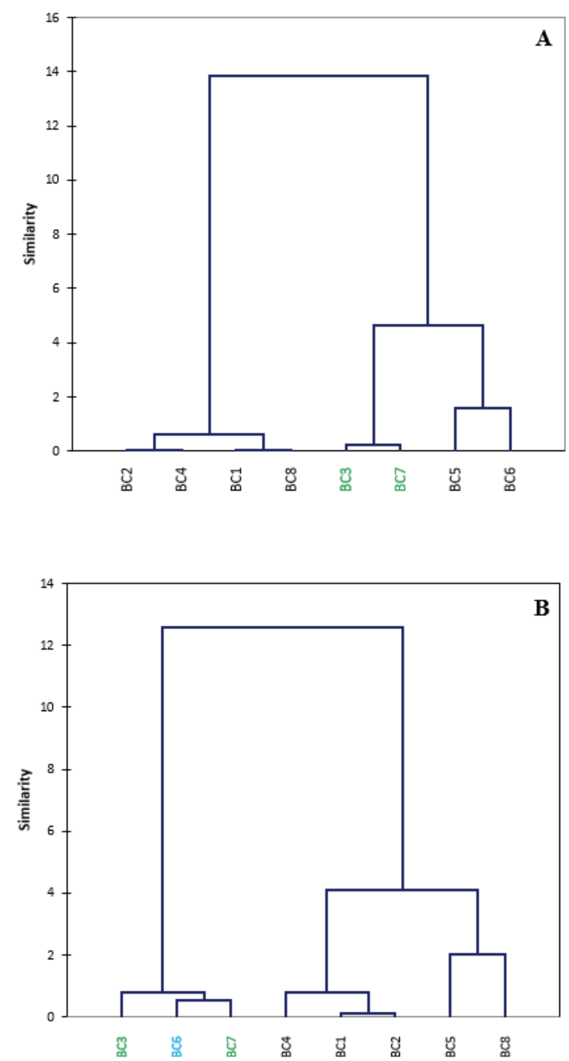

Figure 5: Dendrogram of the hierarchical ascending classification (ACH) of the inoculation effects according to the variables studied: **A**) germination rate. **B**) root length under salt stress

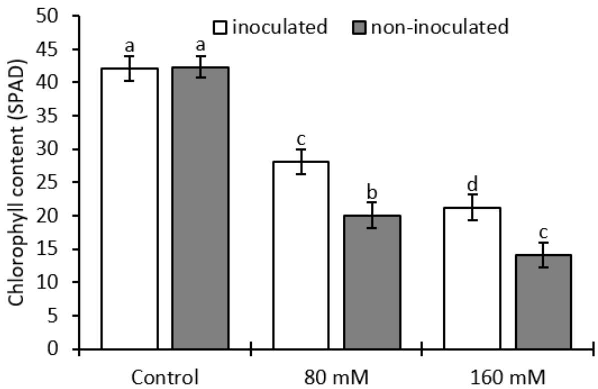

Figure 6: Effect of inoculation on chlorophyll content under salt stress. The error bars represent the standard error of mean; values followed by different letters heading the bars are significantly different ($p < 0.05$)

3.3.2 Proline content

The results obtained from pot experiments conducted in the greenhouse showe that the accumulation of proline varies according to the salinity levels and the inoculation treatment (Fig. 7).

Under different salinity levels, non-inoculated plants accumulate an important amount of proline (53.02 and 59.25 µg ml^{-1}), which is significantly different from the 48.28 and 54.91 µg ml^{-1} recorded in inoculated plants at 80 and 160 mM respectively. In this experiment, it was found that stress application resulted in non-inoculated plants losing chlorophyll content while simultaneously gaining proline content. Gharsallah et al. (2016) and Bresson, (2013) argue that this is due to the reorganization of the enzymatic function of the salt-treated plants, in fact, glutamate which is a common precursor of chlorophyll pigments and proline, is more commonly used in proline biosynthesis. According to Abiala et al. (2018), when plants are challenged by abiotic stress they frequently produce a stress response to try to mitigate the effects of the stressor and cellular solutes such as proline and sugars rise to confer desiccation tolerance.

3.3.3 Glycine betaine content

For all treatments, GB content increased with increasing salt concentration (Fig. 8), it increased from 327,83 to 361,75 µg ml^{-1} in non-inoculated plants, respectively for 80 and 160 mM. However, this increase was also significant for the inoculated plants (303,56 to 335,86 µg ml^{-1}).

Based on these findings , and the fact that salt stress in non-inoculated plants is also manifested by an increase in their GB content, it has recently been proposed by a number of authors (Amaresan et al,. 2016), that GB and proline are osmoregulators produced by a wide range of species, and are involved in stress resistance mechanisms. In fact, proline, soluble sugars, and GB accumulation, at the cellular level, during saline stress are primarily responsible for the maintenance of a high internal osmotic pressure (Chakraborty et al., 2012). This study revealed that when the salt stressed plants were inoculated with PGPR, the proline and GB accumulation did not significantly increase, indicating that they were less stressed. This may perhaps be due to a reduction in the growth inhibitory effect of salt on wheat plants through the enhanced activity of rhizospheric bacteria that can provide a variety of molecules which increases the tolerance of this plant.

Numerous microorganisms live in the rhizosphere of plants, and although for a long time, they were only thought of as diseases. This perception has been disproved by current research on the soil microbial popula-

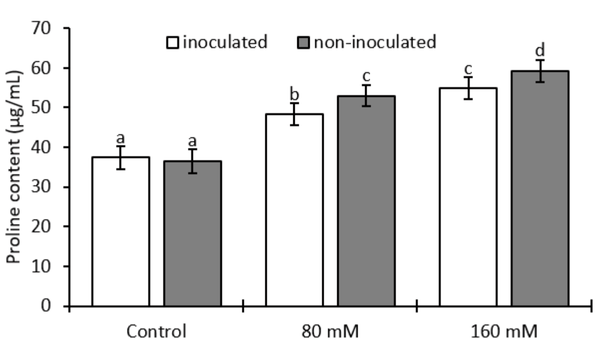

Figure 7: Effect of inoculation on proline content under salt stress. The error bars represent the standard error of mean; values followed by different letters heading the bars are significantly different ($p < 0.05$)

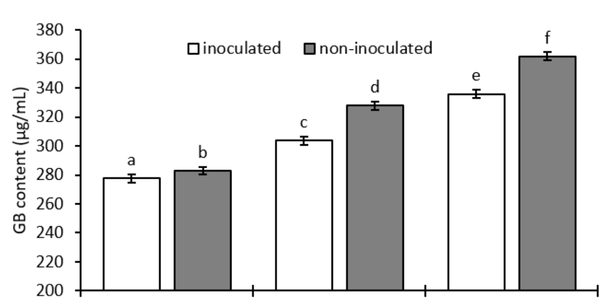

Figure 8: Effect of inoculation on glycine betaine content under salt stress. The error bars represent the standard error of mean; values followed by different letters heading the bars are significantly different ($p < 0.05$)

tion. Previous studies on the soil microbial community by Ambrosini et al., (2016) ; Bremer and Krämer, (2019) show that rhizospheric microorganisms are also capable of producing certain phytohormones andother similar compounds, which can enhance plant growth. Zerrouk et al. (2020); and Egamberdieva et al. (2019) have also found that the majority of rhizobacteria can boost plant tolerance by assisting with the uptake of specific nutrients, through symbiotic N_2 fixation, inorganic phosphate solubilization and organic phosphate mineralization.

4 CONCLUSIONS

Eight rhizobacteria's effects on the germination and the physiology status of wheat under salt stress were examined *in vitro* and in a greenhouse. Overall, the findings show that salt stress can be totally or partially offset by, inoculation with the tested rhizobacteria. We may deem the BC3, BC6, and BC7 strains as the most successful in reducing the negative effects of salt stress improvement in seed germination and a decrease in osmoregulators contents were seen, especially, after inoculation with BC3, BC6 and BC7 strains, so we can qualify them as the most effective to counteract the adverse effects of salt stress. From the research that has been carried out, we have demonstrated that inoculation with PGPR can modify the behavior of the plant and appeared as a biological solution to alleviate salt stress conditions in salinity-impacted regions mainly in arid regions.

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Dissemination of the quarantine weeds of the genus *Ambrosia* in the steppe zone of Ukraine

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Abstract: The article presents the results of many years of research for the period 2003-2020, which were aimed at studying the dissemination and expansion of plant groups of quarantine species of *Ambrosia* weeds in the eastern steppe of Ukraine. It has been established that the intensive dissemination of the species of the quarantine ragweed weed occurs both naturally and anthropically. Monitoring of the dissemination and growth of ragweed species is noted in all types of phytocenoses of the steppe. The increase in the areas of dissemination of aggressive species of ragweed weeds poses a widespread threat to all plant populations. Studies show a tendency to increase the number of ragweed plants in such plant groups as agrophytocenoses, phytocenoses of planted forests, phytocenoses of urban areas, meadows and pastures. For the period 2011-2002 the number of ragweed plants increased in meadows by 7.5 times, in pastures by 14.8 times, in agrophytocenoses by 2.95 times, in phytocenoses of urban areas by 1.68 times, in phytocenoses of planted forests by 1.28 times. Due to the lack of control over the dissemination and appropriate eradication measures, the largest increase in the number of ragweed plants over the past 10 years has been observed in meadows and pastures. It is recommended in phytocenoses of planted forests and urban areas in meadows and pastures, total mowing of ragweed plants before their flowering 5-7 times during the growing season in order to prevent replenishment of the seed stock of this weed in the soil during the growing season.

Key words: *Ambrosia*; quarantine weed; phytocenosis type; dissemination; number

Razširjanje karantenskih plevelov iz rodu *Ambrosia* v območju step v Ukrajini

Izvleček: Članek predstavlja rezultate večletne raziskave, izvedene v obdobju 2003-2020, katere namen je bil preučevanje razmnoževanja in razširjanja karantenskih plevelnih vrst iz rodu *Ambrosia* v vzhodnih stepah Ukrajine. Ugotovljeno je bilo, da poteka intenzivno razmnoževanje in razširjanje karantenskih plevelnih vrst ambrozije naravno in antropogeno. Sledenje razmnoževanja in rasti vrst ambrozije je bilo izvedeno v vseh stepskih fitocenozah. Povečanje območij razširjenosti teh agresivnih vrst plevelov predstavlja veliko grožnjo vsem populacijam rastlin. Raziskave kažejo tendenco povečanja števila rastlin ambrozije v rastlinskih združbah kot so agrofitocenoze, združbe gojenih gozdov in urbanih območij in travišča (travniki in pašniki). V obdobju 2011-2002 se je število rastlin ambrozije povečalo v travnikih za 7,5 krat, na pašnikih za 14,8 krat, v agrofitocenozah za 2,95 krat, v združbah urbanih območij za 1,68 krat in za 1,28 krat v združbah gojenih gozdov. Zaradi pomanjkanja nadzora razmnoževanja in neprimernih ukrepov iztrebljanja je največje povečanje v številu rastlin ambrozije v zadnjih 10 letih opaženo na travnikih in pašnikih. Za zatiranje ambrozije v gozdnih združbah, združbah urbanih območij, na travnikih in pašnikih je priporočena košnja ambrozije pred cvetenjem, 5-7 krat v rastni sezoni, da se prepreči nastanek semenske banke v tleh med rastno sezono.

Ključne besede: *Ambrosia*; karantenski plevel; vrste fitocenoz; razmnoževanje; številčnost

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1 INTRODUCTION

One of the factors that reduce the efficiency of technologies for growing agricultural crops is the weediness of agrocenoses of varieties and hybrids. The coefficient of harmfulness of weeds growing in agrocenoses depends on the degree of weediness of agrocenoses and on biological weed species, the rate of their growth and development (Aksyonov, 1997).

Cultivation of field crops is always accompanied by the appearance of dominant groups of weeds in their agrocenoses, which have a multifaceted negative effect on plants of varieties and hybrids, complicating the phytosanitary situation in agricultural production (Sibikeeva and Borisov, 2013).

One of the reasons for the decline in the effectiveness of weed control is the high ecological plasticity of some of them (Aksyonov, 2010).

A particularly high ecological plasticity is characteristic of plants of foreign origin, that are, quarantine weeds (Zakharenko & Zakharenko, 2004).

All over the world there is an acute problem of the expansion of alien plants, causing colossal economic damage. Their invasion leads to floristic pollution of the territory and is a serious environmental problem. They often become harmful weeds of fields and other lands, some pose a danger to human health (Abramova, 2011a).

The problem of invasions of alien weed species is relevant for Ukraine and especially for the steppe zone of the country.

The overwhelming majority of invasive species are from North America, less often East Asian or Mediterranean species.

Of the invasive species, the most dangerous are weed species from the genus *Ambrosia*.

It was revealed that ragweed species with a wide ecological range are introduced into a variety of cenoses - from one-year pioneer communities to floodplain pastures and disturbed steppes, forming a series of replacement communities (Abramova, 2011b).

Among all types of weeds of the genus *Ambrosia,* which grow in Ukraine, one of the most harmful weeds is the species *Ambrosia artemisiifolia* L., classified as a type of quarantine weed.

Ambrosia is extremely harmful. It causes biological and ecological damage to the environment and causes allergies in humans.

The current state of the flora of Ukraine, which is under the constantly growing anthropic pressure, is characterized by a change in the species composition and structure of vegetation, a significant increase in its role as an anthropophilic element. Large economic activity of human creates the preconditions for the transformation of local vegetation into depleted and less valuable, partially or completely formed from synanthropic species, many of which are quarantine weeds. The invasion and further active dissemination, and in some cases the dissemination of species of the adventive fraction of the flora, occurs both naturally and anthropically (Fisyunov et al., 1970).

The geographical location of Ukraine in Europe is favorable for various types of transport links and transportation of goods, both by land, air and sea transport. Active economic ties contribute to the intensive propagation of various groups of goods (including agricultural products) imported from other countries. But the process of delivery of imported products in the form of seeds of agricultural crops, seedlings of fruit and flowers, overshadows the fact that quarantine organisms enter Ukraine and their further dissemination and aggressive behavior is dangerous (Maryushkina, 2001).

Unfortunately, such a vivid example for Ukraine is the entry into its territory of the North American species of weeds *Ambrosia artemisifolia* L., introduced to Ukraine at the beginning of the 20th century. Weed plants of the species *Ambrosia artemisifolia* L. spread at a high speed and, therefore, it is no coincidence that they were included in the A-2 list - quarantine microorganisms that are limitedly disseminated in Ukraine (Maryushkina, 1986).

In the steppe zone of Ukraine, the emergence and dissemination of quarantine weeds poses a widespread threat, since aggressive quarantine species, such as *Ambrosia artemisifolia* L., grow in all types of phytocenoses of agricultural crops and beyond, and their dissemination areas are increasing every year.

For the first time in Ukraine, *Ambrosia artemisifolia* L. was discovered in 1914 in the village Kudashevka, Dnepropetrovsk region (German agronomist Krikker grew it as a substitute for cinchona), and in 1925 – in the vicinity of Kiev (on the territory of an elevator) (Dobrochaeva et. al., 1987).

Currently, this quarantine weed is common in all regions of the country. The largest areas of dissemination of *Ambrosia artemisifolia* L. are noted in the regions of the steppe zone of Ukraine: Zaporozhskaya – 1338.5 thousand hectares, Donetskaya – 1087.8 thousand hectares, Dnepropetrovskaya – 425.0 thousand hectares, Kirovogradskaya – 306.2 thousand hectares, Khersonskaya – 290.7 thousand hectares, Nikolaevskaya – 77.9 thousand hectares (Podberezko, 2012).

The most important task of land users, due to the high severity of weeds of the genus *Ambrosia*, is to prevent the dissemination of contamination of areas with this dangerous quarantine weed.

During flowering, each individual plant of *Ambrosia* forms billions of pollen grains (pollen), which are carried by the wind over long distances, causing hay fever in sensitive people: loss of performance is observed, swelling of the mucous eyes and upper respiratory tract appears, asthma develops. The ragweed pollen contains special proteins - antigens E and K. Through the mucous membrane, they enter the lymph and blood, causing disease. Leaf allergens cause dermatitis (Mirkin, 1986; Nadtochyi, 2007).

Weeds of the genus *Ambrosia*, as plants imported from another continent, do not have natural enemies in Ukraine (animals do not eat ragweed, agricultural crops cannot compete with ragweed in agrocenoses) and are distinguished by significant biological activity (Gavrilyuk and Aksyonov, 2013).

Introduced to new habitats, invasive weed species find themselves far from the press of their predators, parasites and diseases that could keep their populations in a balanced state. As a result of the loss of natural biological control, these types of weeds often become very harmful in those places where they have settled and spread (Neronov V. M. and Lushchekina, 2001).

Weeds of all *Ambrosia* species are able to suppress and displace not only cultivated but also wild plants, thus capturing new areas and areas. In one year, the weed of the genus *Ambrosia* can spread over an area of almost 5 km (Makoveev and Luchinskyi, 2008c.)

The economic damage from weeds of the genus *Ambrosia* in the areas of its mass distribution is extremely high.

Weeds of the genus Ambrosia consume significant reserves of productive moisture for the development and formation of a powerful aboveground mass and root system. For example, weeds of the species *Ambrosia artemisifolia* L. consume soil moisture for the formation of a unit of dry matter, on average, 2 times more in comparison with cereal crops, which leads to drying out of the soil; decrease in soil fertility, suppression of cultivated plants in crops. With a low level of agricultural technology for growing crops, ragweed in agrocenoses outgrows plants of varieties and hybrids, strongly suppresses them. This leads to a sharp decrease in productivity, and in some cases to the complete death of crops of cultivated plants. Biochemical studies have shown that plants of the genus *Ambrosia* synthesize chlorogenic and isochlorogenic acids, an ester of glucose and caffeic acid, which suppress the germination and growth of many plant species ((Maryushkina, 1986; Glubsheva and Karpushina, 2009).

The damage that genus *Ambrosia* inflicts on agriculture has a number of economic factors as well: a decrease in the quality indicators of seed, a deterioration in the quality of crop production; decrease in the productivity of pastures; an increase in additional costs for seed cleaning and the purchase of herbicides, additional agrotechnical measures in cultivation technologies, including the application of herbicides (Luchinskyi and Knyazeva, 2010b; Makoveev and Luchinskyi, 2008b).

The vector of competitive relations in agrocenoses between plants of varieties, hybrids and plants of genus *Ambrosia*, which aggravate the harmfulness of the weed, is strongly influenced by the climate of the area, the weather conditions of the growing season, and the cultivated crop. In addition, fluctuations in abundance of autonomous origin are inherent in many weeds (Coble, 1981; Cousens and Mortimer, 1995).

Even if there are two plants of the weed of the genus *Ambrosia* per 1.0 m^{-2} in the agrocenosis, the yield of soybean grain can be reduced to 15.2 % in comparison with agrocenoses in which these do not grow. A further increase in the number of genus *Ambrosia* plants in soybean agrocenoses is accompanied by significant decreases in yield. If there are 10 *Ambrosia* plants in the agrocenosis per 1.0 m^2, the soybean yield decreases up to 30.0 %. When weeds of *Ambrosia* grow in the agrocenosis of soybeans with density of 35-40 plant m^{-2}, the maximum yield decreases is 55.0-63.0 %.

Due to the low competitiveness of soybeans in relation to weeds in soybean agrocenoses, *Ambrosia* plants produce almost twice as many seeds as in sunflower and corn agrocenoses, which is one of the reasons for the low yield of soybeans in Ukraine (Storchous, 2017).

The weak competitive ability of cultivated plants to weeds, including ragweed, is one of the main reasons for the formation of low yield levels by varieties and hybrids.

Thus, it is known that the low competitiveness of oil flax to weeds is due to slow growth in the initial phases of flax development, as well as a botanical feature of oil flax plants – small leaves on the plant (Dryakhlov, 2002).

Sunflower has a relatively high competitiveness in relation to weeds. Nevertheless, one of the reasons for the decline in sunflower yields is its high infestation with quarantine weeds of the genus *Ambrosia*.

The most harmful (quarantine) weed in sunflower crops is *Ambrosia artemisifolia* L. The economic threshold of harmfulness, depending on the botanical and biological characteristics of sunflower plants, is within 5.5-8.4 weeds of *Ambrosia artemisifolia* L. plant m^{-2}. With infestation of crop plants with this weed in sunflower crops in the amount of 5 plant m^{-2}, the loss of yield when applying different doses of fertilizers increases from 0.15 to 0.41 t ha^{-1}, with the number of weeds of 10 plant m^{-2} losses increase from 0.69 to 1.09 t ha^{-1}. With an increase in the number of *Ambrosia artemisifolia* L. plants in sunflower agrocenoses to 20-30 plant m^{-2}, a decrease in the level of profitability of cultivation of this oilseed crop is noted (Luchinskyi S. and Luchinskyi V., 2010a; Makoveev et. al., 2008a).

If there are 1-2 ragweed plants in corn crops per 1 m^2, 250-500 thousand seeds get into the soil during the growing season, and more than 1 billion grains of pollen of this weed get into the air. The decrease in the yield of corn grain at this level of contamination of crops with ragweed is up to 0.7 t ha^{-1}. With an even higher density of ragweed plants in maize agrocenoses (3-5 plant m^{-2}), the yield decreases by 35.0 %.

Control over the dissemination of ragweed plants growing in agrocenoses is relevant and rather difficult. There are many effective scientific developments and developed methods for controlling ragweed using the following methods: manual (when each ragweed plant is pulled out by hand), manual and mechanical mowing, chemical, biological, agrotechnical.

The agrotechnical method is the most effective. Agrotechnical methods of controlling ragweed plants include: compliance with crop rotations, basic tillage (disc plowing of stubble after harvesting the main crop, disc loosening of the soil in 2-3 tracks, plowing, cultivation as weeds grow, etc.), pre-sowing tillage (cultivation and harrowing as weeds emerge in the cotyledon phase).

Unfortunately, the use of agrotechnical and other methods of combating ragweed does not provide a high efficiency of suppression of this type of weed in agro- and phytocenoses.

Given the exceptional viability, resistance of ragweed to unfavorable environmental conditions, the incomplete effectiveness of measures aimed at suppressing ragweed plants, it becomes necessary to conduct studies on the spread and control of weeds of the genus *Ambrosia* in the steppe of Ukraine.

2 MATERIALS AND METHODS

In order to study cultivated plant groups and establish the abundance and dissemination of weeds of the genus *Ambrosia* in the Steppe of Ukraine, long-term studies were carried out during 2003-2020.

The survey of the species composition of weeds and the ways of weediness and soil infestation was carried out using the route-expedition method.

The species composition of weeds growing in fields, gardens, parks, forest belts, meadows, and pastures, their seed productivity and abundance were studied using conventional methods (Ivashchenko, 2001; Kamyshev, 1970; Fisyunov et al., 1974; Fisyunov, 1983).

During the survey of the species composition of weeds, phytocenoses were classified into:

agrophytocenoses – fields, vegetable gardens;

sylvophytoculturecenoses – artificial forest plantations, forest protective belts (phytocenoses of planted forests);

urbophytoculturecenoses – parks, gardens, flower beds (phytocenoses of urban areas).

Meadows and pastures were identified as separate cultural phytocenoses.

The abundance of weed plants in culture phytocenoses was determined by the two most common methods:

- the number of weed species was assessed visually (a point scale of N. F. Komarov was applied);
- direct counting of the number of weed specimens per unit area (1.0 m^2).

When studying the species diversity of weeds, the classical comparative ecological and morphological method was used, based on the analysis of mass herbarium material, observations and accounting in nature (Siniwardana, 1984).

The species composition of weeds was determined using atlases and keys (Kondratyuk, 1985; Maysuryan, 1978).

About 260 short-term expeditionary route trips were made, about 500 sheets of herbarium were collected, stored at the Lugansk National University named after Taras Shevchenko.

The survey of culture phytocenoses was carried out during the periods:

- the beginning of the growing season (spring);
- mid-summer;
- end of the growing season (autumn).

3 RESULTS AND DISCUSSION

When examining the dissemination areas of ragweed, it was found that three species of weeds of the genus Ambrosia grow in the Steppe of Ukraine: ragweed *Ambrosia psilostachya* L., ragweed *Ambrosia trifida* L., ragweed *Ambrosia artemisiifolia* L.

The study of the dissemination areas of weeds of this genus made it possible to carry out a botanical description of the plants of the established ragweed species.

Ragweed *Ambrosia psilostachya* L. is a perennial plant up to 180 cm high with creeping rhizomes, characterized by the highest frost resistance.

Seedlings with a developed, thickened hypocotyledonous part, colored in reddish-purple color of various shades.

Leaves are opposite and alternate, deeply divided or pinnately dissected. The first leaves are opposite, whole or divided. Lobes of leaves lanceolate or linear-lanceolate, acute, pointed, serrated.

Stem straight, branched, rounded, rough. The surface of leaves, stems and branches is covered with short, stiff hairs.

Flowers are heterosexual, form heads, collected, in turn, in a brush. Male flowers are larger with a bell-shaped corolla, tightly collected in a brush 7.0-15.0 cm long, include up to 100 heads. The anther is oblong with a curved tip at the apex. Female flowers are few, single. The female flowers are found in the axils of the upper leaves or at the base of the male inflorescences.

Head inflorescences in brushes. Shape of achene cover (false seed) are converse ovoid with a blunt nose at the top. The surface of the achene is heavily pubescent with green, easily abraded hairs, wrinkled and tuberous. The color of the achene is dark brownish-greenish or dark gray.

Plants of this ragweed species survive in the most difficult growing conditions. The propagation of plants by root suckers and seeds leads to a constant expansion of the growing areas of the weed.

Ragweed of *Ambrosia trifida* L., is an annual plant capable of rapidly growing green mass. Plant height from 1.0 to 3.0 m.

Weed seedlings with spoon-shaped or elliptical cotyledons, which are 1.2-4.0 cm long and 0.6-1.5 cm wide. The stem below the cotyledons is brilliant green with purple spots; first pair of leaves lanceolate with serrated edges; the second pair is deeply three-lobed and roughly hairy.

Leaves opposite from below, alternate from above; three-five-lobed, distinctly three-lobed, with sometimes oblong-lanceolate lobes. The edges are coarsely toothed; surface with a rough sandy texture.

The stem is straight, furrowed, slightly branched, coarse-haired, up to 3.0-4.0 cm thick, woody by the end of the growing season. Covered with short, coarse, coarse hairs.

The flowers are male or female, greenish. Male inflorescences in the form of brushes, located in the terminal ears, each of them is surrounded by 5-12 bracts and has three noticeable black ribs. Length up to 10.0 cm. Female flowers are one-flowered, located in clusters of one to four at the base of male flowers or in the axils of leaves, are 0.6-1.3 cm long. Flowers are pollinated by the wind, producing a large amount of pollen.

Achenes with a smooth surface, ovoid, grayish-brown, having a length of 0.6-0.8 cm, a width of 0.2-0.3 cm. The surface is smooth.

The root has a rod-like shape, branched.

Giant ragweed *Ambrosia trifida* L. was discovered somewhat later than common ragweed plants, but quickly spreads over many regions of Ukraine. Seeds are spread across the territory of Ukraine with grain, which is supplied from the southern, steppe regions. Three-part ragweed infests spring cereals, row crops, forage grasses, vegetable gardens, orchards, meadows. It grows abundantly on moist soils and low relief areas along the banks of rivers, gullies, ravines, floodplain lands, on the sides of railways, highways and dirt roads.

Ragweed of *Ambrosia artemisiifolia* L. is similar in appearance to common wormwood (*Artemisia vulgaris* L.). *Ambrosia artemisiifolia* L. is an annual, heat- and light-loving, drought-resistant plant. The height of the weed plants is from 0.2 m to 2.0 meters. Under favorable conditions, the plants reach a maximum height of 2.0 m.

This type of ragweed is propagated by achenes.

The upper leaves of *Ambrosia artemisiifolia* L. plants are alternate, feathery, dark green in color. The lower leaves are opposite, twice pinnately dissected, with elongated-lanceolate areas, pubescent below, have a bluish color.

The plant stem is strong, straight, spreading, branched in the upper part and has pubescence.

Flowers are dioecious, small, yellow in color, collected in dioecious green heads. Male flowers form cluster-like inflorescences located at the ends of stems and twigs. The female flowers are placed one at a time in the leaf axils or under the male inflorescences. Receptacle bristly-scarious.

The fruit is an achene. The achene is located inside the envelope and has an inverse ovoid shape. The achenes mass of 1000 is 1.5-2.0 g.

The root system of *Ambrosia artemisiifolia* L. is strong, pivotal, highly branched, and goes deep into the ground to a depth of 4 meters or more.

Ambrosia artemisiifolia L. infests all field crops, occurs in gardens, forest edges, household plots, along roadsides and ditches.

It was found that under the conditions of the Steppe zone of Ukraine, in particular in the northeastern part of the steppe, the species of the weed *Ambrosia artemisiifolia* L. grows in all types of cultivated communities that have been studied. The number of *Ambrosia artemisiifolia* L. plants varied depending on the type of plant grouping and the time of counting.

During the research during 2003-2020 there is an increase in the number of plants of *Ambrosia artemisiifolia* L. in all studied cultural phytocenoses of the eastern Steppe of Ukraine both in the first and in the second half of summer. The level of infestation of phytocenoses with *Ambrosia artemisiifolia* L. was determined by the type of phytocenosis and the period of the growing season of plants in phytocenoses. The level of emergence of ragweed plants was significantly lower in 2003-2010. The smallest number of plants of the quarantine species of the weed of *Ambrosia artemisiifolia* L. is observed in meadows and pastures. On average for the period 2003-2010 the number of plants of this kind of weed in meadows was 4 plant m^{-2} in the first half of summer, 7 plant m^{-2} in the second half of summer, 1 and 5 plant m^{-2} on pastures, respectively (Table 1).

The weediness of meadows and pastures with the weed *Ambrosia artemisifolia* did not exceed 1 point. The frequency of occurrence of this species of weeds in meadows and pastures was very random.

Compared to the phytocenoses of meadows and pastures, the level of the number of ragweed plants was higher in agrophytocenoses, and in phytocenoses of planted forests and phytocenoses of urban areas it was at maximum. So, in the second half of growing season, the number of ragweed plants in 2003-2010 in agrophytocenoses was 24 plant m^{-2}, phytocenoses of planted forests 142 plant m^{-2}, and in phytocenoses of urban areas 128 plant m^{-2}. The number of ragweed plants in phytocenoses of planted forests and urban areas exceeded the number of weeds in agrophytocenoses, respectively, by 591.7 % and 533.3 %.

In surveys carried out for the period 2011-2020 a clear tendency of an increase in the dissemination of ragweed was established in all types of phytocenoses. Especially during this period, the maximum number of plants of this quarantine weed, 216 plant m^{-2}, was noted in phytocenoses of urbana areas in the second half of growing season.

The agrotechnical methods used in the technologies of growing field crops do not fully ensure the effectiveness of suppressing ragweed plants in agrophytocenoses. The average number of plants in these years of study in the second half of summer in agrophytocenoses was 71 plant m^{-2}.

The dynamics of the increase in the number of plants of the quarantine ragweed weed in all studied types of phytocenoses is shown in Figure 1.

In comparison with the period 2003-2010 the largest increase in the number of quarantine weed of *Ambrosia artemisiifolia* L. for the period 2011-2020 was noted in meadows and pastures. The lack of control over the spread of ragweed in these phytocenoses and, accordingly, the absence of measures to combat ragweed has led over the past 10 years to the uncontrolled spread of quarantine weeds in meadows. The number of weeds in meadows increased 7.5 times (or 751 %), on pastures - 14.8 times (or 1480 %).

The rates of dissemination of ragweed in agrophytocenoses remain quite high. The number of plants of *Ambrosia artemisiifolia* L. in agrophytocenoses for the period 2011-2020 increased 2.95 times (or 295 %). Until 2011, this type of quarantine weed in agrophytocenoses was quite rare and grew mainly on the sides, in some farms of the region, then already in 2011-2020. According to our monitoring data, ragweed plants were identified in stands of row crops, spring grain crops and even in agrocenoses of winter wheat, the most competitive agricultural crop in relation to ragweed among all crops grown in the eastern Steppe of Ukraine.

With the maximum number of plants of *Ambrosia artemisiifolia* L. in phytocenoses of planted forests and urban areas, it is observed for the same period of time 2011-2020 the smallest increase in the number of this type of quarantine weed. The increase in ragweed weeds in phytocenoses of planted forests was 1.28 times (or 128 %), in phytocenoses of urban areas – 1.68 times (or 168 %).

Apparently, the wider dissemination of the quarantine species of the weed of *Ambrosia artemisiifolia* L. in phytocenoses of planted forests is constrained by competitive relations between the main plant components of artificial forest plantations, forest protective belts and ragweed plants. However, this type of weed has already significantly entrenched itself in phytocenoses of planted forersts and a significant stock of ragweed seeds in the soil is capable of creating both high potential and actual weediness of agrocenoses. The close placement of artificial protective forest plantations near crop rotation fields is a significant factor that contributes to an increase in ragweed infestation of fields, since sufficient control over the growth and distribution of ragweed in phytocenoses of planted forests is not carried out. The ongoing control

Table 1: The level of infestation of phytocenoses with *Ambrosia artemisiifolia* L. of the eastern Steppe of Ukraine, plant m^{-2}

	Average for 2003-2010		Average for 2011-2020	
Phytocenosis type	first half of the growing season	second half of the growing season	first half of the growing season	second half of the growing season
agrophytocenoses	8	24	27	71
sylvophytoculturecenoses	79	142	136	175
urbophytoculturecenoses	64	128	98	216
meadows	4	7	23	53
pastures	1	5	31	74

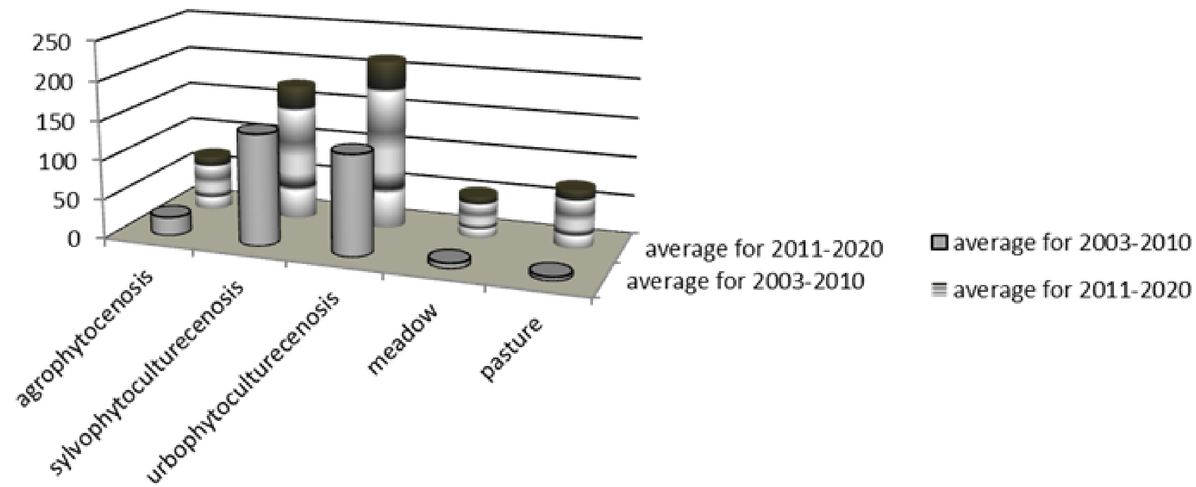

Figure 1: Dynamics of an increase in the number of ragweed plants in phytocenoses of the eastern steppe of Ukraine for the period 2003-2020 (data from the second half of the growing season are given)

measures to suppress ragweed in agrophytocenoses are clearly insufficient to reduce the level of the presence of *Ambrosia artemisifolia* L. in crop rotation fields.

In urbophytoculturecenoses, the wider dissemination of *Ambrosia artemisiifolia* L. is limited to manual measures used in parks, gardens, flower gardens by the population to control this type of weeds, as well as, to a certain extent, by the competition for survival factors between the main components of parks, gardens with ragweed plants.

Nevertheless, the level of the number of plants in phytocenoses of planted forests and urban areas shows that these types of plant communities in the eastern steppe of Ukraine are the main sources of the uncontrolled distribution of ragweed.

If in the fields, it is possible to suppress *Ambrosia artemisiifolia* L. plants with the help of glyphosate herbicides, then in parks and settlements the problem of quarantine weed control is not always effectively solved. At the beginning of the period of appearance of ragweed species, when they grew as single individuals in phytocenoses of planted forests and urban areas, no attention was paid to the plants of these weeds. As the growing areas expanded and the number of weeds of various ragweed species increased, ragweed became widespread. As a result of the spread of ragweed pollen during the flowering period, the population in cities and villages began to suffer massively from hay fever. Today, it is not easy to eradicate the quarantine weed *Ambrosia artemisiifolia* L. completely, because the soil has significant reserves of seeds that remain viable for more than 50 years, and introduced natural pests are ineffective in destroying ragweed, and the weed itself plastically adapts to local conditions.

In the initial period of the appearance of ragweed in the Ukrainian Steppe, herbologists attributed this type of weeds to the early spring type of weeds. Our long-term observations show that, depending on the weather conditions of the growing season in the eastern steppe of Ukraine, seedlings of ragweed plants appear simultaneously with the emergence of late spring weeds or in 1-2 weeks after the appearance of their seedlings. In the second half of summer, the quantitative and population indicators of ragweed species increase significantly, especially in those territories where systematic measures of control over the number and state of ragweed populations are not applied.

One of the preventive effective methods of suppressing the growth of weeds of all species of plants of the genus *Ambrosia* in agrophytocenoses is the observance of crop rotations and the requirements for the implementation of agrotechnical techniques in the technology of growing crops; in sylvophytoculturecenoses, urbophytoculturecenoses, in meadows and pastures, the main method of combating ragweed plants is the constant total mowing of the plants of this quarantine weed before their flowering in order to prevent the spread of weeds and replenish seed stocks in the soil.

4 CONCLUSIONS

1. The studies have shown that the agrobiological features of the extremely aggressive quarantine species of genus *Ambrosia* contribute to the rooting and further spread of its populations in new territories of the eastern steppe of Ukraine. These species form almost mono-

dominant communities, displacing native weed species in plant populations.

2. The spread of ragweed species is determined by the hardiness of the quarantine weed plants, adaptability to growing conditions and to conditions of persistent drought, which is consistently characteristic of the second half of the growing season in the steppe of Ukraine.

3. The absence of proper control over the growth and distribution of ragweed species in phytocenosesof planted forests leads to an increase in the number of ragweed plants in agrophytocenoses of agricultural crops.

4. *Ambrosia artemisiifolia* L. is no longer a quarantine species, which includes restrictedly distributed species. Currently, this weed is disseminated unlimitedly, it is a cosmopolitan, measures to limit the expansion of ambrosia are not yet effective.

5. In addition to constant monitoring of phytocenoses of planted forests and urban areas, meadows and pastures (groups where chemical methods of weed control cannot be applied), with the growth of plant of genus *Ambrosia*, constant total mowing of plants before flowering is one of the effective and recommended ways to control and prevent the further spread of this invasive species of weed.

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Classification of pomegranate cultivars by multivariate analysis of biochemical constituents of HPLC

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Classification of pomegranate cultivars by multivariate analysis of biochemical constituents of HPLC

Abstract: Pomegranate fruits are highly diverse and may be divided into geographical groupings based on their characteristics. Genetic research has verified these categories in recent years and further categorized variants into geographic-genetic groupings. This study aimed to assess the biochemical contents of eight varieties of pomegranate fruit seed and the categorization of pomegranate using multivariate statistical analysis. Polyphenolic chemicals are key secondary metabolites in pomegranate, and their presence influences the quality and sensory qualities of the fruit they produce. Fruit extracts from the Faqyan cultivar contained the highest total phenolic content of all studied cultivars. Pomegranate cultivars such as Shaqlawa, Halabja Sour, and Faqyan were shown to have the highest antioxidant activity. Gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, cinnamic acid, rutin, apigenin, rosmaric acid, and quercetin were the most abundant phytochemical components in the study. According to the results of multivariate analysis, pomegranate cultivars were divided into four major groups. The pomegranate fruit seed is the most abundant source of antioxidants and beneficial phytochemical elements. Finally, the Sidakan Sweet and Shaqlawa cultivars included a significant content polyphenolic compounds.

Key words: antioxidant; gallic acid; chlorogenic acid; *Punica granatum*, phenolic compounds

Razvrščanje sort granatnega jabolka z multivariatno analizo biokemičnih sestavin izmerjenih s HPLC

Izvleček: Plodovi granatnega jabolka so zelo raznoliki in jih lahko razdelimo v geografske skupine na osnovi njihovih lastnosti. Genetske raziskave so v zadnjih letih potrdile te skupine in jih nadalje razčlenile v geografsko genetetske podskupine. Namen te raziskave je bil ugotoviti biokemično sestavo plodov osmih sort granatnega jabolka z multivariatno analizo. Polifenoli so glavna sestavina sekundarnih metabolitov v granatnem jabolku, njihova prisotnost vpliva na kakovost in senzorične lastnosti plodov. Izvlečki plodov iz sorte Faqyan so vsebovali največ polifenolov izmed vseh preučevanih sort. Sorte granatnega jabolka kot so Shaqlawa, Halabja Sour in Faqyan so se izkazale z največjo antioksidacijsko aktivnostjo. Galna, kavna, klorogenska, *p*-kumarna, cimetna in rožmarinska kislina, rutin, apigenin, in kvercetin so bile najboj pogoste fitokemične komponente v tej raziskavi. Na osnovi multivariatne analize so bile sorte granatnega jabolka razdeljene v štiri glavne skupine. Sočen ovoj semen granatnega jabolka je najbolj pogost vir antioksidantov in drugih ugodnih sestavin. Sorti Sidakan Sweet in Shaqlawa sta se izkazali z značilno veliko vsebnostjo polifenolov.

Ključne besede: antioksidanti; galna kislina; klorogenska kislina; *Punica granatum*; fenolne spojine

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1 INTRODUCTION

The pomegranate (*Punica granatum* L.) which is originally described throughout Mediterranean region is a fruit crop that belongs to the Punicaceae family and has gained popularity in recent years due to its multi-functionality and high nutritional value when consumed as part of a healthy diet. In present it is grown worldwide in a variety of geographical regions, meeting the nutritional and medicinal requirements of people in a variety of countries. (Holland et al., 2009). Countries such as Iran, India, Egypt, China, Israel, Tunisia, Syria, Lebanon, Turkey; Greece; Cyprus; Italy; France; Spain; Chile; Portugal; the United States; Oman; and most recently, South Africa are growing in commercial pomegranate orchards a variety of pomegranate cultivars (Caleb et al., 2012; Holland et al., 2009). Among the many nutrients found in the edible part of the fruit are high concentrations of acids, sugars, polyphenolics, and essential minerals (Al-Maiman & Ahmad, 2002). The fruit is most commonly consumed as fresh arils or processed products, primarily juice. The color of the pomegranate fruit's outer peel does not indicate how ripe it is or whether if it is ready for consumption (Holland et al., 2009). Fruit quality assessment and classification are frequently based on factors such as the color of the aril, the total soluble solids content, and the presence of organic acids (Cristosto et al., 2000; Martinez et al., 2006). The extraordinarily high antioxidant capacity of pomegranates, directly correlated with the high amount and unique composition of phenolic compounds in the fruit, is credited with the fruit's favorable health properties. (Borochov-Neori et al., 2011; Fischer et al., 2011; Gil et al., 2000).

Pomegranate seeds, which are a byproduct of pomegranate juice manufacturing, include a variety of nutraceutical components, including sterols, alpha-tocopherol, punicic acid, and hydroxybenzoic acids, among other substances (Kwan & Kowalski, 1978), suggesting that the extracts of pomegranate seed residue might be used as a nutraceutical resource, according to the research (Revilla & González-SanJosé, 2002) On the other hand, according to some researchers Pomegranate aril and juice contain a significant amount of polyphenolics, primarily composed of ellagitannins (punicalagin), gallic acid, ellagic acid, anthocyanins, catechins, caffeic acid, and quercetin derivatives. (Viuda-Martos et al., 2010). The amounts of these substances are dependent on the cultivars, the environment conditions, of the growing location where pomegranate ochards are established (Melgarejo et al., 2000). Many different pomegranate cultivars from Iran, Turkey, the United States, Italy, and South Africa have been investigated thus far for their polyphenolic content in their juice. However, prior to this study, no profile comparison of polyphenol composition and antioxidant capacity in sweet and sour pomegranates from different regions of Iraq's Kurdistan region was conducted.

Kurdistan is a regional supplier of pomegranate, and it has more favorable growing circumstances than any other region where the fruit crop is grown.

Pomegranates grown in the neighborhood are diverse and have been adapted to the many natural states of Kurdistan's environment. As a result, the loss of genetical diversity in crop species as a result of commercialization has prompted the need to safeguard the genetic resources that are currently available. Unfortunately, there is currently no information available on the diversity in chemicalcomposition of pomegranate fruit in Kurdistan, aside from a recent article by Mohammad et al. (2018) that looked at the physic-chemical properties of selected fruit cultivars at commercial harvest. In this study, the goal was to develop; using chemical analyses, as a classification model that would allow to classify Kurdistan pomegranates according to cultivars without regard to the effects of climate or geographical origin, and that would be based on the phenolic composition of the seed arils of pomegranates.

2 MATERIAL AND METHODS

2.1 POMEGRANATE SAMPLES

Fruits of selected cultivars of pomegranate (*Punica granatum* L.) (4 kg) were manually collected at commercial maturity from commercial Kurdistan region vineyards in the eight geographical regions (see Table 1). Samples were transported on ice and stored at -20 °C until required and destemmed while frozen.

2.2 POLYPHENOLICS

2.2.1 Extraction and analysis of polyphenolics:

In order to extract polyphenolics, 2 g of the powdered sample was removed and after adding 4 ml of methanol solvent containing 1 % acetic acid, the extraction process was performed under ultrasonic waves for 20 minutes. The phenolic acids studied in this study were isolated, identified, and quantified applying HPLC (high-performance liquid chromatography) device model 1100 series (Agilent USA), prepared with an injection loop of 20 microliters, four solvent gradient pump, degassing system, Column oven (set at 25 °C), and diode array detector, set at 250, 272 and 310 nm, respectively. Isolation on the Ceylon octadecyl column (inner diameter 4.6 mm,

Table 1: Sampling locations of the different pomegranate cultivars studied

Cultivar	province	Height (m)	Latitude	Longitude
'Sidakan Sweet'	Erbil	1502.0	36.5579	44.8283
'Sidakan Sour'	Erbil	777.0	36.6085	44.5239
'Shaqlawa'	Erbil	975.0	36.7990	44.6704
'Balakayati'	Erbil	1145.0	36.4098	44.3201
'Halab Sweet'	Sulaymaniyah	928.0	36.2995	44.4136
'Halab Sour'	Sulaymaniyah	927.0	36.4098	44.3202
'Faqyan'	Erbil	870.0	36.54	44.539
'Hiran'	Erbil	650.0	36.283	44.496

length 25 cm, a particle size of 5 micrometers ZORBAX Eclipse XDB). In order to process the data, Chemstation software was applied.

2.3 TOTAL ANTIOXIDANT

Total antioxidant was measured according to the method of Brand Williams et al. (1995) (Brand-Williams et al., 1995) by DPPH standard.

2.4 TOTAL PHENOLIC CONTENT

Following the colorimetric oxidation/reduction reaction of phenolics, the total phenolics content was measured using the Folin Ciocalteau technique described by Singleton et al. (1999), with minor modifications. Polyphenolics were extracted by adding 10 ml 85 percent methanol to 1 g fine powder of samples and mixing thoroughly. After that, by the addition of 2.5 ml of Folin-Cicalteau reagent and 2 ml of 7.5 % sodium carbonate. For 1.5 to 2 hours, the samples were shaken vigorously. A spectrophotometer was used to measure the absorbance of the samples at 765 nm (PG Instruments T80 UV, UK). The calibration curve was created using gallic acid. The results were presented in milligrams of GAE per 100 grams of fresh mass (FM).

2.5 STATISTICAL ANALYSIS

SAS 9.2 software was used to analyze all of the data (SAS, 2009). A one-way analysis of variance (ANOVA) was performed, and significant differences between groups were determined using Tukey's multiple range tests at a $p \leq 0.05$. In addition, distinct genotypes were classified based on the presence of phytochemical substances. In order to analyze the variables, principal component analysis (PCA) and hierarchical cluster analysis (HCA) were carried out with the help of the Excel spreadsheet program XLSTAT (2018).

3 RESULT AND DISCUSSION

3.1 HPLC ANALYSIS OF THE SAMPLES

Figure 1 demonstrates the chromatogram of nine standards that were injected into an HPLC system. Between the eight cultivars studied in this study, the amounts of specific phenolic acid acids (gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, cinnamic acid, and rosmaric acid), as well as flavonoids (rutin, apigenin, and quercetin), were found to be considerably different (Table 2). Gallic acid, chlorogenic acid, and coumaric acid were found as the most abundant phenolic compounds in the extracts of grape fruits. The high concentrations of gallic acid (68.6 mg kg^{-1}), caffeic acid (19.2 mg kg^{-1}), chlorogenic acid (202.2 mg kg^{-1}), *p*-coumaric acid (74.25 mg kg^{-1}), cinnamic acid (3.3 mg kg^{-1}), rutin (6.12 mg kg^{-1}), apigenin (11.26 mg kg^{-1}), rosmaric acid (2.51 mg kg^{-1}) and quercetin (10.17 mg kg^{-1}) were obtained in 'Sidak Sweet', 'Hiran', 'Shaqlawa', , and 'Balakayati' pomegranate seed fruit extracts, respectively. The highest concentrations of gallic acid, caffeic acid, rutin, and apigenin were observed in Sidak Sweet cultivar fruit extracts and p-coumaric acid, rosmaric acid, and cinamic acid concentration in 'Shaqlawa' fruit seed aril grown in Erbil province.

3.2 ANTIOXIDANT ACTIVITY

According to Figure 2, the antioxidant activity was influenced by both the cultivar and the samples' location. According to the results, the highest antioxidant activity was achieved from 'Shaqlawa' fruit extract (80 %), and

Table 2: Content of biochemical compounds in fruits of different pomegranate cultivars

Cultivars	Gallic acid (mg kg-1)	Caffeic acid (mg kg-1)	Chlorogenic acid (mg kg-1)	Rutin (mg kg-1)	p-Coumaric acid (mg kg-1)	Rosmaric acid (mg kg-1)	Quercetin (mg kg-1)	Cinnamic acid (mg kg-1)	Apigenin (mg kg-1)
Sidak Sweet	68.6a	19.2a	95.7c	6.12a	28.08c	0.78f	3.29e	0.57d	11.26a
Sidak Sour	39.45b	10.77b	56.88e	0.72d	10.85e	0.36g	1.77g	o.31g	2.9f
Shaqlawa	28.5c	2.91e	136.5b	0.6f	74.25a	2.51a	9.99b	3.3a	7.83b
Balakayati	28.35d	1.92f	82.26d	3.24b	28.15b	1.06d	10.17a	0.86c	6.02d
Halab Sweet	13.32e	0.26h	18.51f	0.1g	6.71g	1.35b	1.28h	0.54e	2.48g
Halab Sour	13.23f	0.55g	19.97g	0.06h	4.47h	1.17c	2.81f	0.47f	3.79e
Faqyan	11.75g	4.66c	11.96h	0.67e	10.15f	0.3h	4.64c	1.1b	6.89c
Hiran	11.31h	3.73d	202.25a	0.93c	11.69d	1e	3.68d	0.31g	1.49h

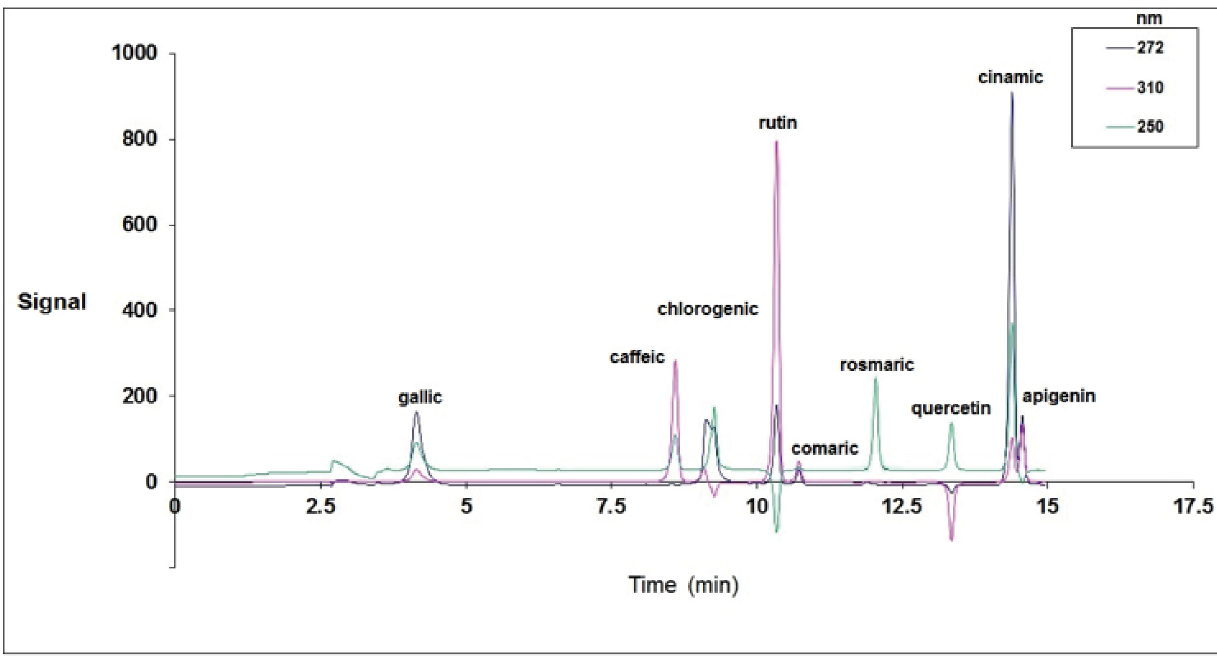

Figure 1: HPLC chromatograms of nine biochemical standards

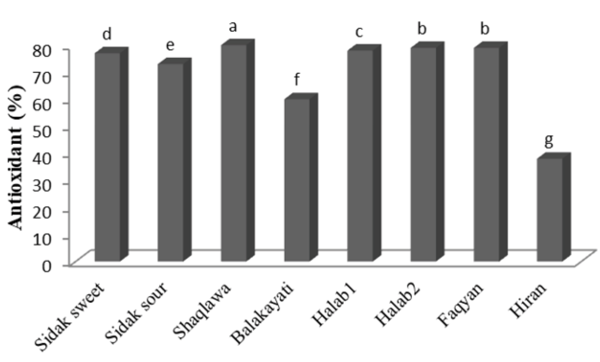

Figure 2: Antioxidant activities of different cultivars of pomegranate seeds by DPPH assay

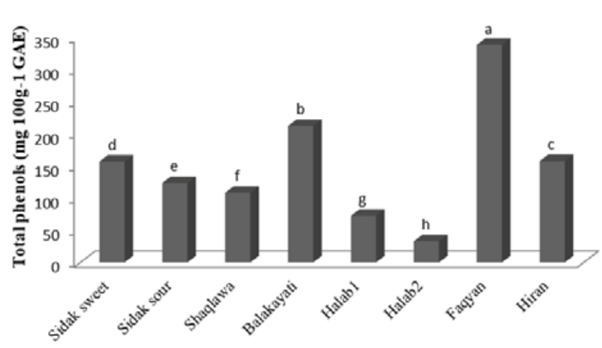

Figure 3: Total Phenolic content (TPC) of different cultivars of pomegranate seed

the lowest antioxidant activity was found in 'Hiran' fruit extract (38 %).

3.3 TOTAL PHENOLIC CONTENT (TPC)

The TPC values of fruit extracts of pomegranate seed cultivars are presented in Figure 3. The amount of TPC in the fruits extracts obtained varied from 33 mg GAE 100 ml^{-1} extract in Halabja sour cultivar to 338.2 mg GAE 100 ml^{-1} extract in Faqyan cultivar. Results showed that TPC of extracts was influenced significantly by sampling location.

3.4 CLASSIFICATION OF POMEGRANATE CULTIVARS

HCA, PCA and were performed to classify the pomegranate cultivar regarding the 11 main traits (TPC, DPPH, gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, cinnamic acid, rosmaric acid, rutin, apigenin, and quercetin). The Ward linkage method carried out the cluster analysis (Figure 4). Based on this analysis, the pomegranate cultivars were classified into four main clusters group. In the first cluster, cultivars of 'Faqyan' were designated as the cultivars with the highest TPC. In the second cluster, a cultivar of 'Halabja' sweet and 'Halabja' sour were determined due to high amounts of antioxidant capacity. As a result of significant concentrations of gallic acid, chlorogenic acid, *p*-coumaric acid, and TPC, the 'Balakayati', 'Sidakan Sweet', and 'Sidakan Sour' were found in the third cluster. Finally but not least, the cultivar of Shaqlawa and Hiran was found to have a significant concentration of chlorogenic acid, coumaric acid, and TPC, which contributed to its classification as the fourth cluster.

PCA graph is shown in Figure 5. A PCA was performed, which reduced the multidimensional structure of the data and produced a two-dimensional map that could be used to explain the observed variation. 65.1 % of the total variation could be attributed to the first two components of the PCA (35.06 % for component 1 and 30.05 % for component 2). Correlations between the first component (PC1) and coumaric acid, quercetin, cinnamic acid, and apigenin are extremely strong posi-

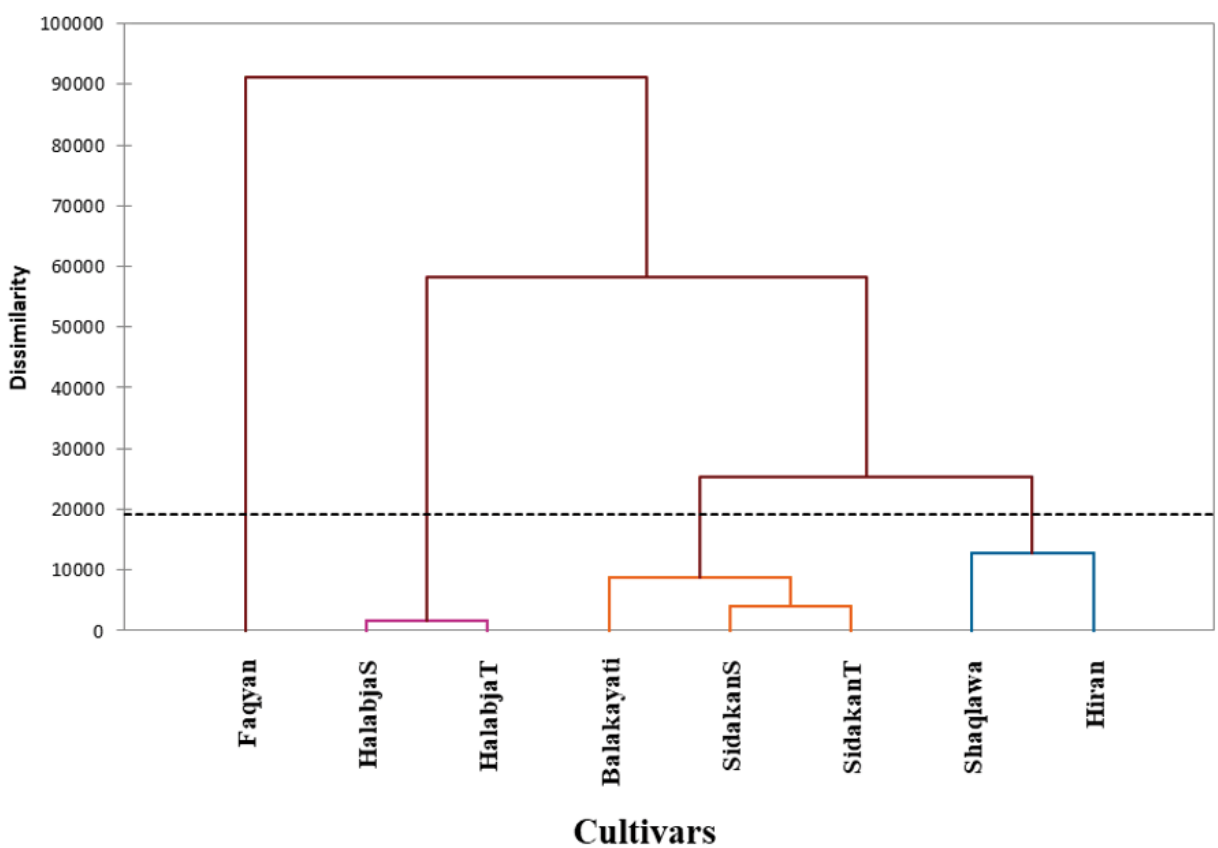

Figure 4: Hierarchical cluster analysis (HCA) of pomegranate cultivars based on the 11 main traits

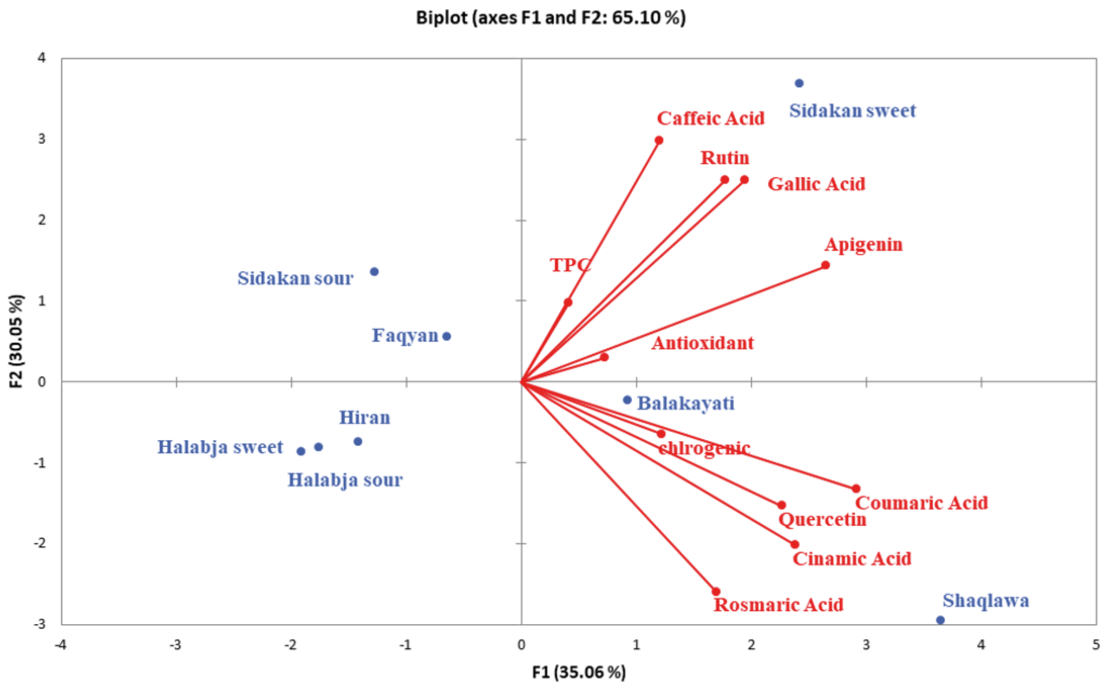

Figure 5: Principal component analysis (PCA) of pomegranate cultivars based on the 11 main traits

tive. Gallic acid, caffeic acid, and rosmaric acid are the primary constituents of the second principal component (PC2), separating the samples.

4 DISCUSSION

Results exhibited considerable variation in genotypes. According to previous research, the levels of polyphenolic compounds of gallic acid, chlorogenic acid, and caffeic acid in pomegranates grown in Chinese farms have been reported in 70-1400, 900-4800, and 1100-2440 mg kg^{-1} DM, respectively (Li et al., 2015). The total phenolic content (TPC) of the juice samples ranged from 0.87 to 1.93 mg of gallic acid equivalents per mL (Russo et al., 2018). Other researchers have also discovered inconsistencies in the concentration of particular phenolic acids in different cultivars of the same plant (Fischer et al., 2011; Gundogdu & Yilmaz, 2012; Lansky & Newman, 2007). These differences were generated not only by variations in cultivar, growing region, and maturity level of the pomegranates under investigation but also by variations in the analytical procedures employed. It is possible to endogenously regulate the biosynthesis of the phenolic composition of fruits during various developmental variations in their life cycle (Gholizadeh-Moghadam et al., 2019) which exogenous agents can influence metabolism pathways. It is obvious that the antiradical activity against DPPH and ABTS radicals increases along with the overall phenolic concentration regardless of the matrix. This is something that can be observed. This indicates that the phenolic compounds that possess antiradical characteristics are the ones that were discovered in the samples (Russo et al., 2018). Exogenous factors such as environmental conditions (temperature, biotic and abiotic stress, light intensity, humidity) and agricultural practices (soil fertility, irrigation) have an impact on the biosynthesis and accumulation of phenolic compounds in medicinal plants, which are fully addressed below (Alirezalu et al., 2018; Ghasemzadeh et al., 2012). The amino acid phenylalanine, which is synthesized via the shikimic acid pathway, is a precursor for several phenolic compounds. Phenylalanine ammonia-lyase is responsible for forming these compounds due to the de-amination of phenylalanine (PAL) (Shahidi & Chandrasekara, 2010). Environmental factors, remarkably light, are one of the most potent factors in phenolic metabolism, and they are significant. The light that impacts PAL increases the synthesis of phenolic compounds in the presence of oxygen(Macheix et al., 2018).

Wang et al. (2003) reported that the amount of phenolic compounds in a solution increases dramatically with increasing temperature and carbon dioxide (CO_2) concentration. There is a possibility that the differences discovered in phenolic compounds between genotypes in this study are related to environmental factors such as geographic variations (altitude, latitude, and height), light intensity, and temperature.

According to the DPPH assay, pomegranate has a range of antioxidant activity ranging from 45 percent to 82 percent, consistent with our present findings. The examination of the antioxidant capacity of the fruit extract revealed that this cultivar possesses significant antioxidant potential due to the presence of simple phenolics, anthocyanins, phenolic acids, and flavonoids in the extract (Çam et al., 2009). Investigators have suggested that carotenes, vitamin C, butylated hydroxytoluene, and phenolic compounds are among the components in fruit extracts with significant radical scavenging capability and that these compounds are found in high concentrations in some fruits (Sayyah et al., 2010; Yildiz et al., 2014). The antioxidant activity of the pomegranate extract is highly correlated with the extract's phenolic contents, including rutin, caffeic acid, chlorogenic acid, and apigenin. This study found a substantial correlation between rutin and caffeic acid and antioxidant activity, which is consistent with earlier studies (Chen & Ho, 1997; Mariangel et al., 2013). Considering a study conducted by Castelluccio et al. (1995) on the antioxidant activity of chlorogenic acid and caffeic acid, they concluded that these compounds were more active than *p*-coumaric acid. Furthermore, Cos et al. (2002) noted that, caffeic acid had the highest scavenging activity in fruits.

According to earlier research, the TPC of pomegranate fruits has previously been observed to range from 53 to 200 (mg GAE ml^{-1}) across pomegranate cultivars obtained from South Africa (Fawole & Opara, 2013). The relationship between latitude and total polyphenolic concentration, total reducing capacity, and DPPH radical scavenging capacity (Li et al., 2015) was positive, revealing that pomegranates grown in high latitude and low latitude longitude regions are more likely to accumulate more polyphenolics and have more significant antioxidant potential in their aril juice. As far as we know, the factors that regulate and control the production of fruit polyphenols have not been identified because the factors that influence polyphenol production among cultivars range from intrinsic genetic factors to various extrinsic environmental factors their interactions have varied over time and space (Hättenschwiler & Vitousek, 2000).

The PCA and cluster analysis were acceptable methodologies for determining cultivar classification among pomegranate varieties. The antioxidant capabilities of pomegranates have been demonstrated in several phytochemical investigations conducted on several pomegranate cultivars. In addition, it has been established by many research groups that the polyphenol content and flavonoids present in the fruits of pomegranate varietals have antioxidant properties.

5 CONCLUSION

There were significant variances in polyphenolic content amongst the different cultivars, which was noticed. This observation demonstrates that the relationship between genotype and environment is one of the most important elements influencing the accumulation and concentration of polyphenolics in pomegranate fruit. Furthermore, these findings demonstrated that pomegranate varieties are prospective sources of natural antioxidants and phenolic compounds employed in the food industry and that multivariate analysis was an appropriate method for classifying the pomegranate samples.

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Peroxidase activity as a biochemical marker of insecticide use in vegetables

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Peroxidase activity as a biochemical marker of insecticide use in vegetables

Abstract: The insecticides use is important for crop improvement and protection, but in excessive amounts, they would induce a dysfunction of metabolic enzymatic systems in plant tissues, leading to undesirable qualitative changes. In this context, we are interested in peroxidase (POD), an important enzyme in plant physiology but whose activity seems to be conditioned by the presence of insecticides in the soil. This work aims to study the impact of locally used insecticides (chlorpyrifos and dimethoate) on the activity of POD in parsley, onion, celery and garlic grown in soils treated or not. POD extraction was performed using Tris-HCl buffer (pH 7.3); its activity was measured using the substrate o-dianisidine in the presence of H_2O_2. Our result showed that POD activity for insecticide treated parsley, celery and onions increased by 30 % 127 % and 341 % respectively, however did not change significantly for garlic. Thus, the action of these chemicals is not trivial because they may alter non-target pathways, especially when doses are not adjusted accordingly. We found that insecticide stress would increase POD activity in all vegetables except garlic, which showed tolerance to insecticides. Our findings suggest that organic farming conditions could minimize peroxidase activity in parsley, celery and onion. We add that overproduction of POD negatively affects the quality and reduces the shelf life of vegetables, thus would be a very interesting biomarker of insecticide stress.

Key words: peroxidase activity; enzymatic browning; insecticides; oxidative stress; crop protection

Aktivnost peroksidaze kot biokemični označevalec uporabe insekticidov v zelenjavi

Izvleček: Uporaba insekticidov je pomembna za izboljšanje in zaščito pridelkov, vendar bi v prevelikih količinah povzročili motnje v delovanju presnovnih encimskih sistemov v rastlinskih tkivih, kar bi povzročilo neželene kakovostne spremembe. V zvezi s tem nas zanima peroksidaza (POD), ki je pomemben encim v fiziologiji rastlin, vendar se zdi, da njeno delovanje pogojuje prisotnost insekticidov v tleh. Namen tega dela je preučiti vpliv lokalno uporabljenih insekticidov (klorpirifos in dimetoat) na aktivnost POD v peteršilju, čebuli, zeleni in česnu, ki rastejo v tleh, obdelanih ali ne. Ekstrakcija POD je bila izvedena z uporabo pufra Tris-HCl (pH 7.3); aktivnost encima POD je bila izmerjena z uporabo substrata o-dianizidina v prisotnosti H_2O_2. Naši rezultati so pokazali, da se je aktivnost POD pri peteršilju, zeleni in čebuli, tretiranih z insekticidi, povečala za 30 %, 127 % oziroma 341 %, pri česnu pa se ni bistveno spremenila. Tako delovanje kemikalij ni nepomembno, saj lahko spremenijo neciljne poti, zlasti če odmerki niso ustrezno prilagojeni. Ugotovili smo, da stres zaradi insekticidov poveča aktivnost POD pri vseh vrtninah, razen pri česnu, ki je pokazal toleranco na insekticide. Naše ugotovitve kažejo, da bi lahko pogoji ekološkega kmetovanja zmanjšali aktivnost peroksidaze pri peteršilju, zeleni in čebuli. Dodajamo, da prekomerna produkcija POD negativno vpliva na kakovost in zmanjšuje rok trajanja zelenjave, zato bi bila zelo zanimiv biomarker insekticidnega stresa.

Ključne besede: aktivnost peroksidaz; encimsko porjavenje; insekticidi; oksidativni stres; zaščita pridelkov

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1 INTRODUCTION

Crops are exposed to a variety of diseases and pests that are responsible for important losses of yields and limited agricultural productivity worldwide. Food and Agriculture Organization (FAO) estimates that annually up to 40 percent of global crop production is lost to pests (FAO, 2021). Plant pathogens can be fungal, bacterial, viral, insects or nematodes and can damage plant parts above or below the ground and alter their quality (Pandit et al., 2022). In order to control these pathogens and protect crops, the intensive agricultural systems rely heavily on the use of chemical pesticides. Nevertheless, the excessive application of pesticides has become a major cause of widespread ecological imbalances. Indeed, these chemicals resulted in serious problems of insecticide resistance, pest resurgence and pesticide residues accumulation in soil, water and plant tissues (Gull et al., 2019). Besides, pesticides may induce physiological variations in plants such as plant growth (Parween et al., 2015), germination (Fatma et al., 2018). Thus, processes of seed germination, cell division and elongation are changed (Gaspar et al., 1991). They also induce metabolic and enzymatic dysfunctions and toxicities on cell membranes (Moriwaki et al., 2017).

For instance, several studies have shown a variation in peroxidase levels after treatment with insecticides (García-Hernández et al., 2005). The peroxidase enzyme (EC1.11.1.7) is an important antioxidant that plays a pivotal role in plant growth and development (Breda et al., 1993). Peroxidases belong to a family of glycoproteins containing iron atoms as a prosthetic group and different quantities of carbohydrate residues (Van Huystee, 1987). Peroxidases are located mainly in the cell wall and in the vacuoles of plant cells; their location varies according to the age, species and developmental stage of the plant (Gaspar et al., 1982). Elevation in POD (peroxidase) activity has been linked to resistance to stress and self-defence mechanisms. Under stress conditions, the rate of respiration increases with upregulation in peroxidase enzyme activity (Aspinall & Paleg, 1981). High levels of POD in plants are involved in multiple deteriorating changes affecting flavor, texture, color and nutrition in processed fruits and vegetables (Bett-Garber et al., 2005). Therefore, knowledge about how they react is an important consideration in food technology.

The use of insecticides is not trivial on the quality of plants and on human health, especially when their dosage and treatment periods are not respected. Moreover, a major problem in Algeria is the unreasonable and random use of insecticides by farmers. In spite of the use of prohibited products such as DDT (dichloro-diphenyl-trichloro-ethane), the overdosing of insecticides and the non-respect of the life span of insecticides are alarming problems, which must be addressed seriously.

Chlorpyrifos and Dimethoate are the most used insecticides in Algeria, they are applied at 0.3-0.7 kg ha^{-1} and 1.5 liters of product/ha respectively on many crops: fruits and vegetables (beans, broccoli, cabbage, cauliflower, peppers, potatoes, spinach, tomatoes) (Worthing & Walker, 1983). The half-life of chlorpyrifos ranges from 60 to 120 days and its persistence appears to be highly dependent on pH, climatic conditions and other soil factors, ranging from two weeks to more than a year. Dimethoate is rapidly absorbed and broken down in the plant by hydrolysis and oxidation (Menzie, 1969). Its half-life in plants varies from 2 to 5 days (Melnikov et al., 1977) and it disappears after an average of 30 days, depending on the plant species and the climatic conditions

The aim of this study is to investigate the effects of insecticides on peroxidase activity in selected vegetables namely parsley, celery, garlic and onion bulbs. Parsley (*Petroselinum crispum* (Mill.) Fuss (*Petroselinum sativum*) a biennial herb is an important dietary source of vitamins and essential metals. Supplementation with parsley at sufficient levels can promote the levels of vitamins and essential metals in the human body (Zhai et al., 2015). Celery (*Apium graveolens* L.) (also called krafes in northern Africa) belongs to the Apiaceae family. It grows annually or perennially throughout Europe and in tropical and subtropical regions of Africa and Asia. Celery is considered the most widely used plant in traditional food and medicine because it contains compounds such as limonene, selinene, furocoumarin glycosides, flavonoids, and vitamins A and C (Kooti et al., 2014; Al-Asmari et al., 2017; Li et al., 2019). Garlic (*Allium sativum* L.) is one of the oldest of all cultivated plants that has been used as a spice or food for over 400 years (Choi et al. 2007). Onion (*Allium cepa* L.) is botanically included in the Amaryllidacea family and a variety of species are found across a wide range of latitudes and altitudes in Europe, Asia, N. America and Africa (Griffiths et al. 2002). Onion is widely used in all parts of the world as a flavoring vegetable in various types of food. These vegetables represent the most important commercial crops and indispensable vegetables in Algeria and other countries thereby provide an important backdrop for evaluating the effects of insecticides in Algeria.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

O-dianisidine and bovin serum albumin (BSA) were obtained from Sigma Aldrich. H_2O_2 (30 % [v/v]) was provided by Prolabo. All chemicals were of the best commercially available quality, and all solutions were prepared using deionized water.

2.2 SAMPLES

Two groups of tissue samples from fresh parsley, celery, garlic, and onion were involved in this study. The first group was provided by a local farmer using chlorpyrifos and dimethoate as insecticides. The second group was provided by a local organic farmer who does not use insecticides. Only uninjured plants were selected.

2.3 PREPARATION OF CRUDE EXTRACT

Peroxidase enzyme extraction was carried out according to Diao et al. (2019). Five grams of each plant were mixed with an electric blender. The resulting mixture was homogenized with 30 ml of Tris-HCl buffer (50 mM, pH 7.3) containing 0.5 M$CaCl_2$ and 5 mM DTT, at 4 °C for 1 hour. After filtration, the extracts were centrifuged (14.000 g, 4 °C, 45 min). The supernatants containing the peroxidase were stored at -20 °C until use.

2.4 TOTAL PROTEIN CONCENTRATION

Protein content of each extract was determined according to the spectrophotometric method of Lowry (1951). The reaction medium contains 3 ml of solution C and 20 µl of the extract; let it stand for 10 minutes in dark, at room temperature, then add 0.3 ml of Folin-Ciocalteu reagent diluted to half. After 15 minutes, absorbance is measured at 750 nm. Concentrations are expressed in grams per 100 grams of fresh matter (g 100 g^{-1}) using the regression equation obtained with BSA.

2.5 ENZYME ASSAY

Peroxidase activity was assayed according to the method of Bradely et al. (1982) modified by Bedouhene et al. (2020). The change in absorbance at 460 nm due to the oxidation of o-dianisidine in the presence of hydrogen peroxide (H_2O_2) and enzyme extract at 25 °C was monitored using Jenway 6405 UV/VIS Spectrophotometer. A standard assay solution contained 15 mM o-dianisidine, 10 mM H_2O_2 in sodium phosphate buffer pH 6.5 was prepared. Twenty-five microliters of the crude extract (contained peroxidase enzyme) were added to the standards solution in total volume of 1 ml. The change of color is due to the oxidation of o-dianisidine in the presence of hydrogen peroxide (H_2O_2). Kinetics of POD activity is followed by monitoring the change in absorbance at 470 nm per min (Abs/min). One enzyme unit (U) is defined as the amount of enzyme producing a 0.001 absorbance change per min under the assay conditions used. The readings were taken for every 1 min for 10 minutes and enzyme extract at 25 °C was monitored using Jenway 6405 UV/VIS Spectrophotometer.

2.6 DATA ANALYSIS

The results were expressed as mean values with their standard deviations. The Two-way ANOVA analysis test was used to estimate the significance of the obtained data for each experiment. The Tukey-Kramer multiple-comparison test was used for analysis of the two sample groups (treated versus untreated) results. Wherever differences are reported as significant, a 95 % confidence

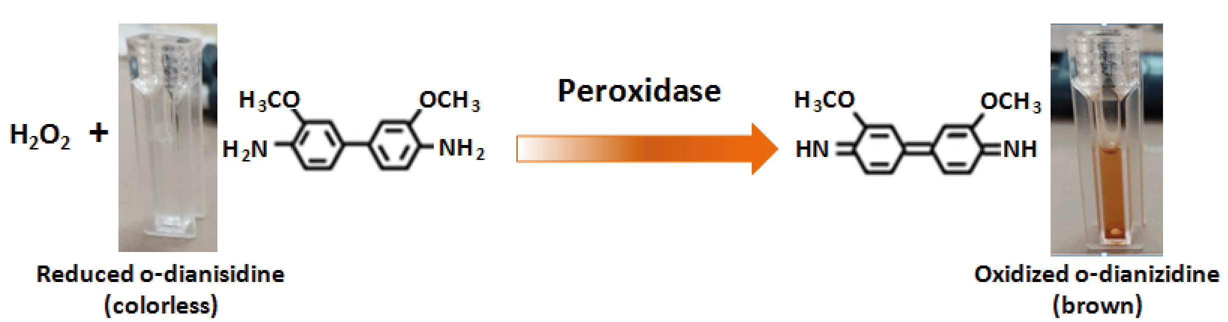

Figure 1: Oxidation of the molecular chromophore (o-dianisidine) by H_2O_2 and peroxidase, and the resultant color change from colorless to brown

level was used. The data analysis was performed using GraphPad Prism software version 5.01 (2010).

3 RESULTS

3.1 PEROXIDASEACTIVITY

Activity was measured in extracts of treated and untreated vegetables with insecticides by spectrophotometry using o-dianisidine as chromogenic agent and hydrogen peroxide (H_2O_2) as substrate (Fig. 1). POD is an enzyme related to plant defence and plays an essential role in resistance to membrane damage, mainly through the enzymatic degradation of H_2O_2. Peroxidase activity was strongly elevated in treated vegetables versus untreated samples (Fig. 2). The four plants showed different levels of POD activity. The level of POD activity was low in garlic treated with insecticides. This finding is supported by the proteins contents results (Table 1).

3.2 COMPRAISON OF PEROXIDASE ACTIVITIES

Peroxidase activities from parsley, celery, garlic and onion bulbsare summarized in Figure 3. Plant samples not subjected to insecticides show POD activities ranging from 201 to 2922, where parsley shows the highest activity, followed by celery and garlic, onion shows the lowest concentration. Higher POD activities ranging from 777 to 3769 $Umin^{-1}g^{-1}$ were observed in samples from insecticide-treated plants. Significantly the highest activity was found in insecticide-treated plant tissues from parsley with 3768.74 ± 141.59 $Umin^{-1}g^{-1}$ and celery with 2680.81

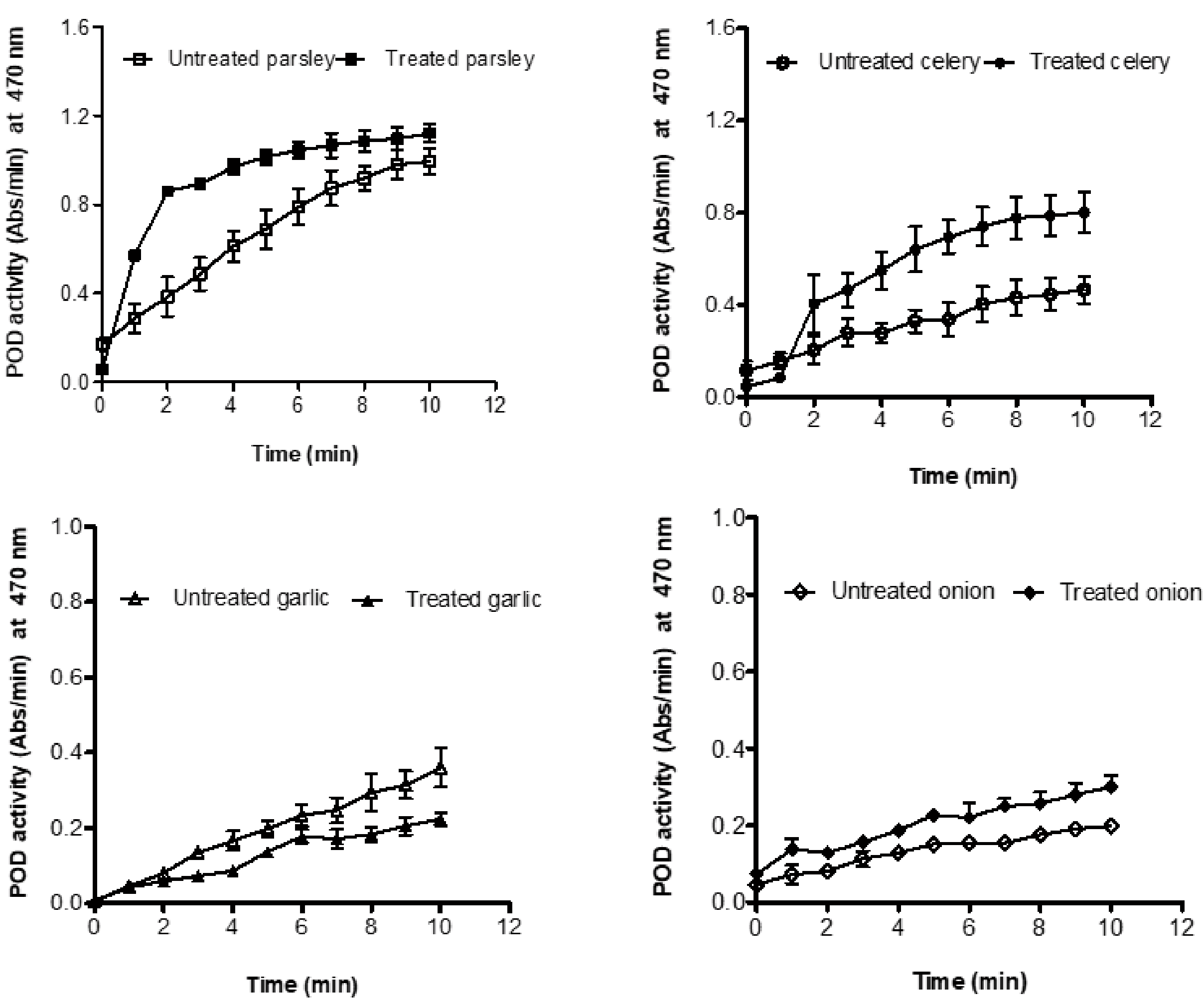

Figure 2: Kinetics of peroxidase activity is followed by monitoring the change in absorbance at 470 nm per min (Abs/min)of crude vegetables extracts (parsley, celery, garlic and onion) treated with insecticides compared to crude vegetables extracts without insecticides (control samples). Data represent mean values ± standard deviation of three determinations

Table 1: Proteins contents in crude vegetables extracts untreated and treated with insecticides

Vegetables	Organs	Total protein (g 100 g^{-1}) in untreated plant	Total protein (g 100 g^{-1}) in treated plant
Parsley	Leaves	1.02 ± 0.011	1.23 ± 0.001 *
Celery	Leaves	0.26 ± 0.001	0.91± 0.014*
Garlic	Bulbs	1.03 ± 0.009	0.73 ± 0.007
Onion	Bulbs	0.15 ± 0.001	0.32 ± 0.003*

Data represent mean values ± standard deviation of three determinations. *Means are significantly different (p< 0.05)

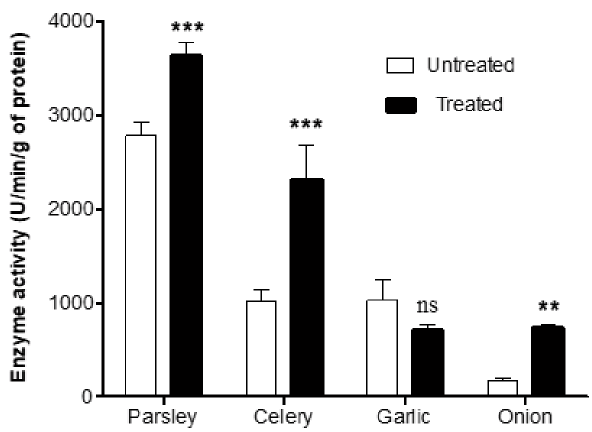

Figure 3: Peroxidase activity of crude vegetable extracts treated or untreated with insecticides. Error bars indicates the standard deviation of determinations. Differences were considered significant at p< 0.05. (ns > 0.05, **p < 0.01, ***p < 0.001)

± 373.66 Umin^{-1}g^{-1} (p< 0.001). Insecticide-treated onions showed lower activity, with a measurement of 776.99 ± 33.62 Umin^{-1}g^{-1} (p< 0.01). Samples derived from garlic did not show a significant increase in POD activity in insecticide-treated 772.84 ± 67.25 Umin^{-1}g^{-1} (p>0.05) compared to the untreated samples that had POD activity of 1253.09± 232.84 Umin^{-1}g^{-1}.

4 DISCUSSION

Use of insecticides leads to a dysfunction of metabolic enzyme systems in plant tissues, and negatively modifies certain physiological functions. In order to show the difference in tolerance behaviour and toxicity level among different vegetables selected against insecticide stress, the activity of the antioxidant enzyme peroxidase was evaluated.

In this work, we compared POD activity in insecticide-treated and untreated parsley, onion, garlic, and celery. The assessment of the oxidation of o-dianisidine in the presence of H_2O_2 revealed that the four plants had significant differences (p<0.05). Our findings are comparable to those of two groups, Hemeda & Klein (1990) and Ponce et al. (2004). They reported differences in POD activity indifferent crude vegetable extracts. García-Hernández (2005) showed high activity of POD in peppers treated with insecticides. On the other hand, the application of insecticides on garlic did not show an increase in POD activity compared to the other plants studied; this could be explained by the fact that the analyzed part is the bulb and not the leaf part. Garlic is described as a biopesticide possessing other defense mechanisms apart from peroxidase, such as poly sulfides. Several studies have shown that garlic possess some insecticidal, fungicidal, acaricidal, nematocidal and bactericidal properties (Lalla et al., 2013; Nwachukwu & Asawalam, 2014). Garlic has received much interest in recent years with respect to environmental concerns about the use of chemically synthesized plant protection products and has been proposed as a green pesticide; a new and environmentally sustainable alternative for application in control programs against various pest species. Indeed, this plant is equipped by evolution to defend itself against pathogens and pests (Mamduh *et al., 2017;* Wang et al., 2019*)*.

Phytotoxicity by excessive use of insecticide has been evaluated in some physiological traits in other cultivars and plants (Mousavizadeh & Sedaghathoor, 2011; Diao et al. 2011; 2019). García-Hernández (2005) reported that the highest insecticides rates caused alterations in the expression of peroxidase. The potential variation in peroxidase activity can be reflected in the growth and yield of plants, playing an important role in some stages of the metabolism, such as the auxin catabolism, and lignin formation (Fang & Kao, 2000). Peroxidase is involved in detoxification of xenobiotic a defense system of plants (Çördük, 2016; Lubos et al., 2011), its increase in plants is thought to be a response to stress, especially when the levels of H_2O_2 which is its substrate is high. The expression of each peroxidase isoform, is linked to the physiological status and the stress of developing conditions in a plant (Lobarzawsky et al., 1991). Hajjar et al. (2018) were able to identify many isoforms of POD using electrophoresis and spectrophotometric approaches. Additionally, they found that each isoform is activated depending on the chemical structure and properties of the insecticide.

Chlorpyrifos and dimethoate are organophosphorus insecticides with a large spectrum activity. Their mechanism of action is to inhibit cholinesterase, which is the cause of potential toxicity in humans (Gupta, 2016; Dhiraj et al., 2020; Nazam et al., 2020). The excessive use of insecticides can underlie health problems in humans; ranging from minor problems(e.g., eye irritation, skin irritation, skin sensitization) (Damalas & Eleftherohorinos, 2011) to neurotoxicity or cancer (Foster & Brust, 1995; Yadav et al., 2019).Exposure to organophosphate insecticides leads to depression of plant growth and nitrogen metabolism (Parween et al., 2011). The highest exposure of the Algerian consumer to pesticide residues through consumption of raw fruit and vegetables was found to be (42 %) for chlorpyrifos (Mebdoua et al., 2017).

Fatma et al. (2018) showed a significant decrease in seed germination of *Allium cepa* in the presence of these insecticides, and the effects were enhanced with increasing their doses. Thus, seed germination, a primary physiological process of plant growth, is strongly influenced by environmental stress. Stunting of plant growth at higher concentrations of applied pesticides indicates a reduction in cell division, cell elongation, and conversion of indole-3 acetic acid to various photo-oxidative products, as these compounds function as potent auxin antagonists (Tevini & Teramura, 1989). Plants possess a complex antioxidant system including enzymes such as catalase (CAT; EC.1.11.1.6), peroxidase (POD; EC. 1.11.1.7), and superoxide dismutase (SOD; EC. 1.15.1.1) to mitigate and repair ROS damage (Pandey & Rizvi, 2010). There are several evidences of insecticide degradation by high activity of oxidoreductase enzymes which reflects the level of toxicity and also the ability to combat stress (Dong et al., 2007; Yildiztekin et al., 2015; Singh et al., 2015).

Several studies have showed that spraying of crops with organophosphorus insecticides was associated with a remarkable stimulation in peroxidase activity (Garcia-Hernandez et al., 2005). Hajjar et al. (2018) found that that the highest level of increase in peroxidase activity was recorded at 20 days after spraying tomato plants with organophosphorus insecticides compared with untreated plants. Furthermore, the effects in interaction and response of peroxidase activity relied significantly on two factors; the insecticide and the dose. The effect of insecticides depended on their formulations and physicochemical properties (vapor pressure and solubility), climatic conditions (temperature, humidity, and sunlight), plant characteristics (genus and species), location of their applications and importantly the number and doses applied (Heshmati et al., 2020). García-Hernández et al. (2005) showed that insecticides applied at low doses did not cause significant differences in peroxidase activity compared to the control without insecticides, but a higher dose significantly increased peroxidase activity. Similar trends have also been reported in studies related to physiological injury by insecticides in hot pepper (Atale et al., 1995; García- Hernández et al., 2000). Furthermore, the results obtained here are consistent with the hypothesis reported by García-Hernández et al. (2005), who reported that insecticide-induced stress influences antioxidant enzymatic activity. The impact of regulated expression of peroxidase in plants has a direct effect on their shelf life. Indeed we noticed that the shelf life of parsley and celery that have not been treated with insecticides is relatively longer than that of treated vegetables. Furthermore, the external morphology of insecticide treated vegetables is altered to appear less shiny.

Some farmers apply insecticides in concentrations that are higher than the recommended amount to control resistant pests, occasionally reporting better control, but the yields are reduced and may have undesirable consequences. In general, the manufacturer's recommended application protocol does not have a negative effect on the plants, and some reports showed that there are certain insecticides that act as growth stimulants when applied at low doses (Ahemad & Khan, 2012; Singh et al., 2015; Yang et al., 2020). Other studies have shown that the excessive use of fertilizers, inappropriate irrigation, and exploitation of metal resources can lead to salt stress to a large extent (Shrivastava & Kumar, 2015; Gull et al., 2019). Under these circumstances, plants are likely to face biotic and abiotic stresses more frequently and simultaneously.

The action of commercial chemicals is not trivial because they modify non-target physiological pathways, especially when the doses are not adapted. Work from this study suggests that insecticide stress influences antioxidant enzyme activity and supports that organic farming conditions minimize peroxidase activity and enzyme browning in parsley, celery and onion. We conclude that POD is a very interesting biomarker of insecticide stress, and that overproduction of POD negatively affects their quality and shelf life.

5 CONCLUSION

Our study showed a significant increase in peroxidase activity on samples from conventional agriculture. These results represent an alarming report on the excessive and unreasonable use of insecticides by farmers, which is why it is important to inform farmers about the danger of these practices. Indeed, the use of chemicals to control pests can be useful on the one hand, but on the other hand can present many risks for human health. In this perspective, the evaluation of peroxidase enzymatic activity could be a reliable tool for the evaluation of the physiological stress resulting from the application of insecticides and will help to prevent the loss of antioxidant potential as well as the quality of vegetables, including the commonly used aromatic plants such as parsley and celery. Thus, we recommend through this study to reduce doses by combining biopesticides and by producing long-term resistant varieties, we also underline the importance of peroxidase which seems to be an interesting marker of insecticide-induced stress. Finally, additional and further studies are required to determine the doses of pesticides that do not significantly influence peroxidase activity.

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Stability of *Vicia faba* L. cultivars and responsible traits for *Aphis fabae* Scopoli, 1763 preference

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Stability of *Vicia faba* L. cultivars and responsible traits for *Aphis fabae* Scopoli, 1763 preference

Abstract: The study aimed to evaluate the responsible traits of preference of *Aphis fabae* to *Vicia faba* cultivars and their stability in multi-environment field tests. The experiment was carried out at the Institute of Forage Crops (Pleven) during the period 2016 to 2018. Aphid infestation was assessed by recording the number per plant at the pod formation, while the chemical composition was determined by standard Weende system methods. Canonical correspondence analysis showed that the aphid density was negatively correlated with the amount of rainfall and humidity until aphids were positively correlated with the temperature. According to GGE biplot analysis cultivar Fb 3270, followed by BGE 029055 and BGE 002106 were stable with a low density of aphids and were defined as tolerant. A significant negative correlation was found between the density of aphids and plant height (r = -0.447). The protein content showed a significant positive correlation (r = 0,686), while phosphorus and cyanogenic glycoside concentration were significantly negatively correlated with the aphid incidence (r = -0.411, r = -0.685, respectively). The results lay the groundwork for further analyses to finely dissect *A. fabae* tolerance in *V. faba* and pave the way for the development of methods to predict potential resistant genotypes in breeding programs.

Key words: *Aphis fabae* preference; faba bean cultivars; stability; morphological and chemical traits

Stabilnost sort boba (*Vicia faba* L.) in odzivne lastnosti črne fižolove uši (*Aphis fabae* Scopoli, 1763)

Izvleček: Namen raziskave je bil ovrednotiti preferenčne odzivne lastnosti črne fižolove uši na sorte boba in njihovo stabilnost v poljskem poskusu v različnih okoljih. Poskus je bil izveden na Institute of Forage Crops (Pleven, Bolgarija) v obdobju 2016-2018. Napad uši je bil ocenjen s štetjem uši na rastlino v razvojni fazi tvorbe strokov, kemijska sestava rastlin je bila določena s standardno Weende metodo. Kanonična korespondenčna analiza je pokazala, da je bila gostota uši v negativni korelaciji s količino padavin in vlažnostjo in v pozitivni korelaciji s temperaturo. Glede na GGE biplot analizo so bile sorte Fb 3270, BGE 029055 in BGE 002106 stabilne z majhno gostoto uši in so bile opredeljene kot odporne. Ugotovljena je bila značilna negativna korelacija med gostoto uši in višino rastlin (r = -0,447). Vsebnost beljakovin v rastlinah je pokazala značilno pozitivno korelacijo s pojavom uši (r = 0,686), medtem, ko sta bili vsebnosti fosforja in cianogenih glikozidov v negativni korelaciji s pojavom uši (r = -0,411; r = -0,685). Rezultati te raziskave dajejo osnovo za nadaljne analize tolerance črne fižolove uši na sorte boba in tlakujejo osnovo za nadaljne analize v razvoju metod za prognoziranje potencialno odpornih genotipov boba v žlahtniteljskih programih.

Ključne besede: preferenca črne fižolove uši na bob**;** sorte boba; stabilnost; morfološke in kemijske lastnosti boba

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1 INTRODUCTION

Faba bean (*Vicia faba* L.) is an important grain legume, protein-rich and widely used for human and animal consumption (Dhull et al., 2022). In addition, faba bean has also a valuable agronomic function considering its high capacity for N_2 fixation.

The most important common bean pest worldwide is the black bean aphid, *Aphis fabae* Scopoli, 1763 (Hemiptera, Homoptera: Aphididae), which causes considerable damage to plants and yield loss reaches 37 % (Munyasa, 2013). Aphids frequently grow and develop rapidly, allowing aphid populations to fastly exceed economic threshold levels (10–15 % attacked plants with single larval colonies, wingless, winged). Numerous colonies of *A. fabae* are very damaging to *V. faba* because of the direct negative impact on plant growth and the quantity and quality of the yield (Shannag & Ababneh, 2007). Injury caused by many aphid species is well documented to change the rate of photosynthesis, plant growth and physiological processes (Cahon et al., 2018; Du et al., 2021; Fuentes et al., 2021). Recurrent chemical applications are inappropriate because they represent a serious threat to pollinators and natural enemies in general. And, biological control approaches that have been attempted so far showed limited effectiveness, which makes studies of methods to control aphid populations extremely important. Insecticides' frequent treatments led to resistance evolution in many insect pests and synthetics are often more toxic to natural enemies than to the pests themselves. In addition, when insecticides negatively impact useful insects, secondary pest outbreaks can result (Straub et al., 2020). Breeding-resistant cultivars are an adequate approach to achieving efficient levels of pest resistance and promoting sustainable agriculture. Many authors reported that the application of resistant cultivars is a substantive and indubitable method for aphid control (Esmaeili-Vardanjani et al., 2013; Josefina et al., 2017). Béji et al. (2015) studied faba bean resistance to *A. fabae* and found that the best parameters describing resistance were pod mass and grain number. Meradsi & Laamari (2016) evaluated the resistance response of *V. faba* to black bean aphid by the relationship between the resistance level and plant morphological characteristics. Golizadeh et al. (2016) reported the relative impact of cultivars‘ resistance to black bean aphids, based on antibiosis and antixenosis.

The application of alternative cropping strategies, specifically the use of different cultivars, is an effective, environmentally friendly alternative to suppress crop pests.

This study aimed to evaluate the responsible traits of preference of *A. fabae* to *V. faba* cultivars and their stability in multi-environment field tests.

2 MATERIAL AND METHODS

The field study was carried out at the Institute of Forage Crops (Pleven; Bulgaria) during the period from 2016 to 2018. Twelve cultivars of faba bean (*Vicia faba* L.), originating from Portugal (Fb 1896, Fb 1903, Fb 1929, Fb 2481, Fb 2486, Fb 3270) and Spain (BGE 002106, BGE 029055, BGE 032012, BGE041470, BGE 043776, BGE 046721) were used. The experiment was laid out in Randomized Block Design (RBD) with three replications and an experimental plot of 4 m^2. The cultivars were planted with a sowing rate of 30 seeds m^2 and kept devoid of insecticide application throughout the experimentation to assess the susceptibility or resistance response to *Aphis fabae*. Aphid infestation occurred naturally. The reaction of different cultivars to *A. fabae* was assessed by recording the aphid number per plant at the 50 % pod formation stage of the faba bean. Therefore, twenty plants were selected randomly from each replication of the cultivar. The average aphid number was calculated based on three-time counts in each stage within 2-3 days. The height of the plants was measured in parallel.

To determine the chemical changes of the aboveground mass of cultivars in the aphid infestation, the plant samples taken of each cultivar were fixed for 15 minutes at 100 °C and dried to a constant mass at 60 °C in a thermostat. The chemical composition was determined by standard methods of the Weende system (AOAC, 2001) and includes crude protein (CP) by the Kjeldahl method (crude protein is calculated on the formulae CP = total N x 6.25) and phosphorus – colourimetrically by hydroquinone (Sandev, 1989). In addition, in fresh plant samples, cyanogenic glycosides contents (mg/100 g dry matter)) was determined according to Ermakov et al. (1987). Chemical compounds in cultivars were determined in the bedding of the pod formation.

To include genotype and genotype x environment (GGE) interplays and to remove interactions between variables, HA-GGE biplot analysis was used (Yan & Holland, 2010). Biplots are often applied to compare multiplex genotypes in different environments (Rubiales et al, 2014; Sánchez-Martín et al, 2014).

To determine the relative effect of the meteorological variables such as rainfall, air temperature and relative humidity on aphid density, canonical correspondence analysis (CCA) was carried out. The Paleontological Statistics Software Package (PAST) (Hammer et al., 2001) was used to perform the analysis.

The data were subjected to one-way ANOVA, and the means were compared by Tukey's test at 5 % probability ($p \leq 0.05$). The Multiple Regression Analysis of Statgraphics Plus (1995) for Windows Ver. 2.1 Software program was used.

3 RESULTS AND DISCUSSION

The meteorological conditions from 2016 to 2018 were different (Figure 1) and affected *Aphis fabae* development and reproduction. The higher average daily temperature in April, May and June months in 2016 (by 2.0 and 0.4 ^{0}C compared to 2017 and 2018), as well as a lower rainfall (by 41.9 and 105.6 mm, respectively), led to an earlier appearance of aphids and a stronger infestation compared to other years. At the same time, the weather conditions were favourable for plant development and did not suffer from a lack of moisture. In 2018, aphids were suppressed by the high amount of rainfall and relative humidity combined with lower temperatures whereat the population density was considerably lower. A similar trend was observed in 2017.

A wide range of the values obtained for aphid density was noted for the 12 *Vicia faba* cultivars studied in the three environments. ANOVA (Table 1) revealed a significant effect of genotype (G), environment (E) and G × E in both variables, being the highest average of a square for G, followed by E and the lowest for G × E.

A canonical correspondence analysis (CCA) was used to illustrate the clear relations of the aphid number to climatic variables (Fig. 2). It was found that the aphid number was negatively correlated with the amount

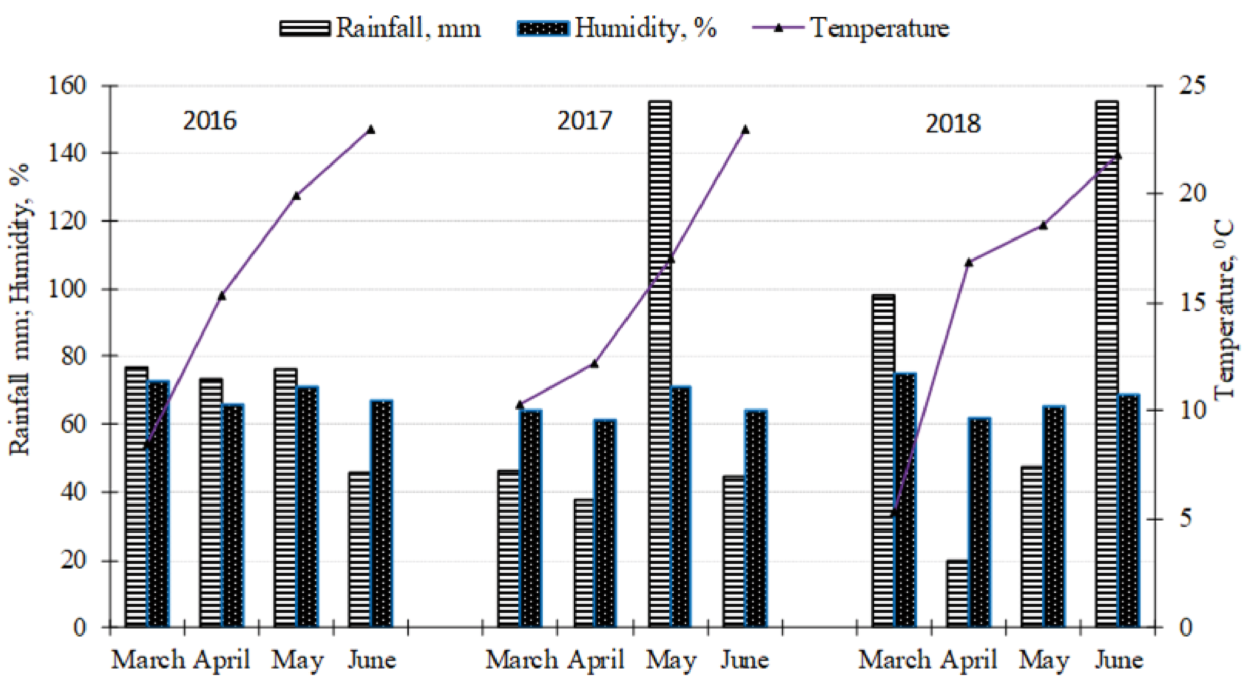

Figure 1: Meteorological characteristics of the period 2016-2018

Table 1: Analysis of variance for *Aphis fabae* number of the 12 faba bean genotypes

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Environment (E)	2	10925763	5462882	367,0335	5,33E-07
Replication/R	6	89303,26	14883,88	0,702751	0,648376
Genotype (G)	11	62417552	5674323	4,554665	0,001222
G^{x}E	22	27408186	1245827	58,82247	5,13E-35
PC1	12	26553829	2212819	104,48	0
PC2	10	854357	85435,7	4,03	0,0002
Residuals	66	1397843	21179,44	#N/A	#N/A

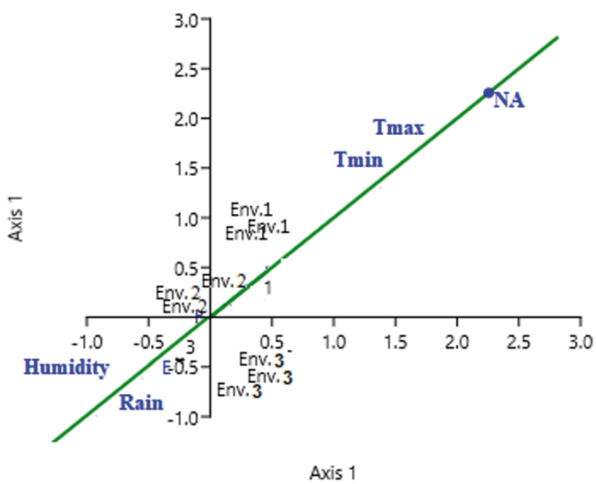

Figure 2: CCA graph based on the correlation of the aphid number for 12 faba bean cultivars according to several climatic parameters. The period analyzed was from March to June, Tmax = maximum temperature; Tmin = minimum temperature; NA – number of *Aphis fabae* Scopoli, 1763

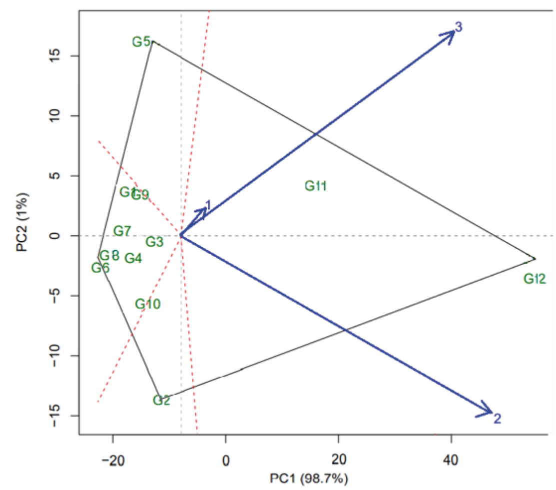

Figure 3: The GGE biplot based on seed damage rate (2016-2018). The cultivars are designated with the symbol "G" and the respective number from 1 to 12, as follows G1- Fb 1896, G2- Fb 1903, G3- Fb 1929, G4- Fb 2481, G5- Fb 2486, G6- Fb 3270, G7- BGE 002106, G8- BGE 029055, G9- BGE 032012, G10- BGE 041470, G11- BGE 043776, G12- BGE 046721. The years are designated with the letter E and numbers 1; 2; and 3 for 2016, 2017 and 2018, respectively, Note: G1 and G9 are heavily overlapped

of rainfall and humidity until *A. fabae* was positively correlated with T min and T max. Furthermore, temperatures were associated with the Environmental 1 drought (2016) and opposed to precipitation and humidity during the Environmental 3 wet season (2018).

Because of the negative effect of rainfall on aphids, the density decreased during rainy seasons, whereas in the driest environments, the aphid number increased. Rainfall and lower temperatures hinder the development of *A. fabae* or fully wash away species from the plants. On the opposite, the temperature had a positive effect, with the number increasing at higher values.

According to the results of the GGE biplot analysis (Fig. 3), the difference in vector length among environments showed phenotypic variances within different environments. Based on the discrimination power (vector length) E2, followed by E3 were the most discriminating, GGE biplot manifested clearly long vectors for E2 and E3, and a shorter vector for E1, respectively.

According to Yan & Holland (2010), an HA-GGE biplot is the preferred GGE biplot as regards test environment and genotype evaluation. A GGE biplot is based on environment-centred data (Gabriel, 1971), which integrates the genotypic main effect with the genotype-by-environment interaction effect of a genotype-by-environment dataset (Yan et al., 2000).

The best part of approximation for the correlation coefficients by the angles is linked to the fit goodness of the biplot although there are no exact relations. The vector correlation is varied depending on the angle between the two environments. Then, the environments were more or less positively correlated since the angles were acute angles. Furthermore, within the environmental group, E2 was apparently less associated with E3, while E1 and E3 were strongly positively correlated.

To determine which of the twelve faba bean cultivars studied were the lowest density of *A. fabae* based on their representation in the biplots, the ranking of the genotypes (considering stability across the environments studied) for both variables assessed is shown in Table 2.

Stability throughout the environments has been taken into account by considering each cultivar (genotype) position in the biplots. Thus, the cultivar with the lowest density of *A. fabae* was G6/Fb 3270 (63.9 winged and wingless individuals/plant) despite exposed environmental interactions, followed by the G8/BGE 029055 (120.9 individuals/plant) and G7/BGE 002106 (214.9 individuals/plant), whose responses were more steady, as shown by their position close to the axis 1. The three cultivars were defined as tolerant.

The results indicated that the cultivar G3 was considered stable similar to G7 given the closeness to the midpoint of the boxplot but presented with a considerably higher value of the density and identified as a sensitive (446,8 individuals/plant). Relatively stable but sensitive with somewhat differences from each other showed G9, G4 and G1 (297.8; 338.9 and 421.4 individuals/plant,

Table 2: Ranking of the twelve faba bean cultivars with the lowest density of *Aphis fabae* (ascending order)

Rank	G	Cultivars
1	G 6	Fb 3270
2	G 8	BGE 029055
3	G 7	BGE 002106
4	G 9	BGE 032012
5	G 10	BGE 041470
6	G 4	Fb 2481
7	G 2	Fb 1903
8	G 1	Fb 1896
9	G 3	Fb 1929
10	G 5	Fb 2486
11	G 11	BGE 043776
12	G 12	BGE 046721

respectively) too. Poor stability and aphid sensitivity showed G2, Q5 and G10 genotypes but G2 and G5 were more affected by the environment.

Genotype G12 (2868,1 individuals/plant) had the highest values for that trait, followed by G11 (1514,9 individuals/plant) which determined them as the most susceptible cultivars. According to the GGE biplot analysis, G11 had the highest value in E3 while G12 - in E2, which were the most favourable for their susceptibility. Genotypes G11 and G12 were less affected by the environment compared to G2 and G5.

The first two principal components (PC1 and PC2) determined 98.7 % of the dispersion.

The tolerance of cultivars Fb 3270, BGE 029055, and BGE 002106 (G6, G8 and G7) might be the result of the combination of different resistance mechanisms. Morphological characteristics such as plant height influenced the abundance of aphids as tolerant cultivars were considerably higher than susceptible ones. The correlation between the aphid density and the height of the cultivar plants was calculated. It was found that *A. fabae* preferred significantly lower plants and a middle negative correlation was found, r = -0.447, p = 0.014 (Fig. 4).

There have been different hypotheses regarding the effects of plant height on the preference of aphids. For example, Meradsi & Laamari (2016) found that morphological characteristics such as plant height did not affect *A. fabae* infestation but resistant cultivars had a longer leaflet than highly susceptible cultivars. Chaudhari et al. (2013) found the degree of association between the aphid population with morphological traits was not significant except for plant height which showed a highly significant negative correlation. On the other hand, Lebbal (2010) mentioned that aphid-resistant and highly susceptible bean cultivars had the same morphological characteristics.

The effect of some chemical traits on aphid preference was examined by regressing models too (Fig. 4).

Aphids feed exclusively on the phloem, their diet is rich in sugar but relatively poor in nitrogen, necessitating the ingestion of large volumes so that the insects can acquire sufficient nitrogen (Douglas et al., 2006). A considerably lower concentration of protein in the aphid-tolerant cultivars was detected. Probably high levels were a key factor supporting high *A. fabae* density and fecundity. A positive significant interaction between aphid density and protein content was detected, r = 0.686, p = 0.022.

The protein preference of aphids observed in that study was consistent with those reported in several previous experiments. Meradsi & Laamari (2016) studied different cultivars of *V. faba* for their resistance against the black bean aphid and found that the low susceptibility of the plant was possibly based on its lower nitrogen and protein and high phosphor content in plant leaves. Chaudhari et al. (2013) reported that resistant lucerne varieties against *Therioaphis maculata* (Buckton, 1899) had a lower total chlorophyll, crude protein, sugar and magnesium contents. The authors found also a highly significant positive correlation between the aphid population and chemical component levels in plants. Comadira et al. (2015) studied the complex relationship between plant N and aphid infestation (*Myzus persicae* (Sulzer, 1776)) and found that in N-deficient barley leaves, the progenitor aphids failed to survive until maturity despite the observed large increase in free amino acids.

The present data revealed the key role of plant protein on the quantity and colonization choice of aphids on faba bean plants.

The phosphorus and cyanogenic glycoside content in the aboveground mass were other reasons for the preferences of aphids. Phosphorus is important for the formation of nucleic acids and phospholipids and is needed for the energy metabolism of photosynthesis (Wang et al., 2020). The results of the regression model showed that the highly preferred cultivars by aphids had a considerably lower phosphorus content, while the high phosphorus level determined a lower *A. fabae* density. A significant negative correlation was found between aphid number and phosphorus levels, r = -0.411, p = 0.013. The same trend was found in cyanogenic glycosides. The low aphid-affected cultivars had a high concentration of cyanogenic glycosides while the preferred plants had low levels. Faba bean leaves contain cyanoglycoside and during their hydrolysis is released hydrogen cyanide which

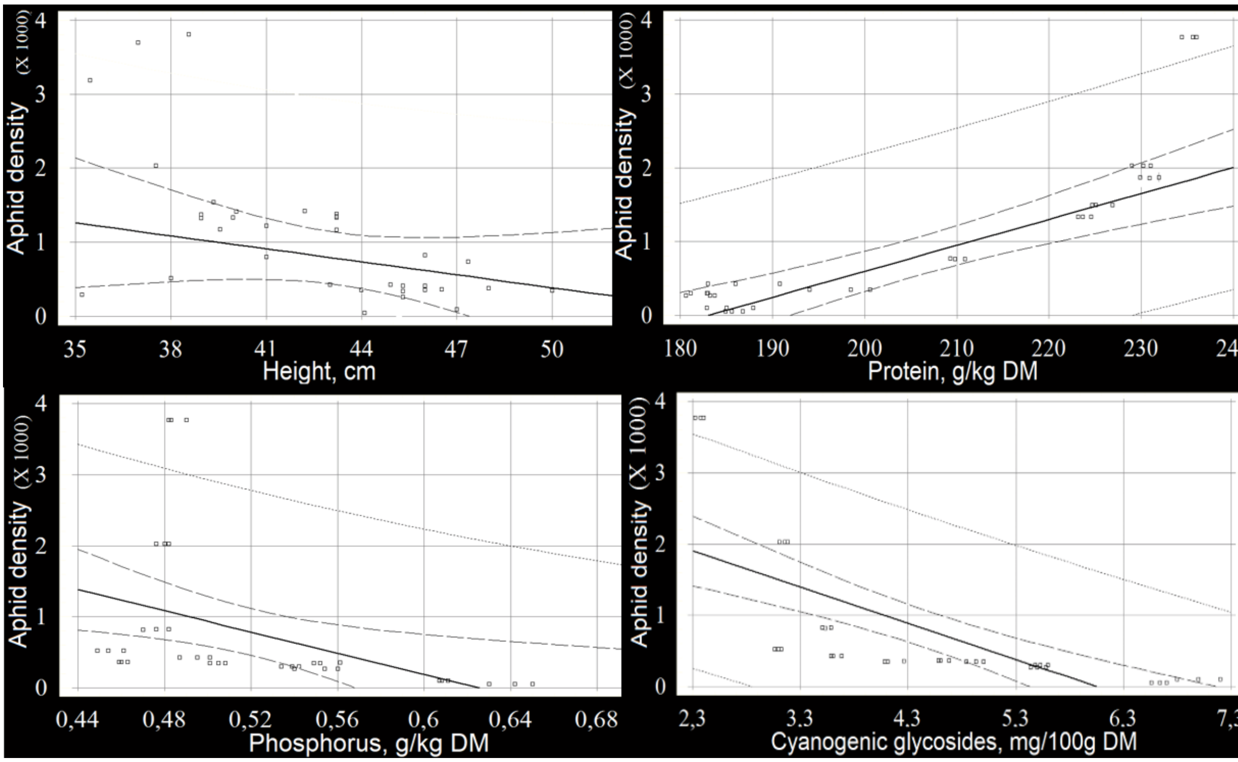

Figure 4: Effect of some traits on the *Aphis fabae* Scopoli density

has an insect-toxic effect in a concentration above 50-75 mg/100g of DM according to Naydenova et al. (2018). These compounds play an important role in plant defence producing bitter taste and toxic hydrogen cyanide which repel pests (Gleadow & Møller, 2014). Therefore, cyanogenic glycoside concentration was significantly negatively correlated with the incidence of black bean aphid (r = -0.685, p = 0.001).

The present results showed that cyanogenic glycosides may play a key protective role against *A. fabae* preventing the colonization and abundance of the species. There was indisputable evidence for the role of cyanogenic glycosides as insect pest deterrents. According to Ballhorn et al. (2008; 2009), resistant bean genotypes had strong cyanogenesis and therefore were efficiently directly defended against insect pests. On the other hand, some authors reported that insect pest damage was responsible for catalyzing the synthesis of cyanogenic glycosides as a defence mechanism (Irmisch et al., 2014; Chunming et al., 2018).

Unlike the cyanogenic glycosides, there have been different hypotheses regarding the effects of concentrations of phosphorus in leaves on the preference of insect pests. For example, Vannette & Hunter (2009) reported that the greater concentrations of P in leaves affected the attractiveness of plants to sap-sucking pests. On the other hand, Azouz et al. (2014) studied how plant mineral status affected the aphid population under field conditions. Authors reported that the susceptible eggplant cultivars had lower potassium, sodium, calcium and phosphorus content and the phytochemical constituents were negatively correlated with the *A. gossypii*Glover, 1877 amount as well as with the level of infestation. Facknath & Lalljee (2005) explained that phosphorus decreases the host's suitability against various insect pests by changing secondary metabolites such as phenolics and terpenes and accumulation of phenolics which acts as a barrier having deterring (antifeedant) or directly toxic (insecticidal) effects on herbivores.

The above graphical representation of the relationships between the aphid density and studied traits allowed statistical results to be obtained (with sufficient approximation) and main dependencies to be established while a regression analysis allowed determining which factors matter most and how these factors influence each other.

The results of the regression analysis (Table 3) showed that the linear component in the regression of aphid numbers according to the chemical traits was significant.

Table 3: Regression analysis and regression coefficients (R) of *Aphis fabae* Scopoli number regarding main chemical traits in *Vicia faba* L. cultivars (Institute of Forage Crops, Pleven, Bulgaria, 2016-2018)

Regression analysis					
Dispersion	df	SS	MS	F-Ratio	P-value
Model	4	2.69668E7	6.74169E6	18.55	0.00001
Residual	31	1.12638E7	363349.0		
Total (Corr.)	35	3.82306E7			
Regression coefficients					
Factors	Coefficients	Standard Error	t Stat	P-value	
Intercept	10734.500	1662.140	6.4582	0.001	
Height	- 47.765*	12.181	-3.921	0.001	
Protein	2.560	31.574	0.081	0.935	
Phosphorus	- 2260.290	1903.530	-1.187	0.244	
Cyanogenic glycosides	- 516.057*	245.832	-2.099	0.004	

Based on the complex trait study was obtained regression equation (1) indicated the impact of each trait on the variation of chemical content:

$$Y = 10734.5 - 47.765*X1 + 2.560*X2 -2260.290*X3 - -516.057*X4 \quad (1)$$

where *Y* - was *Aphis fabae* number; *X1* - height; *X2* - protein; *X3* – phosphorus; *X4* - cyanogenic glycosides

Results showed that on black bean aphid infestation, the highest negative significant influence had cyanogenic glycosides (r = -516.1) followed by height (r = -47.8). Protein content had a positive influence (r = 2.6), while phosphorus - was negative but the effect on aphid attack was not significant.

4 CONCLUSIONS

Canonical correspondence analysis showed that the aphid number was negatively correlated with the amount of rainfall and humidity until *A. fabae* was positively correlated with Tmin and Tmax.

According to GGE biplot analysis cultivar Fb 3270, followed by BGE 029055 and BGE 002106 were stable with a low density of aphids and were defined as tolerant.

A significant negative correlation was found between aphid number and plant height (r = -0.447). Among biochemical constituents, crude protein content showed a significant positive correlation (r = 0,686) while phosphorus and cyanogenic glycosides concentration were significantly negatively correlated with the incidence of black bean aphid (r = -0.411, r = -0.685, respectively).

Cultivars with high phosphorus and cyanogenic glycosides concentration and low crude protein content can be included in future breeding programmes to improve resistance to *A. fabae*.

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Establishment of an *in vitro* method for micropropagation of ironwort, (*Sideritis raeseri* Boiss. & Heldr.)

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Establishment of an *in vitro* method for micropropagation of ironwort (*Sideritis raeseri* Boiss. & Heldr.)

Abstract: Ironwort / Mountain Tea (*Sideritis raeseri* Boiss & Heldr.) is an endangered (EN) plant species in Albania. This study aimed to develop a rapid clonal propagation protocol using *in vitro* methodologies. The ironwort seeds were pre-treated with three concentrations of GA_3 (250, 500, and 1000 mg l^{-1}). During the inoculation stage, two types of culture media, Murashige & Skoog (MS) and Woody Plant Medium (WPM), were tested, and the effects of both GA_3 concentration and culture media used were evaluated. For the subculture stage, three cytokinins (6-b*enzylaminopurine* / BAP, kinetin, zeatin) at four concentrations (0.5; 1.0; 1.5; 2.0 mg l^{-1}), were compared for the RGR index, while for the rooting stage, two different auxins (1-n*aphthaleneacetic acid* / NAA and i*ndole-3-butyric acid* / *I*BA) at four concentrations (0.5; 1.0; 1.5; 2.0 mg l^{-1}) were tested. GA_3 at 500 mg l^{-1} and MS medium resulted as more effective. The highest value of the RGR index during the subculture stage was obtained in the MS nutrient medium supplemented with BAP at 1.5 mg l^{-1}. For rhizogenesis response, IBA was more effective for roots and length number. Based on these results, *in vitro* methodologies can be a promising tool for the mass production of this endangered plant species and with possible applications for enhancing the production of valuable nutraceuticals.

Key words: mountain tea; micropropagation; seed germination; nutrient medium; GA_3 concentration

Vzpostavitev *in vitro* metode za mikropropagacijo albanskega sklepnjaka (*Sideritis raeseri* Boiss. & Heldr.)

Izvleček: Vrsta *Sideritis raeseri* Boiss & Heldr. je ogrožena (EN) rastlinska vrsta Albanije, sorodna vrsti *Sideritis scardica* Gris., poznani kot šarplaninski čaj. Namen raziskave je bil razviti protokol hitrega klonskega razmnoževanja te vrste z *in vitro* metodo. Semena so bila predhodno obdelana s tremi koncentracijami giberilinov (GA_3; 250, 500, in 1000 mg l^{-1}). Na stopnji inokulacije sta bili preiskuševani in ovrednoteni dve vrsti gojišč, Murashige & Skoog (MS) gojišče in gojišče za lesnate rastline (WPM) hkrati z učinki različnih koncentracij giberilinov. V prvi fazi gojenja so bili preiskušeni trije citokinini (6-benzilaminopurin *(*BAP), kinetin, zeatin) v štirih koncentracijah (0,5; 1,0; 1,5; 2,0 mg l^{-1}) in primerjani z indeksom relativne prirasti (RGR). V fazi ukoreninjanja sta bila preiskuševana dva auksina (1-naftalen ocetna kislina *(*NAA) in indol-*3*-maslena kislina (*I*BA) v štirih koncentracijah (0,5; 1,0; 1,5; 20 mg l^{-1}). Giberilini (GA_3) pri koncentraciji 500 mg l^{-1} in MS gojišče so bili najbolj učinkoviti. Največja vrednost indeksa relativne prirasti (RGR) je bila v prvi fazi gojenja dobljena v gojišču MS z dodatkom BAP 1.5 mg l^{-1}. Za nastanek korenin je bil IBA bolj učinkovit tako glede števila kot dolžine korenin. Na osnovi teh rezultatov lahko zaključimo, da je *in vitro* metoda obetajoče orodje za masovno razmnoževanje te ogrožene vrste z možnostjo uporabe pri pospešeni proizvodnji vrednih hranilnih snovi.

Ključne besede: albanski sklepnjak; mikropropagacija; kalitev semen; gojišča; GA_3 koncentracija

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1 INTRODUCTION

Mountain tea (*Sideritis raeseri* Boiss et Heldr.), also known as Ironwort, is an aromatic medicinal native plant of the western Balkans, including south Albania, southeastern parts of North Macedonia, and North Greece (Zekaj et al., 2008). It is considered a Balkan endemic species due to its restricted distribution range of only three countries. Due to its high quantity of bioactive compounds with a high percentage of antioxidants, mountain tea has been widely utilized in alternative medicine since ancient times (Romanucci et al., 2017; Tadić et al., 2021). According to Hodaj et al. (2017), it is also used as an herbal tea to treat digestive system disorders, coughs and as a dietary supplement for avoiding anemia. The European Medicinal Agency (EMA) has approved the use of mountain tea, due to its medicinal values and its marketing in the market or pharmacy, based on its traditional use for at least 30 years in Europe and particularly in the Balkans, where the population has the experience and accurate information on optimal daily dosing (EMA, 2016).

According to Tomasini & Theilade (2019), mountain tea is a culturally and commercially significant important species locally consumed as a tea and used for the treatment of flu symptoms and respiratory problems, as well as harvested for trade. However, due to its widespread use, it has been subjected to uncontrolled and destructive harvest practices in its distribution area of occurrence in Albanian territory, resulting in a decrease in the wild populations of the mountain tea (Bojadzi et al., 2012; Tomasini & Theilade, 2019).

Mountain tea populations have been reduced by 50 % in the Prespa area from 1990 until today, and since its population in Albania has been reduced by 30 % and destructive harvest practices of the natural population continue, it has been assessed as endangered (EN) species by the Albanian government (Shuka & Malo, 2010; MoE, 2013; Shuka et al., 2021). Furthermore, *S. raeseri*, with its closest relative, *S. scardica*, is listed as species of high conservation interest for the western Balkan countries (Aneva & Zhelev, 2018).

In light of the preceding, it is critical to improving the situation through *in situ* and *ex situ* cultivation and conservation techniques. Furthermore, *in vitro* technologies are effectively applied with the goal of rapid mass production via clonal propagation and the establishment of a genetic collection for the conservation of endangered plant species of economic importance. The medicinal and aromatic plants (MAPs), among others, are the main focus of these techniques' application because of their importance and widespread use in pharmacy and medicine (Neergheen-Bhujun et al., 2017; Moraes et al., 2021).

Seasonal variations, growing practices, the expense of production, as well as other factors all impede large-scale phytochemical production from field-grown plants. The application of biotechnological techniques would be of significant interest in this area, not only for biomass production but also for optimizing the production of secondary metabolites (Georgiev et al., 2009; Cardoso & Silva, 2013; Kapoor et al., 2018). Efforts are being made to modify the organogenesis of the secretory structures of MAPs in terms of density or glandular diameter (Vantu & Gales, 2009; Sota et al., 2019; Sota et al., 2020), as well as the possibility of increased synthesis of essential secondary metabolites, using specific physic-chemical parameters under in vitro conditions (Avato et al., 2005; Tousi et al., 2010; Sharma et al., 2015; Radić et al., 2016; Jamwal et al., 2018).

The success of *in vitro* stabilization and multiplication of plant germplasm is determined by several parameters, including the explant chosen, the physical or chemical treatments applied, and any pretreatment used. Some studies (Shtereva et al., 2015; Papafotiou & Kalantzis, 2009; Danova et al., 2013) employed seeds as primary explants and isolated nodal explants for further multiplication via subcultures for *Sideritis* sp. In many cases, when seeds are used as initial explants, pretreatment with GA_3 is seen as effective in order to enhance their germination and faster proliferation under *in vitro* conditions (Khuat et al., 2022; Cornea-Cipcigan et al., 2020; Rout et al., 2017; Arabaci et al., 2014; Gashi et al., 2012). This effect is related to the synthesis of α-amylases, essential enzymes that help and promote breaking seed dormancy (Finch-Savage & Leubner-Metzger, 2006). For *Sideritis leucantha* Cav., a Spanish endemic species, Juan-Vicedo et al. (2021) refined a micropropagation and cryopreservation strategy using shoot explants. Sarropoulou & Maloupa1 (2015) studied the effects of various dikegulac sodium concentrations, a PGR that enhance lateral growth, on *in vitro* regeneration of *S. raeseri* using shoot tips as primary explants. In some of these studies, efforts have been made to find a suitable plant growth regulators (PGRs) ratio in different stages of micropropagation that enhanced *in vitro* regeneration with potential uses for other purposes such as conservation or secondary metabolites production.

Because many *Sideritis* species are indigenous to certain places, they are adapted to the native growing conditions in their natural habitats. Hence, adaptation abilities are likely to vary significantly among species in the genus. This study aimed to stabilize an effective micropropagation protocol by using various concentrations of GA_3 for enhancing seed germination under *in vitro* conditions and confronting some PGRs ratios for *in* *vitro* regeneration and rooting induction on the derived plantlets.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL COLLECTION AND DISINFECTION

As primary explants were used mature seeds of *Sideritis raeseri* Boiss. & Heldr. collected in the National Park of Prespa, Albania. The seeds were left for 30 min in tap water and, after that, were sterilized with 5.20 % sodium hypochlorite solution for 15 minutes.

2.2 GA_3 PRETREATMENT FOR SEED GERMINATION ENHANCEMENT

Before inoculation in culture vessels, the seeds were treated for 24 h in GA_3 solution. Three concentrations of gibberellic acid (GA_3), specifically I: 250 mg l^{-1}; II: 500 mg l^{-1}; III: 1000 mg l^{-1}, were tested and compared. After this treatment, the explants were inoculated in the nutrient medium, and their *in vitro* cultivation was initiated.

2.3 MEDIA COMPOSITION IN EACH STAGE OF MICROPROPAGATION

Inoculation and seeds germination stage: after GA_3 treatment, the explants were inoculated in a nutrient medium for their germination. Two different basal media, specifically Murashige-Skoog (MS) medium (Murashige & Skoog, 1962) and Woody Plant Medium (WPM) (Lloyd & McCown, 1980) were compared, each of them supplemented with 1-naphthaleneacetic acid (NAA) at 0.1 mg l^{-1} and 6-benzylaminopurine (BAP) at 1 mg l^{-1}. Seeds' germination started 6 – 7 days of culture, but germination percentage and morphometric parameters (shoot length and leaves number) were evaluated after 30 days of culture.

Subculture stage: For shoots regeneration, MS basal medium was used, and the effect of three different cytokinins (BAP, kinetin, zeatin) at four concentrations (0.5; 1.0; 1.5; 2.0 mg l^{-1}) were tested and compared. The plant material was weighed before inoculation in each treatment (initial mass - M1), while after 30 days, the biomass obtained in each treatment was weighed (final mass - M2). After that, the relative growth rate (RGR) following the formula: $RGR = (lnM2 – lnM1) / (no.\ of\ days)$ x 100 was evaluated, where *ln* is the natural logarithm, and FM is the fresh mass (Gatti et al., 2017).

Rooting stage: For rhizogenesis induction, MS basal medium was used, and the effect of two different auxins, specifically -naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) at four concentrations (0.5; 1.0; 1.5; 2.0 mg l^{-1}), were tested and compared. In this stage, roots number and lengths were evaluated.

In all cases, all media were enriched with sucrose at 3 % and agar at 0.57 %. The pH was adjusted to 5.7 prior to autoclaving.

2.4 INCUBATION CONDITIONS

The cultures were maintained in the growth chamber at a temperature 25 °C ± 2 °C with a 16 h light / 24 h photoperiod with cool, white fluorescent light of intensity 43.4 mmol m^{-2} s^{-1}.

2.5 STATISTICAL ANALYSES

For each treatment, 30 explants were used, and all experiments were repeated at least three times. Experimental data was elaborated by the Student's Test and the analysis of variance (ANOVA) with JMP 7.0 statistical software. Seeds germination, morphometric parameters, and RGR index in each cultivation stage were measured after 30 days of culture.

3 RESULTS

3.1 EFFECT OF GA_3 AND MEDIA TYPE ON *IN VITRO* SEEDS GERMINATION

In this experiment, seed germination and shoots development of *S. raeseri* affected by GA_3 and the type of culture media were investigated. Seeds started germination after 6 7 days of culture, and shoot / root organogenesis was observed due to the proliferation of zygotic embryos (Fig. 1 a). Within a week, these organs are differentiated (Fig. 1 b, c). The obtained results showed that seeds pre-treatment with GA_3 solution gave high germination rates in all concentrations used.

From the variability chart (Fig. 2), it can be observed that the differences in this parameter were not influenced by the type of basal media used but only by the GA_3 concentration. The results clearly show no statistical differences between MS and WPM media for the same concentration of GA_3. Therefore, treatment with GA_3 at 500 mg l^{-1} was evaluated as the most effective concentration, resulting specifically in an 88.4 % of germination rate for MS medium and 86.4 % for WPM medium.

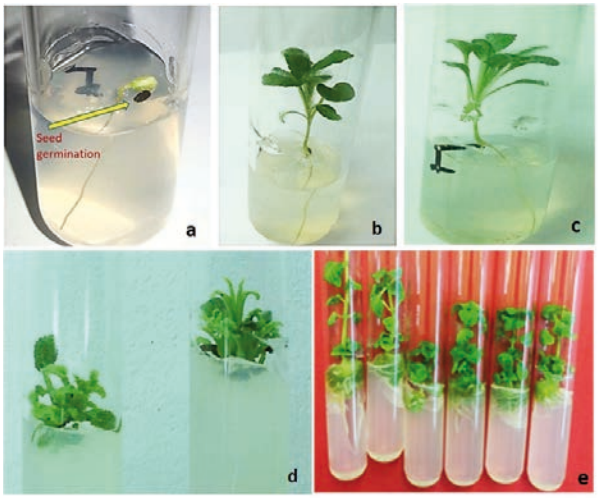


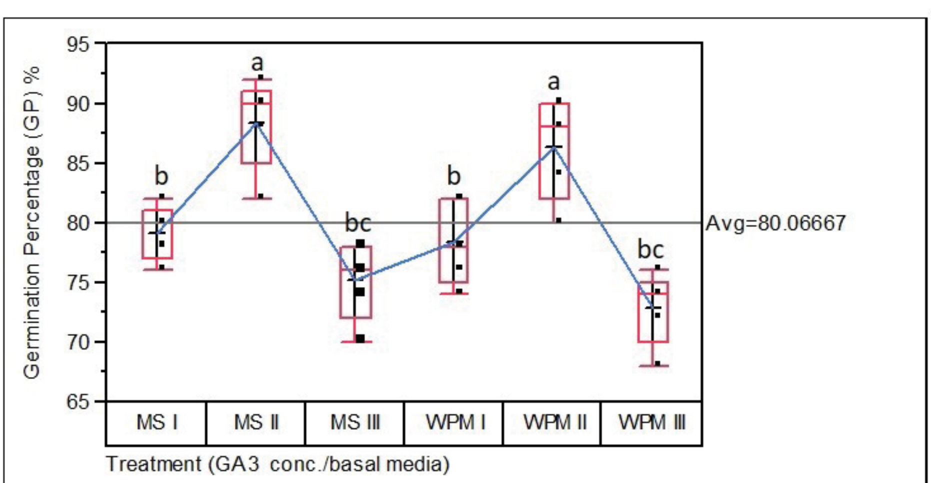
Figure 1: *Sideritis raeseri* Boiss. & Heldr. micropropagation **a)** Seed germination under *in vitro* conditions **b, c)** Shoot and root differentiation **d)** Shoots regeneration during subculture **e)** Rhizogenesis induction

After seeds germination, morphological characteristics such as shoots length (cm) and the number of leaves were monitored to detect if the pre-treatment of seeds or the basal media used caused any significant difference in these monitored parameters. For the above, the obtained results were interesting (Fig. 3). Regarding shoots length (cm), no differences were observed caused by the basal medium type. Even for this parameter, the highest results were obtained in GA_3 solution at 500 mg l^{-1}, precisely 2.31 cm for MS medium and 2.16 cm for WPM medium. The lowest results were obtained in GA_3 solution at 1000 mg l^{-1} for both basal media used (Fig. 2 a). The same trend was observed even for the number of leaves, where the best results were obtained in GA_3 solution at 500 mg l^{-1}. However, significant differences were observed between MS and WPM medium for this concentration (precisely, 17.01 in MS medium and 14.22 in WPM medium). For the other GA_3 concentrations, no differences were observed between MS and WPM media for the leaves number parameter (Fig. 2 b). In an overall analysis, we conclude that pretreatment with GA_3 at 500 mg l^{-1} is the most effective concentration, and cultivation in MS basal medium is more advantageous than WPM.

GA_3 is the plant hormone that is crucial in breaking seed dormancy. From our data, it is clear that GA_3 positively affects *in vitro* organogenesis by stimulating the proliferation of embryos within the seed to give shoots and roots. In all treatments with GA_3, is observed not only the germination rate at high values but also the increase of biomass of the monitored biometric parameters. So, it is evidenced that the GA_3 beneficial role in promoting seed development of *S. raeseri*.

3.2 BIOMASS PRODUCTION UNDER DIFFERENT CONCENTRATIONS AND TYPES OF CYTOKININS

The proliferated plantlets were subcultured for further multiplication, whereas before inoculation in the nutrient medium, the roots were removed, and small shoots were used for this purpose. A few days after cultivation in the subculture stage, new shoots and leaves



Basal media / GA_3 conc.	Mean
MS II	88.40^{a}
WPM II	86.40^{a}
MS I	79.20^{b}
WPM I	78.40^{b}
MS III	75.20^{bc}
WPM III	72.80^{bc}

Figure 2: Variability chart for germination rate depending on the GA_3 concentration and basal media used

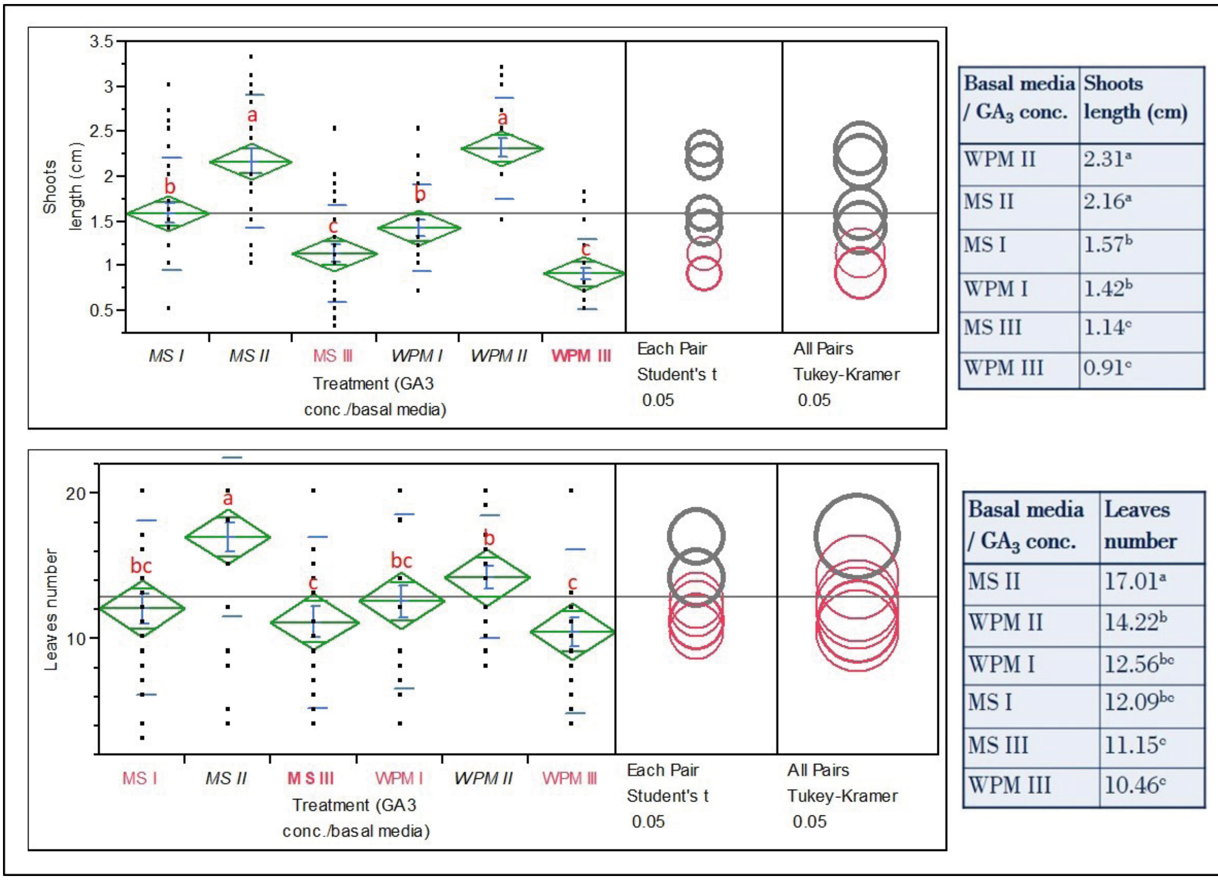


Figure 3: Oneway Analysis of **a)** Shoots length (cm); **b)** Leaves number; depending on the GA_3 concentration and basal media used

were formed in all concentrations or types of cytokinins supplemented in the nutrient media (Fig. 1 d). The initial and final mass results indicated that both type and cytokinin concentration affected *in vitro* regeneration of plantlets during this stage (Tab. 1).

Regarding the variability for the RGR index between different cytokinins for the same concentration (Fig. 4a), it can be said that except for the lowest concentration used of 0.5 mg l^{-1} where kinetin showed the highest effectiveness of the three cytokinins used, in all other concentrations the highest value of RGR was obtained from the use in the nutrient medium of BAP. In most cases, kinetin and zeatin are very close to each other in their effectiveness concerning the value of RGR according to the measurements performed, except for the RGR value at 1.5 mg l^{-1} of cytokinins concentration, where zeatin gave the lowest value. Regarding comparing different concentrations within the same type of cytokinin (Fig. 4b) for kinetin and BAP, the best results for RGR value were obtained when using 1.5 mg l^{-1} of each cytokinin, precisely 5.72 for kinetin and 6.11 for BAP. While for zeatin, the most optimal concentration resulted the one at 2.0 mg l^{-1}. An overall analysis of the obtained data showed that the most effective treatment, depending on the cytokinin type and concentration, was the use of BAP at 1.5 mg l^{-1}, where the RGR value obtained is equal to 6.11.

Table 1: Plantlets' weight before and after subculture stage

	Kinetin				Zeatin				BAP			
Concentration (mg l^{-1})	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2
Initial mass (g) (M1)	1.04	1.12	1.08	1.20	1.10	1.08	1.28	1.15	1.12	1.04	1.10	1.13
Final mass (g) (M2)	3.38	4.21	6.01	5.77	2.81	3.92	4.49	5.57	3.07	4.31	6.88	5.98
RGR index	3.93	4.41	5.72	5.23	3.13	4.30	4.18	5.26	3.36	4.74	6.11	5.55

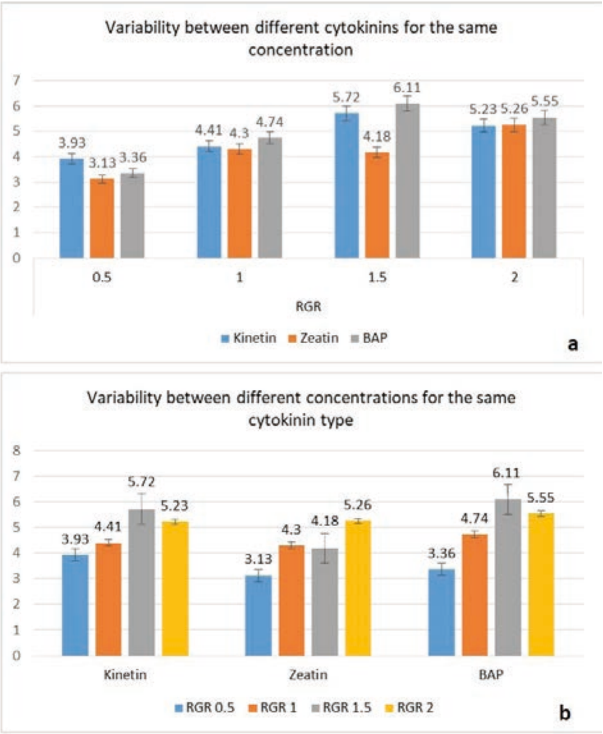


Figure 4: Variability for RGR index between **a)** different cytokinins for the same concentration **b)** different concentrations for the same cytokinin

3.3 *IN VITRO* ROOT FORMATION USING DIFFERENT CONCENTRATIONS OF IBA AND NAA

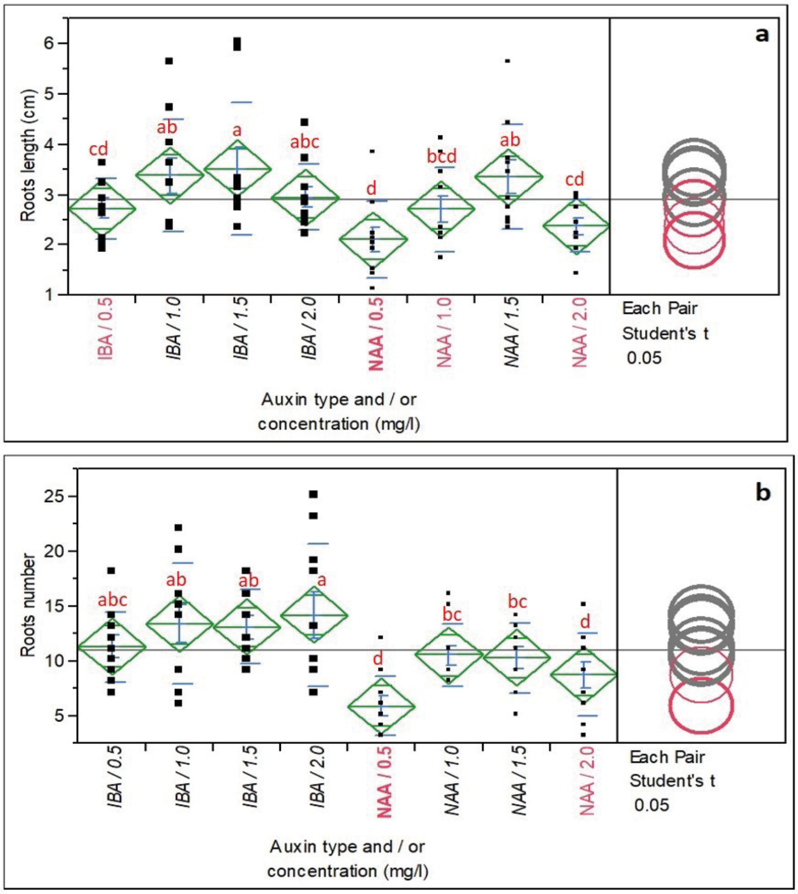
Root formation is a crucial stage for the micropropagation of plants reproduced *in vitro*. In this part of the study, the effect of IBA and NAA added separately in four concentrations on *in vitro* rooting of *S. raeseri* was investigated. The rooting response was observed at a high rate in all treatments (Fig. 1 e), but even in this stage was observed that the monitored morphometric parameters (number of roots and their length) are highly affected by the type and auxin concentration (Fig. 5 a; b). From an overall evaluation, IBA resulted more effective than NAA for both parameters under evaluation. Regarding roots length, the most effective treatment resulted in the use of IBA at 1.5 mg l^{-1}, a value (3.51 cm) statistically different from all the other values obtained, followed by the use of IBA at 1 mg l^{-1} and NAA at 1.5 mg l^{-1}, with mean values respectively 3.38 and 3.35. As for roots number, all the treatments with IBA showed higher values of this parameter, and the best result was obtained at 2.0 mg l^{-1} of IBA, with a respective value of 14.21 roots/plantlet.

4 DISCUSSION

Seed germination is a complex process, and GA_3 plays a crucial role in controlling and encouraging germination in many plant species. In this respect, exogenous applications of GA_3 are primarily used during *in vivo* or *in vitro* plant cultivation for enhancing seeds germination. Our study showed that adding GA_3 to the culture medium, regardless of concentration, increased the percentage of in vitro *Sideritis raeseri* germination, indicating the role of GA in breaking dormancy. Furthermore, the results revealed that the seed treatments significantly affected the germination and seedling growth parameters. Maximum germination and other seedling growth parameters were observed with 500 mg l^{-1} of GA_3. Otherwise, seedlings derived from GA_3-treated seeds showed normal morphology. In this regard, in their report, Cornea-Cipcigan et al. (2020) concluded that exogenous applications of GA_3 stimulated not only the germination of *Cyclamen* sp. but also higher rates of biometric parameters in the obtained plantlets.

Similarly, Gashi et al. (2012) found that using 1000 mg l^{-1} GA_3 + 0.3 % KNO_3 highly stimulated the germination of *Ramonda serbica* Pančić seeds grown in *Petri* dishes under controlled physical conditions. Also, Arabaci et al. (2014) mentioned that the pre-treatment of *Sideritis perfoliata* L. seeds with GA_3 at 100 mg l^{-1} for two hours resulted in a 100 % of germination rate. Furthermore, in their study on seed germination of *Vasconcellea stipulate* V.M. Badillo, Vélez-Mora et al. (2015) found effective the use of GA_3 at 1.44 µM in Nitch & Nitch basal medium, which significantly stimulated seeds germination. Similarly, Ake et al. (2007) obtained positive results on *in vitro* germination of coconut embryos by supplementing the semi-solid medium with GA_3 at 4,6 µM. Meanwhile, Nikam & Barmukh (2009) found effective the soaking of *Santalum album* L. seeds at 4 mM of GA_3 solution and obtained an 80.67 % of germination rate after seeds *in vitro* inoculation in MS medium.

In our study, there are no observed significant differences between MS and WPM basal media used for most of the results. Regarding the efficiency of MS media for *in vitro* regeneration of *S. raeseri* plantlets under *in vitro* conditions, our results are similar to those reported by other authors for micropropagation of different *Sideritis* species (Juan-Vicedo et al., 2021; Sevindik et al., 2019; Shtereva et al., 2015; Papafotiou & Kalantzis, 2009). On the other hand, Yavuz (2016) found compelling the use of B5 medium for *in vitro* regeneration of *Sideritis stricta* Benth. plantlets.



Auxin type and / or concentration	Roots length (cm)
IBA / 2.0	2.94^{abc}
IBA / 1.5	3.51^{a}
IBA / 1.0	3.38^{ab}
IBA / 0.5	2.72b^{cd}
NAA / 2.0	2.37^{cd}
NAA / 1.5	3.35^{ab}
NAA / 1.0	2.71^{bcd}
NAA / 0.5	2.09^{d}

Auxin type and / or concentration	Roots number
IBA / 2.0	14.21^{a}
IBA / 1.0	13.42^{ab}
IBA / 1.5	13.13^{ab}
IBA / 0.5	11.35^{abc}
NAA / 2.0	8.73^{d}
NAA / 1.5	10.31^{bc}
NAA / 1.0	10.58^{bc}
NAA / 0.5	5.94^{d}

Figure 5: Oneway Analysis of a) Roots length (cm); b) Roots number, depending on the auxin type and/or concentration

Cytokinins have been used to stimulate plant growth and development as they favor cell division and cytokinesis, thus stimulating lateral shoots growth. In tissue culture, the types and concentrations of cytokinin added to culture media are the most important factors affecting the *in vitro* multiplication of plant propagules. In the present work, variations in the response of the multiplication parameters of *Sideritis raeseri* were observed depending on the type and concentration of cytokinin. Among three types of cytokinins, i.e., BAP, kinetin, and zeatin, used for in vitro shoot multiplication, BAP at 1.5 mg l^{-1} was the most effective treatment. Shoot multiplication in the present study was obtained by enhancing shootlets' fresh mass, which is crucial in employing tissue culture techniques for *Sideritis raeseri* micropropagation. Similar to our results, other authors, when confronting different types or concentrations of cytokinins, also have reported the effective use of BAP for *in vitro* multiplication of *Sideritis* sp. (Yavuz, 2016; Papafotiou & Kalantzis, 2009). Meanwhile, Juan-Vicedo et al. (2021) evidenced that for *S. leucantha*, the best results for shoot morphogenesis were obtained on a nutrient medium supplemented with 0.44 µM 2-isopentenyladenine. On the other hand, Shtereva et al. (2015) stated that for micropropagation of *S. scardica*, the use of zeatin at 2 mg l^{-1} combined with 0.2 mg l^{-1} indole-3-acetic acid (IAA) was the best combination for shoot proliferation.

For *in vitro* root formation, most plant species require a medium supplemented with essentially auxin-specific PGRs. Usually, IBA, IAA, or NAA are used for the rhizogenesis of plant microshoots. In the present study, IBA was found to be superior over the NAA for *in vitro* root formation of *S. raeseri* since the highest values of root numbers and lengths were observed with IBA. Furthermore, the obtained roots' appearance was healthy and suitable for successful acclimatization. Our findings on the effect of IBA in rhizogenesis induction of *S. raeseri* are in line with the ones reported by Yavuz (2016), who achieved the best rooting rate of *S. stricta* on B5 medium supplemented with 4.5 mg l^{-1} IBA. On the other hand, for *Sideritis leucantha* the best auxin for rooting response resulted 1-naphthaleneacetic acid (Juan-Vicedo et al., 2021). In this respect, Ragavendran et al. (2012) rooted *in vitro* raised shoots of *Passiflora foetida L.* by use of IBA.

5 CONCLUSIONS

An effective micropropagation method of *Sideritis raeseri* Boiss. & Heldr. was recognized using seeds as the initial explant. The *in vitro* germination rate and shoots length were strongly affected by the GA_3 concentration, where the treatment with GA_3 at 500 mg l^{-1} gave the best results. Many plants were obtained in the subculture stage, where the most effective cytokinin was found to be the cytokinin BAP. For the monitored parameters (RGR), it can be concluded that the concentration of 1.5 mg l^{-1} of BAP and kinetin was the most optimal concentration that strongly influences the *in vitro* regeneration of plantlets. At the same time, zeatin was found effective at the concentration of 2 mg l^{-1}. For rhizogenezis induction, IBA was more effective than NAA for roots length and number. Effective clonal growth enables the creation of a plant collection with the potential for utilization in conservation programs, which are very important to apply to this endangered plant species. Also, this may further extend studies toward optimizing protocols for *in vitro* production of secondary metabolites from this important medicinal species.

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Variability of genetic - morphological traits of eleven seed strains of *Mangifera indica* L. growing in Upper Egypt

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Variability of genetic - morphological traits of eleven seed strains of *Mangifera indica* L. growing in Upper Egypt

Abstract: Mango (*Mangifera indica* L.) is one of the tastiest fruits in the world, with numerous advantages beyond their economic value. Eleven genotypes of mango various cultivars were examined for variability, heritability, and genetic advance, as well as multivariate analysis based on cluster and principal component analysis (PCA) for yield and some of its contributing traits during the two growing seasons, 2021 and 2022. All studied traits showed significant differences, and the phenotypic coefficients of variation (PCV) were found to be higher than genotypic coefficients of variation (GCV), supporting the idea that morphological (genetic) traits are more prevalent than environmental influence. All traits had substantial heritability ranging from 75.63 to 99.93 %, and the highest significant genetic advance (119.09 %) was for the number of fruits per tree than other traits. Four clusters were formed, *i.e.*, clusters I and IV had four genotypes, cluster II had two, and cluster III had one genotype. The highest cluster mean values for fruit diameter, fruit mass, yield per tree, and the number of fruits per tree were found in Cluster II, followed by cluster I. Greater genetic divergence was found between 'Zebda' or 'S9' or 'S10' with most other genotypes, indicating that these genotypes may be used to study the characters' broad range of variability and to yield high-quality recombinant lines. In light of the fact that mango is a very heterozygous crop, our current genetic results can be used for the selection of the appropriate parents in hybridization programs and in vegetative propagation to yield selective traits.

Key words: *Mangifera indica*; mango genotypes; genetic variation; principal component analysis; heritability

Spremenljivost genetskih (morfoloških) lastnosti sedmih semenskih linij manga (*Mangifera indica* L.) rastočega v Zgornjem Egiptu

Izvleček: Mango (*Mangifera indica* L.) je eden izmed najokusnejših sadežev na svetu s številnimi prednostmi poleg njihove cene. Preučevanih je bilo enajst genotipov manga različnih sort glede na njihovo variabilnost, dednost in genetsko prednost. Opravljena je bila multivariatna analiza, ki je temeljila na analizi grozdov in glavnih component (PCA) za pridelek in nekaterih z njim povezanih lastnosti v dveh rastnih sezonah, 2021 in 2022. Vse preučevane lastnosti so pokazale značilne razlike, kjer je imel fenotipski koeficient spremenljivosti (PCV) večje vrednosti kot genotipski koeficient raznolikosti (GCV), kar podpira idejo, da so morfološke (genetske) lastnosti prevladujoče nad okoljskimi vplivi. Vse lastnosti so imele znatno dednost, ki je znašala od 75,63 do 99,93 %. Največja značilna genetska prednost (119,09 %) je bila ugotovljena za za število plodov na drevo. Izoblikovale so se štiri skupine in sicer skupini I in IV s štirimi genotipi, skupina II je imela dva in skupina III en genotip. Največje poprečne vrednosti skupine za premer in maso plodu, pridelek na drevo in število plodov na drevo so bile določene v skupini II, ki ji je sledila skupina I. Med vsemi genotipi je bila večja genetska raznolikost ugotovljena pri sortah Zebda, S9 in S10, kar nakazuje, da bi se ti genotipi lahko uporabili za preučevanje značaja širše variabilnosti, kar bi privedlo do zelo kakovostnih rekombinantnih linij. Ob dejstvu, da je mango izredno heterozigotna kulturna rastlina, bi se ti rezultati lahko uporabili za odbiranje primernih staršev v programih križanja in pri vegetativnem razmnoževanju izbranih lastnosti.

Ključne besede: *Mangifera indica*; genotipi manga; genetska variabilnost; analiza glavnih komponent; dednost

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1 INTRODUCTION

Mango (*Mangifera indica* L.) is the king of fruits and, most important, occupies second place in terms of cultivated area after citrus in Egypt. The cultivated area of mango trees reached 265509 feddan (0.42 ha), producing 1,091,535 tons of fruits. The fruiting area in Egypt's Aswan Governorate, where the current study was conducted, got 13573 fed (5700.66 ha) and generated roughly 67076 tons of fruits (SIAERI, 2019). Mangoes are cultivated in over 100 countries, and the top-producing countries are India, China, Thailand, Indonesia, Mexico, and Nigeria (FAO, 2019). Mangoes are naturally heterogeneous and have a wide range of seed genotypes, demonstrating a wide genetic range in shape, color, bearing behaviors, maturation stage, and yield. Several factors, including selection, mutation, genetic drift, and recombination, provide sources of genetic diversity. Most mango cultivars, including some superior clones, are hybrids resulting from natural cross-pollination (Krishna & Singh, 2007; Ramírez & Davenport, 2016). Despite the level of genetic diversity among mango landraces and cultivars, many seeding strains have high productivity. Some of them outperform some of the mango varieties in some crop traits. However, in Egypt, wide mango varieties mainly arise from seedling strains. Mango is an allopolyploid, most probably amphidiploid, and outbreeding plant with chromosome number 2n = 40 (Pierozzi & Rossetto, 2011) and is highly heterozygous as performance varies with the climate and resulting in a high level of genetic diversity. Vasugi et al. (2012) revealed that to develop new varieties, breeding programs that use germplasm with specific traits need precise information. Mangos have been classified based on fruit characteristics, including color, size, shape, mass, peel percentage, stone, pulp, and nutrient composition (Igbari et al., 2019; Arogundade et al., 2022). The morphological characteristics that distinguish mango cultivars make it difficult to distinguish between closely related varieties (Begum et al., 2016). Abdelsalam et al. (2018) presented some mango cultivars in Egypt by employing morphological properties of the fruits and molecular markers techniques to show the diversity of the collected cultivars.

Understanding the variability among a crop's genetic stocks is crucial to breeding programs. Moreover, genetic variability is essential to know the gene source for a particular trait within germplasm to identify desirable cultivars for commercial production and improve yield and other traits (Govindaraj et al., 2015). Also, the progress in breeding programs depends on the genetic variability in the breeding material. Most of the genetic characteristics are governed by more than one gene, which is highly affected by the environment. Hence, the coefficients of variation (both genotypic and phenotypic) and heritability (the degree to which a trait can be attributed to a particular gene) are crucial in determining the inheritance pattern of the traits. The heritability of a genetic character is important in determining the response to the selection (Piepho & Mohring, 2007). Because of the great heritability, the breeder can choose plants depending on how they display their traits (Holland, 2014).

Majumder et al. (2013) studied 60 mango genotypes to determine their variability, heritability, and genetic advance. They found that significant variations were observed in 20 traits. Also, there were considerable differences between the genotypic (GCV) and the phenotypic coefficients of variation (PCV) for almost all the characters, indicating the effect of the environment on the expression of these traits. However, among the studied characters, GCV and PCV were high for fruit yield per plant and the number of fruits per tree. All the studied traits showed considerably high heritability, ranging from 56.2 to 98.2 %, while the genetic advance was high for the top traits. Moreover, the combined influence of genetic advance and heritability offers the most effective conditions for selecting a specific trait.

Estimating each trait's contribution to the total observed variations in the genotypes is important; this enables the identification of the significant traits accounting for the greater share in the observed variations and then enables the breeder to focus on specific traits of interest for crop improvement. Consequently, the current work aimed to evaluate 11 mango genotypes by employing multivariate analysis based on cluster and principal component analysis (PCA) for yield and some of its contributing traits, as well as estimating the genetic variability, heritability, and genetic advance among the yield and its components.

2 MATERIALS AND METHODS

The present study was conducted on 12 years old mango trees grown on clay soil at Qus, Qena governorate, Egypt (25°54'56.2" N 32°45'30.7" E) during two successive seasons of 2021 and 2022. The experimental materials comprised 11 genotypes, *i.e.*, ten seeding strains and one check as the Zebda variety. The trees were spaced at 7 m x 7 m. The experimental design was intended in Randomized Complete Block Design with three replications. A single tree of both strains and varieties with the same uniform size is considered one replicate. Common cultural practices for orchards were provided with standard agronomic methods such as fertilization, irrigation, and pest management, as usual for mango farms. Data was recorded on ten quantitative characters in three replications and a single tree considered as replication for the studied traits as follows: yield per tree (Y), number of fruits per tree (NF), fruit mass (FM), fruit length (FL), fruit diameter (FD), fruit pulp (FP), seed mass (SM), total soluble solids (TSS), total sugars (TS g) and total acidity (AC). The yield per tree was recorded over the study period, fruit mass was measured by weighing balance, total soluble solids were measured by using a handy refractometer (AOAC, 2000), and total acidity was measured as g citric acid/ 100 g pulp according to (AOAC, 2000).

2.1 STATISTICAL ANALYSIS

The mean values of the data were analyzed according to (Sharma, 1998). Data were analyzed separately for each year and combined over two years (Steel & Torrir, 1980). The differences between the means for all studied traits were calculated using revised L.S.D. at 5 % and 1 %. Genotypic and phenotypic coefficient of variation (GCV and PCV) were computed by the formula suggested by Singh & Chaudhury (1985). Heritability, in a broad sense, was estimated according to Falconer (1989). The genetic advance was calculated as per the formula given by (Allard, 1960).

The hierarchical cluster analysis procedure of the program SPSS-V.13 for windows carried out cluster analysis. Principal component analysis (PCA) was performed using Minitab statistical software -V.17. The PCA was used to determine the extent of genetic variation. Eigenvalues were obtained from PCA, which were used to determine the axes' relative discriminative power and associated characters (Pradhan et al., 2015). The genotypes were categorized in a bi-plot figure and compared with the cluster analysis. Simple correlation coefficients between different traits under the study were analyzed by the method of Hayes et al. (1955).

3 RESULTS AND DISCUSSION

3.1 MEAN PERFORMANCE OF MANGO GENOTYPES

As shown in Table 1, it should be called that 2 out of 11 genotypes, *i.e*., S9 and S10, were superior for the number of fruits per tree, yield per tree, fruit mass, and fruit diameter in the two years and showed significant ($p < 0.01$) compared to 'Zebda' (chick genotype No. 11), which was superior from the other genotypes in 5 out of 10 traits namely, fruit pulp, seed mass, TSS, total sugars, and total acidity in two years. Generally, the genotypes S9 and S10 were superior in yield traits, while 'Zebda' was superior in all quality traits. Igbari et al. (2019) evaluated seven mango varieties using 13 morphological traits, and the results exhibited some variability in fruit sizes and shapes, leading to reliable discriminating characters. They demonstrated that while some mango fruit morphological traits showed the greatest diversity, others showed little to no variation and could not be effectively employed as a characterization tool.

3.2 ANALYSIS OF VARIANCE, GENOTYPIC AND PHENOTYPIC COEFFICIENTS OF VARIATION

The individual analysis of variance for each year and the combined analysis are shown in Table 2. The results indicated that mean squares of the studied genotypes were highly significant ($p < 0.01$) every two seasons and combined, indicating wide genetic diversity among the genotypes for all examined traits. Meanwhile, there were no significant differences in combined analysis among years for all traits except for the number of fruits/tree. However, the interaction of genotypes × years was significant ($p < 0.01$) for three out of ten studied traits: number of fruits/ tree, yield/ tree, and fruit mass. These results agree with Hamad (2021) and Serry et al. (2019).

Table 3 displays the heritability and predicted genetic advance, as well as the extent of variability within ten characters across different genotypes, as evaluated by range, genotypic coefficient of variation, and phenotypic coefficient of variation. The highest range of variation was recorded in the number of fruits per tree (141.77-488.73 and 146.90-496.63 fruits), followed by yield per tree (39.27-137.70 and 40.70 - 141.30 kg/tree), fruit mass (220.13-281.80 and 224.43-285.33 g) in first and second years, respectively among the characters (Table 3). A moderate range of variation was found in seed mass percentage (6.33-19.10 %) and (19.10-71.82 %), with a mean of 15.11 and 16.70 % in the first and second seasons, respectively. The remaining contributing characters had a narrow range of variation, indicating a small value of variability among the genotypes. The results of Jena et al. (2021) showed a marked variation in the fruit traits of mango genotypes, reflecting the highly heterozygous nature. Akhtar et al. (2007) stated that characters with a high range of variation should be prioritized in the selection. Galal et al. (2017) and Patel et al. (2015) obtained a wide range of phenotypic variations, high heritability, and genetic advance among the genotypes for the number of traits. Generally, population variability is essential for breeding programs, as substantial variation in the qualities of interest indicates an opportunity for successful improvement through selective breeding.

A high magnitude of GCV percentage and PCV

Table 1: Means of the studied traits for eleven mango genotypes cultivated in Upper Egypt for the years 2021 and 2022

Genotypes	Number of fruits/tree	Yield/tree (kg)	Fruit mass (g)	Fruit length (cm)	Fruit diameter (cm)	Fruit pulp (%)	Seed mass (%)	TSS (%)	Total sugars (%)	Total acidity (%)
					1st Year					
S1	285.83 ± 15.72	61.23 ± 34.74	220.13 ± 26.17	8.57 ± 0.09	7.3 ± 0.07	62.17 ± 2.76	18.73 ± 0.24	13.93 ± 0.1	10.87 ± 0.44	0.34 ± 0.00001
S2	308.80 ± 51.87	69.8 ± 16.48	230.13 ± 9.77	8.7 ± 0.09	7.5 ± 0.04	61.97 ± 0.44	19.1 ± 0.01	14.17 ± 0.32	10.87 ± 0.25	0.31 ± 0.00003
S3	322.33 ± 46.97	70.63 ± 56.14	236.17 ± 11.32	8.97 ± 0.06	7.67 ± 0.06	62.23 ± 0.32	18.07 ± 0.3	14.83 ± 0.02	11.2 ± 0.12	0.305 ± 0.00003
S4	339.00 ± 25.04	82.07 ± 30.77	248.47 ± 10.17	8.67 ± 0.44	7.63 ± 0.09	62.4 ± 0.39	18.27 ± 0.03	15.33 ± 0.02	11.23 ± 0.02	0.291 ± 0.00001
S5	364.73 ± 37.76	91.50 ± 41.97	257.83 ± 4.42	8.93 ± 0.33	7.77 ± 0.06	63.47 ± 0.08	18 ± 0.21	15.27 ± 0.1	11.47 ± 0.04	0.277 ± 0.00005
S6	378.67 ± 38.58	98.80 ± 10.36	260.1 ± 4.81	9 ± 0.31	7.93 ± 0.06	63.87 ± 0.02	17.1 ± 0.36	15.87 ± 0.04	11.3 ± 0.13	0.275 ± 0.00003
S7	390.43 ± 34.40	102.93 ± 34.54	262.77 ± 25.9	9.43 ± 0.12	7.87 ± 0.2	64.4 ± 0.93	17.4 ± 0.16	15.9 ± 0.04	12.23 ± 0.09	0.257 ± 0.00001
S8	408.83 ± 33.82	107.47 ± 44.33	268.67 ± 10.89	9.57 ± 0.09	8.07 ± 0.09	65.23 ± 0.02	17.2 ± 0.16	16.2 ± 0.04	12.57 ± 0.17	0.25 ± 0.00003
S9	428.17 ± 17.42	120.80 ± 05.32	276 ± 24.25	10.03 ± 0.26	8.13 ± 0.06	66.67 ± 0.3	16.6 ± 0.16	16.23 ± 0.16	12.63 ± 0.02	0.252 ± 0.00001
S10	488.73 ± 19.33	137.70 ± 23.85	281.8 ± 9.25	10.67 ± 0.24	8.37 ± 0.02	68.23 ± 0.6	16 ± 0.17	17.13 ± 0.04	13.43 ± 0.15	0.245 ± 0.00001
Chick	141.77 ± 10.94	039.26 ± 0.96	247.17 ± 52.02	11.9 ± 0.04	7.63 ± 0.06	72.17 ± 0.16	6.33 ± 0.04	17.93 ± 0.143	14.47 ± 0.04	0.32 ± 0.0001
Means	350.66	89.29	253.57	9.49	7.81	64.8	15.11	14.99	11.47	0.26
$LSD_{0.05}$	12.97	4.97	4.58	0.38	0.34	1.3	0.7	0.51	0.59	0.05
$LSD_{0.01}$	17.68	6.77	6.25	0.52	0.46	1.77	0.96	0.7	0.8	0.07
					2nd Year					
S1	298.97 ± 13.33	68.6 ± 7.09	224.43 ± 14.16	8.77 ± 0.05	7.23 ± 0.04	62.2 ± 2.01	19.1 ± 0.16	14.53 ± 0.06	10.73 ± 0.24	0.338 ± 0.000112
S2	315.5 ± 22.21	73.2 ± 9.49	238.47 ± 5.97	8.83 ± 0.06	7.4 ± 0.09	62.33 ± 0.44	19 ± 0.25	15.33 ± 0.13	10.8 ± 0.13	0.315 ± 0.000025
S3	326.57 ± 55.44	78.27 ± 7.12	237.23 ± 18.13	9.03 ± 0.02	7.6 ± 0.07	62.13 ± 0.3	18.5 ± 0.16	15.17 ± 0.09	11.07 ± 0.09	0.31 ± 0.0001
S4	347 ± 28.99	88.2 ± 4.69	251.03 ± 7.7	8.97 ± 0.17	7.76 ± 0.06	63.23 ± 0.1	18.2 ± 0.09	15.86 ± 0.06	11.37 ± 0.02	0.295 ± 0.000025
S5	372.63 ± 42.8	94.27 ± 14.06	252.5 ± 23.59	9.13 ± 0.13	7.87 ± 0.1	63.67 ± 0.14	18 ± 0.16	16.1 ± 0.16	11.63 ± 0.003	0.27 ± 0.000025
S6	380.37 ± 35.1	98.5 ± 12.31	258.6 ± 6.31	9.57 ± 0.02	7.8 ± 0.16	64.03 ± 0.49	17.6 ± 0.09	16.03 ± 0.1	11.87 ± 0.04	0.258 ± 0.000004
S7	410.07 ± 36.56	109.37 ± 10.17	265.43 ± 22.66	9.63 ± 0.06	7.9 ± 0.21	65.07 ± 0.44	17.3 ± 0.16	16.33 ± 0.16	12.37 ± 0.2	0.26 ± 0.000025
S8	421.53 ± 27.32	112.5 ± 10.93	269 ± 3.64	10 ± 0.28	7.6 ± 0.39	65.23 ± 0.14	17 ± 0.25	16.47 ± 0.08	12.5 ± 0.01	0.26 ± 0.000025
S9	444.1 ± 19.91	123.63 ± 11.96	275.63 ± 23	10.1 ± 0.36	7.96 ± 0.25	66.93 ± 0.17	16.5 ± 0.04	16.67 ± 0.02	13.13 ± 0.3	0.252 ± 0.00001
S10	496.63 ± 19.88	141.3 ± 31.92	285.33 ± 14.42	11 ± 0.21	8.27 ± 0.08	67.7 ± 0.11	15.77 ± 0.04	17.27 ± 0.02	13.4 ± 0.14	0.250 ± 0.00002
11(chick)	146.90 ± 54.27	40.7 ± 1.11	238.4 ± 15.97	11.53 ± 0.03	7.83 ± 0.01	70.67 ± 0.69	6.7 ± 0.16	17.43 ± 0.13	15.03 ± 0.06	0.323 ± 0.000132

Continued on the next page

Means	360.02	88.72	254.19	9.69	7.75	64.84	16.7	16.11	12.17	0.28
$LSD_{0.05}$	7.43	3.8	4.37	0.45	0.42	1.19	0.64	0.34	0.54	0.05
$LSD_{0.01}$	10.13	5.19	5.97	0.61	0.57	1.63	0.87	0.46	0.73	0.07

Table 2: Mean squares of variance analyses for the studied traits of eleven mango genotypes cultivated in Upper Egypt for the years 2021 and 2022 and combined analysis for the two years

S. O. V	df	NF	Y (kg/tree)	FM (g)	FL (cm)	FD (cm)	FP (%)	SM (%)	TSS (%)	TSg (%)	AC (%)
Year 1											
Replications	2	119.09	226.37	121.26	1.73	0.44	0.58	0.2	0.15	0.38	0.001
Genotypes	10	24464.9**	2405.4**	1113.2**	3.14**	0.28**	30**	37.4**	4.3**	4.0**	0.003**
Error	20	57.95	8.5	7.24	0.05	0.04	0.58	0.17	0.09	0.12	0.0001
Year 2											
Replications	39.18	86.9	96.664	0.79	0.94	0.22	0.14	0.58	0.35	0.001	0.001
Genotypes	25503.8**	2373.4**	1043.2**	2.47**	0.24**	21.57**	36.12**	2.29**	5.10**	0.003**	0.003**
Error	19.03	4.99	6.61	0.07	0.06	0.49	0.14	0.04	0.1	0.0002	0.0001
Combined analysis											
Years (Y)	1	1445.8**	292.74	6.37	0.62	0.62	0.02	0.1	2.64	1.82	1.82
Error (a)	4	1264.46	229.82	110.55	1.42	1.42	0.41	0.2	1.02	0.46	0.46
Genotypes (G)	10	27219.3**	4769.1**	2123.5**	5.53**	5.53**	50.93**	73.42**	6.27**	9.02**	9.02**
G × Y	10	22749.4**	9.61**	32.82**	0.09	0.09	0.59	0.12	0.27	0.11	0.11
Error (b)	40	2.35	0.57	6.76	0.04	0.04	0.53	0.16	0	0.1	0.1

percentage were observed in the number of fruits per tree, followed by yield per tree and fruit mass in both seasons. Meanwhile, the other traits recorded less difference between GCV and PCV and less influence by environmental conditions (Table 3). High estimates of broad sense heritability for these variables show little to no environmental influence, even though they exhibit a little mismatch between PCV and GCV, as evidenced by these results. GCV's high value can be exploited through proper selection. Galal et al. (2017) reported that the higher the genotypic coefficient of variation value, the more potential for character improvement. These findings agree with Majumder et al. (2012); Patel et al. (2015); Sridhar et al. (2018), and Das et al. (2021). They found significant heritability and genetic advance among the genotypes and tiny variations between the genotypic and phenotypic coefficients of variation for practically all variables, indicating that environmental influences were minimal. The high PCV and GCV were obtained for fruit mass, seed width, seed mass, acidity, TSS, and yield/plant. The high PCV and GCV were obtained for fruit mass, seed width, seed mass, acidity, TSS, and yield/plant.

3.3 HERITABILITY AND GENETIC ADVANCE

As our understanding of genetics continues to expand, we can utilize heritability to forecast how choosing superior genotypes will ultimately pan out. From the results presented in Table 3, the heritability percentage ranged from 75.63 (fruit diameter) to 99.93 % (number of fruits per tree). High heritability percentage estimates coupled with the high genetic advance in the number of fruits per tree (99.76 and 119.09 %) in the first season indicate that the environment less influenced this character, showing that these traits were controlled by a small number of genes or, alternatively, that there was an additive genetic influence even if they were polygenic in nature. As this is the case, selecting certain characteristics would be more useful for increasing yield. The high value

Table 3: Range of values and genetic parameters for all studied traits of mango genotypes cultivated in Upper Egypt during the years 2021 and 2022

Characters	Min	Max	GCV (%)	PCV (%)	Hb (%)	GA
			Year 1			
Number of fruits/tree	141.77 ± 10.94	488.73 ± 19.33	2320.07	2325.58	99.76	119.09
Yield/tree (kg)	39.26 ± 0.96	137.7 ± 23.85	894.78	897.95	99.65	17.44
Fruit mass (g)	220.13 ± 26.17	281.8 ± 9.25	145.38	146.33	99.35	14.82
Fruit length (cm)	8.57 ± 0.09	11.9 ± 0.04	10.86	11.03	98.46	0.1
Fruit diameter (cm)	7.3 ± 0.07	8.37 ± 0.02	1.03	1.2	85.85	0.07
Fruit pulp (%)	61.97 ± 0.44	72.17 ± 0.16	15.11	15.41	98.08	1.17
Seed mass (%)	6.33 ± 0.04	19.1 ± 0.01	74.69	75.04	99.54	0.35
TSS (%)	13.93 ± 0.1	17.93 ± 0.143	8.84	9.03	97.83	0.18
Total sugars (%)	10.87 ± 0.44	14.47 ± 0.04	10.88	11.21	97.07	0.24
Total acidity (%)	0.245 ± 0.00001	0.34 ± 0.00001	0.35	0.35	99.3	0.0002
			Year 2			
Number of fruits/tree	146.90 ± 54.27	496.63 ± 19.88	2359.54	2361.3	99.93	39.18
Yield/tree (kg)	40.7 ± 1.11	141.3 ± 31.92	844.32	846.1	99.79	10.26
Fruit mass (g)	224.43 ± 14.16	285.33 ± 14.42	135.93	136.8	99.37	13.53
Fruit length (cm)	8.77 ± 0.05	11.53 ± 0.03	8.27	8.51	97.15	0.14
Fruit diameter (cm)	7.23 ± 0.04	8.27 ± 0.08	0.78	1.02	75.63	0.09
Fruit pulp (%)	62.13 ± 0.3	70.67 ± 0.69	10.84	11.09	97.73	0.99
Seed mass (%)	6.7 ± 0.16	19.1 ± 0.16	71.82	72.11	99.6	0.29
TSS (%)	14.53 ± 0.06	17.43 ± 0.13	4.64	4.73	98.06	0.08
Total sugars (%)	10.73 ± 0.24	15.03 ± 0.06	13.67	13.94	98.07	0.2
Total acidity (%)	0.250 ± 0.00002	0.338 ± 0.000112	0.36	0.36	98.45	0.0004

Genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), heritability (Hb), and genetic advance (GA) of heritability coupled with a moderate degree of genetic advance was recorded for yield per tree, fruit mass in the two seasons, and the number of fruits per tree in the 2nd season. Thus, selection would be sufficient in situations of high heritability value and high or moderate value of genetic advance. This condition develops because of the interaction of additive genes (Das et al., 2021). Sridhar et al. (2018) concluded that high heritability implies that the environment's influence was negligible, allowing the breeder to choose plants based on their phenotypic expression. Hence the selection of the characters would be suitable for improving mango.

Because strong heritability does not always reflect high genetic progress, high heritability coupled with a lower degree of genetic advance was seen in the results of fruit length, fruit diameter, fruit pulp percentage, seed mass percentage, TSS percentage, total sugars, and total acidity, demonstrating that environmental factors and non-additive gene effects (dominance and epistasis) played a more significant role in determining these characteristics than did additive genetic factors (Sridhar et al., 2018; Getachew et al., 2021). All of the analyzed traits of mango genotypes with high heritability in the Das et al. (2021) study had high genetic advance values, indicating that additive genes controlled these qualities, and that selection would favor their improvement. In addition, Jena et al. (2021) concluded that the high heritability of mango traits and closeness of GCV and PCV values indicate they are less environmentally effective. Consequently, a reliable selection is made for breeding based on phenotypic characteristics (Bally and De Faveri, 2021).

3.4 PRINCIPAL COMPONENT ANALYSIS AND GENETIC DISTANCE

Figure 1 shows the various components and the eigenvalues calculated by principal components analysis (PCA). The principal component analysis revealed that four principal components, PC1, PC2, PC3, and PC4, with eigenvalues 4.70, 2.12, 0.12, and 0.06, respectively, have accounted for the total cumulative variability among genotypes. The first two principal components, PC1 (67.1 %) and PC2 (30.25 %), showed eigenvalues of more than one, and cumulatively they explained 97.35 % variability (the highest variance when correlating the most relevant components), where the contribution of PC1 towards variability was the highest (67.1 %). The results showed that fruit mass and total sugars in PC1 and fruit mass and number of fruits in PC2 had the highest loadings. Many authors as Lawson et al. (2019) and Sridhar et al. (2022), established the effectiveness of PCA, proving it could classify mangoes. Lal et al. (2019) reported that

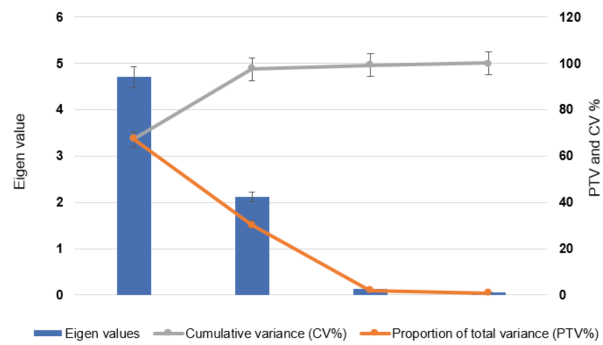

Figure 1: Scree plot of Eigenvalues, variability proportion (PTV), and cumulative variability (CV) for studied traits of eleven mango genotypes

PCA for 17 traits of 60 mango genotypes was reduced to six principal components with eigenvalues up to 1.0, presenting a cumulative variance of 78.78 % variation among the genotypes.

As shown in Table 4 and Figure 2, PC1 has a positive association with total sugars and fruit mass and a negative association with total acidity. The second PC has a positive association with fruit mass and the number of fruits while the negative association with total sugars. The third PC has a positive association with fruit diameter and the number of fruits while a negative association with yield per tree. PC4 has a positive association with fruit mass and a negative association with yield per tree.

The current investigation indicated that five major characters contributed one hundred percent to genetic divergence out of a total of seven yield and contributing traits. The number of fruits per tree and the yield per tree were found to contribute 87.8 % and 8.4 %, respectively, to genetic divergence out of the five major traits studied (Figure 3).

Previous studies (Rajan et al., 2009; Majumder et al., 2012; Singh, 2016; Sridhar et al., 2022) have also reported the maximum contribution of the number to genetic divergence in mango genotypes. Therefore, the

Table 4: Principal component analysis for different traits in mango genotype

Variables	PC1	PC2	PC3	PC4
NF	0.429	0.479	0.515	0.254
Y	0.165	0.0316	-0.254	-0.838
FM	0.627	0.571	-0.054	0.435
FL	0.144	0.0630	-0.092	0.233
FD	0.058	0.240	0.829	-0.278
AC	-0.323	-0.239	0.356	0.313
TS g	0.669	-0.719	0.185	0.047

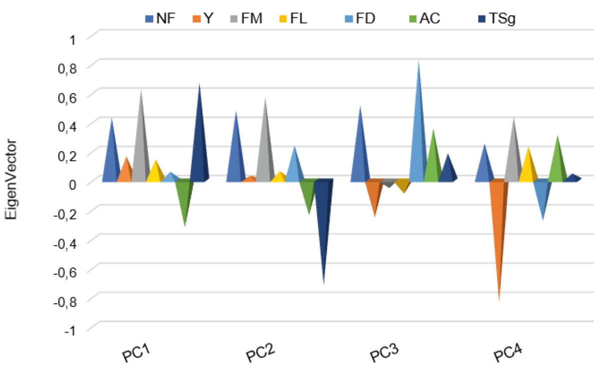

Figure 2: Scree plot of Eigenvector for studied traits of 11 mango genotypes in Upper Egypt

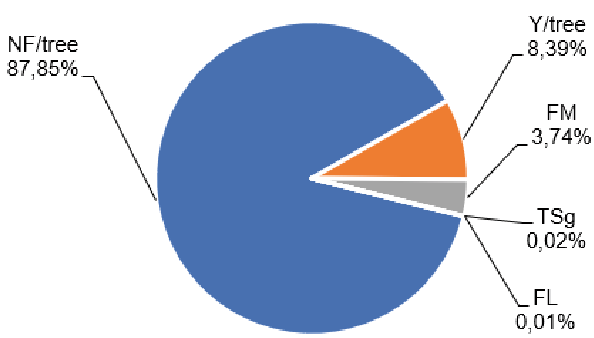

Figure 3: Graphical representation of the proportionate contribution of studied major traits towards genetic divergence

number of fruits would be the critical parameter for selecting divergent genotypes. Clusters I and II exhibited the highest cluster mean values for most studied physical traits (Table 5). The highest cluster mean values for fruit diameter, fruit mass, yield per tree, and number of fruits per tree were found in Cluster II, followed by cluster I. Cluster III represented the highest cluster mean value for total acidity, total sugars, TSS, fruit pulp, and fruit length. Although cluster IV had the maximum number of genotypes (4), no remarkable feature was noticed in this cluster for most different characters, and it had the lowest mean values for fruit diameter, fruit length, fruit mass, total sugars, total soluble solids, and fruit pulp.

In order to decipher the variation among the genotypes, a principal component analysis (PCA) was carried out. Moreover, a scattered diagram of the genotypic distribution pattern on the axis is shown in Figure 4. The scree plot indicates most of the variation is derived from the first and second components in the eigenvalue of the genotypes data. The results of biplot-PCA stated the presence of high genetic variations among genotypes based

Table 5: Average (av) of studied traits for each cluster along with standard deviation (SD), as well as the difference (dif) between each cluster, mean, and total mean

Cluster No.		Fruit diameter (cm)	Fruit length (cm)	Fruit mass (g)	Yield/tree (kg)	Number of fruits/tree	Total acidity (%)	Total sugars (%)	TSS (%)	Seed mass (%)	Fruit pulp (%)
I	av ± SD	7.86 ± 0.001	9.41 ± 0.11	261.86 ± 34.88	101.91 ± 58.42	390.91 ± 43.39	0.26 ± 0.00003	12 ± 0.25	16.03 ± 0.08	17.45 ± 0.15	64.37 ± 0.56
	dif	0.08	-0.18	7.99	10.52	35.56	-0.02	-0.11	0.12	0.79	-0.45
II	av ± SD	8.19 ± 0.04	10.46 ± 0.3	279.7 ± 30.03	130.86 ± 49.3	464.41 ± 59.95	0.25 ± 0.00	13.15 ± 0.15	16.83 ± 0.28	16.22 ± 0.22	67.39 ± 0.68
	dif	0.4	0.86	25.82	39.46	109.06	-0.03	1.05	0.91	-0.44	2.57
III	av ± SD	7.73 ± 0.00	11.72 ± 0.00	242.78 ± 0.00	39.98 ± 0.00	144.33 ± 0.00	0.32 ± 0.00	14.75 ± 0.00	17.68 ± 0.00	6.52 ± 0.00	71.42 ± 0.00
	dif	-0.05	2.13	-11.1	-51.42	-211.01	0.04	2.65	1.77	-10.14	6.6
IV	av ± SD	7.52 ± 0.04	8.82 ± 0.02	235.76 ± 26.82	74 ± 7.93	318 ± 45.06	0.31 ± 0.00029	11.02 ± 0.06	14.9 ± 0.32	18.63 ± 0.18	62.33 ± 0.11
	dif	-0.27	-0.78	-18.12	-17.4	-37.34	0.03	-1.08	-1.02	1.97	-2.49

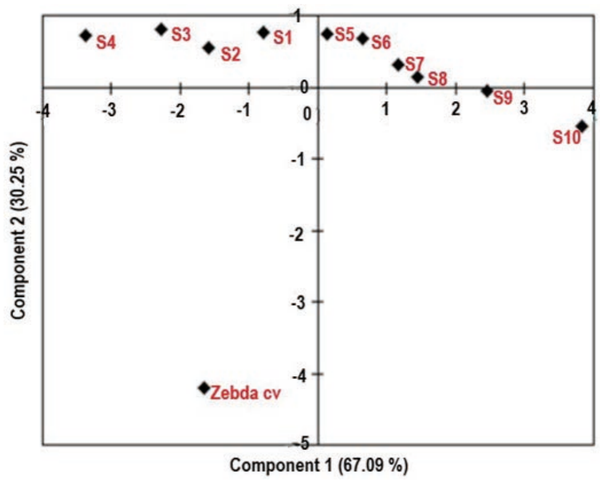

Figure 4: Principal component analysis (PCA) based on the first and second components for the eleven mango genotypes

on the data of studied traits (Figure 4). The first and fourth clusters had four genotypes accounting for 72.8 % of total genotypes (36.4 % each), beside two and one genotypes were classified in the second and third clusters accounting for 18.1 % and 9.1 % of total genotypes, respectively (Table 6).

Pairwise comparisons were conducted between all genotypes, and the mean dissimilarity values were calculated based on five traits of the studied mango fruit. The distance between all eleven mango genotypes was evaluated. The Euclidean distance coefficient ranged from 0.539 (between S1 and S2) to 16.334 (between S2 and ''Zebda'), where the mean distance between groups was found with a maximum (16.334), as shown in Table 7, which indicates to a reasonable variance between the genotypes. Zebda cultivar was found to be much distanced genetically from other genotypes (>10 DC), followed by S10 [from S1 (7.6 DC), S2 (7.5 DC), S3 (7 DC), and S4 (6.3 DC)], S9 [from S1 (6 DC) and S2 (5.9 DC)] as well as both S10 [from S5, (5.4 DC)] and S9 [from S3, (5.4 DC)] that distanced from the genotypes under study. Most other genotypes were scattered over the plot with a medium or close genetic distance. There may have been shared ancestors between the genotypes, as evidenced by the close distance between them and their grouping within a common cluster (Lal et al., 2019). When there is a lot of variance between individuals' genes, it might cause phenomena known as allelic amplitudes to appear in the population's phenotypes. Using varieties from various clusters with high to moderate genetic distances in crossing programs may be advised to create new recombinants with desirable characteristics (Majumder et al., 2013).

Table 6: Optimization grouping between 11 mango genotypes, obtained by cluster analysis, based on five fruit characteristics, using the Euclidian distance

Clusters	No. of genotypes	Percentage	Genotypes included
I	4	36.4 %	GROUP1 (Y1 > = 0, Y2 > = 0): S5, S6, S7 and S8
II	2	18.1 %	GROUP2 (Y1 > = 0, Y2 < 0): S9 and S10
III	1	9.1 %	GROUP3 (Y1 < 0, Y2 < 0): Check
IV	4	36.4 %	GROUP4 (Y1 < 0, Y2 > = 0): S1, S2, S3 and S4

Table 7: Euclidean distance coefficient (DC) among 11 Mango genotypes

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	'Zebda'
S1	0	0.539	1.052	1.732	2.34	3.031	3.846	4.483	6.019	7.642	16.329
S2		0	0.855	1.428	2.123	2.852	3.682	4.325	5.91	7.518	16.334
S3			0	0.893	1.631	2.263	3.16	3.805	5.432	7.023	15.625
S4				0	0.831	1.509	2.39	3.032	4.682	6.253	15.085
S5					0	0.798	1.586	2.229	3.858	5.44	14.409
S6						0	1.068	1.66	3.268	4.817	13.641
S7							0	0.647	2.317	3.879	13.057
S8								0	1.702	3.238	12.527
S9									0	1.631	11.268
S10										0	10.085

The hybridization program would be sensible if performed with 'Zebda' combined with any other studied genotypes and between S10 with S1, S2, or S3 due to higher observed distances to obtain higher values of essential characteristics, as well as a mitigation of the speed of primitive extinction and adaptive genes between genotypes (Govindaraj et al., 2015). Small distances between S1 and S2 (0.539 DC) or S2 and S8 (0.647) may correspond to originating from a common ancestor, or some genetic material may be substituted between the parental roots of these genotypes, making them all combined into one main group (Davis, 1997; Tahir et al., 2021).

3.5 UPGMA CLUSTERING DENDROGRAM

Figure 5 presents the UPGMA tree diagram generated by cluster analysis based on five fruit traits of mango genotypes. Generally, it shows two large classes: low seed mass (SM) trait ('Zebda') and high or medium SM trait (other genotypes). Four groups were formed in a complex selection across the approved cut-off point; 'Zebda' formed a single cluster within a class with a low SW trait and the other ten genotypes in the other class. Genotypes clustered similarly in the dendrogram, cluster analysis, principal component analysis (PCA) graph, and along the two axes of the PCA graph (Figure 4). Once more, 'Zebda' created a single cluster that was very different from the other clusters, suggesting that this genotype could be crossed with others to produce offspring with the desired characteristics. The perusal of the results revealed that the number of fruits per tree and fruit mass exhibited higher estimates of GCV, heritability, and genetic advance, indicating additive gene effects controlling these traits. Therefore, individual plant selection for these traits would be effective in the mango crop. Accordingly, non-additive control the inheritance of all studied traits except the two above traits (number of fruits per tree and fruit mass); hence, other methods used in the breeding that traits other than selection like hybridization, mutations, and vegetative propagation, especially mango is a highly heterozygous crop. The results of genetic studies can be used for the selection of parents in hybridization programs. Hence, direct selection may be followed to im-

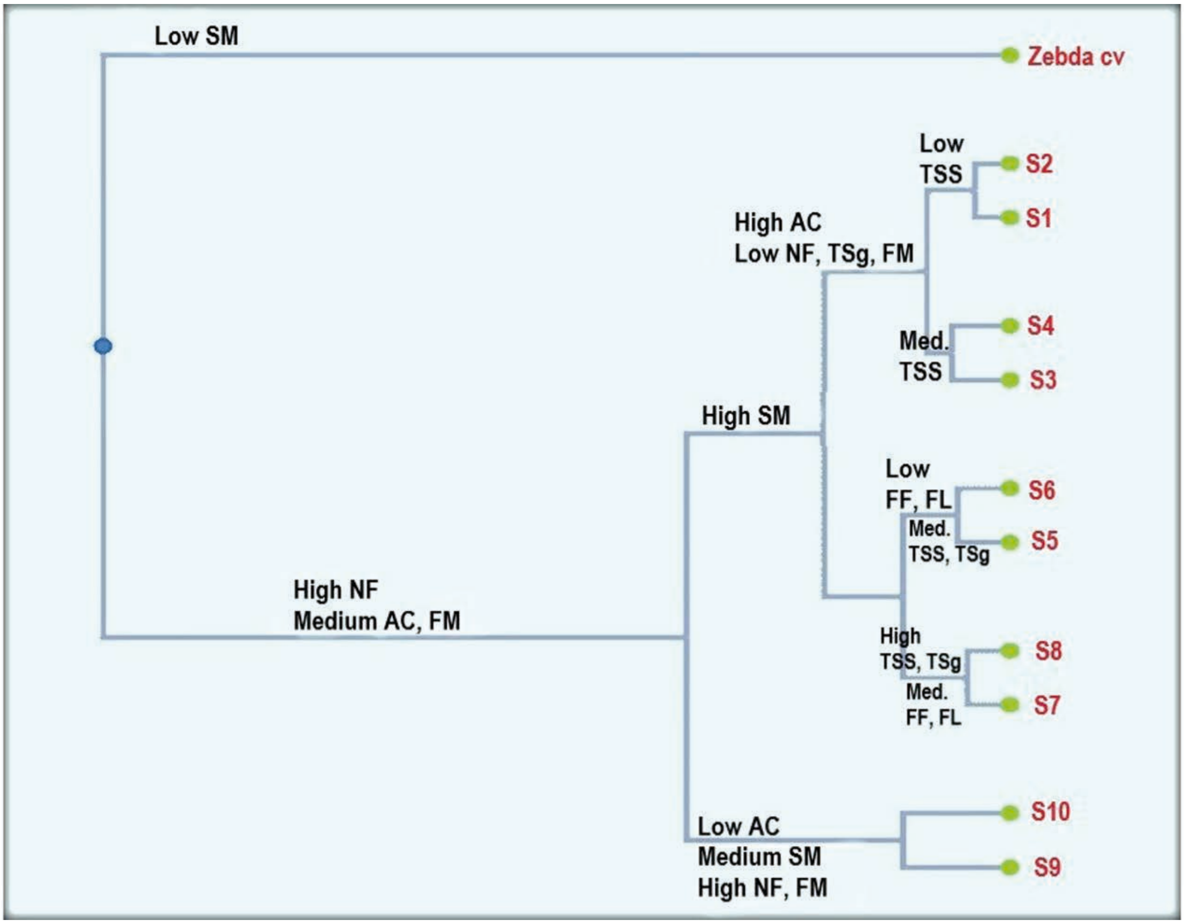

Figure 5: Dendrogram, using average linkage (Between Groups), for eleven mango genotypes based on five fruit traits

prove mango for these characters. Sridhar et al. (2018) and Das et al. (2021) both find results consistent with these conclusions.

3.6 CORRELATION COEFFICIENTS

Understanding how different traits are linked is crucial during the crop improvement selection process (Fasahat et al., 2016). Simple correlation coefficients between different traits in eleven mango genotypes for nine traits in two years (above and below) are demonstrated in Table 8.

In the present study, the highest significantly positive correlation coefficients were obtained between yield per tree and number of fruits per tree (0.987 and 0.995), followed by fruit mass (0.966 and 0.971), fruit pulp (0.905 and 0.940), fruit length (0.865 and 0.910) and total sugars % (0.819 and 0.911) in first and second seasons, respectively. This finding implied that selection procedures aimed at increasing yield per tree would improve these characteristics automatically. On the other hand, seed mass demonstrated a negative and significant correlation with each yield per tree (-0.838 and -0.883), no of fruits per tree (-0.860 and -0.954), fruit mass (-0.833 and -0.913) and fruit pulp (-0.850 and -0.868) as well as TSS % (-0.400 and -0.885) and total sugars (-0.802 and -0.913) in first and second seasons, respectively. The number of fruits per tree showed positive and highly significant with fruit mass (0.955 and 0.973), fruit length (0.868 and 0.911), and fruit pulp (0.910 and 0.942), while it demonstrated insignificant with seed mass (-0.860 and-0.954) in first and second seasons, respectively. These results agree with Samal et al. (2012) and Igbari et al. (2019). They used the Pearson correlation coefficient for mango varieties quality parameters and found positive and negative correlations between many fruit traits. Lawson et al. (2019) also used Pearson's correlation coefficient to explore the relationship between the postharvest quality parameters during mango fruit ripening. A real picture of the genetic relationships between various traits and the direct and indirect contributions of one trait to another is provided by correlation analysis (Jena et al., 2021). In our investigation, positive and negative correlations between quantitative characteristics were strongly reflected.

4 CONCLUSIONS

Eleven genotypes of mango exhibited substantial genetic diversity. The highest range of variation was recorded in the number of fruits per tree, followed by yield per tree and fruit mass. High heritability estimates coupled with a high or moderate degree of genetic advance in the number of fruits per tree, yield per tree, and fruit mass. The correlation was positive and significant between yield per tree, with each of number of fruits per tree, fruit mass, fruit length, fruit pulp, and total sugars. In contrast, seed mass demonstrated a negative and significant correlation with yield per tree, number of fruits per tree, fruit mass, and fruit pulp in the two years of study. For the future experiment, traits contributing maximum to genetic diversity, such as fruits per tree, fruit mass, and yield per tree, should be prioritized as selection parameters, and diverse genotypes identified in the present study may be utilized for attempting heterotic cross combinations and developing hybrid varieties.

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Table 8: Simple correlation coefficients between each pair of nine traits in 1st (above diagonal) and 2nd year (below diagonal) in eleven mango genotypes

Traits	Y (kg)	NF	FM (g)	FL (cm)	FD (cm)	FP (%)	SM (%)	TSS (%)	TSg (%)
Y (kg)		0.987**	0.966**	0.865**	0.846**	0.905**	-0.838**	0.469	0.819**
NF	0.995**		0.955**	0.868**	0.847**	0.910**	-0.860**	0.466	0.847**
FM (g)	0.971**	0.973**		0.773	0.805**	0.850**	-0.833**	0.397	0.788
FL (cm)	0.91**	0.911**	0.869**		0.859**	0.817**	-0.693	0.473	0.711
FD (cm)	0.649**	0.646	0.637	0.662		0.755	-0.638	0.382	0.627
FP (%)	0.94**	0.942**	0.929**	0.856**	0.546		-0.850**	0.639	0.879**
SM (%)	-0. 883**	-0.954**	-0.913**	-0.828**	-0.595	-0.868**		-0.400	-0.802**
TSS (%)	0.883**	0.891**	0.901	0.698	0.45	0.808**	-0.885**		0.483
TSg (%)	0.911**	0.912**	0.889**	0.745	0.428	0.895**	-0.913	0.867**	

**. The mean difference is significant at the 0.01 level

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6 AUTHOR CONTRIBUTIONS

The study's planning and design included the participation of all authors. Mansour, MM, and Osman, SOA, prepared the materials and collected the data; Hussein, NRA, and Zaki H analyzed the data. Mansour, MM, Osman, SOA, and Zaki H wrote the original and subsequent versions of the manuscript. All authors have reviewed and approved the final manuscript.

7 DECLARATIONS

7.1 CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Use of sugars as alternative to chemical control: trials carried out on thrips associated with olive tree

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Use of sugars as alternative to chemical control: trials carried out on thrips associated with olive tree

Abstract: Foliar spraying of infradoses of sugars (glucose, fructose or sucrose) induces plant resistance to pests that are particularly difficult to combat. These include thrips, which can cause flower abortion, stunting and deformation of olives, resulting in significant crop losses. Randomised block trials were conducted during three years (2017 to 2019), on two cultivars Chemlal and Sigoise, in an olive grove in Batna province (Algeria), with the aim of determining the most effective dose and type of sugar on thrips populations, and to evaluate the effectiveness of combining sugar with chemical treatment, as well as the possibility of reducing the dose of the latter. The results showed that sucrose at a concentration of 100 ppm was the most effective and that the efficacy of sucrose was higher than that of glucose and fructose, on both cultivars tested. The combination of sucrose with insecticide resulted in a synergistic effect and a higher efficacy gain than sucrose alone, and that the efficacy of the combination sucrose + insecticide at low dose D1 was identical to the combination sucrose + insecticide at recommended dose D2. It is therefore possible to reduce the chemical insecticide dose while maintaining good treatment efficacy for the control of these pests.

Key words: thrips; *Olea europaea* L.; sucrose; fructose; glucose; 'Sigoise'; 'Chemlal'

Uporaba sladkorjev kot alternative kemijskemu nadzoru: poskus zatiranja tripsa na oljkah

Izvleček: Pršenje s sladkorji v majhnih koncentracijah (glukoze, fruktozeali saharoze) vzpodbuja odpornost rastlin na škodljivce, ki jih je še posebej težko zatirati. Med njimi so tripsi, ki lahko povzročajo odpadanje cvetov in deformacijo plodov oljk, kar znatno zmanjša pridelek. V obdobju treh let, 2017-2019, je bil na dveh sortah oljk, Chemlal in Sigoise, izveden naključni bločni poskus v oljčniku v provinci Batna (Alžirija), z namenom določitve najbolj učinkovitega odmerka in vrste sladkorja za uravnavanje populacije tripsa in ovrednotenje učinkovitosti kombiniranja sladkorja s kemičnimi zaščitnimi sredstvi kot možnosti njihove manjše uporabe. Rezultati so pokazali, da je bila saharoza pri koncentraciji 100 ppm najbolj učinkovita in, da je bila učinkovitost saharoze večja kot glukoze in fruktoze pri obeh preiskušenih sortah. Kombinacija saharoze z insekticidi je imela sinergetski učinek in večjo učinkovitost kot samo saharoza. Učinkovitost kombinacije saharoze in insekticida pri majhnem odmerku D1 je bila enaka kot pri kombinaciji saharoze in priporočenem odmerku insekticida D2. Iz tega sledi, da je mogoče zmanjšati odmerek insekticidov za doseganje dobre učinkovitosti pri uravnavanju teh škodljivcev.

Ključne besede: trips; *Olea europaea* L.; saharoza; fruktoza; glukoza; 'Sigoise'; 'Chemlal'

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1 INTRODUCTION

The olive tree (*Olea europaea* L.) is a typical and emblematic tree of the Mediterranean countries where it is of great importance from the economic, social and landscape point of view (Loumou & Giourga, 2003; Pappalardo et al., 2021). It represents one of the oldest and most widespread crops in Algeria. Thanks to its capacity to adapt to all bioclimatic stages, this species is present in the majority of the Algerian territory (Abdessemed et al., 2018). Nevertheless, the olive tree is susceptible to several insects' attacks and pathogens that cause a decline in olive production (Hadjou et al., 2013; Canale et al., 2019). Climatic variations in recent years have facilitated the introduction, spread and establishment of some pests and diseases in olive production (Ouyang et al., 2020; Vono et al., 2020; Ruggero, 2021). Among these pests, thrips (Thysanoptera), which are tiny sucking biting insects, having a short reproductive cycle with high reproductive potential and have a wide host spectrum including many weeds (Mound, 2018). The damage caused by food bites and viruses transmitted by certain species of thrips is mainly qualitative (discolouration, necrosis, deformation, etc.) and leads to a downgrading of the fruit and therefore to significant economic losses (Bournier, 1983). On olive trees, thrips attacks cause abortion of flowers and young fruits or result in stunted, scarred and deformed olives (Spooner-Hart et al., 2007; Phillips et al, 2020). The specific biology and behaviour of thrips makes chemical control difficult. Indeed, thrips tend to hide in flowers and buds, safe from contact insecticides; eggs inserted in the plant, and nymphs located in the soil, are also safe from treatments. In addition, they have the ability to develop resistance to insecticides (Bielza et al., 2007; Funderburk et al. 2016). Wu et al. (2018) and Reitz et al. (2020), reported that in recent years thrips have been a serious problem for crops, due to their damage and the constraints of pesticide application that make their control difficult. Faced with this situation, the use of alternative methods to chemical control remains necessary, such as the use of intercropping (Gombač & Trdan, 2014), biological control by predatory mites *Neoseiulus* spp., hemipterans *Orius* spp. (Loomans & Murai, 1997), or by predatory thrips *Aeolothrips intermedius* Bagnall 1934 (Trdan et al., 2005)

A new biocontrol method based on exogenous applications of infra-doses of soluble carbohydrates has been developed to reinforce plant immunity against certain herbivores and pathogens (Arnault et al., 2021). This is the new concept of 'Sweet Immunity' or 'sugar-enhanced defence' (Bolouri-Moghaddam & Van Den Ende, 2013; Arnault et al., 2021). Soluble carbohydrates, mainly, sucrose, glucose and fructose, are involved in many stress response mechanisms, biotic or abiotic, where they act not only as metabolites, but also as signals capable of activating signalling pathways leading to gene expression changes (Morkunas & Ratajczak. 2014; Formela-Luboińska et al., 2020). Furthermore, soluble carbohydrates sprayed at low doses can penetrate the cuticle and end up on the plant surface, constituting signals perceived by the insect through contact, then influencing its behavior and selection of the host plant to lay eggs (Derridj et al., 2011). This method induces physiological and metabolic changes in plant tissues and on the leaf surface, as well as resistance to pests (Smeekens et al., 2010)

The action of soluble carbohydrates (glucose, fructose, sucrose, trehalose) sprayed in infra doses (ppm) on the surface of cultivated plants has been studied on different crops for the control of various pests; such as *Cydia pomonella* (L., 1758) on apple, *Thrips tabaci* Lindeman, 1889 on leek, *Ostrinia nubilalis* (Hübner, 1796) on maize, *Tuta absoluta* (Meyrick, 1917), *Meloidogyne javanica* (Treub, 1885) and *Botrytis cinerea* Pers. on tomato (Ferré et al., 2008; Derridj et al., 2012; Arnault et al., 2012, 2015, 2017). These studies revealed that sugar alone has interesting effects and when combined with chemical plant protection products, it allows reducing their doses while keeping a good efficiency. Sugars activate defense pathways but not always in the same way (Arnault et al., 2021), it would however be advisable to analyse for each crop and each targeted phytophage, the most active sugar and dose. Derridj (2009), reports that the species, variety and age of the plant at the time of treatment seem to be important factors for successful resistance induction. The judicious choice of varieties and sugar should make it possible to significantly limit phytosanitary interventions against one or several pests. As no studies have been carried out on thrips associated with olive trees, this work aims to determine the most effective sugar dose (sucrose tested at different doses of 1, 10, 100 and 1000 ppm); the most effective type of sugar to use (sucrose, fructose or glucose); and whether the use of sugar alone or combined with a phytosanitary treatment (insecticide tested at recommended and reduced doses), could be an effective alternative for the control of thrips populations associated with olive tree, on two cultivars (Sigoise and Chemlal), in an olive grove located in the region of Batna (North-East of Algeria).

2 MATERIAL AND METHODS

2.1 STUDY SITE

The study was carried out in an olive grove (Table

1), located in the region of Oued Chaaba, 10 km Southeast of Batna (Northeast Algeria). This region is characterized by a semi-arid climate, hot, dry in summer and cool in winter.

Table 1: Characteristics of the experimental orchard

Geographical coordinates	35° 30′ 17″ N, 6° 4′ 40″ E
Age of the plantation	14 years
Area	7 ha
Number of trees	2000
Topography (altitude)	1082 m
Cultivar	Chemlal (55 %) Sigoise (45 %)
Plantation density	5 x 7 m
Ploughing	in winter
Irrigation	drip irrigation
Fertilisation	livestock manure
Weed control	manual/mechanical
Protection programme	no treatment

2.2 EXPERIMENTAL DESIGN

Six trials were conducted on two olive cultivars (Sigoise and Chemlal), during three years, from 2017 to 2019. Table 2 illustrates the aspects of the two studied varieties.

All trials are based on a randomised Fisher block design with four replications (4 blocks). The modalities are randomly distributed within each block and each modality (elementary plot) consists of two trees, its surface is 35 m^2 (5 m x 7 m).

In the first year of 2017, two trials were carried out on two olive cultivars (Sigoise and Chemlal), the objective was to determine the most effective sugar dose on thrips associated with the olive crop. Once identified, this dose will be used in subsequent trials. A sucrose treatment at different doses, 1, 10, 100 and 1000 ppm (= 0.1, 1, 10 and 100 g 100 l^{-1}) was compared to the control (untreated trees) and a reference treatment (insecticide Acetamiprid). Then during 2018, two trials were set up to determine the effect of sugar type on treatment efficacy. The sucrose treatment was compared to fructose and glucose, tested at the same concentration (100 ppm =10 g 100 l^{-1}), on both cultivars (Sigoise and Chemlal). In 2019, the objective of the trials was to evaluate the effect of the application of infra-doses of sugar (sucrose 100 ppm) associated or not with a chemical treatment (Acetamiprid) and also to study the possibility of reducing the dose of phytosanitary product.

Table 2: Characteristics of the studied varieties

Cultivar		Chemlal	Sigoise
Origin		local (Kabylie, North of Algeria)	local (Mascara, Northwest of Algeria)
Destination		oil	dual purpose (oil+table)
Tree	Port	upright	upright
	Vigour	high	medium
	Foliage density	medium	medium
	Rooting rate	very low	medium
Fruit	Mass (g)	1.05-2.14 (reduced)	2.74-4.79 (medium)
	Form	elongated	ovoid
	Summit	pointed	rounded
	Aspect	smooth	smooth
	Colour at maturity	black	black
Endocarp	Mass (g)	0.43-0.45 (small)	0.55-0.76 (big)
	Form	spherical	ovoid
	Summit	pointed	rounded
	Core surface	rough	rough
Leaves		long (70.19 mm)	medium (50.62 mm)
Quality (acidity %)		very good (0.171 à 0.22)	medium(0.177 à 0.34)
Oil yield (%)		18 à 24	18 à 22
Maturity		end of October	November
Resistance to drought and cold		medium	Low

2.3 APPLICATION OF TREATMENTS

The chemical insecticide (Acetamiprid) was applied when the intervention threshold was reached (10 thrips/100 shoots), which was determined by weekly monitoring (scouting) of thrips by the strike method. Shaking on shoots was carried out weekly from the beginning of flowering (April-May), on 100 actively growing shoots, selected at random in the study plot (Mandrin & Lichou, 2000, Valette, 2007). According to these authors, the monitoring of the number of thrips trapped allows to know the peak of thrips migration, key date for a chemical intervention. According to Allan and Gillett-Kaufman (2018), the peak of thrips collection on olive trees coincided with flowering. Above 10 thrips per 100 shoots, chemical treatment is justified (Valette, 2007). The chemical insecticide used in this study was Acetamiprid, which was tested at the recommended dose (D2 = 50 ml 100^{-1}l) and at a dose reduced by half (D1 = 25 ml 100^{-1}l)

The sugars (sucrose, fructose and glucose) are sprayed in infra-doses (in the ppm range), obligatorily early in the morning, before the start of photosynthesis, at the time when the intercellular spaces of the apoplast are poor in sugars, according to the method advocated by (Derridj, 2009; Derridj et al., 2011, 2012; Arnault et al., 2015, 2021), using a backpack sprayer and trying to wet the whole foliar surface. The treatment with the chemical insecticide was done just after the sugar spray.

2.4 SAMPLING METHOD

Thrips sampling during the 3 years of the study (2017 to 2019) was carried out according to the method recommended by Valette (2007), which consists in randomly shaking 25 twigs per elementary plot (2 trees/elementary plot), in the five directions (north, south, east, west and center) and from top to bottom. Thus, 100 twigs per modality were shaken during each sampling (each modality is repeated 4 times). Sampling was carried out in the morning between 8 and 10 am, 1 day (24 h) before treatment, and 1, 3, 7, 10, 14 and 20 days after treatment. Thrips dropped on the Japanese umbrella were preserved in 70 % alcohol, counted and identified in the laboratory.

2.5 STATISTICAL ANALYSIS

The results of the average number of thrips per twig and the percentages of effectiveness of the treatments were processed by analysis of variance (ANOVA) with Tukey's test using the Excel Stat 2014 software. Results were expressed as mean ± S.E. (Standard Error), and considered significantly different at $p < 0.05$.

Treatment efficacy is calculated using formula (1) of Henderson and Tilton 1955 (Valette, 2007), which determines the effectiveness of the different treatments relative to the control and relative to the pre-treatment data (To)

$$Efficacy\,\% = 100 \cdot \left[1 - \frac{Nu(t_0) \cdot NT(t)}{Nu(t) \cdot NT(t_0)}\right] \quad (1)$$

$Nu(t_0)$: number of thrips before treatment on control; $Nu(t)$: number of thrips after treatment on control; $NT(t)$ number of thrips after treatment on treated plot; $NT(t_0)$:number of thrips before treatment on treated plot.

3 RESULTS AND DISCUSSION

3.1 PRESENTATION OF THE RECORDED SPECIES

The thrips species collected on olive trees in the study area (Table 3) are phytophagous (*F. occidentalis* was the dominant species of all species found). Indeed, the females of these species lay their eggs mainly in flower buds and flowers (Lambert, 1999). Upon hatching, the larvae feed on pollen and floral parts, causing premature flower drop. Heavy infestations cause silvering of the fruits, which dry out and fall prematurely. On olive trees, thrips cause damage to olives in the form of scars and wounds on the surface of the fruit, resulting from the sucking action of thrips, which extract the contents of the plant cells. The wounds result in the loss of the original

Table 3: Thrips species encountered in an olive grove in the Batna region

Suborder	Family	Species
Terebrantia	Thripidae	*Frankliniella occidentalis* (Pergande, 1895)
	Melanthripidae	*Odontothrips confusus* (Piesner, 1926) *Melanthrips fuscus* (Sulzer, 1776)
Tubulifera	Phlaeothripidae	*Haplothrips aculeatus* (Fabricius, 1803)

colour and the acquisition of the characteristic silvery appearance of the wounded olives (Halimi et al., 2022).

3.2 DETERMINATION OF THE SUGAR DOSE

The 1, 10, 100 and 1000 ppm sucrose modalities were compared to the reference treatment 'insecticide' and to the control. The results revealed, for both cultivars (Figure 1), a decrease in thrips populations on all treated modalities from the day after treatment (T+1). This decrease continued until the end of the experiment for the 1, 10 and 100 ppm sucrose treatments. On the other hand, the number of thrips increased from the third day (T+3) in the plots treated with 1000 ppm sucrose. The insecticide treatment resulted in a sharp decrease in thrips populations until day 3 (T+3). Then, the population increased again to reach the level of the "100 ppm sucrose" modality towards the end of the experiment at T+20

In order to better analyze these data, it appears interesting to evaluate their respective effectiveness (Figure 2) and to compare them statistically.

The chemical modality "insecticide" offered a good efficiency from the first day of treatment. The best efficacy was obtained 3 days after treatment (T+3), with 69.64 % ± 2.69 on Sigoise and 81.72 % ± 3.92 on Chemlal, then this efficacy gradually decreased. On the contrary, the efficacy of the sugar treatments, sucrose 1.10 and 100 ppm, increased with time and the best efficiencies were obtained at the end of the experiment at T+20, with respectively 38.39 % ± 1.36, 40.46 % ± 1.72, 48.30 % ± 1.16 on Sigoise and 33.14 % ± 1.39, 41.56 % ± 1.26, 52.48 % ± 1.68 on Chemlal.

Derridj (2009) and Derridj et al. (2011) reported that foliar spraying of sugars at infra doses (in the range of 1 to 10 g 100^{-1}l) on fruit and vegetable plants induces systemic resistances against different pests. These resistances occur on the surface and in the leaves as well as in the roots, against insects, fungal pathogens and nematodes respectively. Indeed, soluble sugars deposited on the plant surface penetrate the plant and can constitute signals that trigger defence cascades within the plant and/or intervene in the plant's physiological regulation pathways. The same authors added that depending on the plant and the pest, the induction of resistance may vary depending on the sugar and its dose. They showed that only sucrose at 10 ppm, fructose at 0.1 ppm sprayed on maize grown under glass had a significant effect on *Ostrinia nubilalis* oviposition, and that the sugar that can induce systemic resistance in tomato to the nematode *Meloidogyne javanica* is sucrose at a concentration of 1 ppm. This dose effect was also observed on *Botrytis cinerea* where the use of 100 ppm sucrose was very effective on tomato against *Botrytis* (100 % reduction of symptoms) and much less on bean (only 23 %). On their side, Arnault et al. (2015) demonstrated that spraying sucrose or fructose at a concentration of 100 ppm was able to reduce codling moth *Cydia pomonella* damage by 55 % in apple orchards.

In our study, after 20 days, the 100 ppm sucrose treatment appeared to be more effective than the treatments at other doses, its efficacy was 48.30 % ± 1.16 on Sigoise and 52.48 % ± 1.68 on Chemlal, and it was even as effective as the treatment with the chemical modality (Figure 2). On the other hand, the least effective treatment, on the two cultivars studied, was the 1000 ppm sucrose, with an efficacy that did not exceed 18 %. Increasing the sugar dose does not increase the effects of

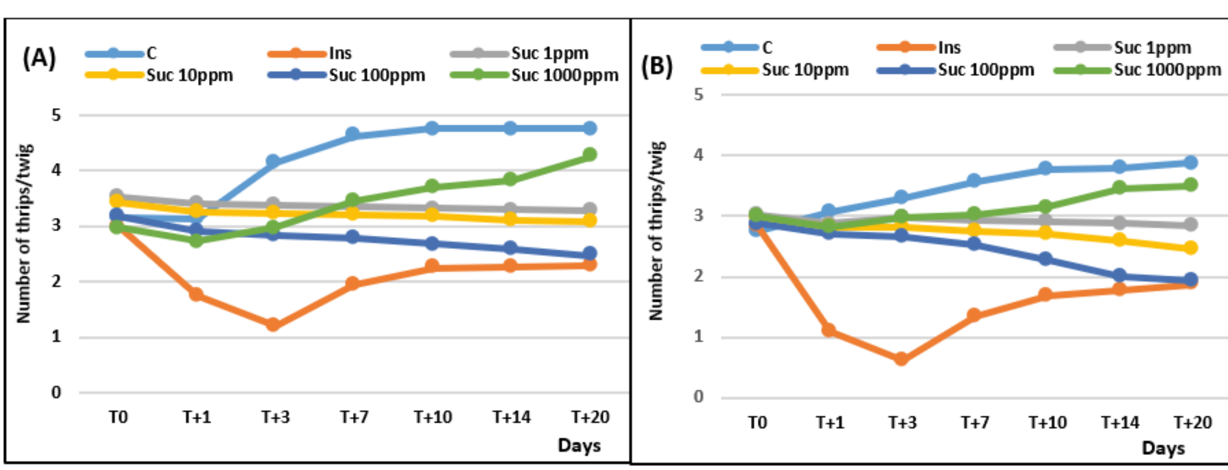

Figure 1: Temporal evolution of the number of thrips per twig according to the different treatments, on the cultivar Sigoise (A) and Chemlal (B) in an olive grove located in the Batna region, in 2017. C (control); Ins= Insecticide; Suc1 = sucrose at 1 ppm; Suc10 = sucrose at 10 ppm; Suc100 = sucrose at 100 ppm; Suc1000 = sucrose at 1000 ppm

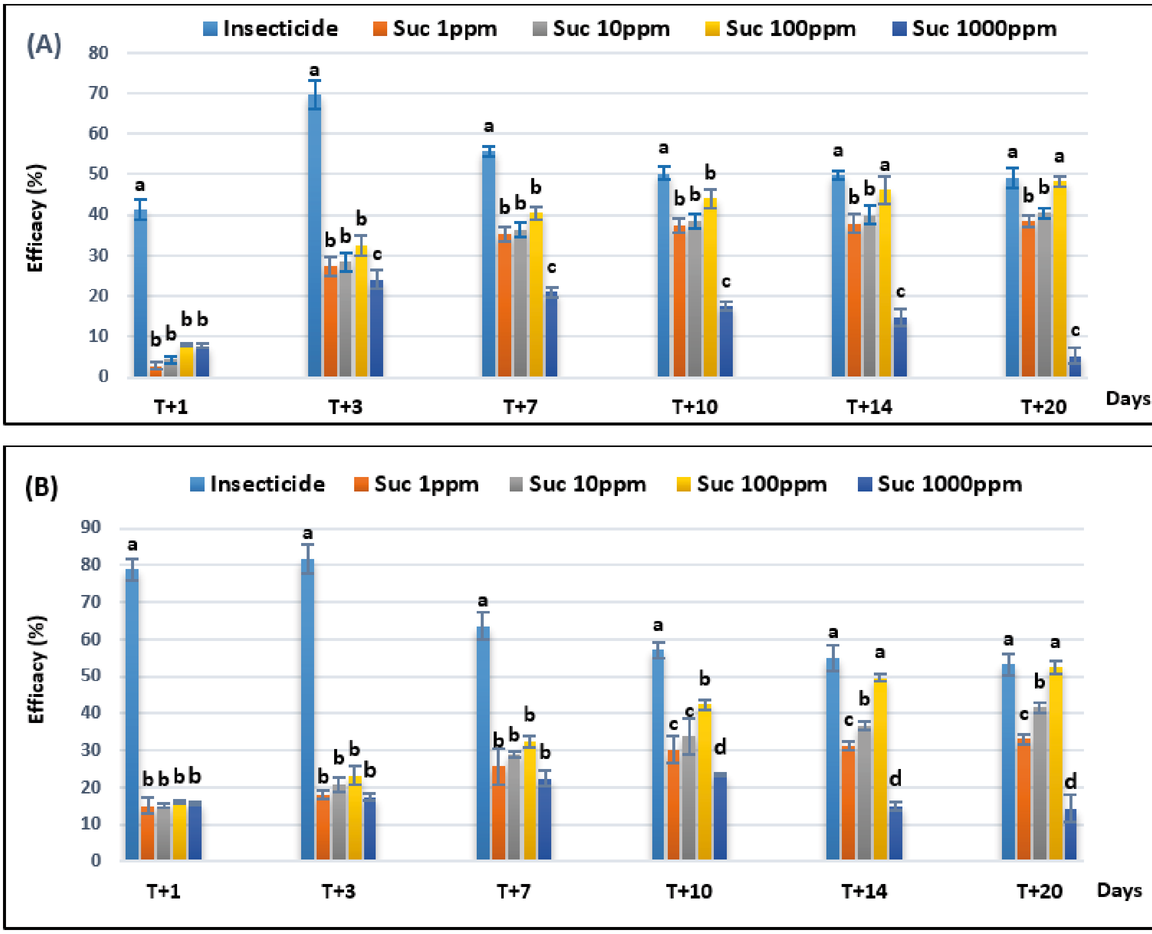

Figure 2: Treatment efficacy, calculated according to the Henderson and Tilton method, at 1, 3, 7, 10, 14 and 20 days after treatment, for the cultivar Sigoise (A) and Chemlal (B), in an olive grove located in the Batna region in 2017. Values with different letters are significantly different (p < 005; Tukey test)

resistance induction and sometimes even cancels them out, and has the disadvantage of having secondary effects (insect feeding, growth and development of epiphytic fungi or bacteria, etc.) on pests on the plant surface (Derridj et al., 2010).

3.3 EFFECT OF THE SUGAR TYPE

The results obtained from the trials conducted in 2018 (Figure 3), showed that treatments with different types of sugar (sucrose, fructose or glucose) and chemical modality resulted in a significant decrease in thrips populations from the first day of treatment. The population levels of the different treated modalities remained significantly lower than the control throughout the experiment.

The results obtained 20 days after treatment (Figure 4) showed that 100 ppm sugar (glucose, fructose or sucrose) sprays on the cultivar Sigoise resulted in a significant reduction of the thrips population compared to the untreated control (2.6 ± 0.78, 3.18 ± 0.67, 2.1 ± 0.78 vs. 4.9 ± 1.16 respectively). On 'Chemlal', glucose, fructose or sucrose treatments at a dose of 100 ppm also resulted in a significant reduction of the thrips population, with a number of 1.4 ± 0.6, 2.13 ± 0.33 and 1.23 ± 0.44 respectively, compared to the untreated control (3.68 ± 0.78)

As reported in the literature, the application of very low doses of sugar to the surface of plants could limit pest attacks by two mechanisms; by modifying the chemical composition of the leaf surface, sugars would disrupt the oviposition behaviour of females, which would not recognize the plant as suitable for the development of their larvae, but also by a systemic effect. Sugars are indeed involved in a cascade of plant defence reactions and can therefore have a generalized effect of stimulating natural defences (Derridj et al., 2011; Arnault et al., 2015; Lambion et al., 2016). Soluble carbohydrates not only act as

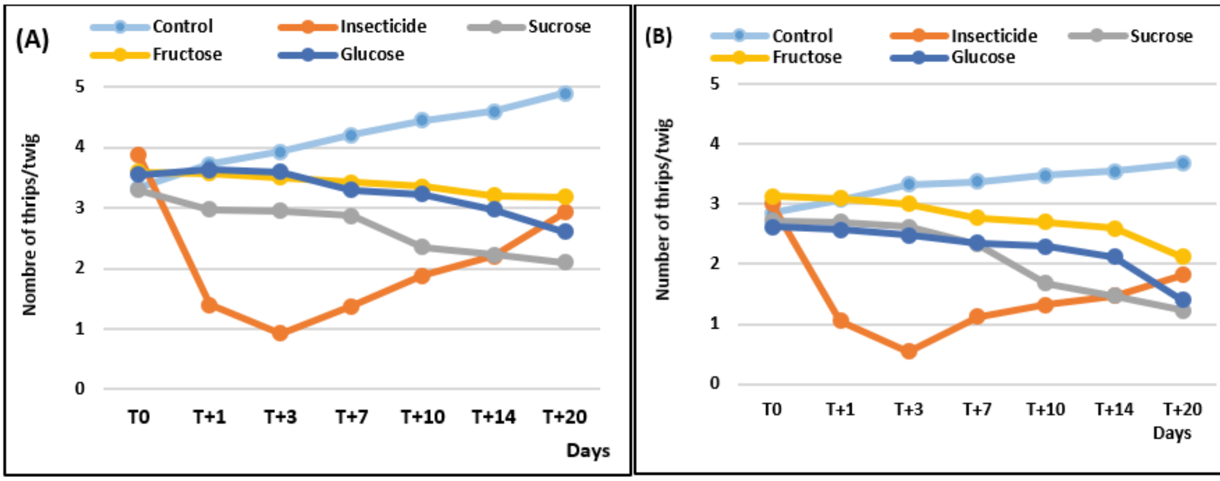

Figure 3: Temporal evolution of the number of thrips per twig according to the different treatments, on the cultivar Sigoise (A) and Chemlal (B) in an olive grove located in the Batna region in 2018

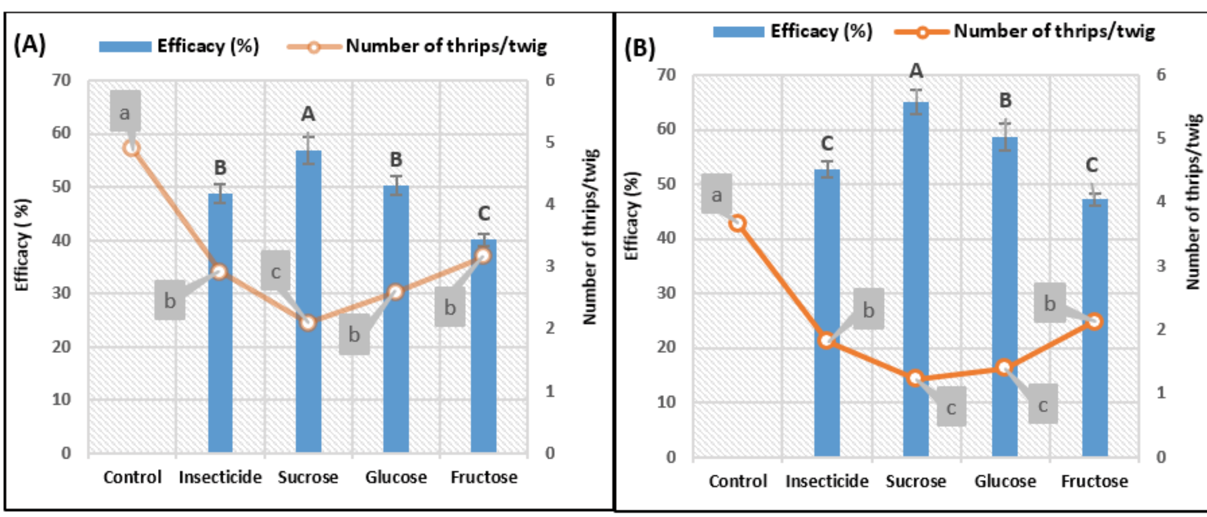

Figure 4: Number of thrips/twig and treatment efficiency, calculated according to the Henderson and Tilton method, at 20 days after treatments, for the cultivar Sigoise (A) and Chemlal (B), in an olive grove located in the Batna region in 2018. The values with the different letters are significantly different [different lower-case letters indicate significantly different mean thrips/twig numbers and different upper-case letters indicate significantly different percentage efficacy ($p < 0.05$; Tukey test)]

carbon skeleton donors and respiratory substrates, but they can also induce metabolic signals influencing the expression of many genes involved in plant defence (Rolland et al., 2006; Morkunas & Ratajczak, 2014; Yoon et al., 2021; Choudhary et al., 2022)

The results acquired from the trials, conducted in 2018 (Figure 4), revealed that the sucrose treatment at 100 ppm offers a more effective protection against thrips (the efficacy is 56.82 % ± 2.55 on 'Sigoise' and 65.14 % ± 2.22 on 'Chemlal'), compared to the treatments with the chemical modality and the other two sugars (glucose or fructose). Indeed, sucrose is the main product of photosynthesis and the main transport carbohydrate in plants (Xu et al., 2018; Aluko et al., 2021). It has been recognized as contributing to various regulatory mechanisms in plants, including growth and development, differential gene expression and stress-related responses (Formela-Luboińska, 2020; Li et al., 2020; Jeandet et al., 2022). High sucrose: hexose ratios can probably trigger a sucrose-specific signal to induce the genes required for the production of a range of protective agents such as anthocyanins and other secondary metabolites (Yoon et al., 2020). The specificity of sucrose as a signalling molecule was demonstrated by the fact that equimolar applications of glucose and fructose did not result in significant accumulation of anthocyanins (Solfanelli et al., 2006).

On the other hand, the 100 ppm glucose treatment was found to be satisfactorily effective for both cultivars tested, with 50.30 % ± 1.70 for 'Sigoise' and 58.64 % ± 2.48 for 'Chemlal'. While fructose at 100 ppm is the least effective sugar, where the efficiency is significantly low compared to the other sugars, which did not exceed 40 % on 'Sigoise' and 47 % on 'Chemlal'. It has been shown that sucrose, glucose and mannitol are the most abundant sugars in olive tree, while fructose is the least present (Bousaadia et al., 2010; Haouari, 2013; DePascali et al., 2022).

Our results are in agreement with those obtained by Valette (2007), who showed that sucrose is the most effective of the three tested sugars (sucrose, glucose and fructose), against thrips on nectarine. Numerous studies have also shown significant protective effects of sucrose at a dose of 100 ppm on different pests such as melon borer and powdery mildew, leek thrips, codling moth, corn borer, tomato leafminer (Derridj, 2009; Derridj et al., 2011, 2012; Arnault et al., 2012, 2015, 2017, 2021)

3.4 EFFECT OF COMBINING SUGAR WITH A PHYTOSANITARY TREATMENT

The obtained results in 2019 trials confirmed the efficacy of sucrose foliar spray, 20 days after treatment (Figure 5), in reducing thrips populations associated with olive. The efficacy was 50.93 % ± 2.52 on 'Sigoise' and 61.83 % ± 2.53 on 'Chemlal'. Sucrose treatment at a concentration of 100 ppm alone induced effects comparable to those recorded with insecticide treatment alone at the recommended dose on the cultivar Sigoise. However, on the cultivar Chemlal, sucrose treatment was more effective than insecticide alone.

Sucrose at 100 ppm improved the efficacy of the chemical modality for both the reference dose (D2) and the halved dose (D1), on both cultivars tested (Figure 5). Indeed, several studies recommend the use of sugars as an additive treatment to phytosanitary treatments. Thus, the work carried out by Derridj et al. (2011), showed that the combination of sucrose at a dose of 100 ppm with a pyrethroid insecticide treatment had a significant effect on the oviposition of the female corn borer *Ostrinia nubilalis*. In plots treated with pyrethroids in combination with 100 ppm sucrose, they observed a 20 % reduction in maize damage compared to maize plots treated with the insecticide alone, where the damage reduction was only 8 %. Similar work by Arnault et al. (2015) also showed that the addition of 100 ppm sucrose to an "organophosphate" chemical treatment increased its effectiveness by 35 %.

Recent experiments (Arnault et al., 2016, 2021; Bouhidel & Lombarkia, 2021), have significantly demonstrated that the addition of sugars, such as sucrose or fructose, can reduce the insecticide dose by up to 50 % while maintaining the same level of efficacy against the pests. This was confirmed by the results obtained in this study, in which the treatment combining 100 ppm sucrose with the insecticide in half dose (D1) was as effective as the sucrose treatment combined with the insecticide in reference dose (D2), on the two cultivars studied,

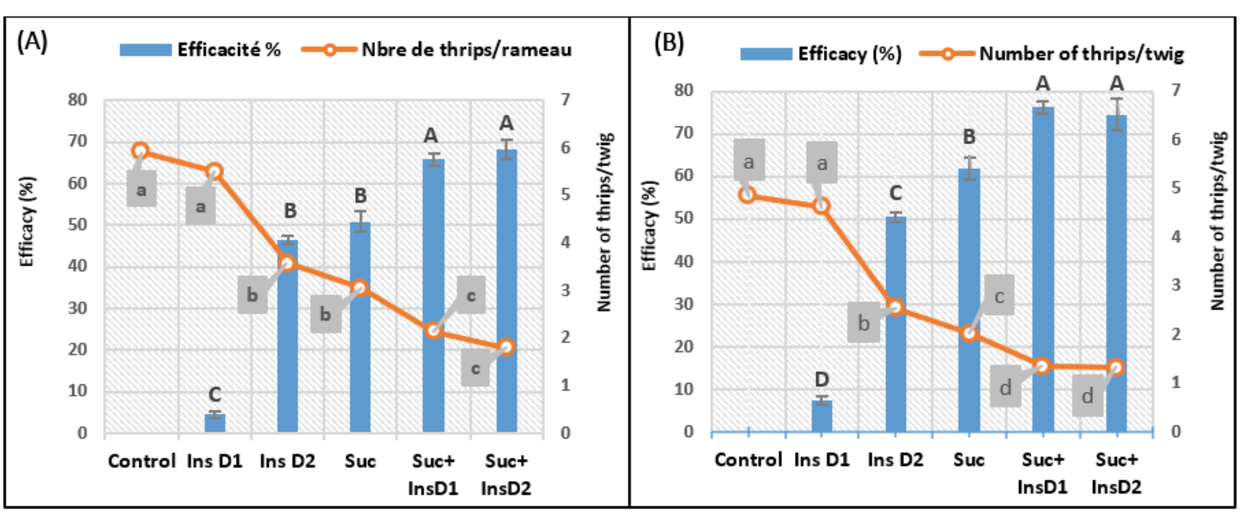

Figure 5: Number of thrips/twig and treatment efficiency, calculated according to the Henderson and Tilton method, 20 days after treatments, for the cultivar Sigoise (A) and Chemlal (B), in an olive grove located in the Batna region in 2019. The values with the different letters are significantly different [different lower-case letters indicate significantly different mean thrips/twig numbers and different upper-case letters indicate significantly different percentage efficacy ($p < 0.05$; Tukey test)]

with an efficacy of 65 to 68 % on 'Sigoise' and 74 to 76 % on 'Chemlal'. The addition of 100 ppm sucrose thus increased the efficacy of a reduced dose of insecticide and resulted in similar efficacy to that obtained with a full dose.

4 CONCLUSION

The conducted trials in the present study showed very promising results. Foliar spraying of sucrose at a dose of 100 ppm showed an improved efficacy on thrips, both alone and in combination with a chemical insecticide. This method reduced the recommended insecticide dose by half while improving the efficacy of the treatment and thus allowing a significant reduction of thrips populations on olive trees.

As a consequence, we can affirm that the use of sugars, which are non-toxic and inexpensive substances, could lower pest population levels to more controllable levels or below economic thresholds, and thus contribute to increase the efficiency of integrated pest management or organic farming methods.

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Water use efficiency, morpho-physiological and biochemical reactions of some bedding plants to drought stress

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Water use efficiency, morpho-physiological and biochemical reactions of some bedding plants to drought stress

Abstract: The purpose of this experiment is to compare the growth and water consumption efficiency of five garden plants (marigold (*Tagetes erecta* 'Red Brocade'), moss-rose (*Portulaca grandiflora* 'Sun Rose'), dahlia (*Dahlia* sp. 'Double Opra'), gazania (*Gazania splendens* 'New Day'), and Indian blanket (*Gaillardia pulchella.* 'Sun Dance')) during the warmer seasons of the year under various levels of drought stress based on field capacity (FC; 25, 50, 75, and 100 %). The interaction effect of plant × drought stress (FC) on the fresh and dry mass of aerial and underground organs was significant. Decreased water availability resulted in a drop in growth parameters (leaf fresh and dry mass and leaf area). In compared to the growth of aerial organs, root biomass increased in response to drought stress. Marigold, Indian blanket, and dahlia plants had the highest root-to-shoot ratio in extreme stress, i.e., FC 25 %. The plant × drought stress interaction significantly influenced flower number, whereas flower diameter was influenced by the main effect of plant and drought stress (not their interaction). The FC 100 % and FC 25 % treatments had the highest and the lowest accumulations of proline and soluble sugars, respectively. Moss-rose, gazania, and marigold ornamental plants had the highest water use efficiency at 75 %, followed by Dahlia at 50 % and moss-rose at 25 %.

Key words: bedding plants; deficit irrigation; root to shoot ratio membrane; peroxidation; photosynthetic capacity

Učinkovitost izrabe vode, morfološki, fiziološki in biokemijski odziv nekaterih okrasnih rastlin na sušni stress

Izvleček: Namen poskusa je bil primerjati rast in učinkovitost izrabe vode petih okrasnih rastlin (žametnice (*Tagetes erecta* 'Red Brocade'), tolščaka (*Portulaca grandiflora* 'Sun Rose'), dalije (*Dahlia* sp. 'Double Opra'), gazanije (*Gazania splendens* 'New Day'), in gailardije (*Gaillardia pulchella* 'Sun Dance') v toplejši rastni sezoni leta pri različnih ravneh sušnega stresa izzvanega z različno poljsko kapaciteto (FC; 25, 50, 75, in 100 %). Vzajemni učinek vrste rastline in sušnega stresa (FC) na svežo in suho maso nadzemnih in podzemnih organov rastlin je bil značilen. Zmanjšana dostopnost vode je povzročila upad parametrov rasti (sveže in suhe mase listov, listne površine). V primerjavi z rastjo nadzemnih organov se je biomasa korenina povečala kot odziv na sušni stress. Žametnica, gailardija in dalija so imele največje razmerje korenine:poganjki pri ekstremnem sušnem stresu, pri FC 25 %. Učinek sušnega stresa je značilno zmanjšal število cvetov in premer cveta pri vseh obravnavanih rastlinah. Obravnavanji s FC 100 % in FC 25 % sta povzročili največjo in najmanjšo kopičenje prolina in topnih sladkorjev v rastlinah. Tolščak, gazanija in žametnica so imele največjo učinkovitost izrabe vode pri FC 75 %, njim je sledila dalija pri FC 50 % in gailardija pri FC 25 %.

Ključne besede: okrasne rastline; deficitno namakanje; razmerje korenina:poganjek; peroksidacija membrane; velikost fotosinteze

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1 INTRODUCTION

Among the most significant environmental stresses affecting the growth and development of agricultural products are climate change and abnormal weather conditions such as drought, long-term hot temperatures, and storms (Wang et al., 2018). Drought stress has the greatest detrimental effects on the global growth and development of crops compared to other environmental stresses. According to the predictions of experts regarding the rising trend of air temperature (up to a 5 °C increase in the coming years), long and dry summers, and a decrease in precipitation (Giordano et al., 2021), it is crucial to choose appropriate strategies, such as screening and cultivating plants with improved water efficiency. Herbaceous bedding plants perform an essential role in parks as well as other green places. The development of high-quality bedding plants is one of the primary priorities of ornamental plant producers. Nevertheless, due to the shallow growth of their roots and high evapotranspiration on the one hand, and the scarcity of water resources in arid and semiarid regions on the other, the production of these plants is perpetually hindered. Several studies have investigated the impact of drought stress on the growth and development of agricultural and horticultural crops such as spiraea, pittosporum (Elansary and Salem, 2015), bougainvillea (Cirillo et al., 2017), callistemon (Álvarez and Sánchez-Blanco 2015), laurus and thunbergia (Toscano et al., 2023), but little is known about the morphological, physiological, biochemical, and water consumption efficiency of herbaceous ornamental plants, particularly when comparing bedding ornamental plants grown under low irrigation conditions.

Drought stress typically disrupts physiological and biochemical processes, resulting in reduced plant growth and performance (Talbi et al., 2020). At the morphological, physiological, and biochemical levels, plants have a variety of actions (mechanisms) in response to drought stress (Larkunthod et al., 2018; Cal et al., 2019). The aforementioned reactions will vary according to the plant species, growth stage, severity of stress, and length of exposure (Mahajan and Tuteja, 2005). Some species elongate their roots to absorb more water and increase the root-to-shoot ratio (Asrar and Elhindi, 2011). This reaction or mechanism preserves the plant's water status and enables photosynthetic processes to continue during drought stress. In a study comparing the responses of geraniums and impatiens to drought stress, the root length of both species increased; however, plant height and the number of flowers per plant decreased solely in the impatient species (Chyliski et al., 2007).

Water use efficiency is one of the most important considerations when selecting plants for areas with limited water and high temperatures throughout the developmental phase. Many reactions and mechanisms influence the efficiency of a plant's water consumption, including cuticle thickness, leaf angle, leaf surface, stomatal opening and closing, root-to-stem ratio, etc. (Mahajan et al., 2005; Giordano et al., 2021). Despite the fact that stomata closure reduces gas exchange and photosynthesis, water consumption efficiency increases. Water use efficiency regulates the relationship between transpiration and photosynthesis. Improved water use efficiency is one mechanism by which plants adapt to drought stress, whereas lower water use efficiency is characteristic of sensitive plants (Jin et al., 2018). Depending on genotype and stress level, water use efficiency can often decline, increase, or remain constant (Cameron et al., 2006). In consideration of this, it is critical to study the water consumption efficiency of ornamental plants under low irrigation conditions.

Many experiments have been conducted on horticultural plants in regards to drought stress; however, the number of these studies on bedding ornamental plants is quite limited; despite their importance to the health of today's industrial and crowded societies, they have received surprisingly little attention. The purpose of this study is to identify the most productive plant based on biochemical parameters and water consumption efficiency by comparing the morphological and biochemical responses of the most important bedding plants to drought stress in an outdoor environment whose growing season (late spring and summer) corresponds with the onset of heat.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND GROWTH CONDITIONS

The reactions to drought of five bedding ornamental flowers, including Indian blanket (*Gaillardia pulchella* 'Sun Dance'), marigold (*Tagetes erecta* 'Red Brocade'), moss rose (*Portulaca grandiflora* 'Sun Rose'), gazania (*Gazania splendens* 'New Day'), and dahlia (*Dahlia* sp. 'Double Opra'), which grow during the hottest seasons of the year, i.e., late spring to late summer, are investigated. These flowers are planted in both pots and outdoor gardens. Four-leaf seedlings of the above-mentioned plants were transplanted into 5-liter pots containing loam soil (Table 1), and two weeks later, drought stress treatments were provided based on the pots' soil moisture content.

The location of the experiment is located between 35 degrees 58 minutes to 39 degrees 47 minutes north latitude (from the equator) and 44 degrees 3 minutes to

Table 1: Physicochemical characteristics of soil substrates used in the experiment

EC (dS m^{-1})	pH	OM (%)	Clay (%)	Silt (%)	Sand (%)	Soil texture	Bulk density (g cm^{-3})	Specific gravity (g cm^{-3})
0.52	6.7	1.4	29	58	13	Loam	1.5	2.7

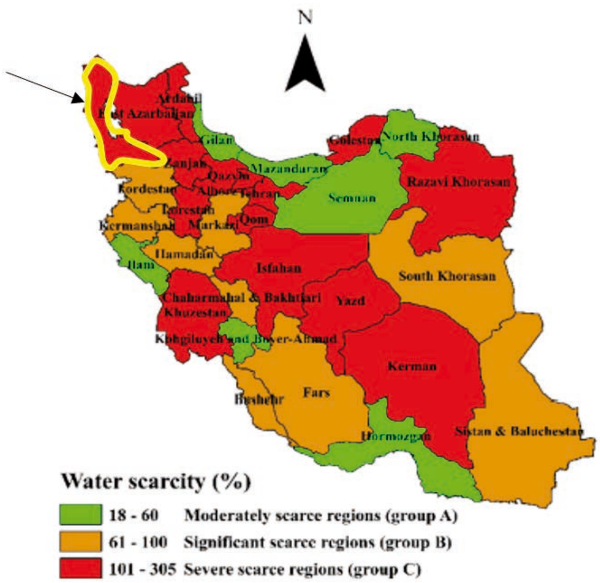

Figure 1: Map of dry climates in Iran (yellow outline (arrow) shows the experimental site) (Qasemipour et al., 2020)

47 degrees 23 minutes longitude from the Greenwich meridian. The height above sea level is 1358 meters (Iran Hydrology, Meteorological Information Bank) (Figure 1).

All cultivated plants were subjected to five levels of field capacity (FC) during the experiment, including FC 100 % (control or no stress), FC 75 % (low stress), FC 50 % (moderate stress), and FC 25 % (severe stress) (Figure 2).

Throughout the cultivation period, environmental characteristics were observed (Table 2). This experiment was carried out as a factorial experiment based on completely randomized design. Each treatment consists of three repetitions, and each repetition has three pots, for a total of 180 pots.

Table 2: Environmental parameters of the experimental site during cultivation

	Mean temperature (˚C)		Relative humidity (%)
Month	Day	Night	
April	13	7	60
May	22	15	38
June	25.8	17	14
July	28	19	10
August	30	19	5
September	25	16	7

2.2 DROUGHT STRESS TREATMENTS (FIELD CAPACITY)

Depending on the pot's field capacity or the percentage of soil moisture, various levels of drought stress were applied. Five pots containing substrate soil were irrigated to saturation with irrigation water, then tightly covered with aluminum foil (to prevent water evaporation) and weighed after the gravity (saturated) water exited. After leaving the saturated water (at 105 °C for 72 hours), the wet and dry mass of the substrate soil were determined, and the obtained humidity was determined to be 100 percent of field capacity (Henry, 1990). Additional treatments were computed using the control's (FC100 %) moisture content (Heidari et al., 2016). Before being irrigated, each pot in the experiment was precisely weighed,

Figure 2: A view of some potted bedding plants under various drought stress or FC levels (Indian blanket (left) and marigold (right))

and the mass loss in each sample is due to the plants' stress levels.

2.3 GROWTH PARAMETERS

At the end of the growth period, all plants were removed from the cultivated beds, and their aerial and underground organs were separated. Exact measurements were taken of root length (the longest root), root volume, the number of leaves per plant, and the fresh and dry mass of roots and aerial organs.

2.4 PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS

Fully developed mature leaves were fixed in liquid nitrogen and stored at -80 °C. The effects of drought stress on the production of osmolytes or compatible metabolites in leaves, such as soluble sugar and proline amino acid, were investigated. For each plant, 0.5 g of leaf tissue was homogenized with 10 ml of 3 % sulfosalicylic acid before being analyzed for proline content. It was heated in a water bath at 100 °C for one hour with 2 ml of glacial acetic acid and 2 ml of centrifuged ninhydrin acid. After cooling the samples, 4 ml of toluene was added to each vial, and the vials were shaken for 15 to 20 seconds. Samples were evaluated for proline content by measuring the 520 nm absorbance of each sample and comparing it to a standard curve for proline concentration (Bates, 1973). Anthrone digested leaf samples with 70 % ethanol, mixed them with the supernatant, and measured absorbance at a wavelength of 625 nm to determine the total soluble sugar content of the leaf sample. As a reference, glucose was used in the preparation of the standard (Irigoyen et al., 1992).

Ion leakage and peroxidation of the leaf cell membrane were measured as indicators of cell and leaf tissue destruction caused by drought stress. Leaf ionic leakage was measured by comparing the electrical conductivity ratio (L1/L2) of leaf tissue under normal conditions (L1) (20 °C for 2 hours) and at high temperature (autoclave for 20 minutes at 121 °C) (L2) (Lutts et al., 1996). Lipid peroxidation of the leaf membrane was also performed using a spectrophotometric method, as was the determination of malonaldehyde content using thiobarbituric acid (TBA) and absorption of the resulting supernatant at wavelengths of 440, 532, and 600 nm (Valentovic et al., 2006).

Photosynthetic pigments, including chlorophyll a, b, and total, as well as carotenoid content of leaves, were measured using a spectrophotometer (Perkin Elmer, UV/VIS, Lambda 25) at 645 and 663 nm (chlorophyll a and b) and 470 nm (carotenoids).

Due to the direct relationship between drought stress and plant calcium uptake, as well as the importance of calcium in the growth and physiology of ornamental plants, the calcium content of the leaf was determined using an atomic absorption device.

2.5 THE RELATIVE WATER CONTENT (RWC) AND WATER USE EFFICIENCY

To determine the relative water content of leaves, young mature leaves from each plant were cut into one-centimeter squares, and 10 pieces of leaves were selected and weighed (FM; fresh mass); The samples were then transferred to petri dishes containing distilled water at 4 degrees Celsius for four hours, after which their mass was measured once more (TM; Turgor Mass). The samples were then placed in an oven at 72 °C for 72 hours and reweighed (DM; dry mass) (Ritchie et al., 1990). The relative water content of the leaf was then estimated using the following formula:

$$RWC = \frac{FM - DM}{SM - DM} \times 100$$

Water use efficiency was also evaluated based on the amount of dry matter generated by each plant per unit of water consumed by that plant in response to various soil stresses or irrigation regimes (Boyer, 1996).

2.6 EXPERIMENTAL DESIGN AND DATA ANALYSIS

This study was analyzed with two factors: bedding plant and irrigation regime, using factorial trials based on a completely random design. SAS Software version 9.1 was used to analyze the data, and Tukey's test was used to compare the means.

3 RESULTS AND DISCUSSION

3.1 GROWTH PARAMETERS

Fresh and dry mass of aerial and underground organs were significantly affected by the interaction effect of plant × drought stress (FC). Decreasing the plant's access to water led to a decrease in growth and developmental parameters, i.e., leaf fresh and dry mass and leaf area; also, the highest leaf area per plant was observed in gazania and marigold plants in the control treatment, or FC 100 %. Unlike the growth of aerial organs, the fresh and dry mass of the root showed an upward trend in response to water stress (Table 3). In other words, at the same time as the severity of drought stress increased, root growth (root fresh and dry mass and root length) of bedding plants increased (probably to search for water needed by the plant). Based on the results of mean comparisons, the highest fresh and dry mass of roots was obtained in the Indian blanket plant at FC 25 %, and the highest root growth was obtained in the marigold flower at FC 25 %.

The root-to-stem ratio, as one of the morphological reactions that is strongly influenced by the plant's ability to access water, was balanced in most plants under 75 to 100 %, while with the increase in stress level, i.e., a 50 % decrease in substrate moisture, the root-to-stem ratio increased. The highest root-to-stem ratio in severe stress, i.e., 25 % FC, was observed in marigold, Indian blanket, and dahlia plants, respectively (Figure 3).

In this experiment, the plant × drought stress interaction had a significant effect on the number of flowers,

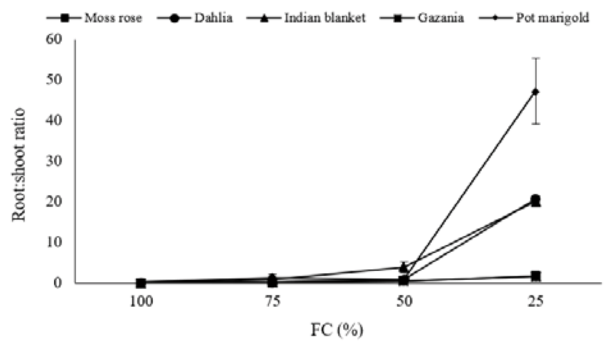

Figure 3: Root to shoot ratio of some bedding plants under various levels of drought stress (field capacity)

but it had no effect on the floral diameter; only the plant type and drought stress had a significant effect on this important ornamental attribute. According to the results, the number of flowers per plant fell as plant access to water reduced, while the minimum number of flow-

Table 3: Growth parameters of some bedding plants under various drought stress levels (FC)

Bedding plant	FC (%)*	Leaf FM** (mg/plant)	Leaf DM*** (mg/plant)	Root FM (mg/plant)	Root DM (mg/plant)	Root length (cm)	Leaf area (mm^2/plant)
Moss-rose	100	5.36 efg****	0.68 ghij	1.67 f	0.04 e	9.67 ij	9.33 gh
	75	2.13 fg	0.38 hij	0.72 f	0.11 e	15.83 fghij	7.25 h
	50	1.70 fg	0.27 ij	0.38 f	0.12 e	18.67 defghi	5.17 h
	25	0.96 fg	0.16 j	0.32 f	0.28 e	30.33 bc	3.95 h
Dahlia	100	13.65 c	3.76 b	16.76 cd	0.17 e	8.20 hij	1138.95 b
	75	11.85 cd	2.32cd	8.45 def	0.60 e	10.22 defgh	608.06 c
	50	6.05 ef	1.26 efgh	1.99 f f	2.38 cde	19.17 bc	443.82 cd
	25	1.54 fg	0.18 j	0.40 f	6.36 b	29.83 j	126.71 efgh
Indian blanket	100	28.99 a	5.45 a	5.47 f	0.61 e	23.17 cdef	352.45 cde
	75	11.85 cd	3.67 b	13.91 cde	3.32 cd	25.83 cd	273.97 defgh
	50	7.54 de	2.07 de	40.05 b	6.89 b	32.42 bc	179.69 defgh
	25	2.04 fg	0.27 ij	55.80 a	10.17 a	37.00 ab	77.46 efgh
Gazania	100	8.54 cde	3.21 b	4.02 f	0.18 e	13.79 ghij	1485.01 a
	75	5.85 efg	1.67 def	1.46 f	0.33 e	16.50 efghij	328.05 cdef
	50	3.57 efg	1.14 fghi	1.03 f	0.44 e	17.33 efghij	292.67 defgh
	25	1.33 fg	0.51 ghij	0.59 f	0.78 e	19.93 defg	23.72 fgh
Marigold	100	20.55 b	3.04 bc	19.44 c	0.17 e	14.33 fghij	1485.01 a
	75	7.93 de	0.91 fghij	8.05 ef	0.56 e	30.17 bc	328.05 cdef
	50	4.89 efg	1.35 efg	6.16 ef	0.93 de	25.67 cde	323.75 cdefg
	25	0.63 g	0.10 j	1.53 f	4.77 bc	45.00 a	23.06 fgh

* FC: Field capacity
** FM: Fresh mass
*** DM: Dry mass
**** Means with the same letter in each column don't have significant difference

ers per plant was obtained at FC 50 % and particularly at FC 25 %. (Figure 4). According to the mean comparisons, the least possible irrigation for marigold and Indian blanket is FC 75 %; however, for moss rose, dahlia, and gazania, it is FC 50 %.

One of the key indicators of flowering bedding plants, flower diameter, was significantly impacted by the plant and various levels of drought stress. As shown in Figure 5A, the relationship between floral diameter and the degree of drought stress is downward and linear. The genotype had a complete impact on flower diameter, with marigolds, gazanias, and dahlias having the highest flower diameters, respectively (Figure 5B).

The interaction between plant genotype and drought stress had a significant impact on the content of photosynthetic pigments, including chlorophylls a, b, and total as well as carotenoids. Based on the findings, all bedding plants showed a decreasing trend in photosynthetic pigment content as drought stress increased. Moss-rose (79 %), Indian blanket (72 %), marigold (68 %), gazania (46 %), and dahlia (40 %) had the highest decrease in total chlorophyll content (the sum of chlorophyll a and b) under severe drought stress (FC 25 %) compared to the control (FC 100 %) (Table 4).

3.2 BIOCHEMICAL AND PHYSIOLOGICAL PARAMETERS

One of the most significant metabolic responses of

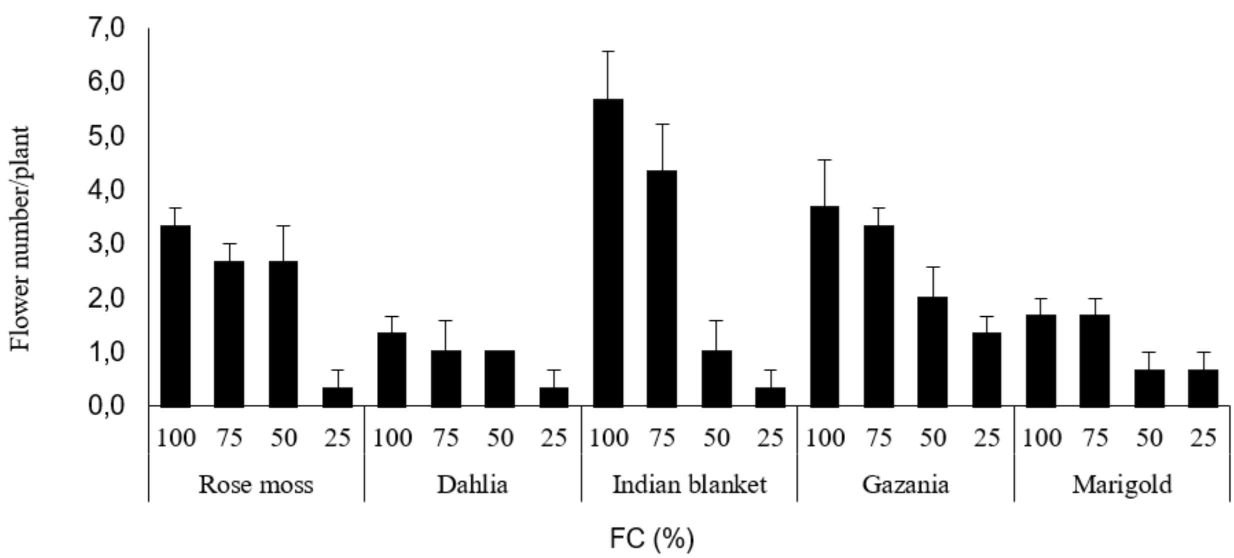

Figure 4: The number of flowers per plant of some bedding plants under various drought stress (FC level)

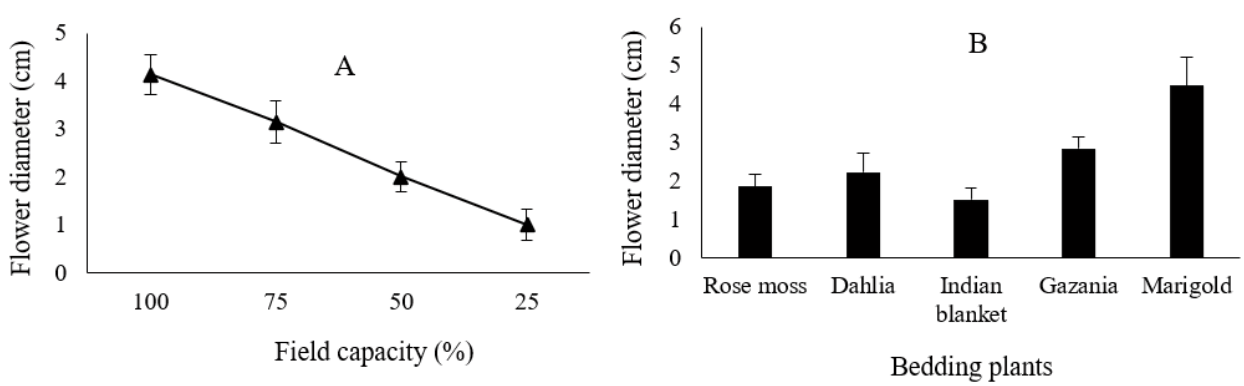

Figure 5: The diameter of bedding plant flowers under various drought stress levels (a) and plant genotype (b)

Table 4: Photosynthetic pigments of some bedding plants under various drought stress levels

Bedding plant	FC*	Chlorophyll a (mg g FM^{-1})	Chlorophyll b (mg g FM^{-1})	Total Chlorophyll (mg g FM^{-1})	Carotenoids (mg g FM^{-1})
Moss-rose	100	0.00034 b**	0.000423 de	0.00073 f	0.0180 i
	75	0.00018 b	0.000249 fg	0.00042 gh	0.0120 ij
	50	0.00013 b	0.000102 hi	0.00021 h	0.0063 j
	25	0.00005 b	0.000066 b	0.00015 h	0.0033 j
Dahlia	100	0.00478 a	0.000801 a	0.00146 a	0.0850 a
	75	0.00068 b	0.000598 bc	0.00123 abc	0.0750 ab
	50	0.00060 b	0.000545 bcd	0.00115 bc	0.0657 bc
	25	0.00045 b	0.000491 cd	0.00087 def	0.0493 e
Indian blanket	100	0.00081 b	0.000536 bcd	0.00134 ab	0.0610 cd
	75	0.00059 b	0.000448 de	0.00106 cde	0.0507 de
	50	0.00047 b	0.000274 fg	0.00075 f	0.0430 efg
	25	0.00024 b	0.000034 i	0.00038 gh	0.0230 hi
Gazania	100	0.00071 b	0.000551 bcd	0.00115 bc	0.0647 bc
	75	0.00049 b	0.000447 de	0.00085 fe	0.0457 ef
	50	0.00044 b	0.000331 ef	0.00077 f	0.0410 efg
	25	0.00034 b	0.000138 fgi	0.00062 fg	0.0317 gh
Marigold	100	0.00043 b	0.000761 a	0.00114 bcd	0.0523 de
	75	0.00037 b	0.000674 ab	0.00104 cde	0.0357 fg
	50	0.00029 b	0.000528 bcd	0.00082 fe	0.0207 hi
	25	0.00013 b	0.000231 fgh	0.00036 gh	0.0130 ij

* Field capacity
** Means with the same letter in each column don't have significant difference

plants to increasing environmental stresses, such as insufficient irrigation, is the accumulation of proline and soluble sugars. As shown in Table 5, the level of soluble sugars and proline was lowest in the control treatment (FC 100 %), or no stress, and the highest in the FC25 % treatment, or severe stress, in all the plants under study. The moss-rose and gazania bedding flowers showed the biggest increases in proline production under severe stress (compared to the control), respectively. The percentage of leaf ion leakage, a sign indicating drought stress is disrupting the cell membrane, significantly rose as the severity of the drought stress increased.

Because of the passive uptake mechanism, humidity and the plant's access to water have a significant impact on the plant's calcium uptake. From this, we may conclude that 100 % FC yielded the highest calcium content in all bedding plants (control). However, there was a decreasing trend of calcium percentage in leaves with increasing stress intensity, with the lowest calcium percentage recorded at 25 % FC (Figure 6). Marigolds (92 %) and gazania (84 %), among the plants studied, showed the greatest reduction in calcium absorption under severe stress (FC 25 %) compared to the control (FC 100 %), and dahlias (48 %), the least.

In the absence of stress, the relative water content of the leaves of moss-rose, dahlia, Indian blanket, gazania, and marigold plants was 86.5, 70, 97, 93.5, and 81 %, respectively. However, as deficit irrigation increased, the relative water content of the leaves decreased, resulting in a decrease of 58, 92, 39, 42, and 63 % in the cases of severe stress (25 % FC) (Figure 7). Low and medium stressors showed no significant difference in any of the studied plants.

3.3 WATER USE EFFICIENCY

Depending on the level of stress and the plant species, the water-use efficiency followed a completely different pattern (Figure 8). The ornamental plants with the highest water consumption efficiency were moss rose, gazania, and marigold (75 %), followed by dahlia (50 %) and moss rose (25 %). The results of this experiment indicate that in arid and water-scarce regions, the regulation

Table 5: Biochemical responses of some bedding plants to various drought stress levels

Bedding plant	FC*	Soluble sugars (μg FM-1)	Proline (μg g FM-1)	Ion leakage (%)
Moss-rose	100	405.00 d**	8.27 j	44.03 de
	75	460.67 c	14.13 hij	51.62 bc
	50	500.33 b	36.13 fg	54.56 b
	25	524.33 a	66.50 d	63.98 a
Dahlia	100	48.90 fgh	5.53 j	29.78 hi
	75	56.97 efgh	7.87 j	33.04 g
	50	61.27 efg	13.21 hij	35.34 g
	25	72.07 e	26.92 ghi	40.77 ef
Indian blanket	100	46.10 gh	10.15 j	28.05 hi
	75	48.23 gh	13.21 hij	36.04 fg
	50	57.57 efgh	47.40 ef	41.97 de
	25	67.80 ef	149.50 b	47.04 cd
Gazania	100	39.13 h	12.00 ij	23.77 j
	75	44.43 gh	18.93 hij	26.51 ij
	50	46.93 gh	29.21 gh	29.27 g
	25	52.53 fgh	181.34 a	35.32 hi
Marigold	100	43.90 gh	11.93 ij	10.03 i
	75	46.00 gh	19.17 hij	11.96 kl
	50	57.97 efgh	53.67 de	14.03 kl
	25	73.73 e	124.36 c	16.51 k

* Field capacity
** Means with the same letter in each column don't have significant difference

of water use for the production of dry matter is crucial. In other words, neither the control treatment (without stress) nor the plants with the highest water use showed the highest water use efficiency. As a result of the water storage organs present in plants such as moss rose, the highest water use efficiency was attained with the lowest water consumption.

4 DISCUSSION

Ornamental bedding plants are commonly impacted by drought, which has a negative impact on plant growth and flowering and, ultimately, on their aesthetic value. To avoid losing their attractive qualities, plants that can withstand water scarcity must be chosen. In order to choose the best plants for urban environments and to develop new cultivars that would be better suited to urban conditions, ornamental growers and breeding programs may benefit from experiments for drought tolerance that are based on measurements of certain factors relevant to the plant's water status. There isn't much knowledge on the application of selection criteria when choosing the right decorative plant species for urban green spaces or when developing plants to be more tolerant of water deficits. The rate of growth or survival of plants is frequently studied to determine how well they can handle drought stress. A more straightforward and efficient strategy may be indirect selection for drought tolerance in breeding, utilizing physiological or biochemical traits as markers. Leaf cell membrane stability, relative water content, and proline content are important factors for evaluating plant

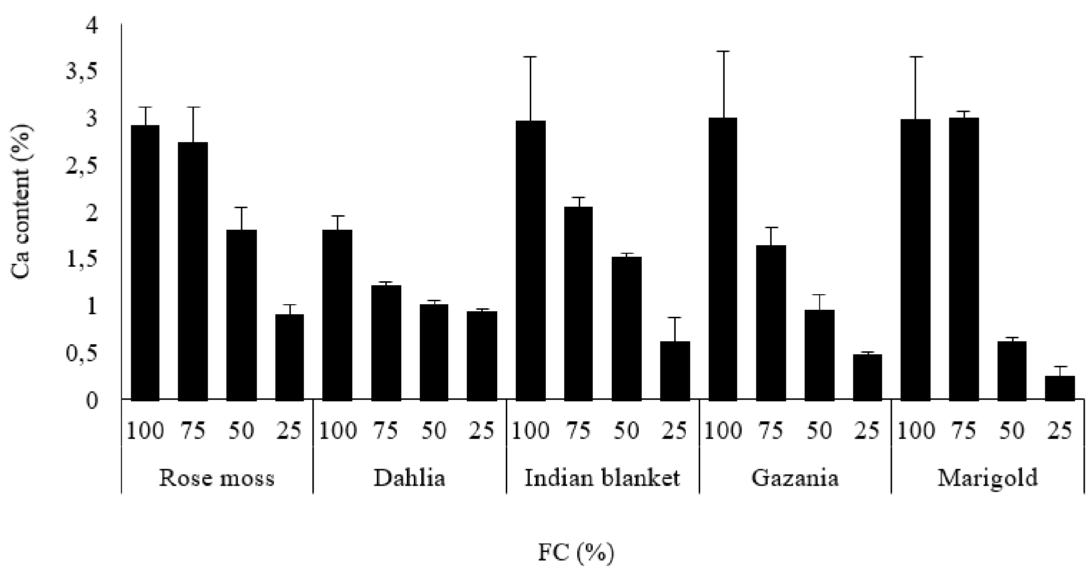

Figure 6: Leaf calcium content of some bedding plants under different drought stress (FC) levels

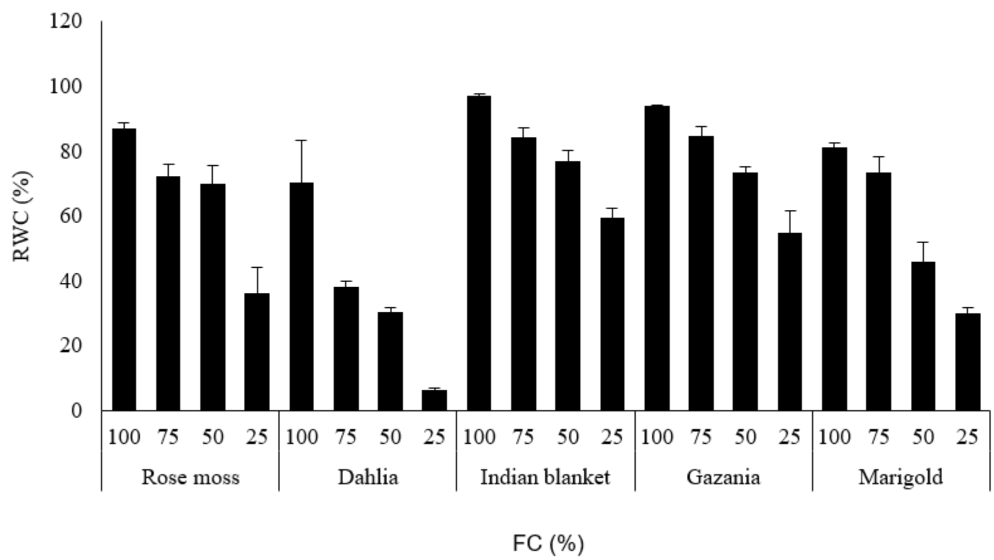

Figure 7: The leaf relative water content (RWC) of some bedding ornamental plants under different drought stress levels

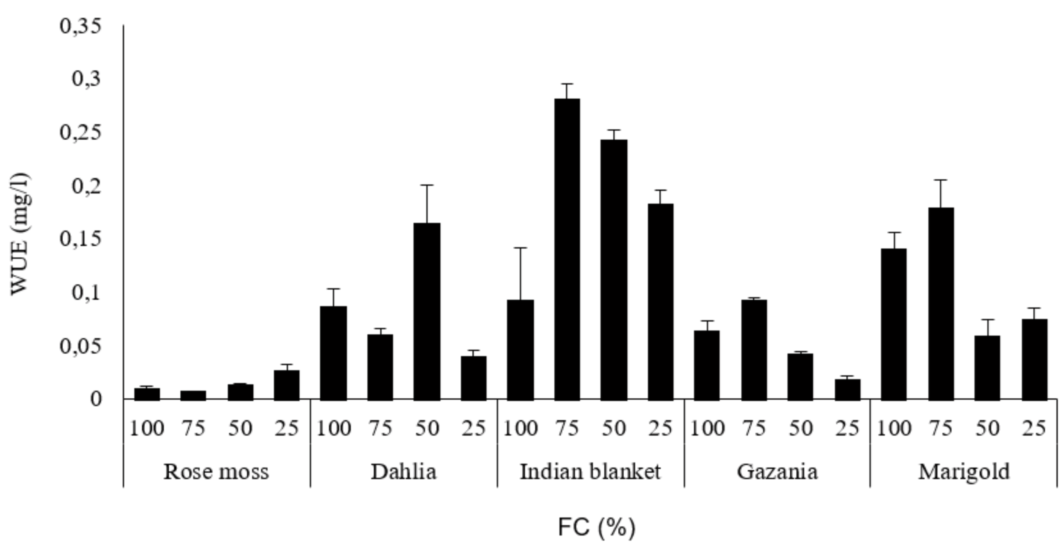

Figure 8: Water use efficiency of some bedding plants under various drought stress levels

reactions to drought stress (Gzik 1996; Quilambo 2004; Grant 2012). Here, we describe an effort to measure the morphological, physiological, and biochemical responses of five popular bedding plants to an imposed water stress. The goal was to determine which of these bedding plants can respond better to water deficit conditions for urban settings. Our investigations on bedding plants support previous findings that different plant species respond differently to drought (Volaire 2003; Kumar et al., 2018).

As previously described, the responses of the aerial (leaf) and underground (root) organs of bedding plants to drought stress showed a completely different pattern. In fact, the investigated bedding plants decreased the fresh and dry mass of the leaves and the leaf area by reducing the plant's access to water, while increasing the fresh and dry mass of the roots and the root length. Reducing leaf area or the phenomenon of leaf area adjustment (to reduce evapotranspiration) and increasing root growth and the root-to-shoot ratio (to improve water absorption) in drought-stressed plants are effective strategies for managing water absorption and consumption (Mahajan and Tuteja, 2005). Also some biochemical mechanisms are involved in conferring tolerance to drought stress in plants. One of the common mechanisms in plants under stress is an increase in the antioxidant activity to limit the oxidative damage, however, numerous factors affect the potential of antioxidant induction (Keyghobadi et al., 2020). The diameter of the flower and the number of flowers are the most significant factors that influence the drought tolerance of bedding plants. Marigold and Indian blanket require 75 % FC for optimal flower development, whereas gazania, rose moss, and dahlia only need 50 % FC. There is a close relationship between the morphological characteristics of plants and their drought tolerance (Bhusal et al., 2021). Rose moss, because of its fleshy leaves (which retain more water), gazania, and dahlia, because of their hairy leaves, have probably been capable of withstanding drought stress better. The decrease in flower diameter with increasing drought stress may also be caused by a drop in cell turgor induced by a shortage of water, which in turn leads to a reduction in cell development and, eventually, a loss in flower diameter. Consequently, the leaf water status, or the leaf's RWC (Figure 5), describes the relationship between plant water content and flower diameters under various drought stress or FC levels. Also, the observed decrease in growth characters may be the result of a decrease in the photosynthesis rate under drought stress, which can be attributed to the closure of stomata or a decrease in the leaf area in response to drought stress. Furthermore, the reduction in growth may be due to the fact that a lot of energy is used to produce enzymes and osmolytes. The decrease in the leaf area under drought conditions can be due to stomatal closure, and reduced water potential, leaf cell turgor pressure, photosynthesis, chlorophyll content, and Rubisco's carboxylase activity. A decrease in the growth rate of plant organs and leaf area due to increased drought stress can also be the result of depressed biosynthesis of growth hormones and induction of inhibitors such as abscisic acid (Keyghobadi et al., 2020).

The results of the current study are in agreement with the outcomes of other investigations in several crops (Toupchi Khosrowshahi et al. 2018; Rafi et al., 2019; Pourasadollahi et al. 2019). Also the existence of genetic diversity for tolerance to stress conditions has been frequently reported in other plant species (Hosseini Boldaji et al. 2012; Zebarjadi et al. 2012). In another study, drought stress effected the growth and antioxidant enzyme activities of *Pandanus* plants, drought stress has significantly affected the growth of *Pandanus* plants, such as LRWC, root-to-shoot ratio, shoot and root biomass, and REL, and led to an accumulation of ROS that damage cell membranes (Mohd Amnan, et al., 2021)

In addition to morphological responses to drought stress, biochemical and physiological responses also play a significant role in improving the plant's status under stress conditions (Hura et al., 2022). Drought stress disturbs physiological and biochemical processes in plants, including cell membrane, disrupting transportation of solutes, photosynthesis rate, nutrient uptake, translocation, and causes electron leakage and excessive accumulation of reactive oxygen species (ROS) (Nalina et al., 2021). Drought stress as an abiotic stress has likely caused an increase in the destruction of the cell membrane and, consequently, an increase in ion leakage (Table 4) and the destruction of photosynthetic pigments, i.e., chlorophyll a, b, and carotenoids, by increasing the production of free radicals. Drought stress changes photosynthetic pigment content. Photosynthetic pigments play important roles in harvesting light. The content of both chlorophyll a and b changed under drought stress. It is generally accepted that the maintenance of cell membrane integrity and stability under water stress conditions is a major component of drought tolerance in plants (Mombeni and Abbasi, 2019). The amount of chlorophyll, the most fundamental photosynthetic property, is significantly altered by water, serving as a unique indicator of chlorophyll photooxidation and degradation (Anjum et al., 2011). Decrease in the photosynthesis rate under drought stress, which can be attributed to the closure of stomata or a decrease in the leaf area in response to drought stress (Bijalwan et al., 2022). However, the increase in the level of compatible metabolites, i.e., proline and soluble sugars, concurrently with the increase in the level of drought stress (Table 4), prompted another biochemical reaction of bedding plants, known as osmotic adjustment (Mahajan and Tuteja, 2005), in order to maintain the plant's stability and absorption capability under low FC levels of the substrate, i.e., 25 and 50 % FC levels. Carbohydrates, the product of photosynthesis, provide a growth and maintenance substrate for non-photosynthetic tissues (Abdallah et al., 2018). Several factors affect sugar transport through the phloem (source, sink, and route between the two), impacting the source-sink interaction (Korner, 2015).The rate of photosynthesis and the amount of sucrose in leaves affect assimilate export from source to sink (Yu et al., 2015). Dry weather reduces photosynthesis and sugar concentration, slowing water transport. Drought also hinders the sink's capacity to utilize assimilates effectively. Drought significantly affects sugar metabolism and phloem loading. On the other hand, drought may change nutrient contents (e.g., sugars and amino acids) (Bijalwan et al., 2022). Often, plant cell membranes are subjected to changes associated with increase in permeability and loss of integrity under environmental stresses. The role of proline in response to drought stress include a very important part in the biosynthesis of cell-wall matrix proteins, such as extensins, that have important roles in cell morphology and provide mechanical support for the cell under stressed conditions. A neglected aspect of proline metabolism concerns its importance during the stress relief phase. In fact, its rapid oxidation is equally important in recycling the free amino acid accumulated during the stress conditions with the production of reducing power, amino nitrogen and energy, all needed in the restoration of cellular homeostasis during the recovery from drought stress (Mombeni and Abbasi, 2019).

In accordance with the results obtained with, in study physiological changes purslane (*Portulaca oleracea* L.) under drought stress, was observed drought treatment for 10 d significantly increased MDA, proline, EL, O_2radical dot−, and activities of SOD and POD. Also drought stress decreased LWC and chlorophyll content. This study indicated that the purslane has a great capability to cope with drought stress and activate many physiological mechanisms, which allow more efficient recovery during rehydration (Jin et al., 2015).

This study indicated a decline in calcium uptake as drought stress increased, particularly at extreme levels of drought stress (FC 25 %). Under normal circumstances, plants have the proper cellular turgor and absorption of nutrient ions, whereas water shortage conditions hamper the absorption of nutrients and consequently prevent shoot and root development. Under drought stress, the nutritional constraints are created by the reduction in the elemental uptake and consequently reduces the production of aerial organs. Therefore, under stress and low cellular turgor, the allocation ratio of the nutrients to roots increases against aboveground parts and the plant will not be able to continue to its normal growth (Keyghobadi et al., 2020). The fundamental reason for the drop in calcium absorption is the correlation between calcium uptake and the percentage of water in the substrate or the availability of water to the roots. In other words, the process of passive absorption of calcium and its direct relationship with the substrate's water capacity (Marschner, 2011) have led to a decrease in this element's uptake. Calcium is essential for the development and quality of horticultural crops, especially ornamental plants. The decreased uptake of this element reduces the appearance, quality, and durability of flowers, as well as their market value. Consequently, plants with the capacity to absorb water more efficiently under conditions of drought stress will be able to produce flowers of higher quality. All bedding plants displayed the highest calcium uptake under the control condition (FC 100 %); however, rose moss and marigold exhibited the highest calcium uptake at FC 75 %, rose moss at FC 50 %, and dahlia at FC 25 %.

Relative water content (RWC) is identified as one of the essential characteristics to determine leaf water status of genotypes to detect heat or drought tolerance ones. Water use efficiency (WUE) is also introduced as an indirect drought-tolerant cultivar selection method for grain yield under drought stress conditions (Bakhshi, 2021). Decrease of RWC is one of the early symptoms of water deficiency in plant tissues and many researchers have reported decrease in RWC under drought stress (Mombeni and Abbasi, 2019). Under extreme drought stress, rose moss exhibited greater water efficiency than the other evaluated bedding plants, most likely due to its fleshy leaves and capacity to retain water. In contrast, a distinct response was observed in other plants. Indian blanket was shown to have the highest water efficiency at all FC levels, including FC 25 %, which had the highest quantity compared to other plants at the same treatment level. It was revealed that dahlias (FC 50 %), gazanias (FC 75 %), and marigold plants (FC 75 %) had the highest water use efficiency. It has been observed that plants leaf relative water content was greater throughout leaf development and reduced as dry matter accumulated when the leaf matured. Water-use efficiency at the whole-plant level is defined as the ratio of dry matter produced and water consumed (Du et al., 2020). That plants water-use efficiency was higher in limited supplies than in well-watered situations. They linked this improved water efficiency to stomatal closure, which reduces transpiration (Khalid et al., 2019). It is reported that high relative water content is a resistant mechanism to drought, and high relative water content is the result of more osmotic regulation or less elasticity of tissue cell wall. Reported that the electrolyte leakage (EL) from a sensitive maize cultivar increased about 11 % to 54 % more than that of a tolerant cultivar after water stress treatment (Mombeni and Abbasi, 2019).

Due to its increased vegetative growth (Table 3), long stem, and morphological characteristics (leaf hairs), the Indian blanket probably has a higher water consumption efficiency than other assessed plants. As an osmotic regulator and stress moderator, FC 25 %, which endured the most severe drought stress, exhibited a considerable increase in proline content. The results of this experiment are in line with the findings of Chyliński et al. (2007) who compared the resistance and reactions of two ornamental plants, impatiens and geraniums.

5 CONCLUSION

Considering the problem of water shortage in many parts of the world and the little knowledge about growth reactions, especially the efficiency of water consumption in ornamental plants in the under irrigation shortage and drought stress conditions, in the current research, 5 important ornamental plants were investigated. The results of this research clearly showed that the resistance of each plant against drought stress depends on the specific morphological, physiological and biochemical reactions of that plant. In addition, the severity of drought stress is one of the most important factors determining plant selection for water shortage conditions. This study confirms that among the investigated bedding plants, Indian blanket has the greatest potential for cultivation in water-limited environments due to increased biomass production, flower number and water use efficiency. Additionally, comparison and evaluation of the growth responses and water use efficiency of commercial varieties of the studied bedding plants as well as the use of more precise tools or protocols to track the moisture status of the root rhizosphere are among the most critical issues that were not possible in the implementation of the current experiment, consequently, it is suggested that these parameters to be taken into consideration in the following research.

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An assessment of the performance of emergency management agency in the natural hazards management among farm households in the south-east zone, Nigeria

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An assessment of the performance of emergency management agency in the natural hazards management among farm households in the southeast zone, Nigeria

Abstract: An assessment of the performance of emergency management agency in mitigating natural hazards among farm households in Southeast Zone, Nigeria was studied. About 240 farm households who were administered questionnaire were chosen from the states' flood- and erosion-prone regions using multi-stage sampling technique. The results indicate that the EmergencyManagement Based-Performance Index's average level of national emergency management activities was 57.33. The total average ratings of the National Emergency Management Agency/State Emergency Management Agency performance indicators based on their usefulness as a measure of natural hazard were estimated as 47.8% which showed that the NEMA/SEMA key performance indicators' degree of effectiveness in hazard management is deemed to be below average of the index. These key performance indicators (KPI) include; distribution of food, provision of seedlings, provision of agrochemical, training of farmers on postharvest crop preservation, use of weather, rehabilitation of water resources, expansion of irrigation facilities, distribution of fingerlings, provision of household items. The highest weighted score assigned to the distribution of food was 2.89, indicating that it is 57.8% successin mitigating natural disasters, while the average weight score allocated to the distribution of seedlings was 2.62, indicating a 52.4% degree of efficacy. However, the study recommends that the funds allotted to NEMA/SEMA should be monitored to ensure it is utilized in achieving its stated aims and objectives.

Key words: KPI; emergency management; naturaldisasters; farm households; Nigeria

Ocena delovanja Agencije za krizno upravljanje v primerih naravnih nesreč med kmečkimi gospodinjstvi na jugovzhodnih območjih Nigerije

Izvleček: Ocenjeno je bilo delovanje agencije za krizno upravljanje za blaženje naravnih ujm med kmečkimi gospodinjstvi v jugovzhodni Nigeriji. Okoli 240 kmečkim gospodinjstvom iz območja držav, ki so podvržene poplavam in erozijam, je bil razdeljen vprašalnik, pripravljen na osnovi večstopenjske vzorčne tehnike. Rezultati so pokazali, da je bila poprečna velikost indeksa kriznega upravljanja, izračunananega na osnovi aktivnosti na nacionalni ravni 57,33. Celokupne poprečne vrednosti indikatorjev Nacionalne agencije za krizno upravljanje/Državne agencije za krizno upravljanje, osnovane na njihovi uporabnosti pri blaženju naravnih nesreč, so bile ocenjene kot 47,8 %, kar je pokazalo, de je učinkovitost NEMA/SEMA ključnih indikatorjev delovanja kriznega upravljanja pod poprečjem indeksa. Ti ključni indikatorji (KPI) so vsebovali: razdelitev hrane, dobavo sadik, dobavo agro-kemikalij, usposabljanje kmetov o ohranjanju pridelkov po spravilu, uporabi vremenske napovedi, obnavljanju vodnih virov, razširitvi možnosti namakanja, razdelitvi mladic rib, dobavi gospodinskih pripomočkov. Največja vrednost uteži, 2,89, je bila ugotovljena pri razdelitvi hrane, kar kaže na 57,8 % uspeh pri spopadanju z naravnimi katastrofami, med tem, ko je bila poprečna vrednost uteži, 2,62, pripisana razdelitvi sadik, kar nakazuje 52,4 % uspešnost. Rezultati raziskave priporočajo, da bi bilo potrebno sredstva namenjena NEMA/SEMA aktivnostim spremljati, da bi zagotovili doseganje zastavljenih ciljev in izzivov.

Ključne besede: KPI; krizno upravljanje; naravne katastrofe; kmečka gospodinjstva; Nigerija

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1 INTRODUCTION

Natural risks are frequently unavoidable environmental physical harms. Drought, erosion, flood, earthquakes, wildfires, and other environmental risks have continued to prevail (FAO, 2021). In Nigeria, erosions, floods, and drought are the most often experienced environmental examples of threats. Floods can be brought on by a variety of things, such as excessive rainfall, quickly accelerated snowmelt, strong winds over water, unusually high tides, tsunamis, or the breakdown of dams, levees, retention ponds, or other water-retention infrastructure. Flooding could be made worse by an increase in impermeable surfaces or by other natural disasters like wildfires, which deplete the quantity of plants that can soak up rain. Floods, soil erosion, gully erosion, coastline erosion, insect invasion, disease outbreaks, and related activities are only a few of the numerous natural and man-made risks that Nigeria's southeast states occasionally encounter (National Disaster Management Framework, 2018). Risks typically affect individuals, their health, and agricultural areas in an unexpected, negative, and immediate way. A hazard response team must be well-organized and prepare well when there are several victims and a need for urgent aid. According to the National Emergency Management Agency (NEMA) Act of 1999, natural or man-made hazards include any conditions brought on by a crisis, epidemic, drought, flood, erosion, earthquake, storm, train, aircraft, oil spill, or other accident, as well as the mass deportation or repatriation of Nigerians from other countries. In 35 of the country's 36 states, Nigeria experienced significant floods in 2012, which affected portions of the nation along key river basins and water courses. The recent floods in Nigeria have been attributed to a combination of natural, environmental, and manmade reasons, including the torrential rains and water releases from the Lagdo dam in Cameroon, the Dand dam in Kowa, the Kiri dam in the River Gongola, among others (UNDP, 2012).

Agriculture, which is also the sector of the economy that is the most heavily impacted by erosion and floods, is a common form of livelihood in rural regions. This claim is consistent with the finding of the Inter-Governmental Panel on Climate Change (IPCC) (2018) that agriculture is extremely vulnerable to the increased frequency, intensity, and unpredictability of extreme weather-related events. Nigeria, like many other nations in Sub-Saharan Africa, has been highly vulnerable to the damaging effects of risks brought on by climate change, according to a 2018 assessment from the IPCC. This is because Nigeria is located in tropical latitude. Despite the growing threat of catastrophes and the potential for catastrophic disasters like drought, flooding, and erosion in the future due to climatic and other environmental causes, the research concluded that these nations have not yet demonstrated complete capability to cope with the issues. As a result of ocean expansion brought on by increasing temperatures, one effect is an increase in mean sea levels, which by 2070 will be around 50 centimeters (IPCC, 2018). A compelling reason to review the methods governments in the region have used to address the crisis, such as the governance structure for risk management practice, is the enormity of the challenges that drought, erosion, and flood risk pose in Sub-Saharan Africa, where most people live on less than USD 1.25 per day (World Bank, 2021). To put it another way, dealing with natural disaster situations is still exceedingly difficult, especially in low-income African nations (UNCTAD, 2018). Thus, hazard's negative effects are more severe in developing countries than in low-income ones. In order to regulate and manage natural hazards, particularly erosions, droughts, and flooding, developing nations frequently struggle with a lack of resources, logistics, and infrastructure. Socio-economic, political, and environmental issues, according to United Nations, Department of Economics and Social Affairs (2020), are to blame for the shortcomings and failures of disaster management in developing nations. He emphasized that there is still a significant degree of poverty and a lack of knowledge about managing the external environment among the socio-economic issues. On the other hand, government authorities still lack the political will and commitment to implement pro-active environmental management policies and programs, particularly in the areas designated as hazard zones (Ovosi, 2010). In response to historical development in hazard management in Nigeria, the National Emergency Management Agency (NEMA) was established under Act No. 12 of the 1999 Constitution, as modified by Act No. 50, to manage disasters in Nigeria. NEMA has therefore been addressing disaster-related concerns by erecting concrete structures in Nigeria since its start. Risk management indicators, such as hazard monitoring and forecasting, early warning systems, community involvement, public education, land-use planning, updating and enforcing safety standards, rescue operations, humanitarian help, and financial assistance are used by NEMA to control these hazards. In order to address this issue, the nation (Nigeria) established the National Emergency Management Agency (NEMA). Act No. 50 of 1999 established NEMA to address concerns relating to disasters in Nigeria. Its goal is to manage situations in Nigeria caused by disasters. Moreover, it oversees initiatives and strategies for successful disaster relief at the municipal, state, and federal levels. According to the literature that is currently accessible, there have not been many studies since NEMA's founding that evaluate the agency's performance, particularly in terms of how well it is accomplishing its goals.

According to many descriptions, the poorest and most vulnerable groups in society are the farm households, which are the ones most severely impacted by climate change-related dangers. More than any other group in society, they are anticipated to benefit from NEMA efforts. So, this article evaluates how well NEMA manages threats brought on by the climate for farm households in Nigeria from the viewpoint of the farmer. While it impacts the most vulnerable populations in developing nations like Nigeria, managing the risks brought on by climate change is really a worldwide issue. This is particularly true for underdeveloped nations with a very limited capacity for adaptation, so that information from one may be applied in another, such as sub-Saharan Africa, whose socioeconomic aspects are quite comparable. NEMA manages these hazards by using performance management indicators, such as crop/livestock management practices, which include distributing food, providing seedlings, providing agro-chemicals, training farmers on postharvest crop preservation, using weather forecasts, and using early warning signals. Meanwhile, under water and irrigation and infrastructure management, the performance indicators include rehabilitation of water sources, irrigation infrastructure renovation, and training farmers on postharvest crop preservation. Fishing net distribution, the provision of fish feed, the distribution of boats to fishermen, the provision of shelters, medical treatment, and financial assistance all fall under the category of procedures for managing fisheries. The provision of clothing and the supply of domestic goods like stoves and cooking utensils are examples of the relevant sector. The management of natural hazards in Southeast States, Nigeria, is discussed in this paper. Natural hazard management refers to the methodical process of using administrative decisions, organizations, operational skills, and capacities to implement policies, strategies, and coping mechanisms of the societies and communities to lessen the impacts of natural hazards and related environmental and technological disasters. This includes all kinds of operations, including as structural and non-structural safeguards against or limiting the negative consequences of risks (via mitigation and readiness). In Nigeria, two emergency management traditions or patterns have developed throughout the years. The "vulture notion" and the "eagle concept" are how these have been described. When compared to the eagle notion, the vulture concept is essentially reactive. The first is comparable to what is sometimes referred to as a "command and control" strategy, but the second is more appropriately described as a "fire-brigade" approach. The NEMA has begun a paradigm transition away from the enduring reactive heritage of hazard management and toward a proactive approach, in keeping with the dominant worldwide mindset. The tradition of hazard is changing from the passive "vulture idea," in which the agency waits for hazards to occur, to the proactive "eagle concept," which uses forecasting and early warning to avoid and mitigate massive displacements of people and disasters.

1.1 LITERATURE REVIEW/THEORETICAL FRAMEWORK

The theoretical basis for this essay is comprised of risk and social management theory, and contingency theory. The requirement for a theoretical framework in this endeavor stems from the fact that it would provide the debate the much-needed analytical grounding. Moreover, analytical systematization would be utilized in a way that would improve patterned explanation of the subject.

1.2 CONTINGENCY THEORY

In an effort to provide a practical paradigm for strategic management, contingency theory of management was developed. This school of thought holds that the use of management principles and practices should depend on the circumstances at hand and that the functional, behavioural, qualitative, and systems tools of management should be used accordingly. The preceding quotation implies that the manager should be able to understand the distinctive relationships between the sub-systems of different companies inside a particular environment and how to approach a specific issue imaginatively. Contingency theory acknowledged that each individual organizational system results from the dynamic and frequently complicated interplay of the subsystems and their biological environment. Thus, the theory asserts that what qualifies as effective management changes depending on the particulars and idiosyncrasies of the organization's overall environment as well as the structure of the organizational sub-systems (Okenwa & Ugbo, 2003).

1.3 RISK AND SOCIAL MANAGEMENT THEORY

As the research issue in this study is the effectiveness of the state emergency management agency in Nigeria, we will use the risk and social management theory. Goldstein (1988) is credited with creating the risk and social management theory, which has since gained backing from a number of other scholars, including Douglas

(1978) and Dynes (1994). Man's transgression of nature and harm done to the environment by man's actions were factors in the birth and development of the risk and social management theory. For instance, Kielland (2012) noted that the risk and social theory in environmental management marks a timely contribution, given that environmental management is now more about calculating and managing the risk to human communities from rapid environmental and technological changes rather than just protecting pristine ecosystems and endangered species from anthropogenic harm. The idea also holds that effective management of mitigation techniques, which try to lessen the adverse effects of a risk or catastrophe occurring, is necessary to assist society's disaster victims (Enwemeka, 2012).

2 MATERIALS AND METHODS

The survey design was adopted for the study. In the first phase, three out of the five states in southeast zone were purposively selected. This was based upon the predominance of erosion and flood occurrences in the states. The contact farmers, 675 who made up the population of the Agricultural Development Programme (ADP), were used as the sample frame. A multiple-stage random sampling procedures were used in picking only 240 households who gave valid information based on the questionnaire administered to them. The information gathered were on the farm household socio economic characteristics, types of natural disasters experienced, farm households' grassroots management practices and NEMA/SEMA activities in the area. To guarantee that the effectiveness of the data instrument; face and content validation were used. Also the consistency and dependability of the questionnaire was carried out via a pilot research. Using the Cronbach Alpha reliability approach, 25 farmers from each state participated in a trial run of the questionnaire to determine its reliability. Data collected were analysed using descriptive statistics, likert scale and United Nation's Activity-Based Performance Index (API).

2.1 ACTIVITY-BASED PERFORMANCE INDEX (API) ESTIMATION PROCEDURES

To determine how well NEMA/SEMA is doing in terms of achieving its goals for hazard management among farm households in disaster zones, activity-based performance index (API) was utilized. As a set of indicating variables or key performance indicators (KPIs) for hazard management, the API entailed compiling hazard management techniques often utilized by the Emergency Management Agency. The variables that proved the success of the program(s), NEMA/SEMA,) served as the KPIs for gauging performance advancement. The success of NEMA and SEMA's actions will mostly be determined by their efficacy, according to KPI. These indicators are actions related to disaster management, and as such, the API gauges how well NEMA/SEMA employs these techniques for hazard management (Below et al., 2012). Experts and stakeholders in risk management and climate change research gave the indicative factors weights on a 5-point scale. According to how successful they were deemed to be as NEMA/SEMA hazard management operations, the weights were given to the indicative factors in ascending order. A free evaluation of each indicator variable's efficacy as a performance indicator was also given by farmers. The household's activity-based performance index has a direct bearing on how well NEMA/ activities SEMA's are used more frequently as a gauge of natural hazard management (Below et al., 2012). As such, the higher the API of the household, the more effective the increased use of these natural management practices is in the management of disaster. Following Below et al., (2012), the approach is specified as:

$$API_j = W_1P_1 + W_2P_2 + W_3P_3 +_{...}+W_nP_{nj} \qquad \text{Eq.1}$$

Where are:

API_j = Activity-based performance index of i_J^{th} household

W_{1n}= Weight of indicating variables;

5- Very effective;

4- Effective;

3- Moderately effective;

2- Poorly effective;

1- Not effective.

$Pn_{1j} = i_J^{th}$ household's assessment of the effectiveness of indicating variable for disaster management (1, if effective, 0 if otherwise).

2.2 LIKERT SCALE RATING

The mean score of respondents in a 4-point scale of 'high incidence = 4, moderate incidence = 3, low incidence = 2, and zero incidence = 1" was used. The mean is 4 + 3 + 2 + 1 = 10/4 = 2.5, using the interval scale of 0.05, the upper limit cut-off is 2.5+0.05 = 2.55, while the lower limit is 2.5-0.05 = 2.45. Based on these limit any mean score above 2.55 was considered high incidence level and any score below 2.45 will be considered low incidence while those between 2.45 and 2.55 were equally considered moderate incidence level. It was also applied in level of intensity.

3 RESULTS AND DISCUSSION

3.1 SOCIO-ECONOMIC CHARACTERISTICS OF RESPONDENTS

The mean age of the farmers was about 52 years, with majority of them (45.0 %) within the age range of 41 to 55 years old. These findings agree with the study of Nwaru (2010) that the respondents were a bit old with average age of about 52 years for smallholder food farmers in Imo State.

The result showed that 29 % of the respondents had no formal education, while about 71.0 % of them had formal education. Out of the 71 % with formal education, about 22.1 % only attended primary schools, 33.0 % attended secondary school while 15.8 % attended tertiary institutions at various levels. The average years of schooling of the respondents were 8 years (Table 1). This shows that the farmers had a very low level of formal education as majority of them barely completed primary education, with a handful of others attempting secondary education. This has severe implications for their ability to access and utilize new and improved techniques and innovations in agriculture. This is consistent with the results of Otitoju (2013) and Nwaru (2010).

Table 1: Frequency distribution of the respondents by their socio-economic characteristics

Variable	Frequency	Percentages (%)
Age		
25-40	22	9.17
41-55	108	45.0
56-65	94	39.17
66-80	16	6.67
Total	240	100
Sex		
Male	120	50
Female	120	50
Total	240	100
Level of Education Mean = 7.83		
Never Attended	70	29.17
1-6 years	53	22.08
6-12 years	79	32.92
12-16 years	38	15.83
Total	240	100
Household Size Mean = 7.5		
1-5	25	10.42
6-10	211	87.92
>10	4	1.67
Total	240	100

Family labour is recognized as major source of labour supply in smallholder food crop production in Africa. This comprises the labour of all males, females and children in a household, who contribute their labour to the household holdings. Majority of the respondents (88 %) fell within the household size of 6-10, followed by 10.42 % of them, which fell within the range of 1-5 persons per households. This is consistent with the results of Abdulai & Huffman (2000); Otitoju (2013); Ozor et al., (2015); Obi et al., (2021). The result shows that the average household size was 7 to 8 persons. The result equally agrees with the findings of Otitoju & Arene (2010) that majority of the respondents (medium scale soya beans farmers in Benue State Nigeria) had an average household size of about 7 people.

3.2 TYPES OF NATURAL DISASTERS EXPERIENCED BY FARM HOUSEHOLDS'

The type of natural disasters experienced by farm households' is shown in Table 2. A number of natural disasters types with different magnitude were identified by the respondents in the areas. These were flood, erosion, water logging, crop failure, pest attacks and disease outbreak. In Anambra State, about 93.8 % and 87.5 % of the farmers identified flood and erosion as major natural disasters they have faced while 75 % and 68.8 % of the respondents indicated disease outbreak and pest attacks. Crop failure and water logging were shown to be the least natural disasters with 62.5 % and 58.8 % of the respondents indicated them as the natural disasters they have experienced so far. In Enugu State, about 86.3 % and 80 % of the farmers identified flood and erosion as major natural disasters they have faced while 63.8 % and 62.5 % of the respondents indicated disease outbreak and pest attacks. Crop failure and water logging were shown to be the least occurring natural disasters with 61.3 % and 58.8 % of the respondents indicating them as the natural disasters they have experienced. In Ebonyi State, about 85 % and 75 % of the farmers identified flood and erosion as major natural disasters they have faced while 65 % and 58.8 % of the respondents indicated disease outbreak and pest attacks. Crop failure and water logging were shown to be the least natural disasters with 43.8 % and 42.5 % of the respondents indicating them as the natural disasters they have experienced. The Federal Government of Nige-

Table 2: Types of natural disasters experienced by farm households'

	Anambra State		Enugu State		Ebonyi State	
Natural disasters	Frequency	%	Frequency	%	Frequency	%
Flood	72	93.75	69	86.25	68	85
Erosion	70	87.5	64	80	60	75
Water logging	47	58.75	49	61.25	34	42.5
Crop failure	50	62.5	47	58.75	35	43.75
Pest attacks	55	68.75	50	62.5	47	58.75
Disease outbreak	60	75	51	63.75	52	65

ria, FGN (2013) reported that floods are the most common and recurring natural disaster in Nigeria. According to Enete et al. (2016), the Nigeria great flood of 2012 is presumably the worst flooding incidence in the country in 50 years, in which large farmlands under cultivation were submerged. Also Obi et al. (2021) reported that gully erosion was one of the greatest environmental disasters in south-eastern Nigeria, where large areas of agricultural lands have been lost completely. This has been corroborated by several other studies (Akinboade, 2013; Ezeigwe, 2015; Ngwu, Mbagwu & Obi et al., 2005). Wei Zhang et al., (2018) reported that the incidence of pests and diseases was a major constraint to increased agricultural productivity of farmers in Nigeria. Most of the time, the farmers are not well equipped to tackle these menace, either due to ignorance or lack of access to appropriate pesticides or insecticides. This results in fluctuation of agricultural yield and productivity, thereby increasing the vulnerability of the farmers to natural disasters.

3.3 FARM HOUSEHOLDS LEVEL OF EXPOSURE TO NATURAL DISASTERS

The farm households' level of exposure to natural disasters is shown in Table 3. The level of exposure of farmers to these natural disasters was examined using level of incidence on a 4-piont likert scale as shown in Table 3.The mean score of "high incidence =4, Moderate incidence =3, low incidence =2 and zero incidence =1" was used to examine the incidence level.In Anambra State, all the variables were on a high incidence level with their mean scores as follows: flood and erosion had mean scores of 3.3 and 3.25, while water logging, crop failure, pest attacks and disease outbreak had 2.68, 2.75, 2.88, and 3 respectively.

Variables observed in Enugu State were also on a high incidence level with their mean scores as follows: flood and erosion had mean scores of 3.24 and 3.1, while water logging, crop failure, pest attacks and disease outbreak have 2.73, 2.65, 2.75, and 2.79 respectively.

In Ebonyi State, variables were equally shown to be on a high incidence level with their mean scores as follows: flood and erosion had mean scores of 3.2 and 3, while water logging, crop failure, pest attacks and disease outbreak had 2.35, 2.39, 2.68, and 2.8 respectively. From the result, it is very clear that these farmers were highly exposed to the incidence of these natural disasters.

3.4 NATURAL DISASTER MANAGEMENT PRACTICES ENGAGED BY FARM HOUSEHOLDS

The natural disaster management practice engaged by farm households is shown in Table 4. At the grassroots level, farmers may not have relied only on Emergency Management Agencies' natural disasters management effort to cushion the effects of natural disasters on them. The frequency distribution of the female farm households according to their increased use of 25 traditional farm practices as measure(s) of natural disaster management is shown in Table 4 below. For purposes of this presentation, these practices were grouped into four broad categories: land and soil management practices, water management practices, crop and livestock practices and institutional measures.

3.4.1 Land/Soil Management Practices.

The results showed that increased land rotation (bush fallow), P1 (96 %) was the most frequently used natural disaster management practices under land and soil management category. This is because there is reduced frequent use of the same lands each year, which helps in climate change management practices. This was followed by avoiding bushfires P2 (88 %). Avoidance of bushfire is intended to achieve land management and traditional use objectives, by keeping the safeguarding

Table 3: Farm households' level of exposure to natural disasters in the three States

Natural Disasters	Mean	Std Dev	Remarks
Anambra State			
Flood	3.3	0.79	High incidence
Erosion	3.25	0.83	High incidence
Water logging	2.68	1.12	High incidence
Crop Failure	2.75	1.10	High incidence
Pest Attack	2.88	1.04	High incidence
Disease outbreak	3	1.01	High incidence
Enugu State			
Flood	3.24	0.85	High incidence
Erosion	3.1	0.95	High incidence
Water logging	2.73	1.10	High incidence
Crop Failure	2.65	1.11	High incidence
Pest Attack	2.75	1.10	High incidence
Disease outbreak	2.79	1.09	High incidence
Ebonyi State			
Flood	3.2	0.88	High incidence
Erosion	3	1.01	High incidence
Water logging	2.35	1.11	High incidence
Crop Failure	2.39	1.10	High incidence
Pest Attack	2.68	1.12	High incidence
Disease outbreak	2.8	1.08	High incidence

of life, property and resources through the prevention, detection, control, restriction and suppression of fire in forest and other vegetation in rural areas. The knowledge of the impacts of bush fire by female farmers is because of the positive effect of fire management plans in the area. Prompt physical weeding and killing/ removal of insects P4 (88 %), and use of insecticides and herbicides P5 (86 %) were the most frequently used natural disaster management practices under land and soil management category. Increased use of these practices helps to check the devastating effect of erosion and flooding. This is particularly important in southeast Nigeria, where large areas of agricultural lands have been lost completely, or have become unsuitable for cultivation or any other productive economic activity, as a result of erosion. (Akinboade, 2013; Ezeigwe, 2015; Ezezika & Adetona, 2011; Ngwuet al., 2005). Also, this measure helps to soften the soil for easy penetration of crops' roots, expose dangerous organisms that could harm the crops, and concentrate vital plant nutrients within the reach of their roots. These practices have relatively low technical skill requirements and cost implications, and as such, could have informed their widespread application by the farming households, also in the same light, raising of mounds P3 (80 %). Raised fields are constructed by excavating parallel canals and piling the earth between them to form long, low mounds with flat or convex surfaces. These raised platforms increase soil fertility, improve drainage in low-lying areas, and improve local micro-environments, primarily by decreasing frost risk.

3.4.2 Organic Manuring Application

Use of organic compost is a sustainable and climate-smart approach to increase soil fertility. The use of composted organic wastes to enhance soil fertility and productivity is gaining huge attention worldwide. Composting is a traditional practice that has been used for centuries. Composting refers to the natural process of rotting or decomposition of organic matter by microorganisms under controlled conditions. It is a biochemical process in which microbial degradation of organic waste results into a product known as organic manure or compost. Composting is a sustainable approach for organic waste management. It not only removes the waste but also transforms waste into nutrient-rich organic product that can be used to enhance soil fertility. Agro forestry practices P8 (75 %) and fertilizer application P7 (74 %) were equally used by the farm households in land/soil management practices. The result agreed with the study from CGIAR research programme on climate change, Agriculture and food security (CCAFS) among over 700 households in East Africa, which found that agro–forestry, was one of the most widely adopted climate change adaptation strategy. It was revealed that 50 % of those households had begun planting of trees as part of their farm practices 10 years ago (Kristjansonet al., 2012). These trees ameliorate the effects of climate change by helping to stabilize erosion, improve water and soil quality, and provide yields of fruits in addition to their usual farm harvest.

3.4.3 Water Management Practices

The result showed mulching P9 (88 %), mulching is very important because it helps in the management of soil erosion, soil quality, soil water, and weeds, pests and diseases control (Lu et al., 2000). Mulching helps to conserve water in the soil, regulate soil temperature and suppress the growth of weeds through the placing of loose sheets, trees/ plants and grasses on the bare soil. This result is consistent with those of Owomboet al. (2014) in Ondo State, Nigeria which showed that farmers used mulching as an adaptation strategy. Use of cover crops P12 (86 %) was used as the water management practices by the farmers.In the report of Bergtold et al. (2017), farmers will adapt and continue to utilize cover crops as management practices against hazard. Further, the cultivation of these cover crops does not entail any additional costs or responsibilities on the farmers, and this may have informed their wide use as a measure of climate change adaptation by the farmers. Similarly, a study conducted by Anyoda et al. (2013) revealed the wide application of cover cropping practices by majority of the farmers (90 %) as an adaptation strategy.

Use of manual/ physical irrigation P10 (85 %) was also a predominant water management practices of the farmers. Success of climate change adaptation depends on availability of fresh water in drought-prone areas. It should be emphasized that most adaptation methods provide benefits even with the lower end of climate change scenarios, such as improved irrigation efficiency. As water becomes a limiting factor, improved irrigation efficiency will become an important adaptation tool, especially in dry season, because irrigation practicesfor dry area are water intensive. Climate change is expected to result in decreased fresh water availability (surface and groundwater) and reduced soil moisture during the dry season, while the crop water demand is expected to increase because of increased evapo-transpiration caused by climate change and the continuous introduction of high-yielding varieties and intensive agriculture

The results further showed that about 21 % of the farmers were involved in water harvesting and storage (P13), about 7 % in the prevention of forest losses along water bodies (P14), and about 29 % in construction and maintenance of drainage channels. These practices are capital intensive, even though the benefits are not exclusive to the particular farmers undertaking them. Most of the time, they are carried out on communal basis in the form of community labour.

3.4.4 Crop/ Livestock Management Practices

Under crop/ livestock management practices, almost 80 % of the respondents used crop rotation (P16). Crop rotation refers to the practice of growing a sequence of plant species on the same land. It is an ancient practice that has been used for thousands of years. Crop rotation has been recaptured the global attention to solve the increasing agroecological problems such as declining soil quality and climate change resulting from short rotation and monocropping. Crop rotation is an effective approach for carbon sequestration as compared to growing same type of crop continuously. Crop rotation is a sustainable approach that increases yield and water use efficiency while reducing soil erosion.The result of multiple/ inter cropping (P17) showed that 87 % of the farmers use it as crop/livestock management practices. This finding agrees with the result of Enete et al. (2011) which showed that multiple/ intercropping was the adaptation practice with the highest profitability index among farmers in Imo States, Southeast Nigeria. According to the author, climate change has resulted in the intensification of multiple/intercropping, even though the practice has been identical with smallholder farming in Nigeria. The intent of this practice is to ensure and minimize the level of crop loss, which the farmers could suffer in the event of adverse weather conditions leading to crop failure. That is, multiple/intercropping provides some measure of security (confidence) to the farmers that at the end of the day, they will go home with some yields. It serves as an insurance against complete crop failure (Benhin, 2006). Enete et al. (2011) noted that different crops have varying degrees of resistance to climate volatility, and such, the cultivation of many crops at the same time could guarantee some harvest for the farmers even in the extreme weather conditions. Under changing planting dates (P19), farmers noted that the trend of uncertainties in extreme weather events had generally increased within the past five years in Southeast Nigeria, to avoid crop production risks due to rainfall variability and drought, staggered planting date is very common to most farmers whereby crops are planted before rain onset (dry land) on uncultivated land. Others were planted immediately after rain, while still other plots were planted a few days after the first rains. Tilling the land commences in fields which were planted prior to cultivation on the third week after the onset of rain which also destroys early geminating weeds and reduces weeding. These were done purposely to distribute risk by ensuring that any rain was utilized to the maximum by the crop planted in dry season.Under use of weather forecast (P20), 65.4 % of the farmers intensively used it as their crop management practices.This is because of the continuous update of weather changes to the farmers via their mobile phones and bill boards from Nigeria Meteorological Agency.Under cultivation of improved varieties (P18), cultivation of diseases resistance crops (P21) cultivation of early maturing crops (P22), the results revealed that 95.0 %, 87.9 % and 88.3 % of the farmers used them as their major crop management practices. These were intensified because of continuous research and government projects from research centres and universities on improved varieties, disease resistant crops like the adoption of bio-fortified cassava and high –yielding varieties of rice to help the farmers through community services. Furthermore, the result showed that only 3 % used cultivation of drought-resistance crop varieties. The result is deduced from the fact that drought is not experienced in Southeast States.

3.4.5 Institutional Measures

Majority of the respondents, 40.2 % agreed that assistance from NEMA/SEMA is one of their natural disaster's management practises, while only 13 % had an on-going insurance cover. It could be a result of unwillingness of the farmers to insure their farm enterprise.

3.5 FARM HOUSEHOLDS USE-INTENSITY LEVEL OF NATURAL DISASTER MANAGEMENT PRACTICES

The level of intensity of use of the practices was examined as shown in Table 3. Under land/soil management practices, all the practices had a high intensity of use score with their mean scores not less than 2.05 (i.e.MS ≥ 2.05) namely; land rotation (bush fallow) (3.61), avoiding bushfire (3.39), raising mounds and ridging across slopes (3.13), prompt physical weeding and killing/removal of

Table 4: Natural disaster management practices engaged by farm households

Natural disaster Management practices	Frequency*	Percentage %
Land/Soil Management Practices		
P1 Land rotation (bush fallow)	230	95.8
P2 Avoiding bushfires	228	87.8
P3 Raising mounds and ridging across slopes	190	79.8
P4 Prompt physical weeding and killing/removal of insects	211	87.9
P5 Use of insecticides and herbicide	207	86.2
P6 Organic manure application	186	77.5
P7 Fertilizer application	178	74.1
P8 Agro-forestry practices	181	75.4
Water Management Practices		
P9 Mulching	211	87.9
P10 Use of manual/physical irrigation	203	84.5
P11 Tree planting	66	27.5
P12 Use of cover crops	206	85.8
P13 Efficient water harvesting and storage techniques	50	20.8
P14 Prevention of forest losses along water bodies	16	6.7
P15 Construction and maintenance of drainage channels	70	29.2
Crop/Livestock Management Practices		
P16 Crop rotation	214	89.1
P17 Multiple/intercropping	207	86.3
P18 Cultivation of improved crop varieties	228	95.0
P19 Changing of planting dates	221	91.2
P20 Use of weather forecast	157	65.4
P21 Cultivation of disease- resistant crops	211	87.9
P22 Cultivation of early maturing crops	212	88.3
P23 Cultivation of drought-resistant crop varieties	29	3.3
Institutional Measures		
P24 Assistance from SEMA	97	40.2
P25 Registration with NAIC (on-going insurance cover)	75	12.9

*Multiple responses

insects (3.32), use of insecticides and herbicides (3.17), organic manure application (3.15), fertilizer application (3.04) and agro-forestry practices (3.14). This is consistent with the study by Ozor et al. (2010) on the mitigation and adaptation to climate change impacts on agriculture in Southern Nigeria, which includes improved use of land management techniques, use of pest and disease resistant crops/species. It also agrees with the study of Mahouna & Barjolle (2018) on farmer's adaptation to climate change and their implications in Benin.

Under water management practices, the following practices were highly intensified mulching (3.34), use of manual/physical irrigation (3.23), tree planting (3.26), use of cover crops (3.2), while efficient water harvesting and storage techniques (1.46), prevention of forest losses along water bodies (1.23), construction and maintenance of drainage channels (1.46) had zero intensity. This agrees with the literature report of Onyeneke (2010) who identified intensified natural disaster management practices by farmers as application of irrigation facilities, ridging and planting of trees. Construction and maintenance of drainage channels, prevention of forest losses along water bodies and efficient water harvesting and storage facilities, which were not majorly used by farm households, could be as a result of being capital projects which they cannot afford. It is also supported by Temes-

Table 5: Farm households' use-intensity level of natural disaster management practices

Natural Disasters Management Practices	Mean	STD. Dev.	Remarks
Land/Soil Management Practices			
P1 Land rotation (bush fallow)	3.612	.65	Moderate intensity
P2 Avoiding bushfires	3.388	.61	Moderate intensity
P3 Raising mounds and ridging across slopes	3.133	.75	Moderate intensity
P4 Prompt physical weeding and killing/removal of insects	3.317	.77	Moderate intensity
P5 Use of insecticides and herbicide	3.167	.77	Moderate intensity
P6 Organic manure application	3.146	.87	Moderate intensity
P7 Fertilizer applicatio	3.042	.84	Moderate intensity
P8 Agro-forestry practices	3.138	.82	Moderate intensity
Water Management Practices	3.342	.79	
P9 Mulching	3.225	.80	Moderate intensity
P10 Use of manual/physical irrigation	3.258	.73	Moderate intensity
P11 Tree planting	3.254	.81	Moderate intensity
P12 se of cover crops	3.200	.60	Moderate intensity
P13 Efficient water harvesting and storage techniques	1.461	.59	Low intensity
P14 Prevention of forest losses along water bodies	1.234	.68	Low intensity
P15 Construction and maintenance of drainage channels	1.467	.74	Low intensity
Crop/Livestock Management Practices			
P16 Crop rotation	3.429	.71	Moderate intensity
P17 Multiple/intercropping	3.258	.61	Moderate intensity
P18 Cultivation of improvedcrop varieties	3.388	.69	Moderate intensity
P19 Changing of planting dates	3.342	.25	Moderate intensity
P20 Use of weather forecast	2.758	.70	Moderate intensity
P21 Cultivation of disease- resistant crops	3.263	.73	Moderate intensity
P22 Cultivation of early maturing crops	3.418	.57	Zero intensity
P23 Cultivation of drought-resistant crop varieties	1.146	.70	Moderate intensity
Institutional Measures			
P24 Assistance from SEMA	1.792	.70	Low intensity
P25 Registration with NAIC (on-going insurance cover)	1.467	.68	Low intensity

Note: MS = Means Score

gen et al. (2014) who identified increase use of irrigation facilities in South Eastern Ethiopia by farmers as one of the major management practices.

The following practices under crop/livestock management practices were highly intensified; crop rotation (3.43), multiple/intercropping (3.26), cultivation of improved crop varieties (3.39), changing of planting date (3.34), use of weather forecast (2.76), cultivation of disease-resistant crops (3.21), cultivation of early maturing crops (3.26) except for cultivation of drought-resistant crop varieties with a mean score of 1.15. It is supported by the study of Nzeh & Eboh (2011) in Enugu State that identified the key indigenous adaptations of farmers to climate change to include change in planting date, change in cropping patterns, change in harvesting date of plants, change in planting distance and introduction of new breeds of crops. In institutional measures, assistance from NEMA/SEMA had a mean score of 1.79 while ongoing insurance coverage from NAIC had zero intensity of 1.47.

3.6 NEMA/SEMA'S ACTIVITY-BASED PERFORMANCE INDEX OF FARM HOUSEHOLDS

NEMA/SEMA's activity-based performance index of farm households is shown in Table 6.

The success of NEMA/natural SEMA's hazard management procedures as measured by its key performance index (KPI) reflects of the average evaluations given by agricultural professionals, farmers, and researchers. As a consequence, the activity-based performance indicator for NEMA/SEMA was on average of 57.33 %. This suggests that out of a possible index score of 120, NEMA/SEMA earned 47.78 %. In order to attain their rated natural hazard management indicators, they need an additional index score of 52.22 %. This is very significant for the efficiency of NEMA/SEMA efforts in the nation. This conclusion conflicts with that of Below et al. (2012), who calculated the efficiency of rural farmers' adaptation strategies to climate change in Tanzania's Morogoro area at 95.6 and 75.3 respectively. In order to support the findings in Table 6, the level of efficacy of each performance measure was also disclosed in accordance with farm families' perceptions. The average weight given to the distribution of food was 2.89, indicating that it is 57.8 % successful in mitigating natural disasters, while the average weight given to the distribution of seedlings is 2.62, indicating a 52.4 % degree of efficacy, and so on.

Table 6: NEMA/SEMA's activity-based performance index of farm households

API	Frequency	Percentage (%)
21 – 40	84	35
41 – 60	20	8.3
61 – 80	80	33.3
81 - 100	56	23.4
Minimum	24	
Maximum	98	
Average	57.33	
Potential score	120	
Total number of observations, N	240	

3.7 WEIGHTED RATINGS OF THE EFFECTIVENESS OF NEMA/SEMA'S ACTIVITIES ON FARM HOUSEHOLDS AS MEASURES OF HAZARD MANAGEMENT.

The weighted ratings of the effectiveness of NEMA/SEMA's activities on farm households as measures of hazard management are shown in Table 7. According to this, an increase in the adoption of these techniques may boost the effectiveness of natural hazard management techniques by an average of 47.78 %. In other words, capacity building for both NEMA/SEMA officials and farmers may still increase the efficacy of these activities by roughly 52.22 %. As a result, there is potential for the creation and use of fresh and creative approaches to the implementation of natural hazard management strategies. The outcome also revealed some differences in the scores from one category of hazard management strategies to another, as well as from one indication to another. Their scores ranged from 50.16 % for crops and animals, through 50.09 % for water/irrigation and infrastructure, to 42.68 % for fisheries and management methods. The management of water resources, crops, and animals was evaluated as having the highest effectiveness in managing natural hazards. This emphasizes the significance of the two as useful indicators for natural hazard management, especially in Southeast Nigeria where agricultural production is heavily dependent on rainfed farming with very few instances of irrigated farming. As a result, measures that will ensure sustainable and timely provision of moisture and water for agricultural production could be useful in managing natural hazards, particularly those associated with climate change issues. The results of the NEMA/SEMA management practice categories revealed that food distribution was the category with the highest level of efficacy in terms of managing crops and animals. The outcome is consistent with the conclusions reached by FAO (2021) that hazard food assistance is a crucial intervention during flood effects. The results of Enete et al. (2016), who assessed the socioeconomic effectiveness of small-holder farmers' flood coping mechanisms, are likewise consistent with this. On the opinion of farm households toward government food intervention programs, food aid received the highest rating. A higher degree of efficacy was also demonstrated by the use of weather forecasts scoring 54.6 %. This suggests that farmers and stakeholders concurred that personal observations of weather changes, friends, radio, television, and phones about weather forecasts, rainfall predictions or changes in rainfall patterns, wind movement, etc. is a very effective tool in natural hazard management by NEMA/SEMA. The results of this study are consistent with a report from the National Metrological Agency Services (NIMET) from 2012, which showed that farmers are aware of the rising trend in temperature and the declining trend in precipitation through personal observation, billboards on weather forecasts, radio, and updates of weather information sent to their phones. With regard to efficacy, seedling distribution scored 52.4 %. The free distribution of agricultural inputs is the intervention that Hemming et al. (2018) contend is the most successful. For instance, as part of a rehabilitation project following the 1992 Southern African Drought, free seeds and fertilizers have been sent to farmers in Malawi practically every year since 1992. Additionally, the effectiveness of providing agrochemicals (score = 48.6 %), training farmers in crop preservation techniques (score = 48.6 %), disease surveillance (score = 48.0 %), restocking small stock (sheep, cattle, and goats) (score = 47.8 %), vaccination and treatment (score = 47.0 %), and pasture preservation (score = 45.0 %) were nearly equal. Construction and upkeep of drainage channels had the greatest level of efficacy among techniques for managing water, irrigation, and infrastructure (score = 60.8 %). Rehabilitation of water resources, provision of irrigation pumps, and irrigation and infrastructural rehabilitation came next, with corresponding levels of efficacy of 58.2 %, 57.6 %, and 51.4 %. This suggests that water and irrigation as well as infrastructure management strategies are capital-intensive projects that were offered by the agencies, particularly amid the country's 2012 disaster. The supply of modest water treatment facilities, the development of capacity for water management, and the extension of current irrigation systems fall under this category. Their levels of efficacy are, respectively, 47.2 %, 47.0 %, and 45.4 %. Distribution of fingerlings, provision of shelters, provision of money and health care, and distribution of fishing nets had the highest levels of effectiveness under Fishery Management Practices, scoring 47.2 %, 44.6 %, and 43.4 % respectively. In contrast, distribution of boats to fishermen anddistribution of fish feeds had levels of effectiveness of 39.6 % and 38.6 % respectively. In the category "Other Relevant Sector," the provision of clothing scored at 41.8 %, while the provision of domestic goods including stoves and cooking utensils received a score of 39.4 %.

4 CONCLUSION AND RECOMMENDATION

Emergency management agencies have similarly utilized a mix of cataclysmic event key execution pointers to battle catastrophic events for farm households. These Key Performance Indicators rehearses incorporate likewise dissemination of nourishment, arrangement of seedlings and agro–synthetic concoctions, preparing of ranchers on postharvest crop safeguarding, field preservation, water system foundation limit expanding on water treatment, conveyance of fingerlings, arrangement of garments and family things like stove, cooking utensil and so forth. The financial qualities of the respondents, for example, age, gender, training, salary field understanding, nearness of cataclysmic event and potential advantages from NEMA/SEMA altogether affected the view of homestead family on NEMA/SEMA's exercises in their territories. Perceiving the significance of NEMA/SEMA's Activities in padding the impacts of catastrophic event, there is requirement for more exertion of the Agency to work together with these influenced homestead family units for important cataclysmic event the board mediations. Along these lines in perspective on the aftereffects of the examination, Southeast States apparently are generally inclined to debacles, while the board of such catastrophe has remained relatively poor. The different estimates embraced so far by NEMA/SEMA appeared not to have the ability to meet the degree of execution duty, which could be successful in overseeing calamity in the State. Hence, the administration of crisis in the States remains without a doubt unacceptable. To control natural hazards among farm households in South-East Nigeria, this study evaluated the effectiveness of emergency management organizations. The effectiveness of the agency is a function of a number of indices under four categories: crops/livestock management, water/irrigation infrastructure management practices, fishery and other relevant sectors. The outcome indicates that the NEMA Based-Performance Index's average level of national emergency management activities was 57.33. The total average ratings of the NEMA/SEMA performance indicators based on their usefulness as a gauge of natural hazard were at 47.78 %. Based on their average weighting of 47.78 %, the NEMA/SEMA key performance in-

Table 7: Weighted ratings of the effectiveness of NEMA/SEMA's activities on farm households as measures of hazard management

NEMA/SEMA Practice	Weight	Level of effectiveness %
Crop/Livestock management practices		
KP1 Distribution of food	2.89	57.8
KP2 Provision of seedlings	2.62	52.4
KP3 Provision of agro- chemicals	2.42	48.6
KP4 Training of farmers on postharvest crop preservation	2.43	48.6
KP5 Use of weather forecast	2.73	54.6
KP7 Disease surveillance	2.40	48.0
KP8 Restocking of small stock (Sheep, goat &cattle).	2.39	47.8
KP9 Vaccination & treatment	2.35	47.0
KP10 Pasture conservation	2.25	45.0
Subtotal	25.08	50.16
Sub average	25.08	50.16
Water &irrigation and infrastructural management practices		
KP11 Rehabilitation of water resources	2.91	58.2
KP12 Irrigation and infrastructural rehabilitation	2.57	51.4
KP13 Expansion of existing irrigation scheme	2.27	45.4
KP14 Capacity building for water management	2.35	47.0
KP15 Provision of small water treatment plants	2.36	47.2
KP16 Provision of Irrigation pump	2.47	57.6
KP17 Construction and maintenance of drainage channels	2.60	60.8
Sub total	17.50	50.09
Sub average	2.51	50.09
Fishery management practices		
KP18 Distribution of fingerlins	2.36	47.2
KP19 Distribution of fishing nets	2.17	43.4
KP20 Provision of fish feed	1.93	38.6
KP21 Distribution of boat to fishermen	1.98	39.6
KP22 Provision of shelters, healthcare & money	2.23	44.6
Sub Total	10.67	42.68
Sub Average	2.67	42.68
Other Relevant Sectors		
KP23 Provision of clothes	2.04	41.8.
KP24 Provision of household items like stoves and cooking utensils	2.02	39.4
Sub total	4.06	40.6
Sub average	2.03	40.6
Total weighting	57.34	-
Average weighting	2.39	47.78
Potential weight	120	
Total number of observation	-	240

Note: KPI; Key performance indicator

dicators' degree of effectiveness in hazard management is deemed to be below average. The average weight assigned to the distribution of food was 2.89, indicating that it is 57.8 % successful in mitigating natural disasters, while the average weight allocated to the distribution of seedlings was 2.62, indicating a 52.4 % degree of efficacy. The South-East States are therefore regarded to be more vulnerable to catastrophes considering the study's findings, while disaster management has remained comparably subpar. The different NEMA/SEMA mechanisms that have been put in place thus far did not appear to be able to handle the amount of performance responsibility necessary to manage hazards in the state. As a result, there is no question that risk management in the southeast states are inadequate. The research consequently advises the necessity for multi-agency trainings and exercises as well as more proactive performance indicators for the agency to improve the effectiveness of emergency management agencies in managing natural hazards (flood, erosion).

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Characterization of nuclear DNA content and chromosome numbers of *Tulipa luanica* Millaku, *T. kosovarica* Kit Tan, Shuka & Krasniqi and *T. albanica* Kit Tan & Shuka

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Characterization of nuclear DNA content and chromosome numbers of *Tulipa luanica* Millaku, *T. kosovarica* Kit Tan, Shuka & Krasniqi and *T. albanica* Kit Tan & Shuka

Abstract: The Balkan Peninsula is considered an important centre of native tulip species. *Tulipa kosovarica* and *Tulipa luanica* are new species recently discovered in Kosovo, and *Tulipa albanica* in Albania. The current study aims at the investigating the nuclear DNA content and chromosome number of these three tulipa species in order to provide for the first time data on their genome size and differences among these three *Tulipa* species. Analysis of nuclear DNA content was performed by flow cytometer (Partec CyFlow Space) in mature fresh leaves for each *Tulipa* species. Samples for chromosome analysis were taken from the root tip meristem of the bulbs. Results showed significantly higher amounts of nuclear DNA (2C) in *T. luanica* compared to *T. kosovarica* and *T. albanica*. The chromosome number for these three species was 2n = 2x = 24, while the chromosome sizes of *T. luanica* resulted larger, compared to that of *T. kosovarica* and *T. albanica*. A correlation between the nuclear DNA content and chromosome size was found among these tulipa species. Moreover, nuclear DNA content and chromosome sizes of *T. luanica, T. kosovarica* and *T. albanica* showed clear differences among these species.

Key words: tulip; DNA content; chromosome number; endemica

Določitev vsebnosti jedrne DNK in kromosomskega števila treh vrst tulipanov, *Tulipa luanica* Millaku, *T. kosovarica* Kit Tan, Shuka & Krasniqi in *T. albanica* Kit Tan & Shuka

Izvleček: Balkanski polotok je pomemben center samoniklih vrst tulipanov.Vrsti *Tulipa kosovarica* in *Tulipa luanica* sta novi vrsti nedavno odkriti na Kosovu, in vrsta *Tulipa albanica* v Albaniji. Namen raziskave je bil preučiti vsebnost jedrne DNA in kromosomskega števila teh treh vrsta tulipanov in tako prvič določiti velikost njihovega genoma in razlike med temi tremi vrstami. Analiza jedrne DNA je bila narejena s pretočnim citometrom (Partec CyFlow Space) v odraslih svežih listih vseh treh vrst. Vzorci za analizo kromosomov so bili vzeti z rastnih vršičkov korenin čebulic. Rezulatati so pokazali značilno večjo vsebnost jedrne DNA (2C) pri vrsti *T. luanica* v primerjavi z vrstama *T. kosovarica* in *T. albanica*. Kromosomsko število vseh treh vrst je bilo 2n = 2x = 24, med tem, ko je bila velikost kromosov vrste *T. luanica* večja v primerjavi z velikostjo pri vrstah *T. kosovarica* in *T. albanica*. Pri vseh treh vrstah je bila ugotovljena korelacija med vsebnostjo jedrne DNK in velikostjo kromosov. Vse tri vrste so se po vsebnosti jedrne DNK in velikosti kromosov jasno razločevale.

Ključne besede: tulipan; vsebnost DNK; kromosomsko število; endemit

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1 INTRODUCTION

The genus *Tulipa* includes the most well-known, adored, and economically significant flowering plants in the world. In the World Checklist for *Tulipa*, 418 are named and 112 were accepted there (Govaerts 2008). Tulips species are grown in wild in North Africa, Southern Europe, the Middle East, and Central Asia, including China. According to Botschantzeva (1962), the Tien Shan and Pamir-Alay Mountain ranges in Central Asia are considered as primary centers while the Caucasus as a secondary gene center for *Tulipa* species. In the Balkan peninsula Greece, Kosovo, Bulgaria, Albania, Macedonia and Serbia have the highest number of tulip species (Govaerts, 2010; Millaku et al., 2018). Up to date in Kosovo there are reported five native tulipa species, *T. serbica* Tatić & Krivošej, *T. australis* L., *T. gesneriana subsp. T. scardica* L., *T. luanica* Millaku and *T. kosovarica* Kit Tan, Shuka & Krasniqi (Millaku & Elezaj, 2015; Shuka et al., 2010, 2012). While the *Tulipa* genus is represented by two stenoendemic species, *T. kosovarica* and *T. serbica*, which are found only in the serpentines of Kosovo. *T. scardica* and *T. kosovarica* grow in serpentine soil, well-drained and exposed to full sun but protected from high winds, while *T. luanica* grows in limestone soil (Osmani et al., 2018). These serpentine soils were characterized by high concentrations of metals, and the levels of metals such as Ni, Co and Cr ranged from 1500 to 1600 mg kg^{-1} for Ni, 130 to 140 mg kg^{-1} for Co, and 380 to 450 mg kg^{-1} for Cr (Osmani et al., 2018). This previous study reported that the enzyme δ-aminolevulinic acid dehydratase activity and concentrations of δ-aminolevulinic acid, malondialdehyde and glutathione showed differences among these three tulipa species, especially *T. kosovarica* and *T. albanica* (serpentine sites) in comparison with *T. luanica* (limestone site).

Despite the existence of a large body of literature on *Tulipa*, taxonomy is generally considered to be difficult. Moreover, molecular analysis is a good attempt to understand the relationships within *Tulipa* species better. Nuclear DNA content can conveniently be measured by flow cytometry using propidium iodide, a stoichiometric DNA stain that intercalates in the double helix (Zonneveld, 2009). It is well known that morphological characteristics of plants are influenced by the vegetative stage of the plant as well as by a variety of environmental conditions. Therefore, the identification of the genetic diversity within and among plants species based on the morphological plant characterization is considered insufficient (Hunter, 2018). DNA-based markers are an advanced tool widely used to assess genetic relationships and diversity in plant species (Kumar, 1999; Hunter, 2018), novel molecular marker techniques have been developed in diverse plant species (Wang et al., 2015) and the transferability of molecular markers between species has been investigated for many species so far (Raveendar et al. 2015; Berisha et al. 2015). The recent advances in sequencing technologies enabled the discovery of functional genes in many plant species (Abbasi et al., 2015; Chai et al., 2017). Furthermore, these initiatives have enabled the development of novel and alternative molecular markers known as gene-targeted markers (GTMs), which are based on the untranslated sections of expressed sequence tags (ESTs) (Poczai et al., 2013) or gene-targeted functional markers (GTFMs), which are gene markers implicated in phenotypic trait variation as a result of their functional gene sequences (Arnholdt-Schmitt, 2005). In addition of above mentioned techniques, flow cytometry helps in the estimation of nuclear DNA content and the ploidy level (Dolezel et al., 2021; Dolezel et al., 2004; Vlacilova et al., 2002). Flow cytometry is a method that can conveniently measure the nuclear DNA content by using propidium iodide, a stoichiometric DNA stain. A genus may contain numerous species with similar chromosomal numbers but different DNA 2C-values (Ohri, 1998). Flow cytometry is a fast and practical method for elucidating systematic relationships among species within the genus. This technique was efficiently employed in ecological, physiological, molecular biology and genome evolution studies, as well as in plant breeding (Dolezel et al., 2021).

The chromosomal karyotype parameters of Iranian *Tulipa* species were studied by Abedi et al. (2015) and Masoud et al. (2002). Their findings demonstrated that while the majority of Iranian species had three distinct chromosome types—m, sm, and st—and were diploid (2n = 2x = 24), their karyotype parameters varied**.** In the Netherlands, the genome size of a variety of *Tulipa* species was studied with results ranging from diploid (30 pg) to tetraploid (123 pg) (Zonneveld, 2009).

Based on our previous investigation, *T. kosovarica* and *T. albanica* grow on serpentine soils while *T. luanica* grows in limestone soil (Osmani et al., 2018). The serpentine soils were characterized by higher concentrations of metals compared with limestone soils. Since these three endemic species are relatively new species discovered in recent years and they grow in different habitats, traditional taxonomy based on geographic distribution and morphological characteristics will be augmented with more information on DNA content and chromosome number to clarify the relationships among these *Tulipa* species. The main objective of this study was to determine nuclear DNA content and chromosomes number of *T. kosovarica*, *T. luanica* and *T. albanica,* and in line with this to have more information about differences between these species.

2 MATERIAL AND METHODS

The plant samples were collected during flowering time in their natural habitats (Figure 1): *Tulipa albanica* at Surroi locality in Albania (altitude 625 m a.s.l., geographical coordinates: 42°02'30" N and 20°20'15" E), *Tulipa kosovarica* at Mrasor locality in Kosovo (altitude 450 m a.s.l, geographical coordinates: 42°30'59" N and 20°34'08" E) and *Tulipa luanica* at Pashtrik locality in Kosovo (altitude 1100 m a.s.l, geographical coordinates: 42°16'17" N and 20°28'24" E). More than 30 plant samples (leaves and bulbs) from each plant species were collected. The sample collections and preparation procedures were carried out at a regulated temperature of 4 °C.

2.1 FLOW CYTOMETRY ANALYSIS

FCM (flow cytometry) is a great method for examining the optical characteristics of small particles suspended in liquid, such as fluorescence and light scatter. The nuclear DNA content and polyploidy analysis of our sample set was carried out using Partec CyFlow Space flow cytometer (Munster, Germany) at analyzes the laboratory of genetics and plant cytogenetics at Namik Kemal University in Tekirdag, Turkey. Fresh mature leaves of three tulip species were collected during two seasons (2016 and 2017) during their flowering time in their natural habitats and were analyzed within the optimal time frame after their collection. The collection, storage and transport of leaves was carried out based on standard procedure and under temperature control (4 °C) with preservation of humidity. The analysis of nuclear DNA content was done with three replicates within the sample (from the same leaf) and from 30 plant individuals (leaves), at least 5000 nuclei were analyzed for each type and sample. Suspension of intact nuclei was prepared using commercial kits manufactured by Partec (Munster, Germany). Homogenization of the leaves (50 mg) was done together with the leaves of the standard plant in a petri dish, where 0.5 ml of extraction buffer was also added. This homogenate was filtered using 50 µm nylon filters and then transferred to standard test tubes of the apparatus where 1.5 ml of DAPI (4',6–diamidin–2-phenylindole) was added; samples were left in the dark for 60 minutes at 4 °C. (Tuna et al., 2001). DAPI was used as a fluorochrome for DNA labeling because it has more affinity for binding to the nitrogenous bases A and T. *Secale cereale* L., which has 16.55 pg / 2C DNA, was used as a standard. The results were processed with the FloMax analysis software program and expressed in pg 2C DNA (picograms of diploid DNA) (Figure 2). The amount of nuclear DNA was calculated based on the following formula:

2C DNA = [(average G1 peak of sample / average G1 peak of standard)] × amount of 2C DNA standard (pg DNA)

2.2 KARYOTYPE ANALYSIS

Samples for chromosome (karyotype) analysis were taken from the root tip meristem of the bulbs. First, the bulbs were collected from their natural habitats, and then they were grown in a vegetative room. After germination, approximately 2 mm root tips were cut and treated with 0.05 % colchicine for 5 hours, then fixed with acetic acid/ethanol in a ratio of 1:3 for 24-48 hours. Hydrolysis of

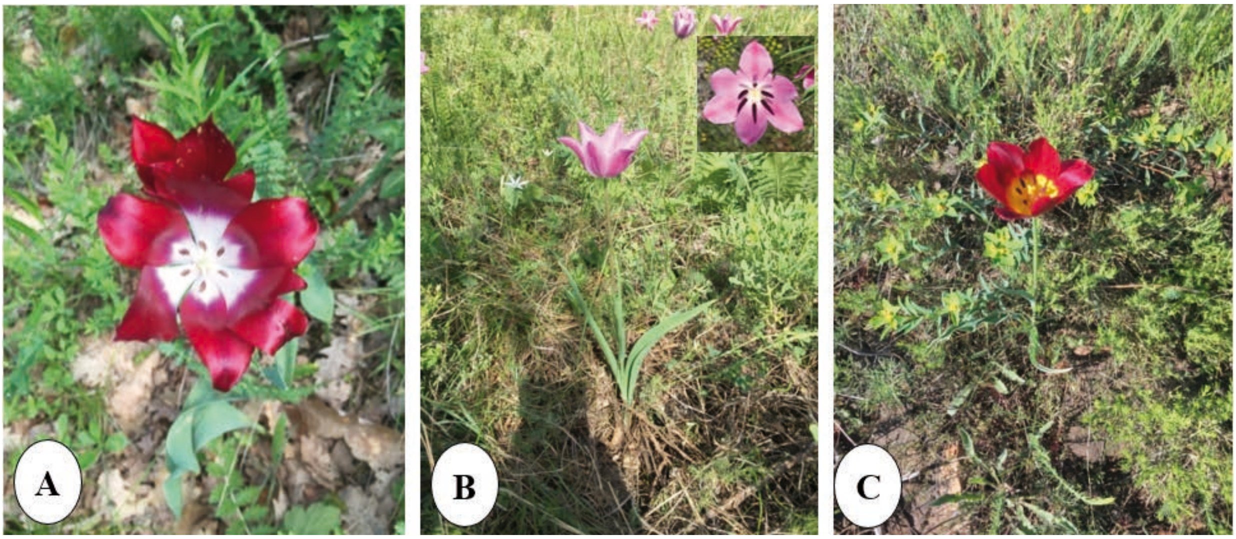

Figure 1: Tulipa species during flowering time in their natural habibitats: A) *T. kosovarica*, B) *T. luanica* and C) *T. albanica*

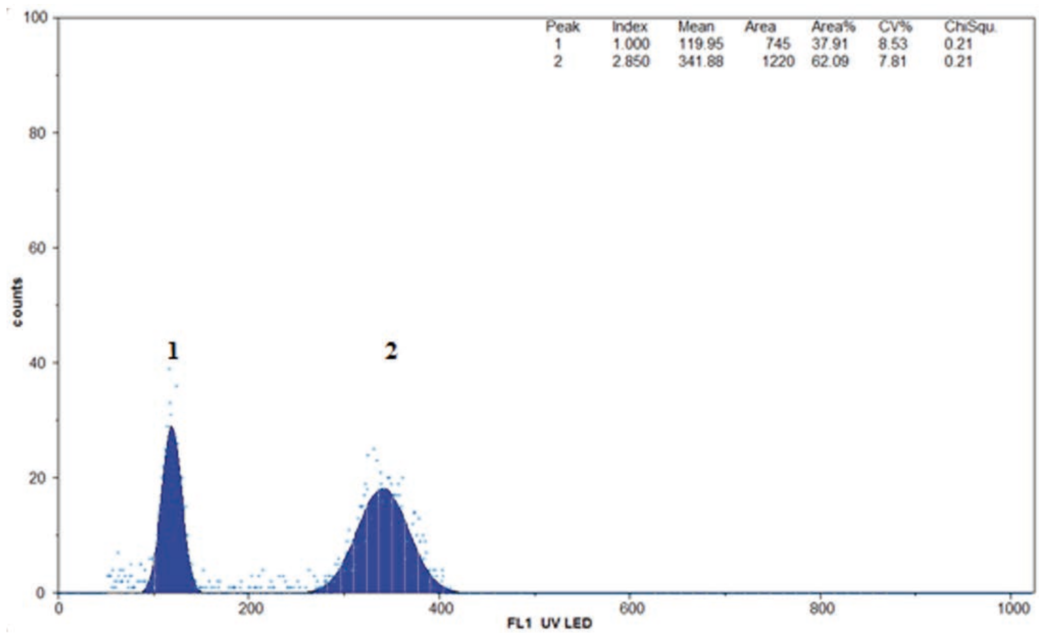

Figure 2: Flow Cytometer histogram for the amount of nuclear DNA (2C) in: 1) standard plant and 2) *Tulipa luanica*

the tip of the roots was done using 1N HCl for 12 minutes at a temperature of 60 °C, while staining was done by using acetocarmine. Karyotyping, determination of centromere position and chromosome type was performed in the metaphase according to the nomenclature of Levan et al. (1964).

2.3 DATA ANALYSES

Statistical analysis of the results was carried out with Sigma stat 32 programs 2004 STAT Software. The data presented in the paper represent the average of at least four independent experiments with ± S.E. Each continuous variable, a distribution form was determined, and the significant differences between means were checked by Student's *t* test.

3 RESULTS AND DISCUSSION

Seven different taxa of *Tulipa* are found in Kosovo, three of which are stenoendemic (*T. luanica, T. kosovarica,* and *T. serbica*), one of which is a local endemic (*T. scardica*), while the other three taxa, *T. gesneriana* and *T. sylvestris* which is represented by two subspecies (*T. sylvestris* subsp. *australis* and *T. sylvestris* subsp. *sylvestris*), have a wider distribution (Millaku et al., 2018; Millaku & Elezaj, 2015; Shuka et al., 2010, 2012). The presence of a high number of *Tulipa* species/taxa in Kosovo and their sympatric area in serpentine substrate in the Deva locality, in the south of Kosovo, close to the border with Albania, makes it an important regional and global habitat of native species of the genus *Tulipa*. According to Millaku et al. (2018), considering tulip species high variability, the application of molecular analyses is of crucial importance to accurately classify *Tulipa* species of Kosovo and of the Balkans and in their taxonomic differentiation.

3.1 NUCLEAR DNA CONTENT

The results indicate a significantly higher amount ($p < 0.001$) of nuclear DNA (2C) in *T. luanica* (47.49 pg) compared to *T. kosovarica* (45.71 pg) and *T. albanica* (43.86 pg) (Table 1). Based on differences in the amount of nuclear DNA expressed as a percentage, we can presume that *T. luanica* and *T. kosovarica* differ by 3.75 %, *T. luanica* and *T. albanica* differ by 7.64 %, while *T. kosovarica* and *T. albanica* differ by 4.05 %. Our results regarding the genome size (2C DNA) of *T. luanica*, *T. kosovarica* and *T. albanica* are in accordance with those reported by other authors; Zonneveld (2009) reported that the genome size of plants of the genus *Tulipa* sp. with diploid number (2n) of chromosomes varied from 32-69 pg 2C DNA. Our genome size results (2C DNA) showed a range of 43.86 – 47.49 pg in the three tulip species, which are within the values reported for tulips with diploid chromosome number by Zonneveld (2009). In our study the 2C DNA content of the *T. albanica* resulted 43.86 pg, while in the same speciesthe 2C DNA content was previously reported to be 54.15 pg (Shuka et al. 2010). The observed differences in the genome size in the same tulipa species can be due to the fact that, in our analysis, we used adult leaves collected at the time of blooming from naturally grown plants, while according to Shuka et al. (2010), the plant material used for the amount of DNA estimation was taken both by the germinated seeds and the adult leaves. Fresh leaves that have nearly completed growth are often preferred; very young leaves might not be as suited because they contain more inhibitors than older leaves (Dolezel et al., 2007). The genome size (2C DNA) reported for other tulips that grow in the Balkans (Albania and Kosovo) was 61.5 pg in *T. schrenkii* Regel and 69 pg in *T. scardica* Bornm. (Zonneveld, 2009). If we compare species of the genus *Tulipa* that extend from east to west, from North Pakistan to the Balkans, a gradual increase in the amount of nuclear DNA is observed, from 32 pg to 69 pg (Zonneveld, 2009). The transfer of DNA sequences from the nucleus into mitochondria and chloroplasts may have been one of the causes for smaller genome sizes (Karimzadeh et al., 2010).

The results of the number of base pairs calculated using a value of 978 mega base pairs (Mbp) for one picogram, showed that *T. luanica* had a greater number of mega base pairs compared to the *T. kosovarica* and *T. albanica* (Table 1). Based on these results, *T. luanica* has about 1740 Mbp more than *T. kosovarica* and about 3550 Mbp more than *T. albanica*, while *T. kosovarica* has about 1809 Mbp more than *T. albanica*. Genome size results were also given with flow cytometer histograms for each tulip species. According to previous studieson genome size, 1pg is equal to several thousand genes or about 978 Mbp (mega base pairs) (Zonneveld, 2009; Dolezel et al., 2003). According to our results, *T. luanica* has a larger genome, a larger number of genes and base pairs, compared to other tulip species under study, *T. kosovarica* and *T. albanica,* the latest are grown on serpentine soils. According to Knight et al. (2005) and Temsch et al. (2010), large genomes are a burden for plant organisms and limit their adaptation. On this regards, plant species that grow under stressful habitats face greater risk; therefore, we presume that the two plant species *T. kosovarica* and *T. albanica* have adapted in terms of genome size to live in serpentine environments, which are considered potentially more stressful, while *T. luanica* in limestone environments has a larger genome.

3.2 CHROMOSOME NUMBER AND KARYOTYPE CHARACTERISTICS

Analysis of the results for chromosome number and karyotype characteristics for *T. luanica, T. kosovarica and T. albanica* are presented in table 2 and figure 4. Based on these results we can conclude that the number of chromosomes in the three species under study, *T. luanica*, *T. kosovarica* and *T. albanica,* is 2n = 2x = 24 (Figure 3). The size of the chromosomes in *T. luanica* ranged from 5 - 12 µm, where two pairs of chromosomes are metacentric (I and VIII), two pairs are submetacentric (X and XII) and the other eight are subtelocentric (II, III, IV, V, VI, VII, IX and XI). In *T. kosovarica* the size of the chromosomes ranged from 5 - 10 µm, where two pairs of chromosomes are metacentric (X and XII), five are submetacentric (II, IV, V, VI and XI) and the other five subtelocentric (I, III, VII, VIII and IX). In *T. albanica* the size of the chromosomes ranged from 5 - 8 µm, where two pairs are metacentric (V and VIII), three pairs submetacentric (VI, X and XII) and the other seven chromosomes are subtelocentric (I, II, III, VI, VII, IX and XI).

Our results for chromosome number and karyotype characteristics of *T. albanica* and *T. luanica* were in accordance with those reported by other authors (Shuka et al., 2010; Millaku & Elezaj, 2015). According to Zonneveld (2009), most of the species of *Tulipa* have the same basic chromosome number, 2n = 2x = 24. In addition, many species in this genus have the same chromosome number, differences in DNA 2C value, when present, have proven to be very effective in delimiting infrageneric

Table 1: The amount of nuclear DNA in picograms (pg) in the leaves of *T. luanica, T. kosovarica and T. albanica*, the difference being expressed as a percentage (%) between these species and the approximate number of mega base pairs (Mbp)

		2C ADN pg	Difference in %	Equivalent in Mbp
T. luanica		47.49 ± 0.53	TL : TK = 3.75 %	46445.22
T. kosovarica		45.71 ± 0.29	TL : TA = 7.64 %	44704.38
T. albanica		43.86 ± 0.59	TK : TA = 4.05 %	42895.08
Significance	TL : TK	< 0.001		
	TL : TA	< 0.001		
	TK : TA	< 0.001		

TL – T. luanica; TK – T. kosovarica; TA – T. albanica. The results are expressed as mean and standard error (±). Significant differences were found between different species for $p < 0.001$

Table 2: Number of chromosomes and karyotypes of *T. luanica*, *T. kosovarica* and *T. albanica*

Species	Chromosome number	Chromosome size	Karyotype characteristics		
			Metacentric	Submetacentric	Subtelocentric
T. luanica	24	5-12 µm	I, VIII	X, XII	II, III, IV, V, VI, VII, IX, XI
T. kosovarica	24	5-10 µm	X, XII	II, IV, V, VI, XI	I, III, VII, VIII, IX
T. albanica	24	5-8 µm	V, VIII	VI, X, XII	I, II, III, VI, VII, IX, XI

divisions in a number of taxa (Ohri, 1998). Genome size has been demonstrated to differ between taxa that share identical chromosome numbers. Moreover, Greilhuber (1998, 2005) has clearly shown that intraspecific variation of genome size is much less than assumed. In this case, results show that the size of the genome (2C DNA) and chromosomes of *T. luanica* are larger compared to *T. kosovarica* and *T. albanica*. A correlation was also found between the 2C DNA content and chromosome size; the larger the genome, the larger are the chromosomes in these three types of tulips. In addition, morphological differences between these species reported from Millaku and Elezaj (2015) show that capsule and seed size of *T. luanica* are bigger in comparison with *T. kosovarica* and *T. albanica*. These findings are consistent with a considerable amount of evidences that suggest that the size of reproductive organs might be related to genome size and that the variations in genome size, both increases and

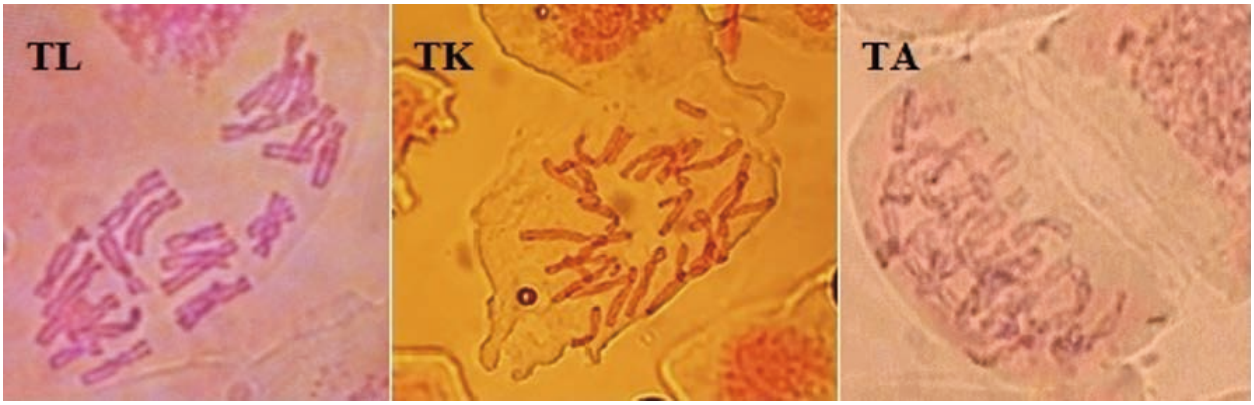

Figure 3: Karyotyping of chromosomes in metaphase for *T. luanica* (TL), *T. kosovarica* (TK) and *T. albanica* (TA)

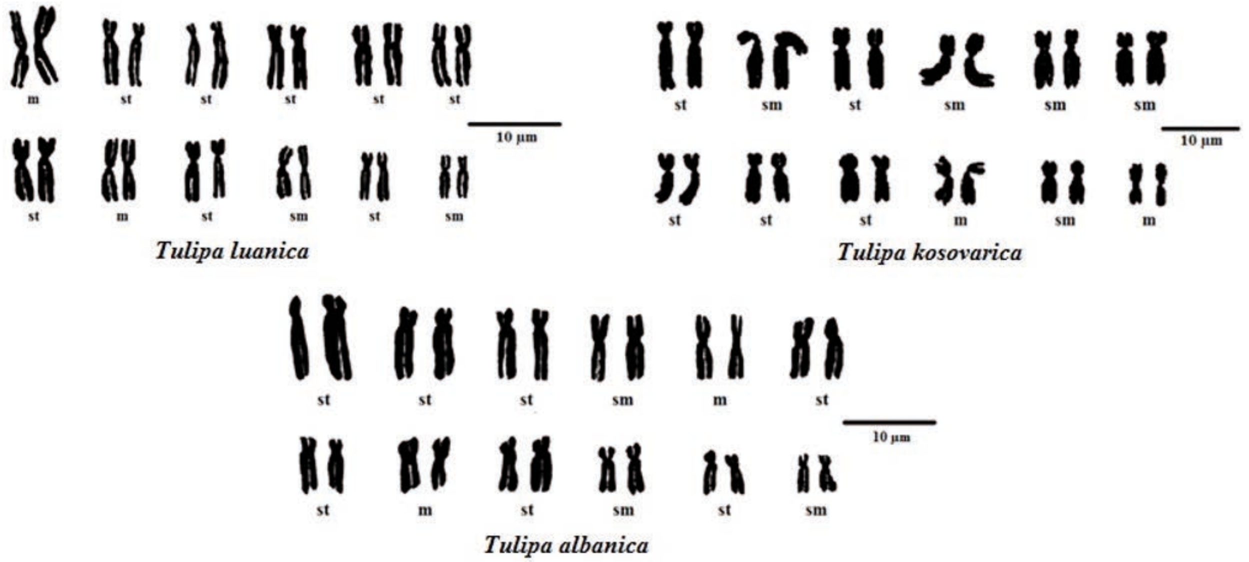

Figure 4: Karyogram and presentation of chromosomes in metaphase

decreases, might have contributed to the evolution and diversification of the genus, even within closely related species (Seijo & Fernandez, 2003).

4 CONCLUSIONS

The combination of analysis of nuclear DNA content carried out in flow cytometry and the number of chromosomes resulted very useful to determine the relationship status among three *Tulipa* species. Moreover, nuclear DNA content and chromosome sizes of *T. luanica, T. kosovarica* and *T. albanica* showed clear differences among these species. The nuclear DNA content and chromosome size of *T. luanica* resulted larger, compared to that of *T. kosovarica* and *T. albanica*. Furthermore, we presume that the two plant species *T. kosovarica* and *T. albanica* have adapted in terms of genome size to live in serpentine environments, which are considered potentially more stressful, while *T. luanica* which is grown in a less stressful limestone environment resulted in a larger genome size.

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Comparative analysis of antioxidant potential in leaf, stem, and root of *Paederia foetida* L.

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Comparative analysis of antioxidant potential in leaf, stem, and root of *Paederia foetida* L.

Abstract: *Paederia foetida* L. is widely used for the treatment of myriad ailments. Thus, searching for plant parts having greater antioxidant potential would make it easy to get suitable materials for herbal drugs. The present effort was made to explore the antioxidant potentials in the plant parts of *P. foetida* grown under natural conditions by means of physiological and biochemical analyses. The young leaves showed the highest reservoir of non-enzymatic antioxidants such as chlorophylls (0.96 mg g^{-1}), carotenoids (0.43 mg g^{-1}), anthocyanins (53.99 µg g^{-1}), phenolics (728.24 µg g^{-1}), flavonoids (4178.05 µg g^{-1}), and proline (1.46 µmol g^{-1}) as compared to others. Total antioxidant activity was found to be the highest in young leaves (84.82 %) followed by young stems (80.24 %) and matured leaves (79.78 %). Analysis of enzymatic antioxidants resulted in the superior activity of ascorbate peroxidase (13.58 µmol min^{-1} mg^{-1}) and glutathione S-transferase (3409 nmol min^{-1} mg^{-1}) in young leaves whereas the highest rate of catalase (409.85 µmol min^{-1} mg^{-1}) and peroxidase (3.5 nmol min^{-1} mg^{-1}) activity were found in matured leaves. However, comparatively higher content of reactive oxygen species; hydrogen peroxide, and lipid peroxidation product; malondialdehyde in matured leaves than that of young leaves suggests that young leaf is a suitable source for herbal medicine.

Key words: medicinal plant; antioxidants; free radicals; reactive oxygen species; oxidative stress

Primerjalna analiza antioksidacijskega potenciala listov, stebla in korenin vrste *Paederia foetida* L.

Izvleček: Vrsta *Paederia foetida* L. (smrdljiva trta) se na široko uporablja za blaženje številnih bolezni. Iskanje delov rastline z večjim antioksidacijskim potencialom bi olajšalo pripravo primernih zdravilnih pripravkov. Namen raziskave je bil preučiti antioksidacijski potencial različnih delov rastline, ki je rastla v naravnih razmerah s fiziološkimi in z biokemičnimi analizami. Mladi listi so imeli v primerjavi z drugimi organi največ neencimskih antioksidantov kot so klorofili (0,96 mg g^{-1}), karotenoidi (0,43 mg g^{-1}), antocianini (53,99 µg g^{-1}), fenoli (728,24 µg g^{-1}), flavonoidi (4178,05 µg g^{-1}) in prolin (1,46 µmol g^{-1}). Celokupna antioksidacijska aktivnost je bila največja pri mladih listih (84,82 %), ki so jim sledila mlada stebla (80,24%) in odrasli listi (79,78 %). Analiza encimskih antioksidantov je pokazala največjo aktivnost askorbat peroksidaze (13,58 µmol min^{-1} mg^{-1}) in glutation S-transferaze (3409 nmol min^{-1} mg^{-1}) v mladih listih medtem, ko sta bili aktivnosti katalaze (409,85 µmol min^{-1} mg^{-1}) in peroksidaze (3,5 nmol min^{-1} mg^{-1}) največji v odraslih listih. Primerjalno večje vsebnosti reaktivnih zvrsti kisika, vodikovega peroksida in peroksidacijskih produktov maščob kot je malondialdehid v odraslih listih nakazujejo, da so mladi listi primernejši vir pripravkov pri zdravljenu s to zdravilno rastlino.

Ključne besede: zdravilne rastline; antioksidanti; prosti radikali; reaktivne zvrsti kisika; oksidacijski stres

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1 INTRODUCTION

Medicinal plants possess therapeutic properties and have beneficial pharmacological effects on humans and animals. The medicinal values of these plants are linked with their phytochemical constituents that cause definite pharmacological action in the human body (Khairullah et al., 2021). The phytochemicals such as flavonoids, phenolic acids, isoflavones, carotenoids, phytosterols, saponins, etc. have great antioxidant potential and are of great interest due to their beneficial effects on human health (Thakur et al., 2020). Reactive oxygen species (ROS) are free radicals with one or more unpaired electrons in their outer shell which occur naturally in plants and animals during different metabolic processes (Hasanuzzaman et al., 2020; Adetuyi et al., 2022). Biotic stresses or different ailments and environmental stressors like UV, ionizing radiations, pollutants, heavy metals, and xenobiotics (i.e., antiblastic drugs) enhance the accumulation of ROS in the living cell which have harmful effects on important cellular constituents like proteins, lipids, and nucleic acids (Gómez et al., 2021). Several investigations reported that oxidative stress led by ROS is responsible for the progression of several diseases including cancer, diabetes, metabolic disorders, cardiovascular diseases, arthritis, and stroke, which causing to ultimate cell death in humans (Pizzino et al., 2017; Mahmoud et al., 2021). Human beings set several strategies to counter face the effects of oxidative stress by means of enhanced activities of enzymatic (e.g., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), etc.) and several non-enzymatic antioxidants such as carotenoids, flavonoids, ascorbic acid, α-tocopherol, etc. (Shah and Gupta, 2020). Since endogenous antioxidant defenses are inadequate to mitigate entire damage, antioxidant-rich diets are essential for maintaining good health (Guerra-Araiza et al., 2013). As antioxidants scavenge free radicals from the cells and reduce the damage caused by oxidation, a diet rich in antioxidants might reduce the risk of the above-mentioned diseases and improve overall health conditions. The most familiar exogenous antioxidants are vitamin C, vitamin E, and polyphenols including carotenoids, flavonoids, and phenols (Blázovics, 2022). Although synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, and tert-butyl hydroquinone are widely used, long-term intake of those resulted in several negative impacts on human health (Engin et al., 2011). So, there has been an increased demand for the therapeutic potentials of plants as natural antioxidant source in reducing oxidative injury. However, indiscriminate use of commonly used commercial antimicrobial drugs against infectious diseases has led to an alarming risk in advancing resistance to multiple drugs, thus necessitating the need for searching the alternatives source of synthetic drugs from medicinal plants (Bungau et al., 2021; Hassan et al., 2021). Antimicrobial drugs of plant origin have enormous therapeutic potential and show a higher level of efficacy in the treatment of infectious diseases rather than synthetic drugs having enormous side effects. However, scientists found that foods containing phytochemicals with antioxidant potential have strong protective effects against the risks of cancer and cardiovascular diseases (Okoye, 2021). Therefore, developing a potential herbal source for beneficial phytochemicals and antioxidants is of great interest to the scientific community for sustainable health security.

Paederia foetida (Skunk vine or Gondhabadali) plant belongs to the family Rubiaceae, is a widely distributed medicinal plant in Asia and especially in south-east Asia (Okamoto et al., 2008). It has a broad spectrum of uses in the treatment of ailments like hepatic disorders, rheumatoid arthritis, constipation, diabetes, coughs, asthma, itches, wounds, stomachache, diarrhoea, dysentery, pain, typhoid, pneumonia, toothache, cancer, etc. (Soni et al., 2013). The presence of essential plant metabolites having anti-ulcer, anti-diarrhoeal, antihyperglycemic, antioxidant, antitussive, and anthelmintic activity in *P. foetida* (Soni et al., 2013) suggests the plant is a potential reservoir of herbal drugs. Although some recent investigations suggest the antioxidative, antidiabetic, and antimicrobial efficacy of *P. foetida* (Karmakar et al., 2020; Satapathy and Pattnaik, 2020; Ghosh et al., 2021a), comparative analysis of antioxidants emphasizing entire plant parts is still to be clarified. However, analysis of plant parts having the best enzymatic antioxidant potential is still to be reported in this valuable medicinal plant. Additionally, the natural occurrence of ROS in the plant parts is yet to be determined. Therefore, the present investigation was set to identify the suitable plant part of *P. foetida* with the best enzymatic and non-enzymatic potentials through physiological and biochemical assays. The findings suggest that the young leaf of *P. foetida* is the best source of antioxidants and utilization of which should mitigate the effect of excess ROS produced by ailments and environmental stressors. Thus, the findings might help develop suitable herbal drugs and maintain sustainable health security.

2 MATERIALS AND METHODS

2.1 COLLECTION AND PREPARATION OF PLANT MATERIALS

The stem cuttings of *P. foetida* were grown in the research field of Bangabandhu Sheikh Mujibur Rahman Agricultural University and those were allowed to grow naturally with proper care and management. The primary branching of the healthy and suitable plants was selected for collecting plant samples. The fresh and fully expanded young leaves (YL) of 15 days-aged, matured leaves (ML) of 45 days-aged, young stems (YS) of 15 days-aged, matured stems (MS) of 45 days-aged, and root (R) of 45 days-aged of *P. foetida* were collected from the selected branches. After repeated washing, the collected materials were allowed to dry for 4-5 days in an oven. The dried materials were chopped and crushed into powder and stored in air-tight containers for further analyses.

2.2 CHLOROPHYLLS AND CAROTENOIDS CONTENT DETERMINATION

Chlorophylls content from freshly collected leaf and stem tissues and carotenoids content from leaf, stem and root tissues were determined using the method described by Porra et al. (1989). Briefly, 100 mg of plant tissues were taken in a glass vial and 5 ml of 80 % acetone was added. The vials were made airtight and kept at 4 °C in the dark for 24 hours. After extraction, the separated plant extracts were taken to measure the absorbance through a spectrophotometer at 663, 646, and 470 nm wavelengths respectively. Blank measurement was done using only acetone. The quantification was done according to the formula of Lichtenthaler & Welburn (1983). The chlorophylls and carotenoids content were expressed as milligram per gram of fresh sample (mg g^{-1}).

2.3 ANTHOCYANINS CONTENT DETERMINATION

Anthocyanins content was determined with little modifications as described by Hughes and Smith (2007). Briefly, 1 g shade-dried powder of plant parts was taken in an ice-cold glass vial containing 5 ml methanol. After making the vials airtight, those were kept in dark condition for 24 hours. Then, 2 ml of extracts were centrifuged with 2 ml distilled water and 2 ml chloroform at 5000g at 4 °C for 15 minutes. The absorbance was measured at 530 nm. Quantification was done according to the formula of Murray and Hackett (1991). The anthocyanin content was expressed as microgram of cyanidin-3-glucoside equivalent per gram of dry sample (µg g^{-1}).

2.4 DETERMINATION OF PHENOLICS CONTENT

Phenolics content of the methanolic extracts was determined spectrophotometrically according to the Folin-Ciocalteu method (Ainsworth and Gillespie, 2007). The absorbance of reaction solutions was measured at 765 nm against a blank sample. Quantification was done according to the formula of Abdul-Hafeez et al. (2014). The measurements were compared to a standard curve of gallic acid solutions and expressed as micrograms of gallic acid equivalents per gram dry mass (µg g^{-1}).

2.5 DETERMINATION OF FLAVONOIDS CONTENT

The methanolic extract of plant materials was used for the determination of flavonoids content using the aluminium-chloride colorimetric assay (John et al., 2014). Quercetin at different concentrations was used as the standard solution. The absorbance of the extracts and standard solutions was measured at 510 nm using a UV/Visible spectrophotometer. The results were expressed as micrograms of quercetin equivalents (QE) per gram of dry mass (µg g^{-1}).

2.6 DETERMINATION OF ENZYMATIC ANTIOXIDANT ACTIVITY

Fresh plant tissues of the plant materials (0.5 g) were homogenized in 1 ml extraction buffer containing 1 mM ascorbic acid, 1 M KCl, 0.5 M K-P buffer (pH 7.0), β-mercaptoethanol and glycerol in ice-cold mortar and pestle. The homogenates were centrifuged at 11,500 × g for 15 min, and the supernatant was used as a soluble protein solution for enzyme activity. The protein concentration was determined by the method of Bradford (Bradford, 1976) using BSA as a protein standard. The catalase (CAT) activity was measured according to the method of Hasanuzzaman et al. (2014). The activity of CAT was determined as µmol min^{-1} mg^{-1} protein using the extinction coefficient of 39.4 M^{-1} cm^{-1}. The activity of ascorbate peroxidase (APX) was assessed by following the procedure outlined by Nakano & Asada (1981). The activity of APX was determined as µmol min^{-1} mg^{-1} protein using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. The activity of peroxidase (POD) was measured by following the method of Hemeda and Klein (1990). The activity of

POD was determined as nmol min^{-1} mg^{-1} protein using an extinction coefficient of 26.6 mM^{-1} cm^{-1}. Glutathione S-transferase (GST) activity was measured by following the procedure of Hossain et al. (2010). The activity of GST was determined as nmol min^{-1} mg^{-1} protein using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

2.7 DETERMINATION OF ANTIOXIDANT ACTIVITY (% DPPH SCAVENGING ACTIVITY) AND PROLINE CONTENT

The plant extracts were supposed to showing DPPH radical scavenging activity by following the method of Abdul-Hafeez et al. (2014). Ascorbic acid was used to make a reference solution. The following equation was used to get the inhibition percentage:

% DPPH radical scavenging activity = $[(A_0 - A_1) / A_0] \times 100$. Where A_0 = absorbance of the control and A_1 = absorbance of the sample. The proline content in different plant parts was measured spectrophotometrically using the acid ninhydrin assay, as described by Bates et al. (1973). The proline content was determined as μmol g^{-1} fresh mass using a standard curve.

2.8 DETERMINATION OF HYDROGEN PEROXIDE (H_2O_2) AND MALONDIALDEHYDE (MDA) CONTENT

The H_2O_2 content was measured following the method of Ghosh et al. (2021b). The leaf samples (0.1 g) were homogenized in 1.5 ml 0.1% trichloroacetic acid (TCA) and the homogenate was centrifuged at 11,500 × g at 4 °C for 15 min. 0.08 ml of supernatant was taken and added 0.2 ml of 100 mM KP buffer (pH 7.5) and 0.8 ml of KI. The tubes were kept in ice for 1 hour and then at room temperature for 20 min to stabilize the reaction. The absorbance was measured at 390 nm. The concentration of H_2O_2 was calculated by using the absorption coefficient of 156 mM^{-1} cm^{-1} and expressed as μmol g^{-1} fresh mass (FM).

MDA content was measured by using thiobarbituric acid (TBA) as the reactive material following the method of Ghosh et al. (2021b). The absorbance was measured at 532 nm and 600 nm. The concentration of MDA was calculated by using the extinction coefficient of 156 mM^{-1} cm^{-1} and expressed as μmol of MDA g^{-1} fresh mass.

2.9 STATISTICAL ANALYSIS

All the experiments were conducted by following CRD (Completely Randomized Design) with four replications. Statistical analysis was performed using Statistix 10 software. The least significant difference (LSD) value at 5 % level of significance and student t-test were used for showing significant differences. The correlation coefficient matrix was visualized using the 'metan' package of R software.

3 RESULTS AND DISCUSSION

3.1 ACCUMULATION OF CHLOROPHYLL PIGMENTS VARIES WITH THE DEVELOPMENTAL STAGES OF PLANT PARTS IN *P. foetida*

Since chlorophylls (chl) as one of the important primary metabolites regulating ROS *in vitro* (Vaňková et al., 2018; Choi et al., 2016), we determined and compared chlorophylls contents in the plant parts of *P. foetida*. This study revealed that the young leaf showed the highest chl content than other parts. *The young leaf, matured leaf, young stem, and matured stem contained 0.62 mg* g^{-1}*, 0.51 mg* g^{-1}*, 0.14 mg* g^{-1}*, and 0.11 mg* g^{-1} *chl a respectively* (Fig. 1a). In case of chl *b*, the young leaf, matured leaf, young stem, and matured stem contained 0.222 mg g^{-1}, 0.177 mg g^{-1}, 0.067 mg g^{-1}, and 0.059 mg g^{-1} chl *b* respectively where the young leaves had the highest chl *b* followed by the matured leaf, young stem, and matured stem (Fig. 1b). The total chl content in the young leaf, matured leaf, young stem, and matured stem was 0.964 mg g^{-1}, 0.784 mg g^{-1}, 0.240 mg g^{-1}, and 0.198 mg g^{-1} respectively. Likewise, in chl *a* and chl *b*, the young leaves had the highest chl content followed by the matured leaves, young stems, and matured stems (Fig. 1c). We also calculated the ratio of chl *a* and chl *b* (chl *a/b*) and found the ratio of 2.826:1, 3.064:1, 2.291:1, and 1.977:1 in young leaves, matured leaves, young stems, and matured stems respectively (Fig. 1d).

Along with being photosynthetic pigments, *chlorophylls* are naturally strong *antioxidants* acting as free radical scavengers (Nazarudin et al., 2022; Pérez-Gálvez et al., 2020). The previous investigation found that chlorophyll extract of *Sauropus androgynous* (L.) is vital to protect against the consequences of oxidative stress in rats (Suparmi et al., 2016), suggesting that the plant parts potentiated with high chlorophyll content should be essential for managing ROS in animals' ailments. The previous investigation reported that Chl content increased from tender leaf, peaked in photosynthetically matured leaf, and then further declined after getting its maximum (Czech et al., 2009). In our case, the highest chlorophyll content in fully expanded young leaf (YL) agreed with that finding (Fig. 1). The total chl content in the leaf of *P.*

foetida was found as 1.2643 ± 0.0396 mg g^{-1} FM in *P. foetida* (Nayak et al., 2015) which was consistent to the results of the present study (Fig. 1). The Chl *a*, chl *b,* and total chl contents of the leaves of *P. foetida* were also measured by Islam et al. (2018) and the results showed little variation than the present study (Fig. 1) which may be due to varied growing, developing, and climatic conditions. The fresh leaves of twenty-one medicinal plants comprising trees, shrubs, and herbs, were investigated for quantification of chlorophyll content which resulted in a wide variation in chl *a*, chl *b,* total chl content, and chl *a/b* ratio (Ghosh et al., 2018). Along with those findings, our observation on the variation of chlorophyll content in different plant parts of *P. foetida* (Fig. 1) suggests that chlorophyll content varies with the species, age, and developmental stages of the plant. The highest chlorophyll content in the young leaf rather than older matured leaf, and the young and matured stem indicates the potential parts of *P. foetida* having greater antioxidant potential.

3.2 VARIED ACCUMULATION OF CAROTENOIDS IN THE PLANT PARTS OF *P. foetida*

Since carotenoids are established as the crucial metabolites for having strong antioxidants (Pérez-Gálvez et al., 2020), we determined those in the plant parts. The carotenoid content was found the highest (0.43 mg g^{-1}) in the young leaves which significantly differed from the other parts. Matured leaves had the second-highest carotenoid content which was 0.28 mg g^{-1}. Carotenoid content of the young stem and matured stem were 0.16 mg g^{-1} and 0.17 mg g^{-1} respectively which were nearly similar and there was no significant difference between them. Roots had the lowest carotenoid content which was 0.058 mg g^{-1} (Fig. 2). The findings of the previous investigations showed higher carotenoids content in the leaf of *P. foetida* (Ghosh et al., 2021a; Islam et al., 2018) which makes an agreement to the present findings where higher carotenes content resulted in the leaves (Fig. 2). A little variation of

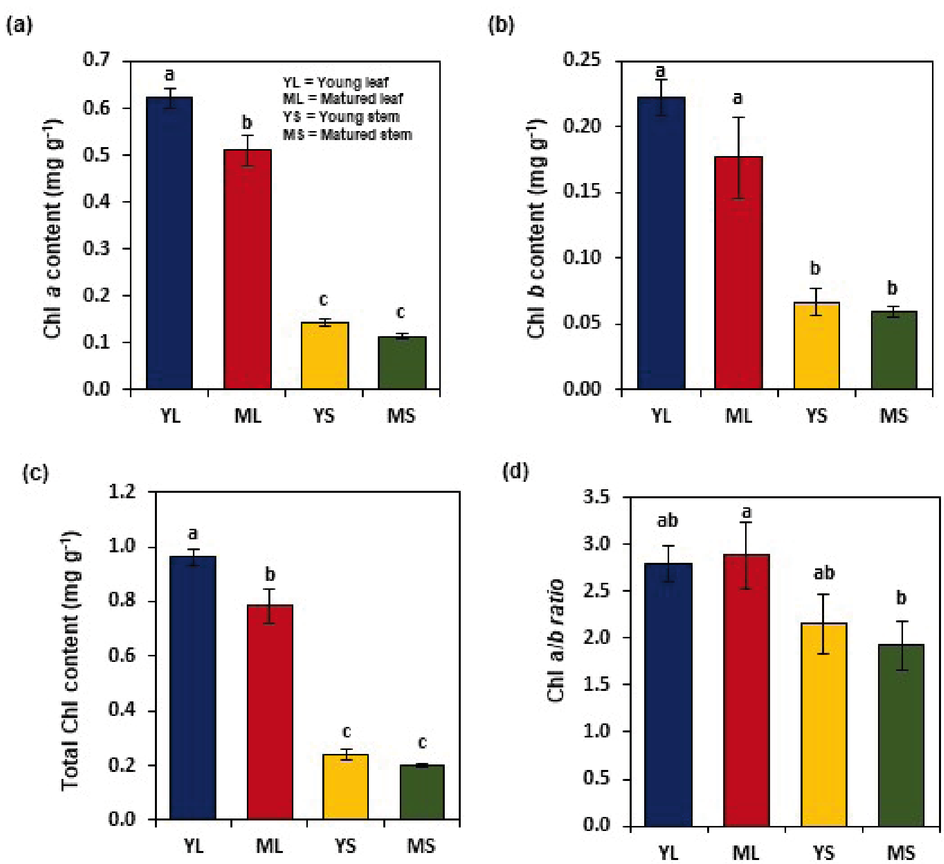

Figure 1: (a) Chl *a* content, (b) Chl *b* content, (c) Total chl content, and (d) Chl *a/b* ratio in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, YS, and MS denote young leaf, matured leaf, young stem, and matured stem respectively

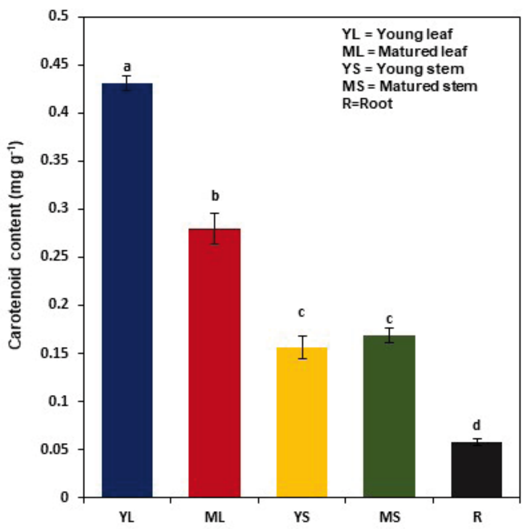

Figure 2: Carotenoid content in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, YS, MS, and R denote young leaf, matured leaf, young stem, matured stem, and root respectively

carotenoids content was reported by Nayak et al. (2015) which may be due to the difference in growing conditions, cultivar, and assay techniques. Carotenoid content was reported in the methanolic extracts of different plant parts of *Hypericum foliosum* Aiton including young leaves, old leaves, stem bark, stems, root, seed capsules, and flowers (Rainha et al., 2011). The study claimed that total carotenoids were found higher in stem and stem bark followed by leaf, root, and seed. In contrast, our study regarding various levels of carotenoids in different plant parts of *P. foetida* (Fig. 2) suggests that the plant is a good source of carotenoids where young leaves have greater efficacy than other plant parts.

3.3 THE PLANT PARTS OF *P. foetida* SHOW VARIATION IN THE ACCUMULATION OF POLYPHENOLS

As polyphenols possess very good antioxidative and pharmaceutical properties (Khan et al., 2021), we determined total phenolics, anthocyanins and flavonoids contents and compared those in the plant parts of *P. foetida*. The young leaves contained the highest amount of phenolics (728.243 µg g^{-1}) followed by the matured leaves (667.945 µg g^{-1}), young stems (651.748 µg g^{-1}), matured stems (589.455 µg g^{-1}), and roots (442.178 µg g^{-1}) (Fig. 3a). Phenolics has been reported to be very effective against cardiovascular diseases by means of having anti-inflammatory, antioxidants, and antiplatelet effects (Khan et al., 2021). A lot of investigations reported the presence of total phenolics in different medicinal plants (Sharma et al., 2022; Pandey and Sharma, 2022; Alfarrayeh et al., 2022; Khan et al., 2022;) and the results of which are consistent with our findings (Fig. 3a). Phenolics content in *P. foetida* was greatly affected by plants parts where leaf accumulated higher phenolics than that of stem and root (Ghosh et al., 2021a). The phenolic contents in this study showed little variation from the findings of others with *P. foetida* (Rosli et al., 2021; Ojha et al., 2018). The variation is due to the age and growing conditions of the plants. As the young leaf of *P. foetida* is very sensitive and more heavily exposed to stressful conditions than other plant parts, it accumulates higher phenolics for better protection against environmental stresses. Along with the findings of the above-mentioned studies, the result of the present study indicates that *P. foetida* is a good source of phenolic contents.

Along with phenolic contents, the anthocyanins content also varied in the plant parts of *P. foetida* in this study (Fig. 3b). The young leaves contained the highest amount of anthocyanins which was 53.99 µg g^{-1} followed by the young stems, matured leaves, matured stems, and roots which contained 39.02 µg g^{-1}, 26.48 µg g^{-1}, 22.09 µg g^{-1}, and 11.67 µg g^{-1} anthocyanins respectively. Anthocyanins are a family of natural pigments considered to be responsible for the color and taste of many fruits and vegetables (Zhang and Jing, 2022; Sunil and Shetty, 2022; Bocker and Silva, 2022). Anthocyanins found in different fruits were reported to have strong antioxidant and anti-inflammatory properties which could inhibit lipid peroxidation (Reis et al., 2016). Previous investigations in other medicinal plants resulted in the presence of this metabolite at various ranges (Sharma et al., 2022; Puzerytė et al., 2022; Joshi et al., 2017). As anthocyanins are a pigment molecule and related to sunlight, roots were found to have the lowest amount of anthocyanin (Fig. 3b). Very recent efforts on *P. foetida* suggested that as compared to roots both leaves and stems are good sources of anthocyanins (Ghosh et al., 2021a). Along with these, the highest level of anthocyanins in the young leaves (Fig. 3b) suggesting that the leaves of *P. foetida* are a good reservoir of anthocyanins.

The young leaves also showed significantly higher flavonoids content than other plant parts (Fig. 3c). There was no significant difference between the flavonoids content of matured stems and roots. The flavonoids contents in the young leaves, matured leaves, young stems, matured stems, and roots were 4178.053 µg g^{-1}, 3871.95 µg g^{-1}, 2662.075 µg g^{-1}, 1372.71 µg g^{-1}, and 1258.22 µg g^{-1} respectively (Fig. 3c). Due to photosynthesis in leaves,

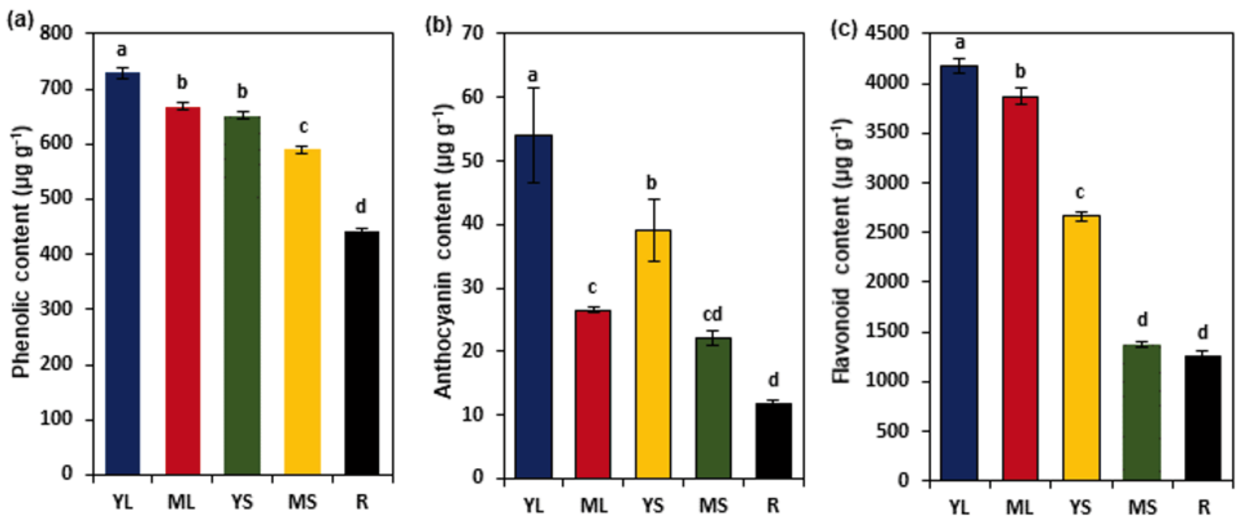

Figure 3: (a) Phenolics (b) anthocyanins, and (c) flavonoids contents in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, YS, MS, and R denote young leaf, matured leaf, young stem, matured stem, and root respectively

flavonoids biosynthetic pathway precursors are more abundant in leaves (Andersen and Markham, 2005). So, flavonoids content was higher in the leaves of *P. foetida* than in other plant parts. Flavonoids content was also reported in *P. foetida* in several studies (Ghosh et al., 2021a, Rosli et al., 2021; Karmakar et al., 2020). Along with those, our findings regarding higher flavonoids content in both young and matured leaves (Fig. 3c) support that the leaves irrespective of whether young or matured are the best source of flavonoids in *P. foetida*.

3.4 ACTIVITY OF ENZYMATIC ANTIOXIDANTS IN THE PLANT PARTS OF *P. foetida*

Enzymatic antioxidants play a crucial role in mitigating the negative impacts of free radicals in cellular and metabolic processes (Hasanuzzaman et al., 2020), thus we determined the activity of CAT, GST, APX, and POD in different plant parts of *P. foetida*. Since matured stems and roots showed lower performances in non-antioxidant activity (Figs. 2 & 3), we focused on the young leaves, matured leaves, and young stems for the determination of enzymatic antioxidant activity.

It was found that the matured leaves showed the highest CAT activity followed by the young leaves and young stems (Fig. 4a). The young leaves, matured leaves, and young stems had the catalase activity of 189.420 µmol min^{-1} mg^{-1}, 409.852 µmol min^{-1} mg^{-1}, and 96.910 µmol min^{-1} mg^{-1} protein respectively. In contrast to CAT activity, APX activity was found to be the highest in the young leaves (Fig. 4b). The young leaves, matured leaves, and young stems showed APX activity as 13.58 µmol min^{-1} mg^{-1}, 8.51 µmol min^{-1} mg^{-1}, and 3.905 µmol min^{-1} mg^{-1} protein respectively (Fig. 4b). The activity of POD was found to be the highest in matured leaves followed by the young stems, and young leaves (Fig. 4c). Young leaves, matured leaves, and young stems showed POD activity as 1.3 nmol min^{-1} mg^{-1}, 3.5 nmol min^{-1} mg^{-1}, and 2.5 nmol min^{-1} mg^{-1} protein respectively. On the other hand, the young leaves showed the highest Glutathione S-transferase (GST) activity than the other parts (Fig. 4d). The young leaves, matured leaves, and young stems had the GST activity of 3039.697 nmol min^{-1} mg^{-1}, 774.568 nmol min^{-1} mg^{-1}, and 167 nmol min^{-1} mg^{-1} protein respectively.

In the enzymatic defense system, catalase (CAT) is very ubiquitous to all living organisms which catalyzes the decomposition of hydrogen peroxide into water and oxygen (Vitolo, 2021). The enzyme is very crucial for defending cells against oxidative damage. The enhanced activity of SOD and CAT during oxidative stress in rat by the exogenous application of aqueous root bark, stem bark and leaves extracts of *Vitex doniana (Adetoro et al., 2013) suggesting the potentiality of plant extracts in mitigating oxidative stress in animals.* Nayak et al. (2015) found no induction of CAT activity in the leaves of *P. foetida*, the result of which was incompatible with our findings where CAT activity was greatly induced in all the plant parts (Fig. 4a). This may be due to the difference in growing conditions, cultivar, and assay techniques. Our study was supported by several investigations where CAT activity was sufficiently reported in medicinal plants (Güneş et al., 2019; Kumar et al., 2012). Along with that, the higher CAT accumulation in the matured leaves of *P. foetida* (Fig. 4a) rather than that of the younger leaves suggesting the activity of CAT varies with the developmental phases of plants. However, the presence of a higher level of CAT in both young and matured leaves suggests that the leaves of *P. foetida* might be a potential source of exogenous catalase. The key member of the ascorbate reduced glutathione (ASA-GSH) cycle, APX was reported to protect chloroplasts and other cell constituents from damage caused by hydrogen peroxide and hydroxyl radicals (Asada, 1992). Though APX activity was reported in medicinal plants (Güneş et al., 2019; Kumar et al., 2012), no previous study regarding the determination of APX activity was made in *P. foetida*. However, a higher level of APX accumulation in *P. foetida* supporting the potential source of this enzymatic antioxidant where the young leaves showed better potential than others (Fig. 4b). Alongside, POD which represents a family of isoenzymes is actively involved in oxidizing ROS (Khan et al., 2014). Though the incidence of POD activity was reported in the medicinal plant species by previous investigation (Güneş et al., 2019), the natural occurrence of POD in *P. foetida* was not reported so far. The variation of POD activity in different plant parts of *P. foetida* implies that the plant is a very good source of exogenous POD, where matured leaves showed better potential than other plant parts. Along with the above-mentioned antioxidants, GST is another key antioxidant enzyme that can quench reactive molecules with the addition of glutathione (GSH) and protect the cell from oxidative damage (Kumar and Trivedi, 2018). Although enzymatic antioxidant GST has been reported in the stress acclimation of land plants (Horváth et al., 2015; Labrou et al., 2015), the natural occurrence of GST was unexplored in medicinal plants. However, GST accumulation in the plant parts

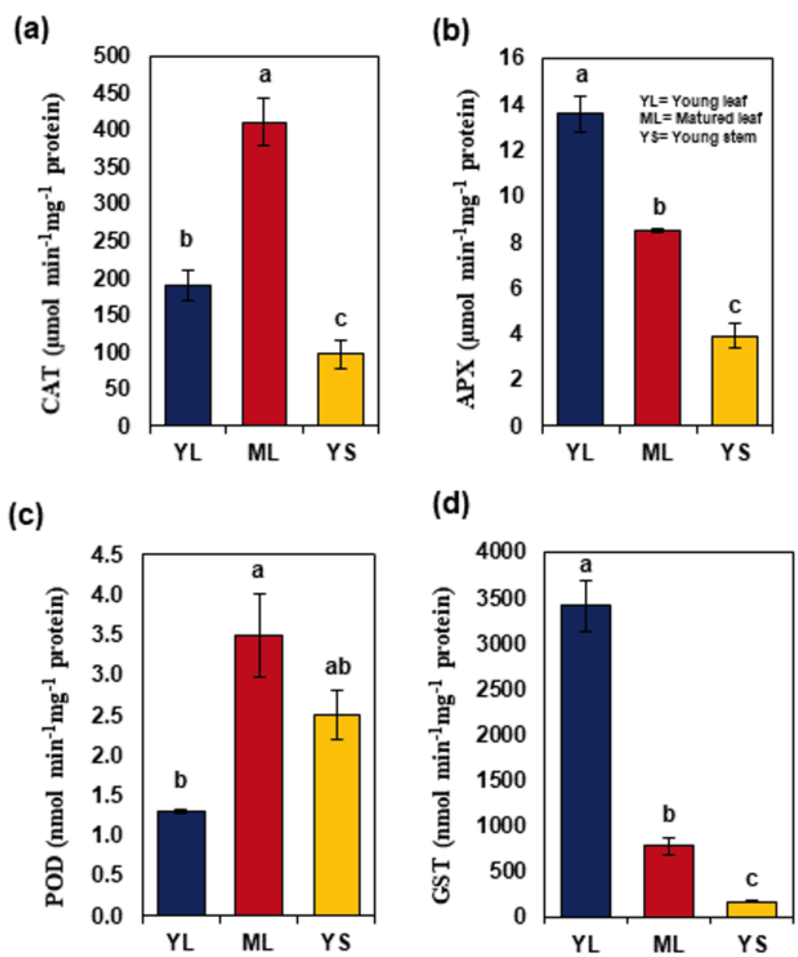

Figure 4: (a) Catalase, (b) ascorbate peroxidase, (c) peroxidase, and (d) glutathione S-transferase in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, and YS denote young leaf, matured leaf, and young stem respectively

of *P. foetida* suggesting the potent natural source of GST where young leaves showed better potential than others.

3.5 TOTAL ANTIOXIDANT ACTIVITY (% DPPH SCAVENGING ACTIVITY) IN THE PLANT PARTS OF *P. foetida*

The antioxidant activity in terms of % DPPH scavenging activity in the plant parts of *P. foetida* was recorded as 84.82 %, 79.78 %, 80.24 %, 48.18 %, and 23.37 % respectively (Fig. 5). So, we could see that the younger portion of the plant; young leaves and stems were very rich in antioxidant activity. According to Sahoo and Bhatnagar (2015), a significant antioxidant activity of 84-85 % was reported in *P. foetida*, the results of which agreed with the results of the present study where both leaves and stems showed about 80 % DPPH scavenging activity (Fig. 5). Similar observation was recorded by another effort in *P. foetida* (Upadhya, 2013). In contrast to fresh leaves, the shade-dried leaves of *P. foetida* exhibited a dose-dependent DPPH free radical scavenging manner, where about 60 % inhibition was recorded by 500 mg ml^{-1} *P. foetida* extract (Uddin et al., 2014). Rutnakornpituk and Boonlue (2013) recorded 74.72 % DPPH scavenging activity in ethyl acetate crude extract of *P. foetida* and the results of which were consistent with the present study. Along with those, in our observation, although no significant differences were found among the young leaves, matured leaves and young stems, the young leaves showed the highest level of total antioxidant activity by means of % DPPH scavenging ability (Fig. 5) and data of which were consistent to the increased levels of non-enzymatic and enzymatic antioxidants in young leaves (Figs. 1, 2, 3, 4).

3.6 PROLINE CONTENT IN THE PLANT PARTS OF *P. foetida*

Since osmolyte proline acts as an antioxidant and has been found to directly react with ROS (Kaul et al., 2008, Sharma and Dietz, 2009), we determined proline accumulation in the plant parts of *P. foetida*. The young leaves contained the proline content of 1.465 µmol g^{-1} followed by the young stems (1.105 µmol g^{-1}) and matured leaves (0.974 µmol g^{-1}) (Fig. 6). Osmolyte proline is frequently employed as a non-enzymatic antioxidant to combat the negative effects of various ROS and attributed as an efficient scavenger of hydroxyl radicals and singlet oxygen (Naliwajski and Skłodowska, 2021). However, proline could act as an antioxidant and be involved in the protection of oxidative damage in a wide array of organisms including fungi, plants, and animals (Krishnan

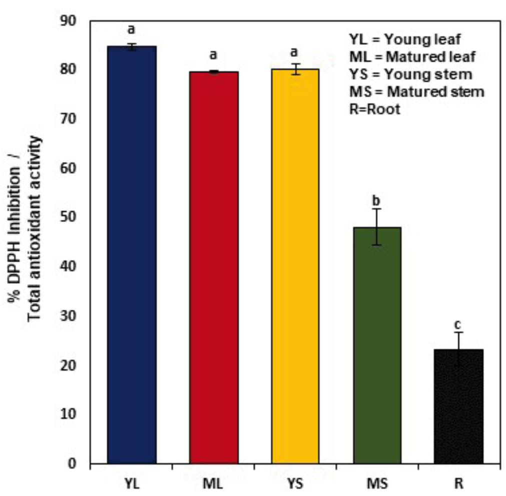

Figure 5: Total antioxidant activity or % DPPH scavenging activity in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, YS, MS, and R denote young leaf, matured leaf, young stem, matured stem, and root respectively

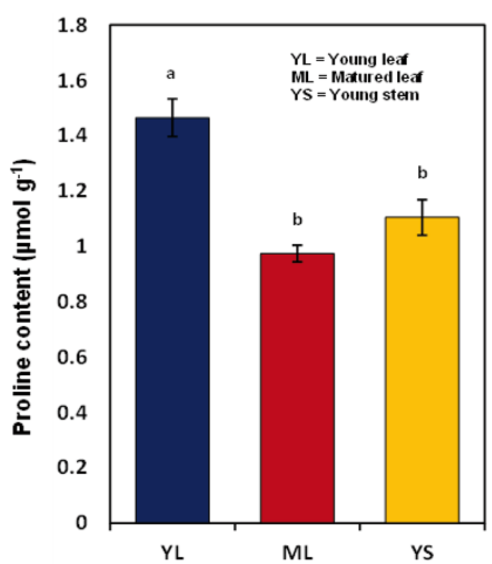

Figure 6: Proline content in different parts of *P. foetida.* Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, and YS denote young leaf, matured leaf, and young stem respectively

et al., 2008, Chen et al., 2005). Additionally, proline was shown to be reported in the protection of human skin cells from photo-oxidative stress suggesting that proline is essential for human ailments (Wondrak et al., 2005). Although proline accumulation in non-stressed conditions was not emphasized more in medicinal plants, a very recent study showed a higher amount of proline accumulation in vulnerable and threatened medicinal plants *Blepharis sindica* T. Anders (Lal et al., 2021). In our study, the highest proline accumulation was found in young leaves rather than matured leaves and stems under non-stressed conditions. The variation of accumulation is due to the various role of proline in the plant's ontogenic process (Kishor et al., 2015). As proline is crucial for living organisms including plants and animals, the plant parts having greater proline accumulation would be good reservoir for herbal drugs.

3.7 H_2O_2 AND MDA CONTENT

To compare the occurrence of ROS in young and matured leaves, we measured H_2O_2 and lipid peroxidation product MDA in those plant parts. The matured leaves had higher H_2O_2 and MDA contents (28.75 µmol g^{-1} FM and 28.60 µmol g^{-1} FM respectively) than in young leaves (27.66 µmol g^{-1} FM and 23.51 µmol g^{-1} FM respectively) (Fig. 7). Though there was no significant difference in H_2O_2 content of young and matured leaves, they show significant difference in case of MDA content at 5 % level of significance. ROS is naturally produced in the plant's body and at lower concentrations acts as signalling molecules in response to growth, development and stress responses, whereas appears as detrimental at higher concentrations (Huang et al., 2019). H_2O_2 is one of the most important members of ROS and enhanced accumulation of which causes lipid peroxidation and membrane injury in plants (Sachdev et al., 2021; Hemantaranjan et al., 2014). MDA is an indicator of lipid peroxidation and oxidative stress which causes membrane leakage (Nahar et al., 2022; Tsikas, 2017). Along with plants, oxidative stress is linked with the occurrence of many human diseases including cancer, brain misfunctioning, diabetes, heart disease, etc. (Law et al., 2017). Therefore, plant parts having a lower occurrence of ROS might be suitable for health concerning issues. In our study, the higher accumulation of H_2O_2 in matured leaves than in young leaves was consistent with the elevated level of MDA in matured leaves (Fig. 7).

3.8 CORRELATION ANALYSIS

The relationship between antioxidative parameters (chlorophyll *a*, chlorophyll *b*, total chlorophylls, chlorophyll *a/b* ratio, carotenoids, anthocyanins, phenolics, flavonoids, DPPH scavenging activity, proline, CAT, GST, APX, POD) was determined through the values of the correlation coefficient where positive values were indicated as red and negative values as blue. The relationship ranged from -1 to 1, whereby -1 means a perfect negative and 1 means a perfect positive linear relationship between variables and 0 indicated no relationship between studied variables (Fig. 8). The results indicated a signifi-

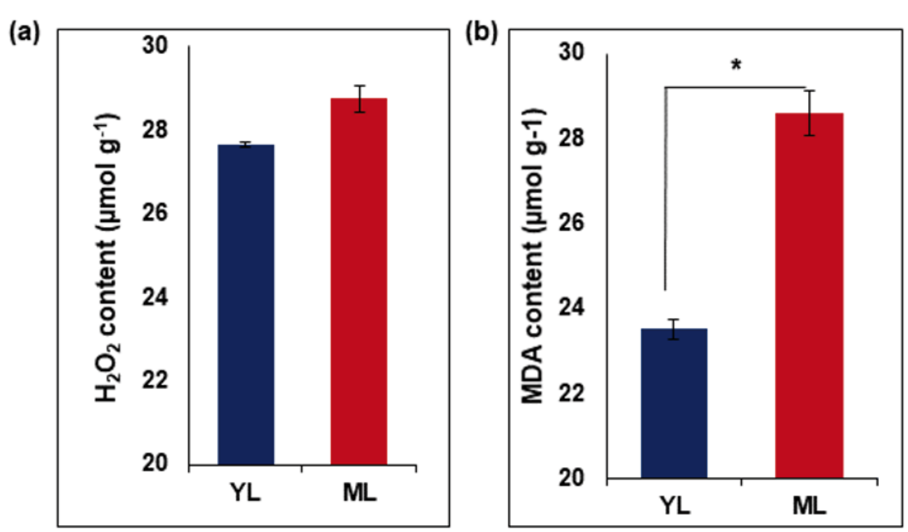

Figure 7: (a) H_2O_2 content and (b) MDA content in young and matured leaves of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Aster mark indicates significant difference between the treatments ($p < 0.05$). YL and ML denote young leaves and matured leaves respectively

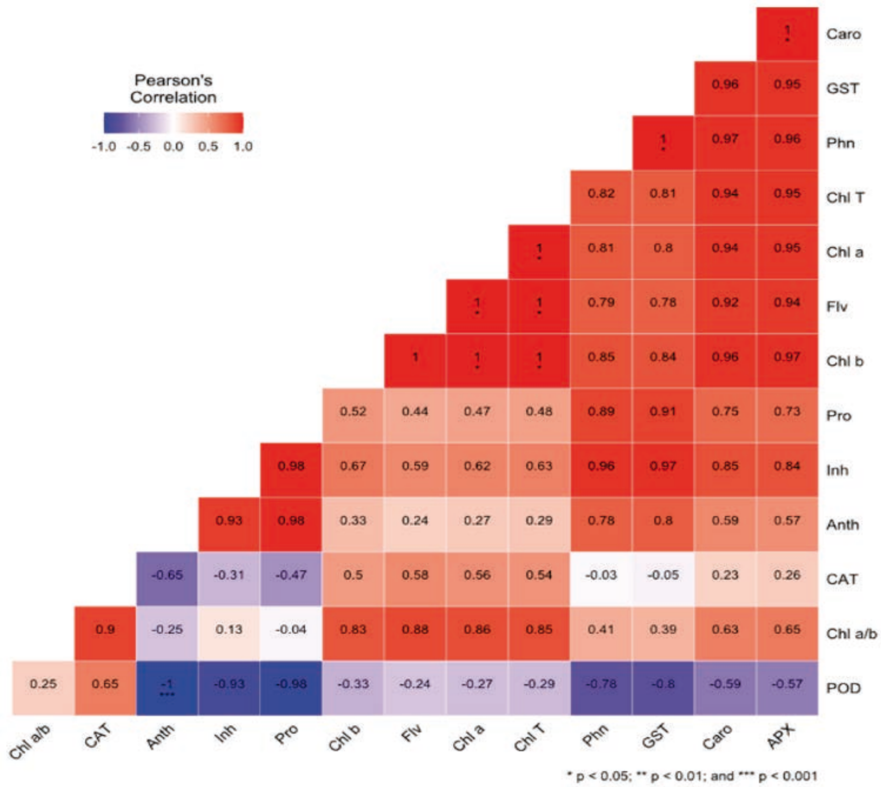

Figure 8. Correlation analysis for showing relationship between antioxidative parameters. The parameters included Chl *a* (chlorophyll *a*), Chl *b* (chlorophyll *b*), Chl T (total chlorophylls), Chl *a*/*b* (chlorophyll *a*/*b* ratio), Caro (carotenoids), Anth (anthocyanins), Phn (phenolics), Flv (flavonoids), Inh (% inhibition of DPPH; Total antioxidant activity), Pro (proline), CAT (catalase), GST (glutathione S-transferase), APX (ascorbate peroxidase), and POD (Peroxidase). The positive values are in red, and the negative values are in blue. It ranges from -1 to 1, whereby -1 means a perfect negative and 1 means a perfect positive linear relationship between variables and 0 indicates no relationship between studied variables

cant positive correlation between various antioxidative parameters. Among all phytochemical parameters, chl *a* chl *b*, total chl, and flavonoids were positively and highly correlated ($r = 1$) at 5 % level of significance. Carotenoids content was positively and highly correlated with APX ($r = 1$) and negatively correlated with POD ($r = -0.59$). GST and phenolics were also highly correlated ($r = 1$) at 5 % significance level. CAT was almost positively correlated with APX and POD and negatively with GST. Anthocyanins content was negatively correlated with POD and CAT. Carotenoids, APX, flavonoids, chlorophylls, phenolics and total antioxidants were positively correlated with each other. Although some paraments showed a negative correlation with each other, most of them maintained a positive correlation with total antioxidant activity in terms of % DPPH scavenging activity. Our findings are consistent with the findings of others where Chl *a* and Chl *b* contents of stem amaranth made a positive correlation with total antioxidant activity (Sarker et al., 2020). Likewise, phenolic compounds in plants made a positive relationship with the antioxidant activity of the tissue (Doğan et al., 2014; Güne et al., 2019). In our observation, positive correlation among most of the parameters (Fig. 8) suggesting that both enzymatic and non-enzymatic antioxidants in the plant parts of *P. foetida* contribute synergistically for boosting up the total antioxidant activity in the plant.

4 CONCLUSIONS

According to the present study, young leaves showed the best potential for non-enzymatic antioxidants like chlorophylls, carotenoids, anthocyanins, phenolics, flavonoids, and proline. Among enzymatic antioxidants, GST and APX activity was found to be the highest in young leaves whereas CAT and POD activity were superior in matured leaves. Although there were no significant differences, the total antioxidant activity in terms of % DPPH scavenging activity was found the highest in young leaves followed by young stems and matured leaves. Based on overall observation, it can be concluded that the medicinal plant *P. foetida* is a very good source of both enzymatic and non-enzymatic antioxidants. The young leaf of the plant might be a suitable option for the preparation of the natural herbal drug. However, further *in vitro* and *in vivo* studies with animal models are required to see the efficacy of the plant parts as potential human drugs.

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Biodecolorization of azo dye Acid Blue 92 (AB92) by *Ceratophyllum demersum* L.: process optimization using Taguchi method and toxicity assessment

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Biodecolorization of azo dye Acid Blue 92 (AB92) by *Ceratophyllum demersum* L.: process optimization using Taguchi method and toxicity assessment

Abstract: This study evaluated the ability of the submerged aquatic plant *Ceratophyllum demersum* to remove the Acid Blue 92 (AB92) dye. The effect of some operational parameters such as the reaction time, initial dye concentration, initial plant biomass, and pH, on dye removal efficiency was studied. Based on Taguchi's results, the optimized conditions for dye removal were time 7 days, dye concentration 20 mg l^{-1}, initial plant biomass 4 g, and initial pH 5. Fourier-transform infrared spectroscopy (FTIR) results confirmed the interaction between dye molecules and plants. Based on the results of this study, *C. demersum* had a reusability to remove the dye, this fact confirming the mechanism of biodegradation in the dye removal process. Also, the effect of AB92 on the physiological responses of *C. demersum* was investigated. Minimum relative growth rate, tolerance index, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids at a concentration of 20 mg l^{-1} of AB92 were observed. The concentration of cyanidin glycoside, lipid peroxidation, and antioxidant activity increased in both concentrations of 10 and 20 mg l^{-1}. It can be concluded that both concentrations of AB92 induced antioxidant activity and the risk of oxidative stress for *Ceratophyllum*.

Key words: azo dye; Acid Blue 92; bioremediation; *Ceratophyllum demersum*; oxidative stress

Biorazbarvanje azo barvila Acid Blue 92 (AB92) z navadnim rogolistom (*Ceratophyllum demersum* L.): optimizacija Taguchijeve metode in ocena strupenosti

Izvleček: V raziskavi je bila ovrednotena sposobnost navadnega rogolista (*Ceratophyllum demersum* L.) za odstranjevanje barvila Acid Blue 92 (AB92). Preučevan je bil učinek parametrov kot so reakcijski čas, začetna koncentracija barvila, začetna biomasa rastline in pH na odstranjevanje barvila iz vode. Na osnovi Taguchijeve metode so bili najboljši pogoji za odstranitev barvila 7 dni pri koncentraciji barvila 20 mg l^{-1}, začetni masi rastlin 4 g in začetnem pH 5. Fourierjeva transformacijska infrardeča spektroskopija (FTIR) je potrdila interakcijo med molekulami barvila in rastlino. Na osnovi te raziskave je bilo ugotovljeno, da ima navadni rogolist sposobnost biodegradacije pri odstranitvi barvila iz vode. Preučevan je bil tudi učinek barvila na fiziološke procese v rogolistu. Pri koncentraciji 20 mg l^{-1} barvila so bile opažene minimalne vrednosti relativne prirasti, tolerančnega indeksa, vsebnosti klorofila a, klorofila b, celokupnega klorofila in celokupnih karotenoidov. Koncentracija cianidin glikozida, peroksidacija maščob in antioksidacijska aktivnost so se povečale pri obeh koncentracijah barvila,10 in 20 mg l^{-1}. Ugotovljeno je bilo tudi, da sta obe koncentraciji barvila vzpodbudili antioksidacijsko aktivnost in, da sta predstavljali nevarnost za oksidacijski stres v rogolistu.

Ključne besede: azo barvilo; Acid Blue 92; bioremediacija; *Ceratophyllum demersum*; oksidacijski stres

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1 INTRODUCTION

Discharge of colored effluents into rivers and lakes leads to reduced water quality, reduced oxygen transfer to water, and decreased solubility of gases (Pillai et al., 2014; Varjani et al., 2021). Dyes used in the textile industry are macromolecules not completely decomposed by conventional wastewater treatment processes due to their structure and nature. The dye "AB92" is from the group of mono-azo dyes and is in the category of anionic dyes. One of the largest and oldest classes of industrial dyes is Azo dyes, which contain about 70 % of textile dyes (Lang et al., 2013). Azo dyes have numerous desirable factors, making them widely useful not only for dyeing textiles and leather but also for application in new technologies. These factors include easy production, high molar absorption coefficient, good stability against light and moisture, and a wide range of colors (Singh & Arora, 2011). Phytoremediation, a term for natural technologies based on using plants to purify the environment and the final refining step after the initial treatments, is highly considered today. It is a relatively new technology that, in addition to being environmentally affable is considered economical, suitable, and particular (Bhat et al., 2022). Aquatic plants are more efficient in phytoremediation than terrestrial plants due to faster growth, higher biomass production, and a higher ability to absorb contaminants. They are also more effective in purification due to direct contact with contamination (Phillips et al., 2015; Sharma et al., 2015). The aquatic plant *C. demersum* has been introduced as a high-efficiency plant species for phytoremediation (Gałczyńska et al., 2019). The genus *Ceratophyllum* is globally distributed and is one of the most important and predominant aquatic plants in rivers and wetlands in Iran (Chorom et al., 2012; Mohan et al., 2017). Physical traits of this plant such as thin cuticle, specific leaf structure, and a lack of roots, facilitate the uptake of xenobiotics through the large surface of this plant without dependence on the root-to-stem transfer system (Rezania et al., 2016). Although *Ceratophyllum* has been very successful in the bioremediation of heavy metals (Krems et al., 2013; Nabi, 2021; Qadri et al., 2022), research on its ability to purify organic contaminants such as synthetic dyes and its effect on the physiological parameters of this plant is very limited. In previous studies, the biomass potential of this plant in bioremediation of synthetic dyes, Basic Blue 41 and Methylene Blue was observed at 94 % (Keskinkan & Lugal Göksu, 2007) and 96 % (Ewadh, 2020) respectively. In the present study, the ability of the submerged aquatic plant *Ceratophyllum demersum* to remove the monoazo dye (AB92) from polluted water has been investigated. To optimize the biological removal process, the effect of different conditions such as test time, the fresh mass of the plant, initial concentration of the dye, and pH was investigated at four levels simultaneously by the Taguchi test. Using the Taguchi test, all the existing interactions between different factors were investigated and finally, the most effective level of each factor and the most effective factor in the biorefining of AB92 by this plant were identified. By using FTIR analysis, the possible interaction between the dye and the functional groups of the plant was identified. Also, the effect of dye on some physiological variables including the content of relative growth rate (RGR), tolerance index (TI), photosynthetic pigments, the content of non-enzymatic antioxidants including carotenoid concentration and anthocyanidin glycoside concentration, free radical scavenging capacity (DPPH) and amount malondialdehyde (MDA) production was investigated.

2 MATERIALS AND METHODS

2.1 PLANT CULTİVATİON AND TREATMENT

Ceratophyllum aquatic plant was collected from Sustan wetland in Lahijan ("N, 50 ° 0'14" E, 37 ° 12' 26) and transferred to the laboratory. Samples were washed and disinfected with 0.5 % NaClO solution and transferred to plastic containers containing 10 % Hoagland medium (Hoagland & Arnon, 1950). Plastic containers were transferred to the culture chamber for better growth and were placed in basic conditions with a temperature of 25 ± 2 °C and a light-dark period of 16/8 hours. Treatment was done after one week (Movafeghi et al., 2016).

2.2 DYE ANALYSİS

Industrial dye AB92 [Mono azo, Anionic; C.I. number: 13390; Molecular Formula: $C_{26}H_{16}N_3Na_3O_{10}S_3$; Mw (g mol^{-1}): 698.58] was purchased from Alvan Sabet Company (Iran). The absorption spectrum of the dye was measured at wavelengths of 200 to 800 nm with a spectrophotometer (CamSpec M501 Single Beam UV/Visible, United Kingdom). AB92 dye has a maximum absorption at a wavelength of 571 nm. Different concentrations of dye were prepared, their adsorption was measured at maximum wavelength, and a calibration diagram was drawn. The amount of dye removal was calculated after the treatment period using Eq. (1). In bioremediation experiments for each treatment, a negative control (without plant) was considered to calculate the adsorption of dye to the wall of the test vessel and the effect of non-biological factors (physical-chemical) on dye removal. Finally, the percentage of net removal was calculated from the difference between the amount of removal in the presence of the plant and the conditions without the plant (Eq. (1)).

$$\text{Dye removal} = \frac{C0 - Cn}{C0} \times 100 \quad (1)$$

Cn: the final concentration of dye,
C0: initial concentration.

2.3 ORTHOGONAL ARRAY

Signal-to-noise ratio analysis was used to detect and obtain the optimal conditions for the experiment. The best level for each factor was introduced after performing this analysis. Signal refers to factors that can be controllable by the user, and noise refers to uncontrollable factors. S / N ratio analysis identifies the conditions in which the S / N ratio is the highest as the optimum condition (Silver, 1991). The signal-to-noise ratio was calculated based on Eq. (2).

$$\frac{S}{N} = \frac{-10\log\left(\frac{1}{Y_1^2}+\frac{1}{Y_2^2}+\frac{1}{Y_3^2}+\cdots+\frac{1}{Y_n^2}\right)}{n} \quad (2)$$

S: signal, N: noise, n: the number of experiments, Y: the result of each experiment

In this study, the effect of four parameters including time, plant biomass, initial dye concentration, and pH were investigated in the removal at four levels. The result of designing the experiment by the Taguchi method was a table with 16 experiments (L_{16}). The conditions of each experiment are shown in Table 1. All experiments were repeated three times. Other environmental conditions, including temperature (25 °C) and solution volume of samples (1 l), were considered constant factors.

2.4 INVESTİGATİON OF REUSABİLİTY OF *C. DEMERSUM* TO REMOVE DYE AB92

4 g of *C. demersum* were exposed to concentrations of 10 and 20 mg l^{-1} of AB92 and the percentage of dye removal was measured for 4 weeks. The culture medium containing the dye was changed weekly.

2.5 FTIR ANALYSIS

FTIR spectroscopy was used to investigate the interaction of acid blue dye 92 with *C. demersum*. For this purpose, 4 g of *C. demersum* was exposed to a dye solution at a concentration of 20 mg l^{-1} for 7 days. First, fresh plant tissue was homogenized with 3 ml of 2-propanol and 7 ml of diethyl ether. The reaction mixture was filtered, and distilled water was added and shaken for 20 seconds. After the separation of the two phases, the organic phase was collected. After evaporation of the solvent, samples were collected and used in FTIR spectroscopic analysis. All preparation steps for FTIR analysis were done for the control sample (dye concentration = 0 mg l^{-1}, fresh mass = 4 g, time = 7 days).

Table 1: Parameters and their values corresponding to their levels were studied in Experiments

	Level			
Parameter	1	2	3	4
A. Time (day)	1	3	5	7
B. Concentration (mg l^{-1})	5	10	15	20
C. Biomass (g)	0.5	1	2	4
D. pH	2.5	5	7	9.5

2.6 GROWTH ASSESSMENT

To evaluate plant growth rate, relative growth rate (RGR) was used based on plant fresh mass. The fresh mass of the plant was weighed after 20 days of treatment of plants with two concentrations of AB92 (10 and 20 mg l^{-1}), and the final mass of the samples was recorded, and the RGR was calculated using Eq. (3) (Radić et al., 2010).

$$\text{RGR (day} - 1) = [Ln(final\ mass) - Ln\ (initial\ mass)]\ /\ day \quad (3)$$

The tolerance index was calculated based on changes in relative growth rate in the presence of AB92 compared to control conditions by Eq. (4) (Forni et al., 2001).

$$\text{Tolerance index } (TI) = \frac{\text{RGR of treatment}}{\text{RGR of control}} \quad (4)$$

2.7 MEASUREMENT OF PHOTOSYNTHETIC PIGMENTS

The plant samples were treated with 0 (as control), 10, and 20 mg l^{-1} of AB92, and the quantity of some physiological parameters were measured after 7 days.

To measure the content of photosynthetic pigments, 500 mg of fresh plant tissue was homogenized in 80 % acetone. The samples were kept in the dark for 24 hours at a temperature of 4 °C and then centrifuged for 10 minutes at 4500 rpm. The absorbance at 470, 645, and 662 nm was read by the spectrometer. The amounts of photosynthetic pigments were determined based on the method of Lichtenthaler (1987) and were reported in μg g^{-1} FM.

2.8 MEASUREMENT OF MEMBRANE LİPİD PEROXİDATİON

The amount of peroxidation of membrane lipids was measured based on the concentration of malondialdehyde (MDA). 500 mg of fresh plant tissue was homogenized with 1 % trichloroacetic acid (TCA). The obtained extract was centrifuged at 4500 rpm for 5 minutes. Then 20 % TCA solution containing 0.5 % thiobarbituric acid (TBA) was added. The reaction mixture was first heated in a water bath at 100 °C for 30 minutes and then immediately cooled on ice and centrifuged at 4500 rpm for 7 minutes. The absorbance of the sample (MDA + TBA) was read at 532 nm. To calculate the concentration of MDA, the extinction coefficient of 155 $\mu mmol^{-1}$ cm^{-1} was used and finally, the amount of MDA was calculated using the following formula (5) and expressed as $nmolg^{-1}$ FM (Heath & Packer, 1968).

$$MDA\ (nmol / g\ FM) = A / \varepsilon B \qquad \text{Eq. (5)}$$

$$A = A600 - A532$$

A600: absorption of non-specific aldehydes at 600 nm, A532: absorption at 532 nm, B: cuvette width (1 cm), ε: extinction coefficient (155 $\mu mmol^{-1}$ cm^{-1}).

2.9 CYANİDİN GLYCOSİDE ASSAY

500 mg of the fresh mass of the plant was homogenized in acidic methanol (including methanol and hydrochloric acid in a ratio of 99 : 1). After that, the resulting extract was centrifuged for 15 minutes at 10,000 rpm, and the absorbance was read at 550 nm. The concentration of cyanidin glycoside was calculated using the extinction coefficient of 33,000 mol^{-1} cm^{-1} and was reported in μmol g^{-1} FM (Wagner, 1979).

2.10 MEASURİNG THE FREE RADİCAL SCAVENGİNG ABİLİTY

The free radical scavenging ability was measured based on the electron-donating ability of the extract to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical (Sampath & Vasanthi, 2013). 100 mg of plant sample were homogenized in 80% methanol and then centrifuged at 10,000 g for 10 min. The reaction mixture included 3 ml of methanolic extract and 1 ml of DPPH ethanolic solution (0.1 **μM**). After placing the samples in the dark for 30 minutes, the absorbance of DPPH ethanolic solution without plant extract was measured at 517 nm as a control solution against 80 % methanol as a blank. Using the following equation, the percentage of DPPH free radical scavenging was calculated Eq. (6).

$$DPPHsc\ \% = ((A_0 - A_1)) / A_0 \times 100 \qquad \text{Eq. (6)}$$

A_0: absorbance of control, A_1: absorbance of sample.

2.11 STATİSTİCAL ANALYSİS

Minitab 15 software and the Taguchi test were used to design the experiments. The experiments were performed in a completely randomized design with three replications. Compare means were done at a 95 % confidence level using one-way ANOVA and Duncan test. Deviation from the mean of the data was indicated by the standard error (SE). SPSS software (version 21) was used for statistical analysis of data, and Microsoft Excel software (2016) was used to draw graphs.

3 RESULTS AND DISCUSSION

3.1 BIODECOLORIZATION OF AB92

Bioremoval of AB92 in the aquatic media via *C. demersum* was investigated. Analysis of plant remediation was carried out on the four factors including time, initial concentration of dye, plant biomass weight, and pH. The results of decolorization obtained from 16 treatments under the influence of the four factors obtained from the Taguchi test are presented in Table 2. The highest amount of refinement was obtained in experiment 13 with an average of 58.83 % and the signal/noise rate was 35.39.

Based on the results of quality analysis, the highest dye removal efficiency was obtained at level 4 of treatment time (7 days), plant biomass (4 g), level 4 of initial concentration of dye (20 mg l^{-1}), and level 2 pH (pH = 5) (Fig. 1).

The importance of the variables in the AB92 dye decolorization process by *C. demersum* has been shown in Table 3. The results based on S/N showed "time" as the most effective factor and "pH" as the least influential factor in this process.

C. demersum showed a significant ability to remove AB92 dye from the culture medium, which was proven by successive experiments. With increasing treatment

Table 2: Experimental layout using the L16 orthogonal array and experimental results for percent of dye removal

Experimental number	A	B	C	D	Dye removal (%)				
					1	2	3	Mean	S / N
1	1	1	1	1	9.7	9.23	8.82	9.25	19.30
2	1	2	2	2	12.73	11.97	12.46	12.38	21.85
3	1	3	3	3	13.75	14.06	13.76	13.85	22.83
4	1	4	4	4	15.83	14.65	15.36	15.28	23.66
5	2	1	2	3	23.36	23.97	24.13	23.82	27.53
6	2	2	1	4	22.76	21.23	21.96	21.98	26.83
7	2	3	4	1	27.56	26.89	26.17	26.87	28.58
8	2	4	3	2	30.32	29.98	30.73	30.34	29.63
9	3	1	3	4	37.45	36.1	37.32	36.95	31.35
10	3	2	4	3	44.09	43.86	43.79	43.91	32.85
11	3	3	1	2	42.18	42.77	43.63	42.86	32.63
12	3	4	2	1	39.21	39.64	39.95	39.66	31.95
13	4	1	4	2	59.9	58.12	58.48	58.83	35.39
14	4	2	3	1	56.97	57.08	56.37	56.80	35.08
15	4	3	2	4	52.33	54.79	51.83	52.98	34.47
16	4	4	1	3	53.13	53.56	52.49	53.06	34.49

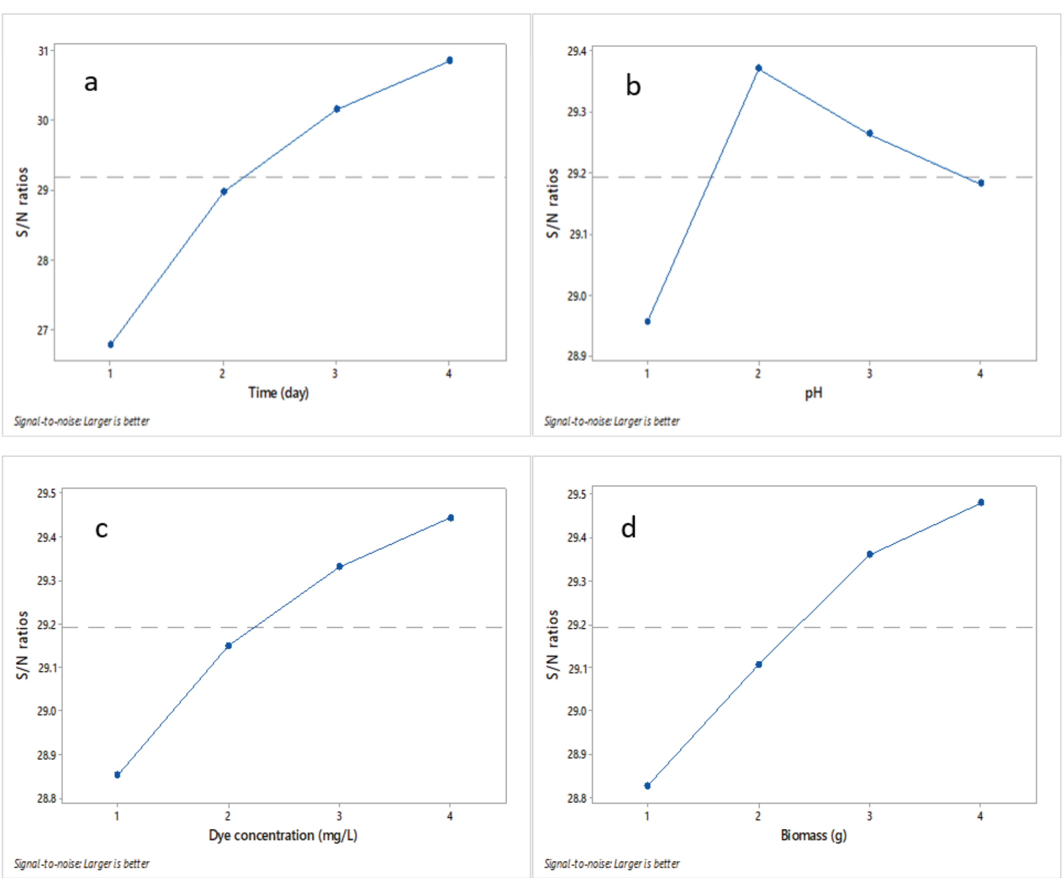

Figure 1: Effect of time (a), pH (b), dye concentration (c), and biomass (d) on dye removal

Table 3: Response to the Taguchi analysis of dye removal data

Parameter	Mean S / N ratio					
	Level 1	Level 2	Level 3	Level 4	Delta	Rank
A	21.91	28.15	32.2	34.86	12.95	1
B	28.4	29.16	29.63	29.94	1.54	3
C	28.32	28.95	29.73	30.12	1.81	2
D	28.73	29.88	29.43	29.08	1.15	4

time, the removal rate of AB92 dye increased significantly. The data obtained from the Taguchi test showed that the time factor had the highest effect on the decolorization process of AB92 dye by *C. demersum* compared to the other factors. Increasing the fresh mass of the plant by providing more surface to remove the dye increases the contact surface of the plant with the dye and consequently increases the efficiency of the process of adsorption and biodegradation of the dye. In submerged aquatic plants, stems play an important role in nutrient uptake; therefore, increasing the decolorization with increasing fresh mass seems logical. Increased decolorization efficiency due to increasing the mass of samples treated with dyes has been reported in previous studies (Daneshvar et al., 2007; Dhote & Dixit, 2009; Khataee et al., 2010). According to the results of the Taguchi test, with increasing the concentration of AB92 dye, its removal by *C. demersum* increased. It seems that two factors are involved in increasing the uptake of the dye by increasing the initial concentration. The first factor, increasing the concentration of the dye, provides the driving force needed to overcome the mass transfer resistance between the solid and liquid phases of the plant. In other words, higher concentrations facilitate the diffusion of the dye. Another factor is that increasing the concentration of the dye increases the likelihood of physical contact and collision between the molecules of the dye and the plant surface, and increases the number of available molecules of the dye at the plant binding site, resulting in increased decolorization (Aravindhan et al., 2007; Daneshvar et al., 2007). In the present study, among the 4 experimental pH ranges, the highest percentage of dye removal was determined at acidic pH of 5. The pH of the environment affects the rate of ion absorption by plants by controlling ionization and mobility. Many factors are involved in this, but the most important factor can be the molecular structure of the dye and the structure of the cell wall of plants. The pH of the environment also affects the solubility of the dye (Solís et al., 2012). AB92 dye is an anionic dye and most ionization occurs at acidic pH. On the other hand, most cell wall molecules have hydroxyl groups that are protonated at acidic pH, thus the electrostatic interactions of cell wall molecules with AB92 anion molecules increase at acidic pH (Ena et al., 2007). The ability of different organisms used in the purification of dyes following their sequential use is considered one of the most important factors in their selection for bioremediation (Ihsanullah et al., 2020).

3.2 INTERACTION BETWEEN PARAMETERS

The results of the interaction between the time factor and other factors (dye concentration, plant biomass, and pH) are shown in (Fig. 2 a-c). In all cases, the lowest mean of refinement (less than 20 %) was observed on the first day and the highest refinement (more than 50 %) was observed on the seventh day of treatment. The results of the interaction between the two factors of biomass and dye concentration showed an inverse ratio between these two factors, so the highest amount of refinement was observed at high concentrations of dye and low values of plant biomass (Fig. 2 - d).

3.3 REUSABILITY EXPERIMENTS

The results obtained from both concentrations of the dye indicated that this plant had an acceptable ability to purify the dye and its ability to refine in the fourth stage was better than in the first stage. Thus, the minimum percentage of purification related to the experiment on the seventh day of the first week, which was obtained at concentrations of 10 and 20 mg l^{-1}, was 51 % and 40 %, respectively. The maximum percentage of purification related to the seventh day of the fourth week at concentrations of 10 and 20 mg l^{-1} were 56 % and 42 %, respectively. In addition, during the decolorization process in the four stages, no morphological changes were observed in the plants due to the accumulation of the dye (Fig. 3).

C. demersum showed an acceptable ability to be reused to remove the dye AB92, which can confirm the occurrence of the biodegradation process in removing the pollutant and distinguish it from other processes, especially adsorption. Because in the adsorption process

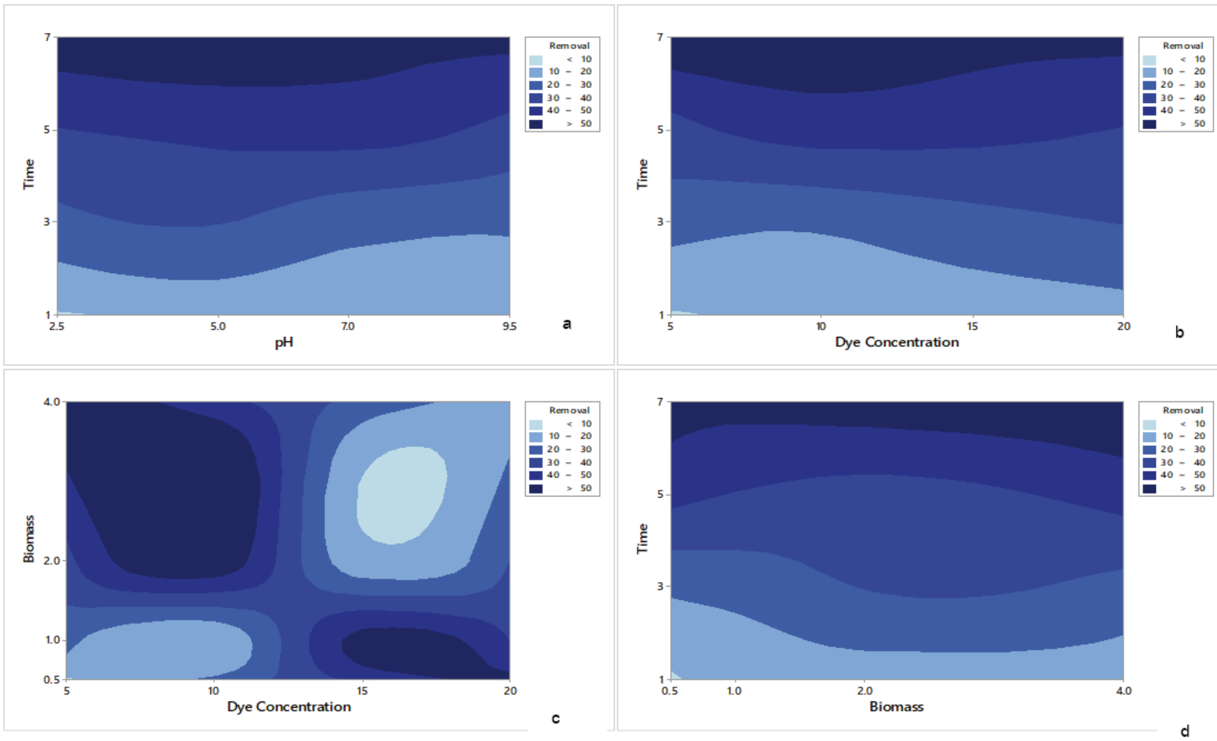

Figure 2: Interaction (a) time and concentration of dye (b) time and pH (c) time and plant biomass (d) plant biomass and dye concentration

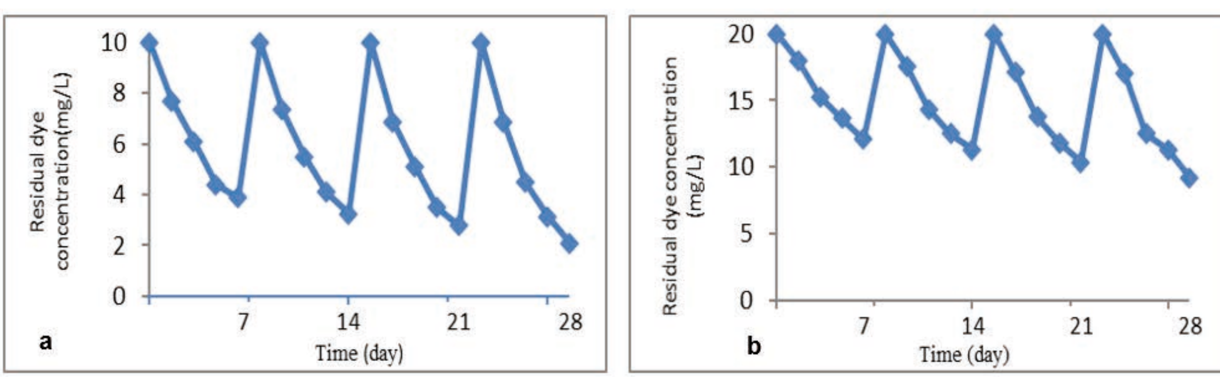

Figure 3: Biological decolorization profiles during repeated batch operations. T = 25 ◦C; [AB92] = a 10 m l^{-1} and b 20 ml^{-1}; [Biomass] = 4g; pH = 7

due to surface capacity limitation, the gradient of the contaminant concentration is quickly balanced and the continuous addition of dye to the environment will not increase the adsorption efficiency (Khataee et al., 2013; Srinivasan & Viraraghavan, 2010). Also, this could have happened due to the positive relative growth rate of *Ceratophyllum* in the fourth week. The positive effect of plant mass on purification efficiency was also confirmed based on the Taguchi test analysis (Table 3). It is also possible that mechanisms such as increasing the antioxidant defense system and increasing the activity of enzymes effective in color decomposition have contributed to increasing the refining capacity of the *Ceratophyllum* plant in the fourth week, and the proof of this requires a more detailed study. In previous studies, the ability of aquatic plants *Nasturtium officinale* Aiton (Torbati et al., 2015), *Hydrocotyle vulgaris* L. during successive purification with acidic blue dye 92 and the aquatic plant *Spirodela polyrrhiza* (L.) Schleid. (Movafeghi et al., 2016) exposed to azo dye Direct Blue 129 has been reported.

3.4 SPECTRAL ANALYSIS OF IR

The IR spectrum of the AB 92 showed some peaks, which correspond to functional groups (Fig. 4a). The peak observed at 3425 cm^{-1} can be related to O-H stretching as in R-OH compounds or N-H stretching as in amines and amides and 2922 cm^{-1} for asymmetric –CH3 stretching vibrations. The peak at 1618 cm^{-1} for N = N stretching confirms the azo nature of the dye. Peaks at 1566 cm^{-1} correspond to C-N stretching as in amides, 1454 cm^{-1} for C–H in plane C–H bend, 1400 cm^{-1} for C-H deformation as in cis-alkene, 1340 cm^{-1} for O-H stretching of phenols. Peaks at 1127 Cm^{-1} for disubstituted benzene ring. This confirms the aromatic nature of the dye, 1046 cm^{-1} for S–O stretching as in sulphonic acids. The IR spectrum plant before and after decolorization has been shown in Fig. 4. In the plant before treatment (control plant) several peaks were observed at 3424, 2920, 1725, 1628, 1464, and 1117 cm^{-1}. The peaks at 3424 cm^{-1} can be related to NH_2 stretching of amino acids or O-H stretching, 2920 cm^{-1} for C–H stretching of CH_2. The peaks at 1725 cm^{-1} for C = O stretching, 1628 cm^{-1} for N–H deformation of primary amines, 1464 cm^{-1} for C–H stretching of alkane CH_3, and 1117 cm^{-1} for C-N vibrations in aliphatic amines. The IR spectrum plant after treatment with AB92 dye, the transfer of peaks related to plant functional groups from 3424, 2920, 1725, 1464, and 1117 cm^{-1} to 3417, 2922, 1726, 1460, and 1154 cm^{-1}, respectively. Also, the appearance of additional peaks at 1649, 1544, 1381,1327, 1154, and 1077 cm^{-1} representing N = N and stretching, C = C vibration of aromatic homocyclic compounds, CH_3 deformation of alkanes, C-N stretching vibrations in aromatic tertiary amines, O-H stretching as in alcohols, and for S = O stretching as in R–SO_3H compounds (Fig. 4).

The results of the FTIR spectrum showed that the control plant has various functional groups including amine, hydroxyl, and carboxyl groups. The displacement of these peaks in the spectrum of the treatment plant may have been due to the formation of interactions between the *Ceratophyllum* plant and the molecules of the AB92 dye. Studies have shown that the functional groups of amines, hydroxyl, carbonyl, and carboxyl in plants can play an important role in the interaction between plants and dye molecules (Liu & Wang, 2023; Sah et al., 2022). This could indicate the occurrence of the first phase of

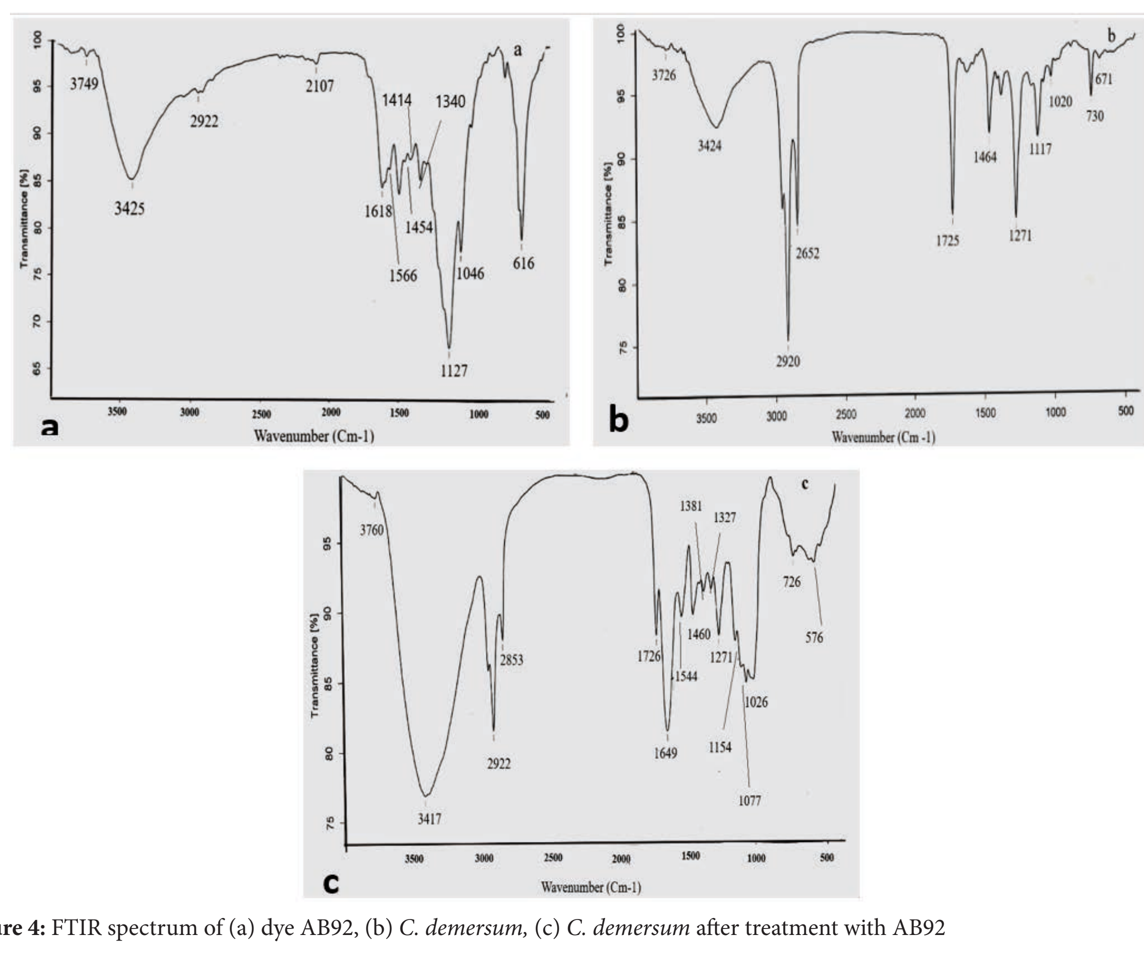

Figure 4: FTIR spectrum of (a) dye AB92, (b) *C. demersum,* (c) *C. demersum* after treatment with AB92

detoxification, i.e. the activation phase of the dye. In this process, organic non-biomolecules obtain a hydrophilic functional group such as hydroxyl, amino, carboxyl, etc. because of enzymatic transformations of oxidation, reduction, hydrolysis, etc. These functional groups increase the reactivity and polarity of the molecule, as well as increase the susceptibility of the contaminant molecule to enzymes and accelerate the change of the contaminant (conjugation or oxidation) (Kvesitadze et al., 2006). Similar results were observed for the adsorption of Basic Red 46 dye (Mahmoodi et al., 2010) and cation dye by biosorbents (Zhang et al., 2013). The IR spectrum of the plant after dye removal significantly differed from that of the AB92 dye and of the control, like the disappearance of the peaks at 1618, 1566, 1414, 1340, 1127, and 1046 cm^{-1} in the treated plant, which was present in the spectra of the dye. Also, the appearance of several new peaks at 1649, 1649, 1544, 1381, 1327, 1154, and 1077 cm^{-1}, supports the biotransformation of the dyes within the *Ceratophyllum*. Thus, it can be suggested that the plant could play the expected role in dye biodegradation. Such bioremediation can be consistent with previous research (Khataee et al., 2013; Vafaei, et al., 2012).

3.5 GROWTH ASSESSMENT

With increasing the concentration of AB92 dye, the relative growth rate and tolerance index showed a significant decrease compared to the control. The tolerance index reached a minimum of 0.26 at a concentration of 20 mg l^{-1} (Table 4).

The relative growth rate is an important parameter to observe the physiological effects of the toxicity of chemicals (Duman & Koca, 2014). The results showed that by increasing the concentration of AB92 dye, the growth rate of *C. demersum* decreased. Previous studies have shown that organic and inorganic xenobiotics can accelerate the aging process and stimulate premature plant death (Parent et al., 2008). This can be a plant defense response to persistent stressors because in these conditions' stressors are stored in the old organs and by separating these areas, toxic compounds are removed from the living parts of the plant. In previous studies, the toxic effects of other environmental pollutants had been shown on the growth of *C. demersum*. For example, reduced growth of *C. demersum* versus increased concentrations of the heavy metals nickel and cadmium have been previously reported (Chorom et al., 2012). In previous studies, a decrease in the growth of this plant against high concentrations of non-ionic surfactant 4-tert-octylphenol (OP) has been reported (Chiu & Wu, 2017). Also, reduced growth of aquatic plants *Nasturtium officinale* (Torbati et al., 2015), *Lemna minor* L. (Khataee et al., 2012), and aquatic fern *Azolla filiculoides* Lam. (Khataee et al., 2013) with increasing concentration of acidic dye 92 were reported.

Table 4: Effects of different concentrations of AB92 (0 – 20 mg l^{-1}) on relative growth rate (RGR) and Tolerance index (TI) in *C. demersum* treated for 7 days (mean ± standard error, n = 3)

AB92 (mg l^{-1})	RGR	TI
0	2.98± 0.09 ^{a}	1 ^{a}
10	1.94± 0.27 ^{b}	0.65± 0.09 ^{b}
20	0.78 ± 0.06 ^{c}	0.26± 0.02 ^{c}

3.6 PHOTOSYNTHETIC PIGMENTS CONTENTS

The effect of different concentrations of AB92 on the concentration of chlorophyll a and chlorophyll b is shown in Table 5. The amount of chlorophyll a and chlorophyll b showed a significant decrease only at the concentration of 20 mg l^{-1} of the dye compared to the control. The results showed that with the increase in the dye concentration, the total chlorophyll concentration decreased significantly. At a concentration of 20 mg l^{-1}, the concentration of total chlorophyll decreased by 16.88 % compared to the control (Table 5). At a concentration of 10 mg l^{-1}, the total carotenoid concentration decreased by 21.4 %, and at a concentration of 20 mg l^{-1}, it decreased by 45 % compared to the control (Table 5).

According to the results of this study, probably, at the concentration of 20 mg l^{-1}, the production and accumulation of free radicals increased and caused damage to the photosynthetic apparatus, and subsequently caused the reduction of photosynthetic pigments. Previous

Table 5: Effects of different concentrations of AB92 (10–20 mg l^{-1}) on chlorophylls and total carotenoid content (μg g^{-1} FM) in the *C. demersum* treated for 7 days (mean ± standard error, n = 3)

AB92	Chlorophyll a	Chlorophyll b	Total chlorophyll	Total carotenoid
0	0.52 ± 0.0080 ^{a}	0.24± 0.0066 ^{a}	0.77 ± 0.0118 ^{a}	0.20 ± 0.0107 ^{a}
10	0.50 ± 0.0067 ^{a}	0.21 ± 0.1369 ^{ab}	0.73 ± 0.0092 ^{b}	0.16 ± 0.0076 ^{b}
20	0.44 ± 0.0036 ^{b}	0.20 ± 0.0097 ^{b}	0.64 ± 0.1268 ^{c}	0.11 ± 0.0135 ^{c}

studies have shown that chlorophylls are more unstable than carotenoids and are easily subjected to oxidative decomposition by singlet oxygen from photosynthesis (Weinberg et al., 2003). Carotenoids can quickly receive the energy from triplet chlorophyll excitation and thus prevent the formation of singlet oxygen and protect chlorophyll from oxidative degradation (Li et al., 2009; Santabarbara et al., 2007). The increase of AB92 showed a similar effect on the carotenoid concentration of aquatic plants *Hydrocotyle vulgaris* (Torbati et al., 2015) and *Azolla filiculoides* (Khataee et al., 2013). The reduction of carotenoids in the *Ceratophyllum* plant under flurochloridone treatment has been previously reported (Zhou et al., 2020).

3.7 LIPID PEROXIDATION ASSAY

The extent of oxidative damage was calculated based on the concentration of malondialdehyde (MDA) as a product of lipid peroxidation. MDA concentration increased by 39.9 % at a concentration of 10 mg l^{-1} of AB92 and 69.5 % at a concentration of 20 mg l^{-1} compared to the control (Table 6). Polyunsaturated fatty acids are exposed to attack by reactive oxygen species, which results in the production of small hydrocarbon fragments such as ketones and malondialdehyde. For this reason, malondialdehyde is considered an indicator of lipid peroxidation. Lipid peroxidation causes damage to the cell by reducing the fluidity of the membrane and increasing the leakage of substances. This research also confirmed this study. Similar results were observed in the treatment of aquatic plants *Azolla filiculoides* (Khataee et al., 2013) and *Nasturtium officinalle* (Torbati et al., 2015) exposed to AB92 and in *Spirodela polyrrhiza* (L.) Schleid. exposed to Direct Blue92 (Movafeghi et al., 2016). Also, an increase in the production of reactive oxygen species has been observed in the treatment of *Ceratophyllum* plants with heavy metals cadmium, lead, and zinc (Hak et al., 2020; Mishra et al., 2008; Mishra et al., 2006).

3.8 THE EFFECT OF AB92 ON ANTHOCYANIDIN GLYCOSIDE CONCENTRATION

With increasing the concentration of AB92, the amount of anthocyanidin glycosides increased. At a concentration of 10 mg l^{-1} of the dye, the amount of anthocyanidin glycosides increased by 33.6 %, and at a concentration of 20 mg l^{-1}, it increased by 81.8 % compared to the control (Table 6). Glycoside anthocyanidins are one of the most effective scavengers for most types of oxidizing molecules, including free radicals (Kong et al., 2003). Previous studies have shown that glycoside anthocyanidins produced in plants have more antioxidant activity than alpha-tocopherol (El-Alfy et al., 2005). An increase in the amount of anthocyanidin glycosides has been reported in the aquatic fern *Azolla filiculoides* (Masoudian et al., 2020) and *Lemna minor* (Masoudian et al., 2022) under oxidative stress conditions.

3.9 THE EFFECT OF AB92 ON FREE RADICAL SCAVENGING ABILITY

By increasing the concentration of AB92, the free radical scavenging ability of the plant increased. The free radical scavenging ability increased by 6.88 % and 14.29 % in the concentration of 10 mg l^{-1} and 20 mg l^{-1} of the dye, respectively (Table 6). The increase in antioxidant activity could probably be due to the increase in the anthocyanidin glycoside. It seems that the presence of AB92 in the culture medium of the plant has caused oxidative stress. The mentioned plant has tried to reduce stress by raising the oxidant defense system, and the increase in free radical scavenging activity confirms this.

4 CONCLUSIONS

Due to its cost-effectiveness and low side effects, phytoremediation technology is one of the most useful methods in pollutant purification. To use this technology, it is essential to identify plant species capable of removing various pollutants. This research showed that the aquatic plant *C. demersum* can significantly remove AB92 from polluted water. The results based on S/N showed treatment time had the most effect and pH factor had the least effect among the investigated factors Also, the highest percentage of removal of dyes was observed at level 4 of treatment time (7 days), level 4 of plant biomass (4 g), level 4 of initial concentration of dye (20 mg l^{-1}), and level 2 pH (pH = 5). The reusability of the plant in four consecutive periods confirmed the process of biological degradation of the dye. The FTIR results confirmed the possible interaction between the dye molecules and the plant's functional groups. Both dye concentrations caused negative effects on growth parameters and damage to the membrane of *C. demersum* through oxidative stress. The stress was more intense in the concentration of 20 mg l^{-1}, compared to 10 mg l^{-1}. *C. demersum* increased non-enzymatic antioxidant (anthocyanidin glycoside) content to defend against oxidative stress.

Table 6: The changes of MDA (µmol g^{-1} FM), anthocyanine glycoside (µmol g^{-1} FM), and antioxidant activity (%), in the *C. demersum* treated by 0, 10, and 20 mg l^{-1} of AB92 (mean ± standard error, n = 3)

AB92	MDA	Anthocyanidin glycoside	Antioxidant activity (%)
0	14.84 ± 0.3871 ^{c}	19.19 ± 0.2626 ^{c}	22.72 ± 0.9709 ^{c}
10	20.77 ± 0.5161 ^{b}	25.64 ± 0.2523 ^{b}	29.60 ± 0.6277 ^{b}
20	25.16 ± 0.3413 ^{a}	34.89 ± 0.4276 ^{a}	37.01 ± 0.7191 ^{a}

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