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

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

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

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

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

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

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1 INTRODUCTION

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

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

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

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

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

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

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

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

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

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS

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

2.2 EXPERIMENTAL DESIGN

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

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

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

2.3 MALONDIALDEHYDE DETERMINATION

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

$$C_{MDA} = (D1–D2)/155*m$$

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

2.4 THE TOTAL CONTENT OF ASCORBIC ACID

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

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

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

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

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

2.5 ANTHOCYANIN DETERMINATION.

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

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

$$C_{ан} = (D*V*R*K)/(l*m)$$

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

2.6 CHALCONES DETERMINATION.

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

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

The calculation was performed using the following formula:

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

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

2.7 STATISTICAL ANALYSES

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

3 RESULTS

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

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

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

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

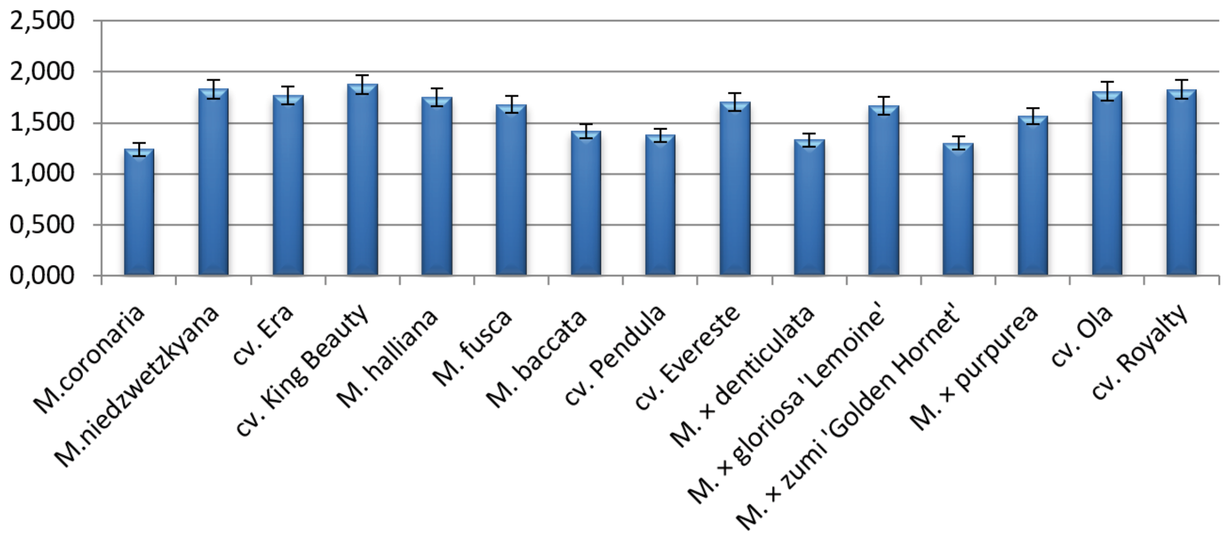


Figure 1: Difference in malondialdehyde accumulation in shoot bark in % for January-February 2023-2024, %

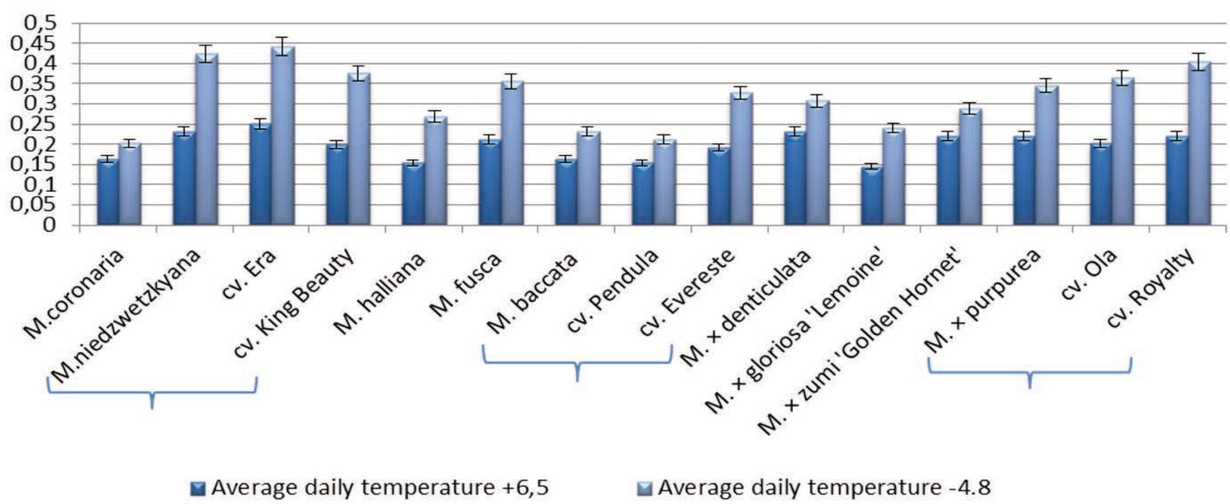


Figure 2: Content of malondialdehyde in the bark of annual sprouts of representatives of the genus *Malus* spp., mg.100 g^{-1}

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

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

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

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

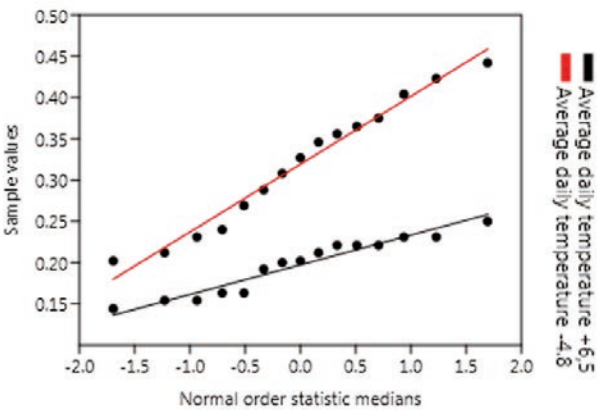


Figure 3: Normal probability plot

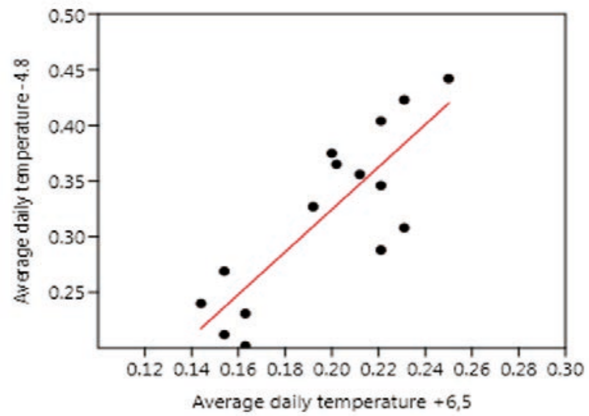


Figure 4: Multivariate linear regression

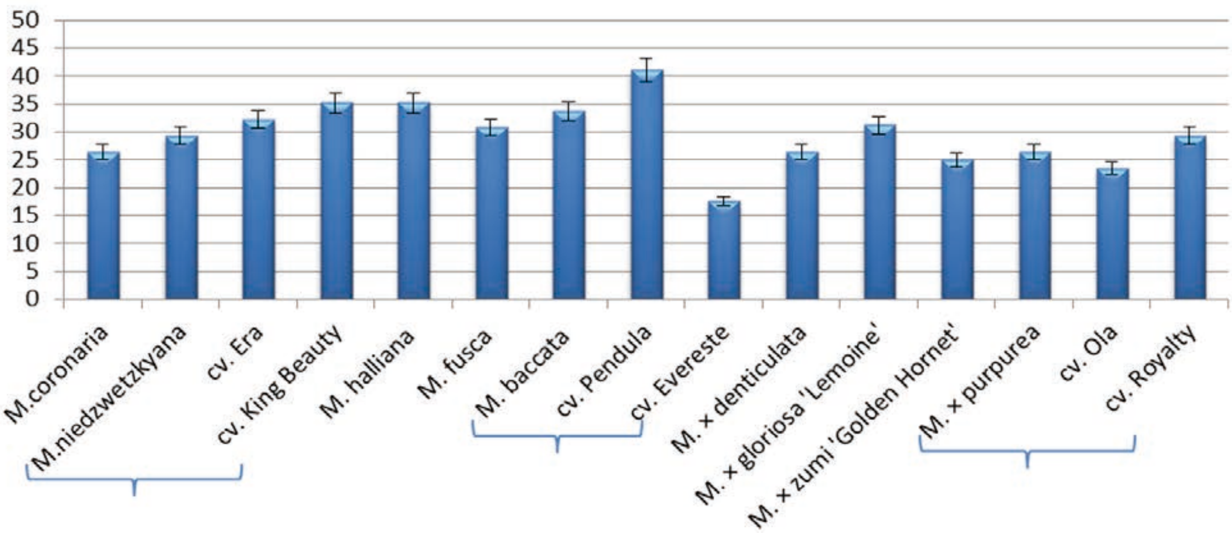


Figure 5: Ascorbic acid content in the bark of annual sprouts of representatives of the genus *Malus* spp., mg.100 g^{-1} (average value for 2023–2024)

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

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

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

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

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

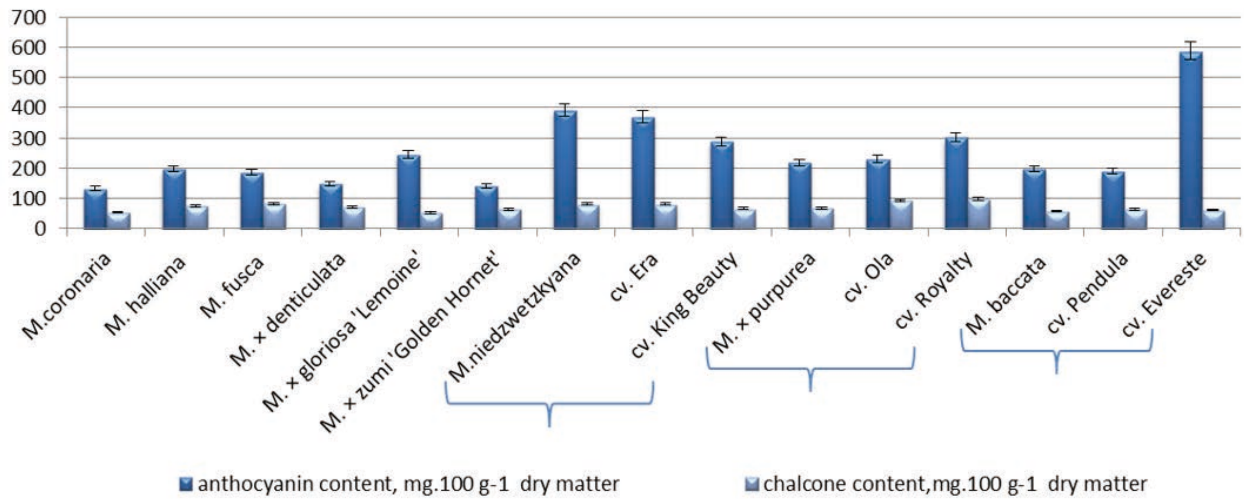


Figure 6: Content of anthocyanins and chalcones in the bark of annual sprouts of representatives of the genus *Malus* spp. (average value for 2023–2024)

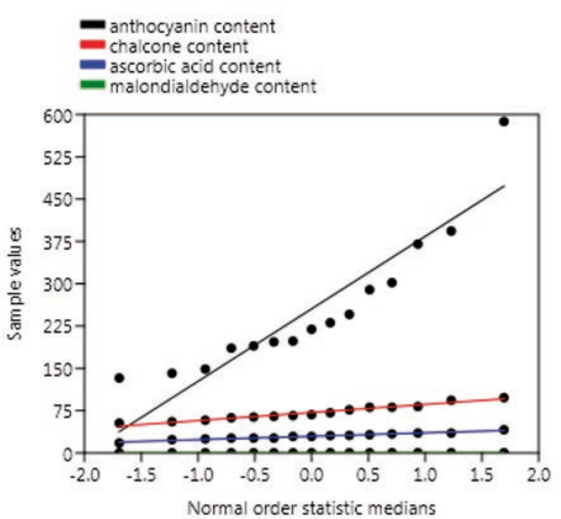


Figure 7: Normal probability plot between content malondialdehyde, vitamin C, anthocyanins, and chalcones in the bark of annual sprouts of the genus *Malus* spp.

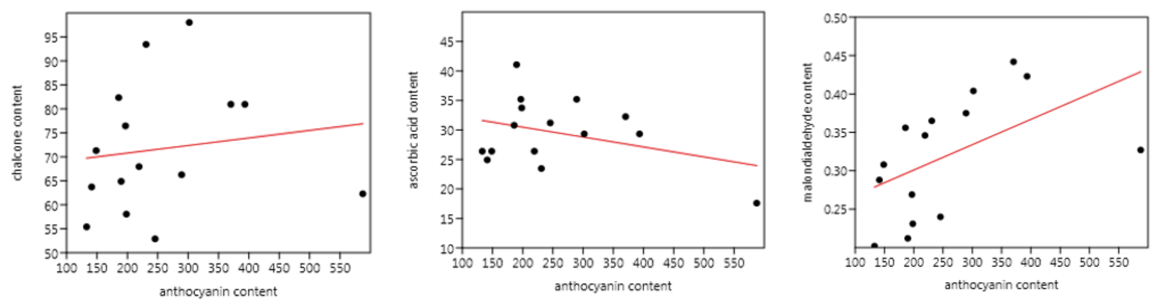
correlation coefficient, and a direct relationship between anthocyanins and malondialdehyde (Fig. 8).

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

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

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

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



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

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

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

Figure 8: Multivariate linear regression

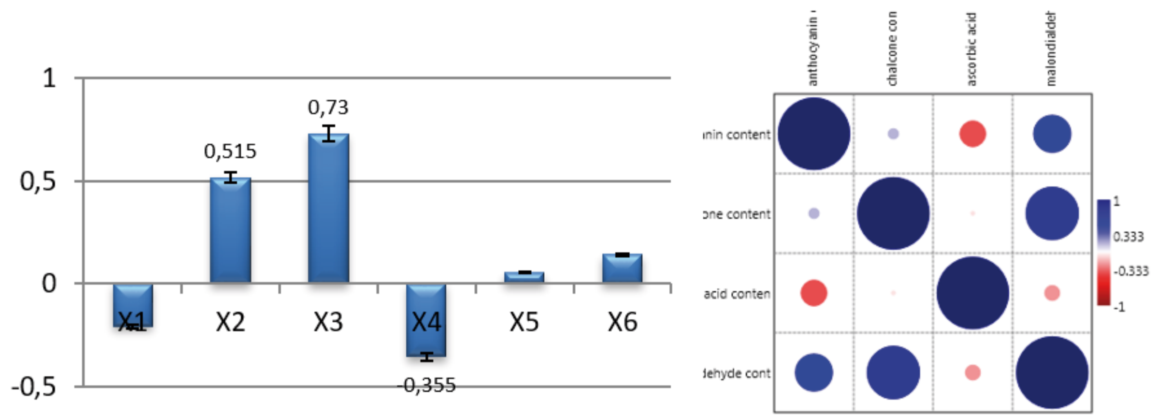


Figure 9: Pearson correlation coefficient: X1 – dependence of malondialdehyde on ascorbic acid content; X2 – dependence of malondialdehyde on anthocyanin content; X3 – dependence of malondialdehyde on chalcone content; X4 – dependence of ascorbic acid on anthocyanin content; X5 – dependence of ascorbic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

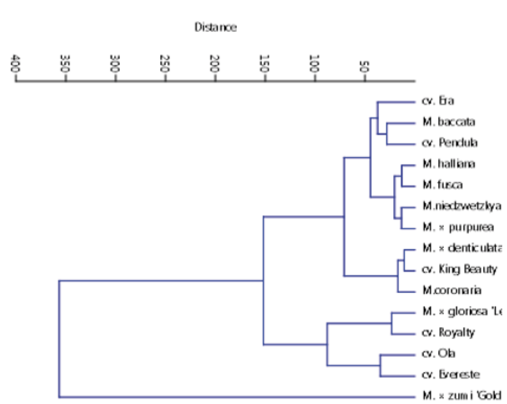


Figure 10: Dendrograms of the cluster analysis of the content of malondialdehyde, ascorbic acid, anthocyanins and chalcones in the bark of annual sprouts of representatives of the genus *Malus* spp.

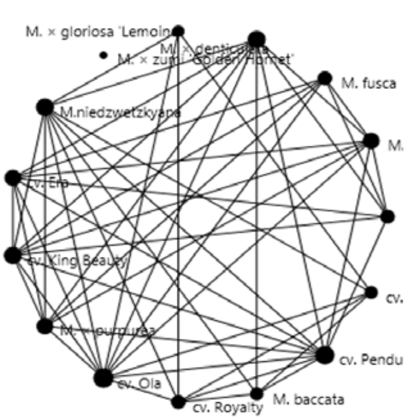


Figure 11: Network section similarity index Gower of the content of malondialdehyde, ascorbic acid, anthocyanins and chalcones in the bark of annual sprouts of representatives of the genus *Malus* spp.

4 DISCUSSION

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

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

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

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

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

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

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

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

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

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

5 CONCLUSIONS

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

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

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

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