Botanica SERBICA

(2024) 48 (2): 185–193

DOI: https://doi.org/10.2298/BOTSERB2402185K
journal homepage: botanicaserbica.bio.bg.ac.rs

Original Scientific Paper

The protective role of exogenous proline in pepper callus exposed to long–term cold stress

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ABSTRACT:
Cold stress is one of the main abiotic stress factors which restricts pepper growth and development. Thus, identifying alternative strategies is critical to reduce cold damage in peppers. This study evaluated the effect of exogenous proline in reducing cold stress damage in pepper callus. For this purpose, callus was obtained from the hypocotyl explants of germinated seedlings under *in vitro* conditions. 0,12 and 24 mM proline were applied to the callus and developed under the same photoperiodic settings at 4°C, 8°C, 16°C and 24°C. Low temperatures increased H_2O_2 and MDA production with the highest H_2O_2 and MDA amounts determined at 4°C. Proline applications decreased the content of H_2O_2 at low temperatures, whereby 24 mM proline caused a significant decrease in the amount of H_2O_2 at 4°C. The lowest MDA accumulation was determined in the 12 mM proline application. The data indicated that the total phenolic content of pepper callus decreased with decreasing temperatures. However, the application of proline increased the total phenolic amounts with the increase in its concentration. DPPH radical scavenging activity, FRAC and total protein content decreased with decreasing the temperature to 4°C and 8°C. However, both exogenous proline applications increased DPPH radical scavenging activity, FRAC and total protein at 4°C and 8°C. The results indicated that the metabolic pathways are triggered by the application of exogenous proline.

Keywords:
Capsicum annum, free radical, lipid peroxidation, oxidative stress, tissue culture, tolerance

UDC: 582.926.2:577.112.388

Received: 23 November 2023
Revision accepted: 26 June 2024

INTRODUCTION

Today, where hunger is an important issue, agriculture holds a significant place for the countries of the world. In our developing and changing world, the population increases every year. Increasing the production of nutrients is thus essential in meeting the nutritional needs of this increasing population and the amount of product grown per unit area should be higher. On the other hand, changing climatic and environmental conditions affect the metabolic and physiological mechanisms in plants, turning into stress factors which cause serious losses in agricultural production and crop yield. To address this problem, plants must be grown in a healthy environment and protected against various stress factors. Developing different strategies to increase efficiency and production has become essential.

The pepper, which belongs to the Solanaceae family, is a widely used and high-value vegetable worldwide due to its colour, aroma and pungency properties (Giuffrida *et al.* 2013; Bogusz Junior *et al.* 2015). The pepper, whose production reached 36 million tons in 2020, is the second most exported fresh vegetable (Url 1 2020). China leads in pepper production, followed by Turkey and Mexico (Url 2 2018). In addition, peppers contain numerous bioactive compounds used as active ingredients in the treatment of various diseases. Therefore, in addition to meeting increased nutritional needs, the medicinal and aromatic properties of peppers are other factors which encourage intensive agricultural production.

Frequent and severe cold stress (at non-freezing temperatures), which is exacerbated by the effects of climate change, affects the growth and development of plants, including peppers, causing a serious reduction in agricultural production, productivity and quality. While some temperate zone plants can tolerate low temperatures by activating their genetic adaptation mechanisms, tropical and subtropical plants are adversely affected by low temperatures. The pepper is a warm season vegetable and develops well at an ideal growth temperature of between 20 and 28°C and is sensitive to cold as it does not have a cold acclimation mechanism (Tang *et al.* 2022). At temperatures below 15°C, growth and yield decrease (Knott & Deanon 1967). Cold stress causes serious damage to the plasma membrane of the cell due to rapidly developing dehydration (Yadav 2010). The increase of reactive oxygen species (ROS) in plants under cold stress causes the destruction of organic molecules such as DNA, proteins, lipids and carbohydrates. Low temperatures also disrupt the functioning of the female organ of the flower, causing a decrease in the amount of pollen and its vitality, thus preventing reproduction (Shaked *et al.* 2004). Therefore, it is important to understand the cold tolerance mechanism of the pepper and to further develop its cold resistance.

In vitro tissue culture techniques are more advantageous for elucidating the basic mechanisms, as they enable work in small areas and under controllable optimum conditions. Studies to date have shown that by supporting *in vitro* culture media with various natural and synthetic plant growth regulators, the biosynthetic activities of cells can be controlled and the accumulation of various metabolites can be achieved. It has been reported that various physical and chemical substances can also be used as elicitors to increase production (Murthy *et al.* 2015). This allows for the development of plants and materials with higher tolerance and resistance to stresses using tissue culture methods (Halder *et al.* 2019).

A promising approach to improve tolerance to different stressors is the exogenous application of natural metabolites such as amino acids at appropriate concentrations (Godoy *et al.* 2021). Studies have shown an increase in the amount of endogenous proline in plants exposed to stress (Trovato *et al.* 2008; Koç *et al.* 2011; Wang *et al.* 2015; Jin *et al.* 2016; Nguyen *et al.* 2020). Proline, which is a very good osmoprotectant, is seen as an effective signal molecule because of its heterocyclic ring and distinct secondary amino group (Burritt 2012; Sing *et al.* 2015; Hossain *et al.* 2019). On the other hand, it has been reported that high amounts of proline have adverse effects on the growth and development of plants and on the functions of proteins (Abdelhamid *et al.* 2013; Elewa *et al.* 2017). Therefore, it is important to determine the appropriate proline concentrations which exert a positive effect on stress. This study was conducted to investigate the changes in the content of total phenolic, total protein, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) as well as DPPH radical scavenging activity (%) and ferrous reducing antioxidant activity (FRAC) in pepper calluses developed at 4°C, 8°C, 16°C and 24°C after treatment with 12 and 24 mM proline. According to a survey of the literature, there is no record of the effect of proline application on these parameters in pepper callus under long-term cold stress.

MATERIAL AND METHODS

Seed germination and culture conditions. Maraş-187 (*Capsicum annuum* L.) pepper seeds obtained from the Eastern Mediterranean Agricultural Research Institute were sterilised with 70% ethanol for 3 min and with 5.25% commercial NaClO for 15 min, and finally rinsed three times with sterile distilled water. They were then cultured in magenta boxes containing sterile agar medium (Ellialtioğlu *et al.* 1998). Hormone-free Murashige and Skoog (MS) (pH 5.7) (Murashige & Skoog 1962) basic nutrient medium supplemented with 3% sucrose and 0.7% agar was used for the germination of the pepper seeds and the magenta boxes were incubated in the dark in a growth chamber at 24 ± 2°C for one week.

Explant source and sowing of the explants in the callus medium. The seedlings grown from the seeds germinated under sterile conditions in a magenta box were subjected to a photoperiod (24 ± 2°C) of 16 hours light and 8 hours dark in the growth chamber (Digitech GLO-PG42). After the 4-week incubation period was completed, the seedlings were used as an explant source. The hypocotyl regions of the seedlings were cut in a laminar flow cabinet. Hypocotyl explants were then prepared by cutting the hypocotyl region into approximately 15 mm pieces. Callus was obtained by placing the hypocotyl explants horizontally in an MS nutrient medium with a pH of 5.7 containing 0.1 mg/L kinetin and 1.0 mg/L 2.4-D, 3% sucrose and 0.7% agar (Ellialtioğlu *et al.* 1998). The callus tissues were cultured in the growth chamber for two weeks at a photoperiodic setting of 16 h light/8 h dark. The developed callus tissues were transferred to magenta boxes containing the MS nutrient medium mentioned above and developed at the same photoperiodic setting in the growth chamber. The callus tissues grown in the magenta boxes were transferred into MS medium (0.1 mg/L kinetin with 1.0 mg/L 2.4–D, 3% sucrose, 0.7% agar), containing 12 and 24 mM proline and were cultured under the same photoperiodic settings at 4°C, 8°C, 16°C, and 24°C for two weeks. Control groups without proline were prepared, and then developed at 4°C, 8°C, 16°C, and 24°C for two weeks.

Measurement of hydrogen peroxide (H_2O_2). The callus samples were homogenised with 0.1% TCA and the ho-

mogenate was centrifuged at 12000 g for 15 min. The supernatant was reacted with a potassium phosphate buffer (10 mM, pH 7.0) and 1 M KI (Velikova *et al*. 2000). The absorption was read at 390 nm. The amount of H_2O_2 was calculated using a standard curve obtained with different concentrations of H_2O_2.

Measurement of malondialdehyde (MDA) content. The reaction mixture containing 20 % trichloroacetic acid (TCA) and 0.5% thiobarbituric acid (TBA) was added to the supernatant obtained from the callus samples, which were homogenised with 1% (TCA) solution and centrifuged. The mixture was incubated at 95°C. It was then cooled in an ice bath and the samples were centrifuged again. Absorbance measurements of the supernatants were made at 532 and 600 nm (Heath & Packer 1968). The amount of MDA was calculated using the extinction coefficient '155 mM^{-1} cm^{-1}'.

Total soluble protein assay. Protein extraction from the callus samples was performed using the procedure proposed by Kurkela *et al*. (1988) and the supernatant was used for the determination of the total soluble protein content. The Bradford method was used for the determination of the total protein amounts (Bradford 1976) and the bovine serum albumin (BSA) was used to set up the standard curve.

Determination of the total phenolic compounds. The Folin-Ciocaltaeu method was used for the determination of the total phenolic contents in the callus samples (Singleton & Rossi 1965). The change in absorbance at 765 nm was measured and gallic acid was used to obtain the standard curve.

Determination of DPPH radical scavenging activity. The callus samples were homogenised with methanol and the extract was incubated with 0.1 mM DPPH at 24°C for 30 min (Blois 1958). The reduction of DPPH radicals was determined at 517 nm spectrophotometrically and was calculated using the following formula: % DPPH radical scavenging activity = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extracts.

Ferrous reducing antioxidant capacity (FRAC). The antioxidant capacity of the callus samples was determined spectrophotometrically following the method proposed by Vijayalakshmi & Ruckmani (2016). To determine reducing power, 0.1 g of callus was extracted with 1.5 mL of methanol. The reaction mixture, which consisted of 250 µL of extract, 0.2 M phosphate buffer pH 6.6 and 1% (m/v) potassium hexacyanoferrate (K3 $Fe(CN)_6$, was incubated at 50°C for 20 min. The reaction was stopped by the addition of 10% (m/v) TCA and centrifuged at 3000 g for 10 min. The supernatant upper layer was mixed with 1.25 mL of distilled water and 250 µL of 0.1% (m/v) of ferric chloride ($FeCl_3$) and incubated at 24°C for 10 min. The absorbance of the reaction mixture was read at 700 nm. The FRAC values of the extracts were expressed as *µg* of ascorbic acid equivalent (AAE) per gram of sample.

Statistical analysis. The data were analysed using analysis of variance (ANOVA) with the SPSS 24 software package. The data were expressed as the means ± SD of 3 replicates. Different letters indicate significant differences at $P < 0.05$ as determined by Tukey's test.

RESULTS

Low temperatures caused a significant increase in the production of H_2O_2 and ultimately led to lipid peroxidation in the pepper callus. The amount of H_2O_2 and MDA increased with decreasing temperatures with the highest H_2O_2 and MDA amounts observed at 4°C ($P < 0.05$) (Fig. 1). This was followed by the amounts of H_2O_2 and MDA at 8°C and 16°C, respectively. The application of different concentrations of proline (12 and 24 mM) reduced the levels of H_2O_2 and lipid peroxidation ($P < 0.05$). However, the lowest levels of production of H_2O_2 were observed in the callus treated with 24 mM proline. When the combined applications with both proline concentrations were compared in terms of H_2O_2 content, it was determined that 24 mM proline reduced the amount of H_2O_2 more than 12 mM proline in callus exposed to +4°C ($P < 0.05$) (Fig. 1). The MDA amounts in the callus treated with 12 and 24 mM proline also showed a decrease. However, it was determined that 12 mM proline applications had a more positive effect on biological membrane protection than 24 mM proline applications ($P < 0.05$). The lowest amount of lipid peroxidation was observed at 4°C and 8°C with 12 mM proline ($P < 0.05$) (Fig. 1).

The lowest total protein levels were found at 4°C and 8°C compared with 16°C and 24°C, but the differences were not statistically significant (Fig. 2). 12 mM proline application increased the amount of total protein at all temperatures compared to the control groups (no proline) ($P < 0.05$). The highest levels of protein were observed at 16°C and 24°C with 12 mM proline ($P < 0.05$). However, the highest total protein increase of 34% was recorded for the 12 mM proline +8°C application compared with the 8°C application alone. It was also determined that the amount of total protein increased at 4°C and 8°C with 24 mM proline compared to the control group ($P < 0.05$) (Fig. 2).

The data indicated that the total phenolic content was reduced by decreasing temperatures in pepper callus ($P < 0.05$). The level of phenolic compounds in the callus growing under optimal conditions (24°C) was found to be much higher than in the callus growing under cold

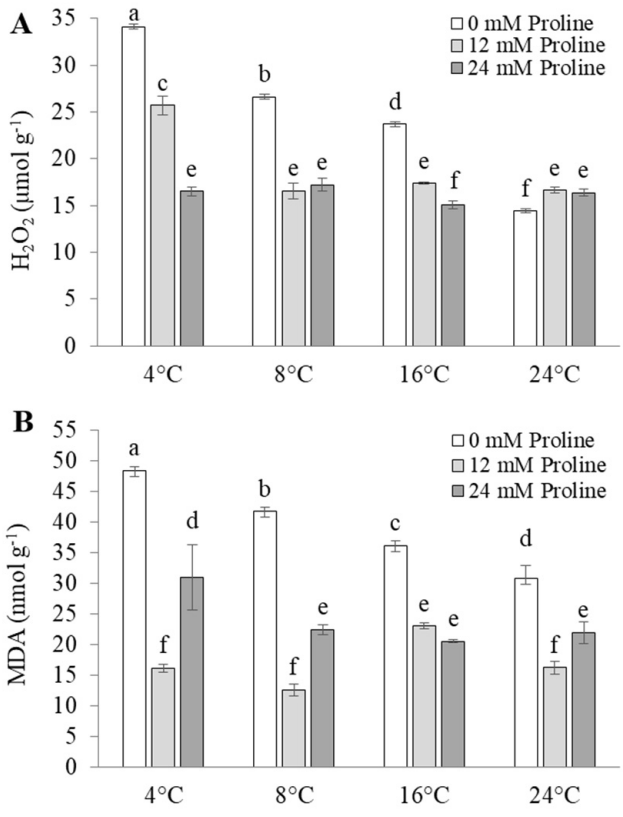

Fig. 1. The effect of temperature and exogenous proline on H_2O_2 (A) and MDA (B) amount in pepper callus

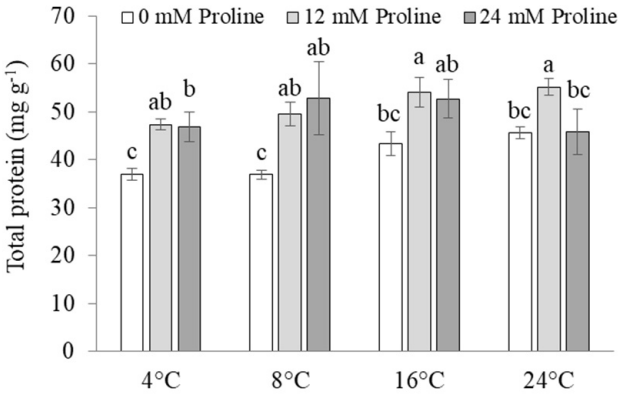

Fig. 2. The effect of temperature and exogenous proline on total soluble protein in pepper callus

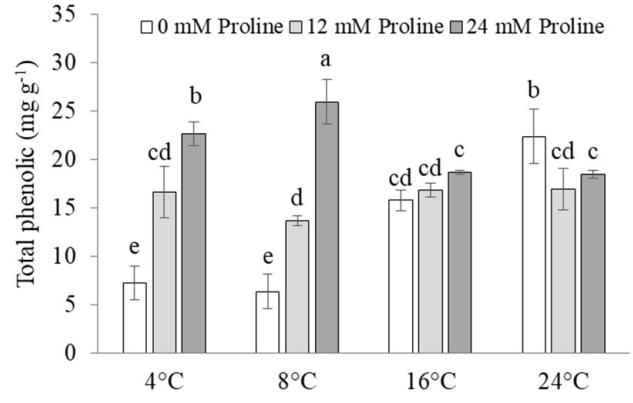

Fig. 3. The effect of temperature and exogenous proline on total phenolic content in pepper callus

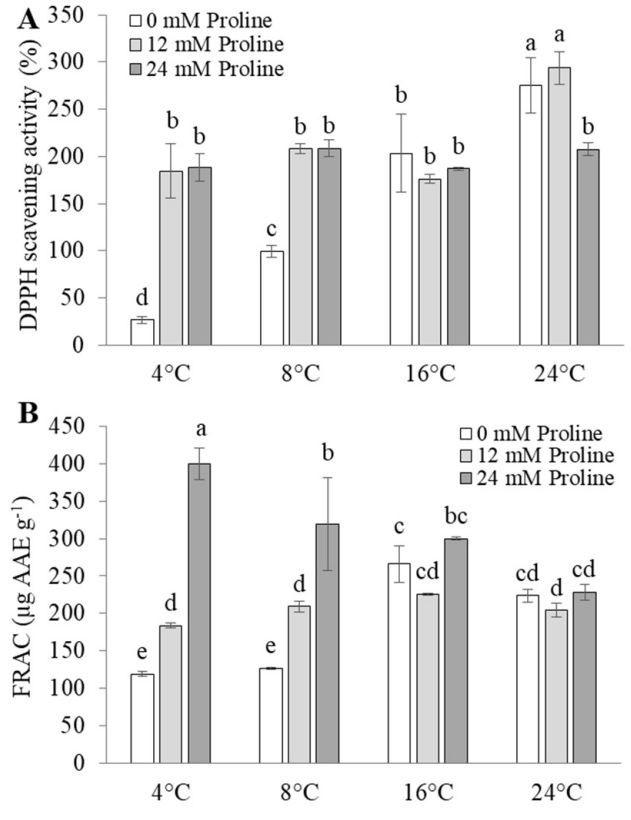

Fig. 4. The effect of temperature and exogenous proline on DPPH scavenging activity (%) (A) and FRAC (B) in pepper callus.

stress ($P < 0.05$) (Fig. 3). The total phenolic content in the callus grown at 24°C was 22.4 ± 2.8 mg g^{-1}. The exogenous application of proline increased the levels of total phenolics in pepper callus, with higher concentrations of proline resulting in larger amounts of total phenolics. Compared to applications at 4°C and 8°C alone, an increase of approximately 130% and 115% in the total phenolic content was determined with 12 mM proline, respectively ($P < 0.05$) (Fig. 3). Compared with the amount of total phenolic compound at 16°C alone, 12 mM proline did not cause a statistically significant increase in the total phenolic content at the same temperature. However, increasing the proline concentration to 24 mM resulted in a significant increase in the total phenolic level compared to 12 mM. The highest increases in total phenolic content of 213% and 308% respectively

were observed at 4°C and 8°C with the 24 mM proline application, compared to the 4°C and 8°C applications alone ($P < 0.05$) (Fig. 3). However, the amount of total phenolic content decreased with the application of 24 mM proline at 24°C.

The results confirm that the DPPH (%) was much higher in the callus growing under optimal conditions (24°C) than in the callus growing under cold stress (4°C, 8°C and 16°C) ($P < 0.05$) (Fig. 4). The data from the current study showed that the DPPH radical scavenging activity (%) decreased with decreasing temperatures to 4°C. The lowest DPPH radical scavenging activity (%) was found at 4°C ($P < 0.05$). However, both 12 mM and 24 mM proline had a significant effect on the DPPH radical scavenging activity (%) compared to the applications without proline. The highest DPPH radical scavenging activity (%) was determined at 24°C in the 12 mM proline application ($P < 0.05$), while the highest DPPH radical scavenging activity (%) increase of 612% was determined at 4°C in the application with 24 mM proline ($P < 0.05$) (Fig. 4). Both proline applications increased DPPH radical scavenging activity (%) at 4°C and 8°C.

The most important indicator of potential antioxidant effect is the reduction capacity of a compound. Similar to the DPPH radical scavenging activity (%), a decrease in the amount of FRAC was detected with decreasing temperatures. It was shown that the callus samples at 16°C were more capable of reducing Fe^{3+} ions, compared to the other samples exposed to cold stress (4°C and 8°C). The reducing power in the callus samples exposed to 24 mM proline was significantly higher than in 12 mM proline at all temperatures (Fig. 4) ($P < 0.05$). However, both proline applications increased FRAC at 4°C and 8°C compared to the application at 4°C and 8°C alone. The highest increase in antioxidant power was found in the 24 mM proline application, with a 234% increase compared to the application at 4°C (Fig. 4) ($P < 0.05$).

DISCUSSION

Cold stress causes oxidative damage in plants by inducing the peroxidation of membrane lipids, disrupting the tertiary structures of proteins, breaking DNA chains, inactivating enzymes and generating ROS, which cause photoinhibition in the photosynthetic apparatus (Posmyk *et al*. 2005; Guo *et al*. 2006; Mo *et al*. 2010). Free radicals, which are unstable due to their unpaired electrons, tend to become stable by reacting easily with other substances. These radicals cause cell damage by removing electrons from other molecules and initiating lipid peroxidation. One of the most important effects of cold stress on plants is the deterioration of membrane integrity, especially due to lipid peroxidation and dehydration. The increase in the amount of MDA, which is formed as the final product by the degradation of unsaturated fatty acids at low temperatures, is a good indicator that the structural integrity of the membranes in plants is impaired. In this study, higher H_2O_2 levels were detected in applications exposed to cold stress alone (4°C, 8°C, and 16°C) compared to the control group (24°C). The production of H_2O_2 and MDA increased with decreasing temperatures to 4°C and 8°C. The results in Fig. 1 show that cold stress stimulates high H_2O_2 production and induces oxidative damage in pepper callus and high MDA synthesis, thus causing membrane disruption. These results are consistent with the findings and results of studies conducted by researchers who observed high amounts of MDA and H_2O_2 in rice, *Solanum lycopersicum* and *Abelmoschus esculentus* under cold stress (Han *et al*. 2017; Liu *et al*. 2020; Phornvillay *et al*. 2020).

Plants have developed various physiological stress responses to protect them against adverse environmental conditions. Low temperatures induced a general decrease in the amount of total phenolics, DPPH radical scavenging activity (%) and FRAC (except at 16°C) in the callus cultures. The results show that prolonged and continuous cold stress causes H_2O_2 and MDA accumulation in pepper callus, as well as a weak antioxidant defence mechanism.

Phenolic compounds tend to accumulate under stress conditions. Phenols, with their antioxidant properties, protect plants against abiotic stress factors by inhibiting reactions caused by free radicals as hydrogen atom donors (Sakihama *et al*. 2002). However, in this study, prolonged cold stress decreased the amount of total phenolic compounds in the pepper callus. Similar results were reported in studies conducted on *Vitis vinifera*, soybean seedlings and *Abelmoschus esculentus* seeds where it was determined that there was a reduction in the total phenolic content in the seedlings exposed to lower temperatures (Posmyk *et al*. 2005; Weidner *et al*. 2009; Amarowicz *et al*. 2010; Król *et al*. 2015; Phornvillay *et al*. 2020). On the other hand, Genzel *et al*. (2021) found that the number of bioactive compounds such as flavonoids increased in peppers exposed to cold and salt stress. High phenolic content was also observed in *Rehmannia glutinosa* and Japonica and Indica rice seedlings exposed to cold stress (Chung *et al*. 2006; Rayee *et al*. 2020). Short-term cold stress applications have also been reported to increase the level of phenolic compounds and antioxidant activity in studies on lettuce (*Lactuca sativa* L.) (4°C, 1–3 days) and cabbage (10°C, 1–3 days) (Oh *et al*. 2009; Eom *et al*. 2022). In a recent study on *Ocimum basilicum* L. plants exposed to cold stress, the highest phenolic content and DPPH activity were determined at 4°C for 12 h (Rezaie *et al*. 2020). In another study, it was reported that cold stress did not change the phenolic content of pea roots (Rudikowskaya *et al*. 2008). The reason for these differences may be attributed to the type, intensity, and duration of the stress, the plant cultivar and plant material (root, stem or leaf) (Saleh 2007).

However, the antioxidant capacities of pepper callus exposed to 8°C and 16°C were higher than at 4°C, while the MDA and H_2O_2 content was found to be lower than at 4°C. The results show that the antioxidant activity at 4°C also causes high oxidative damage as it was probably insufficient to cope with the high free radical production in pepper callus exposed to cold stress. Additionally, total protein decreased slightly following exposure to 4°C and 8°C. Similarly, a decline in total protein content was observed in the study conducted on the mung bean (*Vigna radiata* L.) exposed to cold stress (Saleh 2007). This decrease in total protein content may result from damage to the protein synthesis system or from the synthesis or activation of proteolytic enzymes such as protease (Pinedo *et al*. 2000). Low temperatures (0–10°C) reduce hydrophobic bindings, which are critical for obtaining three-dimensional conformations of proteins (Adiva & Waisel 1975). The results indicate that pepper callus is susceptible to cold stress under culture conditions at 4°C.

DPPH is one of the free radicals which damage the cell membrane and is reduced to the non-radical form by the proton donation of antioxidants (Singh *et al*. 2021). 12 and 24 mM proline induced both an increase in the DPPH radical scavenging activity (%), antioxidant power, and phenolic compound levels along with a significant decreases in the amounts of H_2O_2 and MDA in pepper callus exposed to 4°C and 8°C. These results were consistent with those of Posmyk & Janas (2007) who reported that exogenous proline decreased lipid peroxidation. The fact that proline application decreases MDA and H_2O_2 content indicates that proline acts as a ROS scavenger with its antioxidant properties and stimulates the antioxidant defence system. Since proline possesses antioxidant properties, it may have contributed to increasing this tolerance by causing the conversion of DPPH• radicals to stable DPPH-H molecules as a ROS scavenger and a hydrogen donor. The increase in the level of phenolic compounds at 4°C and 8°C indicates that exogenous proline effectively stimulates phenolic metabolites which protect cells from oxidative damage. It was determined that the application of proline to fruit-bearing branches of citrus trees exposed to low temperatures (-3°C for three hours) caused an increase in the amounts of chlorogenic acid, gallic acid, p-coumaric acid, ferulic acid and flavonoids (Mohammadrezakhani *et al*. 2019). Another study conducted on sugar beet exposed to drought stress indicated that exogenous proline application caused an increase in the total phenolic content (Alkahtani *et al*. 2021). Burguieres *et al*. (2006) reported that phenols protected cells from potential oxidative damage and increased the stability of the cell membrane during stress in *Pisum sativum*. These findings indicate the possible major role of phenolic compounds in the detoxification of free radicals and the preservation of membrane integrity. Phenolic compounds have a strong antioxidant effect as radical scavengers which donate hydrogen to free radicals. Our data indicated that proline was effective in increasing the tolerance of pepper callus to low temperatures. Although antioxidant activity in plants is mostly associated with phenolic compounds, antioxidant capacity is another parameter which indicates the antioxidant activity of a compound (Subramanian *et al*. 2013). The data indicate that the amount of FRAC is positively correlated with the total phenolic content, and the application of proline before cold stress contributed to the antioxidant capacity and prevented the lipid peroxidation of the membrane. In the current study, the increase in the level of FRAC due to proline applications showed that proline may have stimulated the synthesis of reducing compounds such as flavonoids, anthocyanins, and endogenous proline.

The data from this study indicated that proline applications increased the amount of total protein. Similarly, it was determined that the application of Pro to maize seeds increased membrane stability and protein concentrations under salt stress conditions (Rady & Hemida 2016). It has been pointed out that proline is a signal molecule which increases the stability of proteins and maintains membrane integrity with its secondary amino group and cyclic structure (Burritt 2012; Sing *et al*. 2015; Trovato *et al*. 2019). As a natural osmoprotectant, it has been reported that proline facilitates protein folding, inhibits aggregation and provides high-efficiency protein expression (Ignatova & Gierasch 2006). In another study, it was found that the stable hydration shell of proline was effective in protecting cell proteins from denaturation and aggregation and maintaining the functional activities of the proteins (Fedotova & Dmitrieva 2016).

CONCLUSION

The exogenous proline application generally increased the antioxidant activity and the total protein content in pepper callus and increased the tolerance of the pepper callus against prolonged cold stress. However, it was observed that pepper callus responded differently to various exogenously applied proline concentrations. Although these data indicate that the application of exogenous proline is an effective approach to reduce the negative effects of stress, they also show that the effect of proline may vary depending on the plant and stress type, application time, method and proline concentration. Proline, as a metabolic signalling molecule, affects plant growth and production. Therefore, a better understanding of the complex relationships of proline with phytohormones and its role in the enzymes and genes responsible for the synthesis of these hormones and osmolytes is needed. Clarifying these and similar aspects related to stress in the regulatory network will enable the development of plants resistant to both extreme low and

high temperatures and will contribute to meeting the nutritional needs of the world's population, which will increase in the future.

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REZIME

Botanica SERBICA

Zaštitna uloga egzogenog prolina u kalusu paprike izloženom dugotrajnom hladnom stresu

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Stres hladnoće je jedan od glavnih abiotičkih faktora koji ograničava rast i razvoj paprike. Stoga je identifikacija alternativnih strategija ključna za smanjenje štete. Ova studija je procenila efekat egzogenog prolina na smanjenje oštećenja izazvanog niskim temperaturama u kalusu paprike. U tu svrhu, kalus je dobijen iz hipokotilnih eksplantata proklijalih sadnica u *in vitro* uslovima. Prolin u koncentracijama 0, 12 i 24 mM je primenjen na kalus i razvijen pod istim fotoperiodičnim podešavanjima na 4°C, 8°C, 16°C i 24°C. Niske temperature su povećale proizvodnju H_2O_2 i MDA, a najveće količine H_2O_2 i MDA su detektovane na 4°C. Primene prolina su smanjile sadržaj H_2O_2 na niskim temperaturama, posebno 24 mM prolina je izazvalo značajno smanjenje količine H_2O_2 na 4°C. Najmanja akumulacija MDA utvrđena je u primeni 12 mM prolina. Najmanja akumulacija MDA utvrđena je u primeni 12 mM prolina. Podaci pokazuju da se ukupni fenolni sadržaj kalusa paprike smanjuje sa padom temperature. Međutim, primena prolina povećavala je ukupne fenole sa povećanjem njegove koncentracije. Aktivnost uklanjanja radikala DPPH, FRAC i sadržaj ukupnih proteina opadali su sa padom temperature na 4°C i 8°C. Međutim, obe primene egzogenog prolina povećale su aktivnost uklanjanja radikala DPPH, FRAC i ukupne proteine na 4°C do 8°C. Rezultati su pokazali da se primenom egzogenog prolina aktiviraju metabolički putevi.

Ključne reči: *Capsicum annum*, slobodni radikali, lipidna peroksidacija, oksidativni stres, kultura tkiva, tolerancija

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