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# The effects of titanium dioxide nanoparticles and spermine on physiological responses of licorice (*Glycyrrhiza glabra* L.) to cold stress

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## Abstract

Cold stress induces biochemical and physiological changes in plants. Therefore, it is important to identify and apply compounds that can increase plant tolerance to cold. Nowadays, the use of nanoparticles as a suitable solution to increase plant resistance to environmental stresses is considered. Besides, polyamines play an important role in regulating plant defense responses to environmental stresses. This study aimed to investigate the effects of spermine and titanium dioxide nanoparticles ($TiO_2$ NPs) on physiological responses of licorice (*Glycyrrhiza glabra* L.) plants to cold stress. Results showed that cold stress increased the content of total protein, phenolic compounds, glycine betaine, soluble sugars, glycyrrhizin, proline, hydrogen peroxide and malondialdehyde and also the activity of antioxidant enzymes, but decreased photosynthetic pigments content and growth indices. Spermine increased most of the features measured at low temperature but decreased the content of hydrogen peroxide and malondialdehyde. The production of glycyrrhizin was stimulated by cold stress and also by the application of spermine and $TiO_2$ NPs. The use of $TiO_2$ NPs and spermine alleviated the negative effects of cold stress and improved licorice plant responses.

**Keywords:** Antioxidant**;** *Glycyrrhiza glabra*; Polyamine; ROS

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## Introduction

Licorice (*Glycyrrhiza glabra* L.) is one of the most important medicinal and economical plants of the Leguminosae family. It has roots and rhizomes that are a rich source of biologically active and natural compounds. An important group of these compounds is triterpene saponins, which have antimicrobial, insecticidal and anti-inflammatory activities (Fukai *et al.* 1998).

Any plant in a particular temperature range has the maximum growth and desirable performance and any deviation from it, especially lowering the critical temperature, causes stress and decreases vegetative and reproductive growth (Yang *et al.* 2011). Reactive oxygen species (ROS) are produced during normal oxidative processes in the cell such as respiration, photosynthesis and oxidative phosphorylation, but their concentration increases during aging and in response to biotic and abiotic stresses. Low temperatures also cause oxidative stress and ROS accumulation. Because under cold stress the plant's ability to remove oxygen radicals is impaired, two enzymatic and non-enzymatic defense systems are used against the extensive ROS accumulation (Hoyle and Santos 2010).

Titanium (Ti) as a beneficial element stimulates growth and increases the absorption of some nutrient elements (Kuzel *et al.* 2003). There is a paucity of literature on the effects of titanium dioxide nanoparticles ($TiO_2$ NPs) on the physiological responses of plants. Numerous

reports showed that $TiO_2$ NPs reduced the destructive effects of stresses by increasing the activity of antioxidant enzymes and decreasing the free radicals of oxygen and malondialdehyde. $TiO_2$ NPs can increase dry weight, chlorophyll biosynthesis, sunlight absorption, rubisco activity, photosynthesis rate and nitrogen content (Hong *et al.* 2005; Gao *et al.* 2006).

Many researchers have pointed to the positive role of polyamines in confronting environmental stresses. Polyamines play important roles in regulating plant physiological activities and improving these activities under stress conditions. The antioxidant effect of polyamines is mainly related to their cationic properties which act to remove free radicals and thus can inhibit lipid peroxidation (Kusano *et al.* 2007). Kubis (2008) reported that spermidine prevented cell membrane damage under cold stress in barley plants, thereby reducing electrolyte leakage and amino acid withdrawal from the cell. To increase stress resistance and alleviate its negative effects, the application of $TiO_2$ NPs and a polyamine may be useful. Thus, the present study was aimed to investigate the effects of $TiO_2$ NPs and spermine on the antioxidative response of licorice plants under cold stress conditions.

## Materials and Methods

### Plant material and growth conditions

*Glycyrrhiza glabra* seeds were obtained from the Pakan-Bazr Seed Production Company (Isfahan, Iran). The experiment was conducted to study the effects of spermine (1 mM) alone and also together with two concentrations of $TiO_2$ nanoparticles (2 and 5 ppm) on biochemical and physiological properties of licorice plants under cold stress and normal conditions (4 ºC and 26 ºC, respectively). The cultures without any $TiO_2$ NPs and spermine were considered as the control. $TiO_2$ nanoparticles with the average particle size of 10-25 nm were supplied by the US Research Nanomaterials, Inc. Seeds were cultured on MS media containing 3% sucrose, 0.7% agar and were kept in a germination chamber with 16/8 h light/dark cycle at 26±2 ºC. The twenty-eight-days old plants were maintained under control condition (26 ºC) and cold treatment (4 ºC) for two days. All treatments were replicated three times. Samples were randomly selected from each treatment. Samples were harvested from all treatments 30 days after cultivation and stored at -70 ºC for further assays.

### Measurement of the growth characters and chemical substances

#### Ti content

Titanium concentration in the seedlings was measured by the method of Gibson *et al.* (2006). Bioaccumulation of Ti in the digested samples was evaluated by using inductively coupled plasma mass spectrometry (ICP-MS, HP-4500, USA).

#### Growth characters

For determining the fresh weight, eight plants in each jar were measured 30 days after seed planting with a digital scale and then their mean was calculated. To obtain the turgid weight, the plants were cut and immersed in the distilled water for 72 h in the dark. Then, the total weight of the pieces was measured and their average was calculated. To measure the dry weight, the pre-treated plant parts were placed in the oven at 70 ºC for 48 h. Then,

they were removed and their weight was measured again. RWC was determined according to the following formula:

$$RWC (\%) = (FM-DM)/(TM-DM)100$$

Where FW is the fresh weight, DW is the dry weight and TW is the turgid weight.

**Photosynthetic pigments content**

Leaf pigments content was measured by the Lichtenthaler and Wellburn (1983) method and the pigment concentration was calculated as mg $g^{-1}$ FW.

**Malondialdehyde and hydrogen peroxide content**

The lipid peroxidation rate was measured based on the method of Heath and Packer (1968) using fresh leaf samples. Malondialdehyde (MDA) concentration was read at 532 nm and the amount of malondialdehyde was calculated using the extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$ and expressed in terms of µmol $g^{-1}$ FW. The content of hydrogen peroxide ($H_2O_2$) was measured according to Velikova *et al.* (2000). The absorbance was read at 390 nm. Hydrogen peroxide content using its extinction coefficient of 0.28 $mol^{-1}$ $cm^{-1}$ was calculated and expressed in µmol $g^{-1}$ FW.

**Free proline and glycine betaine content**

Free proline content was determined according to Bates *et al.* (1973) and presented in mg $g^{-1}$ fresh weight. Glycine betaine was measured according to the method of Grieve and Grattan (1983) and reported in µmol $g^{-1}$ DW.

**Total phenolics content**

The total phenol content of the methanolic extracts was measured following the method of Plessi *et al.* (2007). Determination of the total flavonoid content of the extracts was done according to the aluminum chloride colorimetric method of Chang *et al.* (2002). Total anthocyanin content was determined as described by Wagner (1979).

**Soluble sugars**

The content of soluble sugars was measured using the method of Irigoyen *et al.* (1992). The absorbance was read at 485 nm for glucose, 480 nm for rhamnose and 490 nm for mannose. The concentration of each sugar was calculated using the standard curve.

**Total soluble protein and antioxidant enzyme activity**

Protein content was measured by the Bradford (1976) method. The absorbance of the samples was read at 595 nm. Total protein content was calculated using the standard curve of bovine serum albumin (BSA) and reported in mg $g^{-1}$ FW. The activity of superoxide dismutase (SOD) was determined following the method of Winterbourn *et al.* (1976). Peroxidase (POD) and catalase (CAT) activities were determined by the method of Chance and Maehly (1955). Determination of polyphenol oxidase (PPO) activity was assessed according to Kar and Mishra (1976). Ascorbate peroxidase (APX) activity assay was measured according to the Nakano and Asada (1981) method.

**Glycyrrhizin content**

Glycyrrhizin was extracted according to Orujei *et al.* (2013). The extract was quantified by high-performance liquid chromatography (HPLC) using a reverse-phase column (150×4.6 mm i.d.; 5 μm) SB-C8 (Agilent Zorbax, USA). The mobile phase flow rate was 1 mL $min^{-1}$ and glycyrrhizin was detected at 254 nm and 25 ºC and the glycyrrhizin content was calculated using the standard chromatogram.

**Experimental design and statistical analysis**

The experiment was carried out as a split-plot design based on randomized complete blocks with three replications. The main plots included normal (26 ºC) and cold (4 ºC) conditions and sub-plots included the following four treatments: control (without TiO2 NPs and spermine); 1 mM spermine; 1 mM spermine + 2 ppm $TiO_2$ NPs; 1 mM spermine + 5 ppm $TiO_2$ NPs. However, for the case of Ti content, the treatment of 1 mM spermine was excluded and only three treatments were evaluated at both temperatures. The data were subjected to analysis of variance followed by Duncan's test for comparing means at $p \leq 0.05$ using the SPSS (Ver. 23) software.

**Results**

**Ti content**

To ensure the absorption of TiO2 NPs by plants, the Ti concentration was measured on the thirty-day-old seedlings incubated at both temperatures, separately. The amount of Ti accumulation was higher in seedlings under cold stress than that in control seedlings. After application of $TiO_2$ NPs and spermine together, Ti accumulated in the seedlings. Also with increasing $TiO_2$ NPs concentration, the Ti accumulation rate increased (Figure 1).

**Growth characteristics**

Cold stress significantly decreased fresh, dry and turgid weight and also relative water content (RWC) compared to the normal temperature. The significant decreases in fresh, dry and turgid weight were induced by spermine at both temperatures compared to the control. Comparison of the mean values showed that the combined effect of spermine and $TiO_2$ NPs significantly reduced the fresh, dry and turgid weight compared to the control plants, except the turgid weight at low temperature (Figure 2). However, the RWC increased under optimum conditions, whereas it decreased significantly under cold stress conditions compared to the control plants. Spermine significantly increased RWC at both temperatures (Figure 2).

**Photosynthetic pigments content**

Cold stress significantly reduced the content of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid compared to the control seedlings at the optimum temperature (Figure 3). Spermine treatment increased the content of photosynthetic pigments at both optimum and low temperatures, except for chlorophyll b at the optimum temperature. The combined effect of spermine and $TiO_2$ NPs significantly increased the content of chlorophyll a, total chlorophyll and carotenoid in both thermal conditions, except total chlorophyll at 2 ppm $TiO_2$ under normal temperature conditions. The combined effect of $TiO_2$ NPs and spermine did

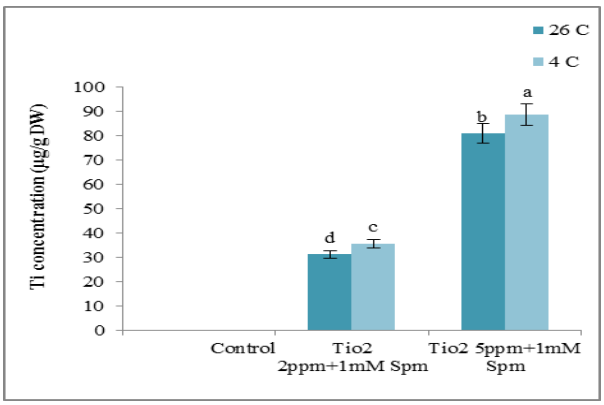


Figure 1. Ti content of *Glycyrrhiza glabra* seedlings treated with TiO2 nanoparticles and spermine (Spm) at optimum (26 ºC) and low (4 ºC) temperatures. Means with different letters above the bars indicate significant differences at p≤ 0.05.

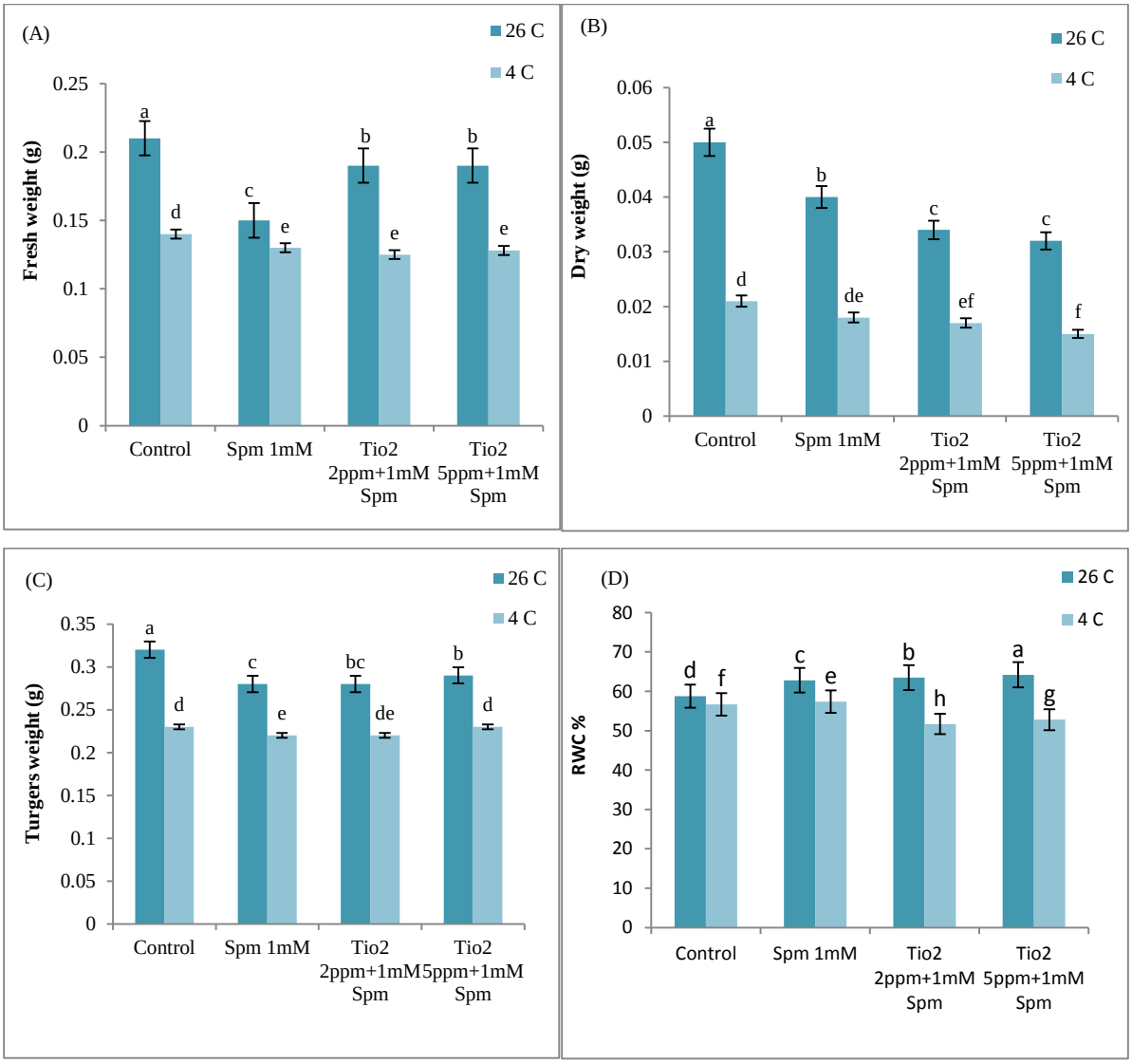


Figure 2. Mean comparison of growth characteristics in *Glycyrrhiza glabra* seedlings treated with TiO2 nanoparticles and spermine (Spm) at optimum (26 ºC) and low (4 ºC) temperatures. (A): Fresh weight, (B): Dry weight, (C): Turgid weight, (D): Relative water content (RWC). Means with different letters above the bars indicate significant differences at p≤ 0.05.

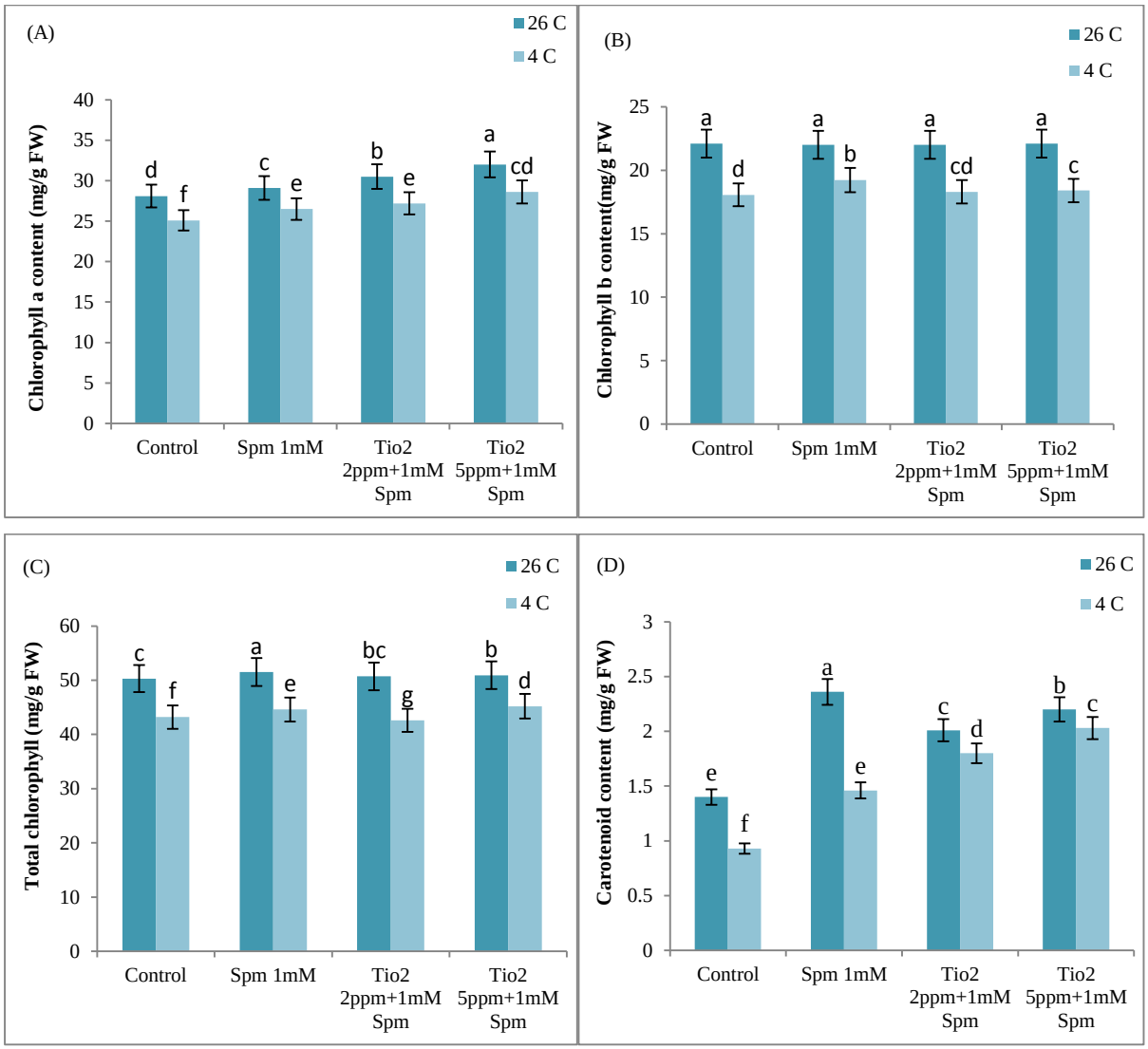


Figure 3. Mean comparison of photosynthetic pigment content in *Glycyrrhiza glabra* seedlings treated with TiO2 nanoparticles and spermine (Spm) at optimum (26 ºC) and low (4 ºC) temperatures. (A): Chlorophyll a, (B): Chlorophyll b, (C): Chlorophyll t, (D): Carotenoid. Means with different letters above the bars indicate significant differences at p≤ 0.05.

not induce a significant change in the content of chlorophyll b under optimum temperature. However, under cold stress conditions, the content of chlorophyll b significantly increased when the TiO2 concentration was 5 ppm.

**Malondialdehyde and $H_2O_2$ content**

Results from the measurement of malondialdehyde content showed that cold stress significantly increased the amount of this compound compared to the control plants (Figure 4A). However, spermine caused a significant decrease of malondialdehyde content at both thermal conditions. The combined effect of $TiO_2$ NPs and spermine reduced malondialdehyde content under both temperatures. Results from the measurement of hydrogen peroxide content showed that cold stress increased the amount of this compound

compared to the normal temperature, but spermine reduced the amount of hydrogen peroxide at both thermal conditions. The combined effect of $TiO_2$ NPs and spermine caused a significant decrease in the amount of $H_2O_2$ (Figure 4B).

**Free proline and glycine betaine content**

The proline content was significantly increased by cold stress (Figure 5A). Spermine did not affect the proline content at the optimum temperature but increased it at the low temperature. The combined effect of $TiO_2$ NPs and spermine at both optimum and low temperatures caused a significant increase in the proline content in comparison with the control plants. Cold stress significantly increased also the content of glycine betaine in the control plants (Figure 5B). Spermine treatment significantly increased glycine betaine content compared to control at both optimum and low temperatures. Application of $TiO_2$ NPs and spermine together at both optimum and low temperatures caused a significant increase in the content of glycine betaine in comparison with the control plants.

**Total phenolic content**

Cold stress caused a significant increase in the content of total phenol, flavonoid and anthocyanin compared to the control plants at the optimum temperature. Besides, spermine significantly increased total phenol, flavonoid and anthocyanin contents at both optimum and low temperatures. Comparison of means showed that the combined effect of $TiO_2$ NPs and spermine at both temperatures increased significantly total phenol, flavonoid and anthocyanin content (Figure 6).

**Soluble sugars**

Results from the measurement of the soluble sugars indicated that the amount of glucose, rhamnose and mannose was significantly increased by cold stress in the control plants. Also, spermine alone or together with $TiO_2$ NPs at both temperatures also increased the content of these sugars compared to the control plants (Figure 7).

**Total soluble protein content and antioxidant enzyme activity**

Results showed that the amount of total protein was increased significantly by cold stress. Application of spermine increased the amount of protein at both temperatures compared to control. The combined effect of both concentrations of $TiO_2$ NPs with spermine at both temperatures significantly increased total protein content compared to the control plants (Figure 8A). A significant increase in the activity of antioxidant enzymes was observed under cold stress conditions. Spermine treatment significantly increased superoxide dismutase activity at both temperatures but increased polyphenol oxidase activity only under cold stress conditions. Spermine significantly reduced peroxidase activity at the optimum temperature but increased it at the low temperature. Exposure to spermine at the optimum temperature, significantly reduced ascorbate peroxidase activity, while it had no significant effect at the low temperature. In the presence of spermine, catalase activity significantly increased at the optimum temperature but reduced at the low temperature. Application of $TiO_2$ NPs and spermine together under normal and cold stress conditions significantly increased the activity of these

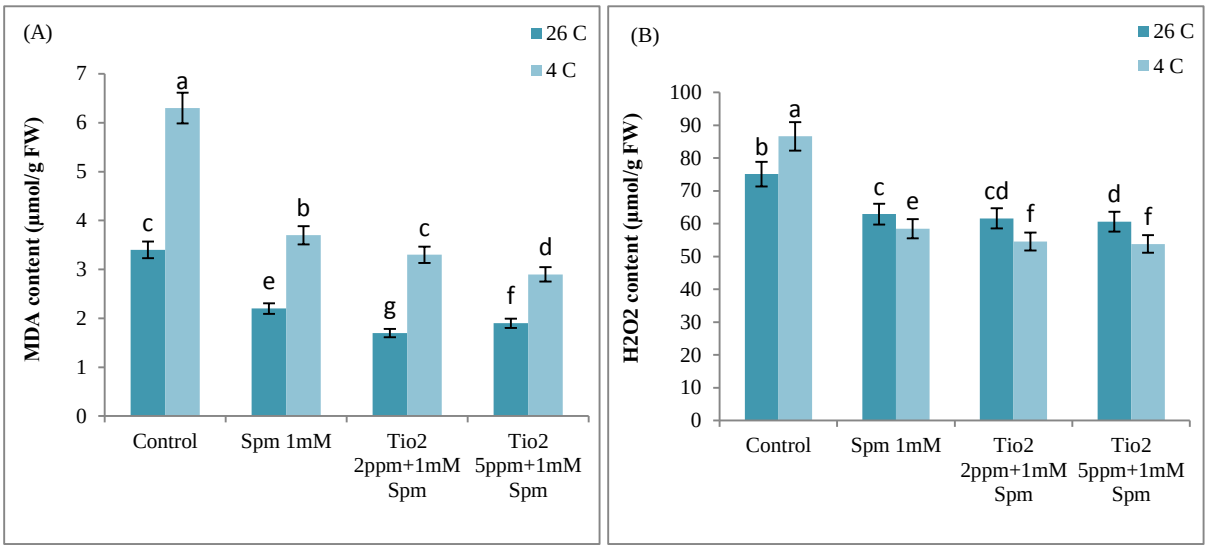


Figure 4. Mean comparison of cell injury indicators in *Glycyrrhiza glabra* seedlings treated with TiO2 nanoparticles and spermine (Spm) at optimum (26 ºC) and low (4 ºC) temperatures. (A): MDA, (B): $H_2O_2$. Means with different letters above the bars indicate significant differences at $p \leq 0.05$

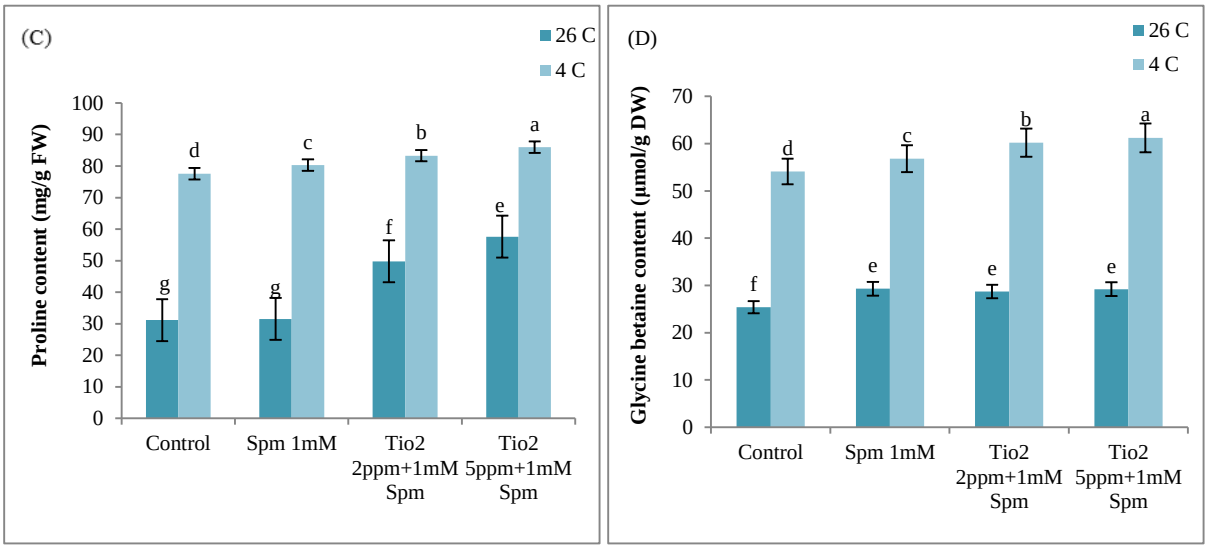


Figure 5. Mean comparison of oxidative damage in *Glycyrrhiza glabra* seedlings treated with TiO2 nanoparticles and spermine (Spm) at optimum (26 ºC) and low (4 ºC) temperatures. (A): Proline, (B): Glycine betaine. Means with different letters above the bars indicate significant differences at $p \leq 0.05$.

antioxidant enzymes (Figure 8B-F).

**Glycyrrhizin content**

Based on the results obtained from HPLC analysis, it was found that cold stress conditions significantly increased the glycyrrhizin content compared to the normal temperature in the control plants (Figure 9). Spermine caused a significant change in the glycyrrhizin content at the optimum temperature but did not affect it at the low temperature. The combined effect of $TiO_2$ NPs and spermine at both thermal treatments caused a significant increase in the glycyrrhizin content compared to the control plants.

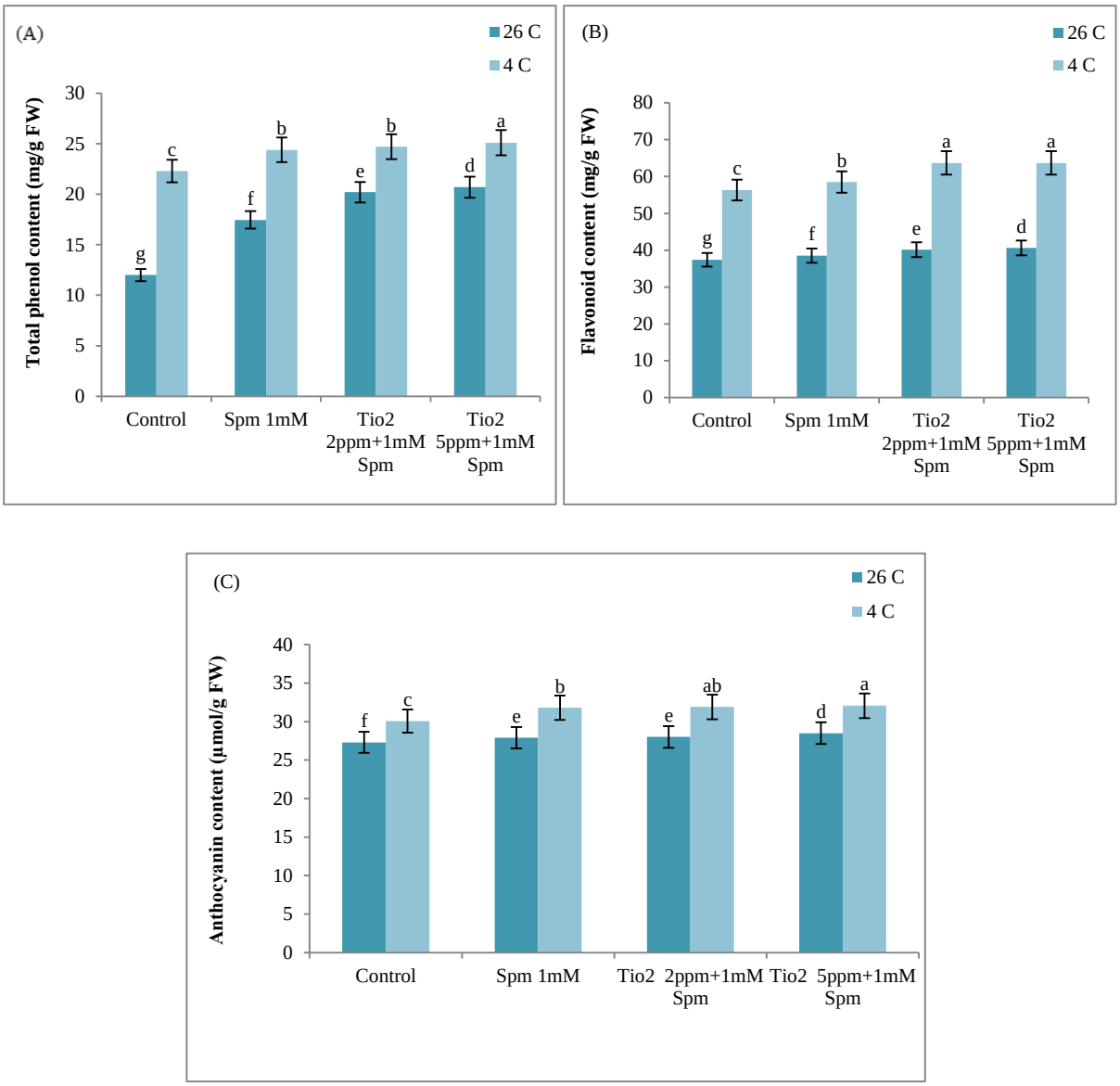


Figure 6. Mean comparison of total phenolics content in *Glycyrrhiza glabra* seedlings treated with TiO2 nanoparticles and spermine (Spm) at optimum (26 ºC) and low (4 ºC) temperatures. (A): Total phenol, (B): Total flavonoid, (C): Total anthocyanin. Means with different letters above the bars indicate significant differences at $p \leq 0.05$.

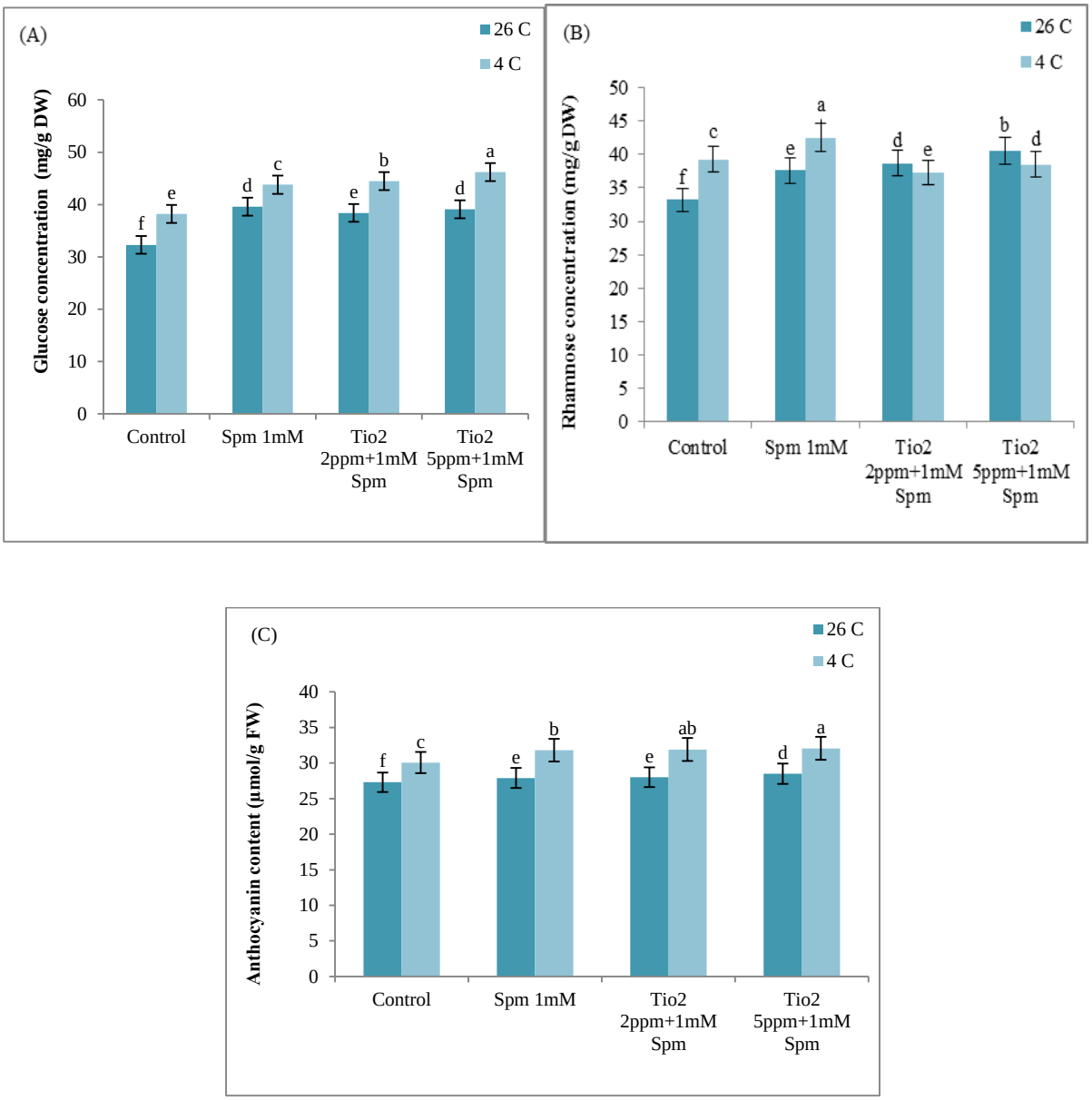


Figure 7. Mean comparison of soluble sugars content in *Glycyrrhiza glabra* seedlings treated with TiO2 nanoparticles and spermine (Spm) at optimum (26 ºC) and low (4 ºC) temperatures. (A): Glucose, (B): Rhamnose, (C): Mannose. Means with different letters above the bars indicate significant differences at $p \leq 0.05$.

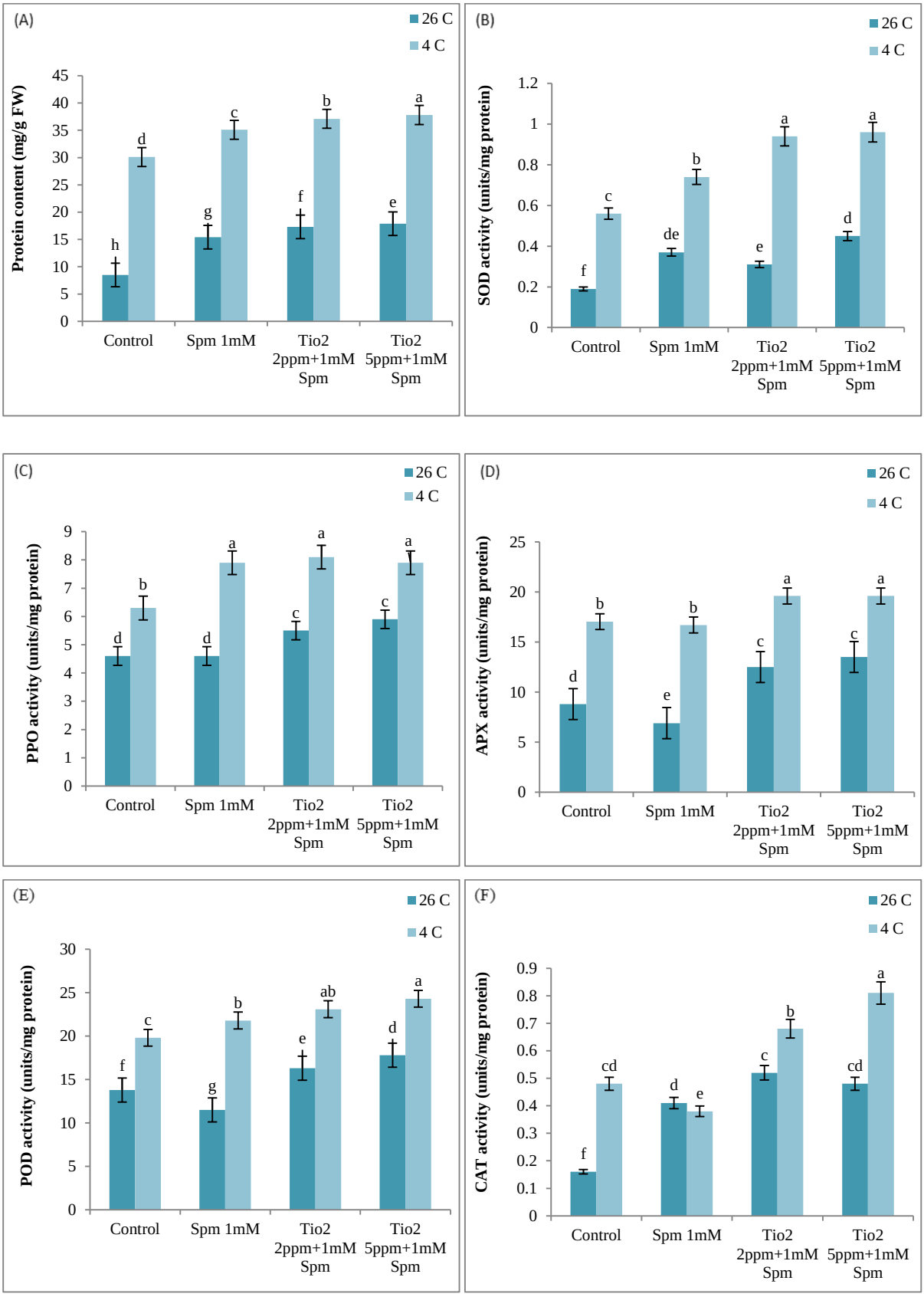


Figure 8. Mean comparison of total protein content and antioxidant enzyme activity in *Glycyrrhiza glabra* seedlings treated with TiO2 nanoparticles and spermine (Spm) at optimum (26 ºC) and low (4 ºC) temperatures. (A): Protein content, (B): SOD activity, (C): PPO activity, (D): POD activity, (E): APX activity, (F): CAT activity. Means with different letters above the bars indicate significant differences at $p \leq 0.05$.

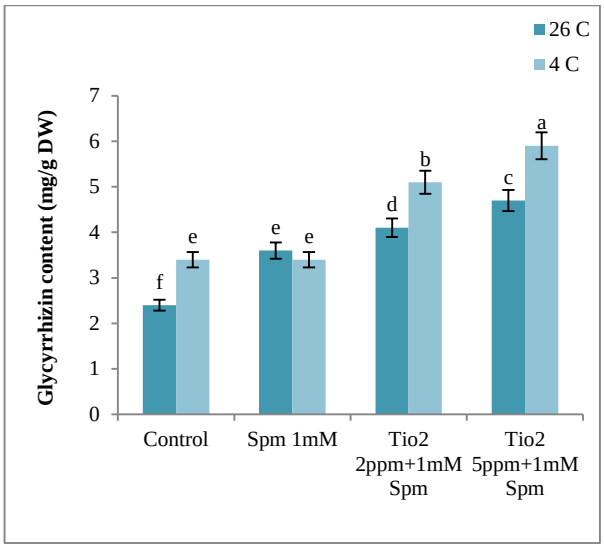


Figure 9. Glycyrrhizin content of *Glycyrrhiza glabra* seedlings treated with TiO2 nanoparticles and spermine (Spm) at optimum (26 ºC) and low (4 ºC) temperatures. Means with different letters above the bars indicate significant differences at $p \leq 0.05$.

**Discussion**

Results from ICP analysis of *Glycyrrhiza glabra* seedlings treated with $TiO_2$ NPs confirmed Ti absorption and accumulation. Ti content of the seedlings increased with an increase in the concentration of nanoparticles. The mechanism responsible for the entry of nanoparticles into the root cells has not been studied sufficiently. However, it may be that nanoparticles penetrate the cell via plasmodesmata. Nanoparticles exert their different functions depending on the size and/or the shape of the particles, the applied concentrations, the specific conditions of experiments, the plant species and the mechanism of uptake. Due to the small size of the $TiO_2$ NPs, they probably pass through plant cell wall cavities and distribute in the cellular compartments (Larue *et al.* 2012).

According to results, cold stress, $TiO_2$ NPs and spermine decreased the fresh and dry weight of the licorice plants. It seems that lowering the temperature probably decreases the production of the plant material and consequently the general growth of the plants by decreasing the rate of respiration and photosynthesis. The increased amount of total phenols after the influence of polyamines was probably one of the reasons for the decline in plant growth. In general, the accumulation of phenolic compounds is one of the defensive responses to biotic and abiotic stresses that results in a decrease in growth (Hirt and Shinozaki 2004).

Our results showed that the content of photosynthetic pigments was decreased by cold stress. The decrease in temperature stops the process of chlorophyll production. Besides, $TiO_2$ NPs and spermine application significantly increased the content of the photosynthetic pigments except for chlorophyll b at the optimum temperature and the low temperature when the $TiO_2$ NPs concentration was 2 ppm. $TiO_2$ NPs can improve chlorophyll structure, increase light

absorption and facilitate chlorophyll formation. It also affects chemical activities and ultimately photosynthesis by transferring light energy to the active electrons (Mahmoodzadeh *et al.* 2013). On the other hand, it has been reported that spermine inhibits chlorophyllase degradation, which is due to its ability to bind to chloroplast proteins. Therefore, further increase of protein-chlorophyll stability delays chlorophyllase degradation (Serafini-Fraccasini *et al.* 2010).

In the present study, cold stress increased MDA content compared to the control plants indicating lipid peroxidation in the licorice seedlings under cold stress. Lipid peroxidation, which leads to the destruction of membranes, imposes oxidative stresses in plants under various stresses including the cold stress. Also, the application of $TiO_2$ NPs and spermine reduced the amount of MDA under cold stress. This finding is in line with Mohammadi *et al.* (2013) who reported that 5 ppm $TiO_2$ NPs reduced MDA production and electrolyte leakage in chickpea under cold stress. The decrease of MDA content in the plants treated with the low concentration of $TiO_2$ NPs results in the stability and improvement of their membrane physical properties. The antioxidant effects of polyamines are mainly related to their cationic properties, which act to remove free radicals and inhibit lipid peroxidation. Besides, polyamines express cold resistance genes in plants (Groppa and Benavides 2008). Also, according to our results, $H_2O_2$ content increased under cold stress compared to normal temperature. One of the changes that occur during plant exposure to cold stress is ROS accumulation (Hassibi *et al.* 2007). Based on our results, the application of $TiO_2$ NPs and spermine may have caused the resistance of seedlings to the cold stress injury, which possibly depend on the efficiency of purifying hydrogen peroxide at low concentrations of $TiO_2$ NPs. Spermine as an antioxidant can bind to $H_2O_2$ due to its cationic structure and reduces its content.

Our results showed that proline content increased under cold stress conditions and also after $TiO_2$ NPs and spermine application. Proline has been identified in many plants and usually accumulates in large quantities in response to stresses. To maintain membrane integrity under stress conditions, protein denaturation must be prevented. Thus, proline by interacting with the enzymes, maintains the protein structure and activity (Nasrollahi *et al.* 2014). Accumulation of proline content in plants exposed to nanoparticles may be a defense mechanism to protect cellular structures from damage due to increased ROS and production of MDA. This may be due to the role of polyamines in supporting proteins and enzymes involved in the proline synthesis, photosynthesis preservation and nutrient modulation. Our results also showed a significant increase in glycine betaine content under cold stress compared to the optimum temperature. Also, the application of $TiO_2$ NPs and spermine increased the amount of this compound in both thermal conditions. Significant evidence indicates that glycine betaine accumulates in the cytoplasm and chloroplasts in response to osmotic stress and acts as a non-toxic material (Hoekstra *et al.* 2001).

The increased total phenol content in response to cold stress and also to $TiO_2$ NPs and spermine application is another result obtained from the present study. Phenolic compounds accumulate in plants in response to biotic and abiotic stresses. Changes in the activity of biosynthetic or

degrading enzymes of phenolic compounds affect the amount of these compounds in the cell. Increased activity of phenylalanine ammonia lyase enzyme (PAL), the first enzyme in the phenol biosynthesis pathway, has been reported under many stresses. In a research study on tobacco plants, application of putrescine under salinity stress, increased PAL actvity (Hajiboland and Ebrahimi 2011). Therefore, the reason for the increase of phenolic compounds in our study may be the increased activity of this enzyme due to spermine application. Our results also showed that the application of $TiO_2$ NPs increased total phenol content under both thermal conditions. There are also some reports about increased phenolic compounds after $TiO_2$ NPs application. Kamalizadeh *et al.* (2015) showed that the low concentration of $TiO_2$ NPs induced the production of phenolic compounds. The results obtained from the present study also showed the increase of flavonoids under cold stress and $TiO_2$ NPs and spermine application. It has been reported that nanoparticles increase the flavonoid content, which can protect plants against oxidative stress. On the other hand, by increasing flavonoids, polyamines prevent oxidative stress and increase cold resistance by scavenging ROS. Anthocyanin content was also increased in the present study by cold stress, which was probably due to increased activity of PAL. Anthocyanin has antioxidant activity that protects cells against ROS. These compounds play a role in preventing lipid peroxidation and uptake of ROS during oxidative stress (Roychoudhury *et al.* 2007).

Results from this study showed that the soluble sugars increased under cold stress conditions and also by $TiO_2$ NPs and spermine compared to the control plants. Soluble sugars act as osmotic regulators, stabilizing cell membranes and maintain cellular turgor. $TiO_2$ NPs increase photosynthesis and efficiency by increasing light absorption and enhance the plant carbohydrate production potential (Abdel Latef *et al.* 2017)

In agreement with several studies, our results indicated that total protein content under cold stress conditions was significantly higher than that of the control plants. The application of $TiO_2$ NPs and spermine also increased protein content under both temperatures. Plants adapt their metabolism to tolerate stresses such as low temperature by synthesis and accumulation of proteins. In a study on two olive cultivars, the total protein content in the cold-tolerant olive cultivar was higher than that of the susceptible cultivar (Afshar Mohammadian *et al.* 2012). Magdy *et al.* (2016) showed an increase in protein content in cotton leaves by $TiO_2$ NPs under drought conditions. On the other hand, polyamines play a role in reducing degradation and enhance the biosynthesis of proteins by eliminating oxygen active radicals (Noah Pisheh and Sheranti 2011).

In accordance with many previous reports, the activity of antioxidant enzymes increases in response to the cold stress, and also the application of $TiO_2$ NPs and spermine. The study by Sariri *et al.* (2016) on tea plants indicated that cold stress could increase SOD, APX and CAT activities. Increased activity of the APX enzyme has been also reported in cold-affected rice plants (Hassibi *et al.* 2007). In another study on bitter orange and grapefruit plants, CAT activity increased as a result of sub-zero temperatures (Sala and Lafuente 1999).

Cold stress increases oxidative stress in plant tissues and plants often increase the activity of their oxidative enzymes to counteract the effects of stress (Habibi and Hajiboland 2011). Exposure to metal oxide nanoparticles produces ROS, thereby causing oxidative stress and activating plant reactions to detoxification such as enzymatic activity (Ma *et al.* 2015). The increase of enzymatic activity could be one of the main mechanisms of protection against increased ROS production (Movafeghil *et al.* 2018). $TiO_2$ NPs can increase antioxidant enzyme activity and reduce the accumulation of oxygen radicals and malondialdehyde (Song *et al.* 2013). Also, polyamines as the signaling molecules, initiate a chain of defense reactions that increase plant tolerance to environmental stresses by increasing the activity of antioxidant enzymes (Syed Sarfraz *et al.* 2011).

Our results also showed that cold stress-induced glycyrrhizin production in the licorice seedlings. The application of spermine and $TiO_2$ NPs also significantly increased the amount of this substance as compared to the control plants. There have been several reports of glycyrrhizin production using different elicitors in licorice. Winida *et al.* (2011) showed that glycyrrhizin content was increased in the licorice plant at 100 mM methyl jasmonate. The increase of glycyrrhizin content has been attributed to the increased expression of the *β*-amirin synthase gene. Hosseinia *et al.* (2018) showed that drought stress increased the expression of glycyrrhizin biosynthetic pathway genes as well as increased levels of this substance in the licorice roots.

## Conclusions

The present study showed that cold stress decreased growth and photosynthetic pigments in the licorice plants, but increased the content of malondialdehyde, $H_2O_2$, proline, glycine betaine, phenolic compounds, sugars and proteins and also the activity of antioxidant enzymes. The application of $TiO_2$ NPs and spermine together could alleviate the adverse effects of cold stress. The production of glycyrrhizin was stimulated by cold stress and application of spermine and $TiO_2$ NPs. Thus, it seems that the application of $TiO_2$ NPs and spermine can be useful to increase stress resistance and to alleviate its negative effects in licorice plants.

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## Conflict of Interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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# اثرات نانوذرات دی اکسید تیتانیوم و اسپرمین بر پاسخ‌های فیزیولوژیکی گیاه شیرین بیان به تنش سرما

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**چکیده**

تنش سرما منجر به القای تغییرات بیوشیمیایی و فیزیولوژیکی در گیاهان می‌شود. بنابراین شناسایی و کاربرد ترکیباتی که قادر به افزایش تحمل گیاه نسبت به تنش سرما باشند، حائز اهمیت است. امروزه استفاده از نانوذرات به عنوان یک راه حل مناسب جهت افزایش تحمل گیاهان نسبت به تنش‌های محیطی مورد توجه قرار گرفته است. همچنین پلی آمین‌ها نقش مهمی در تنظیم پاسخ‌های دفاعی گیاه به تنش‌های محیطی دارند. لذا هدف از این مطالعه، بررسی اثرات کاربرد نانوذرات دی اکسید تیتانیوم و اسپرمین بر پاسخ‌های فیزیولوژیکی گیاه شیرین بیان در برابر تنش سرما بود. نتایج حاصل از این مطالعه نشان داد که تنش سرما موجب افزایش محتوای پروتئین، ترکیبات فنلی، گلایسین بتائین، قندهای محلول، گلیسیریزین، پرولین، پراکسید هیدروژن و مالون دی آلدئید و نیز افزایش فعالیت‌های آنتی اکسیدانی شده، لیکن محتوای رنگیزه‌های فتوسنتزی و شاخص‌های رشد را کاهش داد. اسپرمین اغلب ویژگی‌های سنجش شده را در دمای پائین افزایش داد، لیکن محتوای پراکسید هیدروژن و مالون دی آلدئید را کاهش داد. تولید گلیسریزین توسط تنش سرما و نیز تیمار نانوذرات دی اکسید تیتانیوم و اسپرمین افزایش یافت. کاربرد نانوذرات دی اکسید تیتانیوم و اسپرمین موجب تخفیف آثار زیانبار تنش سرما و بهبود پاسخ‌های گیاه شیرین بیان گردید.

**واژه‌های کلیدی**: آنتی اکسیدان؛ پلی آمین؛ شیرین بیان؛ گونه های اکسیژن فعال