

Effects of TiO₂ nanoparticles and spermine on antioxidant responses of *Glycyrrhiza glabra* L. to cold stress

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Abstract – Licorice (*Glycyrrhiza glabra* L.) is known as an important medicinal plant throughout the world. Glycyrrhizin is one of the most important specialized metabolites produced by licorice. In order to study the effect of TiO₂ nanoparticles (TiO₂ NPs) and spermine on physiological and biochemical traits of licorice under cold stress conditions, a factorial experiment was conducted in a completely randomized design with three replications. Plants were exposed to optimum temperature (26 °C) as control and low temperature (4 °C) as cold stress conditions and also treated with TiO₂ NPs (2 and 5 ppm) and spermine (1 mM), separately. Results from physiological and biochemical analyses of the aerial parts of licorice seedlings showed that the growth parameters and the content of photosynthetic pigments decreased in response to low temperature. TiO₂ NPs and spermine treatments increased plant resistance to cold stress and decreased the level of oxidative damage by reduction of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) contents. In other hand, TiO₂ NPs and spermine caused increase of phenolics, total protein and osmolytes contents under cold stress conditions. An increase in glycyrrhizin content was significantly induced by low temperature, TiO₂ NPs and spermine.

Keywords: antioxidative defense, glycyrrhizin, licorice, low temperature, polyamine

Introduction

Licorice (*Glycyrrhiza glabra* L., Fabaceae) is a plant species native to Eastern Europe and Southwest Asia, including Iran. The roots of the plant are valuable sources of useful secondary metabolites such as glycyrrhizin and phenolic compounds used in the pharmaceutical and food industries. The triterpenoid saponin of glycyrrhizin is the major active constituent of licorice root and is 30–50 times sweeter than sucrose. Glycyrrhizin and its derivatives possess anti-inflammatory and antioxidant activities and are involved in plant defense responses (Yin et al. 2017).

Various environmental conditions such as climatic changes, seasonal variations, light, temperature, humidity, and soil conditions can affect the content of plant secondary metabolites including saponins. The plants exhibit several mechanisms to protect cells against environmental stresses and attempt to withstand changes in metabolism by reducing the damage caused by stresses (Yordanov et al. 2003). Stress factors rarely induce only isolated effects and there are usually concurrent effects. Cold stress is one of the limiting factors of survival and growth and plays an important

role in ecological distribution of organisms. All organisms have developed various mechanisms for adaptation against the cold. Cold adaptability involves reprogramming gene expression in a variety of metabolic pathways such as biosynthesis of proteins and fats and accumulation of osmolyte compounds such as proline, glycine betaine, and polyamines (Kabiri et al. 2012).

Polyamines are important polycations like putrescine, spermidine and spermine that play important roles in a wide range of physiological processes including growth and development, stimulation of cell division, DNA synthesis and proteins, rooting control and response to environmental stresses such as cold, heat, drought and salinity (Navakoudis et al. 2007). It was demonstrated that exogenous application of 1 mM spermine reduced chilling injury during low temperature storage of grape berries, leading to maintenance of fruit quality and shelf life (Harindra Champa et al. 2015).

Nanoparticles (NPs) in agricultural systems can potentially be used as an appropriate candidate for change in the growth, development, productivity, and quality of plants. They have small particle size and increase contact with materials. Accordingly, plants are most commonly used to de-

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termine the effect of nanoparticles (Nair et al. 2010). Titanium dioxide nanoparticles are used in many industries due to their specific physical and chemical properties. Nano-titanium dioxide enhances the ability of plants to absorb sunlight that affects the production and conversion of light energy to active electrons and chemical activities and increases the efficiency of photosynthesis (Akbari et al. 2014). Several reports have shown that titanium dioxide nanoparticles reduce the destructive effects of stresses by increasing the activity of antioxidant enzymes and reducing the free radicals of oxygen and MDA (Zheng et al. 2007). However, effects of TiO₂ NPs on plants differ depending on plant species, applied concentration, duration and type of Ti structure. TiO₂ NPs in an anatase crystal structure are toxic in all of the tested organisms at high concentrations. In contrast, because of the lipophilicity of TiO₂ NPs in the rutile form, they can produce larger aggregates in an aqueous medium, which promotes a reduced effect on biological organisms and a lower toxicity than the anatase form (Clément et al. 2013). In most previous reports, interactions of different forms of TiO₂ NPs with several plant species at different concentrations were studied under optimum conditions. TiO₂ NPs up to 2 ppm improved growth and photosynthesis efficiency in *Lycopersicon esculentum* Mill., but had negative effects on growth and fresh weight of the roots (Song et al. 2013). Very few studies have previously been conducted on TiO₂ NPs application under various environmental stresses. For example, the effect of TiO₂ NPs (as anatase form at 2–500 ppm concentrations) on *Cicer arietinum* L. genotypes (Fabaceae) was investigated under cold stress conditions. Results from this study revealed that low concentrations of TiO₂ NPs (up to 5 ppm) alleviated cold-induced damages in the studied genotypes (Mohammadi et al. 2013). Cold stress conditions induces oxidative processes in plant cells by production of reactive oxygen species (ROS), which interact nonspecifically with many cellular components, triggering peroxidative reactions and causing significant damage to essential macromolecules and especially damaging the membranes as the primary site of cold injury. TiO₂ NPs by induction of plant antioxidant systems alleviate accumulation of MDA, which can be considered an evaluation factor of membrane damage (Stam-poulis et al. 2009).

In order to increase resistance to stress, modify negative effects of stress, and increase the qualitative properties of growth, application of TiO₂ NPs and polyamines may be useful. Thus, in this study the effects of TiO₂ NPs and spermine on physiological and biochemical responses of licorice plants were investigated under cold stress conditions.

Materials and methods

Plant material and growth conditions

Seeds of *Glycyrrhiza glabra* plants were provided from the Pakan-Bazr Seed Production Company (Isfahan, Iran). The seeds were disinfected using NaClO 20% for 10 min and then washed 3 times in sterile distilled water. TiO₂NPs were purchased from US Research Nanomaterials (USA) and had

an average diameter of 10–25 nm (99% anatase). TiO₂NPs were dispersed in filtered double-distilled water in an ultrasonic bath for at least 20 min and then added to the cultures prior to autoclaving at 120 °C for 15 min. Spermine (Sigma) solution was first sterilized by a 0.2 µm filter and then added to MS (Murashige and Skoog1962) liquid culture medium after autoclaving. The medium pH was adjusted to 5.7–5.8 before autoclaving. Eight seeds were cultured in each jar containing MS basal medium, 3% sucrose and 0.7% agar. The TiO₂ nanoparticles were used at 0, 2 and 5 ppm and spermine at 0 and 1 mM concentrations, separately. Cultures without any TiO₂ NPs and spermine were considered controls. All cultures were incubated in a growth chamber at 26 ± 2 °C, with an irradiance of 4000 Lux provided by white light fluorescent lamps, under a 16:8 h light/dark photoperiod for 30 days. The 30-day-old seedlings were divided into two groups of thermal treatment, one group was maintained under control condition (26 °C) and the other group at 4 °C as cold treatment for 2 days. Then, the aerial parts of treated seedlings were used for all assays. All experiments were conducted in triplicate.

Analysis of growth parameters

The 30-day-old seedlings were used to measure fresh, dry and turgid weight. Fresh weight was measured using a digital scale. In order to determine the turgid weight, seedlings were washed in distilled water and maintained in the dark for 72 h and then their weight was measured. In order to determine dry weight, seedlings were placed in an oven at 70 °C for 48 h and then their weight was measured. Relative water content (RWC) was determined according to following formula:

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

Where, FW: Fresh weight; DW: Dry weight; TW: Turgid weight.

Analysis of biochemical characteristics

Aerial parts of the treated seedlings were cut and washed in distilled water. Then they were oven-dried at a constant 70 °C for 24 h and digested on a hot block with concentrated HNO₃ for 1 h at 60 °C. The samples were cooled to room temperature. Bioaccumulation of Ti in the digested samples was evaluated by inductively coupled plasma mass spectrometry (ICP-MS, HP-4500, USA) (Wu et al. 2007).

To measure the glycyrrhizin content 0.4 g of the dried samples was subjected to extraction using 1 mL 80% (v/v) methanol at 60 °C for 6 h and then centrifuged at 4000 g for 10 min at room temperature. The supernatant was transferred to a new tube and 1 mL 80% methanol was added and centrifuged again. The extract was quantified by high performance liquid chromatography (HPLC) using a reverse-phase column (150×4.6 mm i.d.; 5µm) SB-C8 (Agilent, USA, Zorbax). The mobile phase flow rate was 1 mL min⁻¹ and glycyrrhizin was detected at 254 nm and 25 °C. The glycyrrhizin content was calculated using a standard chromatogram (Orujei et al. 2013).

Photosynthetic pigments were extracted by grinding 0.5 g of the fresh sample in 0.5 mL acetone (80% V/V). The absorption was recorded at 647, 663 and 470 nm. The pigment content was calculated as mg g⁻¹ FW (Lichtenthaler and Wellburn 1983).

Lipid peroxidation was determined by measuring MDA content, following the method described by Heath and Packer (1968) with slight modifications. The amount of MDA was calculated by using an extinction coefficient of 155 mM⁻¹ cm⁻¹. The generation of H₂O₂ was measured according to Velikova et al. (2000). The content of H₂O₂ was calculated using the molar extinction coefficient 0.28 mol⁻¹ cm⁻¹ ($\epsilon_{530} = 0.28 \text{ mol}^{-1} \text{ cm}^{-1}$).

Free proline content was determined according to Bates et al. (1973). Glycine betaine content was determined according to Grieve and Grattan (1983) using a standard curve, and expressed in $\mu\text{mol g}^{-1}$ DW.

The content of total phenols of the methanolic extracts was evaluated following the method of Plessi et al. (2007). Flavonoid content was measured using the aluminum chloride method and calculated by quercetin standard curve (Chang et al. 2002). Total anthocyanin content was determined as described by Wagner (1979).

To measure the content of soluble sugars (glucose, rhamnose and mannose), 100 mg of the fresh samples was homogenized with 10 mL 95% ethanol, and extracts were centrifuged at 6000 g for 15 min. Then, the upper phase of the centrifuged samples was supplemented with 3 mL anthrone (150 mg anthrone in 100 mL 72% H₂SO₄) and maintained at 100 °C for 10 min in boiling water. Then, the absorbance was read at 485 nm (glucose), 480 nm (rhamnose) and 490 nm (mannose) (Irigoyen et al. 1992). The content of soluble sugars was determined using glucose, mannose and rhamnose standard curves and expressed as mg g⁻¹ DW.

To extract protein, 0.5 g of the fresh samples was ground in liquid nitrogen and homogenized in phosphate buffer (50 mM, pH 7.0) containing 1% (w/v) polyvinyl pyrrolidone at 4 °C. The homogenate was centrifuged at 13000 g and 4 °C for 20 min. The supernatant was used for enzyme and protein assays. Total protein content was determined according to Bradford (1976) and expressed in mg g⁻¹ FW.

The activity of superoxide dismutase (SOD) was evaluated by inhibiting the photochemical reduction of 3-nitro blue tetrazolium (NBT) by an enzyme-containing plant extract (Winterbourn et al. 1976). One unit of enzyme activity, the amount of enzyme that inhibits NBT reduction by up to 50%, was considered. Peroxidase (POD) and catalase (CAT) activities were evaluated based on 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 performed according to the Nakano and Asada (1981) method.

Statistical analysis

All data were analyzed using a factorial randomized complete block design with three replications. Error bars

of graphs show the standard error (S.E.) of the mean values. The data were subjected to analysis of variance (ANOVA) followed by Duncan's test at $P < 0.05$ using with the SPSS statistical software program (SPSS Inc, ver. 23, SPSS Inc., Chicago, IL).

Results

Analysis of growth parameters

Cold stress significantly decreased fresh, dry, and turgid weights. However, TiO₂ NPs increased these parameters at both optimum and low temperatures. With increasing TiO₂ NPs concentration up to 5 ppm, fresh, dry, and turgid weights increased at both optimum and low temperatures. Significant decreases in fresh, dry and turgid weights were induced by spermine at optimum and both temperatures. Mean comparison of the relative water content showed that cold stress caused significant reduction of RWC compared to control. TiO₂ NPs (2 and 5 ppm) and spermine significantly increased RWC at optimum temperature compared to control. A RWC level of 65% was reached with 5 ppm TiO₂ NPs under optimum temperature. RWC was significantly increased only at 2 ppm TiO₂ NPs under cold stress conditions compared to control (Fig. 1).

Analysis of biochemical characteristics

Results from ICP analysis of Ti NPs-treated seedlings confirmed Ti accumulation in aerial parts, which was increased by increasing nanoparticle concentration. In addition, low temperature increased Ti content compared to optimum temperature at different applied Ti concentrations (Fig. 2a).

The mean comparison of glycyrrhizin content showed that cold stress increased glycyrrhizin content compared to control. The highest glycyrrhizin content was observed at 5 ppm TiO₂ NPs under cold stress conditions. Spermine caused a significant change in the glycyrrhizin content at optimum temperature, but had no effect at low temperature (Fig. 2b).

The content of photosynthetic pigments was decreased by cold stress. Plants treated with TiO₂ NPs showed a significant increase in Chl *a*, Chl *b*, total Chl and carotenoid contents at both optimum and low temperatures compared to control plants. The increase of nanoparticle concentration did not cause any significant changes except in total Chl and Chl *b* contents at optimum temperature, but did however increase significantly the content of all photosynthetic pigments at low temperature. In addition, spermine increased the content of photosynthetic pigments at both optimum and low temperatures, except for Chl *b* at optimum temperature (Fig. 3).

The amount of MDA was increased significantly by cold stress. TiO₂ NPs (2 and 5 ppm) and spermine caused significant decrease in MDA content at low temperature (Fig. 4a). On the other hand, the amount of MDA increased at 5 ppm TiO₂ NPs and optimum temperature. Results also showed

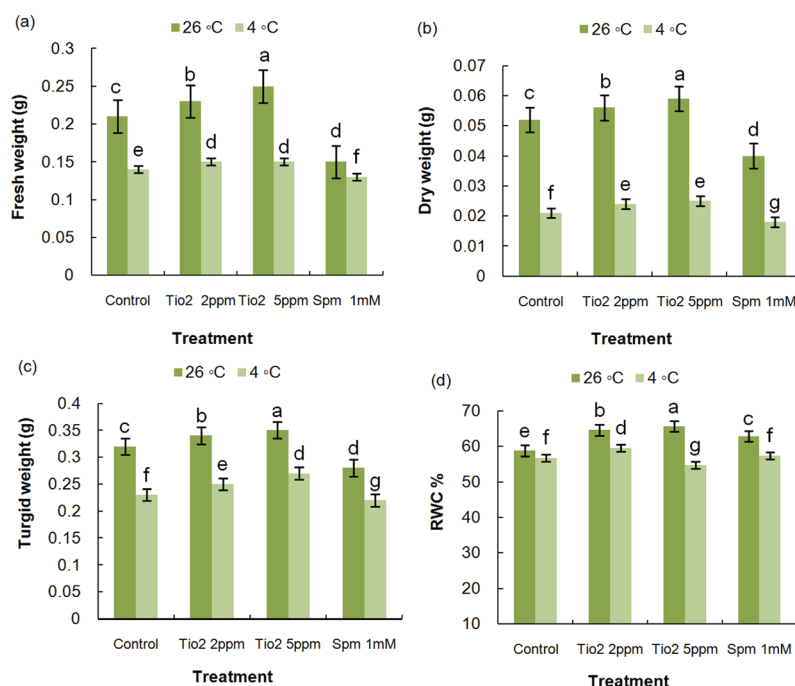


Fig. 1. Growth parameters of *Glycyrrhiza glabra* seedlings exposed to TiO₂ nanoparticles (TiO₂) and 1 mM spermine (Spm) under optimum temperature (26 °C) as control and low temperature (4 °C) as cold stress conditions: a – fresh weight, b – dry weight, c – turgid weight, d – Relative water content (RWC). Mean ± standard error of 3 replicates is presented. Different letters above the bars indicate significant differences at P < 0.05.

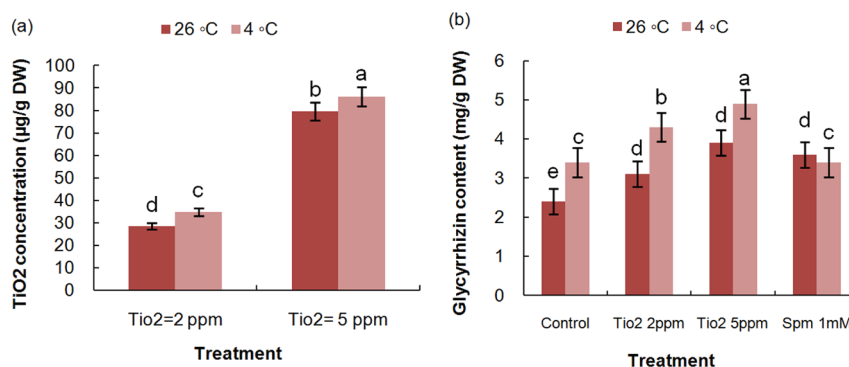


Fig. 2. TiO₂ and glycyrrhizin contents of *Glycyrrhiza glabra* seedlings dry weight (DW) exposed to TiO₂ nanoparticles (TiO₂) and spermine (Spm) under optimum temperature (26 °C) as control and low temperature (4 °C) as cold stress conditions: a – TiO₂, b – glycyrrhizin. Mean ± standard error of 3 replicates is presented. Different letters above the bars indicate significant differences at P < 0.05.

that the highest content of hydrogen peroxide was observed at low temperature without any treatments (Fig. 4b). TiO₂ NPs caused a significant decrease of hydrogen peroxide content under both temperatures. In addition, spermine reduced the amount of hydrogen peroxide at both temperatures. The lowest amount of hydrogen peroxide was observed in the spermine-treated seedlings at low temperature and in the 5 ppm TiO₂ NPs-treated seedlings at optimum temperature.

The mean comparison of proline content showed that cold stress caused significant increase in proline content as compared to control. In addition, TiO₂ NPs increased proline content at both optimum and low temperatures as compared to control. Spermine had no effect on proline content at optimum temperature, but increased it at low temperature (Fig. 5a). In addition, glycine betaine content significantly increased under cold stress conditions. TiO₂ NPs and

spermine treatments significantly increased glycine betaine content compared to control at both optimum and low temperatures (Fig. 5b).

Evaluation of the phenolic compounds showed that cold stress significantly increased the content of total phenol, flavonoid and anthocyanin. TiO₂ NPs significantly increased total phenol, flavonoid and anthocyanin contents at both optimum and low temperatures. TiO₂ NPs (except for 2 ppm) did not cause any significant change in anthocyanin content under cold stress conditions. The highest amounts of total phenol and flavonoid were achieved with 5 ppm TiO₂ NPs application and the lowest contents of total phenol, flavonoid and anthocyanin were observed under normal conditions. In addition, spermine significantly increased total phenol, flavonoid and anthocyanin contents at both optimum and low temperatures (Fig. 6).

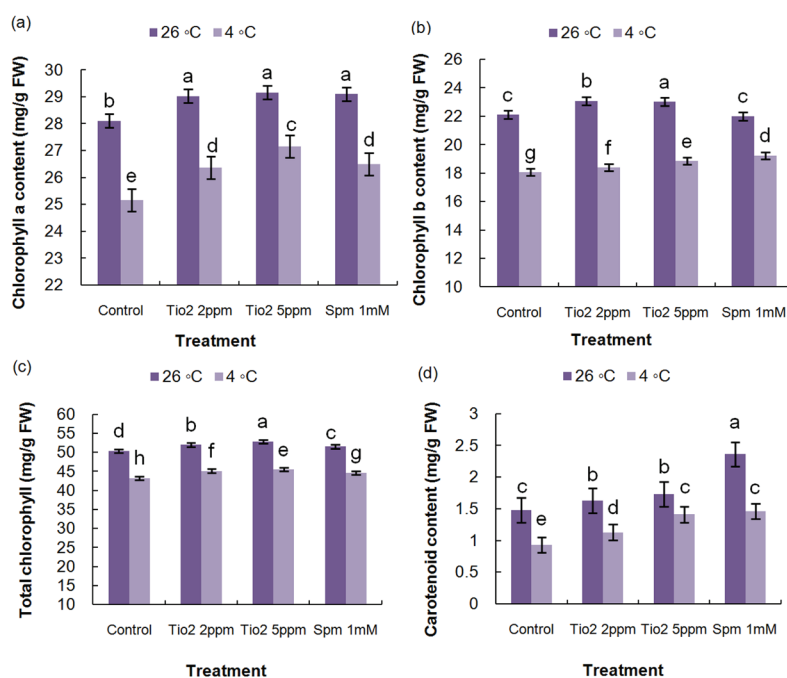


Fig. 3. Photosynthetic pigment content of *Glycyrrhiza glabra* seedlings fresh weight (FW) exposed to TiO₂ nanoparticles (Tio2) and 1 mM spermine (Spm) under optimum temperature (26 °C) as control and low temperature (4 °C) as cold stress conditions: a – chlorophyll a, b – chlorophyll b, c – total chlorophyll, d – carotenoid. Mean ± standard error of 3 replicates is presented. Different letters above the bars indicate significant differences at P < 0.05.

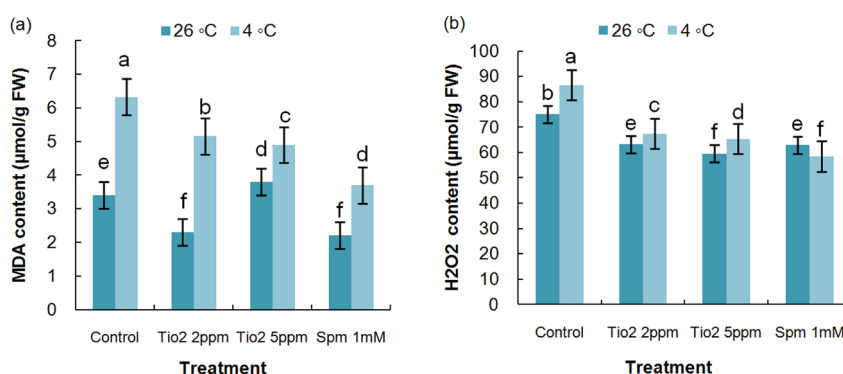


Fig. 4. Oxidative damage in *Glycyrrhiza glabra* seedlings fresh weight (FW) exposed to TiO₂ nanoparticles (Tio2) and 1 mM spermine (Spm) under optimum temperature (26 °C) as control and low temperature (4 °C) as cold stress conditions: a – malondialdehyde (MDA), b – H₂O₂. Mean ± standard error of 3 replicates is presented. Different letters above the bars indicate significant differences at P < 0.05.

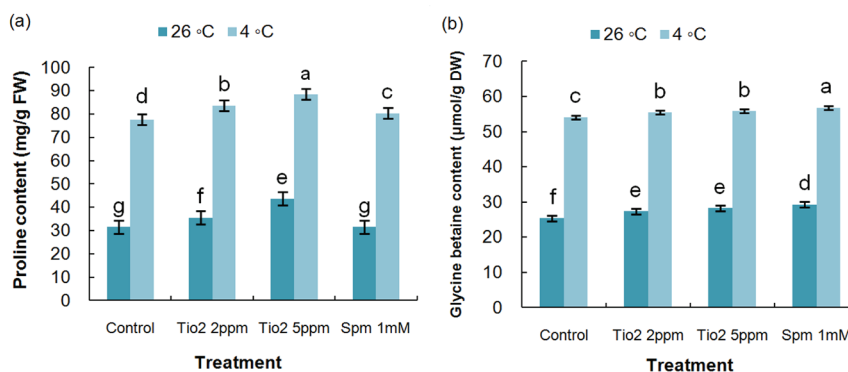


Fig. 5. Osmolyte content of *Glycyrrhiza glabra* seedlings exposed to TiO₂ nanoparticles (Tio2) and 1 mM spermine (Spm) under optimum temperature (26 °C) as control and low temperature (4 °C) as cold stress conditions: a – proline in fresh weight (FW), b – glycine betaine in dry weight (DW). Mean ± standard error of 3 replicates is presented. Different letters above the bars indicate significant differences at P < 0.05.

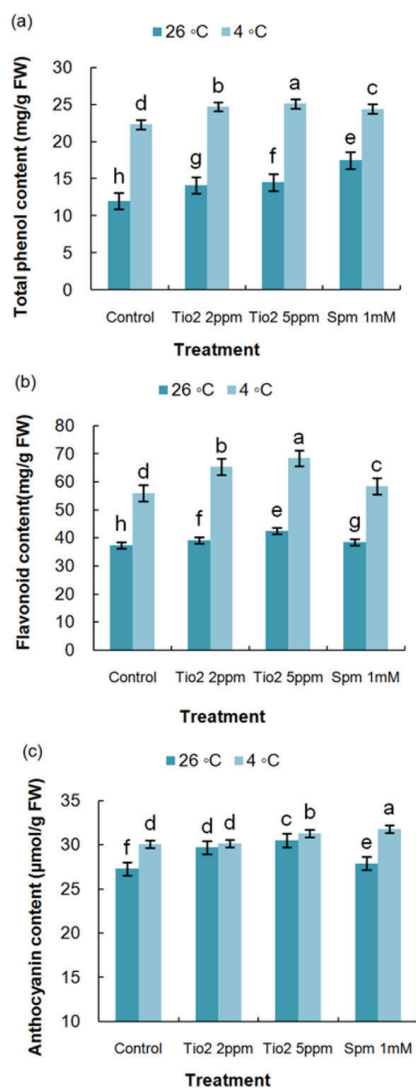


Fig. 6. Phenolic content of *Glycyrrhiza glabra* seedlings fresh weight (FW) exposed to TiO₂ nanoparticles (Tio2) and 1 mM spermine (Spm) under optimum temperature (26 °C) as control and low temperature (4 °C) as cold stress conditions: a – total phenol, b – total flavonoid, c – total anthocyanin. Mean ± standard error of 3 replicates is presented. Different letters above the bars indicate significant differences at P < 0.05.

Based on our results cold stress significantly increased glucose, rhamnose and mannose concentrations compared to control. At both temperatures, application of 2 and 5 ppm TiO₂ NPs and spermine significantly increased the content of soluble sugars as compared to control (Fig. 7).

The mean comparison of total protein content showed a significant change in protein content under cold stress conditions (Fig. 8a). At low temperature, total protein content was significantly higher than that at optimum temperature. TiO₂ NPs increased the protein content at both optimum and low temperatures. The minimum content of total protein was obtained in control seedlings at optimum temperature. Application of spermine increased the amount of protein at both temperatures compared to control. A significant increase in the activity of antioxidant enzymes was

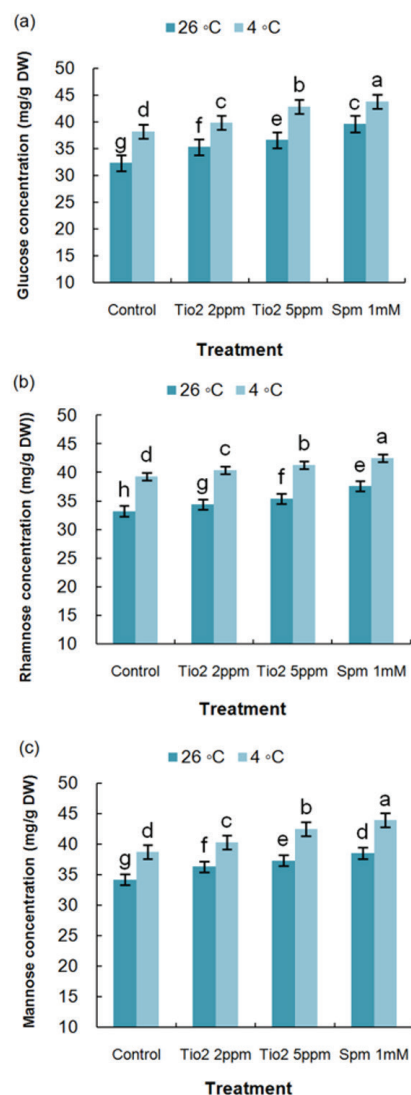


Fig. 7. Soluble sugar content of *Glycyrrhiza glabra* dry weight (DW) exposed to TiO₂ nanoparticles (Tio2) and 1 mM spermine (Spm) under optimum temperature (26 °C) as control and low temperature (4 °C) as cold stress conditions: a – glucose, b – rhamnose, c – mannose. Mean ± standard error of 3 replicates is presented. Different letters above the bars indicate significant differences at P < 0.05.

observed under cold stress conditions. In addition, TiO₂ NPs application increased the activity of these enzymes at both temperatures. However, except for 2 ppm concentration, TiO₂ NPs did not change POD activity. In addition, spermine significantly increased SOD activity at both temperatures (Fig. 8b). Spermine increased PPO activity under cold stress conditions; however, at optimum temperature it did not have a significant effect (Fig. 8c). Spermine significantly reduced POD activity at optimum temperature, but increased it at low temperature (Fig. 8d). Exposure to spermine at optimum temperature significantly reduced APX activity, but at low temperature had no significant effect (Fig. 8e). In the presence of spermine, CAT activity significantly increased at optimum temperature, but not at low temperature (Fig. 8f).

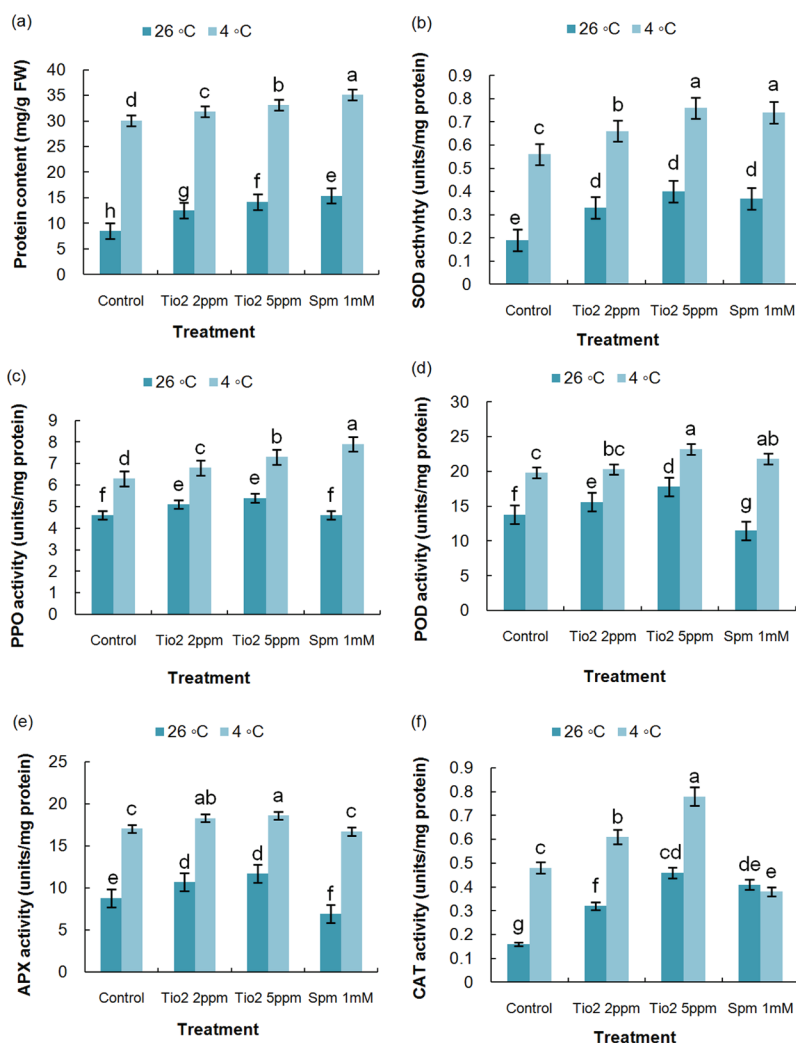


Fig. 8. Total protein content and antioxidant enzyme activity of *Glycyrrhiza glabra* seedlings exposed to TiO₂ nanoparticles (Tio2) and 1 mM spermine under optimum temperature (26 °C) as control and low temperature (4 °C) as cold stress conditions: a – total protein content in fresh weight (FW), b – superoxide dismutase (SOD) activity, c – polyphenol oxidase (PPO) activity, d – peroxidase (POD) activity, e – ascorbate peroxidase (APX) activity, f – catalase (CAT) activity. Different letters above the bars indicate significant differences at P < 0.05.

Discussion

Titanium as a beneficial element stimulates plant growth and increases the absorption of certain elements such as nitrogen, phosphorus, calcium, magnesium, iron, manganese and zinc. The absorption of these elements depends on the moisture content, plant variety or species, soil pH and the status of the nutrients in the soil (Kuzel et al. 2003). It has been reported that titanium dioxide nanoparticles increase the dry weight, chlorophyll biosynthesis, rubisco activity, and photosynthesis rate and nitrogen level in spinach (Zheng et al. 2007). Our results from ICP analysis of *Glycyrrhiza glabra* seedlings treated with TiO₂ NPs confirmed Ti accumulation in aerial parts, which increased with an increase in the concentration applied. The mechanism responsible for nanoparticle entry into the root cells has not been studied sufficiently. However, it may be that nanoparticles penetrate the cell via plasmodesmata. Because of the small size of TiO₂ NPs, they might pass through cell wall pores and be dis-

tributed in subcellular compartments (Fleischer et al. 1999). Nanoparticles including TiO₂ NPs exert their different functions depending on their size and shape, applied concentrations, experiment conditions, plant species and mechanism of uptake (Nekrasova et al. 2011). Our results showed increase of Ti accumulation at low temperature. It is expected that TiO₂ NPs uptake can induce physiological responses to cold stress. The higher Ti accumulation at low temperature could be due to the direct or indirect effects of cold stress on plasma membrane and morphoanatomical characters of plants (Kazemi Shahandashti et al. 2014).

Based on our results, cold stress and TiO₂ NPs application increased the content of glycyrrhizin in spermine-treated seedlings. Several reports showed that the amount of enzymatic and nonenzymatic antioxidants like glycyrrhizin of licorice plants tended to increase under drought stress (Nasrollahi et al. 2014). Squalene synthase (SQS), β -amyrin synthase (BAS), cycloartenol synthase (CAS) and lupeol synthase (LUS) enzymes are known to be involved in

the synthesis of glycyrrhizin. Hosseini et al. (2018) in a study on licorice under drought conditions found that drought stress increased the expression of glycyrrhizin biosynthetic pathway genes and increased the amount of this substance in roots. In addition, the content of glycyrrhizin increased in response to elicitors such as jasmonates and salicylic acid (Winida et al. 2011). Increasing evidence indicated that glycyrrhizin exerts antioxidant effect and reduces oxidative damage (Kim and Lee 2008).

Growth reduction is one of the clearest plant responses to cold stress. Results from the present study showed that both dry and fresh weights of *G. glabra* seedlings were significantly reduced by cold stress. The most important response of plants sensitive to cold stress is the reduction of photosynthesis efficiency. This can lead to light damage or diminish PSII activity even at moderate levels of light, which is associated with decrease of carbon metabolism and electron transfer products (Joshi et al. 2007). Our results also showed decrease of photosynthetic pigments and growth parameters under cold stress conditions. TiO₂ NPs can improve the structure of chlorophyll, increase light absorption and facilitate the formation of chlorophyll. In agreement with our results, TiO₂ NPs increased chlorophyll biosynthesis in bean plants, because of their positive effects on nitrogen uptake (Raliyal et al. 2014). According to our results, spermine increased photosynthetic pigments at low temperature, which is consistent with the results of Cohen et al. (1979) indicating that polyamines prevent chlorophyll degradation under stress conditions.

According to our results, under cold stress conditions the highest amount of H₂O₂ was generated. Abiotic stresses could stimulate the NADPH oxidase activity in the plasma membrane, and produce H₂O₂ as a potent ROS (Hao et al. 2006). In TiO₂ NPs and spermine-treated seedlings, generation of H₂O₂ was significantly reduced. Reactive oxygen species are produced in chloroplasts and mitochondria in response to environmental stresses and are toxic to the cells. In the absence of certain protective mechanisms, they can seriously disrupt natural metabolism through oxidative damage to membrane lipids, proteins and nucleic acids (Davey et al. 2005). One of the main products of lipid peroxidation is MDA, which can cause protein damage by reactions with lysine amino groups, cysteine sulfhydryl groups and histidine imidazole substitutes (Refsgaard et al. 2000). MDA has been well recognized as a parameter reflecting damage by cold stress (Davey et al. 2005). Results from the present study showed significantly increased MDA content in conditions of cold stress. The decrease of MDA content in the seedlings treated with the low concentration of TiO₂ NPs has been attributed to stabilized composition and improved physical properties of their cell membranes (Mohammadi et al. 2013). TiO₂ NPs can also improve the activity of antioxidant enzymes, that may be the other reason behind reduced lipid peroxidation and improved membrane integrity under cold stress (Lei et al. 2008). The increase of MDA formation and lipid peroxidation in seedlings treated with higher concentrations of TiO₂ NPs may be due to the genotoxicity and cy-

tototoxicity of these particles (Ghosh et al. 2010). Results from the present study indicated that the effects of TiO₂ NPs are concentration-dependent and can be cytotoxic to licorice seedlings at 5 ppm concentration. TiO₂ NPs at 5 ppm that increased MDA content at optimum temperature may exceed the special threshold. The role of polyamines in protecting plants against lipid peroxidation is a protective mechanism of the cell to prevent oxidative damage. This group of compounds possesses antioxidant properties, the ability to neutralize acid, cell wall and membrane stability, and prevent degradation under stress conditions (Liu et al. 2007). Results from the present study showed that spermine significantly reduced the amount of MDA under cold stress, which can be attributed to the role of polyamines in ROS elimination.

The most important biochemical reaction of plant cells to environmental stresses including drought, salinity, and cold is the accumulation of organic metabolites like proline, glycine betaine and sucrose. Osmotic regulators such as proline also maintain cellular water, and turgor pressure may contribute to protecting macromolecules, cellular structures, and neutralizing free radicals (Kiara and Roy 1999). There are several studies on proline production in plants and multiplier increase in proline accumulation under stress conditions (Ahmadi and Ardekani 2006). Our results showed that cold stress significantly increased proline content more than twofold compared to control. Proline content was also increased by TiO₂ NPs at both optimum and low temperatures. It seems that TiO₂ NPs not only improve the activity of antioxidant enzymes, but also induce proline synthesis, which is responsible for maintaining cell turgor under cold stress conditions. TiO₂ NPs increased the amount of proline under water-deficit conditions in wheat (Aliabadi et al. 2016) and cotton (Magdy et al. 2016) plants. Our results indicated that spermine had no significant effect at optimum temperature, but significantly increased proline content at low temperature. Application of spermine and putrescine in rice caused proline accumulation in leaves under water deficit conditions and reduced the harmful effects of the stress (Farooq et al. 2009). In addition, accumulation of glycine betaine in higher plants in response to salinity, drought and cold stress has been widely reported (Wani et al. 2013). It has been reported that glycine betaine may be involved in inhibiting ROS accumulation, protecting photosynthetic apparatus, activating some stress-related genes, and protecting the membrane (Chen and Murata 2008). Glycine betaine also reduces the harmful effects of environmental stress by protecting the fourth structure of proteins and thereby maintaining enzyme activity. The results of this study also showed a significant increase of glycine betaine content under cold stress compared to control. In addition, the application of TiO₂ NPs and spermine increased the amount of this compound at both temperatures.

Phenolic compounds are derivatives of the phenylpropanoid pathway and are a part of the non-enzymatic and antioxidant defense system of the plant cells. These compounds can act as scavengers of free oxygen radicals or essential antioxidants to protect plants against the progression

of the oxidative chain (Ksouri et al. 2007). In this study, the amount of phenolic compounds increased under cold stress conditions. TiO₂ NPs and spermine treatments at both optimum and low temperatures increased the amount of these compounds. The increase of phenolic compounds is probably due to their antioxidant roles in response to cold stress as well as ROS neutralization. Anthocyanins are one of the most important antioxidant compounds that not only eliminate free radicals but also prevent their production in plants (Tripathi et al. 2006). Previous studies indicated a strong relationship between cold stress and anthocyanin accumulation in different plants such as grape, maize, blood orange, and apple (Ubi et al. 2006). Our results also showed the increase of antocyanin content under cold stress conditions.

In other hand, plant cells synthesize and accumulate some proteins, sugars and organic acids to protect plants in response to cold stress. Accumulation of soluble proteins can reduce the intracellular free water and prevent the formation of ice crystals in the intrinsic medium and prevents cell damage (Griffith and Yaish 2004). Titanium nanoparticles increase photosynthesis with increased light gain and carbohydrate production. Application of 0.01% TiO₂ NPs was able to increase the soluble sugar content in green bean plants (Abdel Latef et al. 2017). Polyamines play an important role in the synthesis of carbohydrates and act as plant growth regulators in some biological processes associated with carbohydrate biosynthesis. The results of this study indicated increased amounts of soluble sugars and protein under cold stress conditions. In addition, spermine and TiO₂ NPs treatments increased the content of these compounds at both optimum and low temperatures.

The concentration of H₂O₂ as a potent ROS increased under cold stress conditions. In the presence of spermine and TiO₂ NPs, the reduction of H₂O₂ may be due to increasing proline content, which has antioxidant feature, and to an elevated antioxidative defense system that could also minimize ROS generation. The ROS levels under stress conditions are controlled by the activity of antioxidant enzymes including peroxidase, catalase and superoxide dismutase and by small molecules present in different cellular compartments. The activity of these enzymes increases in response to biotic and abiotic stresses. It was found that the activity of SOD, APX, and CAT enzymes in tea leaves increased under cold stress

conditions (Sariri et al. 2016). According to our results, increasing the activity of antioxidant enzymes play an important role in protecting licorice seedlings against cold stress by eliminating ROS. Exposure to metal oxide nanoparticles causes ROS production, thereby causing oxidative stress and activating plant reactions for detoxification like enzymes activation. The activity of the enzymes may change depending on the type of nanoparticles, concentration, and the time exposure (Movafeghil et al. 2018). Our results showed that TiO₂ NPs at both applied concentrations increased the activity of antioxidant enzymes. Increased SOD, CAT and POD activities in response to TiO₂ NPs application and consequently reduced H₂O₂ and MDA have been reported the in several studies (Song et al. 2013). By the application of spermine in our experiment, the activity of the antioxidant enzymes was enhanced under cold stress conditions, which is agreement with findings observed in other plants.

Conclusions

Results showed that a 2-day cold treatment reduced growth, but increased oxidative stress and activated antioxidative and osmoprotective responses of licorice seedlings. Cold treatment also increased the content of glycyrrhizin in the seedlings. When supplemented into the cultivation medium, TiO₂ NPs enhanced the ability of seedlings to tolerate cold stress by decreasing MDA and H₂O₂ contents, and increased antioxidative and osmoprotective responses. TiO₂ NPs also enhanced the accumulation of glycyrrhizin in control and cold-treated seedlings. The addition of spermine in the cultivation media also reduced MDA and H₂O₂ contents in licorice seedlings, probably by increasing the activity of antioxidant enzymes, but had a negative effect on the growth of control and cold-treated seedlings. In comparison to TiO₂ NPs, spermine had a less pronounced effect on glycyrrhizin content of licorice seedlings. However, further research is needed for accurate evaluation of cold stress and TiO₂ NPs effects on glycyrrhizin content of the *G. glabra* roots. The results obtained from present study may contribute to a better understanding of the effect of cold stress on licorice plants, and could help the development of a strategy for overproduction of glycyrrhizin for use in food and pharmaceutical industries.

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