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Titanium dioxide nanoparticles treatment on morphological and physiological characteristics of licorice (*Glycyrrhiza glabra* L.) under drought stress

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Drought stress is one of the factors limiting growth and development in plants, which negatively affects the morphological traits and physiological processes of plants. These effects are reduced using a variety of compounds and nanomaterials, including titanium dioxide nanoparticles (TiO_2 NPs). In recent years, the use of TiO_2 NPs has been the focus of plant physiologists due to their special biological properties. This beneficial nanoparticle stimulates the growth and quality of crops. The foliar application effects of TiO_2 NPs on morphological and physiological traits of licorice (*Glycyrrhiza glabra* L.) under drought stress were investigated in a factorial experiment with a completely randomized design with three replications under greenhouse cultivation at Malayer University in spring 2019. TiO_2 NPs were used at concentrations of 0, 0.02, and 0.04 mM with the application of drought stress and irrigation cut-off at 24 h, 7 days, and 14 days. The measured morphological and physiological traits were root length, shoot length, root diameter, stem diameter, root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW), shoot dry weight (SDW), chlorophyll content (a, b, and total) (Chl. a, Chl. b) and, carotenoids, anthocyanin, proline, total phenol, total flavonoids, soluble carbohydrates, and insoluble carbohydrates. The results indicated that values of all traits, except root length, shoot length, and SDW, decreased with drought stress, while it increased physiological traits, including anthocyanins, carotenoids, flavonoids, and soluble and insoluble carbohydrates. TiO_2 NPs positively influenced all physiological traits, except insoluble carbohydrates and total phenols.

Key words: Licorice (*Glycyrrhiza glabra* L.), titanium dioxide nanoparticles, morphological and physiological characteristics, drought stress.

INTRODUCTION

Licorice (*Glycyrrhiza glabra* L.) is a perennial plant from the family Fabaceae and the subfamily Papilionaceae. It is one of the medicinal plants worldwide that has long been considered by humans and is known as the ancestor of medicinal plants due to its long history (Sharma et al., 2023). Licorice root is mentioned as a

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medicine in most pharmacopeias .For about 400 years, the root and rhizome of this plant have been used medicinally and have been registered in pharmacopeias of the United States, China, and other countries. Licorice is used in Asian and European traditional medicine to treat stomach ulcers, respiratory infections, etc. (Wang and Nixon, 2001). Plants encounter various environmental stresses during their lifetime, which limit the chances of development and survival in plants (Renziehausen et al., 2024). In many parts of the terrestrial globe, favorable growing conditions persist only for a short time, and plants are forced to perform their basic stages of growth in such a short time. In some areas with appropriate growth conditions, increasing density and the number of plants cause their competition to obtain food, water, and light (Larcher, 2003).

In general, stress is an unfavorable condition that does not necessarily result in immediate death and occurs permanently or temporarily in one place but influences the vital functions of organisms (Walter et al., 1985). Drought stress is one of the main environmental stresses that negatively affect plant growth and development worldwide (Farooq et al., 2017). Plants have developed various mechanisms to cope with drought stress by a series of physiological and biochemical responses (He et al., 2015), among which antioxidant defense plays a key role in reducing oxidative damage resulting from drought stress (Wu et al., 2015). Additionally, reduced plant growth under drought stress has been proven to be usually due to reduced photosynthesis or nutrient uptake; therefore, maintenance of photosynthetic efficiency and antioxidant capacity plays an important role in plant tolerance to water deficit (Tripathi et al., 2017).

Titanium dioxide (TiO_2) is an inorganic oxide that exists in various forms. Titanium dioxide nanoparticles (TiO_2 NPs) have very wide applications in various sciences and can be considered as one of the most widely used nanoparticles (NPs), a considerable volume of which enters nature. In recent years, the use of TiO_2 NPs has been the focus of plant physiologists due to their special biological properties (Mohammadi et al., 2016). Although TiO_2 NPs are widely used in daily life, more studies should be conducted on their uptake and transport in plants, as well as their positive and negative effects on plant growth and development (Feizi et al., 2012). Tio_2 exists in three crystalline forms, including anatase (quadrilateral with a density of 3.894 g cm^{-3}), rutile (quadrilateral with a density of 4.25 g cm^{-3}), and brookite (amorphous with a density of 4.12 g cm^{-3}) (Salari et al., 2007; Ahmad et al., 2007). TiO_2 NPs possess all the properties of Tio_2, and their small particle size increases their contact surface with materials, producing more efficiency and effectiveness (Karimi and Mirjalili, 2009). TiO_2 has a positive impact on plant physiology, particularly in response to biotic and abiotic stresses, depending on various factors like size, concentration, exposure of the NPs, and other variables. Further, TiO_2 has many applications, such as being used as nano-fertilizers, adsorption of heavy metals from industrial wastewater, and antimicrobial activity (Rasoli et al., 2016). TiO_2 NPs can improve the efficiency of photosynthetic apparatus and increase the plant's ability to absorb sunlight, thereby affecting the production and conversion of light energy into active electrons and chemical activities and increasing photosynthetic efficiency in plants (Akbari et al., 2014). Spraying of TiO_2 NPs increased the amount of chlorophyll (chl.) index, proline and protein content, concentrations of potassium, zinc, iron, manganese, leaf surface, and reduced the amount of minimum chlorophyll fluorescence. Also, total soluble carbohydrate content increased in parallel with TiO_2 NPs concentration, while the treatment did not affect the amount of FV/FM [refers to the ratio of variable fluorescence (Fv) to maximum fluorescence (Fm)] (Mohammadi et al., 2016). These NPs can be absorbed and accumulated in plant tissues and positively affect seed germination, seedling growth, photosynthesis, plant response to stress, and yield traits (Du et al., 2017). TiO_2 NPs have recently been used in nanotechnology to increase plant tolerance under adverse conditions (Tripathi et al., 2017). This study aimed to investigate the foliar application effects of TiO_2 NPs on morphological and physiological processes in licorice under drought stress. Other researchers examined the effects of nano titanium dioxide on the morphological and biochemical characteristics of some strawberry cultivars under hydroponic cultivation conditions. The results showed significant differences among the studied cultivars in terms of the evaluated traits. Traits such as fruit number, leaf surface area, dry weight of aerial organs, dry weight of roots, soluble solids, and antioxidants increased (Fatemeh et al., 2023). TiO_2 NPs have a positive impact on plant physiology, particularly in response to biotic and abiotic stresses, depending on various factors like size, concentration, exposure of the NPs, and other variables. Further, TiO_2 NPs have many applications, such as being used as nano-fertilizers, adsorption of heavy metals from industrial wastewater, and antimicrobial activity (Rakhi et al., 2024).

MATERIALS AND METHODS

The seeds of licorice were obtained from Pakan Bazr Company (Isfahan) on June 7, 2016. TiO_2 NPs were procured from Micronutrient Nanofertilizers, Sepehr Parmis Company (JarfaPajouhan Science Company). Drought stress was applied by the interruption of irrigation. Licorice-containing pots were kept in the Malayer greenhouse with a 16 h light/8 h dark photoperiod and 38 to 40°C.

Soil composition consisted of 49% sand, 24% silt, 27% clay, and 2% animal manure. The seeds were first soaked in water for 24 h, 10 seeds were then placed in each pot containing about 7 kg of soil, and covered by 1 cm of soil.

At first, the surface soil was moistened every day. After the germination stage and the emergence of the stems from the soil surface, the pots were watered every second day. Treatments were

started after 64 days. This factorial experiment was performed in a randomized design to evaluate the susceptibility of the licorice plant to different concentrations of Ti (0.0, 0.02, and 0.04) under drought stress treatment within 14 days. To apply drought stress, the pots were watered on the first day, and the foliar application of TiO_2 NPs was applied 24 h after the first harvest took place. For the rest of the pots, interruption of irrigation was applied for 7 and 14 days before the harvest, during which these pots were sprayed with TiO_2 NPs every 2 days. The whole collected samples were transferred to the laboratory for measurements of morphological traits.

The measured morphological traits were shoot and root length by a ruler, stem and root diameter by a caliper, and fresh and dry weights of shoot and root scale (0.001 g accuracy).

Chlorophyll and carotenoid contents were measured according to Lichtenthaler (1987). To this end, 0.2 g of wet tissue was ground in a porcelain mortar containing 5 ml of 80% acetone and then filtered on Whatman No. 1 paper. Next, the volume of the obtained extract was made to 10 ml using acetone. The optical density (OD) of the extract was read against 80% acetone as a blank at wavelengths of 646.8, 663.2, and 470 nm by spectrophotometry. The concentrations of these pigments were calculated using Equations 3 to 4:

Chl. a (mg/ml) = (12.25 A663.2) − (2.79 A646.8)

Chl. b (mg/ml) = (21.51 A646.8) − (5.1 A663.2)

Car. (mg/ml) = (1000 A470-1.8 Chl. a-85.02 Chl. b)/19

Measurement of anthocyanin and total phenol content

Anthocyanin levels were measured by the method of Hara et al. (2003). To do this, 1 g of leaf tissue in 10 ml of acidic methanol (including 99.5% methyl alcohol and pure hydraulic acid at 99:1) was filtered using Whatman No. 2 filter paper and then centrifuged at 12,000 rpm for 12 min. The absorbance of the supernatant was determined at 530 nm using an acidic methanol blank with a spectrophotometer, and the results were compared as absorbance/g fresh weight.

The powdered sample (0.1 g) was extracted with 80% methanol in a bain-marie at 70°C for 3 h. A quantity of total phenol was measured using the Folin-Dennis reagent. This method is based on redox reactions. The folate reagent is stable only at an acidic pH, but it can be reduced at pH. Thus, the phenolic extract solution containing this reagent should be shaken vigorously so that it is reduced before degradation. Total phenol was measured based on gallic acid as a standard.

Measurement of proline, soluble carbohydrates (SCs), and insoluble carbohydrates content

Proline was extracted and measured according to Bates et al. (1973). To prepare the Ninhydrin reagent, 1.25 g of ninhydrin was dissolved in 30 ml of glacial acetic acid by heating gently. Then, 20 ml of 6 M phosphoric acid was added and mixed thoroughly. This solution is stable at 4°C for 24 h. To measure proline content (PC), 0.5 g of wet tissue in 10 ml of 3% sulfosalicylic acid was ground in a porcelain mortar and homogenized completely. The volume of the resulting extract was recorded after being filtered with Whatman No. 2 paper. Next, 2 ml of the filtered extract was mixed with 2 ml of ninhydrin solution plus 2 ml of glacial acetic acid and placed in a water bath (100°C) for 1 h. The reaction was then completed by placing the test tubes in an ice bath. Afterward, 4 ml of toluene was added to the content of each tube and stirred vigorously for 15 s. The supernatant, containing toluene and proline, was separated from the aqueous phase, and its absorbance was determined by spectrophotometry at 520 nm. PC in each sample was calculated based on the absorbance, and certain concentrations in a standard curve were calculated based on mg/g wet weight (Bates et al., 1973).

The measurement of SCs by the phenol-sulfuric acid method (Kochert et al, 1978) is based on the acid hydrolysis of SCs and the formation of a furfural compound that produces a color complex with phenol. To do the test, 0.1 g of the dried sample was weighed with a scale to the nearest 0.001, poured into a test tube with 10 ml of 70% ethanol, and then refrigerated for 1 week to dissolve SCs. After 1 week, 0.5 ml of the supernatants in the samples was harvested and their volume was made to 2 ml with distilled water. Then, 1 ml of 5% phenol was added to the solution and mixed well by vortexing. Thereafter, 5 ml of concentrated sulfuric acid was added to each sample by pressure under a hood. A yellow solution was obtained that changed color over time and turned into light brown. The solution was kept at ambient temperature to cool down for 30 min and produce the final color. Finally, the resulting color intensity was used to measure glucose by a spectrophotometer at 485 nm. The absorbance level will be proportional to the color intensity of the solution, which in turn will be proportional to the concentrations of SCs.

The insoluble carbohydrate (starch) was measured by filtering the ethanol solution of plant samples used for SCs, and the residual precipitate on filter paper was used to measure the leaf starch. First, the precipitate was dried, weighed, and poured into a test tube, with adding 10 ml of distilled water was added, and then incubated in a bain-marie at 100°C for 15 min. Then, the solution was filtered, and the filtrate was made to a volume of 25 ml with distilled water added. Next, 1 ml of 5% phenol was added to the solutions and mixed well by vortexing. Afterward, 5 ml of concentrated sulfuric acid was added to each sample by pressure under a hood. A yellow solution was obtained, and finally, the absorbance level of starch in the samples was measured at 485 nm by spectrophotometry.

Statistical analysis

This experiment was conducted in a factorial design within a completely randomized design. For statistical analysis, the SPSS software was used, and Duncan's multiple range test was applied for mean comparison.

Ethical consideration

The data presented in this study are obtained from a research project conducted in the Malayer University. Experiments were carried out based on procedure and guidelines approved by the Ethics committee in research of Malayer University.

RESULTS

According to ANOVA results of experimental data, the interaction effect of TiO_2 NPs treatment and drought stress was significant on all morphological traits of the plant at a level of 1%, except for root diameter and root dry weight (RDW) (Table 1). The interaction effect of TiO_2 NPs treatment was significant on the physiological traits of chlorophyll a, chlorophyll b, anthocyanin, PC, and insoluble carbohydrates at 1% level, and on total phenol trait at 5% level (Table 2). This interaction had no significant effects on carotenoids, flavonoids, and SCs (Table 2).

Table 1. Results of ANOVA for the interaction effects of drought, TiO_2 NPs, and harvest time on morphological traits of licorice in pot conditions.

SOV	df	MS												
		Total fresh weight (mg)	RFW (mg)	SFW(mg)	Root length (mm)	Shoot length (mm)	Root diameter (mm)	Stem diameter (mm)	RDW (mg)	Root turgor weight (mg)	SDW(mg)	shoot turgor weight (mg)	Root RWC (%)	Shoot RWC (%)
Drought (a)	1	5.526**	0.567**	0.247^{ns}	210.436**	4.125^{ns}	4.188**	0.19*	0.092**	1.284**	5.467^{ns}	0.123^{ns}	601.408*	230.71^{ns}
TiO2 (b)	2	0.084^{ns}	0.068^{ns}	0.025^{ns}	69.174**	36.049**	1.65**	0.044^{ns}	0.02**	0.155**	0.008^{ns}	0.532*	394.598^{ns}	1022.625**
Harvest time (c)	2	1.444**	0.311**	0.066^{ns}	210.127**	182.751**	0.297^{ns}	0.001^{ns}	0.106**	1.058**	0.004^{ns}	0.274^{ns}	2766.859**	221.79^{ns}
a×b	2	0.131^{ns}	0.218**	0.17^{ns}	71.96**	78.475**	0.411^{ns}	0.013^{ns}	0.001^{ns}	0.125*	0.013^{ns}	1.393**	661.792*	295.793^{ns}
a×c	2	1.709**	0.207**	0.164^{ns}	86.352**	20.721*	1.575**	0.298**	0.045**	0.545**	0.001^{ns}	0.101^{ns}	77.655^{ns}	850.162**
b×c	4	0.41*	0.085*	0.287*	24.554*	29.578**	0.99*	0.102^{ns}	0.006^{ns}	0.165**	0.022**	1.378**	2783.755**	246.296^{ns}
a×b×c	4	0.243^{ns}	0.187**	0.51**	34.73**	31.318**	0.557^{ns}	0.145*	0.006^{ns}	0.21^{ns}	0.021**	1.028**	2163.54**	232.644^{ns}
Error	36	0.133	0.029	0.088	8.271	6.65	0.308	0.045	0.003	0.03	0.005	0.119	155.916	168.029
CV%	-	25.13	37.00	31.32	19.75	14.87	16.44	18.79	30.13	25.66	27.25	23.12	20.87	22.93

Table 2. Results of ANOVA for the interaction effects of drought stress, TiO_2 NPs, and harvest time on physiological traits of licorice in pot conditions.

SOV	df	MS									
		Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Carotenoid (mg/g FW)	Total chlorophyll (mg/g FW)	Anthocyanin (µmol/g FW)	Flavenoid (µmol/g FW)	Total phenol (µg/g DW)	Proline (µmol/g FW)	Soluble carbohydrates (g/g DW)	Insoluble carbohydrate (g/g DW)
Drought (a)	1	0.109*	0.628**	34.219**	0.213*	0.057**	479.861*	0.005^{ns}	14.151^{ns}	10801.075**	329.401^{ns}
TiO2 (b)	2	0.266**	0.834**	8.446*	0.405**	0.072**	687.133**	0.026*	25.747**	3846.49*	299.964^{ns}
Harvest time (c)	2	0.062*	0.133^{ns}	0.492^{ns}	0.374**	0.032**	2582.494**	0.082^{ns}	352.631**	7382.088**	7447.407**
a×b	2	0.03^{ns}	0.015^{ns}	3.956^{ns}	0.013^{ns}	0.041**	261.146^{ns}	1.312**	108.551**	16737.42**	651.136*
a×c	2	0.624**	0.524**	47.956**	0.189**	0.19**	1257.887**	1.173**	140.482**	56804.015**	812.537**
b×c	4	0.087**	0.104^{ns}	5.743*	0.027^{ns}	0.1**	2247.137**	0.138^{ns}	76.238**	17758.442**	2613.847**
a×b×c	4	0.144**	0.647**	0.736^{ns}	0.215**	0.029**	120.735 ^{ns}	0.224*	49.737**	1571.935^{ns}	1680.928**
Error	36	0.016	0.064	1.855	0.035	0.004	97.705	0.073	3.878	860.294	144.144
CV%	-	12.671	19.163	12.735	8.944	18.746	13.666	3.913	22.427	8.496	8.648

Change in the shoot length and root length

Figure 1A shows that the highest and the lowest root lengths were measured using 0.04 mM of TiO_2 NPs and the control treatment, respectively both in the second harvest treatment (after 7 days of irrigation cut-off). Figure 1 shows that TiO_2 NPs had a significant positive effect on plant root, length and neutralized the effect of stress in the second harvest (7 days of irrigation cut-off).

Figure 1B shows that the treatments with 0.04 and 0.02 mM of TiO_2 NPs in the second harvest treatment (14 days of irrigation cut-off) and the control treatment in the third harvest have the highest shoot length. The lowest value of this trait

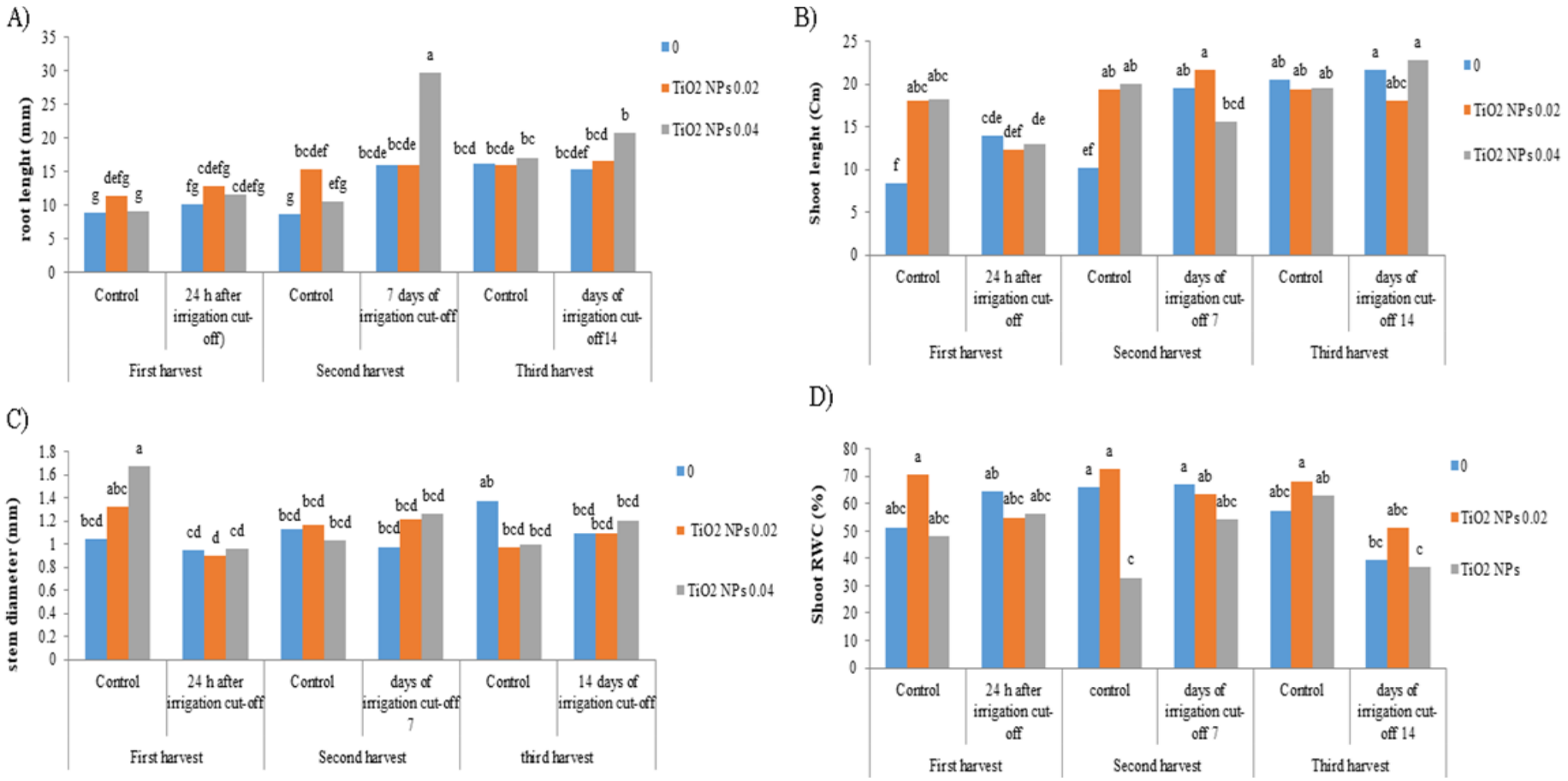


Figure 1. Interaction effects of drought, TiO_2 NPs, and harvest time on (A) root length, (B) shoot length, (C) stem diameter and, (D) the shoot relative water content in licorice.

was recorded in the control treatment with no use of TiO_2 NPs in the first harvest. The maximum shoot length in the treatments with 0.04 and 0.02 mM of TiO_2 NPs indicates their positive effect on the shoot length. Figure 1C shows that the stem diameter is uppermost in the first harvest treatment (control) and 0.04 mM of TiO_2 NPs. The first harvest treatment (24 h after irrigation cut-off) with 0.04 mM of TiO_2 NPs led to the lowest stemdiameter. The stem diameter decreased in the stress conditions using 0.04 mM of TiO_2 NPs in the first harvest before applying drought stress. Figure 1D shows the highest relative water content (RWC) of the shoots in the first (control), the second (control), and the third (control) harvest treatments, all with 0.02 mM of TiO_2 NPs. The lowest values are observed in the second (control) and the third (14 days of irrigation cut-off) harvest treatments, both with 0.04 mM of TiO_2 NPs. Figure 2A shows that the highest root fresh weight (RFW) is in the second harvest treatment (control) with 0.02 mM of TiO_2 NPs. The lowest value belongs to the first harvest treatment (control) without TiO_2 NPs and the first harvest (24

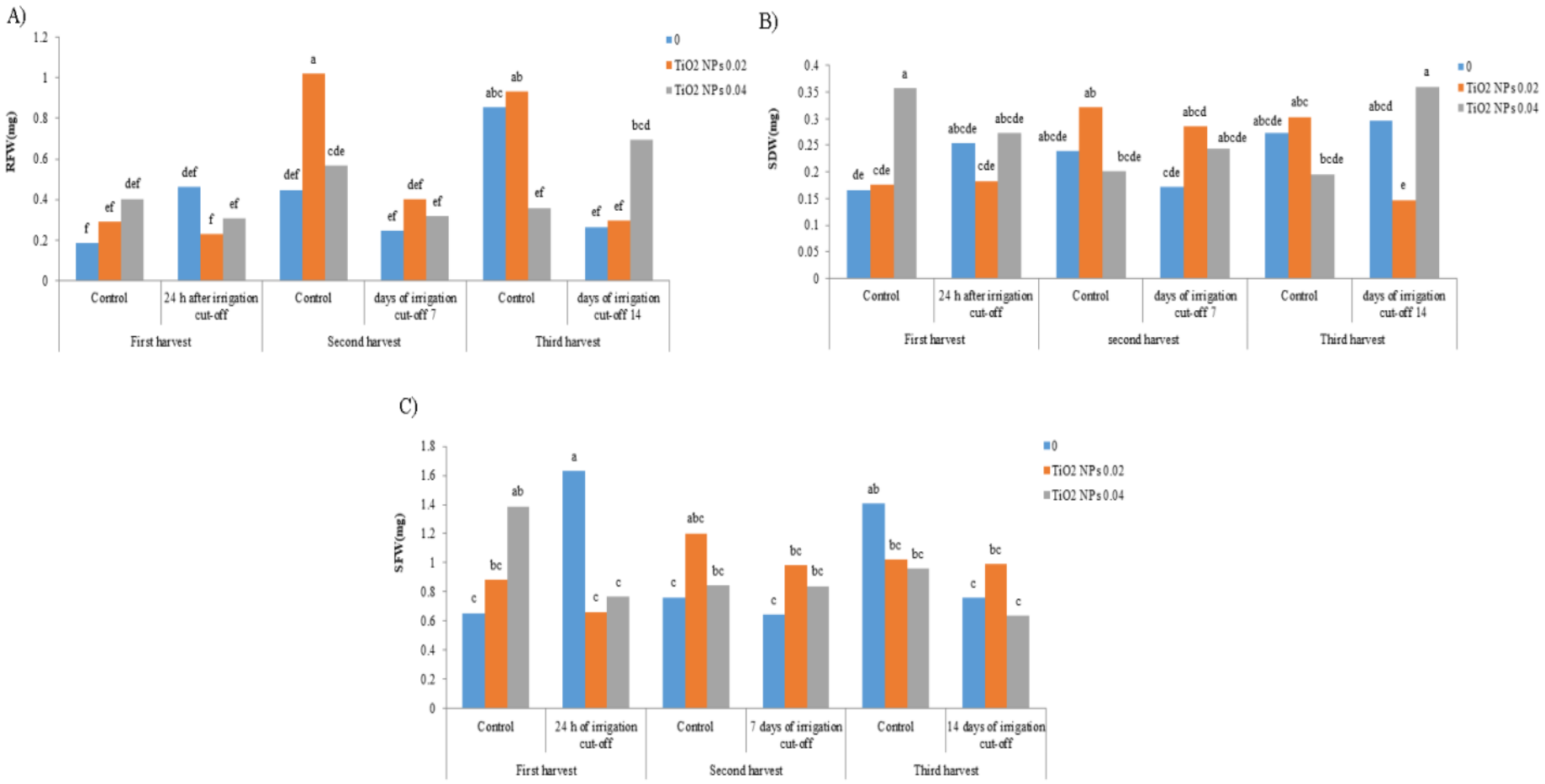


Figure 2. Interaction effects of drought, TiO_2 NPs, and harvest time on (A) root fresh weight , (B) shoot dry weight, and (C) shoot fresh weight in licorice.

h after irrigation cut-off) with 0.02 mM of TiO_2 NPs. The RFW decreased significantly with increasing stress intensity (Figure 2-b). Figure 2B shows that the highest shoot fresh weight (SFW) is related to the first harvest treatment (24 h after irrigation cut-off) without the use of TiO_2 NPs, and the lowest value belongs to the third harvest treatment (14 days of irrigation cut-off) with 0.4 mM of TiO_2 NPs. Figure 2C shows that the highest shoot dry weight (SDW) belongs to the first (control) and the third (14 days of irrigation cut-off) harvest treatments, both with 0.04 mM of TiO_2 NPs. The lowest value belongs to the third harvest treatment (14 days of irrigation cut-off) with 0.02 mM of TiO_2 NPs. The greatest SDW with 0.02 mM of TiO_2 NPs in the first and third harvests means that this concentration of TiO_2 NPs has an increasing effect on the plant SDW. Drought stress caused a

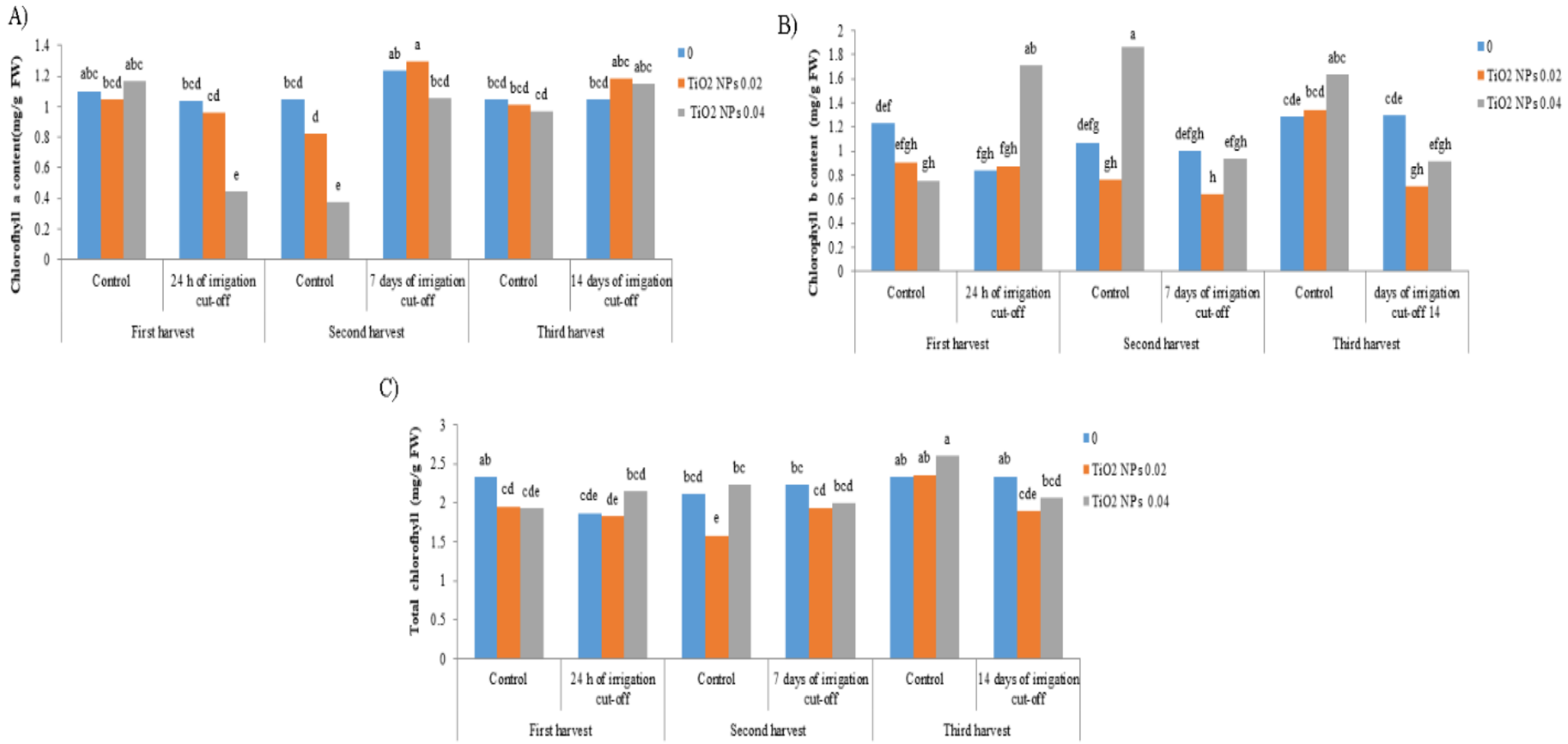


Figure 3. Interaction effects of drought, TiO_2 NPs, and harvest time on (A) chlorophyll a, (B) chlorophyll b, and (C) total chlorophyll content of licorice.

significant reduction in the SDW in the third harvest (7 days of irrigation cut-off).

Changes in the plant chlorophyll content

Figure 3A shows that the highest chlorophyll is seen in the second harvest treatment (7 days of irrigation cut-off) with 0.02 mM of TiO_2 NPs. The second (control) and the third (24 h of irrigation TiO_2 NPs contain the lowest chlorophyll as levels. Figure 3B shows the highest chlorophyll b content belongs to the second harvest treatment (control) with 0.04 mM of TiO_2 NPs, and the lowest value is observed in the second harvest treatment (7 days of irrigation cut-off) with 0.02 mM of TiO_2 NPs. Figure 3 shows that TiO_2 NPs cut-off) harvest treatments both with 0.04 mM of positively affected chlorophylls a and b contents and yielded the highest values in these treatments. Figure 3B shows that the highest chlorophyll b content is related to the third harvest treatment(control) with 0.04 mM of TiO_2 NPs, and the lowest value is seen in the second harvest treatment (control) with

0.02 mM of TiO_2 NPs.

Changes in plant anthocyanin and phenol contents

Figure 4A shows the amount of anthocyanin is uppermost in the first harvest (control) with 0.02 mM of TiO_2 NPs, in the third harvest (14 days of irrigation cut-off) without TiO_2 NPs, and in the second harvest (control) with 0.02 and 0.04 mM of TiO_2 NPs (7 days of cut-off). The lowest value belongs to the third harvest treatment (control) without TiO_2 NPs. The highest amount of anthocyanin at 0.02 and 0.04 mM of TiO_2 NPs indicates a positive effect on the anthocyanin content (Figure 4A).

Figure 4B shows that the highest and the lowest total phenol concentrations are observed in the first (control) and the second (7 days of irrigation cut-off) harvest treatments, both with 0.04 mM of TiO_2 NPs. As shown in the figure 4B, the anthocyanin content has increased with increasing irrigation cut-off compared to the control, indicating that increasing drought stress has increased anthocyanin levels as an antioxidant, and TiO_2 NPs had positive effects at concentrations of 0.02 and 0.4.

Changes in insoluble carbohydrates and PC

Figure 4C shows the third harvest treatment (14 days of irrigation cut-off) without TiO_2 NPs contains the highest amount of insoluble carbohydrates. The first harvest (control) and the first harvest (24 h after irrigation cut-off), both with 0.04 mM of TiO_2 NPs, represent the lowest levels of insoluble carbohydrates, and the highest level (500 g per dry weight) was observed in the second harvest (7 days of irrigation cut-off).

Figure 4D shows the highest PC in the third harvest treatment (control) with 0.02 mM of TiO_2 NPs. The lowest PC belongs to the second harvest treatment (control) with 0.04 mM of TiO_2 NPs (control), without the use of TiO_2 NPs (24 h of irrigation cut-off), the second harvest (control) without the use of TiO_2 NPs, 0.02 and 0.4 mM of TiO_2 NPs, 7 days of irrigation cut-off without the use of TiO_2 NPs, and 0.02 mM of TiO_2 NPs, and the third harvest (14 days of irrigation cut-off) with 0.02 mM of TiO_2 NPs.

DISCUSSION

In different plants, the root system expands to cope with drought so that the plant can absorb more water from the soil. The root growth increases to counteract water deficit, and root length may have an important effect on plant life performance as they spread their roots to deeper parts of the soil containing more moisture (White and Castillo, 1989). The results showed that TiO_2 positively affected root length and neutralized the stress effect to some extent (Figure 1). TiO_2 at higher concentrations and TiO_2 NPs can affect some growth traits due to the small particle size and the easier penetrability into the roots (Haghighi and Daneshmand, 2013). Through foliar application and root nutrition, TiO_2 NPs cause the nutrient uptake, in particular nitrogen, thereby stimulating root growth (Saber et al., 2013).

During the plant shoot, TiO_2 NPs at concentrations of 0.02 and 0.4 mM had a significant effect on the second and third harvests, respectively, leading to the highest growth (Figure 2). Jaberzadeh et al. (2013) reported that foliar application of TiO_2 NPs increased wheat stem length and grain yield under drought stress.

The plant stem diameter decreased after 14 days of drought stress conditions in the third harvest, and in the first harvest before the application of drought stress. TiO_2 NPs at a concentration of 0.04 mM positively influenced the stem diameter (Figure 3), which plays a significant role in terms of osmolyte storage during the vegetative season and the transferability of these materials during growth; a larger stem diameter increases the optimal production potential in the plant (Tabatabai and Shakeri, 2012). Blum et al. (1997) presented evidence that the Azargol genotype was less affected by stress due to its larger stem diameter. In a study on thyme (*Thymus vulgaris*), plant height, main branch diameter, sub-branch diameter, and leaf RWC decreased in response to drought stress (Fabriki and Davoudnia, 2018). Appropriate concentrations of TiO_2 NPs can probably be used as external stimuli to improve stem diameter growth and essential oil content in plants (Mohammadi et al., 2016).

The plant RFW significantly decreased by drought stress in the third harvest, and TiO_2 NPs at 0.02 mM had a positive effect on the control plants in the second and third harvests, with the former showing the highest RFW (Figure 2a). A study on chickpeas revealed that decreased leaf area, stomatal closure, decreased water absorption and declined water and nutrient transfer following a decrease in moisture in the root zone, and in general the application of drought tolerance mechanisms by the plant, resulted in reduced production and transfer of photosynthetic materials to the roots, which quantitatively reduced root density and fresh weight (Alkire and Simon, 1993).

The SFW in the third harvest decreased with increasing drought stress (Figure 2b). Some processes are very sensitive to increased drought stress while other processes are less influenced by drought stress. Furthermore, shoots and roots are related to each other in different aspects, and a change in the growth of one will also change the other (Alizadeh et al., 2009). Some experiments indicate that drought stress decreases the RFW along with SFW. Fresh weight loss in plants under drought stress conditions seems to be due to the inhibition of cell development and growth caused by a decrease in osmotic pressure (Abdalla and El-Khoshiban,

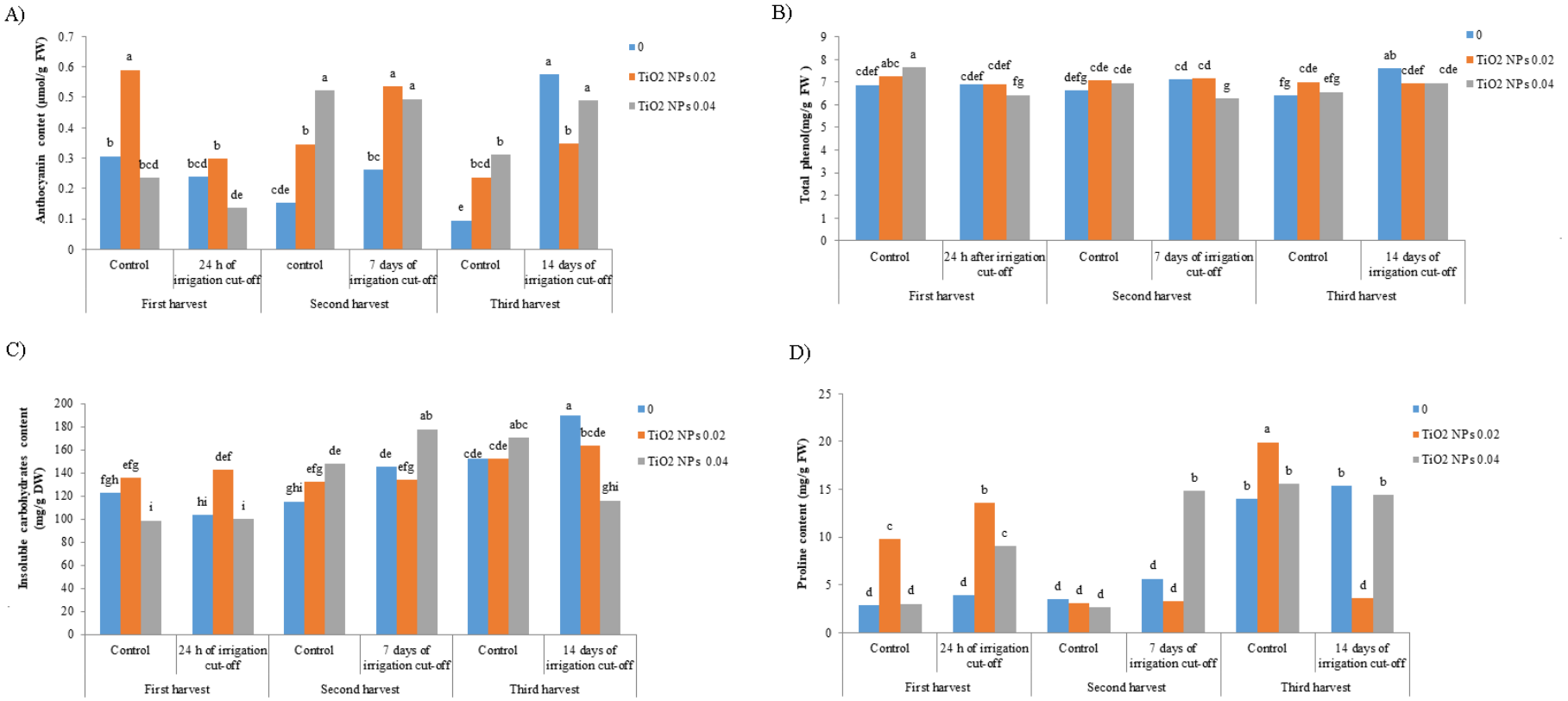


Figure 4. Interaction effects of drought stress, TiO_2 NPs, and harvest time on (A) anthocyanin, (B) phenol, (C) insoluble carbohydrate content, and (D) the proline contents of licorice.

The SDW reached the highest value in the third harvest with 0.04 mM of TiO_2 NPs, which had a high positive effect on SDW and neutralized the effect of drought stress on the plant (Figure 2C). Saber et al. (2013) studied the effect of TiO_2 NPs on *Spinacia oleracea* and reported that the synthesis of amino acids, proteins, chlorophyll, and photosynthesis by increased by TiO_2 NPs through accelerating the activity of reductase enzyme, leading to increases in *S. oleracea* growth dry/wet weight. Improvement of plant condition and an increase in shoot dry weight under drought stress with the use of titanium have been reported by several researchers (Shekari et al., 2016).

TiO_2 NPs at the concentration of 0.02 mM (control) with no drought stress led to the highest RWC, which was significantly affected by TiO_2 NPs with no stress application (Figure 1D). In a study, the foliar application of 0.01% TiO_2 NPs resulted in the highest yield of maize under drought stress, and the lowest RWC of shoots was observed with no foliar application (Khalilvand et al., 2019). Chlorophylls a and b levels were uppermost in the second harvest at 0.02 and 0.04 mM of TiO_2 NPs, respectively, which indicates the positive effect of this nanoparticle on chlorophyll pigments and neutralizing the effect of stress

(Figure 3). The foliar application of TiO_2 NPs in maize increased photosynthetic pigments compared to the control treatment, which was attributed to the stabilization of the chloroplast membrane and its protection by TiO_2 NPs (Morteza et al., 2013). In fact, these NPs can improve the structure of chlorophyll and the reception of sunlight by these pigments. Yang et al. (2006) attributed an increase in the chlorophyll content of the *S. oleracea* plant to elevated nitrogen uptake and metabolism by TiO_2 NPs.

Increasing drought stress in the third harvest resulted in increased anthocyanin as an antioxidant against stress and reached the highest levels in the first, second, and third harvests at 0.02 and 0.04 mM of TiO2 NPs (Figure 4A). A decrease in soil water content at different levels increases anthocyanin levels, which, along with water deficit, may be due to the positive effect of anthocyanins on the removal of free radicals. This means that some compounds, such as secondary metabolites, antioxidants, and anthocyanins, significantly increase in the plant under stress conditions. This increase results from the photoprotective role of anthocyanins by direct removal of free radicals during stress. It is believed that secondary metabolites are produced for plant adaptation to adverse factors and environmental stresses and as a kind of defensive cascade to maintain the balance of vital activities (Kruk et al., 2005). During stress, an increase in anthocyanins in *Craterostigma* leaves has been reported by Hoekstra et al. (2001). Similar to flavonoids, anthocyanins are a protective pigment that protects plants against stress (Chalker-Scott, 2002).

Total phenol as an antioxidant against stress increased in the third harvest compared to the control, with the highest value in the first harvest at 0.4 mM of TiO_2 NPs, which had a significant positive effect on total phenol content (Figure 4B). Any kind of stress on the plant has been shown to increase phenolic compounds, the presence of which reduces the colonization of microorganisms. It has been reported that the antioxidant properties of plants depend on their levels of individual polyphenolic compounds (Wiseman and Halliwell, 1996). Phenolic compounds accumulate in plants in response to numerous biotic and abiotic stresses, such as ultraviolet radiation, low temperatures, reduced nutrition, pathogen invasion, and drought (Yamasaki et al., 1997). Phenolic compounds are known as the most important antioxidant compounds that play their antioxidant role by several mechanisms, such as scavenging free radicals, interrupting oxidation chain reactions, hydrogen donation, free oxygen scavenging, chelation of metal ions, or as a substrate of peroxidase enzymes. In a study on the effect of TiO_2 on sage under drought stress, the highest phenol content was observed under drought stress with the foliar application of TiO_2 NPs at 100 mg/l. In another study, the highest and lowest levels of phenols were found with the foliar application of 200 mg/l and no application of TiO_2 NPs (Ghorbanpour and Hatami, 2015).

Insoluble carbohydrates as an antioxidant reached the highest amount in the control after 14 days of stress (Figure 4C). A number of studies have investigated the role of SCs and their increase under various stress conditions, all of which indicate the role of these compounds in cellular osmotic regulation including research on soybeans (Yamada and Fukutoku, 1986). On the other hand, the stem can reportedly play an important role as a special place for the short-term storage of carbohydrates during stress (Davidson and Chevalier, 1992). In general, the increase in SCs during drought stress (in particular severe stress) can be explained by the following reasons: (1) Degradation of insoluble carbohydrates, leading to an increase in SCs, (2) synthesis of these compounds from non-photosynthetic pathways, and (3) growth arrest (Hissao, 1973). Proline levels in *Melissa officinalis* (Abbaszadeh et al., 2008) and *Satureja hortensis* (Esmaielpour et al., 2013) increased with increasing drought stress. Zarafshar et al. (2015) claimed that the foliar application of TiO_2 NPs during drought stress did not have an increasing effect on PC in drought-exposed seedlings. In a study by Amirjani et al. (2014), increasing the concentration of TiO_2 NPs elevated PC in the shoots of the medicinal plant *Cathrantus roseus*, but it decreased non-significantly at 0.0 µm of TiO_2 NPs compared to the control.

Mohammadi et al. (2016) showed that application of nano-scale TiO_2 at low concentration improved the morphological and physiological traits of the plant compared to other doses, particularly under water scarce conditions, leading to better plant performance. Enhanced chlorophyll and carotenoids contents were recorded in leaves of nano-anatase TiO_2-treated plants under both normal and drought stress conditions, when compared to the control. The levels of H_2O_2 and MDA in plants exposed to nano TiO_2 were lower than that of other treatments, therefore, lipid peroxidation was less pronounced in such plants. In both well-watered and drought stress conditions, the highest values of seed oil and protein contents were obtained in plants treated with nano TiO_2 at 100 mg l^{-1}. Therefore, exogenous application of nano TiO_2 particles at appropriate concentrations can ameliorate drought stress damage to flax seed plants as well as increase the drought tolerance with remarkable improvement in physiological process.

Selda et al. (2024) showed applications of 10 ppm TiO_2-NPs improved growth parameters and the soil plant analysis development (SPAD) index and enhanced stomatal conductance, relative water content, and protein content in grapevine saplings under both drought and well-irrigated conditions. Conversely, oxidative stress parameters, including the membrane damage index, hydrogen peroxide, drought index, and lipid peroxidation levels, were significantly reduced following 10 ppm TiO_2-NP applications under drought conditions. Furthermore, total phenolic content, PC, and ascorbate peroxidase, catalase, and superoxide dismutase activities, which increased significantly with drought stress, were reduced to lower levels, paralleling the alleviation of drought-

induced oxidative stress. This study's results suggest that the primary role of TiO_2 NPs in enhancing drought tolerance is due to their beneficial effects in alleviating damage caused by drought stress. This finding applies not only to grapevines but may also be relevant for other agricultural crops.

Conclusion

Plants are a fundamental component of all ecosystems and play a key role in the fate and transport of NPs in the environment through uptake and bioaccumulation. Nanotechnology can play an important role in improving the present plant management practices. TiO_2 NPs as beneficial NPs can enhance and stimulate growth and the absorption of some elements. In recent years, the use of TiO_2 NPs has been of high interest to plant physiologists due to their biological properties that improve the morphological and physiological traits of plants. Drought stress is one of the most important limiting factors of plants that physiologically causes various environmental stresses and reduces plant yield by 30 to 50%. The results of this experiment and other experiments demonstrate that the drought stress-exposed plant encounters a decrease in the concentration of soil nutritional solutions, and this reduction in nutrient solubility reduces the uptake of these nutrients by the roots and reduces nutrient transfer to the leaves and stems, thereby limiting plant growth. As mentioned earlier, TiO_2 NPs stimulate the uptake of elements, provide the favorable amount of nutrients to the plant, and somehow has a moderating effect on the plant under stress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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