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BASIC AREA
Article

TiO_2 nanoparticles alleviates the effects of drought stress in tomato seedlings

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ABSTRACT: Nanoparticles have been widely used in recent years, to increase plant tolerance under stress conditions. In this study, TiO_2 nanoparticles (NPs) (100 ppm) were applied to tomato plants exposed to drought, and the changes were investigated by physiological, biochemical and proteomic methods. It was determined that TiO_2 NPs treatment increased the relative water content and decreased the proline and malondialdehyde content under drought conditions. As a result of proteomic analysis, it was revealed that the expression of a total of 132 proteins changed as a result of the comparison of the treatment groups (drought *vs*. control, control-100 *vs*. control, and drought-100 *vs*. drought). One of the most striking results of the study was the increase of the amounts of photosynthesis-related proteins and plasmamembrane intrinsic protein in both drought and control groups with TiO_2 NP-treatmtent. The up-regulation of plasmamembrane intrinsic protein is very important for preserving the water potential under drought conditions. Taken together, it was observed that the water potential of the plant was preserved, lipid peroxidation decreased under drought conditions with the application of TiO_2 nanoparticles, and the expression of proteins related to photosynthesis, energy and antioxidant system increased. This study provided clues to the molecular mechanism of the results of many studies available in the literature about nanoparticle treatment under stress condition and showed that TiO_2 nanoparticles have a great potential to be used to increase the stress tolerance of tomato plants under drought conditions.

Key words: *Lycopersicon esculentum*, water deficit, protein expression, lipid peroxidation, exogenous application.

INTRODUCTION

Tomato (*Lycopersicon esculentum* L.) is widely cultivated and an economically important crop plant in the world (Zhao et al. 2021). According to Food and Agriculture Organization of the United Nations (FAO)'s 2020 data, an average of 186.82 million tons of tomatoes were produced that year, and this amount has made tomato the most widely grown vegetable worldwide.

Tomato has the greatest area under cultivation compared to other vegetables and it is naturally exposed to many environmental stressors (Sousaraei et al. 2021). Tomato is known as a drought sensitive plant (Zhao et al. 2021), and drought causes serious yield losses in tomato plants (Cui et al. 2019).

There are two big rational ways to reduce the yield losses of plants under drought conditions. One of them is to develop resistant varieties to stress conditions, but this is a time-consuming and difficult task (Basu et al. 2016), and the other one is to help plants to cope with environmental stresses with exogenous applications. The use of nanomaterials is an innovative trend among approved plant solutions against abiotic stress (Heikal et al. 2022). Nanotechnology, which has been widely used in the agricultural industry in recent years, is seen as an alternative tool that facilitates the ability of plants to overcome environmental stressors.

Nanoparticles (NPs) have been used for many purposes, such as agrochemical, nanofood and nanobiocomposite in modern agriculture (Heikal et al. 2022). Various studies have also been conducted to understand the effects of NP applications on

plants under drought stress (Behboudi et al. 2018, Djanaguiraman et al. 2018). In these studies, NPs treatments enhanced drought tolerance by increasing antioxidant enzyme activities, chlorophyll quality, protein and carbohydrate content and photosynthesis capacity. However, the answer to how NP applications make these effects under stress conditions is still an important problem.

Molecular methods, especially omic techologies such as genomic, transcriptomics and proteomic, are used to figure out this complex mechanism. Thanks to proteomic techniques, quantitative and qualitative changes in protein expression levels can be detected, and specific protein lists can be determined by comparing the protein abundance between control and stressed and/or treatment groups. Proteomics may also provide new insights to improve knowledge of interactions between plants and nanoparticles (Štefanić et al. 2019).

There are some proteomic studies that present the effects of NP treatments on plants under abiotic stress conditions in the literature. In these studies, silver (Jhanzab et al. 2019), aluminum oxide (Mustafa et al. 2016) and zinc oxide NPs (Hossain et al. 2016) have been used in different plants and, as a result, plant, organ and/or treatment specific proteome response have been identified. Hovewer, according to our best knowledge, proteomic studies that reveal the effects of TiO_2 NPs on tomato plants under drought stress have not been reported earlier.

The complex drought stress response of each plant can involve a specific mechanism. Therefore, tolerance mechanisms should be carefully investigated in a versalite way. In the present study, TiO_2 NPs have been used to alleviate negative effects of drought stress on tomato plants. The response of drought-stressed tomato plants was measured in terms of physiological and biochemical parameters. Proteomic analysis was also performed to compare protein expressions among control-drought, control-control-TiO_2 NPs treated, and drought-drought-TiO_2 NPs treated tomato plants.

MATERIALS AND METHODS

Plant material and TiO_2 treatment

TiO_2 NPs were purchased from Thermo Fisher Scientific (Titanium (IV) oxide, NanoArc™, anatase, nanopowder, 99.9%, 32 nm, metals basis) and used without further purification. The concentration of TiO_2 was determined according to previous studies in the literature (Gohari et al. 2020). Stock solution (1 $g.L^{-1}$) was prepared in ultrapure water and continuously sonicated for 60 min. The stock suspension was used to prepare the final concentration (100 $mg.L^{-1}$).

The seeds of tomato (*Lycopersicon esculentum* L. 5MX12956) were surface-sterilised with sodium hypoclorite (2.5%, v/v) for 5 min and rinsed three times with dH_2O before sowing. Sterilized seeds were sown into peat and perlite (2:1, v:v) filled plastic pots. Seedlings were grown in a controlled growth room chamber maintained at 25±1°C and over 17°C day and night temperatures, respectively, with the long day photoperiod of 16:8 hours (light:dark, light density 300 μmol $m^{-2}s^{-1}$) and conditions with relative humidity 65-70% for 20 days. After that, plants were divided into two groups. Half of them were normally irrigated (control), and the other pots were non-watered (drought) for seven days. Leaves of half of the seedlings in each group (control and drought) were sprayed with 100 ppm TiO_2 NPs solution for two days before witholding water, while the controls were sprayed with distilled water. Leaves were rinsed in distilled water before they were used for analysis. Then, leaves of plants were harvested and immediately put into liquid nitrogen and stored at -80°C for further analyses.

Water status of the leaves

The leaf relative water content (RWC) was determined according to Smart and Bingham (1974). Ten discs were weighed (fresh weight, FW) from randomly selected leaves from each application group. Then, they were placed in a distilled water filled in petri dishes for 4 h (25 °C) and weighed. Their turgid weights (TW) were determined. The leaves were dried in an oven (85°C, 24 h) for dry weights (DW). RWC was defined based on Eq. 1:

$$RWC = (FW\text{-}DW) / (TW\text{-}DW) \times 100 \quad (1)$$

Determination of lipid peroxidation

The level of the malondialdehyde (MDA) was determined for the evaluation of membrane damage caused by drought stress and/or TiO_2 NPs treatments. Fresh leaf tissues (0.2 g) were ground into liquid nitrogen and homogenized in 5% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at room temperature for 15 min at 12,000 g. The supernatant was transferred to the tubes by taking equal volumes of 0.5% thiobarbituric acid and 20% TCA solutions and incubated at 96°C for 25 min. After that, the tubes were centrifuged at 12,000 g for 5 min, and the supernatant was measured at 532 and 600 nm. MDA contents were calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$. The results were expressed in nmol MDA·g^{-1} FW (Ohkawa et al. 1979).

Determination of free proline content

Free proline content was determined according to the method of Bates et al. (1973). Leaf tissues were ground into liquid nitrogen, homogenized in sulfosalicylic acid (3%) and centrifuged at 3,000 rpm. Then, equal volume of the supernatant, acetic acid and ninhydrin were mixed well and boiled for 1 h. Cold toluene was added to this mixture, and the toluene phase was measured at 520 nm. The proline concentration was calculated by using a calibration curve and expressed as µmol proline·g^{-1} FW.

Determination of titanium content

Inductively coupled plasma-mass spectroscopy (ICP-MS) was used to determine quantification of TiO2 NPs uptake by leaf. Harvesting leaves were washed with distilled water three times. After that, leaf samples (0.5 g) were digested in a 3:1:1 ratio nitric acid/perchloric acid/hydrochloric acid solution and incinerated over a hot plate at 200°C. These samples were then diluted with 50 mL of ultrapure water and analyzed by ICP-MS, Agilent 7500. For each group, three replicates were analysed.

Proteomic analysis

Protein isolation was carried out following the method of Méchin et al. (2007). According to this method, 3 g of leaf samples were put into teflon chambers and 300 mg of polyvinylpyrrolidone (PVP) was added. The leaves were grounded into a fine powder in liquid nitrogen by using the Retsch MM400 system for 90 seconds. The thoroughly powdered leaf samples were taken into tubes, and pre-cooled 10 mL of TCA-acetone buffer (10% TCA, 0,07% 2-ME, 100 mL acetone) was placed on it for each 1 g sample. The tubes were incubated at -20°C for 60 minutes and then centrifuged at 6,000 rpm for 60 minutes at +4°C. After discarding the supernatant, 5 mL of 2-ME acetone (0,07% 2-ME, 100 mL acetone) was added to the pellet and left at -20°C for 1 hour. At the end of the period, the samples were centrifuged at 6,000 rpm for 60 minutes at +4°C. This process was repeated until the pellet turned completely white. The protein precipitate, which turned white in color, was kept in a sterile area at room temperature until the acetone odor disappeared. After the acetone odor was completely gone, 500-750 µL of solubilization solution–Tris (30 mM), urea (7 M), thiourea (2 M), magnesium acetate (5 mM) and 4% CHAPS–was added for each 1 g sample on the protein precipitate, and the proteins were completely dissolved. Protein concentration was determined using Bradford assay with the bovine serum albümin (BSA) standard (Bio-Rad, United States of America). SDS-PAGE analysis was also conducted to qualitative and quantitative evaluation of proteins according to Laemmli (1970).

Tryptic cleavage of proteins

Proteolytic digestion was performed in accordance with the FASP Protein Digestion Kit (ab270519) protocol by taking 200 µg of protein extracts. After the obtained peptides were lyosified with the help of vacuum concentrator, the peptides

were dissolved with 20 µL of 0.1% formic acid buffer. Dissolved peptide samples were centrifuged at 3,000 g, and insoluble fractions were precipitated. The supernatant was carefully collected, and peptide concentrations were calculated using the Qubit protein assay kit (Thermo Scientific, Q33211).

Nano-liquid chromatography mass spectrometry analysis

Of the peptides whose concentration was calculated, 2 µg of samples were analyzed by nano-liquid chromatography mass spectrometry (nLC-MS/MS) using the Ultimate 3000 RSLC nano system (Dionex, Thermo Scientific, CA, United States of America) connected to a Q-Exactive mass spectrometer (Thermo Scientific, United States of America), each sample repeated three times. The entire system was controlled by Xcalibur 4.0 software (Thermo Fisher Scientific, CA, United States of America). High performance liquid chromatography (HPLC) separation was performed using mobile phases A (0.1% formic acid) and B (80% acetonitrile + 0.1% formic acid). The truncated peptides were concentrated and desalted by passing over a trap column. Peptides were then transferred to an Acclaim PepMap RSLC C18 analytical column (75 µm × 15 cm × 2 µm, 100 Å diameter, Thermo Scientific, CA, United States of America) for chromatographic separation. Full scan mass spectrometry (MS) spectra were obtained with the following parameters: resolution 70,000, scan range 250-2,000 m/z, target automatic gain control (AGC) 3×E6, maximum injection time 60 ms, and sputtering voltage 2.4 kV. MS/MS analysis was performed via data-dependent acquisition with the selection of the top 10 precursor ions. MS2 analysis consisted of higher-energy collisional dissociation from collision-induced dissociation with the following parameters: resolution 17 500, AGC 1E6. The maximum injection time was 100 ms, the isolation window was normalized to 2 m/z, and the collision energy (NCE) was set to 27. The instrument was calibrated with a standard positive calibrator (LTQ Velos ESI Positive Ion Calibration Solution 88323, Pierce, United States of America) prior to each analysis.

Nano-liquid chromatography mass spectrometry data analysis

Proteom Discoverer 2.2 software (Thermo Scientific, United States ofAmerica) was used for the analysis and protein identification of the raw data obtained as a result of the analysis, and the following parameters were applied during the analysis: peptide mass tolerance 10 ppm, MS/MS mass tolerance 0.2 Da, mass accuracy 2 ppm, tolerant low 1, minimum peptide length 6 amino acids, maximum peptide length 150 amino acids, constant changes cys-teine carbamidomethylation, unstable changes with methionine oxidation, asparagine deamination phosphorylation of cysteine, threonine, and tyrosine. The unique number of peptides identified for each protein was taken as a minimum, and the data obtained were searched in the Uniprot database.

Statistical analysis

Experiment was carried out completely randomized experimental design with two factors (TiO_2-NP and drought). Each factor had two different levels. Treatments had five replications with five plant each. Data were subjected to analysis of variance (ANOVA), and the means were separated using the LSD multiple range test. Difference of control and drought and difference of control/drought TiO_2 treatment was evaluated with t test, separately. All the statistical analyses were performed using the JMP13 Software package.

RESULTS

In order to understand the possible benefits of TiO_2-NPs treatment to the increasing of stress tolerance to drought, the performance of NP-treated plants was analyzed under drought conditions (Fig. 1). The results showed that TiO_2 NPs treatment caused a significant change in physiological parameters under drought.

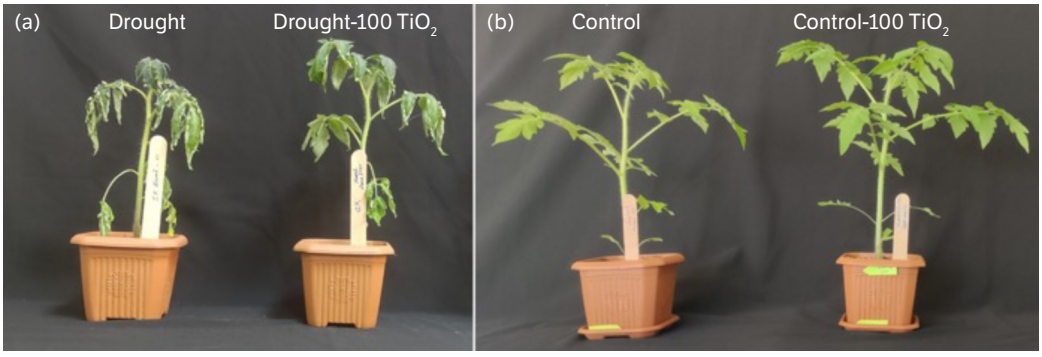
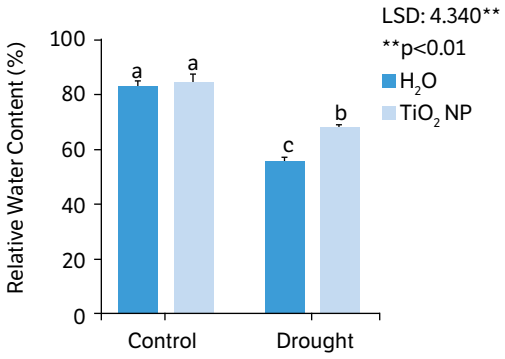


Figure 1. Photos of tomato plants after TiO_2 and drought treatments.

Effect of TiO_2 NPs on relative water content of tomato plants

Drought stress decreased relative water content in all groups compared to control. Treatment of 100 ppm TiO_2 NPs maintained the RWC 18% higher than non-TiO_2 treated plants under drought condition (Fig. 2).

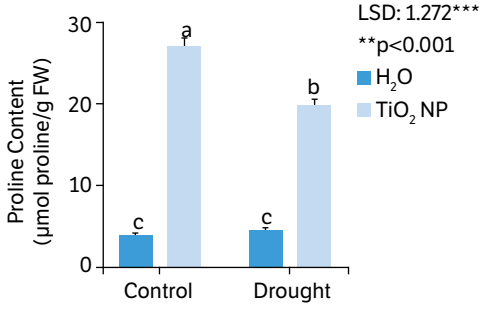


#Different letters indicate statistically significant differences between groups.

Figure 2. Effect of 100 ppm TiO_2 NPs on relative water content of tomato plants under drought stress#.

Effect of TiO_2 NPs on proline content of tomato plants

Proline content increased under drought compared to well-watered plants. The increase in proline content was about 7,56-fold higher than control plants. On the other hand, TiO2 NPs application decreased proline content by 29% as compared to drought, and application of TiO_2 NPs under control conditions did not show any significant increase in proline content (Fig. 3).

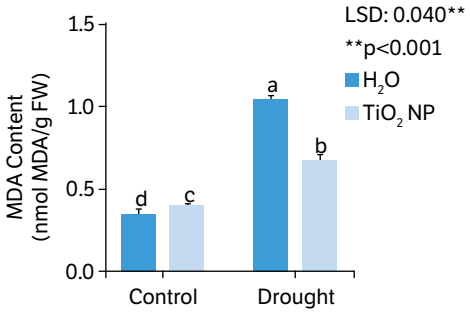


#Different letters indicate statistically significant differences between groups.

Figure 3. Effect of TiO_2 NPs on proline content of tomato plants under drought stress#.

Effect of TiO_2 NPs on MDA content of tomato plants

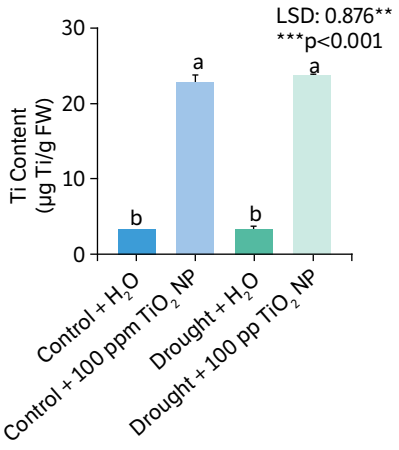
Drought stress increased MDA content compared to well-watered plants. However, spraying tomato plants with 100 ppm TiO_2 NP significantly decreased MDA content under drought stress (Fig. 4).



#Different letters indicate statistically significant differences between groups.
Figure 4. Effect of TiO_2 NPs on malondialdehyde content of tomato plants under drought stress.

Titanium content of tomato plants

Titanium content of the leaves increased after exogenous TiO_2 NPs treatment in both control and drought groups. On the other hand, drought treatment did not change titanium content of tomato plants (Fig. 5).



#Different letters indicate statistically significant differences between groups.
Figure 5. Titanium content of treatment groups#.

Proteomic analysis

Proteins that was extracted from the leaves treated with and/or without TiO_2 NPs were analyzed by using nLC-MS/MS under control and/or drought conditions. A total of approximately 500 proteins was identified, among which proteins with a high protein score and with a 1.5-fold increase/decrease in abundance were selected. However, after eliminating uncharacterized proteins, 132 proteins were identified and compared between application groups. As seen in the Venn diagram (Microsoft Excel 16.0 version, 2016), four proteins were commonly changed in all groups: six proteins were common between drought vs. control and drought-100 vs. drought, two proteins were common between drought-100 *vs*. drought and control-100 *vs*. control, and 17 proteins were common between drought *vs*. control and control-100 *vs*. control groups.

The abundance of 31 (Table 1), 21 (Table 2) and 80 (Suppl. Table 1) proteins were identified by comparing control-100 *vs*. control, drought-100 *vs*. drought, and drought *vs*. control groups, respectively (Fig. 6). The accession numbers, protein names, protein scores, pI values, biological process, molecular functions, cellular compartment and abundance (up or down) information of proteins were shown in the tables by using uniprot databases.

Table 1. List of differentially abundant proteins in leaves of tomato plants treated with 100 ppm TiO_2 NPs under control.

Accession No.	Protein	Score	Mass (kDa)	pI	Biological process / molecular function	Cellular compartment	Abundance (control-100/ control)
A0A3Q7H0L3	14_3_3 domain-containing pro	106	33,7	5,21	Signal transduction	Cytoplasm	2,176
A0A3Q7HD09	23 kDa subunit OEE of PS II	2,080	27,7	7,83	Photosynthesis / oxygen evolving activity	Chloroplast	1,541
A0A3Q7FXR7	Bet_v_1 domain-containing pro	289	16,8	6,04	Response to stress	Cytoplasm	1,566
A0A3Q7I0X4	Chlorophyll a-b binding proein	492	31,1	6,14	Photosynthesis / chlorophyll binding	Chloroplast	1,765
Q2MI71	Cytochrome b6	151	24,1	8,76	Photosynthesis / electron transport	Chloroplast	2,422
P17786	Elongation factor 1-alpha	406	49,3	9,11	Translation elongation factor activity	Cytoplasm	0,646
A0A3Q7FRC7	Elongation factor Tu	308	51,8	6,55	Translation elongation factor activity	Cytoplasm	0,49
C6K2K9	GDP-mannose 3',5'-epimerase	74	42,4	6,27	Vitamine C biosynthesis	Cytoplasm	0,576
A0A3Q7FSD7	Geranylgeranyl diphosphate reductase	95	51,3	8,92	Photosynthesis / chlorophyll biosynthetic process	Chloroplast	0,572
A0A3Q7EHJ9	Glucose-1-phosphate adenylyltransferase	106	56,8	8,16	Starch biosynthetic process / glucose-1-phosphate adenylyltransferase activity	Chloroplast	0,667
A0A3Q7FYH4	Glutmate-1-semialdehyde 2,1-aminomutase	76	51,5	6,99	Chlorophyll biosynthesis / transaminase activity	Chloroplast	0,653
A0A3Q7HK83	Glutathione peroxidase	139	26,2	9,06	Response to oxidative stress / peroxidase activity	Cytoplasm	1,672
A0A3Q7HWS9	Nucleoside diphosphate kinase	123	25	8,95	Nucleoside diphosphate phosphorylation	Cytoplasm	1,557
P23322	OEE protein 1	2,029	34,9	5,96	PS II stabilization / oxygen evolving activity	Chloroplast	1,569
A0A3Q7HGJ9	Phosphoglycerate kinase	1,483	92,7	6,93	Gluconeogenesis	Cytosol	0,631
A0A3Q7EQ38	Phosphoribosylamine-glycine ligase	369	135	5,43	Purine nucleobase biosynthesis / protein binding	Chloroplast	0,613
Q2MIA0	PS I P700 chlorophyll a A1	435	83	7,18	Photosynthesis / electron transfer activity	Chloroplast	3,192
Q2MIA1	PS I P700 chlorophyll a A2	993	82,4	7,17	Photosynthesis / electron transfer activity	Chloroplast	3,098
P54773	Photosystem II 22 kDa protein	420	29,3	8,72	Photosynthesis	Chloroplast	1,85
A0A0C5C9Q9	PS II CP43 reaction center protein	1031	51,9	7,21	Photosynthetic e-transport PS II / e-transport	Chloroplast	2,252
Q2MI75	PS II CP47 reaction center protein	493	56	6,77	Photosynthetic e-transport PS II / e-transport	Chloroplast	2,497
A0A0C5CUN5	Photosystem II D2 protein	911	39,5	5,55	Photosynthetic e-transport PS II / e-transport	Chloroplast	2,178
Q672Q6	PS II OEC protein 3	454	24,6	9,64	Photosynthetic e-transport chain/ e-transport	Chloroplast	1,566

continue...

Table 1. Continuation...

Accession No.	Protein	Score	Mass (kDa)	pI	Biological process / molecular function	Cellular compartment	Abundance (control-100/ control)
A0A0C5CHA2	Photosystem II protein D1	665	38,9	5,25	Photosynthetic e-transport PS II / e-transport	Chloroplast	2,492
A0A3Q7FFY2	PS II stability/assembly factor	95	42,2	6,71	Photosystem II (PSII) biogenesis	Chloroplast	0,628
K4DFV3	Plasmamembrane intrinsic protein 13	77	30,8	7,83	Response to water deprivation / Water chanel activity	Plasma membrane	1,506
P17340	Plastocyanin	416	17	5,2	Photosynthesis / electron transfer activity	Chloroplast	1,636
A0A3Q7H287	PSI subunit V	544	23,1	9,67	Photosynthesis / electron transport	Chloroplast	1,933
Q9LEG3	Putative alcohol dehydrogense	119	27	6,43	Growth-development	Muti-location	1,49
P07179	Ribulose bisphosphate carboxylase small subunit	788	20,3	7,05	Photosynthesis	Chloroplast	3,992
A0A3Q7GYB4	Tr-type G domain- protein	369	53,2	7,39	Mitochondrial translational elongation	Mitochodrion	0,614

Table 2. List of differentially abundant proteins in leaves of tomato plants treated with 100 ppm TiO_2 NPs under drought.

Accession No	Protein	Score	Mass (kDa)	pI	Biological process / molecular function	Cellular compartment	Abundance (drought-100/ drought)
A0A3Q7IAU4	(S)-2-hydroxy-acid oxidase	540	40,7	8,98	Primary metabolism / (S)-2-hydroxy-acid oxidase activity	Peroxisome	1,571
A0A3Q7IR67	14_3_3 domain-containing prot	158	28,2	4,82	Signal transduction	Cytoplasm	0,609
A0A3Q7IH24	23 kDa subunit OEE of PS II	98	25,7	8,69	Photosynthesis / oxygen evolving activity	Chloroplast	0,48
A0A3Q7ILD3	5-methyltetrahydropteroyltri glutamate-homocysteine S-methyltransferase	358	84,7	6,4	Methionine biosynthetic process / Zinc ion binding	Cytosol	1,748
A0A3Q7HH02	60S acidic ribosomal protein P0	157	34	5,16	Cytoplasmic translation / Large ribosomal subunit rRNA binding	Cytosol	1,449
A0A3Q7H036	ACT domain-containing prtein	91	30,6	5,67	Aminoacid metabolism	Muti-location	0,618
A0A3Q7GD18	ATP synthase subunit beta	484	59,6	6,06	ATP synthesis / ATP binding	Chloroplast	1,505
K4BAE6	Catalase	522	56,9	7,21	Response to hydrogen peroxide / catalase activity	Cytoplasm	1,695
A0A3Q7I0X4	Chlorophyll a-b binding proein	492	31,1	6,14	Photosynthesis / chlorophyll binding	Chloroplast	1,646
A0A0C5CED3	Cytochrome b559 subunit	227	9,4	4,94	Photosynthetic e-transport chain / e-transfer	Chloroplast	0,654
Q2MI71	Cytochrome b6	151	24,1	8,76	Photosynthesis / electron transport	Chloroplast	0,584
A0A3Q7J9P7	Cytochrome c domain-containing protein	147	35,7	7,01	Mitochondrial electron transport, ubiquinol to cytochrome c / electron transfer activity	Mitochondrion	0,714
A0A3Q7F980	Fructose-bisphosphate aldolase	1,064	41,9	8	Glycolytic process	Cytosol	1,519
A0A3Q7FSD7	Geranylgeranyl diphosphate reductase	95	51,3	8,92	Photosynthesis / chlorophyll biosynthetic process	Chloroplast	1,461
A0A3Q7HBV3	Germin-like protein	1,539	22	6,77	Response to oxidative stress	Apoplast	0,591

continue...

Table2. Continuation...

Accession No	Protein	Score	Mass (kDa)	pI	Biological process / molecular function	Cellular compartment	Abundance (drought-100/ drought)
A0A3Q7FLA3	Glutamine amidotransferase type-2 domain-containing pro	285	177	6,51	Ammonia assimilation cycle / glutamate synthase activity	Mitochodrion	1,839
A0A3Q7HQA3	Glycine cleavage system P protein	335	113	7,14	Organonitrogen compound biosynthetic process / glycine binding	Mitochodrion	1,578
A0A3Q7F3S0	Nucleoside diphosphate kinase	285	16,2	6,8	Nucleoside diphosphate phosphorylation	Cytosol	0,5
K4DFV3	Plasmamembrane intrinsic protein 13	77	30,8	7,83	Response to water deprivation / water channel activity	Plasma membrane	1,602
A0A3Q7GB48	Thioredoxin dmain-con protein	73	13,6	6,32	Protein-disulfide reductase activity	Cytosol	0,598
A0A3Q7IDX8	Transketolase	458	68,7	5,88	Pentose-phosphate shunt / metal ion binding	Cytosol	1,578

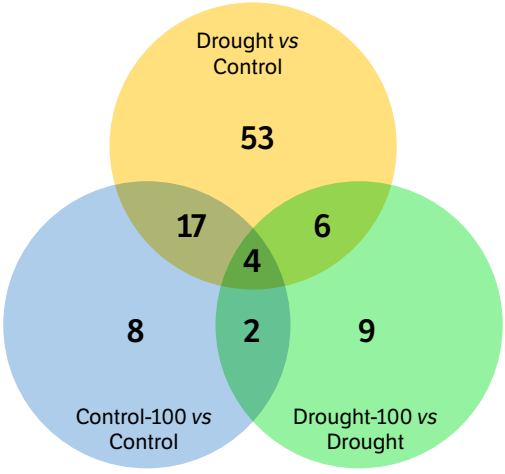


Figure 6. Venn diagram of differently expressed proteins.

All proteins were classified into six functional categories, such as photosynthesis, biosynthesis, translation, energy, stress response, and others (Fig. 7).

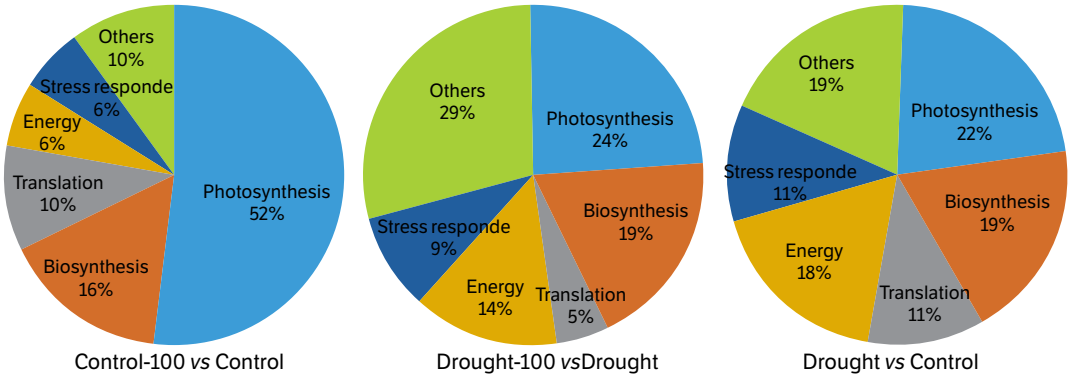


Figure 7. Functional classification of the differentially expressed proteins during control/drought and/or TiO_2 NP treatment in tomato.

DISCUSSION

Drought is accepted as one of the most important environmental stress factors due to the size of the agricultural areas it affects and its negative effects on almost all plant functions. The response of plants to drought stress is very complex and involves changes in morphological, physiological, and biochemical processes (Ramadan et al. 2022).

As it was expected, RWC of tomato plants were reduced by drought stress in this study. It is well known that plants prevent transpiration by closing stomata, increasing stomatal resistance, and decreasing stomatal conductivity under drought stress. These changes are resulted in decrease in RWC and photosynthesis (Sousaraei et al. 2021). While TiO_2 NPs application did not change the RWC in control, it increased in drought-TiO_2-treated group compared to non-TiO_2 treated group. High RWC is often associated with high-stress tolerance for tomato under drought conditions. Therefore, better preserving the water by TiO_2 NPs treatment under drought stress may contribute to enhance drought tolerance of tomato plants. However, there is no record in the literature showing that TiO_2 NPs application increases RWC by influencing the cellular mechanism. The fact that TiO_2 NPs application increased the abundance of plasmamembrane intrinsic protein (Tables 1 and 2) under drought conditions in this study may contribute to explain this situation. The results of Bárzana et al. (2022) support the findings of the present study. They reported that NPs applications lead the alteration of the expression aquaporin genes.

The results in this study showed that drought stress increased proline content compared to well-watered plants. The results are in good agreement with the literature (Çevik et al. 2019, Ali et al. 2021). Proline accumulation is considered as an adaptive metabolic adaptation of plants to drought stress (Borišev et al. 2016).

Proline is known as a multi-functional molecule, and osmoprotectant role is its best known feature. TiO_2 treatment decreased proline content under drought stress. However, no correlation was found between RWC and proline content in TiO_2 and drought-treated plants in this study. The relationships between NP treatment and proline content under stress conditions in the literature are complicated.

Borišev et al. (2016) showed that fullerenol NPs treatment decreased proline content in sugar beets plants, while Ali et al. (2021) reported that chitosan NPs treatment increased proline content in *Catharanthus roseus* plant under drought stress. Ramadan et al. (2022) emphasized that up to 150 ppm TiO_2 treatment did not significantly change the proline content under drought stress. On the other hand, Shah et al. (2021) reported that seed priming of maize seeds with TiO_2 enhanced proline content under salinity stress. These results may indicate that changes in proline content of plants that treated with TiO_2 under environmental stress conditions create stress, application dose or species-specific response.

MDA is the end product of the lipid peroxidation and a good indicator of the membrane damage. The increasing of MDA content under drought may indicate that drought-induced oxidative stress cause oxidation of membranes (Hossain et al. 2015). Treatment of TiO_2 NPs to drought-stressed plants decreased MDA content in this study. The decreasing of MDA content under drought conditions by TiO_2 treatment compared to drought control group may be an indication that plant tolerance increased by TiO_2 NP treatment. In reports on this subject, it has been emphasized that exogenous TiO_2 NPs treatment reduces the H_2O_2 content by increasing antioxidant enzyme activities, and it causes decreasing in the MDA content (Gohari et al. 2020). TiO_2-NPs treatment was found toxic at high level on *Zea mays*, and the authors reported that TiO_2-NPs may act as oxidant, which may cause oxidative stress in plants (Karvar et al. 2022). In the present study, TiO_2-NPs treatment before drought stress may trigger the antioxidant system by causing oxidative stress at low level. This situation may be advantage for plants to get ready against to drought stress. Thus, when the plants were exposed to drought stress, they could create a faster and stronger antioxidant response.

Since proteome changes caused by drought stress in tomato have been shown by many studies in the literature (Çelik et al. 2021, Rai et al. 2021), proteome changes that occur in control and drought conditions as a result of TiO_2 treatment were compared and discussed in this study. TiO_2 NPs treatment increased the acumulation 15 of 16 photosynthesis-related proteins under control condition. The increase in photosynthetic parameters after TiO_2 NPs treatments were also shown in different plants by other researchers under both control and stress conditions (Ahmad et al. 2018, Gohari et al. 2020).

Titanium is known as a beneficial element for crucial mechanisms of plants such as photosynthesis at low concentrations (Ahmad et al. 2018). In the present study, the increasing of the abundance of proteins associated with electron transport, chlorophyll biosynthesis and calvin cycle by TiO_2-NPs treatment may help to enhance photosynthetic activity of tomato plants

compared to non-TiO_2 treated plants under drought stress. In the literature, enhanced activity of the key photosynthetich proteins such as rubisco, chlorophyll a-b binding protein and subunits of photosystems after TiO_2-NPs was well documented (Ahmad et al. 2018). According to the results of this study, especially the photosynthetic electron transport mechanism was affected by TiO_2 treatment. On the other hand, TiO_2 NPs treatment increased the abundance of light harvesting chlorophyll a-b binding (LHCB) protein, which was down-regulated by drought stress. LHCB proteins is one of the most abundand membran protein in the natüre, and over-expression of this protein has been associated with stress tolerance (Xu et al. 2012). According to these findings, increasing of LHBC protein abundance by TiO_2 NPs treatment under drought stress may enhance drought tolerance of tomato plants.

The abundace of biosynthesis-related proteins (5-methyltetrahydropteroyltri glu-tamate-homocysteine S-methyltransferase, geranylgeranyl diphosphate reductase) also up-regulated by TiO_2 NPs treatment under drought stress. 5-methyltetrahydropteroyltr iglutamate-homocysteine S-methyltransferase is involved in methionine biosynthetic process. H. Zhang et al. (2016) used two different cotton cultivars that have difference tolerance to drought in their proteomic study and reported that 5-methyltetra hydropteroyltriglutamate-homocysteine S-methyltransferase protein was expressed much more in drought-tolerant culture, and this could increase drought tolerance. They also defined this protein as a key enzyme that may have important roles in drought tolerance. Geranylgeranyl diphosphate reductase catalyses the reduction of geranylgeranyl diphosphate to phytyl diphosphate, and Tanaka et al. (1999) clearly showed that this protein has a important role in biosynthesis of tocopherol and chlorophyll. Liu et al. (2015) confirmed the findings of Tanaka et al. (1999) and showed that plant tolerance to various abiotic stresses increased when they up-regulated this protein. Up-regulation of 5-methyltetrahydropteroyltri glutamate-homocysteine S-methyltransferase and geranylgeranyl diphosphate reductase proteins with TiO_2-treatment may enhance the methionine, tocopherol and chlorophyll metabolisms for tomato plants to cope with drought stress. Maqsood et al. (2022) showed that exogenous methionine application alleviated drought related inhibition via reducing reactive oxygen species content and enhancing antioxidant capacity. Similar results were also reported for tocopherol by Ma et al. (2020). These findings may indicate that exogenous TiO_2-NPs application may alleviate drought caused inhibition by inducing biosynthesis of methionine and tocopherol.

Exogenous application of TiO_2 NPs up-regulated two (fructose-bisphosphate aldolase and ATP synthase subunit beta) and down-regulated one (cytochrome c domain-containing protein) energy related proteins. There are two different isoforms of fructose-bisphosphate aldolase: cytoplasmic and plastidic (Çevik et al. 2019). The cytoplasmic FBA has a central role in glycolysis pathway (Ziveri et al. 2017). ATP synthase is closely related to energy metabolism; it accelerates synthesis of ATP for methabolic activities (Zhou et al. 2022). Under drought conditions, the energy required by cells increases due to the active stress response. Therefore, exogenous applications that support energy metabolism are very important for plants to cope with drought. In this study, the improvement of energy metabolism by titanium application may be a great advantage for tomato plants.

Plants close their stomata to maintain water status in response to drought stress. It limits CO_2 uptake and increase radical content. However, plants have adapted to eliminate harmful radicals using antioxidant defense systems (Sutulienė et al. 2021). TiO2 treatment up-regulated the expression of catalase protein in this study. Catalase is one of the most important antioxidant system members and catalyze the reduction of hydrogen peroxide into oxygen and water. There are some studies that showed correlation between high catalase activity and drought tolerance in the literature (Nasirzadeh et al. 2021, Islam et al. 2022).

The protection of the membranes from harmful radicals such as H_2O_2 is mostly depended on high catalase activity. Therefore, it can be said that TiO_2 NPs application reduces the harmful effects of radicals by increasing antioxidant enzyme activities. (S)-2-hydroxy-acid oxidase (alternative name of glycolate oxidase 1) is the other protein that up-regulated with TiO_2 NP application under drought stress. (S)-2-hydroxy-acid oxidase catalyses the oxidation of glycolate to generate glyoxylate and TiO_2. This enzyme causes the production of 70% total cellular H_2O_2 in C3 plants. Z. Zhang et al. (2016) proposed that activities of catalase and glycolate oxidase enhanced response to environmental stress to modulate H_2O_2 level. Emamverdian et al. (2021) clearly showed that TiO_2-NPs application increased antioxidant enzyme activities and decreased content of reactive oxygen species (H_2O_2 and $O_2^{\bullet-}$) under cadmium stress. Unfortunately, there is no evidence on how exogenous NP

treatments induce these changes in plants. It is not yet clear whether these changes are caused by the stimulation of specific mechanisms or whether the TiO_2 NPs act as an oxidant and cause the cellular response.

One of the most interesting finding of the present study was increasing of plasma membrane intrinsic protein abundance by TiO_2 NP treatment both control and drought conditions. Plasma membrane intrinsic protein is a member of aquaporins. Aquaporins increase membrane permeability and play an important role in plant water balance. Li et al. (2016) conducted a study to understand the roles of plasma membrane intrinsic protein under control and drought condition in tomato plants. They up-regulated two plasma membrane protein genes expressions and concluded that over-expression of these genes improve the plant water content and maintain osmotic balance. These findings suggest that accumulation of plasma membrane intrinsic protein in plants may improve drought tolerance. However, exogenous TiO_2 NP treatment enhanced the expression of this protein under control and drought condition. This may help to explain how TiO_2 NP enhance water content under drought conditions.

CONCLUSION

This study showed that MDA content decreased and RWC was better maintained with 100 ppm TiO_2 NP application under drought conditions. These two parameters are associated with high-drought tolerance. Proteomic analyses indicated that the antioxidant defense system was stimulated by TiO_2 application, and accordingly lipid peroxidation was reduced. In order to maintain the water potential under drought conditions, it was determined that TiO_2 NPs up-regulated aquaporins rather than increasing the intrinsic osmoprotectants. The increase of plasma intrinsic membrane protein abundance with TiO_2 NPs application may be the key point behind the increase in drought tolerance level of tomato plants after NP treatment under drought conditions. It is considered important to conduct more detailed studies on this subject. In addition, the up-regulation of photosynthesis-related proteins by TiO_2 NP application under control conditions, as well as drought conditions, may indicate that these NPs have the potential to be used to increase photosynthesis efficiency under control conditions.

DATA AVAILABILITY STATEMENT

All dataset were generated and analyzed in the current study.
Supplementary material is at https://doi.org/10.5281/zenodo.7411744.

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