Alleviation of the effects of on drought stress *Verbascum nudicuale* by methyl jasmonate and titanium dioxide nanoparticles

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Abstract

Drought stress causes severe metabolic dysfunctions by formation of oxidative stress that may lead to damage in DNA, inactivation of enzymes, and lipid peroxidation. Plants have developed different morphological, physiological, and biochemical mechanisms to withstand the drought stress. Plant growth regulators (PGRs) are one of the most important endogenous substances involved in the amelioration of tolerance in various plant species. In this study, the possible recovery ability of *Verbascum nudicuale* plants from drought stress conditions was assessed using methyl jasmonate (MJA), titanium dioxide nanoparticles (TiO_2NPs), and their interactions as the PGRs in liquid culture media. Results showed that the growth parameters and photosynthetic pigment content markedly decreased due to polyethylene glycol (PEG)-induced drought stress. The treatment of *V. nudicuale* plants with TiO_2NPs showed a considerably improving effect on pigments synthesis and biomass production during the treatment. However, cultures containing MJA negatively affected the growth parameters and increased the content of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA). Treatment of plants with PGRs showed a considerable improvement in increasing the synthesis of the phenolic compounds and proline accumulation. Alteration in the activity of antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD), and polyphenol oxidase (PPO) significantly varied in different treatments. These findings suggest that the recovery treatment with PGRs proved to be very effective in alleviating the adverse effects of drought stress.

Keywords: biomass; drought stress; plant growth regulators; phenol; *Verbascum nudicuale* Wydl.

Ghasemlou, F., H. Amiri, R. Karamian and **A. Mirzaie-asl.** 2019. 'Alleviation of the effects of on drought stress *Verbascum nudicuale* by methyl jasmonate and titanium dioxide nanoparticles '. *Iranian Journal of Plant Physiology,* 9 (8),2911-2920.

Introduction

Drought stress may result in severe metabolic dysfunctions through inducing oxidative stress which in turn may cause damage in DNA, inactivate enzymes and result in lipid peroxidation (Akte et al., 2016). In response to water deficit, plants undergo a number of changes in physiological and metabolic mechanisms (Akte et al., 2016). Multiple factors activate the resistance response of plants to drought stress, and PGRs are one of the most important endogenous substances involved in the

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Received: May, 2019
Accepted: September, 2019

mechanisms of susceptibility or tolerance of various plant species (Lambert et al., 2011). PGRs are considered as signal molecules leading to the creation of messages that are widely employed to mediate the accumulation of plant secondary metabolites (Raskin, 1992). Methyl jasmonate (MJA) is known as a PGR in the induction of secondary metabolites, that is able to regulate the resistance of plants against both biotic and abiotic stresses by the regulation of contiguous molecular processes (Raskin, 1992). On the other hand, this natural PGR can elicit a number of morphological and physiological responses to environmental stresses (Raskin, 1992).

In recent years, nanomaterials have received particular attention for their positive impact on plant growth due to their small size (Shobha et al., 2014). They can be used as PGR to induce plants defense system. Various nanomaterials including silver, gold, zinc, copper, titanium, silicon, and magnesium nanoparticles (NPs) are commercially available with diverse applications in biology, agriculture, and medicine (Abdel Latef et al., 2018). Titanium is a useful element for the plant and can stimulate the absorption of essential elements such as nitrogen, phosphorus, calcium, magnesium, iron, manganese, and zinc. This increases the ability of the plant to absorb and use water and food and like chloroplasts; it has the ability to absorb light and can play a positive role in enhancing the plant's photosynthesis efficiency (Abdel Latef et al., 2018). Upon entering the cells, nanoparticles (NPs) can produce reactive oxygen species (ROS) and induce oxidative stress that activate defense gene expression (Shallan et al., 2016).

Verbascum is the largest genus within the large family of Scrophulariaceae. Extracts of *Verbascum* plants contain a substantial number of valuable natural products (Alipieva et al., 2014). Phenolic compounds are known as a class of secondary metabolites of these plants which have recently attracted attentions because of their antioxidant and free radical scavenging abilities (Alipievaet al., 2014). The enhanced phenolic compounds production is associated with an increase in the expression of key genes of the phenyl propanoid pathway (Lambert et al., 2007). The present study was aimed to investigate the recovery effects of MJA, TiO_2NPs, and MJA + TiO_2NPs on the biomass production and determine whether upregulation of nonenzymatic and enzymatic antioxidant systems could be enhanced for the survival of *V. nudicuale* Wydl. plants during drought stress induced by polyethylene glycol (PEG).This protocol can enable us to produce enough plant material of this medicinal species required for different purposes such as secondary metabolites production and biotechnological researches.

Material and Methods

Plant material

Seeds of *V. nudicuale*. were collected from Hamedan province, west of Iran from July to August, 2016. Seeds were washed with tap water, surface-sterilized in 70% (v/v) ethanol and 5% sodium hypochlorite for 10 min, and then rinsed three times in sterile distilled water. Seeds were cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with %3 (w/v) sucrose and 1% (w/v) agar. The media pH was adjusted to 5.7-5.8 prior to autoclaving. All cultures were incubated at 25 ± 2° C under a 16:8 h light/dark photoperiod in a culture room. Thirty-day-old plants were then transferred to basal MS liquid media containing different concentrations of PEG-6000 for creating artificial drought conditions at three levels (0, -0.3, and -0.6 MPa osmotic potential) and also 200 µM MJA and 20 mg L^{-1} TiO_2NPs as PGRs. Cultures were maintained on a rotary shaker at 100 rpm for 15 days before they were used for subsequent experiments.

PGR preparation and treatments

Reagents used as specific scavengers, PEG-6000 and MJA, were purchased from Sigma-Aldrich (St. Louis, MO, USA). TiO_2NPs were purchased from US Research Nanomaterials (USA) with an average diameter of 10-25 nm (99% anatase). The required amount of PEG with the molecular mass of 6000 was calculated by Michel and Kaufman (1973) equation and added to the liquid culture media at three levels 0, -0.3, and -0.6 MPa osmotic potential prior to autoclaving at 121° C for 15 min. The nanoparticles were suspended in deionized water using a sonicator prob horn ultrasonic for at least 15 minutes and then added

to the cultures prior to the autoclave, as to PEG. MJA was used at concentration of 200 µM; it was prepared as a stock solution in ethanol and filter sterilized through a Millipore filter (0.22 µm, Sartorius). Sterilized MJA was then added to the liquid culture media after autoclave. PGRs concentration ranges used in these experiments including MJA and TiO_2NPs were chosen by reviewing published studies (Ali et al., 2007; Lambert et al., 2011; Kamalizadeh et al., 2015). The effects of PGRs including MJA, TiO_2NPs, and MJA + TiO_2NPs on 30 day-old plants were measured after 15 days of culture in these media. A factorial experiment based on a completely randomized design was used to study the effect of the PGRs on the measured parameters. All experiments were conducted in triplicate.

Determination of biomass and photosynthetic pigments

The amounts of the shoot growth were measured in terms of fresh weight (FW) and dry weight (DW). Shoots were placed between the folds of blotting paper to remove excess water and FW was weighed. Then, they were oven-dried at 70 °C for 48 h and weighed again for determination of DW. The pigment contents of the leaves were calculated as mg g^{-1} FW by method of Lichtenthaler and Wellburn (1983).

Determination of phenolic compounds content

For total phenolics and flavonoids, leaves were homogenized in 80% methanol (6 mL) and then centrifuged at 5,000 rpm for 20 min and then assessed according to Miliauskas et al. (2004) and Chang et al. (2002), respectively.

Determination of hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and free proline contents

The generation of H_2O_2 was measured according to Velikova et al. (2000). The concentration of H_2O_2 was calculated using the molar extinction coefficient 0.28 M^{-1} cm^{-1} (ε_{530} = 0.28 M^{-1} cm^{-1}). The level of membrane lipid peroxidation was determined by MDA as a final product of lipid peroxidation. The quantity of MDA-thiobarbituric acid (TBA) complexes formed was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Vos et al., 1991). Free proline content was determined according to Bates et al. (1973).

Determination of antioxidant enzyme activity

The extraction procedure of soluble protein for determination of antioxidant enzyme activity was based on the method described by Gadzovska et al. (2007). Determination of total protein content was performed with a Bio-Rad Protein Assay Reagent (Bradford, 1976) using bovine serum albumin (BSA) as standard. Superoxide dismutase (SOD) activity was determined following the method of Beauchamp and Fridovich (1971). Peroxidase activity (POD) was determined by the guaiacol oxidation method (González et al., 1999). Polyphenol oxidase (PPO) activity was determined based on the method of the Raymond et al. (1993). The activity of all three enzymes was shown in the form of min^{-1} mg^{-1} protein.

Statistical Analyses

Experiments were independently repeated three times under the same conditions and all analyses were performed in triplicate. Error bars of graphs showed the standard error of mean value (±SE). The statistical analyses were performed with the SPSS statistical software program (SPSS Inc, version 20). Means were compared using one-way ANOVA (GML procedure). All statistical tests were considered significant at $P \leq 0.05$.

Results

Effect of PGRs on the shoot growth

A decrease in the shoot fresh and dry weights was observed upon the exposure of the control plants to PEG. MJA and MJA + TiO_2NPs decreased shoot fresh and dry weights twice as much as those in the control plants (Fig. I). MJA at 200 µM seemed to have an inhibitory effect on the shoot growth. On the other hand, shoot fresh and

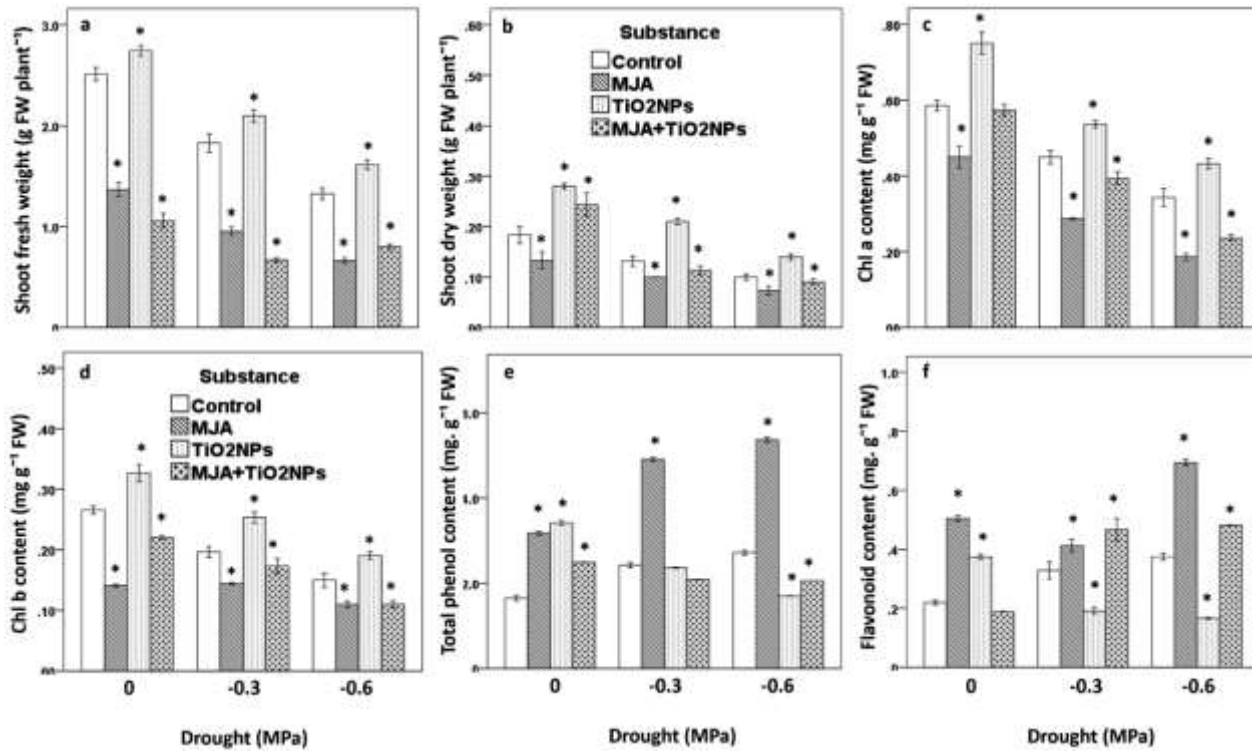

Fig.I. Effects of MJA, TiO_2NPs, and MJA + TiO_2NPs on shoot fresh weight, shoot dry weight, chlorophyll (Chl) a, b, total phenol, and flavonoid contents in the *V. nudicuale* plants under drought stress; asterisk denoted values indicate significant differences between control and treated cultures ($P < 0.05$). DW: Dry weight; FW: Fresh weight

dry weights in the treated plants with TiO_2NPs increased significantly (Fig. I) even in the presence of drought stress.

Effect of PGRs on photosynthetic pigments

Drought stress also influenced chlorophyll (Chl) a and b contents so that at higher drought levels, Chl a and b contents significantly decreased in control plants (Fig. I). A marked difference was observed in Chl a and b contents between the treated and control plants. Similar to biomass production, an increase in the pigment contents was observed in TiO_2NPs -treated cultures even in the presence of drought stress (Fig. I). Pre-incubation of the cultures with MJA and MJA + TiO_2NPs decreased photosynthetic pigments contents, which were significantly lower than those of the control plants (Fig. I).

Effect of PGRs on phenolic compounds content

The amounts of phenol compounds in the treated and control cultures showed marked differences. Studying levels of phenolic compounds in the cultures treated with MJA, TiO_2NPs, and MJA + TiO_2NPs revealed that these treatments induced a sizable increase in the total phenol content in comparison with the control plants in the absence drought stress (Fig. I); however, the total phenol contents did not show any increase in TiO_2NPs and MJA + TiO_2NPs -treated plants at -0.3 and -0.6 MPa osmotic potential. The plants treated with MJA revealed a sizable increase in total phenol contents at all levels of drought stress (Fig. I).

Unlike TiO_2NPs, the plants treated with MJA revealed a sizable increase in total flavonoid contents compared to control cultures at all levels of drought stress (Fig. I). Study of flavonoid contents in the treated cultures showed that MJA + TiO_2NP treatment induced a significant increase in the content of flavonoid compared to non-stressed conditions. Similar to total phenol contents, flavonoid contents in control plants increased with an increase in the drought stress (Fig. I).

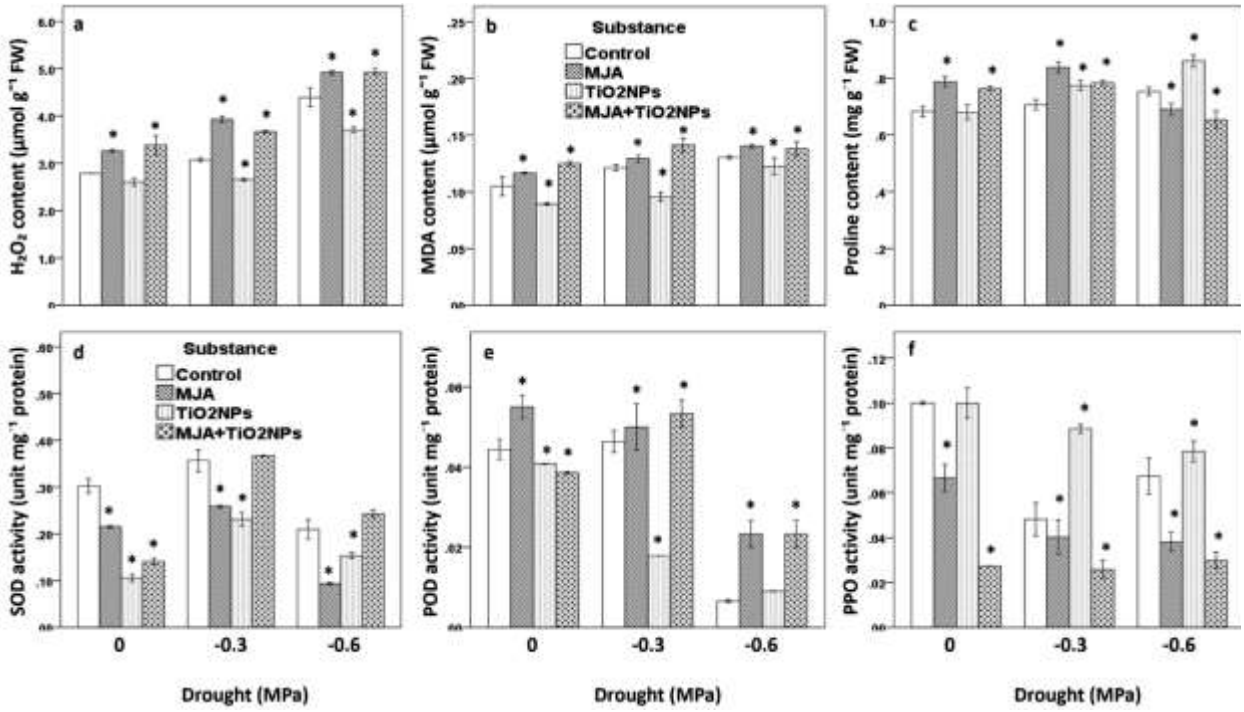

Fig.II. Effects of MJA, TiO_2NPs, and MJA + TiO_2NPs on hydrogen peroxide (H_2O_2), malondialdehyde (MDA), proline content, superoxide dismutase (SOD) activity, peroxidase (POD) activity, and polyphenol oxidase (PPO) activity in the *V. nudicuale* plants under drought stress; asterisk denoted values indicate significant differences between control and treated cultures ($P< 0.05$).

Effect of PGRs on H_2O_2 content

Quantitative measurement of H_2O_2 using spectroscopic analysis showed that exposure to PGRs led to significant changes in H_2O_2 content (Fig. II). Our results showed that H_2O_2 content increased with the drought intensification in control plants. A significant increase in H_2O_2 level was induced by MJA and MJA + TiO_2NPs treatments at all levels. A sizable reduction in H_2O_2 production was observed in TiO_2NPs -treated plants at -0.3 and -0.6 MPa levels which demonstrated super protection effects of this PGR in the scavenging of free radicals even at a higher level of water deficit (Fig. II).

Effect of PGRs on MDA content

Results of PGRs treatment on MDA content in *V. nudicuale* are shown in Fig. II. TiO_2NPs treatment remarkably decreased the MDA content which was similar to the results of H_2O_2 (Fig. II). Changes in MDA content were observed in MJA and MJA + TiO_2NPs-treated cultures, which caused a significant increase in MDA content at all levels of drought stress.

Effect of PGRs on free proline content

Changes in free proline content were observed in TiO_2NPs-treated cultures, which caused a significant increase in free proline content especially in the presence of drought stress (Fig. II). The treated plants with MJA and MJA + TiO_2NPs treatments revealed a sizable increase in proline content compared to control cultures at 0 and -0.3 MPa levels (Fig. II); however, a significant decrease in proline content was observed in the plants exposed to these two treatments compared with controls at -0.6 MPa (Fig. II).

Effect of PGRs on antioxidant enzyme activity

SOD activity in the plants treated with PGRs is shown in Fig. II. A decrease in SOD activity was observed in shoots upon the exposure to MJA and TiO_2NPs treatments. MJA + TiO_2NPs treatment resulted in a reduction of SOD activity in non-stress conditions, unlike -0.3 and -0.6 MPa levels

which did not affect SOD activity (Fig. II). After treatments with PEG, POD activity showed a decrease at -0.6 MPa osmotic potential in control plants. MJA influenced POD activity significantly in *V. nudicuale* plants at all levels (Fig. II). It is worth noting that MJA + TiO_2NPs was found to be effective in the enhancement of POD activity in the treated cultures compared to control plants except in conditions without tension; however, TiO_2NPs showed an increase in POD activity only at -0.3 MPa level (Fig. II). MJA and MJA + TiO_2NPs were shown to differentially decrease PPO activity in *V. nudicuale* cultures compared to control plants. Unlike SOD and POD enzymes, the treatment of plants with TiO_2NPs significantly promoted PPO activity especially at stress conditions (Fig. II); however, as shown in Fig. II, enzyme activity showed a decrease with drought intensification in control plants.

Discussion

Drought stress suppresses plant growth, triggers an oxidative burst, accelerates the degradation of photosynthetic pigments and cell membrane damage, induces an array of antioxidant enzymes expression, elicits membrane lipid peroxidation, and accelerates leaf senescence (Nekrasova et al., 2011). Upon stress perception, plants evolved numerous nonenzymatic and enzymatic antioxidant defenses leading to the creation of messages that activate the ion channels and adjust the activity of kinases, and the production of various ROS. These messages ultimately lead to the expression of particular groups of defense genes that produce a general defense response (Lambert et al., 2011).

Chlorophyll derivatives are involved in many biological activities of plants such as antioxidant and antimicrobial activities (Moiseeva and Mikhailesc, 2000). The decreases in plant growth and photosynthesis that occur in response to drought stress are generally attributed to increases in the content of hormones related to stress (Akte et al., 2016). According to our results, cultures containing MJA seriously decreased chlorophyll content and biomass production (Fig. I), supporting the hypothesis that a specific signaling mechanism is triggered by MJA at its high concentration. In barley and soybean, MJA (100 μM) treatment prohibited the germination and growth, reduced the expression of photosynthesis-related genes, increased the degradation of Rubisco, and finally caused a reduction in chlorophyll and biomass production (Wu et al., 2012). Hence, the negative effects of MJA on photosynthetic pigments content and growth in cultures containing this PGR are in a dose-dependent manner that, at this concentration, is toxic to the plant growth. It has been shown that anatase TiO_2NPs can be effectively internalized and can lead to extensive metabolic changes in plant cells (Shallan et al., 2016). This entrance can be elucidated by the means of osmotic force, cell wall pores, and plasmodesmata (Abdel Latef et al., 2018). However, the various effects of NPs on plants can be explicated depending on the size and/or the shape of the particles, the applied concentrations, the specific conditions of experiments, the plant species, and the mechanism of uptake (Nekrasova et al., 2011). The low concentration of TiO_2NPs increases the production of chlorophyll by increasing nitrogen and magnesium absorption, resulting in increased root growth and mineral absorption (Abdel Latef et al., 2018); however, higher concentrations of TiO_2NPs may cause cyto- and geno-toxicity in plants. Hence, the positive effects of TiO_2NPs on plant growth can be attributed to enhancing photosynthesis as well as better cell growth by regulating cell cycle (Fig. I).

PGRs may cause transient oxidative stress which acts as a hardening process, thereby increasing the antioxidative capacity of the plants (Popova, 2013). It seems that the production of secondary metabolites such as phenolics and the other low molecular weight substances was induced by the defense system of the treated plants (Chalker-Scott, 1999). The phenolic compounds play important roles in the production of biomass and increasing plant resistance to stress by the inactivation of enzymes free radicals and thus act as a scavenger of ROS (Ali et al., 2007). MJA and SA are two key signal molecules widely used as PGRs inducing particular enzymes of the secondary metabolic pathway to form defense compounds such as phenolics and triterpenoid saponins (Ali et al., 2007). Plant tissues containing flavonoid and anthocyanin are usually resistant to stress (Chalker-Scott, 1999), and this emphasizes the protective role of these

compounds in plants. Total phenol and flavonoid production increases in the roots of *Panax ginseng* by MJA and SA treatment and this strongly indicates that these PGRs result in the production of phenolic compounds by altering the phenolic synthesis enzymes (Ali et al., 2007). Kamalizadeh et al. (2015) reported that TiO_2NPs up to 30 mg L^{-1} induced the accumulation of rosmarinic and chlorogenic acids in *Dracocephalummoldavica*. Due to the results obtained from content of phenol substances in the treated plants, it can be concluded that these PGRs cause the trigger signaling cascade and hence increase different phenolic compounds as the main part of the antioxidant mechanism in the plants.

Oxidative stress implies a harmful process, which is mainly related to the oxidizing nature of ROS. Hydrogen peroxide, which is considered as one of the active oxygen species, is a signal molecule that leads to oxidative stress and thus activates a signal transduction pathway (Maksymiec, 2007). Portions of membrane lipid including polyunsaturated fatty acids are hypersensitive to the peroxidation of lipids created by ROS. To counter the adverse effects of ROS plants evolved numerous nonenzymatic and enzymatic antioxidant defenses. The expression of many defense genes in the treated plants by MJA could be explained by ROS mediated responses that were involved mainly in regulating oxidative reactions and inducing antioxidant systems (Shan and Liang, 2010). It is suggested that MJA can induce oxidative stress to activate the antioxidant system of the plants (Ali et al., 2007). The toxic action of NPs on proteins can be expressed by binding NPs to protein SH bonds (Nekrasova et al., 2011). Excess ROS production and significant increase in lipid peroxidation were also reported from various plants exposed to different types of metal oxide NPs (Dimkpa et al., 2012). An increase in the hydrogen peroxide content in the cotton plants was observed as a result of treatment with TiO_2NPs (Shallan et al., 2016). According to our findings, it may be concluded that the role of MJA in activating the defense system was mediated by the increase in the H_2O_2 production followed by activation of the expression of defense genes. In MJA and MJA + TiO_2NPs -treated cultures, the increase in MDA content can be due to the increase in H_2O_2 production. TiO_2NPs showed a decrease in H_2O_2 production followed by a decrease in lipid peroxidation, suggesting that these PGRs application could alleviate drought-induced injury in *V. nudicuale* plants.

The production of osmolytes such as proline is a well-known adaptive mechanism in plants under stress conditions such as drought and high salinity (Ali et al., 2007); hence, the level of proline accumulated could be a basis for selection of water stress-tolerant plants. The alleviating effect of MJA on cell membrane function under water deficit conditions can also be associated with the activation of the synthesis of soluble phenolics and anthocyanins, increasing proline content or up-regulation of antioxidant system along with simultaneous reduction in H_2O_2 and lipid peroxidation levels, which protect cell membranes against the harmful effects of ROS (Ali et al., 2007). The increased accumulation of proline in NPs-exposed plants may have resulted from the plant defense mechanism for protecting the cell structures from the damage of excess ROS and MDA generation (Chiang and Dandekar, 1995). Under drought stress, the accumulation of proline during NPs treatment results in balancing the osmotic potential of cytoplasm with that of the environment (Shallan et al., 2016). In our study, a substantial increase in proline level was observed in cultures treated with all treatments (Fig. II); this indicates the positive effects of PGRs in increasing proline content in order to protect the plant under the drought stress.

The production of antioxidant enzymes such as SOD, POD, and catalase (CAT), which are functionally interdependent, are created by oxidative stress (Nekrasova et al., 2011). Alteration in the activity of SOD, PPO, and POD enzymes significantly varied in different treatments. Our results indicated that the downregulation of the enzymatic antioxidant system observed in the treated plants, possibly owing to a reduced need for the removal of activated oxygen species, was consistent with the recovery in the synthesis of secondary metabolites in *V. nudicuale* plants. Exogenously supplied MJA and SA activate antioxidant enzymes and induce resistant protein expression (Popova et al., 2013); they can significantly reduce the effects of ROS overproduced. It was shown in *Scrophularia striata* cell cultures that, 100 µM MJA decreased

the SOD activity during the first 12 h treatment and then increased its activity. In addition, the POD and CAT activities in the cultures treated with 100 µM MJA significantly increased 12 h after treatment as compared to their control plants (Khanpour-Ardestani et al., 2015). Hence, MJA with hold-up antioxidant enzymes level blocked the ROS effects on membranes. The entrance of heavy metals into plants activates defense systems, induces the accumulation of osmolytes, and changes the hormonal balance and production of SOD, CAT, and POD enzymes; the activation of antioxidant enzymes in response to the metal may differ between plant species (Polevoi, 2001). Both positive and negative effects have been observed after exposure of plants to various kinds of NPs (Kus et al., 2006). For example, in the case of copper ions, it has been shown that these ions inhibit the activity of SOD, POD, and CAT enzymes more strongly, but copper NPs activate them (Nekrasova et al., 2011). In cultures exposed with MJA and MJA + TiO_2NPs, an increase in the activity of POD enzyme was observed at -0.3 and -0.6 MPa levels which may be a defense mechanism to increase plant survival at higher drought levels.

Conclusion

In the present study, we conducted an investigation on the effect of alternative means of improving growth by the use of PGRs and nanoparticles in *V. nudicuale* plants. Results revealed that these treatments can lead to a series of physiological and biochemical changes in *V. nudicuale*. Treatment with TiO_2NPs proved to be very effective in alleviating the adverse effects of drought stress on *V. nudicuale* cultures through increasing their antioxidative potential for drought stress tolerance and recovery. On the other hand, the use of combined PGRs (MJA + TiO_2NPs) can be a good option for the enhancement of plant secondary metabolites synthesis suggested. However, this study needs further scrutiny to understand the molecular mechanism between nanomaterials and other PGRs under abiotic stress. Also, because *Verbascum* species contain a variety of bioactive compounds, further intensive studies are required in animal models to use *Verbascum* as a potentially effective therapeutic material for treating disease.

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