IMPACT OF NANOSIZED TITANIUM DIOXIDE ON AGRONOMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF ANNUAL MEDIC (*MEDICAGO SCUTELLATA L.*)

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ABSTRACT. In order to investigate the effect of exogenous application of nano-TiO₂ on annual medic, a field study was conducted in a factorial design based on randomized complete blocks with four replications. The experimental treatments included six concentrations of nano-TiO₂ (Control, 0.01%, 0.02%, 0.03%, 0.04%, 0.06% g/l) and spraying at two growing stages (pod stage and 10% flowering stage). Results showed that the effects of nano-TiO₂ and spraying times on dry forage yield were significant (p<0.01). Nano-TiO₂ spray appear to influence the malone dialdehyde (MDA) content (p<0.01). With increasing concentrations of nano-TiO₂ the values of aforementioned measured variable significantly decreased. The activities of antioxidant enzymes, including catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) were affected by nanoparticle (p<0.01) and spraying times (p<0.01), as well as their interactive effect of two mentioned factors were significant in terms of guaiacol peroxidase (GPX) (p<0.01) activity and dry forage yield. Among different concentrations of nano-TiO₂, 0.04% and 0.06% have the best effect on all traits. Totally, treatment with nano-TiO₂ were more effective in the pod stage, compared to 10% flowering stage.

Key words: Medic; Nano-TiO₂; Antioxidant enzymes; Dry forage yield.

INTRODUCTION

The equilibrium between the production and the scavenging of ROS may be perturbed by various biotic and abiotic stress factors such as drought, salinity, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks (Bhattachrjee, 2005). Also, oxidative stress is a common mechanism for cell damage induced by nanoparticles (NPs). NPs may induce intracellular oxidative stress by disturbing the balance between oxidant and antioxidant processes (Pulska et al., 2007). These disturbances in equilibrium
lead to sudden increase in intracellular levels of ROS which can cause significant damage to cell structures. They also can damage DNA, proteins, lipids and chlorophyll (Mittova et al., 2000). Nanoparticles (nanoscale particles = NSPs) are atomic or molecular aggregates with at least one dimension between 1 and 100 nm (Roco 2003), that can drastically modify their physical and chemical properties, compared to the bulk material (Nel et al., 2006). Applications of nanomaterials can help faster plant germination/production, effective plant protection with reduced environmental impact as conflicting to traditional approaches (Khot et al., 2012).

There has been research by experts on the applications of titanium dioxide (TiO2) NPs can reduce H2O2, superoxide radicals, and malonyldialdehyde content in the aged chloroplasts of spinach by activating superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) (Lei et al., 2008). Lu et al. (2002) reported that combination of nanosized SiO2 and TiO2 could increase nitrate reductase enzyme in soybean (Glycine max), increase seed abilities of absorbing and utilizing water and fertilizer, encourage its antioxidant system, and actually hasten its germination and growth. Song et al. (2012) reported that Lemna minor cells accumulated high levels of ROS when the seedlings were exposed to TiO2 NPs than when exposed to the same concentration of bulk TiO2. The plant cells increased antioxidant enzyme defense (POD, SOD, and CAT) activity to eliminate the accumulated ROS in plant cells when the TiO2 NPs concentration was lower than 200 mg/L in the culture media.

Considering the mentioned effects of TiO2 NPs on physiological process, this study aimed to evaluate potential effects of TiO2 NPs on antioxidant enzymes activities and dry yield production of annual medic.

**MATERIALS AND METHODS**

**Plant materials and experiment design**

Current investigation was conducted under farm condition at the University of Share Gods, Iran, to investigate the effect of TiO2 nanoparticles on the antioxidants enzymes activity, lipid peroxidation (MDA) and dry yield production of annual medic (Medicago scutellata L.).

The experiment was conducted in a factorial design based on randomized complete blocks with four replications. The experimental treatments included six concentrations of nano-TiO2 (Control, 0.01%, 0.02%, 0.03%, 0.04%, 0.06% g/l) and spraying at two growing stages (pod stage and 10% flowering stage).

**Protein determination**

Protein concentration of the various extracts and solutions was determined by the dye-binding method of Bradford (1976), using bovine serum albumin as standard.

**Enzyme extractions**

A quantity of 0.5 g of fresh foliar tissue from fresh seedlings (uppermost leaves) were harvested, weighed, washed
with distilled water and then were ground in cold mortar; 2.5 ml ice-cold extraction buffer (0.05 M Tris-HCl pH 7.5, 3 mM MgCl₂, 1 mM EDTA) were added on powder. Extraction buffer included 2 mM ascorbate that is served for determining APX activity. The homogenates were centrifuged at 15,000 g for 15 min at 4°C. Three replicates per treatment were used. The supernatant was stored at 4°C and used for CAT, APX, and GPX assays.

**Enzyme assays**

CAT activity was determined by measuring H₂O₂ consumption (Maehly and Chance, 1959) in reaction mixture containing 3 ml of 50 mM phosphate buffer (pH 7.0), 5 μl H₂O₂ %30 (w/v) and 50 μl extraction buffer. CAT activity was measured spectrophotometrically at 240 nm. Extinction coefficient of 0.036 mM⁻¹ cm⁻¹ was used to calculate its activity. The unit for CAT activity was micromoles of hydrogen peroxide oxidized per minute per milligram of protein. All assays were performed in triplicates.

APX activity was measured using modified method originally described by Asada (1992). The reaction mixture contained 3 ml of 50 mM potassium phosphate buffer (pH=7.0), which included 0.5 mM ascorbate and 0.1 mM EDTA. Then, 400 μl H₂O₂ %30 (w/v) and 100 μl extraction buffer were added. APX activity was measured spectrophotometrically at 290 nm. Extinction coefficient of 2.8 mM⁻¹ cm⁻¹ was used to calculate its activity. The unit for APX activity was micromoles of ascorbate oxidized per minute per milligram of protein.

GPX activity was determined according to the method of Chang and Kao (1998). The reaction mixture contained 3 ml 50 mM potassium phosphate buffer, 10 μl H₂O₂ 30% (w/v), 1 ml guaiacol 1% and 0.3 ml extraction buffer. GPX activity was measured spectrophotometrically at 420 nm. Extinction coefficient of (26.6) (mM⁻¹ cm⁻¹) in a minute was used to calculate its activity.

Oxidative damage to leaf lipids, resulting from drought stress, was measured in terms of MDA content using thiobarbituric acid (TBA) - reactive substances (Sairam et al., 1998). Leaf samples of 0.5 g were homogenized in 10 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 g for 5 min. Four milliliter of 0.5% TBA in 20% TCA was added to 2 mL of aliquot of the supernatant. The mixture was heated at 100°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000 g for 10 min, the absorbance of supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The following formula was applied to calculate malondialdehyde (MDA) content using its absorption coefficient (ε) and expressed as nmol malondialdehyde g⁻¹ fresh mass, following the formula:

\[
\text{MDA (nmol g}^{-1}\text{ FM) = [(A532-A600) \times V \times 1000/\varepsilon] \times W,}
\]

where \(\varepsilon\) is the specific extinction coefficient (≈155 mM⁻¹ cm⁻¹), \(V\) is the volume of crushing medium, \(W\) is the fresh weight of leaf, \(A600\) is the absorbance at 600 nm wavelength and \(A532\) is the absorbance at 532 nm wavelength.
Statistical analysis

The experimental design was a factorial experiment, completely randomized in three replications. Graphs were drawn using Microsoft Office Excel 2013 software.

RESULTS AND DISCUSSION

Dry forage yield production

Result showed that the effect of nano-TiO₂ and spraying times in dry forage yield were significant \((p<0.01)\). Also, interactive effect of two mentioned factors were significant \((p<0.01)\) on this trait \((\text{Tab. 1})\). Using TiO₂ at both 10% flowering stage and beginning of poding stage had a significant effect on dry yield production. The greatest dry yield production was observed at 0.04 and 0.06% concentrations of TiO₂, which were applied at 10% flowering stage (respectively, 1753 and 1740 kg/ha). Foliar application at 10% flowering stage at the beginning of poding stage had considerable positive effect on dry forage yield production \((\text{Fig. 1-h})\).

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>CAT</th>
<th>APX</th>
<th>GPX</th>
<th>MDA</th>
<th>Dry forage yield</th>
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<tr>
<td>Replication</td>
<td>3</td>
<td>114/89</td>
<td>97/94</td>
<td>97/54</td>
<td>16/97</td>
<td>17933/29</td>
</tr>
<tr>
<td>Spraying time (T)</td>
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<td>474/3</td>
<td>70/08</td>
<td>158/05</td>
<td>176/3</td>
<td>163216/68</td>
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<tr>
<td>Concentration (C)</td>
<td>5</td>
<td>419/1</td>
<td>545/5</td>
<td>61/78</td>
<td>315/7</td>
<td>220899/38</td>
</tr>
<tr>
<td>T × C</td>
<td>5</td>
<td>11/20</td>
<td>14/48</td>
<td>5/65</td>
<td>3/78</td>
<td>7124/38</td>
</tr>
<tr>
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<td>17/10</td>
<td>9/15</td>
<td>0/97</td>
<td>10/38</td>
<td>1839/29</td>
</tr>
<tr>
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<td>21/72</td>
<td>12/35</td>
<td>15/65</td>
<td>17/30</td>
<td>12/92</td>
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As a result of unique physicochemical properties of engineered nanoparticles which are due to their small size, large surface area, chemical composition, surface reactivity, and charge, shape, and media interactions, they have recently attracted the attention of many researchers \((\text{Abdi et al., 2008; Menard et al., 2011})\). In this respect, Feizi et al. \((2012)\) also reported that application of bulk and nano-sized titanium dioxide improved seed germination and seedling growth in wheat. Nano-anatase TiO₂ treatment improves spinach growth by influence on nitrogen photoreduction \((\text{Yang et al., 2007})\). Owolade et al. \((2008)\) reported that the seed yield of cowpea \((\text{Vigna unguiculata} \text{ L. Walp.})\) was increased when treated (as foliar application) with nano-sized titanium dioxide. Similar yield increases were reported in rice with a corresponding reduction in the incidence Curvularia leaf spot and bacteria leaf blight disease \((\text{Chao and Choi, 2005})\).
Figure 1 - Effects of TiO₂ NPs treatments on some physiological traits and dry forage yield production of annual medic
Antioxidant enzymes activity

Data analysis clarified that the activity of antioxidant enzymes was affected by TiO2 nanoparticles. The simple effect of concentration and stage of TiO2 nanoparticles application on CAT, APX and GPX activities was significant at \( p \leq 0.01 \) whereas the interaction between concentration and stage of TiO2 nanoparticles application was only significant on GPX activity \( (p \leq 0.01) \) (Tab. 1).

Mean comparison clarified that CAT activity was influenced by TiO2 concentration as TiO2 concentration increased, CAT activity significantly increased. The greatest CAT activity was assayed at 0.06% solution whereas the lowest value occurred at 0.01% solution of TiO2. CAT activity also was impressed by stage of TiO2 application. The activity of CAT was greater, when TiO2 used at pod stage compared with 10% flowering stage (Fig. 1-c,d).

Using TiO2 solution at beginning of pod stage when compared with 10% flowering stage had significantly higher effect on APX activity. TiO2 concentration significantly affected APX activity. When TiO2 nanoparticles concentration increases from 0.01 to 0.06%, APX activity also regularly increased. APX activity reached to twice of control treatment when TiO2 concentration reached to 0.06 (Fig. 1-a,b).

The effect of TiO2 concentration and the stage of TiO2 using on GPX activity were signignificant. The greates GPX activity was exhibited in 0.06% concentration of TiO2 which was applied at 10% flowering stage and the lowest value was related to control treatment at the beginning of poding stage (Fig. 1-g).

Exposure of plants to unfavorable environmental conditions can increase the production of ROS to protect themselves against these toxic oxygen intermediates, plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ antioxidant defense systems (Tuteja, 2007). In plants, ROS are normal by products of various metabolic pathways and are also produced under stress conditions in various cellular compartments. ROS are generated by normal cellular metabolism, and it’s production is controlled by various enzymatic and nonenzymatic antioxidant systems. CAT (H2O2 oxidoreductase) is a heme-containing enzyme that catalyzes the dismutation of H2O2 into H2O and O2. In our study, TiO2 considerably increased CAT activity in alfalfa plants. In this respect, Harison (1996) also reported that exogenous application of TiO2 nanoparticles increases CAT activity and plant resistance to environmental stresses. APX is thought to play the most essential role in scavenging ROS and protecting cells in higher plants, algae, euglena and other organisms. APX is involved in scavenging of H2O2 in water-water and ASH-GSH cycles and utilizes ASH as the electron donor (Gill and Tuteja, 2010). GPX decomposes indole-3-acetic acid (IAA) and has a role in the
biosynthesis of lignin and defence against abiotic stresses by consuming H$_2$O$_2$. GPX plays a key role in decreasing H$_2$O$_2$ content accumulation, eliminating MDA resulting cell peroxidation of membrane lipids and maintaining cell membrane integrity (Jaleel et al., 2008).

Higher APX, GPX, and CAT activities within plant cells mean that the plant accumulated a higher level of ROS, but the plant can eliminate this accumulated ROS in the plant cells. If the stress was too strong, the plants' defense system could not stop the production of ROS effectively; as such, some antioxidant enzymes activities would decrease, resulting in severe damage to plants or even plant death (Mittler, 2002). The significant effect of titanium nanoparticles on spinach is probably attributed to the small particle size, which allows its penetration into the seed during the treatment period. Such promotory effect of nanoscale TiO$_2$ on antioxidant system was reported in soybean (Lu et al., 2002). The exposure of TiO$_2$ nanoparticles reduced CAT activity in isopods (P. scaber) (Jemec et al., 2008), but increased its activity in freshwater cladoceran (D. pulex) (Klaper et al., 2009). This is probably due to the difference in defense system among the various test species, the nanoparticles characteristics, and the treatment condition such as the use of cosolvent or sonication pretreatment.

Laware and Raskar (2014) reported that CAT and GPX activities enhance in the presences of 10-30 µgml-1 TiO$_2$ nanoparticles but their activities decrease by higher concentrations of TiO$_2$ nanoparticles. The nano-particles might have reduced ROS stress in treated onion seedling by reducing H$_2$O$_2$, superoxide radicals, and lipid peroxidation products and increasing activities of superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, and catalase enzymes as observed in spinach (Lei et al., 2008). Based on results of TiO$_2$ nanoparticles effect on onion seedling growth it could be stated that nanoparticles might have helped activated and promoted hydrolytic enzymes seed antioxidant system (Lu et al., 2002).

**MDA content**

Mean comparison showed that the stage of TiO$_2$ application had a significant effect on MDA accumulation as using this compound at the 10% flowering stage had a greater effect on MDA accumulation, when compared with beginning of poding. MDA accumulation decresed by incresing TiO$_2$ concentration. The lowest MDA accumulation was observed in 0.06% concentration of TiO$_2$ (Fig. 1-e,f). The peroxidation of lipids is considered as the most damaging process known to occur in every living organism. The determination of MDA is a convenient method of quantifying the extent of lipid peroxidation (Chang and Sung, 1998). In present study, MDA content decreased under higher levels of TiO$_2$. Federici et al. (2007)
attributed lipid peroxidation, one of the recognized oxidative stress mediated consequences, to direct contact of the tissue with TiO\textsubscript{2} nanoparticles and indirect rapid distribution of ROS around the body.

REFERENCES


EFFECTS OF EXOGENOUS APPLICATION OF NANO-TITANIUM DIOXIDE ON ANNUAL MEDIC


