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# 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-$TiO_2$ 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-$TiO_2$ (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-$TiO_2$ and spraying times on dry forage yield were significant ($p$<0.01). Nano-$TiO_2$ spray appear to influence the malone dialdehyde (MDA) content ($p$<0.01). With increasing concentrations of nano-$TiO_2$ 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-$TiO_2$, 0.04% and 0.06% have the best effect on all traits. Totally, treatment with nano-$TiO_2$ were more effective in the pod stage, compared to 10% flowering stage.

**Key words**: Medic; Nano-$TiO_2$; 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 (Pulskamp *et al.,* 2007). These disturbances in equilibrium

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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 ($TiO_2$) NPs can reduce $H_2O_2$, 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 $SiO_2$ and $TiO_2$ 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 $TiO_2$ NPs than when exposed to the same concentration of bulk $TiO_2$. The plant cells increased antioxidant enzyme defense (POD, SOD, and CAT) activity to eliminate the accumulated ROS in plant cells when the $TiO_2$ NPs concentration was lower than 200 mg/L in the culture media.

Considering the mentioned effects of $TiO_2$ NPs on physiological process, this study aimed to evaluate potential effects of $TiO_2$ 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 $TiO_2$ 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-$TiO_2$ (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 $MgCl_2$, 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 $H_2O_2$ consumption (Maehly and Chance, 1959) in reaction mixture containing 3 ml of 50 mM phosphate buffer (pH 7.0), 5 µl $H_2O_2$ %30 (w/v) and 50 µl extraction buffer. CAT activity was measured spectrophotomerically 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 by 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 $H_2O_2$ %30 (w/v) and 100 µl extraction buffer were added. APX activity was measured spectrophotomerically 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 $H_2O_2$ 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 100ºC 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 ε 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-$TiO_2$ 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 (*Tab. 1*). Using $TiO_2$ 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 $TiO_2$, 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 (*Fig. 1-h*).

**Table 1 - Analysis of variance on some physiological traits and dry yield production of annual medic affected by $TiO_2$ NPs at two growing stages (pod stage and 10% flowering stage)**

| S. O. V. | df | MS | | | | |
|---|---|---|---|---|---|---|
| | | CAT | APX | GPX | MDA | Dry forage yield |
| Replication | 3 | 114/89$^{**}$ | 97/94$^{**}$ | 97/54$^{**}$ | 16/97$^{ns}$ | 17933/29$^{**}$ |
| Spraying time (T) | 1 | 474/3$^{**}$ | 70/08$^{**}$ | 158/05$^{**}$ | 176/3$^{**}$ | 163216/68$^{**}$ |
| Concentration (C) | 5 | 419/1$^{**}$ | 545/5$^{**}$ | 61/78$^{**}$ | 315/7$^{**}$ | 220899/38$^{**}$ |
| T × C | 5 | 11/20$^{ns}$ | 14/48$^{ns}$ | 5/65$^{**}$ | 3/78$^{ns}$ | 7124/38$^{**}$ |
| Error | 33 | 17/10 | 9/15 | 0/97 | 10/38 | 1839/29 |
| CV (%) | | 21/72 | 12/35 | 15/65 | 17/30 | 12/92 |

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 (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 $TiO_2$ treatment improves spinach growth by influence on nitrogen photoreduction (Yang *et al.,* 2007). Owolade *et al.* (2008) reported that the seed yield of cowpea (*Vigna unguiculata* 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 (Chao and Choi, 2005).

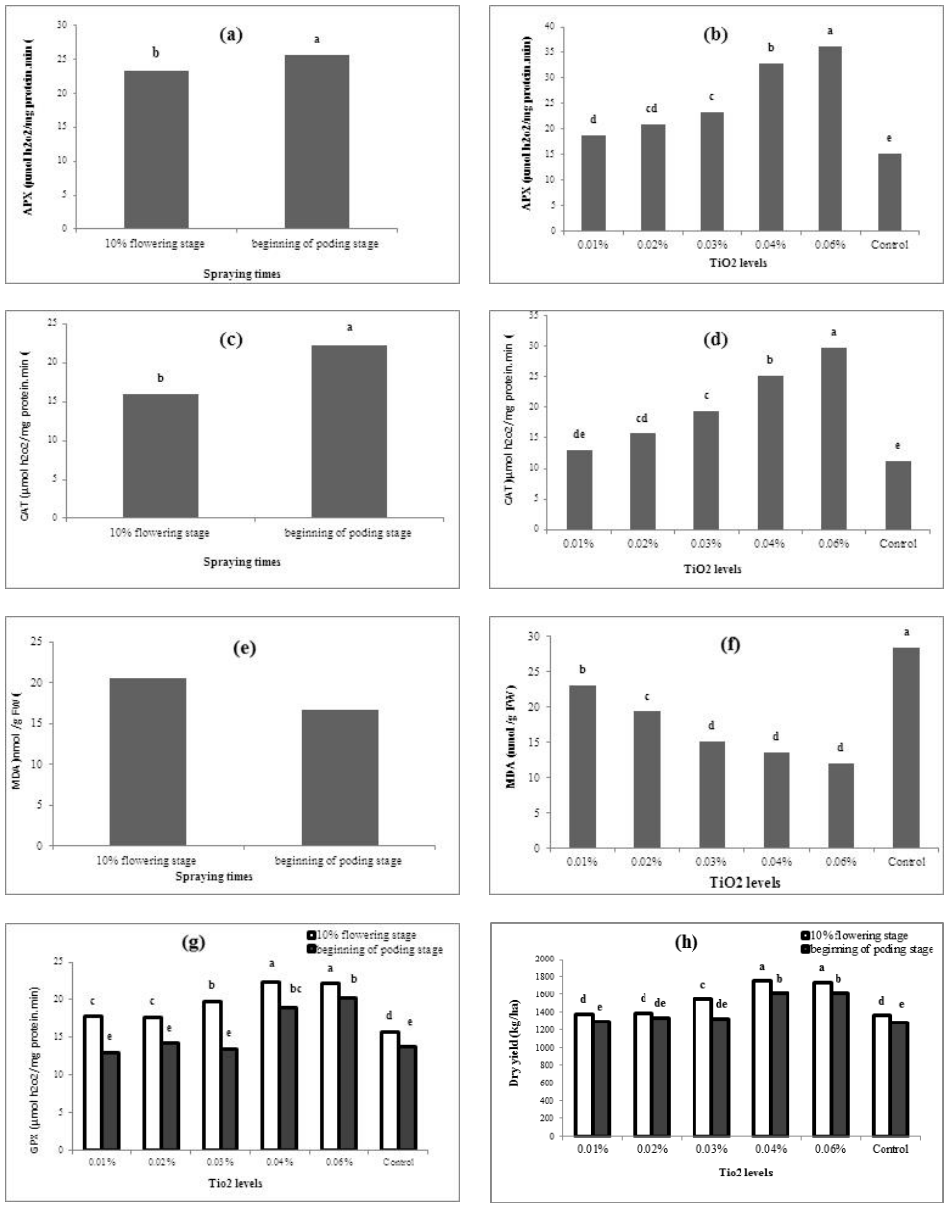


**Figure 1 - Effects of $TiO_2$ NPs treatments on some physiological traits and dry forage yield production of annual medic**

**Antioxidant enzymes activity**

Data analysis clarified that the activity of antioxidant enzymes was affected by $TiO_2$ nanoparticles. The simple effect of concentration and stage of $TiO_2$ nanoparticles application on CAT, APX and GPX activities was significant at $p \leq 0.01$ whereas the interaction between concentration and stage of $TiO_2$ 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 $TiO_2$ 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 $TiO_2$ application. The activity of CAT was greater, when $TiO_2$ used at pod stage compared with 10% flowering stage (*Fig. 1-c,d*).

Using $TiO_2$ solution at beginning of pod stage when compared with 10% flowering stage had significantly higher effect on APX activity. $TiO_2$ concetration significantly affected APX activity. When $TiO_2$ nanoparticles concentration increases from 0.01 to 0.06%, APX activity also regularly increased. APX activity reached to twice of control treatment when $TiO_2$ concentration reached to 0.06 (*Fig. 1-a,b*).

The effect of $TiO_2$ concentration and the stage of $TiO_2$ using on GPX activity were signignificant. The greates GPX activity was exhibited in 0.06% concentration of $TiO_2$ 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 ($H_2O_2$ oxidoreductase) is a heme-containing enzyme that catalyzes the dismutation of $H_2O_2$ into $H_2O$ and $O_2$. In our study, $TiO_2$ considerably increased CAT activity in alfalfa plants. In this respect, Harison (1996) also reported that exogenous application of $TiO_2$ 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 $H_2O_2$ 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 $H_2O_2$. GPX plays a key role in decreasing $H_2O_2$ 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 $H_2O_2$, 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 $TiO_2$ nanoparticles and indirect rapid distribution of ROS around the body.

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