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Influence of titanium and bio-fertilizers on some agronomic and physiological attributes of triticale exposed to cadmium stress

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Abstract

To evaluate the effects of titanium and bio-fertilizers on some agronomic and physiological properties of triticale grown in cadmium (Cd) contaminated soil, a glasshouse experiment was conducted based on a completely randomized design with three replications. The treatments were arranged in a 4×4 factorial experiment with four levels of titanium (control, bulk titanium, 0.01%, and 0.02% titanium nano-particles) and four levels of bio-fertilizers (no bio-fertilizer, *Azotobacter chroococcum, Azospirillum brasilense* and *Azorhizobium caulinodans*). The Cd contamination increased Cd accumulation in the leaves and seeds and also enhanced antioxidant enzymes activity. Cadmium stress decreased grain yield, 1000-grain weight and chlorophyll. Titanium nano-particle (0.02%) caused the maximum increase in grain yield, 1000-grain weight, leaf Cd, seed Cd, chlorophyll content. Superoxide dismutase (SOD) and catalase (CAT) activity increased due to titanium application but malondialdehyde (MDA) contented decreased. Although, there was no significant difference between control and bulk titanium treatments in terms of SOD activity. The effect of 0.02% nano-particle in increasing SOD activity was more pronounced than 0.01% treatment. In addition, CAT activity was more affected by titanium nano-particles than bulk titanium. Furthermore, *Azorhizobium* increased grain yield, 1000-grain weight, leaf Cd, seed Cd and chlorophyll while decreased superoxide dismutase and catalase enzyme activity.

Keywords: Titanium; triticale; antioxidant enzymes; bio-fertilizers.

1. Introduction

Cadmium (Cd) is a non-essential heavy metal, released into the environment by anthropogenic and non-anthropogenic sources (Sandalio *et al.,* 2001). So far, few studies have been conducted to determine the effect of toxic Cd levels on the uptake and distribution of Cd in different parts of plants, particularly in the parts used as food or feed. Crop plants growing in high levels of Cd will show a series of physiological disorders, such as reduction in chlorophyll, sugar and protein content, decrease of photosynthesis and dramatic change of phenol content and related enzyme activities, finally leading to lower yield (Satyakala, 1997). For instance, Muradoglu *et al.,* (2015) indicated that Cd had negative effect on chlorophyll content. Cadmium induces oxidative stress leading to the overproduction of harmful reactive oxygen species (ROS) (Zhang *et al.,* 2010). These ROS may cause damage to cell membranes, proteins, DNA replication and repair. Cadmium is not redox-active metal and cause ROS generation and oxidative stress via indirect mechanisms (Srivastava and Dubey, 2012). This metal deplete cell's major antioxidants particularly thiol containing antioxidants and enzymes (Sharma *et al.*, 2012).

Nano-particles are introduced in a growing number of commercial products. Among them, TiO_2 nano-particles are one of the most produced TiO_2 nano-particles in the world (Breckle, 1991). Titanium has significant biological effects on plants, being beneficial at low and toxic at higher concentrations. Although titanium is not toxic for animals and humans, its effects on plants and bacteria show noteworthy concentration dependence. Whereas for bacteria it acts as an antibiotic (Yaghoubi *et al.,* 2000), for plants, it shows beneficial effects on various physiological parameters at low doses [e.g. biomass yield (Pais, 1993), essential element contents (Giménez *et al.,* 1990), chlorophyll contents (Carvajal *et al.,* 1994) and is toxic at higher ones [chlorosis, retardation of growth (Hruby *et al.,* 2002)]. The positive effects of titanium treatment were found on rape plant development (an increase of chlorophyll content and photosynthesis rate), the yield and thousand-seed weight of winter wheat, and the yield and sugar content of sugar beets (Grenda, 2003). Plants interact with their atmospheric and edaphic environments strongly and are expected to be affected by engineered nano-particles (Ruffini-Castiglione and Cremonini, 2009). In this regard, Lu and co-workers (2002) have shown that a combination of nano-sized SiO_2 and TiO_2 increases the nitrate reductase enzyme in soybean (*Glycine max*) and improves plants abilities to absorb and utilize water and fertilizer. In addition, promotes antioxidant system, during germination and growth. Moreover, it has been found that TiO_2 nano-particles encourage spinach (*Spinacia oleracea)* seed germination and plant growth (Zheng *et al.,* 2005). Navarro and co-workers, (2008) stated that engineered nano-particles could sequester nutrients on their surfaces and thus serve as a nutrient stock to the organisms, particularly those engineered nano-particles having high

specific surface area. Relatively few mechanisms have been demonstrated that explain the increased tolerance to environmental stresses of plants treated with bacteria of the genus *Pseudomonas*. These bacteria can facilitate plant growth indirectly by reducing plant pathogens, or directly by facilitating the uptake of nutrients from the environment, by influencing phytohormone production (e.g. auxin, cytokinin and gibberellins), by enzymatic lowering of plant ethylene levels and/or by production of siderophores (Glick *et al.*, 1997; Kohler *et al.,* 2006). Considering the above states, an experiment was performed to study the combined effects of titanium leaf application and bio fertilizers on antioxidant enzymes activity and some agronomic and physiological attributes in triticale.

2. Materials and Methods

2.1. Study site and climate

The experiment was undertaken at the Agricultural Research farm in Islamic Azad University, Varamin-Pishva branches, Tehran, Iran in 2015. Study site was situated at 31° 51′ E and 20° 59′ N and 1050 m above sea level.

2.2. Experimental design and treatments

A completely randomized design arranged in a 4 × 4 factorial experiment, with three replications was used. The experimental factors included four levels of titanium (control, bulk titanium 0.01% and 0.02% titanium nano-particles) and four levels of bio-fertilizers (no bio-fertilizer application, *Azotobacter chroococcum, Azospirillum brasilense* and *Azorhizobium caulinodans*). Triticale seeds were treated with bacterial suspension of (72 h old, density 2-5x10^8 CFU ml^{-1}) for 30 min before sowing. Ten seeds were sown in 30 × 30 cm plastic pots filled with soil. The $CdCl_2$ (80 mg kg^{-1} of soil) was mixed into the soil before potting.

2.3. Titanium foliar application

Titanium dioxide nano-particles and titanium oxide (bulk) were sprayed on the plants by a calibrated pressurized backpack sprayer (20 l) at stem elongation and flowering stages. Control plants were treated by distilled water.

2.4. Sampling and data collection

At the seed filling stage, flag leaves were collected and immediately frozen in liquid nitrogen and stored at -80º C until laboratory analyses. At maturity stage, plants were harvested at soil surface and seeds were collected and weighted. Grain yield per plant was determined. All the leaves were dried for 48 h, at 85 °C, in laboratory oven, for determining cadmium contents. Leaves and seed samples were separately digested by HNO_3 and $HClO_4$ in tubes placed on an A1 block brought gradually to 205 °C. Cd was determined by atomic absorption spectrophotometry, using an ICP-AES atomic absorption spectrophotometer (Inductively Coupled Plasma Atomic Emission Spectroscopy, SPS 1200VR, Seiko, Japan).

2.5. Determination of chlorophyll content in leaf extract

Chlorophyll was extracted in 80% acetone from the leaf samples according to the method of Arnon (1949). Extracts were filtrated and content of total chlorophyll was determined by spectrophotometry at 645 and 663 nm, respectively. The content of chlorophyll was expressed as mg g^{-1} fresh weight according to the following equation (Arnon, 1949).

$$\text{Total chlorophyll} = [20.2(D645) + 8.02(D663)] \frac{V}{1000W}$$

2.6 Antioxidant enzyme activity

Superoxide dismutase (EC 1.15.1.1) activity was determined by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitro blue tetrazolium according to the method of Giannopolitis and Ries, (1977). The reaction mixture contained 100 µl l µm riboflavin, 100 µl 12 mM L-methionine, 100 µl 0.1 mM EDTA (pH 7.8), 100 µl 50 mM Na_2CO_3 (pH 10.2), 100 µl 75 µM nitro blue tetrazolium in 2300 nitro blue tetrazolium 25mM sodium phosphate buffer (pH 6.8) and 200 µl crude enzyme extract, in a final volume of 3 ml. Glass test tubes that contained the reaction mixture were illuminated with a fluorescent lamp (120 W), and identical tubes that were not illuminated served as blanks. After illumination for 15 min, absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme which caused 50 % inhibition of photochemical reduction of nitro blue tetrazolium.

Catalase (EC 1.11.1.6) activity was estimated by the method of Cakmak and Horst, (1991). The reaction mixture contained 100 µl crude extract, 500 µl 10mm H_2O_2 and 1400 µl 25 mm sodium phosphate buffer. The decrease in the absorbance recorded at 240 nm for 1 min by a spectrophotometer.

The level of membrane damage was determined by measuring MDA as the end product of peroxidation of membrane lipids (De Vos *et al.,* 1991). In brief, samples were homogenized in an aqueous solution of tri-chloroacetic acid (10 % w/v), and aliquots of filtrates were heated in 0.25% thio-barbituric acid to 100 °C, for 30 min. The amount of MDA was determined from the absorbance at 532 nm, followed by correction for the non-specific absorbance at 600 nm. The content of MDA was determined using the extinction coefficient of MDA (ε = 155 μM^{-1} cm^{-1}).

2.6. Statistical analysis

All data were analysed from analysis of variance using the GLM procedure in SAS (SAS Institute Inc., 2002). The assumptions of the variance analyses were tested by checking if the residuals were random, homogenous, with a normal distribution and a mean of about zero. The significance of differences among means was carried out using Duncan's multiple range test at $p < 0.05$.

3. Results and Discussion

The main effects of titanium concentration and bio-fertilizer were significant on all measured traits however, the main effect of bio-fertilizer was not significant on leaf cadmium concentration and MDA content (Table 1). The results revealed that the interaction between titanium

concentration and bio-fertilizer was significant on grain yield, 1000-grain weight and total chlorophyll content. In general, titanium application could increase grain yield and 1000-grain weight (Table 2) but 0.02% titanium dioxide nano-particle application was more effective to improve yield and yield components (Table 2). The highest grain yield was obtained when stressed plants were treated with 0.02% titanium dioxide nano-particle (Table 3).

The results suggest that titanium increases triticale growth, yield and yield components. Among different titanium treatments titanium dioxide nano-particle at 0.02% concentration, produced the highest yield and yield components. Zheng and co-workers, (2005) demonstrated that titanium nano-particles helped the water absorption in spinach and improved growth. The results of Zheng and co-workers, (2005) showed that spinach growth greatly improved by applying 250-4,000 ppm nano-titanium dioxide. In addition, particle size is an important factor affecting particle absorption by plants. Since titanium oxide (bulk) has greater size than titanium dioxide nano-particles it could not absorbed by plants easily rather than nano-particles. This is in agreement with Zheng and co-workers, (2005) who reported that the significant effect of titanium nano-particles on spinach is probably attributed to the small particle size, which allows its penetration into the seed during the treatment period. It seems that bulk titanium could not penetrate into the plants; therefore, the results were not as marked as those of the nano-particles treatments. Increase in growth and yield may be due to positive effects of titanium in different cellular mechanisms, for instance improve of photosynthesis and increase in chlorophyll content are two possible reasons for this. Owolade and co-workers, (2008) reported that grain yield of cowpea (*Vigna unguiculata* Walp) increased when treated (as foliar application) with nano-sized titanium dioxide. They concluded that it may be due to the photo-catalyst ability of the nano-sized titanium dioxide which leads to an increased photosynthetic rate. Similar yield increases were reported in rice with corresponding reduction in the incidence *Curvularia* leaf spot and bacteria leaf blight disease (Chao *et al.,* 2005). In addition, the maximum grain yield and 1000-grain weight were obtained when *Azorhizobium* was applied (Table 2 and 3). Gupta and co-workers (2002) showed that bacterial inoculation protected the plants against the inhibitory effects of heavy metals. It is likely that the siderophore-producing and P-solubilizing isolates might have helped plant root proliferation and enhanced the uptake of soil minerals such as Fe and P by the host plant. The other possible mechanism of plant growth promotion is the microbial production of indole acetic acid (IAA). The IAA produced by bacteria promotes root growth by directly stimulating plant cell elongation or cell division (Glick *et al.,* 1998). In addition, the results demonstrated that the highest Cd concentration in leaf and grain was observed when triticale plants treated by 0.02% titanium dioxide (Table 2). Su and co-workers, (2008) showed that titanium dioxide nano-particles increased assimilate translocation by improving the structure of chlorophyll pigments and absorbing more light. Furthermore, the highest Cd concentration in leaf and grain belong to *Azorhizobium* inoculation treatment. The present study showed that bacteria appear to increase the plant's potential for Cd phytoremediation because they protect the plant against metal inhibition and facilitate Cd accumulation. The present study showed that bacterial inoculation could enhance metal tolerance in triticale, which might be explained by the production of siderophores by bacteria which contain the 1-aminocyclopropane-1-carboxylate ACC deaminase enzyme, protecting the plants against heavy metal toxicity by decreasing the level of ethylene stress (Burd *et al.,* 1998). The lowest chlorophyll content was found in non titanium treated plants (Table 2 and 3). While the highest chlorophyll content was observed in plants treated by 0.02% titanium dioxide (Table 2 and 3). It has been confirmed that heavy metals affect photosystems' functions (Yang *et al.,* 2006). It has been shown that chlorophyll related proteins, which transfer protons in PSII, were decomposed and decreased under heavy metal stress (Peng and Wang, 1991). When soil is contaminated with heavy metals, this leads to an increase in ROS generation in chloroplasts, which destroys chlorophyll molecules, reduces photosynthesis and growth. Ouzounidou, (1995) concluded that the chlorophyll synthesis can be significantly reduced in plants cultivated in soils contaminated by heavy metals. Previous results have revealed that nano-TiO_2 particles at appropriate doses decrease H_2O_2 accumulation, which subsequently prevent chlorophyll degradation and or stimulate its biosynthesis. This phenomenon may protect photosynthetic processes of stressed plants.

According to Priyadarshini and co-workers, (2012) nano-silver particles at 100 mg l^{-1} increased the chlorophyll *a* and total chlorophyll content in *Brassica juncea* seedlings up to 40 and 25%, respectively. They reported that improved quantum efficiency in the leaves of treated seedlings significantly correlates with higher pigments values. Enhanced chlorophyll and carotenoids contents of the some plants through different nano-materials application were previously reported in *Pelargonium zonale* cultivars with nano-silver (Hatami and Ghorbanpour, 2014), in maize (*Zea maize*) with magnetic nano-particles (Racuciu and Creanga, 2006), and in *Pelargonium graveolens* with nano-TiO_2 particles (Ghorbanpour and Hatami, 2015). Moreover, the results showed that the highest total chlorophyll was obtained from *Azorhizobium* inoculation treatment.

In summary, the results of this study showed that the application of *Azorhizobium* improves triticale growth and production. The highest SOD activity and CAT activity were observed in those plants which were treated by TiO_2 and 0.02% titanium dioxide (Table 2). Indicating that SOD plays an important role in decreasing deleterious effects of induced oxidative stress due to heavy metal stress.

It has been reported that titanium dioxide nano-particles can provoke SOD, CAT and glutathione peroxidase activity and conserve plants against free oxygen radicals (Su *et al.,* 2008).

Nano-particles improve root ability in water and nutrient absorption and enhance antioxidant capacity in soybean

plants (Harrison, 1996). The results are in agreement with other researches (Zheng *et al.,* 2008; Hong *et al.,* 2005). The increase in SOD activity may also confirm the increased production of superoxide radicals mediated by heavy metal stress. In addition, the result showed that the lowest SOD activity and CAT activity were observed control condition (Table 2). It appears that nitrogen-fixing bacteria reduce ROS production and ultimately reduce the antioxidant enzymes activity in plants (Reddy *et al.,* 2004). Malondialdehyde content significantly reduced due to titanium application so that in 0.02% nano-particles treatment, the MDA content was lower than that in 0.01%, bulk or control treatments (Table 2). As mentioned earlier, activity of SOD and CAT has been boosted in response to titanium application. As a result, accumulation of MDA lessened due to induction of plant antioxidant systems (Lei *et al.*, 2008). Reduction in MDA content may be due to positive effect of titanium to help antioxidant system for scavenging or neutralizing of ROS. This finding proves that titanium is involving in lipid peroxidation and membrane stability process (Tohidi Moghaddam and Madani, 2016).

Table 1. Analysis of variance on some agronomic and physiological traits of triticale as affected by nano-particles titanium and bio-fertilizers

S.O.V	df	Grain yield	1000-grain weight	Leaf Cd	Grain Cd	Total chlorophyll	Superoxide dismutase	Catalase	Malondialdehyde
Titanium	3	**	**	**	**	**	**	**	**
Bio-fertilizer	3	*	*	ns	**	**	**	**	ns
Titanium × Bio-fertilizer	9	**	**	ns	ns	**	ns	ns	ns
C.V (%)		4.12	7.69	4.11	3.02	2.12	5.84	1.61	2.24

, and ns significant at 0.05, 0.01 percentage and no significant*

Table 2. Main effects of titanium treatments and bio-fertilizers on some agronomic and physiological traits of triticale

Treatment	Grain yield (g per plant)	1000-grain weight (g)	Leaf Cd (mg kg^{-1})	Seed Cd (mg kg^{-1})	Total chlorophyll (mg g^{-1} FW)	Superoxide dismutase (ΔA mg pro min^{-1})	Catalase (ΔA mg pro min^{-1})	Malondialdehyde (nmol g^{-1} FW)
Titanium								
Control	6.43c	24.15c	28.23d	2.81d	1.68d	726.17c	128.18d	8.85a
Bulk titanium	8.87b	26.12b	35.34c	3.49c	1.77c	742.00c	155.78c	7.45b
Titanium dioxide 0.01	10.69a	28.35a	39.95b	4.15b	1.83.b	866.68b	221.95b	5.20c
Titanium dioxide 0.02	10.71a	28.2a	43.91a	5.32a	1.92a	947.65a	251.98a	4.82d
Bio-fertilizers								
Non bio fertilizer	7.26c	23.91d	36.43a	3.58c	1.69d	843.40a	214.12a	8.63a
Azotobacter	9.64c	27.00c	37.26a	4.05b	1.77c	833.54ab	198.48a	8.54a
Azospirillum	9.77a	27.41b	36.75a	3.83b	1.85b	816.57b	179.69b	8.50a
Azorhizobium	10.06a	27.92a	36.99a	4.31a	1.90a	789.00c	165.58c	8.70a

Treatment means followed by the same letter within each common are not significantly different (P < 0.05) according to Duncan's Multiple Range test

Table 3. Interaction between titanium treatments and bio-fertilizers on some agronomic and physiological traits of triticale

Titanium	Bio-fertilizers	Grain yield (g per plant)	1000-grain Weight (g)	Leaf Cd (mg kg^{-1})	Grain Cd (mg kg^{-1})	Total chlorophyll (mg g^{-1}FW)	Superoxide dismutase (ΔA mg pro min^{-1})	Catalase (ΔA mg pro min^{-1})	Malondialdehyde (nmol g^{-1} FW)
Control	Control	4.85h	20.8f	23.81i	2.03j	1.39fg	811.90ab	251.96a	8.60a
	Azotobacter	6.88g	25.4e	24.14ih	2.30i	1.42f	778.87cb	200.83bc	8.57a
	Azospirillum	6.91fg	24.8d	24.31gh	2.42hi	1.46f	754.47cd	183.41cd	8.55a
	Azorhizobium	7.11f	25.6c	24.57g	2.57h	1.51e	736.88cd	76.26dc	8.37b
Bulk titanium	Control	7.24f	23.9cd	29.69f	2.83g	1.54e	848.94a	226.15 ab	7.56c
	Azotobacter	9.33c	26.8b	30.47e	2.87g	1.59de	814.34ab	147.23ef	7.45c
	Azospirillum	9.36c	26.7b	30.32e	3.19f	1.63d	659.01e	118.20fgh	7.39d
	Azorhizobium	9.58c	27.1b	30.64e	3.24f	1.68d	621.36ef	97.47bcd	7.44c
Titanium dioxide 0.01	Control	8.53d	25.6c	33.81d	3.35ef	1.72c	754.47cd	200.83bc	5.23g
	Azotobacter	11.16.b	29.3a	34.29c	3.49ed	1.75c	736.88cd	176.26dc	5.46e
	Azospirillum	11.34b	28.9ab	34.39c	3.65dc	1.78b	637.52ef	135.35fg	5.45e
	Azorhizobium	11.81a	29.6a	34.58c	3.80c	1.82b	642.36ef	33.36fg	5.37f
Titanium dioxide 0.02	Control	8.43e	25.4c	37.35b	4.39b	1.79b	848.94a	226.15 ab	4.85h
	Azotobacter	11.22b	28.8ab	37.71ab	4.52ab	1.81b	814.34ab	251.96a	4.68j
	Azospirillum	11.48ab	29.2a	37.68ab	4.60ab	1.83b	811.90ab	197.47bcd	4.75i
	Azorhizobium	11.74a	29.4a	37.90a	4.73a	1.95a	778.87cb	83.41cd	4.66j

Treatment means followed by the same letter within each common are not significantly different (*P* < 0.05) according to Duncan's Multiple Range test

In general, although several recent studies have considered the antioxidative responses by the cell to abiotic stress and tolerance, other possible events such as non-enzymatic components that notably includes reactions linked to the intracellular ascorbic acid and glutathione in the maintenance of redox homeostasis should be taken into account.

4. Conclusion

The nano-TiO_2 at proper concentrations could act as modifier to alleviation of deleterious effects of heavy metal stress on physiological processes through increasing antioxidant enzyme activity, which resulted in reduced lipid peroxidation and stability of chlorophyll pigments. The nano-TiO_2 at proper concentrations increased triticale yield under Cd stress. The application of bio-fertilizers could reduce the harmful effects of ROS and improve triticale tolerance to Cd contamination.

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