Pak. J. Agri. Sci., Vol. 52(1), 177-182; 2015
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

PHYTO-AVAILABILITY OF PHOSPHORUS TO *Lactuca sativa* IN RESPONSE TO SOIL APPLIED TiO_2 NANOPARTICLES

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Phytoavailability of phosphorus (P) in soil remains a major issue for crop production particularly in developing world. The objective of present work was to study the effects of engineered TiO_2 nanoparticles (nano-TiO_2) on phytoavailability of P and growth response of *Lactuca sativa* (Lettuce). Suspensions of 0, 25, 50, 75 and 100 mg kg^{-1} of nano-TiO_2 having size less than 65 nm were applied to a sandy loam soil. The concentration of phytoavailable P in soil increased up to 56% after 72 h incubation at room temperature (25°C) in Petri dishes when 100 mg nano-TiO_2 kg^{-1} soil was applied. Similar trends were also observed for phytoavailable P in the soil after culturing *L. sativa* plants onto it for 14 days. Shoot and root lengths were increased up to 49% and 62% respectively at 100 mg kg^{-1} of soil applied nano-TiO_2, as compared to the control treatment. Shoot and root P concentrations were increased up to 36 and 175% respectively at 100 mg kg^{-1} of nano-TiO_2, as compared to the control. P uptake per plant was five-folds with reference to the control. *L. sativa* significantly acidified its rhizosphere. This innovative study provides an important base line to further investigate the detailed mechanisms involved in improved P uptake and plant growth in response to application of engineered nanoparticles in the field.
Keywords: P uptake, *Lactuca sativa*, Plant growth, Acidification

INTRODUCTION

Phosphorous (P) is one of the essential nutrients for plant growth and plays an important role in a variety of processes such as energy generation, nucleic acid synthesis, photosynthesis, respiration, redox reactions, carbohydrate metabolism, and nitrogen fixation. Phosphorous availability to plants is a worldwide problem and 30–40% crop production in the world is limited by P deficiency (Vance *et al.,* 2003; Grant *et al.*, 2005). According to the estimates, the world's resources of low cost P being used as fertilizer will be depleted by 2050 (Vance *et al.,* 2003). A major proportion of the applied P is fixed on exchange sites is the soil. However, precipitation of P as calcium compounds in calcareous soil appears more important (Naeem *et al.*, 2013). Multiple strategies have been proposed to cope up with this issue (Ali *et al.,* 2012). Tian *et al.* (2012) have reviewed bioengineering based methods to enhance P use efficiency of crops/pastures, including conventional and molecular assisted breeding, identification and application of key genes for biotech plants. Root exudates in the rhizosphere are very critical for plant nutrition (Cesco *et al.,* 2012; Possinger *et al.,* 2013). Plant growth–promoting rhizobacteria (PGPR) are getting more attention for P solubilization (Turan *et al.,* 2012). Root hairs (Vandamme *et al.,* 2013) and root architecture development (Niu *et al.,* 2013) are also regarded as important factors for improving P uptake by plants. Other methods such as returning of wheat straw in compacted soils (Guo and Wang, 2013) and addition of mineral fertilizers (Hejcman *et al.,* 2013) have also positive impacts on P availability.

In spite of these valuable contributions by the researchers to increase P availability and improve crop production, the ever increasing population of the world is threatening food security and available arable land. So, there is a need to think out of the box solutions to feed the people adequately. In this scenario, Nanotechnology may play a significant role in agriculture as it is being used in many fields. It may have positive or negative effects depending upon the type of use and the kind of nanopartciles (Hänsch and Emmerling, 2010; Ma *et al.,* 2010; Ghosh *et al.,* 2010; Mahajan *et al.,* 2011). Very few studies have been carried out to assess the effects of nanoparticles on mineral nutrition and plant growth. Santner *et al.* (2012) reported 8-40 folds increase in plant P availability in response to Al_2O_3 nanoparticles in solution studies. Ze *et al.* (2011) reported that nano-TiO_2 can considerably stimulate photosynthesis and plant growth. Enhanced nitrogen metabolism and photosynthesis have been observed in spinach after treatment with nano-TiO_2 (Yang *et al.,* 2006 and 2007). Zheng *et al.* (2004) found improved vigor of spinach seedlings with the use of nano-TiO_2. Looking on these effects reported in literature, the

objective of the present work was to assess the effects of nano-TiO_2 application on P availability in soil and, ultimately growth of *L. sativa*.

MATERIALS AND METHODS

Preparation of soil and nanoparticles: Soil was taken from a local nursery, air dried and sieved to 2 mm. Its texture was sandy loam and the pH was 8.5. Nano-TiO_2 particles used for this study were synthesized using liquid impregnation method (LI). Precisely, 3 g of Titania was added into 100 mL distilled water and stirred vigorously on magnetic stirrer. The slurry was allowed to stay for 24 h and dried at 105°C for 12 h. The dried material was ground in agate mortar and calcinated at 400°C for 6 h in a muffle furnace (Zeb *et al.,* 2010). The crystal structure and size of TiO_2 nanoparticles were determined using X-ray Diffraction (XRD, JEOL JDX-II, X-Ray). The surface morphology of TiO_2 nanoparticles was determined by scanning electron microscopy (SEM, JEOL JSM-6460) at 500–10,000 magnifications. The size of the nanoparticles was less than 65 nm. For application into the soil, nanoparticles were suspended in distilled water and were dispersed using ultra-sonicator (JAC Ultra Sonic 1505) for 30 minutes. The desired concentrations of TiO_2 nanoparticles suspensions were prepared to achieve levels of 0, 25, 50, 75 and 100 mg Nano-TiO_2 kg^{-1} of soil.

Pre-culture Experiments: To determine the effects of Nano-TiO_2 on P availability in soil without plant culture, suspensions of nanoparticles were added drop by drop to 40 g of soil in Petri dishes and mixed thoroughly. There were three replicates for each treatment. The Petri dishes were incubated at 25°C for 72 h to achieve equilibrium at exchange sites in the soil. Soil moisture contents for the experiments in Petri dishes were approximately 60% and the soil was super-saturated through continuous supply of water through filter paper placed below the soil and dipped in water on the other side over a period of 72 h. After incubation, soils were dried at 40°C and P contents were assayed using malachite green method (Ohno and Zibilske, 1991) after extraction with 0.5M $NaHCO_3$ (Olsen *et al.,* 1954).

Plant Culture Experiments: For bioassay experiments of *L. sativa*, seeds were grown in soil without adding any fertilizer over a period of two weeks and irrigated with tap water. Seedlings of homogenous size and vigor were selected for culture experiments. Small sized earthen pots were filled with 500 g soil spiked with TiO_2 nanoparticles to achieve levels of 0, 25, 50, 75, 100 mg kg^{-1}. *Lactuca sativa* seedlings were washed with tap water and transplanted into the pots. Plants were irrigated thrice a week to maintain field capacity level in the potted soil for 14 days under natural conditions. For each treatment, there were five replications. After 14 days of culture, plants were harvested and data regarding root, shoot length and weight were recorded.

Determination of soil pH and Olsen–P: Fresh soils after culture were taken for pH determination. Soil pH was measured from a 1:5 soil/water suspension on dry basis. After shaking soil solution mixture for 10 min, it was allowed to settle down. The pH of supernatants determined by pH meter was considered as the soil pH. For Olsen-P, soil was extracted with 0.5M $NaHCO_3$ at 1:20 i.e. soil: solution ratio (Olsen et al. 1954). Soil solution was shaken for 30 min with rotating shaker and left for 15 min to settle down. Olsen-P was measured by malachite green method (Ohno and Zibilske, 1991). One milliliter of the extractant was added with 200 µL of each reagent 1 (Ammonium heptamolybdate + sulphuric acid) and reagent 2 (Polyvinyl alcohol + Malachite green oxalate). Finally, Olsen-P was measured by recording absorbance of reaction mixture using spectrophotometer at 630 nm wavelength. For P analysis, detection limit (DL) and working limit (WL) were measured to estimate error for measurements. The reading for blank and first standard was taken 6 times, then analyzed statistically. The error value found was negligible.

Plants analysis: The plant material was oven dried at 105 °C for 48 h and weighed again for dry weight. The samples were ground and digested in concentrated nitric acid – perchloric acid (HNO_3-$HClO_4$) mixture in 2:1 ratio on hot plate at 180°C. The aliquots were filtered and stored at 4°C for P analysis. The concentration of P in plant materials was determined using Vanadomolybdo phosphoric acid by calorimetric method (Ryan *et al.,* 2001). Phosphorus uptake was calculated from the following relation:

P uptake (mg $plant^{-1}$) = [(Shoot dry weight x shoot P conc.) + (Root dry weight x Root P conc.)]

Statistical Analysis: This study was performed in five replicates and results are presented as the mean ± SD (Standard Deviation). Results were analyzed using single factor ANOVA using data analysis tools in Excel. Statistical significance ($P \leq 0.05$) was established with LSD Fisher test.

RESULTS AND DISCUSSION

Growth response of L. sativa to Nano-TiO_2 application in soil: Application of nano-TiO_2 had significant effects on plant growth parameters like shoot and root lengths and, dry weight (Table 1). The shoot dry weight significantly increased with application of nanoparticles and the value was more than two folds at 100 mg kg^{-1} soil as compared to the control treatment. Shoot length increased from 7.62 to 11.36 cm with application of Nano-TiO_2 at 100 mg kg^{-1} soil. The root length without application of nanoparticles was 4.98 cm. It increased significantly ($P \leq 0.05$) to 8.08 cm with the application of 100 mg Nano-TiO_2 kg^{-1} of soil. Shoot and root lengths were increased up to 49 and 62% respectively, as compared to the control. At the highest concentration (100 mg kg^{-1}), shoot dry weight was doubled as compared to the control.

Table 1. Growth response of *L. sativa* to Nano-TiO_2 application in soil in a pot experiment.

Treatment	Growth Parameters			
TiO_2 (mg/kg)	SDW (g)	RDW (g)	SL (cm)	RL (cm)
0	0.26±0.09	0.13±0.07	7.62±0.91	4.98±0.92
25	0.31±0.04	0.13±0.04	9.14±0.66	5.74±0.29
50	0.34±0.11	0.14±0.07	9.52±0.83	6.24±0.34
75	0.49±0.08	0.18±0.14	9.66±0.44	6.60±0.33
100	0.55±0.20	0.23±0.21	11.36±0.75*	8.08±1.40*

SDW = Shoot Dry Weight, RDW = Root Dry Weight, SL = Shoot Length, RL = Root Length. The values are given as Mean ± SD (standard deviation) for five replicates for each treatment.

Some previous investigations on effects of nanoparticles on plant growth are consistent with our findings. Growth and vigor of spinach seedlings accelerates after treatment with proper concentration of TiO_2 nanoparticles (Zheng *et al.,* 2005; Yang *et al.,* 2006).

Effect of Nano-TiO_2 on availability of phosphorus and its uptake: According to Hesterberg (2010), over 99% of soil P is found in poorly available forms to plants. So the search for methods to increase phytoavailable P is continuously in progress. Effects of nanoparticles on availability of P were assessed in present study. In an experiment only with addition of nano-TiO_2, Olsen-P or phytoavailable P increased up to 56% as compared to the control (Fig 1A). Phosphorus availability was well correlated with applied nanoparticles levels ($R^2 = 0.97$). Results of phytoavailable P in soil after plant culture are presented in Fig 2B. There was significant increase in Olsen-P after culture. All the trends were very similar to that of without plant culture. Moreover, there was no significant effect of *L. sativa* plants as the final values of P were the same (3.4 mg kg^{-1}) at 100 mg Nano-TiO_2 kg^{-1} of soil in with and without culture experiments. Available P and applied levels of nano-TiO_2 showed significant correlation ($R^2 \geq 0.95$) in both cases. Recently, Luo *et al.* (2010) observed higher release of P from sediment in aquatic environment in the presence of nano-TiO_2 and UV irradiance, probably due to enhance photocatalytic activity.

Phosphorus concentrations in shoots and roots are presented in Fig 2. Different levels of TiO_2 nanoparticles significantly ($P \leq 0.05$) enhanced P concentration in shoots and roots over respective controls. The concentration of P in *L. sativa* shoots varied from 392 to 533 mg kg^{-1} DW for control and the highest level of Nano-TiO_2 applied, respectively. The percentage increase with respect to control was 36%. In roots, the P concentrations ranged from 351 to 965 mg kg^{-1} for control and the maximum level of nanopartciles (100 mg kg^{-1}) applied, respectively. The results of P uptake per plant in response to different treatments are presented in Fig 3.

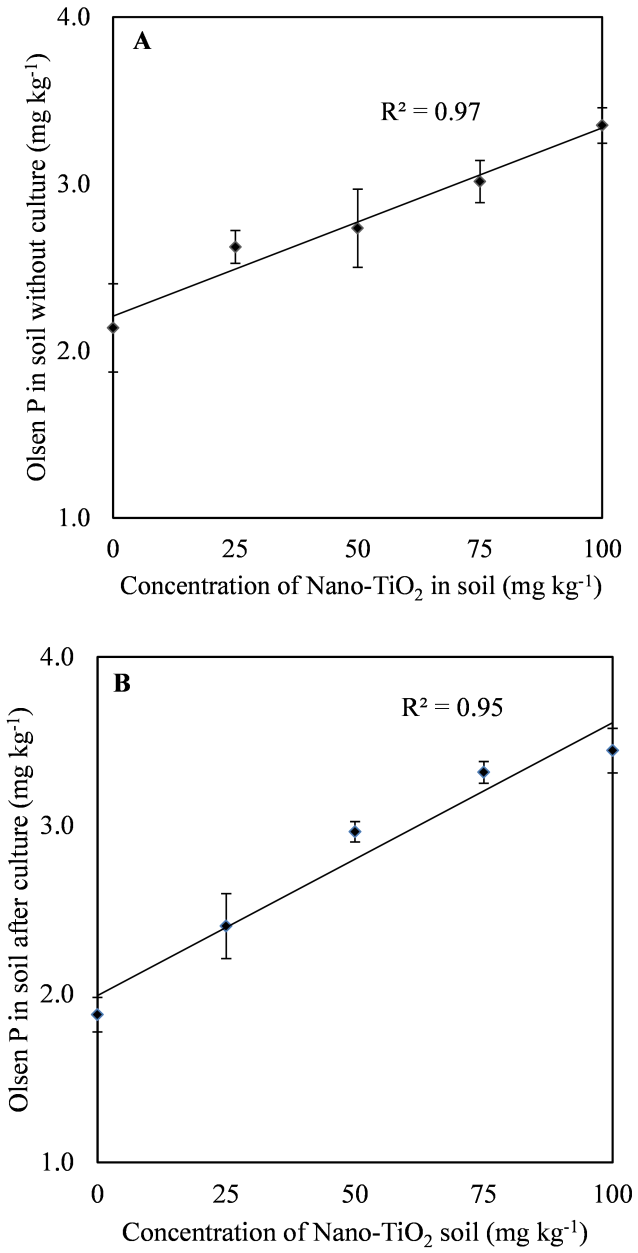

Figure 1. Phytoavailable P in soil in response to nano-TiO_2 application. A) Pre-culture experiment without plant. B) Phytoavailable-P in culture experiment of *Lactuca sativa* exposed to different levels of nanoparticles for 14 days. Results are presented as Mean ± SD (standard deviation) for five replicates each.

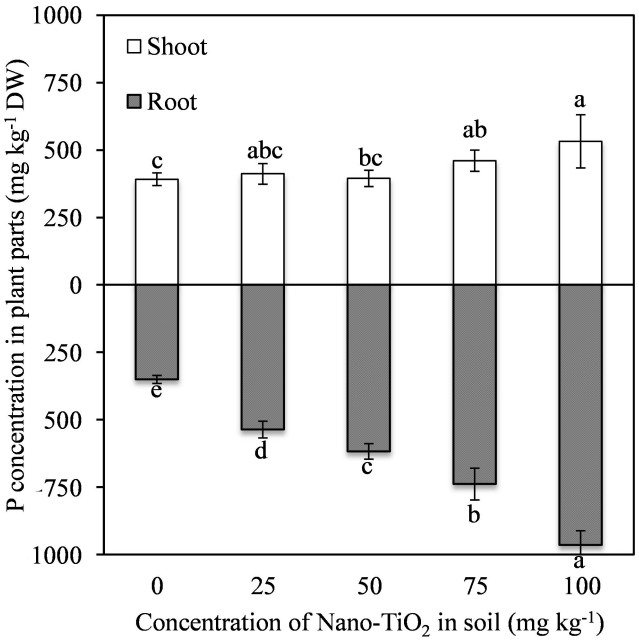

Figure 2. Phosphorus concentrations in plant parts i.e. shoot and root. Different alphabets show significant difference (p<0.05) for treatments.

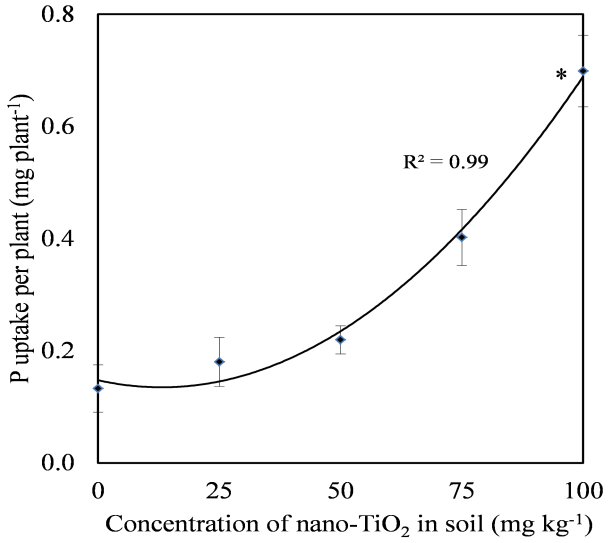

Figure 3. Phosphorus uptake by *Lactuca sativa* plants during a culture period of 14 days. The (*) indicates significant difference (*P<0.05*).

There was significant (p<0.05) increase in P uptake per plant with increasing levels of Nano-TiO_2 applied in soil. It ranged from 0.14 to 0.70 mg P per plant. Amounts of phytoavailable P were also depicted in shoots and roots, and P concentrations were increased up to 36 and 175% respectively, as compared to the control (Fig. 2). Plant uptake was also increased five folds with respect to the control (Fig 3). Santner *et al.* (2012) reported 8-40 folds increase in P uptake by *Brassica napus* from the nutrient solution using Al_2O_3 nanoparticles but interactions in soil are completely different and to achieve 40-fold increase is very difficult.

Effects of Nano-TiO₂ on soil pH :Very interesting results were observed for pH changes in the rhizosphere. The relationship between pH and TiO_2 nanoparticles applied in soil is given in Fig 4. During the experiment without plant, there was no change in soil pH (8.5±0.1) with the application of nanoparticles. *L. sativa* plants only i.e. control were able to acidify their rhizosphere up to pH 6.94 (Fig 4). The pH recorded with the application of nanoparticles @100 mg kg^{-1} was 6.02 which showed acidification of 0.92 pH units only due to presence of nanoparticles in the rhizopshere. These rhizosphere pH changes during two weeks culture with plant were statistically significant (P<0.05).

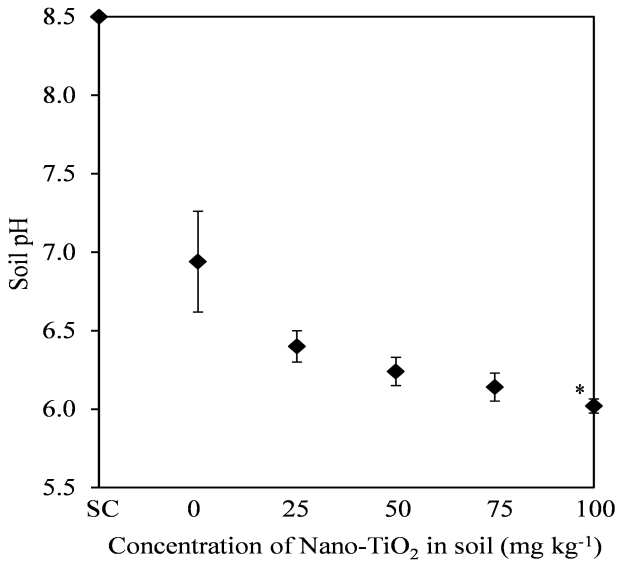

Figure 4. Rhizosphere pH after 14 day culture of *Lactuca sativa* on soil amended with Nano-TiO_2. The (*) indicates significant difference (*P<0.05*).

Our results indicated the significant role of TiO_2 nanoparticles in enhancing P uptake and growth of *L. sativa* while lowering the pH of soil. A hypothesis can be built that the positive effects of nano-TiO_2 were probably due to the release of root exudates with addition of nanoparticles as these can act as catalyst if taken up by the plant (Hong *et al.,* 2005). The results of rhizopshere pH also support this hypothesis as acidification was observed. It is documented that the soil pH in the range of pH 6 leads to maximum phytoavailability of P. Phosphorus solubility can be

increased by excretion of organic acids into rhizosphere and lowering the rhizosphere pH in neutral to alkaline soils (Marschner, 2005). Plants develop a wide range of strategies to modify the Pi availability by various processes such as the acidification of the rhizosphere as a consequence of the cation/anion balance of plant nutrition, respiration, or active processes, exudation of organic acids, and secretion of extracellular phosphatases (Bertrand *et al.,* 1999; Hinsinger, 2001).

Conclusions and perspectives: Finally, we conclude that application of nano-TiO_2 in soil can significantly improve phytoavailable P. The best results were found at 100 mg nano-TiO_2 kg^{-1} soil. Plant uptake was 5-folds with reference to the control for *L. sativa.* At this moment, we can hypothesize that the application of nanoparticles in soil could interfere at exchange sites. The introduction of TiO_2 in soil could provide more adsorption sites to the PO_4^{3-} ions due to higher polarizing power of Ti^{3+} (6.7 C/m^2) as compared to Ca^{2+} (2.2 C/m^2) and make covalent bonds with the PO_4^{3-} groups. Possible entry of nanoparticles in plant can trigger metabolic activity contributing to enhanced exudation leading to acidification. Subsequently, desorption of PO_4^{3-} could occur through a ligand exchange reaction upon plant root exudation, possibly altering the adsorption-desorption equilibrium and releasing P into soil solution which is readily available for uptake. These positive effects under soil conditions can be a source of high prospects in the future for agriculture. This new approach, however, needs more comprehensive studies to explore the underlying exact mechanisms for optimal use of nanoparticles in crop production.

Acknowledgements: The authors gratefully acknowledge the Science and Technology Fund of National University of Sciences and Technology, Islamabad, Pakistan for the financial support for the present research work. We also thank for the help rendered by Dr Muhammad Bilal, CIIT, Abbottabad and Ms Aneela Iqbal, IESE-NUST, Islamabad during the preparation of manuscript.

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