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RESEARCH ARTICLE

The Effect of N-TiO2 on Tomato, Onion, and Radish Seed Germination

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Abstract

The effects of nano-size titanium dioxide (N-TiO_2) on the germination of tomato (*Lycopersicum esculentum* L.), onion (*Allium cepa* L.), and radish (*Raphanus sativus* L.) seeds were assessed in laboratory and greenhouse trials. Seeds were germinated in Petri dishes in a laboratory and in peat:perlite (1:1, v/v) in a greenhouse containing four concentrations of N-TiO_2 (0, 100, 200, and 400 mg L^{-1}). N-TiO_2 at 100 and 200 mg L^{-1} had the most positive effect on germination. In the laboratory, the highest germination percentage of tomato and onion was observed at 100 mg L^{-1} (100 and 30%, respectively), and in radish, 100% germination was obtained with 400 mg L^{-1}. In the greenhouse, seedlings were tallest after exposure to 400 and 200 mg L^{-1} for tomato and onion, respectively, and 400 and 100 mg L^{-1} for radish. N-TiO_2 may serve as a seed-priming agent for horticultural crops.

Key words : germination percentage (GP), mean germination time (MGT), nanotechnology, scanning electron microscopy

Introduction

Materials with a dimension of between 1 and 100 nm are termed nano materials. Nanotechnology widely employs such nano materials in cosmetic and skin care products, antibacterial and air cleaning products, transportation, energy, and agriculture due to their unique physico-chemical properties, namely high stability, anticorrosion, and photocatalyst activity (Castiglione et al. 2010), a high surface area due to their tiny size – relative to their bulk materials – which increases solubility and results in higher surface reactivity (Castiglione and Cermonini 2009). Since nano materials can penetrate cell membranes faster and easier (Sheykhbaglou et al. 2010), their application in agriculture as a fertilizer holds great potential (Mishra et al. 2014). New forms of fertilizer are always being sought to improve the efficiency of agriculture.

The effects of nano materials on several plant species has been investigated, although this field of study is still relatively new to applied horticulture. Nano materials have been shown to have both positive and negative effects. The germination and growth of soybean (*Glycine max* L.) was improved when a mixture of nano-size silicon dioxide (N-SiO_2) and nano-size titanium dioxide (N-TiO_2) were incorporated into the growth medium, increasing the ability to absorb water and fertilizers due to enhanced nitrate reductase (NiR) activity and the stimulation of antioxidant systems (Lu et al. 2002). N-TiO_2 also promoted the growth of spinach (*Spinacia oleracea* L.) by protecting the chloroplast from aging during long-term illumination, an abiotic stress (Lee et al. 2010; Lu et al. 2002). The growth of *Larix olgensis* A. Henry was increased following the application of N-SiO_2 (Lin et al. 2004). Carbon nanotubes (CNTs) are another nano material that has greatly impacted seed germination and plant growth. Alimohammadi et al. (2011) reported a mixed response to CNTs in tomato (*Lycopersicon esculentum* Mill.): while biomass increased, root weight and chlorophyll content decreased. Cañas et al. (2008) compared the effects of two types of CNTs (functionalized and non-functionalized single-walled) on six horticultural crop species (cabbage (*Brassica oleracea* L.), carrot (*Daucus carota* L.), cucumber (*Cucumis sativus* L.), lettuce (*Lactuca sativa* L.), onion

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(*Allium cepa* L.), and tomato). These materials showed mixed responses: non-functionalized CNTs inhibited root elongation in tomato but increased it in onion and cucumber while functionalized CNTs inhibited root elongation in lettuce.

Some reports have shown the negative effect of nano materials on the root growth of five plant species (corn (*Zea mays* subsp. *mays* L.), cucumber, soybean, cabbage, and carrot) that were inhibited by using nano-aluminum oxide (Yang and Watts 2005). Lin and Xing (2007) investigated the toxicity of five nano materials (multi-walled CNTs, N-Al, N-Al_2O_3, N-Zn, and N-ZnO) on six plant species (radish (*Raphanus sativus* L.), rape (*Brassica napus* L.), ryegrass (*Lolium rigidum* L.), lettuce, corn, and cucumber) and reported the toxic concentrations of all these nano materials. Root elongation of all crops was significantly inhibited by 2,000 mg L^{-1} N-Al_2O_3 and 2,000 mg L^{-1} N-Zn; 2,000 mg L^{-1} N-ZnO significantly inhibited the seed germination of ryegrass and corn. N-ZnO also terminated the root elongation of all tested crops. Fe (zero-valent iron nano particles, or N-ZVI) and Ag nano particles at 250 mg L^{-1} and 10 mg L^{-1}, respectively inhibited ryegrass, barley (*Hordeum vulgare* L.) and flax (*Dactylis glomerata* L.) growth (El-Temsah and Joner 2010). These nano materials reduced shoot growth more than germination percentage and N-Fe had a more inhibitory effect than N-Ag. The germination of these three crops was completely inhibited at 1,000 – 2.000 mg L^{-1} of N-ZVI, and the order of sensitivity to NZVI (from highest to lowest) was: ryegrass > barley > flax (El-Temsah and Joner 2010). Ag nanoparticles at 75 μg L^{-1} prolonged *Arabidopsis thaliana* (L.) Heynh. Vegetative development and shortened its reproductive growth (Geisler-Lee et al. 2014). Collectively, these studies indicate that different crop plants are responsive in quite different, and sometimes contradictory, ways to different nano materials, although this seems to depend very strongly on the species, and on the concentration and form of nano material used.

Titanium (Ti) is a beneficial element that has significant effects on plants, and, like many other heavy metals, at a low concentration it shows a promoting effect while at a high dose it has a toxic effect. Ti has several biological effects. When this is chelated with organic acids such as ascorbic, citric and malic acid (Kuzel et al. 2003), it increases Fe and Mg contents in plant tissues (Gimenez et al. 1990), and it increases NiR activity, chlorophyll *a* and *b* biosynthesis, and affects other enzymatic activities that are related to the defense mechanism of plant organisms (Kuzel et al. 2003). Thus, when Ti is applied to plants, it results in a high level of protection against biotic and abiotic stresses (Kuzel et al. 2003). Sarmiento et al. (1995) used titanium ascorbate in germination media to evaluate the chemical induction of lipoxygenase activity in tomato and red pepper (*Capsicum annuum* L.) and observed that there was a relation between Ti and lipoxygenase biosynthesis. However, the effect of N-TiO_2 as a priming agent in seed germination has not been studied, and this is a rare report of such a use in horticultural plants. Only few studies to date have investigated other aspects of N-TiO_2 on plant growth in which it was proposed to be more effective on herbaceous plants than on woody plants. Seeger et al. (2009) observed that N-TiO_2 had no effects on willow (*Salix* sp.) trees after short-term exposure. Haghighi et al. (2012) reported the effect of N-Ti and Ti in growth of tomato under N deficiency, observing that N-Ti compensated for the unfavorable effect of N deficiency in tomato. Feizi et al. (2013a, b) showed that 60 mg L^{-1} of N-TiO_2 improved germination-related parameters of sage (*Salvia officinalis* L.) and fennel (*Foeniculum vulgare* Mill.), respectively, but not shoot, root and seedling elongation and biomass while lower concentrations had no effect and higher concentrations had a toxic effect.

The effects of nano materials on plant species depend on cultural conditions and on concentration. Sexual propagation is an easy and common way to propagate horticultural plants, and achieving a high rate and percentage of germination with vigorous seedlings is a core focus of horticultural nursery production. Considering that some positive effects on seed germination using nano materials have been achieved, it was the objective of this study to expand the ways in which seed priming of three horticultural crops within the Solanaceae, Alliaceae, and Brassicaceae could be improved by N-TiO_2.

Material and Methods

The effect of N-TiO_2 particles on seed germination and seedling growth of tomato, onion, and radish was studied. Two studies were arranged on the basis of a completely randomized design (CRD) with four replications in laboratory and greenhouse conditions with 100 seeds per replicate. In this study, the chemical vapor deposition (CVD) method was used to produce N-TiO_2 particles by using acetylene as a carbon source (Soleimani et al. 2012). The optimum temperature for making N-TiO_2 particles was 1,300°C in an atmospheric argon environment. This temperature strongly depends on the flow rate of Ar and acetylene. When dealing with CVD instrumentation system at a large scale, there is a significant thermal gradient inside the tubing furnace due to the high flow rates of gaseous species. Therefore, to limit the thermal gradient, it is necessary to promote the temperature inside the middle zone of the furnace (Soleimani et al. 2012). After production (using different methods such as reflux in an acid environment), the N-TiO_2 particles were approximately 98% purified. During acid treatment, carbon-carbon bonds are broken down, resulting in the formation of functional groups such as -OH or -COOH. This process leads to the separation of nano particles such as Fe or amorphous carbon (Maleki et al. 2011). The purity of the N-TiO_2 was tested using Raman spectrometry, pattern X-ray diffraction (XRD), thermo gravimetric analysis (TGA), and N_2 adsorption isotherms (Mohammadi et al. 2011). A uniform mixture was prepared by sonication (500 KHz for 1 h). Depending on the intensity of the environment, N-TiO_2 is also stable in O_2,

Table 1. The effect of N-TiO_2 on growth aspects of seedlings of three horticultural species in the laboratory stage

N-TiO_2 (mg L^{-1})	GP (%)	GR (days)	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	MGT (days)
Lycopersicon esculentum							
0	92 ± 0.02 b†	5.70 ± 0.01 b	3.03 ± 0.12 b	2.54 ± 0.33 b	0.40 ± 0.12 ab	0.03 ± 0.45 b	3.72 ± 0.09 b
100	100 ± 0 a	6.45 ± 0.20 a	28.75 ± 4.78 a	4.50 ± 1.29 b	0.55 ± 0.09 b	0.04 ± 0.02 b	3.94 ± 0.14 ab
200	100 ± 0 a	6.36 ± 0.10 a	26.75 ± 11.35 a	7.75 ± 2.62 a	0.34 ± 0.09 b	0.07 ± 0.02 a	3.96 ± 0.09 ab
400	95.5 ± 6.60 ab	6.07 ± 0.20 ab	21.25 ± 6.29 a	6.00 ± 3.36 ab	0.58 ± 0.18 a	0.06 ± 0.02 a	4.00 ± 0.19 a
Allium cepa							
0	20 ± 0.02 a	1.29 ± 0.01 a	1.10 ± 0.12 b	0.90 ± 0.33 b	††	††	0.88 ± 0.09 a
100	30 ± 15.49 a	2.20 ± 0.67 a	12.33 ± 4.72 a	4.83 ± 1.44 a	††	††	2.00 ± 1.16 a
200	29 ± 17.30 a	1.87 ± 0.61 a	11.50 ± 3.52 a	4.50 ± 1.01 a	††	††	1.71 ± 1.19 a
400	23 ± 11.40 a	1.78 ± 0.75 a	2.46 ± 0.70 b	3.36 ± 0.70 a	††	††	1.40 ± 0.56 a
Raphanus sativus							
0	96 ± 0.05 a	19.50 ± 0.07 a	19.50 ± 1.16 a	4.06 ± 0.29 a	4.42 ± 0.22 a	0.35 ± 0.01 b	0.04 ± 0.17 b
100	94 ± 6.90 a	10.14 ± 0.93 b	10.14 ± 0.93 b	2.23 ± 0.10 b	2.10 ± 0.47 b	3.06 ± 0.01 a	0.18 ± 0.71 a
200	90 ± 7.60 a	10.31 ± 1.06 b	10.31 ± 1.06 b	1.59 ± 0.92 c	3.88 ± 2.20 a	2.76 ± 0.03 a	0.16 ± 0.86 a
400	100 ± 0 a	9.62 ± 1.17 b	9.62 ± 1.17 b	1.67 ± 0.36 bc	4.42 ± 0.97 a	3.01 ± 0.01 a	0.18 ± 0.36 a

†Differences within a column followed by the same letter are not significantly different at $P < 0.05$ (LSD test; analyzed separately for each species).
††Shoots were not observed.
GP = germination percentage; GR = germination rate; MGT = mean germination time

light, or heat. The stability of N-TiO_2 in O_2 or heat depends on the temperature. At normal environmental conditions, N-TiO_2 only adsorbs O_2. Also, under atmospheric conditions, the thermal stability of N-TiO_2 is ~ 500°C. The stability of N-TiO_2 strongly depends on the wavelength and intensity of light. Usually, the large molecular weight N-TiO_2 significantly reduces the effects of these external parameters (Maleki et al. 2011). N-TiO_2 particles with a diameter 8 to 15 nm, and a length of more than 10 µm were applied at four concentrations: 0, 100, 200, and 400 mg L^{-1}. The pH of all solutions was 6.5.

Seeds of all three species were sterilized in a commercial 50% (v/v) sodium hypochlorite (2.5 g/100 g) solution for 10 min, rinsed thoroughly with distilled water, and then transferred to Petri dishes or pots and then 50% benomyl (Merck, NJ, USA) was added to medium as a sterilant. In the first laboratory experiment, seeds of each crop were placed over filter paper inside 6-cm Petri dishes and were soaked with N-TiO_2 particles. Petri dishes were placed at 25 ± 2°C and a 16-h photoperiod at 93.9 µmol m^{-2} s^{-1}.

Germinated seeds were counted daily for 12 days in the laboratory and germination percentage (GP) was calculated on the last day. Seeds with a 2-mm-long radical were considered as germinated seeds. Mean germination time (MGT) was calculated based on the following formula (Hartman et al. 2002):

$$MGT = \sum ni.ti/T$$

where n_i is the number of seeds germinating on the t_ith day of germination testing and T is the total number of seeds that germinated during the experiment.

At the end of the laboratory experiment, the radical and plumule length and fresh weight (FW) of each germinated seed was measured. Plants were placed in an oven at 70°C for 48 h and then weighed with sensitive scales to two decimal places.

In the greenhouse stage, an experiment was conducted with four replications for each of the three plants. After soaking for 48 h in water, seeds were transferred to pots containing cocopeat:perlite (1:1, v/v). Pots were placed in a greenhouse with a day/night temperature of 25 ± 2/15 ± 2°C. In the greenhouse stage, dry weight (DW), FW, and seedling root and shoot length were measured after 12 days, as was done for the laboratory experiment.

Electron microscopy (SEM-Oxford 360) was performed to monitor the possible relation between seed coat morphology and root structures and N-TiO_2 penetration.

Experiments were designed in a completely randomized design with four treatments and four replicates per treatment. All data were subjected to one-way ANOVA with Statistix 8 (Tallahassee, FL, USA) and the means were compared by LSD at $P < 0.05$ which indicated significant differences between treatments.

Results

Effect of TiO_2 on seed germination and seedling growth of tomato

N-TiO_2 improved the GP of seeds, especially at 100 and 200 mg L^{-1} (Table 1). The same results were observed for GR: highest GR (6.45) was observed at 100 mg L^{-1}. Both root length and shoot length increased by 67.22 and 89.46%, respectively, when exposed to 200 mg L^{-1} N-TiO_2. Highest FW was obtained with 400 mg L^{-1} N-TiO_2. MGT increased as the concentration of N-TiO_2 increased (Table 1). The germination of seeds peaked (100%) after the first three days then reached a plateau (Fig. 1A). Shoot length decreased when N-TiO_2 was applied but root length, and shoot FW and DW increased in the greenhouse (Table 2). Longest roots (4.5 cm) and highest FW and DW (0.91 g and 0.74 g, respectively) of

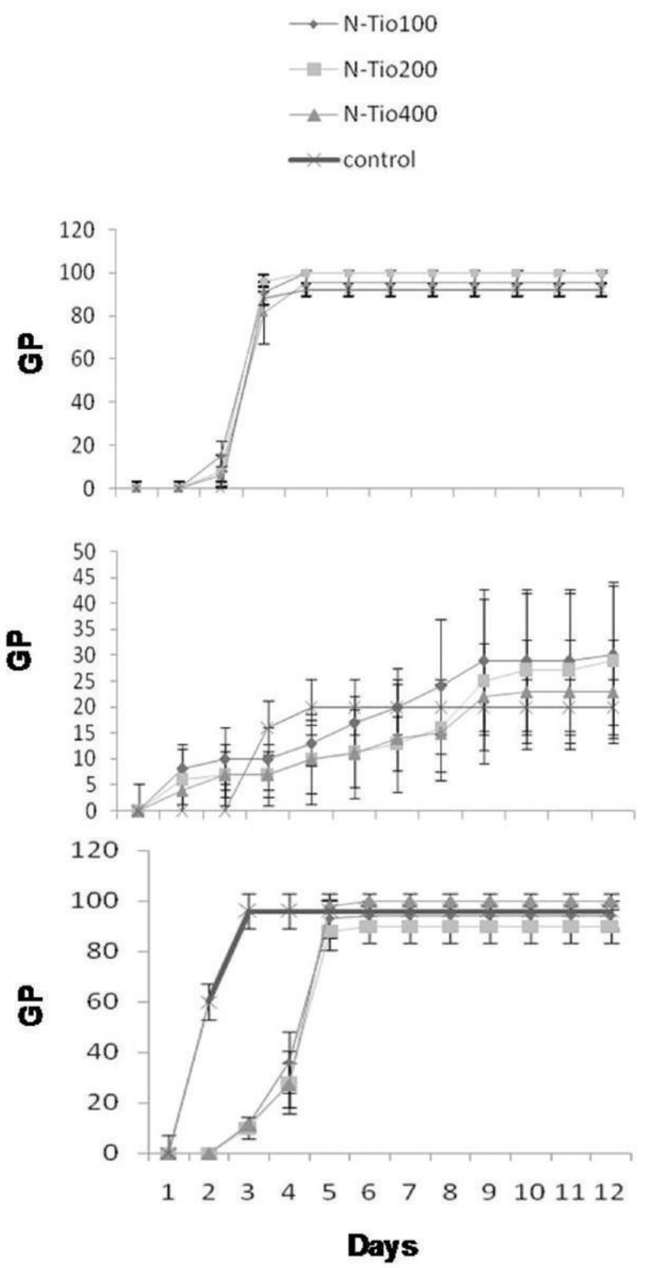

Fig. 1. The effect of different concentrations of N-TiO$_2$ on the germination percentage (GP) (%) of tomato (A), onion (B) and radish (C) during the laboratory experiment.

greenhouse-grown seedlings were observed after applying 400 mg L^{-1} N-TiO$_2$ (Table 2).

Effect of TiO$_2$ on seed germination and seedling growth of onion

Although GP and GR were not significantly affected by N-TiO$_2$, the highest mean was obtained at 100 mg L^{-1} N-TiO$_2$ (Table 1). Highest shoot and root length (12.33 cm and 4.83 cm, respectively) was observed after exposure to 100 mg L^{-1} N-TiO$_2$ (Table 1). MGT did not change after the application of N-TiO$_2$ (Table 1). Onion seeds germinated until the 12th day. Control seeds (not exposed to N-TiO$_2$) began to germinate from the second day while seeds that were exposed to N-TiO$_2$ began to germinate from the first day. GP reached a plateau at around 20% in the control after 4 days but ~ 30% after N-TiO$_2$ was applied (Fig. 1B). The application of N-TiO$_2$ had little effect on the growth of onion in the greenhouse stage. There were no significant differences in shoot length and FW and DW of greenhouse-grown seedlings between treatments (Table 2).

Table 2. The effect of N-TiO$_2$ on growth aspects of seedlings of three horticultural species in the greenhouse stage

N-TiO$_2$ (mg L^{-1})	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
		Lycopersicon esculentum		
0	6.90 ± 1.03 a†	2.40 ± 1.09 b	0.64 ± 0.68 bc	0.03 ± 2.67 d
100	3.62 ± 0.75 c	1.87 ± 0.85 b	0.53 ± 0.08 c	0.34 ± 0.05 c
200	4.87 ± 0.62 bc	3.62 ± 1.25 ab	0.73 ± 0.03 b	0.57 ± 0.04 b
400	6.37 ± 1.25 ab	4.50 ± 0.70 a	0.91 ± 0.06 a	0.74 ± 0.04 a
		Allium cepa		
0	9.34 ± 0.92 a	5.17 ± 1.72 a	0.30 ± 3.14 a	0.02 ± 2.43 a
100	0.55 ± 1.10 b	2.50 ± 5.0 a	0.38 ± 0.76 a	0.02 ± 0.05 a
200	1.50 ± 1.47 b	5.75 ± 4.19 a	0.13 ± 0.18 a	0.25 ± 0.20 a
400	1.12 ± 1.31 b	5.00 ± 5.83 a	0.08 ± 0.11 a	0.07 ± 0.09 a
		Raphanus sativus		
0	1.20 ± 0.98 b	5.45 ± 1.32 a	2.52 ± 2.09 a	0.14 ± 0.37 a
100	9.04 ± 1.40 a	1.99 ± 0.20 b	0.79 ± 0.20 b	0.21 ± 0.13 a
200	7.17 ± 2.90 a	1.72 ± 0.50 b	0.56 ± 0.25 b	0.09 ± 0.06 a
400%	9.03 ± 0.79 a	2.21 ± 1.00 b	0.74 ± 0.15 b	0.18 ± 0.11 a

†Differences within a column followed by the same letter are not significantly different at $P < 0.05$ (LSD test; analyzed separately for each species).

Effect of TiO$_2$ on seed germination and seedling growth of radish

GP did not change significantly following the addition of N-TiO$_2$. GR, shoot length, and root length decreased after the application of N-TiO$_2$ although the FW and DW increased, reaching a maximum at 400 and 100 mg L^{-1} N-TiO$_2$, respectively (Table 1). MGT increased after N-TiO$_2$ was applied but the differences between Ti concentrations were not significant (Table 1). The highest number of germinated seeds was observed on the 5th day (Fig. 1C). In greenhouse experiments, N-TiO$_2$ increased shoot length the most at 100 mg L^{-1} although root length and FW of greenhouse-grown seedlings decreased after the application of N-TiO$_2$ (Table 2).

Discussion

Titanium is a beneficial element that can increase Fe and Mg contents in plant tissues, NiR activity, and chlorophyll *a* and *b* biosynthesis (Kuzel et al. 2003). These physiological effects of titanium have greater effects when used at very low concentrations. In our study, N-TiO$_2$ accelerated the germination of tomato more than radish and of radish more than onion (Fig. 1A - C).

Zheng et al. (2005) observed that when 2.5% N-TiO$_2$ was used on naturally aged spinach, it could accelerate metabolism, including photosynthesis and enzyme activity, as well

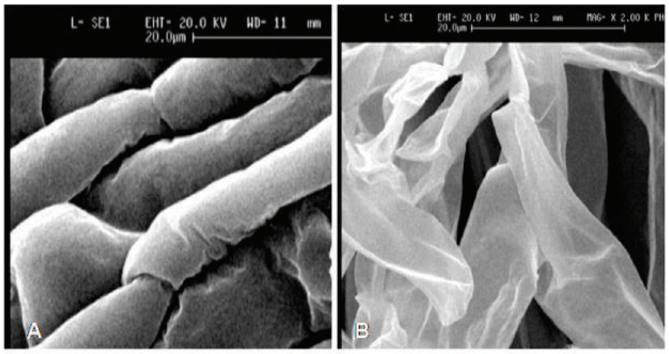

Fig. 2. Scanning electron microscope images of root (A) and testa (B) of tomato.

as germination characteristics such as germination rate and vigor index. They claimed that the penetration of N-TiO$_2$ into cells induced an oxidation-reduction reaction by superoxide ion radicals during germination. This process resulted in the quenching of free radicals in germinating seeds. Therefore, production of oxygen during this process also promoted germination. The improvement in germination can thus be related to root and testa morphology, which would facilitate the penetration of N-TiO$_2$ into these structures, as evidenced by SEM (Fig. 2 - 4). The fine pores of testa would allow nano materials like N-TiO$_2$ to be in closer physical contact with these porous coats, enhancing their effects. Therefore the surface area of the root and seed coat in contact with N-TiO$_2$ would increase, as would their influence (Fig. 2).

Increased water absorption as a result of treatment with N-TiO$_2$ improved spinach (Zheng et al. 2005) and flax (Clément et al. 2013) seed germination but not wheat seed germination (Feizi et al. 2012). The length and weight of tomato seedlings and onion were affected by N-TiO$_2$ as was reported for rape seedlings (Su et al. 2009). Yang et al. (2006) claimed that improved growth of spinach was due to enhanced photosynthesis. Their study showed that N-TiO$_2$ could affect nitrogen metabolism and greatly enhance the activity of some vital enzymes such as NiR, glutamate dehydrogenase, glutamine synthase, and glutamic-pyruvic transaminase, which play critical roles during growth. Active oxygen radicals, including superoxide and hydroxide anions, may be activated by N-TiO$_2$ (Khot et al. 2012) and increase the penetrability of the seed capsule to water and oxygen, thus speeding up germination (Feizi et al. 2013a, b). Clément et al. (2013) noted that the antimicrobial nature of N-TiO$_2$ could contribute to improved root growth in flax. The observations by Fan et al. (2014) support this notion when they observed reduced root growth, decreased *Rhizobium*-legume (pea) symbiosis, and delayed nodulation and nitrogen fixation.

In some cases, a reduction in some growth parameters was observed which may indicate a toxic effect of N-TiO$_2$. A high concentration of N-TiO$_2$ limited pea growth parameters, including GR, germination index, and root length (Su et al. 2009). In both laboratory and greenhouse experiments in this study, radish was sensitive to the applied concentrations of N-TiO$_2$, thus a lower concentration may have been more effective and could be the subject of future studies.

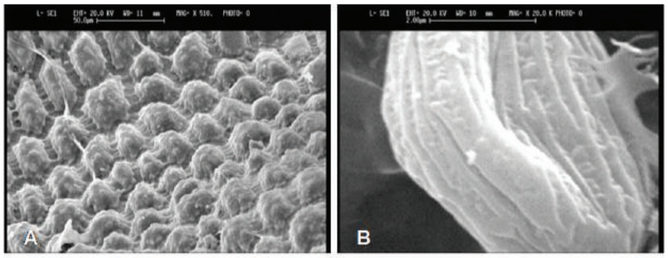

Fig. 3. Scanning electron microscope images of root (A) and testa (B) of onion.

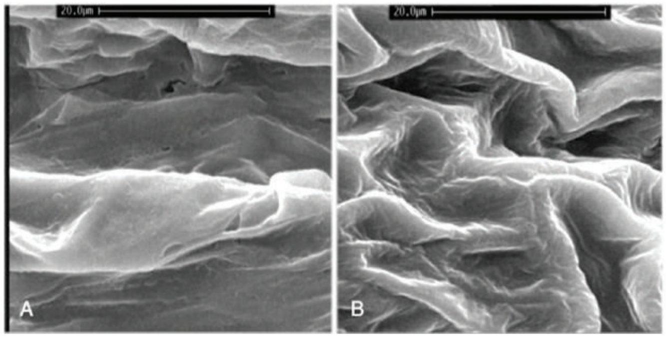

Fig. 4. Scanning electron microscope images of root (A) and testa (B) of radish.

Castiglione and Giorgetti (2010) studied the effect of N-TiO$_2$ on a monocot (corn) and a dicot (purple broad vetch; *Vicia narbonensis* L.) and observed that at 4% N-TiO$_2$, germination diminished and root elongation was restricted in *V. narbonensis* at a lower concentration (0.2% N-TiO$_2$) than corn. They related the toxic effect of N-TiO$_2$ to the irregularity of chromosomes in mitotic cells. Although there are very few studies on the effect of N-TiO$_2$ on seed germination, the general conclusion that can be made is that each plant is affected differently by N-TiO$_2$. N-TiO$_2$ had no effect on willow (Seeger et al. 2009), but enhanced germination in rape seed (Su et al. 2009) and spinach (Zheng et al. 2005).

The method and timing of application also strongly influence the effectiveness of N-TiO$_2$. Seeger et al. (2009) found that willow trees were not sensitive to short-term exposure of N-TiO$_2$. Another study evaluated the impact of different concentrations of N-TiO$_2$ by soaking rape seeds for different lengths of time: soaking seeds in a suspension of N-TiO$_2$ for 2 h improved the germination and growth of rape seeds (Su et al. 2009). In this study, the method of application of N-TiO$_2$ also affected germination: soaking tomato, onion, and radish seeds in the laboratory stage was more effective than immediate drenching of seeds in N-TiO$_2$ solution in the greenhouse stage. For instance, for the same characters, application of N-TiO$_2$ in the laboratory caused a 67% increase in root length but in the greenhouse it increased root length by about 46%.

Conclusion

N-TiO$_2$ has a positive effect on tomato, onion, and radish for two reasons: a greater contact area, and the nano size of TiO$_2$. N-TiO$_2$ can accelerate seed germination depending on the species, surface structure of testa, timing and method of N-TiO_2 application, and N-TiO_2 concentration. N-TiO_2 most positively affected tomato seed germination. The recommended concentrations for the application of N-TiO_2 are 200 mg L^{-1} for tomato, 100 mg L^{-1} for onion, and less than 100 mg L^{-1} for radish in the laboratory. Soaking is more effective than immediately drenching, but more studies are still required to confirm these results.

Contributions by authors and conflicts of interest

Both authors made a substantial contribution to the manuscript and the research presented. MH designed the study and conducted the experiments. JATdS and MH analyzed the data, interpreted the results and co-wrote the paper. Both authors have seen and agreed to the submitted and revised manuscript. Both authors take full public responsibility and abide by the four-clause definition of authorship as defined by the ICMJE. The authors declare no conflicts of interest.

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