Chemosphere 91 (2013) 506–511

Contents lists available at SciVerse ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Phytotoxicity and stimulatory impacts of nanosized and bulk titanium dioxide on fennel (*Foeniculum vulgare* Mill)

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HIGHLIGHTS

- ▶ Low dosage of nanosized TiO_2 enhanced indices of seed germination of fennel.
- ▶ Germination percent highly improved following exposure to 60 ppm nanosized TiO_2.
- ▶ Nano-TiO_2 can apply for improvement the seed germination of fennel.

ARTICLE INFO

Article history:
Received 2 July 2012
Received in revised form 4 December 2012
Accepted 8 December 2012
Available online 26 January 2013

Keywords:
Nanosized TiO_2
Phytotoxicity
Medicinal plant
Seed improvement

ABSTRACT

The objective of the this study was to compare concentrations of nanosized TiO_2 at 0, 5, 20, 40, 60 and 80 mg L^{-1} with bulk TiO_2 for phytotoxic and stimulatory effects on fennel seed germination and early growth stage. After 14 d of seed incubation, germination percentage highly improved following exposure to 60 ppm nanosized TiO_2. Similar positive effects occurred in terms of shoot dry weight and germination rate. Application of bulk TiO_2 particles in 40 ppm concentration greatly decreased shoot biomass up to 50% compared to the control. Application of 40 ppm nanosized TiO_2 treatment improved mean germination time by 31.8% in comparison to the untreated control. In addition, low and intermediate concentrations of nanosized TiO_2 enhanced indices such as germination value, vigor index and mean daily germination. In general, there was a considerable response by fennel seed to nanosized TiO_2 presenting the possibility of a new approach to overcome problems with seed germination in some plant species, particularly medicinal plants.

1. Introduction

There has been a rising demand for nanotechnology-based products in recent years, particularly in areas directly related to humans (Lee et al., 2012). Nanotechnology has many applications in agricultural research, such as in reproductive science and technology, the transfer of agricultural and food waste to energy and other helpful by-products through enzymatic nanobioprocessing, disease prevention and various other plant treatments using nanocides (Carmen et al., 2003).

Titanium is the ninth most abundant element and the second most abundant transition metal found in the earth's crust (about 6.320 ppm). An important effect of titanium compounds on plants used for various crops is improvement of yield (about 10–20%). Other effects of titanium on plants are increased contents of some essential elements in plant tissue; an increase in enzyme activity such as peroxidase, catalase, and nitrate reductase activities in plant tissue and research has shown increased chlorophyll in paprika (*Capsicum anuum* L.) and green alga (*Chlorella pyrenoidosa*) (Hruby et al., 2002). Nanosized TiO_2 is a frequently used nanoparticle, consequently there has been an exponential increase in data collection on the effects of TiO_2 nanoparticles on different species but there is much less information on the effects of nanoparticles on plants compared to animals. Studies of effects of TiO_2 nanoparticles on plants provide information about the positive and stimulating effects as well as any negative impact (Klancnik et al., 2011). Research by Lin and Xing (2007) evaluated phytotoxicity of five

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http://dx.doi.org/10.1016/j.chemosphere.2012.12.012

types of metallic nanoparticles in six plant species and indicated that seed germination was not affected except for the inhibition of nanoscale zinc on *Lolium multiflorum* and nanoscale zinc oxide on maize (*Zea mays*). They indicated that inhibition of root growth varied significantly among nanoparticles and plants and that it was partially correlated to nanoparticle concentration. Foltete et al. (2011) stated that altered TiO_2 nanocomposites were tested in the liquid phase on the plant model *Vicia faba*, which was exposed to three nominal concentrations: 5, 25 and 50 mg altered TiO_2 nanocomposites per liter for 48 h. Plant growth, photosystem II maximum quantum yield, genotoxicity (micronucleus test) and phytochelatins levels showed no change compared to controls (Foltete et al., 2011). Zheng et al. (2005) confirmed that nanosized TiO_2 helped water absorption in spinach seeds and as result accelerated seed germination. Lu et al. (2002) shown that a combination of nanosized SiO_2 and TiO_2 could increase nitrate reductase enzyme in soybean (*Glycine max*), increase its abilities of absorbing and utilizing water and fertilizer, promote its antioxidant system, and in fact accelerate its germination and growth. Also, the positive effects of TiO_2 could be probably due to the antimicrobial properties of engineered nanoparticles, which can enhance strength and resistance of plants to stress (Navarro et al., 2008). Research has demonstrated that Pd-nanoparticles altered kiwifruit pollen morphology and entered the grain more rapidly and to a greater extent than soluble Pd(II). At particulate Pd concentrations well below those of soluble Pd(II), pollen grain demonstrated rapid losses in endogenous calcium, inducing pollen plasma membrane damage. Research has also demonstrated that Pd-nanoparticles caused increased toxicity to kiwifruit pollen compared to soluble Pd(II) (Speranza et al., 2010).

F. vulgare commonly known as fennel is a well known and important medicinal and aromatic plant widely used to treat carminative, digestive, lactagogue, diuretic, respiratory and gastrointestinal disorders (Manzoor et al., 2012). Efficient seed germination and early seedling establishment are important processes in commercial agriculture. Rapid and uniform seedling emergence leads to successful plant establishment, as a deep root system is formed before the upper layers of the soil dry out, harden, or reach supra-optimal temperatures (Chen and Arora, in press). Some reports have demonstrated that nanoparticles can induce phytotoxicity and have a negative impact on seed germination and growth but the unique properties of nanoparticles can be used to improve seed germination and crop performance. This use of the potentially positive effects of nanoparticles may be a helpful approach to decrease consumption of chemical agents in agriculture that would help to lower environmental pollution. In previous work it was demonstrated that using nanosized TiO_2 in low concentration (2 and 10 ppm) could encourage seed germination of wheat in comparison to bulk TiO_2 and untreated control groups, but in high concentrations (100 and 500 ppm) it had an inhibitory or no effect on wheat seed (Feizi et al., 2012). However poor seed germination is a common occurrence in medicinal plants and there are no studies on the effects of nanoparticles on medicinal plants particularly fennel, which is one of the most important medicinal and aromatic plants cultivated in the world. This study was therefore done to investigate possible phytotoxicity and/or beneficial stimulatory effects of nanosized TiO_2 concentrations compared to bulk TiO_2 particles on fennel seed and seedling growth.

2. Materials and methods

2.1. Description of materials

Fennel (*F. vulgare*) dry seeds were taken from the Research Farm of Agricultural Faculty, Ferdowsi University of Mashhad. Nanosized TiO_2 powder was AEROXIDE® TiO_2 P25, supplied by Degussa GmbH Company. Specific surface area of nanosized TiO_2 was 50 $m^2\,g^{-1}$, average primary particle size was 21 nm and purity was > 99.5%. The size of TiO_2 nanoparticles (Fig. 1) was determined through Scanning Tunneling Microscope (STM) in Central Laboratory of Ferdowsi University of Mashhad.

X-ray diffraction (XRD) pattern of nanoparticles TiO_2 was shown in Fig. 2. XRD measurement showed that the TiO_2 nanoparticles used in the study were made by 80% anatase and 20% rutile. Analysis of particles in X-ray diffraction indicates Tetragonal particles and the crystalline nature of TiO_2 particles.

Bulk TiO_2 particles were supplied by AppliChem GmbH Company, they had 99% purity and particle size was measured by Scanning Electron Microscope (SEM) in Central Laboratory of Ferdowsi University of Mashhad (Fig. 3). XRD measurement showed that the bulk TiO_2 particles used in the study were made by 100% anatase. Analysis of particles in X-ray diffraction indicated tetragonal particles and the bulk TiO_2 particles had a crystalline nature (Fig. 4).

2.2. Plant culture and exposure

Tests were done to assess the effect of different concentrations of bulk and nanosized TiO_2 on fennel seed germination in a completely randomized design with four replications. The treatments in the experiment were five concentrations (5, 20, 40, 60 and 80 ppm) of bulk and five concentrations (5, 20, 40, 60 and 80 ppm) of nanosized TiO_2 and an untreated control (without any TiO_2 types). The Experiment was performed in a germinator with an average temperature of 25 ± 1 °C at the College of Agriculture, Ferdowsi University of Mashhad, Iran in 2012.

Seeds of similar size were randomly selected and sterilized using ClONa (5%) for 3 min and then carefully washed with distilled water three times. In order to obtain properly dispersed and stable TiO_2 suspensions of each concentration, an ultra-sonication treatment was applied to bulk and nanoparticles TiO_2 powders dispersed in water for 15 min. The seeds were placed on paper in four groups of 25 seeds in Petri dishes, and after that 3 ml of each concentration treatments was added to each. For the control, only distilled water was added to the Petri dishes. Germination tests were performed according to the rule issued by the International Seed Testing Association (ISTA, 2009). All concentrations of TiO_2 and the control were tested at the same time to ensure uniform conditions of light and temperature across all tests. Number of germinated seeds was noted daily for 14 d. Seeds were considered germinated when the radicle showed at least 2 mm in length (ISTA, 2009). Mean germination time was calculated based on Matthews and Khajeh-Hosseini (2007) (Eq. (1)):

$$\mathrm{MGT} = \frac{\sum F.X}{\sum F} \quad (1)$$

where F is the number of seeds newly germinated at the time of X, and X is the number of days from sowing.

Germination rate was determined based on Maguire (1982) (Eq. (2)):

$$\text{Germination rate} = (a/1) + (b - a/2) + (c - b/3) + \ldots + (n - n - 1/N) \quad (2)$$

where $a, b, c, \ldots, n$ are numbers of germinated seeds after $1, 2, 3, \ldots, N$ days from the start of imbibition.

Seedling vigor was computed based on Vashisth and Nagarajan (2010) (Eqs. (3) and (4)):

$$\text{Vigor index I} = \text{Germination }\% \times \text{Seedling length (cm)} \quad (3)$$

$$\text{Vigor index II} = \text{Germination }\% \times \text{Seedling weight (g)} \quad (4)$$

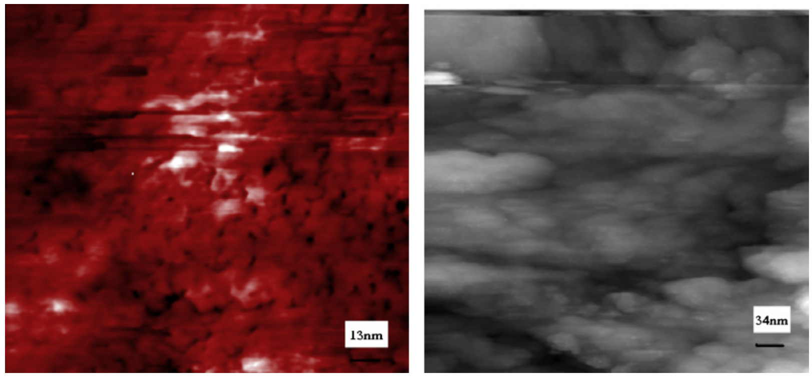

Fig. 1. Images of nanosized TiO_2 by Scanning Tunneling Microscope (STM).

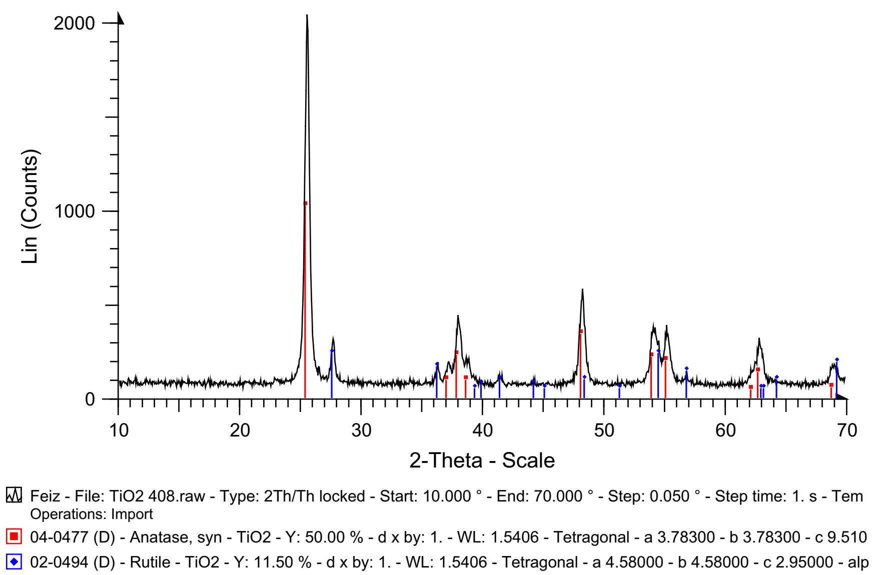

Fig. 2. X-ray diffraction (XRD) pattern of nano TiO_2 particles.

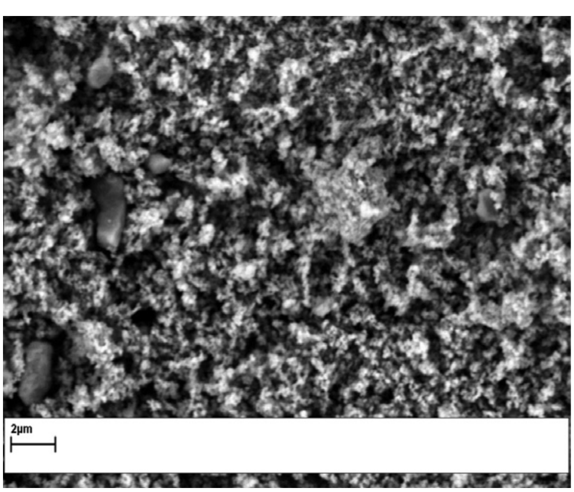

Fig. 3. Image of bulk TiO_2 particles by Scanning Electron Microscope (SEM).

Evaluations of Mean Daily Germination (MDG), Pick Value (PV) and Germination Value (GV) were calculated by the following equations (Hartmann et al., 1990):

$$\text{MDG} = \text{Germination }\%/\text{Total experiment days} \tag{5}$$

$$\text{PV} = \text{Maximum germinated seed number at one day}/\text{day number} \tag{6}$$

$$\text{GV} = \text{PV} \times \text{MDG} \tag{7}$$

2.3. Statistical analysis

A one-way analysis of variance (ANOVA) was performed between treatment samples in a completely randomized design in four replications. Data were analyzed using MSTAT-C software. Significant levels of difference for all measured traits were calculated and means were compared by the multiple ranges Duncan test at 5% level.

3. Results and discussion

Results demonstrated that treatments applied in this experiment had significant effects on most studied traits. Use of TiO_2 nanoparticles extraordinarily enhanced fennel seed germination, while seed germination percentages decreased from exposure to concentrations of bulk TiO_2 particles compared to the control

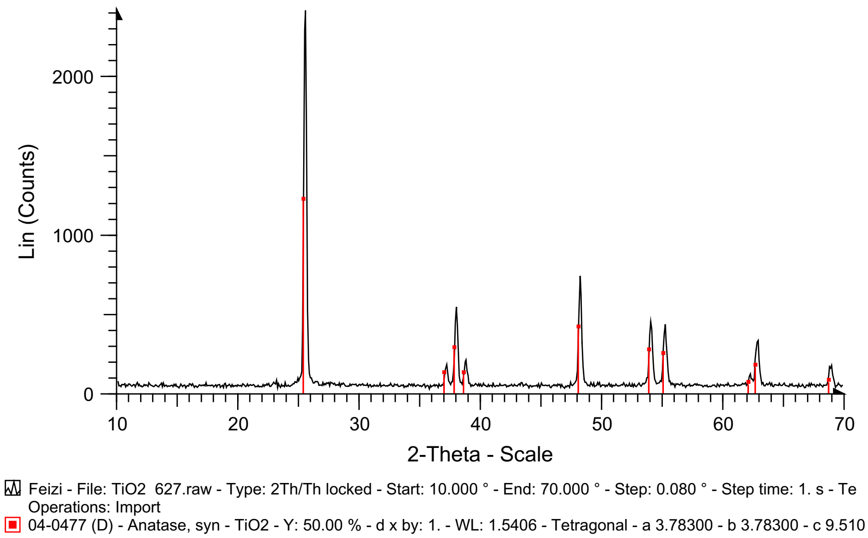

Fig. 4. X-ray diffraction (XRD) pattern of bulk TiO_2 particles.

group (Fig. 5). Final seed germination percentage showed the most value (76%) in 60 ppm TiO_2 nanoparticles, whereas the lowest value (41%) was in 60 ppm bulk TiO_2 particles. However, fennel seeds exposed to TiO_2 nanoparticles improved germination percentage 39.5% compared to the unexposed control group and was 83% more than those concentrations of bulk particles. The key reason for this increased growth rate could have been the photo-sterilization and photo-generation of "active oxygen like superoxide and hydroxide anions" by nano-TiO_2 that enhanced seed stress resistance and encouraged capsule penetration for intakes of water and oxygen needed for quick germination (Khot et al., 2012). Zheng et al. (2005) reported that nanosized TiO_2 contributed to water absorption by spinach seeds and as result accelerated seed germination. An earlier study was done (Feizi et al., 2012) demonstrating that although the highest germination percentage (98%) was in both 2 ppm bulk and nanosized TiO_2 concentrations, the two treatments had no significant effect on the seed germination percentage. The reason for this can be attributed to differences between physoio-biological natures of wheat and fennel seeds during the germination stage. Wheat seeds have no naturally occurring inhibitory factors affecting germination (germination percent in control group was 92%) but fennel seeds naturally have weak germination that possible be for some reasons such as climatic conditions (altitude, latitude, precipitation, soil properties, and

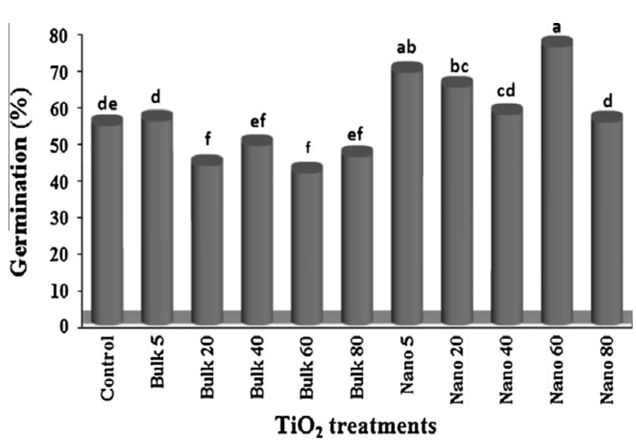

Fig. 5. Effect of bulk and nano TiO_2 concentrations on germination percentage of fennel seed (SD = 5.04, LSD = 10.26).

etc.), native seed lots, growth condition of parental plant and other factors that can weaken seed germination (germination percent in control group was 54.5% in the present study). Clément et al. (in press) reported that the soaking of flax seeds in the suspensions of anatase nanoparticles at concentration 100 mg L^{-1} had positive effects on seed germination and root growth. These positive effects could be due to antimicrobial properties of anatase crystalline structure of TiO_2 that increase plant resistance to stress.

Shoot, root and seedling elongation were not significantly affected by bulk and nanosized TiO_2 concentrations. Application of bulk TiO_2 particles in 40 ppm concentration greatly decreased shoot biomass up to 50% compared to the control seeds but at the concentration of 40 ppm nano-TiO_2 did not demonstrate such reduction in shoot biomass (Table 1). The greatest shoot biomass was found in 5 ppm bulk particles (1.18 mg) and 80 ppm nanoparticles (1.16 mg) of titanium dioxide. The highest root biomass was achieved from concentrations of 20 ppm bulk-TiO_2, 5 and 20 ppm nano-TiO_2 but an increased concentration of bulk particles of 80 ppm significantly reduced root weight. It is probable that increasing the concentration of bulk-TiO_2 induced aggregation of particles and resulted in clogging of root pores that interrupted water uptake by seeds. In addition, application of 40 ppm concentration of bulk and nanosized TiO_2 decreased fennel seedling biomass. It seems that nano TiO_2 could stimulate process of seed germination like water and oxygen uptake result in improves seed germination percentage but in later growth stages, seedling might respond as different. Also it is possible that the seeds promote by nano TiO_2 and then cultivate in soil in field. In this condition it is possible physico-chemical properties of soil modify adverse effects on plant growth and weights. Lin and Xing (2007) confirmed the phytotoxicity of nano-Al and Al_2O_3 significantly affected root elongation of ryegrass and corn, respectively whereas, nano-Al facilitated root growth in radish and rape. Although root length and weight are not standardized in toxicity tests, they may be helpful to compare the toxicity effects after seeds exposure to nanoparticles since low values can be related to non-acute toxicological or stress effects (Barrena et al., 2009). In an experiment, Barrena et al. (2009) stated that it seems that in the case of Fe-nanoparticles treatment, the development of thicker roots was favored, whereas in the case of Au, root growth was mainly due to elongation. The root growth in length but not in width might be an avoidance mechanism of the seed to a stress issue produced by the presence of nanoparticles (Barrena et al., 2009).

Table 1
Influence of bulk and nanosized TiO_2 concentrations on seed elongation and biomass of fennel seedling.

TiO_2 concentration (ppm)		Shoot length (cm)	Root length (cm)	Seedling length (cm)	Shoot dry weight (mg)	Root dry weight (mg)	Seedling weight (mg)
Control	0	4.06a[A]	5.81a	9.87a	1.07ab	0.38b	1.46ab
Bulk TiO_2	5	3.76a	5.25a	9.02a	1.18a	0.36bc	1.55a
	20	4.12a	6.21a	10.34a	1.12ab	0.54a	1.66a
	40	4.01a	5.51a	9.52a	0.53d	0.32bcd	0.85d
	60	3.89a	7.00a	10.89a	0.99ab	0.34bcd	1.33abc
	80	3.74a	6.25a	9.99a	0.95abc	0.2d	1.15bcd
Nano TiO_2	5	3.79a	5.55a	9.34a	1.01ab	0.4ab	1.42ab
	20	4.38a	5.93a	10.31a	0.8bcd	0.4ab	1.21bc
	40	4.05a	6.65a	10.71a	0.64cd	0.22cd	0.86d
	60	4.11a	5.93a	10.05a	0.81bcd	0.27bcd	1.09cd
	80	3.87a	7.39a	11.27a	1.16a	0.29bcd	1.45ab
SD		0.423	0.798	1.000	0.184	0.085	0.187
LSD		0.847	1.619	2.035	0.375	0.166	0.382

[A] Means, in each column, followed by similar letter are not significantly different at the 5% probability level – using Duncańs Multiple Range Test.

Many germination-related events (gene transcription and translation, respiration and energy metabolism, early reserve mobilization and DNA repair) could also take place during seed treatment (Varier et al., 2010), although often restricted due to reduced water supply compared to regular germination (Chen and Bradford, 2000; Li et al., 2005). The key to increased seed germination rate is the penetration of nanomaterial into the seed. Khodakovskaya et al. (2009) reported that MWCNTs can penetrate tomato seed and increase the germination rate by increasing water uptake. MWCNTs increased seed germination up to 90% (compared to 71% in control) in 20 d; it also increased plant biomass.

In general, lower mean germination time represents earlier germination. These results revealed that exposure of fennel seeds to 40 ppm nanosized TiO_2 obtained the lowest mean germination time (3.99 d) but higher concentrations did not improve mean germination time. Thus, 40 ppm concentration of nanosized TiO_2 treatment reduced mean germination time by 31.8% in comparison to the untreated control, whereas 40 ppm concentration of bulk TiO_2 contributed to a reduction of mean germination time of about 21% in comparison with the control (Fig. 6). Zheng et al. (2005) stated that the considerable effect of nanosized TiO_2 on spinach germination in tests was probably because of small particle size, which allowed nanoparticles to penetrate the seed during the treatment period, exerting its enhancing functions during growth.

In most cases, exposure of fennel seeds to low concentrations of nano TiO_2 particles led to enhanced germination rate (Table 2). The highest germination rate was found in 5 ppm nano-TiO_2 particles (6.39 seed d^{-1}) and increasing concentration decreased the germination rate. 60 ppm bulk-TiO_2 treatment showed the lowest germination rate (2.45 seed d^{-1}). All of bulk TiO_2 particle treatments inhibited germination rates compared to the control (Table 2). Using bulk TiO_2 particles significantly decreased germination value of seeds while nanosized TiO_2 had a positive effect on germination value. It is most probable that nanoparticles could penetrate into the seed coat and exert a beneficial effect on the process of seed germination but bulk particles, having a larger size, cannot easily enter the same pathway, therefore may accumulate in the pores of a seed coat and clog up water and oxygen transition. Based on studies on nanoparticles effect on seed germination mechanism it could state nanoparticles might helped the water absorption by the seeds (Zheng et al., 2005), increase nitrate reductase enzyme, increase seed abilities of absorbing and utilizing water and fertilizer, promote seed antioxidant system (Lu et al., 2002), reduced anti oxidant stress by reducing H_2O_2, superoxide radicals, and malonyldialdehyde content, and increasing some enzymes such as superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, and catalase activities (Lei et al., 2008) result in improve seed germination in some plant species. Foltete et al. (2011) examined altered TiO_2 nanocomposites (ATN) in the liquid phase on *V. faba*, exposed to three nominal concentrations: 5, 25 and 50 mg ATN/L for 48 h. They concluded plant growth, photosystem II maximum quantum yield, genotoxicity (micronucleus test) and phytochelatins levels showed no change compared to controls.

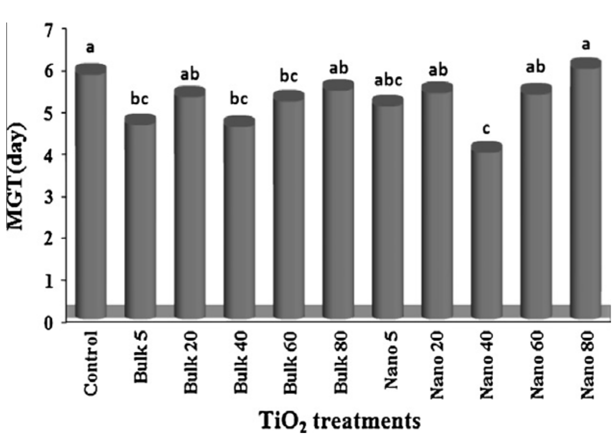

Fig. 6. Effect of bulk and nano TiO_2 concentrations on mean germination time of fennel seed (SD = 0.64, LSD = 1.319).

Exposure of seeds to all concentration of bulk TiO_2 significantly diminished MDG compared to the untreated control. Application of nanosized TiO_2 not only had negative effect on MDG, but also the greatest MDG was found in 60 ppm nanosized TiO_2 treatment. However, the Pick Value (PV) of fennel seedlings was not affected by different concentrations of bulk and nanoparticles. Application of bulk-TiO_2 concentrations had a negative effect on vigor index I but the stimulating effect of nanoparticle treatments was seen on vigor index I of fennel seeds. Additionally, use of 5 ppm nanosized TiO_2 showed the greatest vigor index II value compared to other doses and treatments (Table 2). Hence, it seems that increasing the vigor indices could improve seed emergence and seedling establishment by nanoparticles treatments which could probably be practical. It has been stated that the biological activity and biokinetics of nanoparticles depends on parameters such as size, shape, chemistry, crystallinity, surface properties (area, porosity, charge, surface modifications, coating), agglomeration state, biopersistence, and dose (Casals et al., 2008). Zheng et al. (2005) showed that the growth of spinach plants was greatly improved at concentrations of 250–4000 ppm nano TiO_2 than concentrations of bulk-TiO_2. Ghosh et al. (2010) observed adverse effect of TiO_2 nanoparticles for another plant species, *Nicotina Tabacum*. They reported that TiO_2 nanoparticles induced DNA injure in *N. tabacum* simply at high concentration of TiO_2 nanoparticles (319 mg L^{-1}).

Table 2
Influence of bulk and nanosized TiO_2 concentrations on growth features of fennel seedling.

TiO_2 concentration (ppm)		Germination rate (seed d^{-1})	Germination value	MDG	PV	Vigor index I	Vigor index II
Control	0	4.05bc[A]	5.958abc	4.428abc	1.68a	541.6cde	79.87ab
Bulk TiO_2	5	2.54de	7.72 abc	2.928f	1.24a	500.7de	86.44ab
	20	3.81bcd	3.69c	2.928f	1.31a	447.1e	71.19bc
	40	3.13cde	4.032c	3.142ef	1.54a	474.3e	42.14d
	60	2.45e	5.154bc	3.142ef	1.22a	450.8e	55.40cd
	80	2.70de	4.048c	3.285def	1.23a	460.2e	52.40cd
Nano TiO_2	5	6.39a	4.153c	4.821ab	2.14a	645.7abc	97.96a
	20	3.29bcde	10.24a	4.285bcd	1.54a	671.3ab	79.26ab
	40	4.50b	6.817abc	3.758cdef	2.04a	608.3bcd	51.03cd
	60	3.23bcde	8.028abc	5.428a	1.74a	762.8a	82.72ab
	80	2.80cde	9.49ab	4bcde	1.48a	624bc	80.17ab
SD		0.756	2.619	0.598	0.452	85.28	11.76
LSD		1.532	5.329	1.209	0.90	138.7	23.95

[A] Means, in each column, followed by similar letter are not significantly different at the 5% probability level – using Duncańs Multiple Range Test.

4. Conclusions

Nanoscience technology is leading to the development of a range of inexpensive applications for enhanced plant growth. Applications of nanomaterial can promote earlier plant germination and improve plant production. To our knowledge, this work is the first publication related to the effects of bulk and nanosized TiO_2 particles in fennel (*F. vulgare*). Fennel seeds have naturally weak germination (about 54%) contributing to some problems for its cultivation. Surprisingly, using TiO_2 nanoparticles promoted fennel seed germination percentage, while seed germination percentages decreased following exposure to concentrations of bulk TiO_2 particles compared to the control group. Exposure of fennel seeds to 40 ppm nanosized TiO_2 obtained the lowest mean germination time but higher concentrations did not improve mean germination time. Low and intermediate concentrations of nanosized TiO_2 improved seedling growth indices but higher concentration (80 ppm) had an inhibitory effect on seed and seedling. Application of bulk TiO_2 reduced some traits in the study such as germination rate, GMD, GV and vigor index compared to nanosized TiO_2 and the control. Nanomaterial can improve plant germination in certain plants but can have adverse affects on others. In such cases, nanomaterial can be applied under controlled conditions (such as in greenhouse-grown plants) to promote germination in plants of interest. Nevertheless, on the basis of these results it is highly recommended that the influence of low dose nanomaterial be assessed in order to encourage seed germination and seedling growth of different medicinal and aromatic plant species. Although this study demonstrated the potential of nanomaterial for agricultural application, further exploration and research is needed to elucidate and expand these possibilities.

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