Influence of Engineered Metal Oxide Nanoparticles on Seed Germination, Seedling Development and Chlorophyll Content

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***Abstract**: Biotechnology and nanobiotechnology combined may result in rapid and significant progress in the area of agricultural industry for their efficient delivery and production of abundant nutritious food. Nanotechnology, the vast field of 21st century, has a very significant impact on world's economy, industry, and people's life. It deals with the physical, chemical, and biological properties of matter considered at nanoscale and their implications for the welfare of world population. The continual use of engineered metal oxide nanoparticles in agriculture, including in various consumer applications, will undoubtedly contaminate the environment, potentially impacting the agriculture and food/feed quality, and may pose unknown risk to human health and safety. The world agriculture is facing many challenges like climate change, urbanization, sustainable use of natural resources, and environmental issues like runoff, and accumulation of pesticides, inorganic fertilizers, etc. These problems are further intensified by an alarming increase in food/feed demand that will be needed to feed an estimated population of 9 billion by 2050.This review summarized the effects of 5 nanoscale of metal oxide nanoparticles (MONPs); aluminium oxide (Al_2O_3), copper oxide (CuO), iron oxide (Fe_2O_3), titanium dioxide (TiO_2) and zinc oxide (ZnO) at different concentrations (0, 5, 10, 20, 40, 80, 160 and 320 mg/l of each MONP) on the seed germination, seedling growth and development of three legume plants named broad bean, common bean and alfalfa in vitro and under aseptic condition. Result indicated that MONPs were differentially influenced the levels of seed germination, seedlings growth and development as well as the chlorophyll content in a dose dependent which varied with type of each MONP and plant type. It was found that maximum seed germination of broad bean, common bean and alfalfa was observed between 40 and 80 µg/ml of each MONP. Iron oxide did not show any toxicity to seed germination at 160 µg/ml. Copper oxide was the most toxic at 160 µg/ml followed by ZnO and Al_2O_3. Unlike seed germination, growth of the seedling was increased gradually with increasing the concentrations of each MONP up to the 80 mg/l. Under in vitro conditions, findings showed that the use of the investigated MONPs can improve the quality of the seed germination and seedling length in comparison with control seedlings. Generally, CuO demonstrated highly potential toxic effects at chlorophyll synthesis at 80 mg/l followed by ZnO and Fe_2O_3 stimulated the plant elongation to the concentration of 80 mg/l. Comparatively few studies have* *been conducted on NPs are beneficiary to seed germination, plant growth and developments. It was concluded that MONPs affect the physiological expression of plant types differently and need further studies to better understand the impact of MONPs on bioaccumulation and the symbiotic interaction between micro- and their macro-symbionts to fix the atmospheric nitrogen. Therefore, extensive researches are required to understand the mechanism for nanoparticles toxicity and their effects on natural agroecosystem.*

Keywords: *Legumes; metal oxide nanoparticles; seed germination; seedling growth and development; nanotoxicology*

1 Introduction

Nanotechnology has great potential to improve environmental quality. It can improve detection and sensing of pollutants as well as help in the development of new remediation technologies. It opens a large scope of novel application in the field of agricultural biotechnology, because nanoparticles (NPs) interact with soil microorganisms causing many biochemical changes, depending on the NPs properties. Also, it has the potential to revolutionize the agriculture with new tools to enhancing the ability of plants to absorb nutrients. The combination between biotechnology and nanobiotechnology may result in rapid and significant progress in the area of agricultural industry for their efficient delivery and production of abundant nutritious food. The term “nanoecotoxicology” has been developed as a separate scientific discipline with the purpose of generating knowledge about the effects of NPs on humans and the environment. The role of nanotechnology in plant and soil systems demonstrates that NPs may assist in the controlled release of agrochemicals for nutrition and protection against pests and pathogens, delivery of genetic material, sensitive detection of plant disease and pollutants, and protection and formation of soil structure.

Nanoparticles are entities that are defined with a size range between 1 and 100 nm. Formation of NPs allows the development of efficient methodologies for minimizing the formation of pollutants and reducing their emissions. The efficacy of NPs is determined by their chemical composition, size, surface covering, reactivity, and the dose at which they are effective. The high reactivity of NPs has continued to raise concerns about their potential toxicity in biological systems. They can be engineered, or generated by industrial combustive processes or of natural origin. Engineered NPs production is growing fast because, of their physical and chemical properties. They are used in different sectors from cosmetics to from textile industry, from medicine to agriculture from the automotive industry to food. The cross-use of NPs can determine the pollution and accumulation of various environmental compartments. Their effect on human health and ecosystems is still not clear.

Nanoparticles have interactions at molecular level in living cells and nano-agriculture involves the employment of NPs in agriculture with the ambition that these particles impart some beneficial effects to the crop [1]. The use of NPs in plant growth and for the control of plant diseases is a recent practice [2-3]. Nanopartcles fall in the transition zone between individual molecules and the corresponding bulk materials, which generates both positive and negative biological effects in living cell [4]. The amount of research has been increasing on the biological effects of NPs on higher plants. Lu et al. [5] studied the effect of mixtures of nano SiO_2 and nano TiO_2 on soybean seed. They found that the mixture of NPs increases nitrate reductase in soybean increasing its germination and growth and ZnO on growth of *Vigna radiata* and *Cicer arietinum* seedlings using plant agar method [6] and Pea nut [7]. USEPA [8] grouped the engineered nanomaterials into four types: (1) carbon-based materials, usually including fullerene, single walled carbon nanotube (SWCNT) and multiwalled carbon nanotube (MWCNT); (2) Metal-based materials such as quantum dots, nanogold, nanozinc (nano-Zn), nanoaluminum (nano-Al), and nanoscale metal oxides like TiO_2, ZnO and Al_2O_3; (3) dendrimers, which are nano-sized polymers built from branched units capable of being tailored to perform specific chemical functions; and (4) composites, which combine nanoparticles with other nanoparticles or with larger, bulk-type materials.

However, this study is problematic as pointed out by Murashov [9], because the authors did not take into account a fact that e.g., soluble Al^{3+} is a potent root toxicant and known to inhibit root growth. The authors warned that care must be taken in toxicity testing when the effects may be related to simple solubility. Lin and Xing [10] investigated the phytotoxicity of 5 types of NPs (multiwalled carbon nanotube, aluminium, alumina, zinc and zinc oxide) on seed germination and root growth of 6 higher plant species. In the root elongation test, all plants were affected when suspended in 2000 mg/L nano-Zn or nano-ZnO. Lee et al. [11] adopted a plant agar for homogeneous exposure of NPs. Copper NPs dispersed in plant agar media were toxic to both tested plants, namely wheat and mung bean, and also were bioavailable. The amount of cupric ions released from Cu NPs had negligible effects. Cańas et al. [12] investigated the effects of functionalized and nonfunctionalized single-walled C nanotubes on root elongation of 6 crop species (cabbage, carrot, cucumber, lettuce, onion, and tomato). Both CNTs and fCNTs affected root elongation of four crop species, but phytotoxicity varied between CNTs and fCNTs, with CNTs affecting more species. On the other hand, there have been reported the positive effects of NPs on plants. For example, several authors [13]; [14]; [15], etc. have shown that nano-sized TiO_2 can have a positive effect on growth of spinach when administered to the seeds or sprayed onto the leaves. Nano-TiO_2 was shown to increase the activity of several enzymes and to promote the adsorption of nitrate, accelerating the transformation of inorganic into organic nitrogen. Normal-sized TiO_2 does not have these effects. The aim of this study is to measure the influences of some engineered MONPs on the seed germination, seedling development and chlorophyll content at different concentrations.

2 Materials and Methods

Five types of metal oxide nanoparticles (MONPs) were used in this study. They are: Al_2O_3, CuO, Fe_2O_3, TiO_2 and ZnO which obtained from Sigma-Aldrich in powder forms. For all except TiO_2, suspensions were placed in an ultra-sound water bath for 30 min to break aggregates before diluting them to the exposure concentrations. For TiO_2, suspension was prepared in dimethylsulphoxide (supplied by Sigma Aldrich). For stock solution preparations, 5, 10, 20, 40, 80, 160 and 320 mg MONPs were mixed with 100 ml deionized water in 250 ml flask and vigorously stirred for 15 min. and then suspensions were stored at 4°C. Different concentrations of 0, 5, 10, 20, 40, 80, 160 and 320 μg/ml (mg/l) of each of MONP were applied to study their effects on the seed germination, seedling development and chlorophyll content of three legume plants: broad bean (*Vicia faba*), common bean (*Phaseolus vulgaris*) and alfalfa (*Medicago sativa*) *in vitro* and under aseptic condition. Seeds of broad bean (BB-02), common bean (CB-07) and alfalfa (MS-07) were immersed in a 10% sodium hypochlorite solution for 10 min to ensure surface sterility.

The seed germination experiment was carried out with 5 sets according to the number of MONPs, each set consists 7 Petri dishs (number of concentrations per MONP) lined with sterilised (hot air-dried) Whatman no. 1 filter paper. Surface sterilised seeds were germinated in each concentration of MONPs. Similar experiment without MONPs was conducted as control. Five seeds were placed per Petri dish and observed for germination. Petri dishes were covered and sealed with tape, and placed in an incubator at 25°C. Daily observation of seed germination was recorded for 5 days of incubation. Then, the seed germination rate was calculated.

Similar desgin was carried out in test tube of 33 mm in diameter and 200 mm length containing basal medium without growth regulators to study the effects of tested different concentrations of various MONPs on seedlings growth and development of the 3 plants. The seeds were germinated and uniform seedlings were selected for experimental continuation. Seedling growth in terms of seedling height and total chlorophyll content were recorded after 15 days and results were compared to see effect of MONPs on seed germination and early seedling growth. Chlorophyll contents were measured by extracting 0.5 g of fresh leaf in 3 ml of 80% acetone with a small amount of quartz sand. according to Lichtenthaler & Wellburn [16] using UV-VIS spectrophotometer. All experiments were conducted with 3 replicates at 25°C.

3 Results and Discussion

Germination is normally known as a physiological process beginning with water imbibition by seeds and culminating in the emergence of the rootlet. Limited studies reported both positive and negative effects of nanoparticles on higher plants. It was pointed out that a mixture of nano-SiO_2 and nano-TiO_2 could increase nitrate reductase in soybean, enhance its abilities of absorbing and utilizing water and fertilizer, stimulate its antioxidant system, and apparently hasten its germination and growth [17]. Nano-TiO_2 was reported to promote photosynthesis and N metabolism, and then greatly improve growth of spinach at a proper concentration [13]; [14]; [18]; [19]. However, after investigating the phytotoxicity of nano-Al_2O_3 powders with or without phenanthrene coating, Yang and Watts [20] concluded that uncoated alumina particles inhibited root elongation of corn, cucumber, soybean, cabbage and carrot. This study attracted attentions from scientists and media, and was used to claim that nanoparticles can exert a negative effect on plants [9]. But, the authors did not identify dissolution of nano-Al_2O_3 in solution, thus, failed to clarify if the phytotoxicity was from nano-Al_2O_3 or aluminium ion in the aqueous solution [9].

In the present study, it was found that seed germination and early seedling growth clearly indicate that all MONPs at lower concentration up to 40 mg/l promoted seed germination, seedling growth and chlorophyll content for all plants, but at higher concentrations, seed germination, seedling growth and chlorophyll content were reduced.

For seed germination, it was found that broad bean seeds (Figure 1a) had the highest germination rate in comparison with control untreated seeds at concentration 40 μg/ml of MONPs which showed significantly high germination percentages and maximum germination per cent of seed germination was observed in 80 μg/ml concentration of Fe_2O_3, whereas CuO treated seeds showed the lowest germination. At high concentrations of MONPs (80 μg/ml) showed significant enhancement seed germination except in the seed treatment with CuO and ZnO which cause a decline in the germination rate of broad bean seeds. However, there was no significant between the seed germination in case of Al_2O_3 and TiO_2 at higher concentrations (80 and 320 μg/ml) which showed decrease in germination rates.

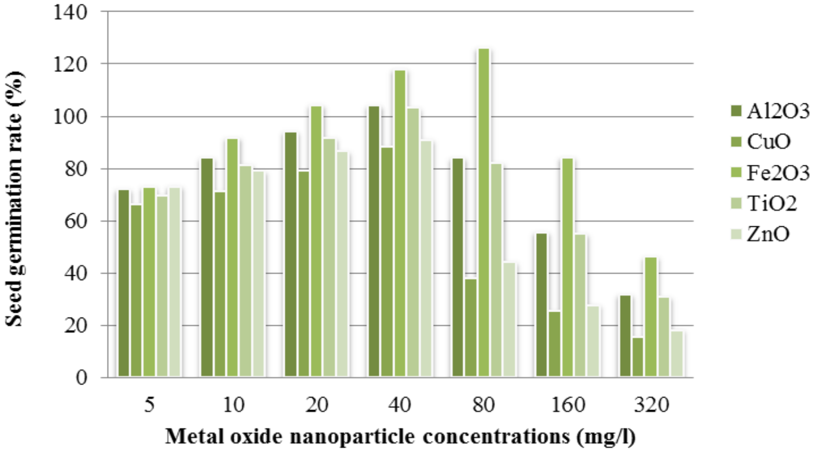

Figure 1a
Nanoparticles impacts seed germination of broad bean (BB-02)

Figure 1b illustrates the rate of germination of common bean seeds when incubated with different MONPs ant different concentrations. The results indicated that common bean seeds were less adapted to these NPs than broad bean. Meanwhile, maximum seed germination was found at the concentration 40 µg/ml and also Fe_2O_3 was the more stimulator for seed germination and CuO and ZnO were the most toxic NPs. Similarly, no significant difference found between Al_2O_3 and TiO_2 in relation with seed germination.

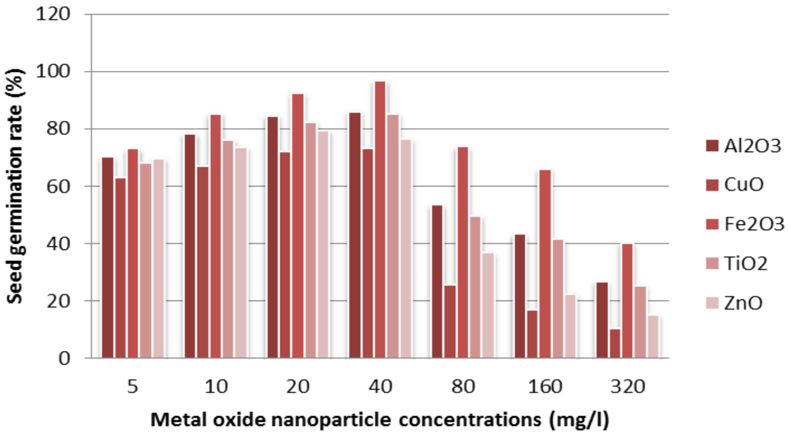

Figure 1b
Nanoparticles impacts seed germination of common bean (CB-2)

Similar trend as to broad bean and common bean was found in case of seed germination of alfalfa (Figure 1c), where maximum rate of germination was found in seed treated with concentration of 40 µg/ml and at alfalfa seeds were more adapted to the MONPs at 80 µg/ml than common bean. It was noted that the germination rate was higher at high concentrations of TiO2 than Al2O3. Also, CuO was the most toxic followed by ZnO.

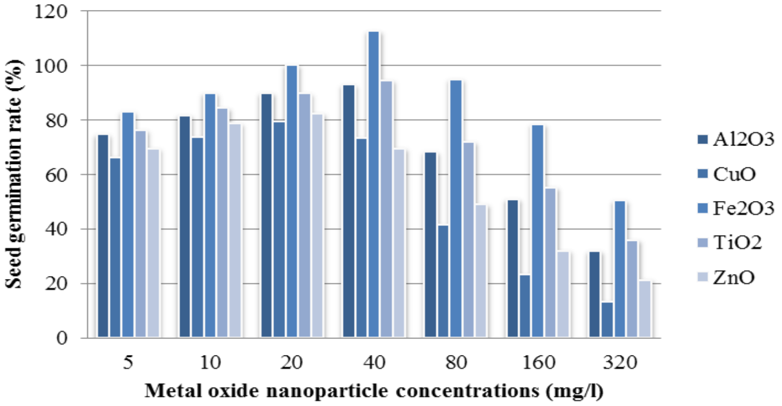

Figure 1c
Nanoparticles impacts seed germination of alfalfa (MS-07)

For early seedling growth and development, it was found that the seedlings grown in agar basal medium supplemented with different concentrations of various MONPs which generally increased length of the seedlings of all 3 plant types when compare to the control seedlings (Figures 2a, 2b, and 2c).

Figure 2a shows that the seedling of broad bean grown in amended agar medium with MONPs increased the seedling length in comparison with those grow in control agar basal medium. The enhancement was observed up to the concentration of 40 µg/ml of each of MONPs. Over this concentration, seedlings were stimulated by Fe_2O_3 followed by Al_2O_3 and TiO_2, where the CuO and ZnO lowering the elongation of the seedlings.

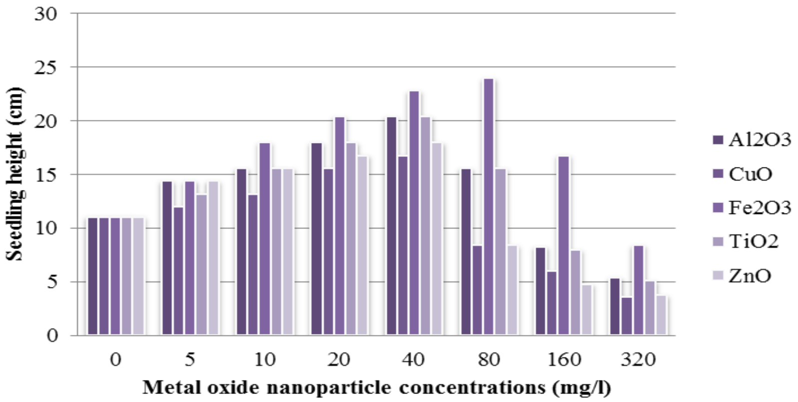

Figure 2a
Nanoparticles impacts seedling height of broad bean (BB-02)

The elongation of common bean seedlings (Figure 2b) was lower than the in the seedlings of broad bean. Similar trends was found at the concentration 80 µg/ml where the seedlings were activated by Fe_2O_3 more than Al_2O_3 which had similar effect as in case of treated seedlings with TiO_2. Meanwhile CuO and ZnO had similar inhibitory effects. At concentration 160 µg/ml, only Fe_2O_3 can help the seedling elongation where the other MONPs decline the rate of growth lower than in control cases.

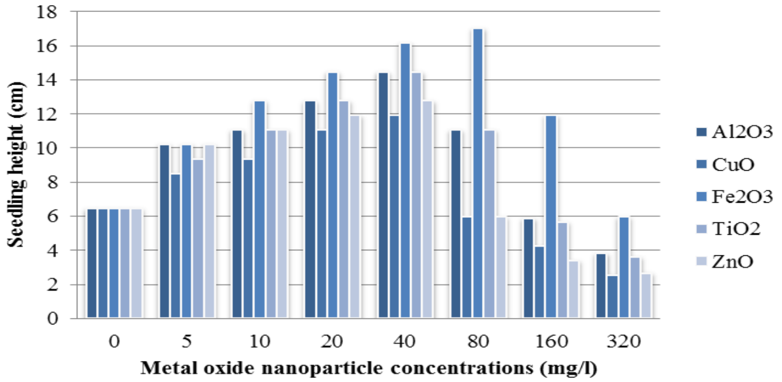

Figure 2b
Nanoparticles impacts seedling height of common bean (CB-2)

Seedlings of alfalfa (Figure 2c) were more adapted to the concentrations of MONPs added to the basal agar medium than common bean but also the maximum growth and the elongation rate were found at the concentration 40 µg/ml and also, Fe_2O_3 was the most stimulator even at the concentration is 160 µg/ml. It was noted that more concentration cause more inhibition of seedling elongation. The increased seedling growth rate may be due to the enhancing the water uptake by the treated seeds.

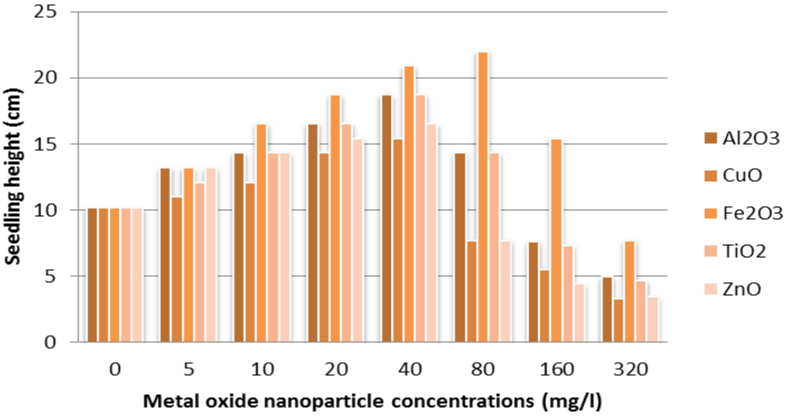

Figure 2c
Nanoparticles impacts seedling height of alfalfa (MS-07)

The effect different concentrations of MONPs on total chlorophyll content were observed in the 3 plants (Figure 3a, 3b and 3c). The chlorophyll content of different crop plants was tolerant to moderate and high concentrations of MONPs used and, therefore, chlorophyll production was not affected till 15th day.

Figure 3a domenstrates the effect of MONPs on total chlorophyll production in the leaves of broad bean seedling. It was found that chlorophyll synthesis was stimulated up the the concentration 40 µg/ml. At 80 and 160 µg/ml, Fe_2O_3 was the most stimulator MONP, while CuO and ZnO were the most toxic MONPs. The results indicated that Al_2O_3 and TiO_2 had the same positive effect on the chlorophyll synthesis of the seedlings grown at 80 µg/ml and decreased the synthesis at concentration 160 µg/ml. The chlorophyll synthesis was highly inhibited at the maximum concentration 320 µg/ml.

In case of common bean grown in basal agar medium supplemented by different concentrations of various MONPs (Figure 3b), it was found that the seedlings well established and the amount of chlorophyll synthesis was higher than in the case of broad bean. With the similar trend that maximum chlorophyll synthesis was at the concentration 40 µg/ml and Fe_2O_3 stimulated the production of chlorophyll at the concentration 80 and 160 µg/ml.

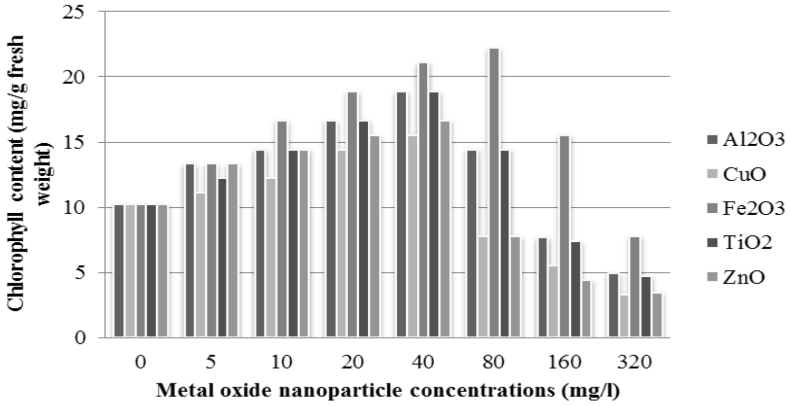

Figure 3a
Nanoparticles impacts chlorophyll content in leaf of broad bean (BB-02)

CuO and ZnO MONPs were the most inhibitors even at high concentration 80 µg/ml. Al_2O_3 and TiO_2 had the same positive effect on the chlorophyll synthesis of the seedlings grown at 80 µg/ml and decreased the synthesis at concentration 160 µg/ml.

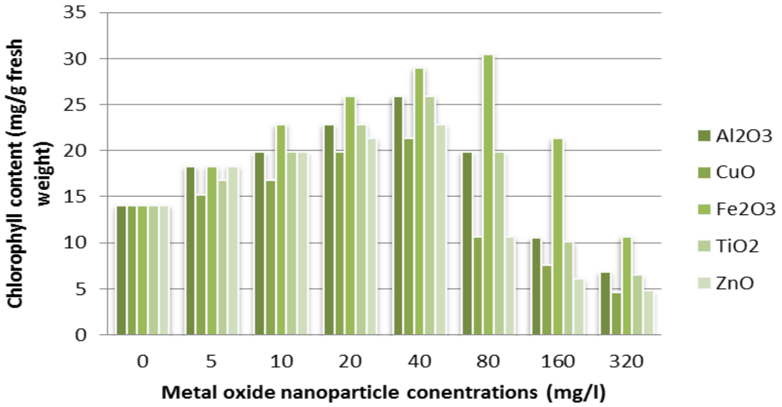

Figure 3b
Nanoparticles impacts chlorophyll content in leaf of common bean (CB-2)

Figure 3c shows the effect of MONPs on the chlorophyll production in the leaflets of alfalfa at different concentrations. It was found that the degree of chlorophyll synthesis is gradually increased more than in control plant seedlings to reach the maximum at the concentration 40 µg/ml. Fe_2O_3 stimulated the production of chlorophyll at the concentration 160 μg/ml. Al_2O_3 and TiO_2 had the same positive effect on the chlorophyll synthesis of the seedlings grown at 80 μg/ml and decreased the synthesis at concentration 160 μg/ml. CuO and ZnO decreased the synthesis of chlorophyll.

A study reported that chlorophyll content of maize plants was found to be increased by low concentration (10-50 μl/l) while it was found to be inhibited by higher concentrations of magnetic nanoparticle [21]. In concentration 60 ppm of silver nanoparticles chlorophyll a and chlorophyll b increases by 49% and 33% compared to the control in common bean. In corn (*Zea mays*) treated crop, the chlorophyll A and B increases by 46% and 26% compared to control respectively [22].

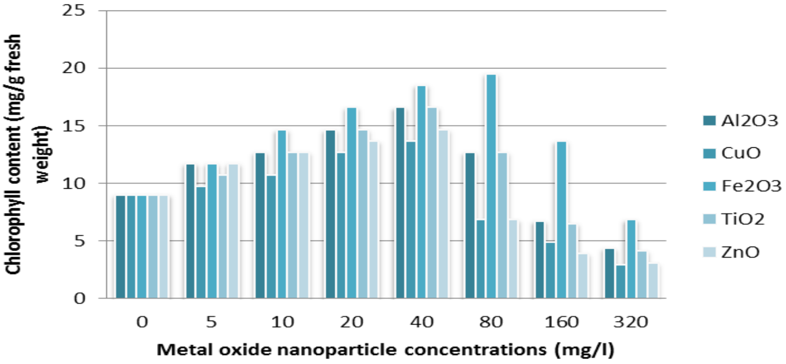

Figure 3c
Nanoparticles impacts chlorophyll content in leaf of alfalfa (MS-07)

Raskar and Laware [23] found that seed germination increased in lower concentrations, however showed decrease in values at higher concentrations. Germination indices showed increased values in lower concentrations; however these decreased significantly at higher concentrations.

Four sets of Boswellia ovalifoliolata seeds were germinated on Murashige and Skoog (MS) basal medium with various concentrations (10 to 30 mg/ml) of biologically synthesized silver nanoparticles (SNPs). It was found that 95% of treated seed were germinated when compare to control seeds (water) which took longer time (10 to 20 days) to sprout, whereas all treated seeds sprouted within 10 days. The maximum height (10.6 cm) observed in seedlings treated with SNPs 4 mg/ml. The possible contribution of SNPs is to facilitate the penetration of water and nutrients through seed coat and accelerate the seed germination and seedling growth of *Boswellia ovalifoliolata* [24].

Effects of multi-walled carbon nanotube, aluminum, alumina, zinc, and zinc oxide on seed germination and root growth of radish, rape, ryegrass, lettuce, corn, and cucumber were investigated by Lin and Xing [10]. They found that seed germination was not affected except for the inhibition of nanoscale zinc (nano-Zn) on ryegrass and zinc oxide (nano-ZnO) on corn at 2000 mg/L. Inhibition on root growth varied greatly among nanoparticles and plants. Suspensions of 2000 mg/L nano-Zn or nano-ZnO practically terminated root elongation of the tested plant species. Fifty percent inhibitory concentrations (IC_{50}) of nano-Zn and nano-ZnO were estimated to be near 50 mg/L for radish, and about 20 mg/L for rape and ryegrass. The inhibition occurred during the seed incubation process rather than seed soaking stage. These results are significant in terms of use and disposal of engineered nanoparticles.

4 Conclusion

Generally, our observation recorded that all seeds treated with NPs completed the germination within 3 to 5 days. However 5 to 7 days required for control seeds. This mean that to some extend the MONPs can positively influence the germination rates of the 3 seed types. Generally, for all 3 seedling types, it was found that Fe_2O_3 was the most stimulator even at the concentration 320 µg/ml.

Under *in vitro* conditions, results showed that the use of the investigated MONPs can improve the seed germination. Generally, CuO demonstrated highly potential toxic effects at levels over 40 and 80 µg/ml followed by ZnO and Fe_2O_3 stimulated the production of seed germination to the level 160 µg/ml. Researchers in nanotechnology can do a lot to benefit society through applications in agriculture and food systems. More studies are needed concerning the plant growth when exposed to various NPs, and such information will be useful for ecological and human health risk assessments. Therefore, extensive researches are required to understand the mechanism for nanoparticles toxicity and their effects on natural agroecosystem. Terrestrial ecosystems are important ecological receptors that have not received sufficient attention with respect to potential toxicity of NPs, especially to microorganisms in soil-plant systems. It is important to focus our concern on the impact of NPs on soil microbial communities, because of their antimicrobial activity. The results of the present study may be helpful to improve the % of seed germination and seedling growth in seeds especially in dormant seeds. By using this technique it can increase the amplification of plants particularly endemic trees with hard seed coat which are in the verge of extinction.

5 References

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