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Ecotoxicological effect of *Lecanicillium lecanii* (Ascomycota: Hypocreales) based silver nanoparticles on growth parameters of economically important plants

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ABSTRACT

Effect of *Lecanicillium lecanii* (Ascomycota: Hypocreales) based silver nanoparticles on seedling emergence, plant growth parameters such as shoot length, leaf surface area, chlorophyll content and phyllosphere microflora of economic important pulses such as **c**owpea (*Vigna unguiculata*), black gram (*Vigna mungo*), green gram (*Vigna radiata*), sorghum (*Sorghum vulgare*) and horse gram (*Macrotyloma uniflorum*) was probed in the present study Moreover the effect on soil heterotrophic microbial populations and soil macronutrients nitrogen, phosphorous and potassium was also investigated. No distinct effect was recorded on seedling emergence, length of the shoot and leaf surface area of all the tested plants. Similarly no effect was observed in the heterotrophic bacterial, fungal and actinomycetes population phyllosphere and chlorophyll content except sorghum. NPK analysis of the treated pot soil showed a sharp increase in phosphate and potassium content than control and no difference in nitrogen level.This study has brought out the safety aspect of nanoparticles to non target organism

Key words: Ag nanoparticles, ecotoxicity, chlorophyll, NPK.

INTRODUCTION

Nanotechnology is currently employed as a tool to explore the darkest avenues of medical sciences in several ways like imaging, sensing, targeted drug delivery, gene delivery systems and artificial implants (Gleiter, 2000). Hence, nanosized organic and inorganic particles are finding increasing attention in medical applications due to their amenability to biological functionalization. Based on enhanced effectiveness, the new age drugs re-nanoparticles of polymers, metals or ceramics, which can combat conditions like cancer and fight human pathogens like bacteria (Key and Maass,2008) Increasing numbers of commercial products, from cosmetics to medicine, incorporate manufactured nanomaterials (MNMs) that can be accidentally or incidentally released to the environment (Monica and Cremonini,.2009). Concern over the potentially harmful effects of such nanoparticles has stimulated the advent of nanotoxicology as a unique and significant research discipline. However, the majority of the published nanotoxicology articles have focused on mammalian cytotoxicity or impacts to animals and bacteria, and only a few studies have considered the toxicity of MNMs to plants. Developmental phytotoxicity of NMS is a critical knowledge gap because nanoparticles entering wastewater streams may predominantly be incorporated intosewage sludge and applied to agricultural fields (Lee *et al*, 2008). In the present study, the effect of biologically synthesized sanoparticles on seedlings emergence and plant growth parameters such as such as shoot length, leaf surface area, chlorophyll content and phyllosphere microflora of economic important pulses such as cowpea (*Vigna unguiculata*), black gram (*Vigna mungo*), green gram (*Vigna radiata*), sorghum (*Sorghum vulgare*) and horse gram (*Macrotyloma uniflorum*) was probed in the present study Moreover the effect on phyllosphere heterotrophic microbial populations and soil macronutrients nitrogen, phosphorous and potassium was also studied.

MATERIALS AND METHODS

Fungal strain and growth condition

Lecanicillium lecanii (MTCC No.2619) strain was obtained from MTCC Chandigarh and maintained on Sabouraud Maltose Yeast Extract broth. For inoculum preparation, a loopful of fungal culture was inoculated in a 250 ml of Erenlymer flask containing 100 ml sterile SMYB broth. The flask was incubated at 30^0 C, for 7 days on a rotary shaker at 150 rpm, and the cells were harvested by centrifugation at 3000 rpm for 15 min. Thus obtained biomass was dried in an oven at 60^0C for 24 hr.

Biosynthesis and characterization of silver nanoparticles

In a typical procedure of nanoparticle synthesis, the dried biomass was washed thrice with Milli-Q-deinosied water to remove any further medium components. The dried biomass was brought in contact with 200 ml of deionised water for 72 hr and agitated under same conditions as described earlier. After the incubation the cell filtrate was obtained by passing it through Whatmann filter paper no.1. Silver nitrate $AgNO_3$, 10^{-3} M (1mM) final concentration was mixed with 50 ml of cell filtrate in a 250 ml of Erlenmeyer flask and agitated at 30° C under dark conditions. Control was run along with experimental flask. Change in color was observed from pale yellow to brown in the silver nitrate solution incubated with *Lecanicillium lecanii*.

The synthesized silver nanoparticles were analyzed periodically using UV-Vis spectrophotometer. The absorbance of the nanoparticles was measured in the range 400-800nm, which includes the plasmon absorbance peak of the silver nanoparticles centered at 430 nm. Further the samples were characterized by SEM. It was observed that the nanoparticle solution was extremely stable for more than six months and no signs of aggregation even at the end of this period.

Evaluation of ecotoxicity impacts

Collection of seeds and nanoparticle treatment

The seeds of *Vigna unguiculata*, *Vigna mungo*, *Vigna radiata*, *Sorghum vulgare* and *Macrotyloma uniflorum* were obtained from Agriculture department, PAJANCOA, Karaikal in sterile polythene bags and all seeds were cleaned thrice in sterile distilled water and immersed in the synthesized nanoparticle suspension for 1 hr. After the treatment, the treated seeds were allowed to shade dry.

Pot assay

The phytotoxicity of NPs was evaluated by the seed germination technique. The germination index has been extensively used as an indicator of phytotoxicity in soils (Tiquia and Tam, 1998). The pots of 14 cm diameter and 12 cm in height were filled with the fertile loam soil upto ¾ th height of the pot. The treated seeds were seeded in the respective pots. Control and replications were maintained in each treatment. Water was sprinkled on the pots regularly. The seedling emergence was observed periodically and the shoot length and leaf surface area were noted after periodic time intervals.

Chlorophyll estimation

The amount of chlorophyll present in the leaves was estimated at 30 th day of treatment followed by Weber (1986) method.

Evaluation of total microbial population on phyllosphere microflora

About 1gm of leaves from respective treated plants were collected on 30th day after nanoparticle treatment, and kept in sterile polythene bags, brought to the laboratory for microbial analysis. Using sterile blade, the respective leaves were cut into 1 cm^2 (100 pieces) with sterile blade. The cut pieces were transferred to 99 ml of sterile distilled water, kept under shaking condition for 1 hr. Serial dilution was made from the suspension. 1 ml of respective aliquot was poured into sterile petriplate. 20 ml of sterile molten nutrient agar, starch casein agar, sabouraud dextrose agar was poured, allowed to solidify.The plates were incubated at respective temperatures (Bacteria 24 hr at 37 °C, Fungi 28 °C for 3 to 4 days, Actinomycetes 37 ° C for 7 days). After the incubation period, the colony count was recorded.

Effect of nanoparticles on soil N,P,K level

After the 15th and 30th day of nanoparticle treatment, about 500g of the soil sample in respective pots was analyzed for NPK level.. The soil analysis was performed in Chennai Mettex Lab Private limited, Chennai.

RESULT AND DISCUSSION

The synthesis of nano particle was confirmed initially by colour change of reaction mixture from pale yellow to brown. This could be observed after the 7th day of incubation (Fig. 1). The change to colour brown suggests silver nanoparticle formation. Moreover, the reaction mixture was remaining as

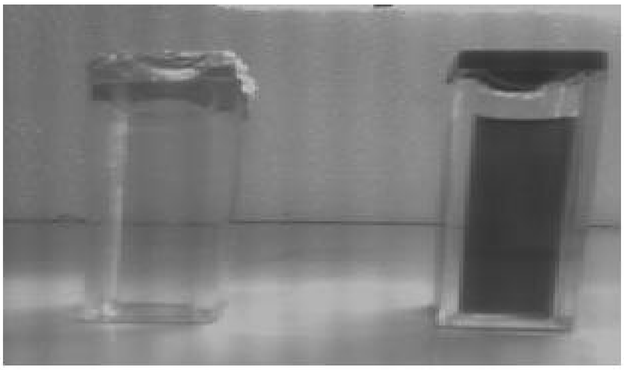

Figure 1. Synthesized silver nanoparticles in the reaction mixture

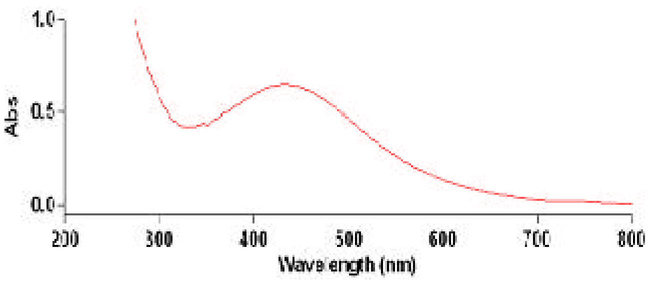

Figure 2. UV absorption spectra of silver nanoparticles

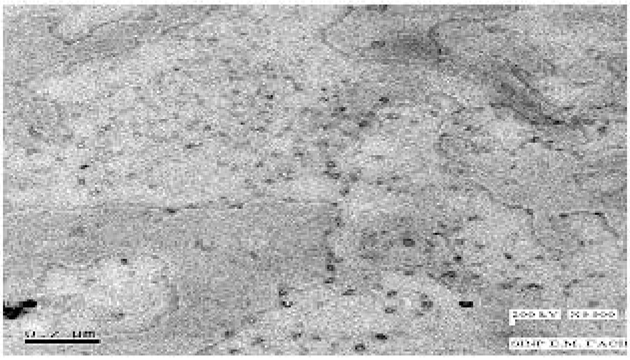

Figure 3. TEM *view of* Ag-based microbial *nanoparticles*

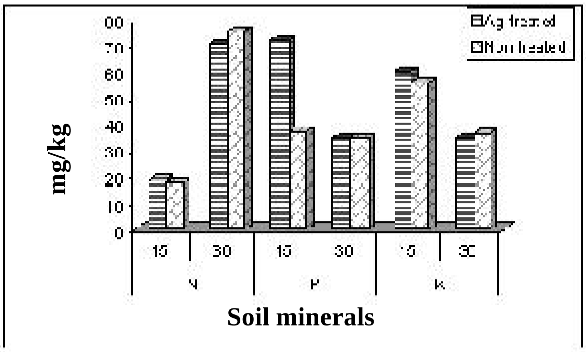

Figure 4. Microbes-based Ag nanoparticles treatment on the soil minerals (N, P, K) after 15 and 30 days of application

brown in colour for months. No aggregation and turbidity was observed in the entire synthesized nanoparticle which reveals the stability of nanoparticle. After the confirmation of silver nanoparticle by the colour change from pale yellow to brown. The nanoparticle was further characterized by UV-vis spectroscopy and TEM. A strong silver plasmon absorption maximum was recorded at 410-420 nm in UV-Vis spectroscopy (Fig. 2). Further characterization was carried out using TEM which reveals spherical silver nanoparticle with the size range of 1.6nm (Fig. 3)

Ecotoxicological effects

Two different measurements were performed in this test (root elongation and germination per cent) using five different seeds (horse gram, sorghum, green gram, black gram and cowpea). Table 1 summarizes the results obtained for the cucumber seeds germination test. After 25 days of germination, cowpea, green gram, and horse gram and black gram, sorghum, and shoot length was slightly lengthened and reduced respectively in microbes-based Ag nanoparticles treatment (Table 1). Both in control and Ag nanoparticles treatments, horse gram, sorghum, green gram, black gram and cowpea germinated with in 3, 5, 2, 4 and 2 days respectively. In the germination tests, in some cases a slight positive effect of Ag was observed, which can be due to a generally-favorable biological response to low exposures of toxins and other stressors (hormesis effect). Moreover, while the germination index was similar regardless of the NPs, their presence induced growth of larger roots. This might indicate that the seeds were slightly stressed by the environment, possibly resulting in harmful effects on long term exposition (Barrena *et al.*, 2009).

In general, the leaf area of the tested crops was greatly reduced while their seeds were treated with *L. lecanii*-based nanoparticles (Table 2). Among the tested plants,

Table 1. Shoot length (cm) of tested plants in relation to various tested time (days)

Treatment	Shoot length (cm)				
	Days after treatment				
	5	10	15	20	25
Cowpea (Treated)	4.4^{ab}	14.6^{ab}	22.4^{ab}	24.8^{ab}	27.5^{ab}
Cowpea (Control)	4.4^{ab}	14.5^{ab}	22.4^{ab}	24.3^{ab}	26.2^{ab}
Black gram (Treated)	2.5	7.9	11.3^{a}	13.2^{a}	15.7^{a}
Black gram (Control)	2.1	9.9±0.1	14.0^{a}	17.7^{a}	20.1^{a}
Green gram (Treated)	3.8	13.9±0.1	20.5	22.9	24.1^{a}
Green gram (Control)	3.3	12.7	19.5	21.4	22.8^{a}
Sorghum (Treated)	0.5^{ab}	2.2^{ab}	7.2^{ab}	8.3^{ab}	9.5^{ab}
Sorghum (Control)	0.6^{ab}	1.9^{ab}	5.2^{ab}	8.2^{ab}	9.7^{ab}
Horse gram (Treated)	2.1	7.3	11.5	14.1^{a}	17.0^{a}
Horse gram (Control)	2.4	7.5	11.2	12.5^{a}	14.5^{a}

^{ab} – non-significant at P >0.05 level by DMRT

^{a} - In column, the mean carries the same letter is statistically significant at P >0.05 level by DMRT

Table 2. Leaf surface area (cm^2) of tested plants at respective time intervals (days)

Treatment	Leaf surface area (cm^2)				
	Days after treatment				
	5	10	15	20	25
Cowpea (Treated)	3.78	7.77	9.60	17.28	33.00
Cowpea (Control)	3.50	7.80	9.66	15.81	34.96
Black gram (Treated)	3.12	5.94	8.70	12.5	25.74[a]
Black gram (Control)	3.6	7.77	9.5	14.4	28.2[a]
Green gram (Treated)	2.52	4.93	5.78	8.61	14.79[a]
Green gram (Control)	2.4	4.16	6.24	8.7	11.5[a]
Sorghum (Treated)	4.7	8.4	15.29[a]	19.32[a]	26.76[a]
Sorghum (Control)	6.0	9.45	12.5[a]	22.92[a]	34.8[a]
Horse gram (Treated)	2.9	7.76	10.25[a]	14.92[a]	22.93[a]
Horse gram (Control)	3.6	8.4	13.7[a]	18.3[a]	28.2[a]

[a] - In column, the mean carries the same letter is statistically significant at P >0.05 level by DMRT

nanoparticles showed more impact in sorghum and horse gram than other plants. *Phyllosphere microbial population like b*acterial, fungal and actinomycetes reduced in horse gram, sorghum, green gram, black gram and cowpea (except bacteria) in (Table 3) treated with *L. lecanii*-based nanoparticles.

Ag nanoparticles treatment significantly ($P < 0.05$) reduced the chlorophyll-B content in horse gram, sorghum and cowpea (Table 4). An opposite trend recorded for green gram. Similarly, total chlorophyll was reduced in green gram. Chlorophyll-A content was significantly ($P < 0.05$) increased by Ag-nanoparticles in green gram and sorghum. Though, in general the carotene content has not been changed, its content significantly ($P < 0.05$) reduced and increased in sorghum and green gram (Table 4). From figure 4, it is very clear that in control plot, the mineral, N contents increased from 15 to 30 days. However, other two minerals like P and K level decreased drastically during the same observation period. Similar results were observed in the Ag application plots. Moreover, Ag application reduced soil mineral level no bar with observation periods. Compared with literature data, while photoactive ZnO or TiO2 (Warheit *et al.,* 2007), bactericide Ag (Shrivastava *et al.,* 2007), hydrophobic Carbon nanotubes (Smith *et al.,* 2007) and fullerenes (Oberdorster, 2004), or Cadmium oxide particles (Braydich-Stolle *et al.,* 2005), show environmental toxicity, it appears that Au and Iron oxide NPs are significantly less toxic (Barrena *et al*., 2009).

The present study clearly reveals non toxic or poor phyto and ecotoxic effect of silver nanoparticles on seedlings

Table 3. Effect of total heterotrophic microbial population on phyllosphere microflora

Treatment	CFU / cm^2)		
	Bacteria	Fungi	Actinomycetes
Cowpea (Treated)	173×10^5	50×10^5	56×10^5
Cowpea (Control)	172×10^5	52×10^5	57×10^5
Black gram (Treated)	197×10^5	49×10^5	39×10^5
Black gram (Control)	199×10^5	50×10^5	40×10^5
Green gram (Treated)	161×10^5	53×10^5	49×10^5
Green gram (Control)	163×10^5	55×10^5	50×10^5
Sorghum (Treated)	166×10^5	43×10^5	68×10^5
Sorghum (Control)	167×10^5	45×10^5	70×10^5
Horse gram (Treated)	169×10^5	111×10^5	52×10^5
Horse gram (Control)	170×10^5	113×10^5	53×10^5

Table 4. Effect of microbes-based Ag nanoparticles treatment on chlorophyll content (g/l) of tested plants

Treatment	Chlorophyll Content (g/l) after 30th day			
	Total Chlorophyll	Chlorophyll A	Chlorophyll B	Carotene
Cowpea (Treated)	0.0945	0.0335	0.0603	0.4194
Cowpea (Control)	0.0945	0.0335	0.0705	0.4194
Black gram (Treated)	0.0945	0.0335	0.0603	0.4204
Black gram (Control)	0.0945	0.0335	0.0603	0.4204
Green gram (Treated)	0.0381	0.0410	0.0111	2.1974
Green gram (Control)	0.0412	0.0406	0.0143	2.0992
Sorghum (Treated)	0.0377	0.0411	0.0107	2.2102
Sorghum (Control)	0.0118	0.0113	0.0042	2.6898
Horse gram (Treated)	0.0945	0.0335	0.0706	0.4194
Horse gram (Control)	0.0945	0.0335	0.0603	0.4194

emergence,plant growth parameters, phyllosphere microflora and soil macronutrients. Further studies will be helpful to assess toxicity of released nanoparticles on plant growth and other components of ecosystem.

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