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Effect of seed treatment and spray with biosilver nano particles and bio control agents on defence related enzymes activity in rice seedlings and plant growth parameters *in vitro*

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Abstract
In the present study Silver nano particles were synthesized using two potential isolates of *Trichoderma*, *Psudomonas fluorescens* and one isolate of pathogen *Rhizoctonia solani* cell free culture filtrates incubated at different days 0, 5, 10 and 15. These synthesized nano particles were characterized by using Zeta potential, Dynamic light scattering (DLS) and Scanning electron microscopy. Four different defence related enzymes, *viz*., Peroxidise, Poly Phenol Oxidase (PPO), Phenyl Ammonia Lyase (PAL), Tyrosinase were assessed from rice seedlings obtained from two nano treatments, two biocontrol agents and carbendazim applied either as seed treatment or spray. Experiment was conducted in pot culture. Observations were recorded on tenth and fifteenth day for seed treatment and after 24 h and 48 h for spray treatments. Enzymes analysis was done for leaves and roots separately. When the rice seedlings, from either 10% bionano treated seeds or from foliar sprayed plants, were assessed for the accumulation of plant defense related enzymes *viz*., peroxidases, polyphenol oxidase, phenyl alanine ammonia lyase and tyrosinase, substantial accumulation was observed as evidenced by the absorbance values indicating induction of resistance mechanism in rice seedlings. The present investigation also revealed increased germination of rice seeds, shoot length, root length, S:R ratio, fresh weight, dry weight and vigour index of rice seedlings These nano preparations alone could increase the growth parameters either on par with respective biocontrol agents or sometimes even better when the rice seeds of cv. NLR 34449 were treated with nano preparations and sown in pathogen uninoculated pots indicating their utility in increasing plant growth in place of even biocontrol agents and fungicide seed treatment.

Keywords: Peroxidise, Carbendazim, polyphenol oxidase, Biocontrol agents and fresh weight

Introduction
Rice (*Oryza sativa* L.) is the monocotelidinary plant, belongs to the family graminae. It is the most basic important staple food for a large area of the world's human population. India is the world's second largest producer of rice after China. Rice is an important food crop and also it earns more money for the country, so the production rate of rice needs to maintain constant. It is an important cereal crop affected by various fungal, bacterial and viral diseases. Use of fungicides to control the disease leads to several adverse effects i.e. resistance development in the pathogen, residual toxicity, environmental pollution, higher cost etc. Therefore, it has needed to adopt ecofriendly approaches for better health of crop and for yield. The practical use of biocontrol agents and biosilver nano particles as control agents is receiving increased attention and this is partly due to their non-toxicity to humans, their systemicity and biodegradability. Furthermore, bio control agents and biosilver nano particles are less-phytotoxic and ecofriendly. Investigations on mechanisms of disease suppression by biocontrol agents have suggested that the active principles present in them may either act on the pathogen directly, or induce systemic resistance in host plants resulting in reduction of disease development (Fotoohiyan *et al*. 2015; Ahmed *et al*. 2000) [12, 1]. Induction of plant's defense genes by application of inducing agents is called induced resistance (Hammerschmidt and Ku´c 1995) [15]. When plants are treated with non-pathogenic or some less harmful pathogens or biocontrol agents [eg. rhizobacteria-induced systemic resistance (RISR)], it then go onto triggers the production of defense-related gene products (Harman *et al*. 2004) [17]. The defense gene products include peroxidise (PO), polyphenol oxidase (PPO) which catalyze the

formation of lignin and phenylalanine ammonia-lyase (PAL) that is involved in phenolics and phytoalexins synthesis (Ramamoorthy *et al*. 2002) [28]. A wide variety of plant tyrosinase behaviors have been reviewed and described. (Mayer and Harel 1991; Van Gelder *et al*. 1997) [23, 33]. A molecular oxygen scavenging function in the chloroplast (Vaughn *et al*., 1988) [34] and a role in plant defense (Constable *et al*. 1995) [7] have been suggested. Its implication in the secondary metabolism of betalains has been proposed (Steiner *et al*. 1999) [32] but the physiological function of tyrosinase in higher plants is yet to be fully determined. Environmental stimuli such as UV rays, pathogen attack, and gravity can induce very rapid changes in hydrogen peroxide (H_2O_2) levels, leading to a variety of physiological responses in plants. Catalase, which degrade H_2O_2 into water and oxygen, is the major H_2O_2- scavenging enzyme in all aerobic organisms (Yang *et al*. 2002) [37]. So, these are the enzymes whose activity needs to be increased to prevent fungal and bacterial diseases and this can be done by seed treatment with various plant extracts, chemicals, biocontrol agents and nanoparticles. The objective of the present study is to observe the potential for induction of systemic resistance in rice by biosilvernano particles and biocontrol agents.

Engineered nanoparticles have three different unique characteristics such as size, structure and properties. These nanoparticles received a particular attention for their positive impact in improving many sectors of economy, including consumer products, pharmaceutics, cosmetics, transportation, energy and agriculture etc., and are being increasingly produced for a wide range of applications within industry (Novack and Bucheli 2007) [26]. The majority of the reported studies point to the positive impacts of nanoparticles on plant growth with a few isolated studies pertaining to negative effect. Numerous studies have demonstrated that TiO_2 nanoparticles promoted photosynthesis and N metabolism and thus greatly improved growth of spinach at low concentration 20 mg/l (Yang *et al*. 2006) [36]. (Lin and Xing 2008) [21] investigated phytotoxicology of nanoparticles (multi-walled carbon nanotube, aluminum, alumina, zinc and zinc oxide) on seed germination and root growth of six higher plant species such as radish, rape, rye grass, lettuce, corn and cucumber. Seed germination was not affected except for the inhibition of nanoscale zinc (nano-Zn) on rye grass and zinc oxide (nano-ZnO) on corn at 2000 mg/l. Inhibition on root growth varied greatly among nanoparticles and plants. (Mahajan *et al*. 2011) [22] studied the effect of nano-ZnO particles on the growth of plant seedlings of mung (*Vigna radiata*) and gram (*Cicer arietinum*). The results revealed that at certain optimum concentration, the seedlings displayed good growth over control and beyond that retardation in growth was observed. Similar results were also reported with the application of nano scale-iron oxide on soybean yield and quality. The results showed that nano-iron oxide at the concentration of 0.75 g/l was increased leaf + pod dry weight and pod dry weight. The highest grain yield was observed with using 0.5 g/l nano-iron oxide that showed 48% increase in grain yield in comparison with control (Sheykhbaglou *et al*. 2010) [31]. Silver nanoparticles (AgNPs) are currently one of the most widely commercially used nanomaterials (Chen and Schluesener 2008) [4]. AgNPs toxicity has been reported in bacteria (Choi *et al*. 2008) [6]. Meyer *et al*. (2010) [25] studied the intracellular uptake and associated toxicity of three silver nanoparticles with different sizes in *Caenorhabdities elegans*. They observed growth inhibition by all AgNPs at concentrations in the low mg/l levels. (Seif *et al*. 2011) [30] studied the effect of nanosilver and silver nitrate on abscission and yield of seed in borage. They showed that increasing the concentration of silver nitrate from 100 to 300 ppm caused a decrease in seed yield. On contrast, increase in the concentration of nanosilver from 20 to 60 ppm has led to an improvement in the seed yield and the lowest amount of seed yield was found with control plants. (Harajyoti and Ahmed 2011) [16] studied Phytotoxicity of *Oryza sativa* by directly exposing it to silver nanoparticles solutions and Transmission Electron Microscope (TEM) revealed that various particle sizes deposited inside the root cells. It was found that during penetrations of particles inside the root cells, they damaged the cell wall and vacuoles to enter. It may be due to the penetrations of large particles entering through small pores of cell walls.

Material and Methods

Synthesis of biosilver nanao particles

Two potential isolates of *Trichoderma* such as ET-1 and RT-4, Two potential isolates of *P. fluorescens* such as PF-2 and PF-5, *R. solani* used for the synthesis of silver nano particles. Seven day old pure culture of selected isolates of *Trichoderma* spp, *P. fluorescens* and one isolate of *R. solani* were individually inoculated in 250 ml conical flasks containing 100 ml of Potato Dextrose Broth (PDB) for fungal cultures and Nutrient broth for bacterial cultures. The culture flasks were incubated at 28 ± 1 °C. The culture filtrate obtained at 5 DAI, 10 DAI, and 15 DAI through 2 layers of sterilized Whattman No-1 filter paper and used for the synthesis of silver nanoparticles.

90 ml of aqueous solution of 1mM Silver nitrate ($AgNO_3$) (equivalent to 170 ppm) was mixed with 10 ml of either *Trichoderma* spp, *R. solani*,or *P. fluorescens* culture filtrates for the extra cellular synthesis of silver nanoparticles in a 250 ml conical flask. The whole mixture was incubated at room temperature for 24 hrs. The color change of silver nitrate from colorless to brown color was considered as indicator of formation of silver nanoparticles through reduction of silver ions from Ag^+ to Ag^0.

UV-Visible Spectroscopy

The spectra of the surface Plasma resonance of AgNPs in the reaction mixture were recorded using UV–Vis spectrophotometer (Shimodzu, UV-2450) at wavelengths between 200 to 800 nm.

Characterization of silver nano particles by Scanning Electron Microscope:

The bulk powdered sample was fixed on to aluminium stub using double sticky carbon tape. An accelerating voltage of 15 kv was used, and the instrument was operated in variable pressure mode. The analysis area was 700 x 300 micrometers. Elements were quantified using emperically ZAF factors.

The EDS data are quantified in SEM on several single particles with a focused beam (Elzey *et al*. 2011) [11].

SEM images further confirmed development of nano structures from Ag-nano particles synthesized by the *Gelidiella acerosa*.

SEM images showed silver nano particles shape and distribution in solution (Meichias *et al*. 2012) [24].

Particle Size and Zeta Potential Analysis

The aqueous suspension of the synthesized silver nanoparticles was filtered through a 0.22 μm syringe driven filter unit and the size of the distributed silver nanoparticles

was measured by using the principle of Dynamic Light Scattering (DLS) technique made in a Nanopartica SZ-100 series compact scattering spectrometer.

Assay of ISR enzymes

Rice seeds of cv. NLR 34449 were soaked in 10 ml of biosilver nano solution (100%), Spore suspension (10^5spores/ml) of *Trichoderma*, *P. fluorescens* cell suspension (10^7cells/ml) and Carbendazim 0.1% for 10 minutes separately. Treated seeds were sown in pots under green house conditions and grown for 15 days. Untreated plants were taken as control.

In the same manner the untreated seeds were sown in the pots separately and after 15 days, 10 ml of nano solution (100%), Spore suspension (10^5spores/ml) of *Trichoderma*, *P. fluorescens* cell suspension (10^7cells/ml) and carbendazim 0.1% were individually sprayed at 24 hours or 48 hours time interval. The leaf and root samples from seed treated or sprayed plants were taken for analysis of defense related enzymes. Observations were also collected on root length, shoot length, fresh weight, dry weight, germination percentage and vigour index.

a) Assay of Peroxidase

Analysis of Peroxidase activity was carried at the wavelength of 420 nm, the "TEST solotion" was prepared by mixing 0.03 ml of enzyme solution with 0.11 ml of 100 mM Potassium Phosphate Buffer, pH 6.0 (at 20 ºC), 0.11 ml of 5% (w/v) Pyrogallol Solution, 0.05 ml of 0.50% (w/w) Hydrogen Peroxide Solution (H_2O_2) and 0.70 ml water. The "BLANK" was prepared by same composition only 0.03 ml of mM Potassium Phosphate Buffer of pH 6.0 was added instead of enzyme solution (Chance and Maehly 1955) [3].

b) Assay of Polyphenol oxidase (PPO)

Analysis of PPO activity was done by according to Duckworth and Coleman 1970 at wave length 420 nm at 25 ºC. The "TEST" solution was prepared by mixing 0.03 ml of enzyme solution with 0.97 ml of 20 mM Catechol solution (which was prepared in 50 mM Potassium Phosphate Buffer, pH 6.8 at 25 °C). The "BLANK" was prepared with same amount of catechol and 0.03 ml of 50 mM Potassium Phosphate Buffer, pH 6.8. without enzyme solution.

c) Assay of Phenylalanine Ammonia Lyase (PAL)

PAL activity was measured by, 0.03 ml of enzyme solution incubated with 0.67 ml of 3 mM L-Phenylalanine Solution (prepared in 150 mM Tris HCl Buffer, pH 8.5 at 30 °C) and 0.30 ml of water and absorbance was measured at 270nm wavelength. This reaction mixture was treated as "TEST" and another one was prepared which was called "BLANK" with same composition only 0.03 ml 150 mM Tris HCl Buffer, pH 8.5 was used instead of enzyme solution (Hodgins 1971).

d) Assay of Tyrosinase

Tyrisonase activity was measured at 280 nm and the reaction cocktail containing 10 ml 1 mM L-Tyrosine Solution, 10 ml 50 mM Potassium Phosphate Buffer, pH 6.5 (at 25 ºC) and 9 ml water was first prepared. Then the reaction cocktail was oxygenated by bubbling 99.9% O_2 through it for 3-5 min. before using it. Then "TEST" was prepared mixing 0.03 ml of enzyme solution and 0.97 ml of reaction cocktail and "BLANK" was prepared by using same amount of reaction cocktail and 0.03 ml of 50 mM Potassium Phosphate Buffer, pH 6.5 (Wong *et al*. 1971).

Results and Discussion

Synthesis of bio nano particles

In the present investigation, two potential antagonistic isolates of *P. fluorescens, i. e.,* PF-2 and PF-5, and two potential antagonistic isolates of *Trichoderma, i. e.,* ET-1 and RT-4 were evaluated for their ability to convert silver in to nano silver.

The bio silver nano conversion was attempted using Potato Dextrose Broth (PDB) culture filtrates of two isolates of *Trichoderma* (ET-1 and RT-4), Nutrient Broth (NB) culture filtrates of two isolates of *P. fluorescens* (PF-2 and PF-5), only mycelial mat and spore suspension (10^{-8} spores/ml) of ET-1 isolate. Bionano conversion was also attempted using uninoculated autoclaved PDB and NB for comparison. Conversion of silver nitrate into silver nano particles was also assessed using culture filtrate of rice sheath blight pathogen *R. solani*.

When 10 ml of cell free culture filtrates of different ages such as 5, 10, 15 and pure nutrient broth, pure potato dextrose were mixed with 90 ml of silver nitrate after 24 hours incubation brown color development indicates the silver nano formation. Mycelia mat, spore suspension of *Trichoderma* also used for the synthesis of silver nano particles. They also synthesized the silver nano particles.

Characterization of silver nano particles by UV-VIS Spectrophotometry:

Three types of absorbance peaks were obtained when synthesized bio silver nano particles tested using UV-VIS spectrophotometer, *i.e.,* one at 220 nm indicating pure silver nitrate, second at 260 nm indicating partial conversion and third at 400-450 nm indicating nano particles presence. The silver nano conversion was confirmed using UV-Vis spectrophotometer when one of the three absorbance peaks, *i. e.*, between 400 to 450nm corresponded to bionano silver (Fig 1).

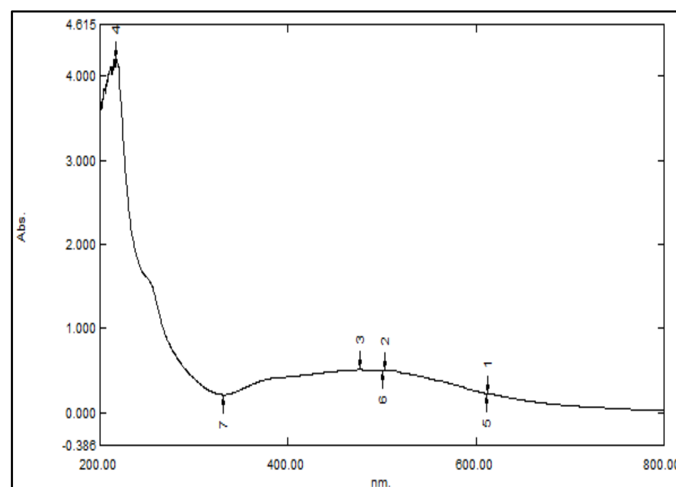


Fig 1: UV-VIS Spectrum of Biosilver nano particles (Silver nano particles prepared from 5days old culture filtrate of ET-1 isolate of *Trichoderma)*

Scanning electron microscopy of bionano silver particles

Scanning Electron Microscopy (SEM) of the synthesized bionano silver particles indicated that the particle size was least with ET-1 nano followed by PF-2 nano and maximum with *R. solani* nano (Plate 1a to c). Analysis of size of nano silver particles using SEM was done earlier by (Elzey *et al*. 2011; Melchias *et al*. 2012) [11, 24].

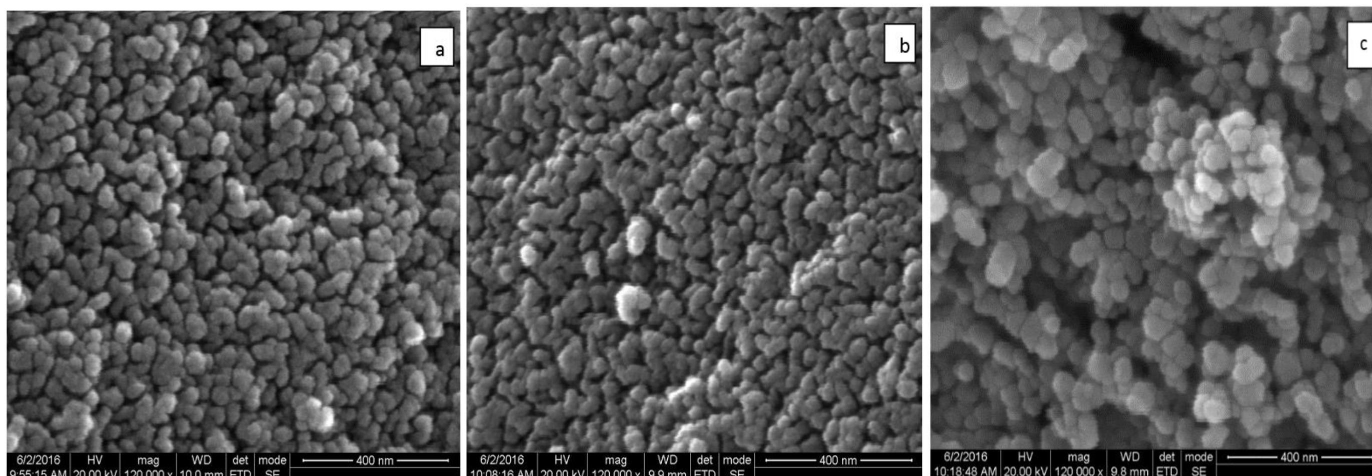
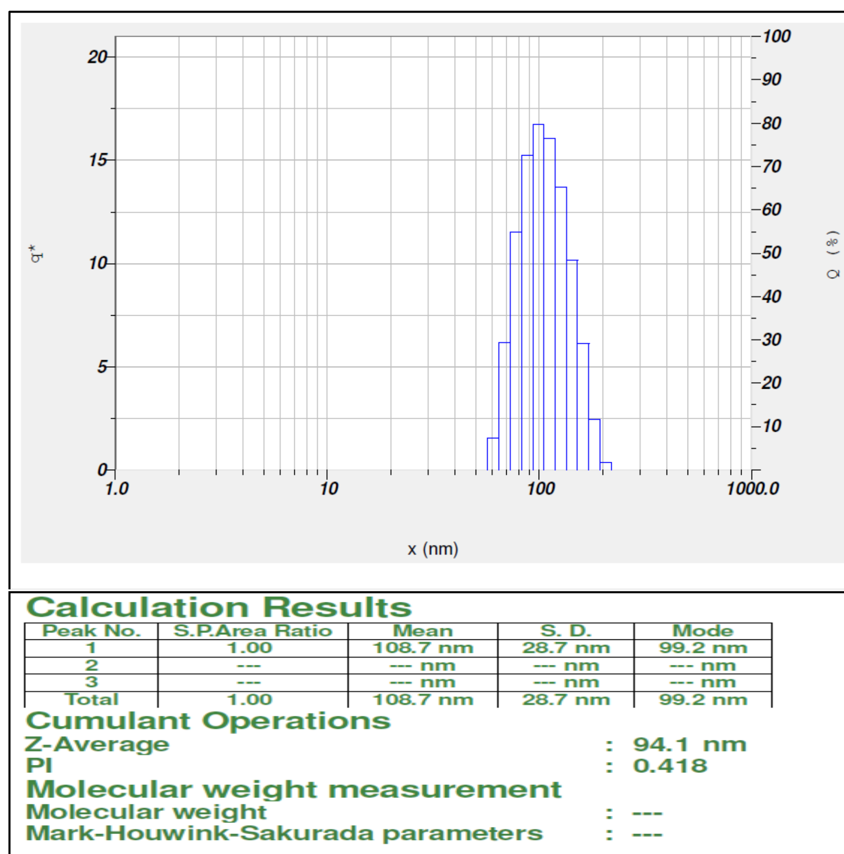


Plate 1: a to c. Scanning electron microscopy images of ET-1, PF-2, *R. solani* biosilver nano particles synthesized from 5 days old culture filtrate

It is also clear that the particles are of monodispersed nature in ET-1 nano and highly polydispersed in *R. solani* nano.

Measurement of size of silver nano particles by dynamic light scattering

Dynamic light scattering (DLS) technique measures the size of nano particles by scattering light intensity. Below 100 nm particles are considered as nano particles. DLA technique is used to determine the size distribution profile of small particles such as nanoparticles in suspension or polymers in solution (Berne and Picora, 2000) [2]. If the system is monodisperse, there should only be one population where as a polydisperse system would show multiple particle population. Bionano particles synthesized in the present investigation using five day old culture filtrates of *Trichoderma* isolate ET-1, *P. fluorescens* isolate PF-2 and *R. solani* were analysed using DLS technique for particle size measurement (Fig 2).



Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	108.7 nm	28.7 nm	99.2 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	108.7 nm	28.7 nm	99.2 nm

Cumulant Operations

Z-Average : 94.1 nm

PI : 0.418

Molecular weight measurement

Molecular weight : ---

Mark-Houwink-Sakurada parameters : ---

Fig 2: Dynamic light scattering particle size of PF-2 N-5

In case of silver bionano prepared from PF-2 and *R. solani* culture filtrates, particle size ranged from 80 to 137.4nm (98.2±28.7nm) with PF-2 nano and 98.8 to 223.7nm (160.9±62.1nm) with *R. solani* nano. This indicated that PF-2 nano and *R. solani* nano were more of polydisperse in nature. In case of silver nano prepared from ET-1 culture filtrate, the particle size ranged from 6.3 to 6.9nm (6.7±0.3nm) indicating monodisperse nature of ET-1 nano preparation. Further, as per the definition of nano, only ET-1 nano preparation stands fit within the nanoparticle group as the particle size was below 100nm while other two were just at or above nano size.

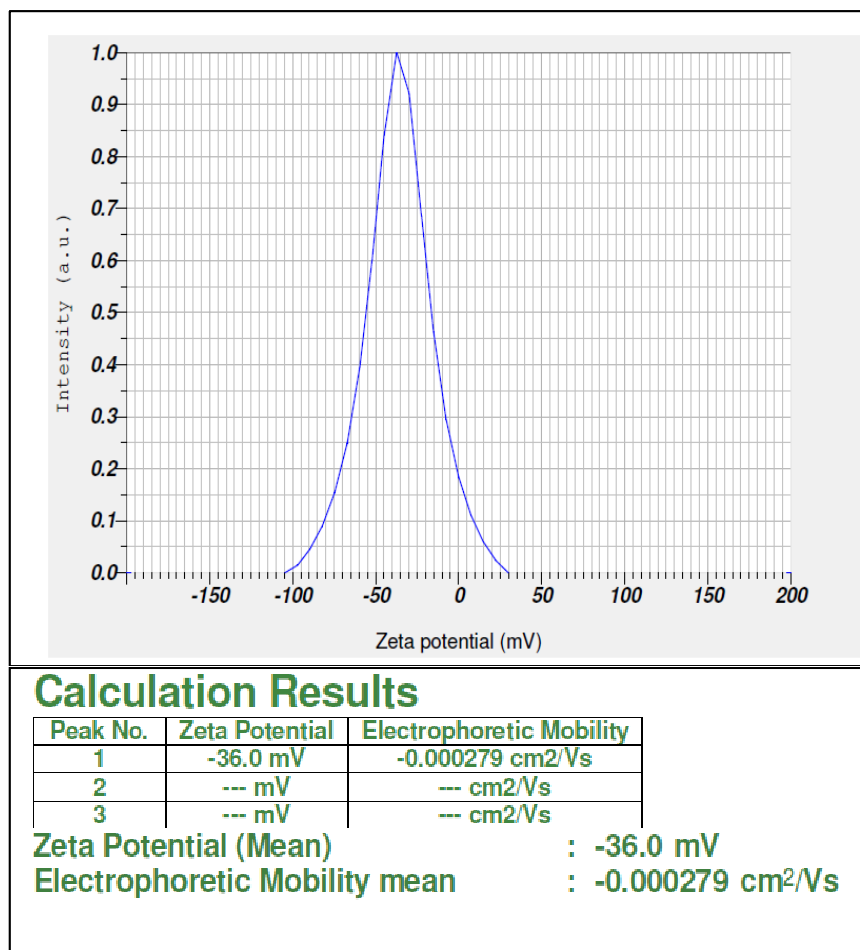
(Dirk *et al*. 2011; Roy *et al*. 2013) [9, 29] measured the size of synthesized silver bionano particles by DLS.

Zeta potential of silver nano particles

Zeta (Z) potential is the electro kinetic potential (mV) in colloidal dispersions. It is a key indicator of the stability of colloidal dispersions. The magnitude of zeta potential indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, *i.e*., the solution or dispersion will resist aggregation. When the particle is small, attractive forces may exceeded the repulsion and dispersion may break and flocculate colloids with high zeta potential (+ve or –ve) are electrically stabilized while colloids with low zeta potential tend to coagulate or flocculate.

The stability behaviour of particles as assessed by zeta potential is of the order i) from 0 to ± 10 – rapid coagulation or flocculation, ii) from ±10 to ±30 – incipient instability, iii) from ±30 to ±40 – moderate stability, iv) from ±40 to ±60 – good stability and v) greater than ±60 – excellent stability (Greenwood and Kendall 1999) [13].

In the present study, *P. fluorescens* PF-2 nano particles prepared from 5 day old 3culture filtrate had the zeta potential -34.4 mV, *i.e*., with moderate stability, Rs nano particles prepared from 5 day old culture filtrate had the zeta potential -36.0 mV which are also moderately stable (Fig. 3) and the *Trichoderma* ET-1 nano particles from 5 day old culture filtrate had the zeta potential -0.3mV, *i.e*., rapid coagulation and flocculation.



Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-36.0 mV	-0.000279 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : -36.0 mV

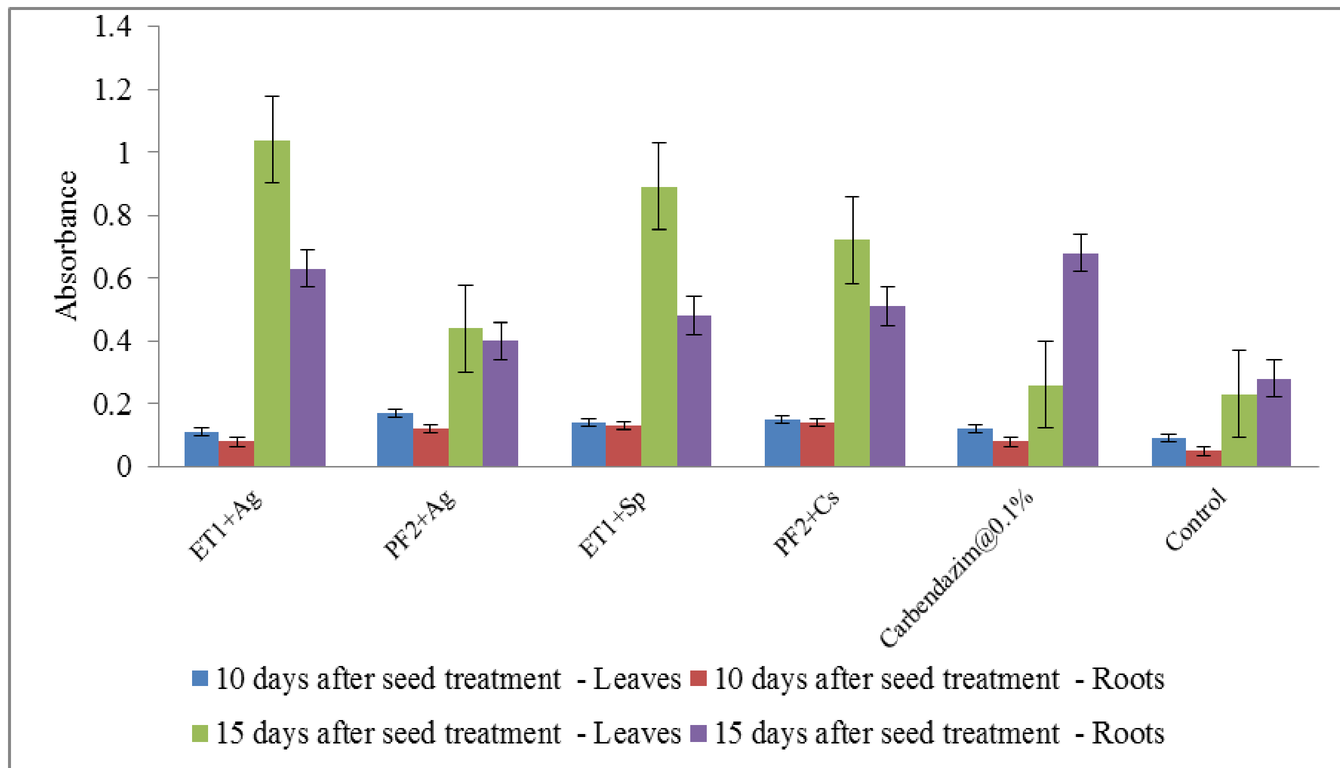
Electrophoretic Mobility mean : -0.000279 cm^2/Vs

Fig 3: Zeta potential of *R. solani* N-5d

Assay of ISR enzymes

In the present investigation four different defence related enzymes, *viz*., Peroxidise, Poly Phenol Oxidase (PPO), Phenyl Ammonia Lyase (PAL), Tyrosinase were analysed from rice seedlings obtained from two nano treatments, two biocontrol agents and carbendazim applied either as seed treatment or spray. Experiment was conducted in pot culture. Observations were recorded on tenth and fifteenth day for seed treatment and after 24 h and 48 h for spray treatments. Enzymes analysis was done for leaves and roots separately. The data was presented in Fig 4a and 4b.

Peroxidases were found to be higher in all the treatments compared to untreated control when absorbance values for peroxidises were compared (Fig. 4a and b). In general, seed treatment effect on peroxidise accumulation was found higher on fifteenth day compared to the absorbance values on tenth day. Further, peroxidase accumulation in seed treated treatments was more or less same in leaves and roots. In spray application treatments also peroxidise accumulation was more 48 hours after spray compared to 24 hours after spray. However, no specific trend was observed in relation to leaf or root as evidence by higher peroxidise accumulation in roots with some of the treatments while in others more in leaves. The present investigation thus revealed accumulation of peroxidases with the application of either nano or bio control agents. (Deborah 2005) [8] reported that in transgenic plants over expressing prx8 gene (pathogen related cell wall peroxidase gene) showed increased tolerance to abiotic stresses such as heat, cold, high salinity and metal ion toxicity.



Note: ET-1+Ag indicating biosilver nanoparticles prepared from ET-1 isolate of *Trichoderma* spp (5 days old culture filtrate).
PF-2+Ag indicating biosilver nanoparticles prepared from PF-2 isolate of *Pseudomonas fluorescence* (5 days old culture filtrate).
ET-1+Sp indicating ET-1 isolate spore suspension
PF-2+Cs indicating cell suspensiuon of PF-2 isolate of *Pseudomonas fluorescence*

Fig 4a: Effect of seed treatment with silver nano particles on Peroxidase activity in rice seedlings

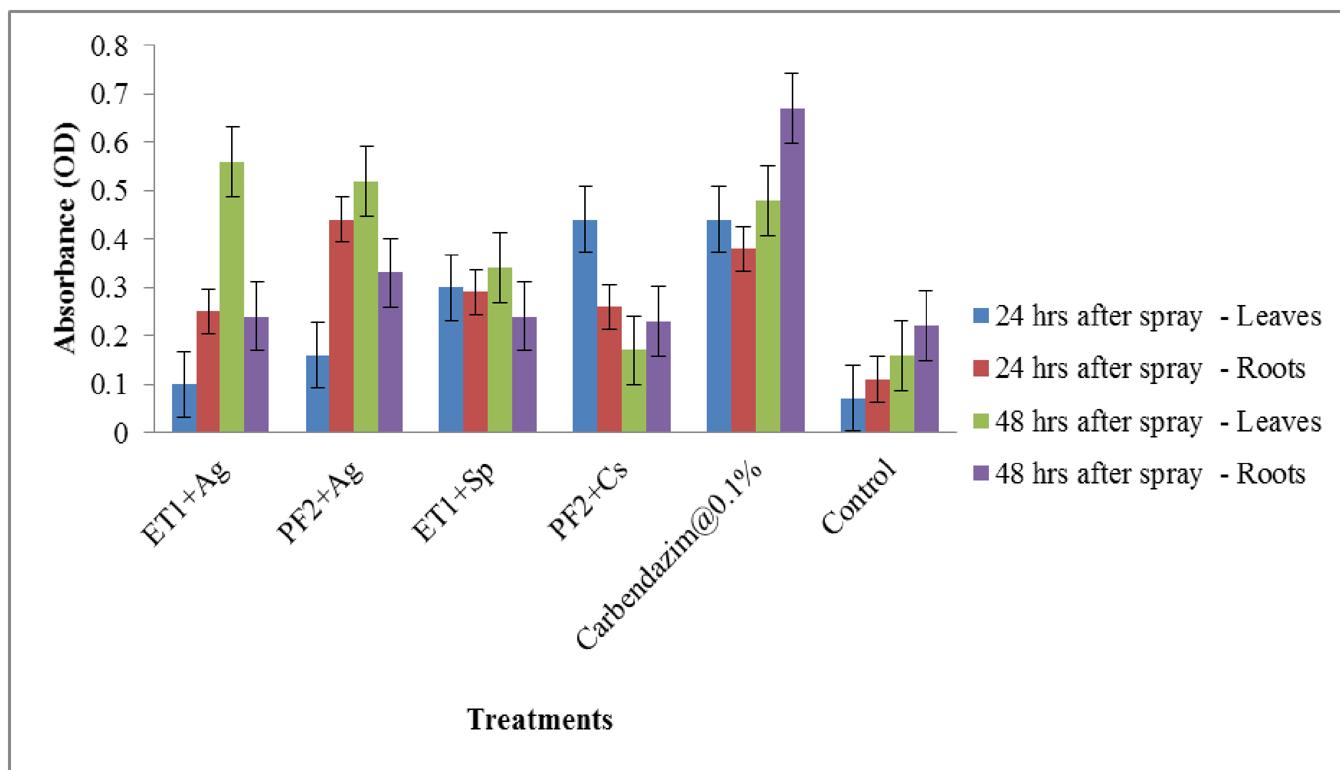


Fig 4b: Effect of spray with silver nano particles on Peroxidase activity in rice seedlings in pot culture

When the accumulation of PPO was observed, seed treatment with nano or bio control agents resulted in more or less equal absorbance values on tenth day in leaf and roots. However, the PPO accumulation was higher compared to untreated control. On fifteenth day after sowing, PPO accumulation was found to be higher in leaves than in roots. Further, nano preparations showed substantially higher accumulation of PPO in leaves and roots compared to bio control agents. Spray accumulation showed higher accumulation of PPO in leaves than that in roots. Twenty four hours after spray

accumulation of PPO was higher than that at 24 h. Further the accumulation in leaves and roots was more or less equal except in carbendazim treatment, where in, roots accumulated lesser amount of PPO's. Similar to observations at 24 hours, 48 hours after spray treatment also resulted in higher accumulation in seedlings raised from nano or bio control agent treatments compared to control. Studies describing correlations of high PPO levels in cultivars or lines with high pathogen resistance continued to provide support for a pathogen defence role of PPO (Raj *et al.* 2006) [27]. Correlations of increased PPO levels associated with rhizosphere bacteria and disease resistance were also reported (Chen *et al.* 2000 and Ramamurthy *et al.* 2002) [5, 28]

Accumulation of PAL also showed similar increase in treatments compared to control. PAL activity with seed treatment was more in leaves than that in roots and the trend was similar both on tenth day and on fifteenth day after sowing. However, the absorbance values of PAL were higher on fifteenth day compared to that on tenth day. Spray treatment also resulted in increased PAL activity but the activity decreased after twenty four hours. The activity of PAL is induced in response to various stimuli such as tissue wounding, pathogen attack, light, low temperature and hormones (Hahlbrock and Grisebach 1979) [14].

The tyrosinase activity was more in seedlings treated with bio nano, bio control agents, or carbendazim compared to seedlings from untreated seeds. Further, the accumulation was more in leaves than that in roots. The activity of tyrosinase with spray treatment decreased after twenty four hours. However, with seed treatment the activity was increased after ten days up to fifteen days. Tyrosinase is a member of PPO family that has both monophenolase activity and diphenolase activity.

Effect of bio silver nano particles on growth parameters of rice seedlings in pot culture

Effect of selected silver nano preparations from ET-1 and PF-2 culture filtrates (from 5day old culture filtrate) was assessed on rice seedling growth using seed treatment method. Rice seeds of cv. NLR 34449 were dipped in individual 10% silver nano preparation for 10 min and sown in pots of 22.5cm dia. Observations were recorded on germination percentage, shoot length, root lengh, shoot: root ratio (S:R), fresh weight, dry weight and vigour index. Treatment with nano was compared with seed treatment with ET-1 spore suspension (10^8 spores/ml), PF-2 cell suspension (10^8 CFU/ml), commonly used rice seed dressing fungicide carbendazim @ 0.1% and untreated control. The data was presented in Table 1. Germination per cent was maximum in ET-1 nano treated seeds (100%) followed by PF-2 nano (95%), ET-1 spore suspension (95%) and carbendazim @ 0.1% (95%) which were superior to untreated control (80%). PF-2 cell suspension (90%) was on par with control (80%).

Table 1: Effect of silver bionano particles on growth parameters of rice seedlings in pot culture.

S. No.	Treatments	Germination (%)	Shoot Length (cm)	Root Length (cm)	S:R Ratio	Fresh Weight (g)	Dry Weight (g)	Vigour Index
1	ET1 N-5d	100^{a}	27.48^{a}	2.56^{ab}	10.70^{b}	1.20^{a}	0.50^{a}	3090^{a}
2	PF-2 N-5d	95^{ab}	24.30^{bc}	2.60^{a}	9.35^{d}	1.00^{b}	0.30^{c}	2786^{b}
3	ET1Spore suspension	95^{ab}	24.64^{bc}	2.09^{f}	11.79^{a}	1.20^{a}	0.40^{b}	3114^{a}
4	PF-2+Cell suspension	90^{abc}	25.50^{ab}	2.54^{abc}	10.02^{c}	0.9^{c}	0.30^{c}	2800^{b}
5	Carbendazim@0.1%	95^{abc}	18.70^{d}	2.48^{bcd}	7.53^{e}	0.50^{d}	0.11^{d}	2116^{c}
6	Control	80^{bc}	17.30^{d}	2.44^{cde}	7.09^{f}	0.40^{e}	0.10^{e}	1974^{c}
	C.D (P=0.01)	10.6	2.42	0.08	0.08	0.06	0.008	243
	SEm (±)	3.05	0.82	0.02	0.02	0.02	0.003	82.78
	C.V (%)	8.04	8.02	1.70	0.44	5.12	2.22	6.99

The shoot length of rice seedlings was significantly higher with ET-1 nano or PF-2 nano or spore/cell suspension compared to untreated control (17.3 cm) and carbendazim (18.7 cm). Maximum shoot length was obtained with ET-1 nano seed treatment (27.4 cm) followed by sed treatment with PF-2 cell suspension (25.5 cm) with insignificant difference between them. Further, PF-2 cell suspension was on par with seed treatment with ET-1 spore suspension (24.6 cm) and PF-2 nano preparation (24.3 cm).

Root length was significantly higher from seeds treated with PF-2 nano (2.6 cm). Least root length was observed in seed treatment with ET-1 spore suspension (2.09 cm), while all others including check were in between PF-2 nano and ET-1 spore suspension in affecting the root length.

In order to assess the impact of seed treatment over all the plant growth, shoot length (S): root length (R) (S:R ratio) was calculated. Result indicated that untreated seeds resulted in seedlings with lowest average S:R ratio of 7.09 which differed significantly with that of seedlings from different treatments. ET-1 spore suspension (11.79) resulted in significantly maximum S:R ratio followed by ET-1 nano seed treatment (10.7) and PF-2 cell suspension seed treatment (10.02). The present investigation indicated superior performance of the bio control agents, *i.e*. *Trichoderma* and P. *fluorescens* either as such as seed treatment or their nano preparations as seed treatment in increasing S:R ratio compared to even carbendazim seed treatment.

The fresh weight of the plant was significantly higher with ET-1 nano seed treatment (1.2 g) and ET-1 spore suspension seed treatment (1.2 g). Seed treatment with PF-2 nano and PF-2 cell suspension showed significantly higher fresh weight than with control or carbendazim but significantly lower compared to seed treatment with ET-1 nano or ET-1 spore suspension.

When bio mass of seedlings was assessed as dry weight similar result was obtained as observed with fresh weight.

Vigour index was maximum in ET-1 spore suspension treated seeds (3114) followed by ET-1 nano (3090), PF-2 cell suspension (2800), PF-2 nano (2786), carbendazim (2116) compared with control (1974). ET-1 spore suspension was on par with ET-1 nano, PF-2 cell suspension and Pf-2 nano were on par with each other, while carbendazim and control were on par with each other.

(Hojjat *et al*. 2016) [19] obtained similar results when five levels of silver nanoparticles (0, 10, 20, 30 and 40 µg mL-1) were used in lentil. Germination percentage did not affect significantly amongst the various treatments. Lentil seedling under Silver Nano particle recorded significantly higher root

length, shoot length, dry mass and speed of germination over control.

Summery and Conclusion

When the cell free culture filtrates of ET-1, RT-4 (prepared in PDB), PF-2 and PF-5 (prepared in NB) were mixed with silver nitrate solution (170ppm) @ 10:90 (culture filtrate: silver nitrate solution), with all the mixtures, colour of the silver nitrate solution changed to light brown colour in 48 hours with colloidal appearance indicating conversion of silver nitrate to bionano silver. It is interesting that similar observations were made even with the culture filtrate of *R. solani*, autoclaved-uninoculated Potato Dextrose broth and autoclaved-uninoculated Nutrient broth indicating that every organism (potato in PDA and beef extract in NB) has the ability of nanoconversion of heavy metals like silver.

When nano conversion was attempted with three different ages of culture filtrates (*i.e*., 5, 10 and 15 days), the result indicated that ability to convert silver nitrate to nano silver varied with type of microbe and age of the culture filtrate.

When washed mycelial mat of ET-1 was exposed to silver nitrate solution, nano conversion was observed indicating that the ability of nanoconversion is instantaneous.

The silver nano conversion was confirmed using UV-Vis spectrophotometer when one of the three absorbance peaks, *i. e.*, between 400 to 450nm corresponded to bionano silver.

In the present study, *P. fluorescens* PF-2 based bionano silver particles prepared from 5 day old culture filtrate had the zeta potential -34.4 mV indicating moderate stability, R. solani based nano particles prepared from 5 day old culture filtrate had the zeta potential -36.0 mV indicating moderate stability and the *Trichoderma* ET-1 based nano particles from 5 day old culture filtrate had the zeta potential -0.3mV indicating rapid coagulation and flocculation.

Dynamic light Scattering spectroscopy revealed that bionano silver particle size ranged from 80 to 137.4nm (98.2±28.7nm) with PF-2 based nano and 98.8 to 223.7nm (160.9±62.1nm) with *R. solani* based nano indicating that PF-2 nano and *R. solani* nano were more of polydisperse in nature. In case of bionano silver prepared from ET-1 culture filtrate, the particle size ranged from 6.3 to 6.9nm (6.7±0.3nm) indicating its monodisperse nature.

Results of Scanning Electron Microscopy also indicated that the particle size in ET-1 based bionano silver was the smallest and monodisperse in nature while other two were of poly disperse in nature with bigger sizes.

Peroxidases activity were found to be higher in all the treatments compared to untreated control when absorbance values for peroxidises were compared. However, there was no specific trend was observed in relation to leaf or root incase of peroxidase as evidence by higher peroxidise accumulation in roots with some of the treatments while in others more in leaves. When the PPO accumulation was observed at 24 hours, 48 hours after spray treatment resulted in higher accumulation in seedlings raised from nano or bio control agent treatments compared to control. Accumulation of PAL also showed similar increase in treatments compared to control. Tyrosinase is a member of PPO family that has both monophenolase activity and diphenolase activity.

When the rice seedlings, from either 100% bionano treated seeds or from foliar sprayed plants, were assessed for the accumulation of plant defense related enzymes *viz*., peroxidases, polyphenol oxidase, phenyl alanine ammonia lyase and tyrosinase, substantial accumulation was observed as evidenced by the absorbance values indicating induction of resistance mechanism in rice seedlings.

The present investigation revealed increased germination of rice seeds, shoot length, root length, S:R ratio, fresh weight, dry weight and vigour index of rice seedlings with 10% silver bionano preparations applied as seed treatment (wet seed treatment for 10 minutes). These nano preparations alone could increase the growth parameters either on par with respective biocontrol agents or sometimes even better when the rice seeds of cv. NLR 34449 were treated with nano preparations and sown in pathogen uninoculated pots indicating their utility in increasing plant growth in place of even biocontrol agents and fungicide seed treatment.

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