Pseudomonas plecoglossicida Based Silver Nanoparticles: Characterization, Effect on Growth and Antioxidative Enzymes on *Pisum sativum* L.

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Summary

The current work focuses on the synthesis of silver nanoparticles utilizing rhizobacteria and screening for their effectiveness in terms of plant growth promotion and antioxidative defence ability in inoculated *Pisum sativum* L. plants. *Pseudomonas plecoglossicida* Nishimori, et al. (2000), isolated from Kundrathur region of Chennai, Tamil Nadu, India, was used for the synthesis of silver nanaoparticles. The synthesized silver nanoparticle was characterized by X-Ray diffraction analysis (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Dynamic Light Scattering (DLS) and Field Emission Scanning Electron Microscopy (FE-SEM) analysis. The efficacy of the plant growth-promoting bacteria synthesized nanoparticle was assessed in terms of growth and antioxidant activity in *Pisum sativum* plants. *P. plecoglossicida* exhibited Indole-3-acetic acid (IAA) production of 7.8 mg/l, P solubilization index of 35 and a high Hydrogen Cyanide (HCN) production. Silver nanoparticles synthesized by WRR55 were validated by peak values of 28.04, 32.45 and 46.44 by XRD analysis. The FTIR spectra revealed the features of the C-O extension, the C-H extension of alkenes and the NH extension of amides. DLS indicated that the particle is nanoscale based on the peak that was obtained between 10 and 100 nm. The produced silver nanoparticles have a spherical shape and a particle size between 30 and 50 nm, according to Field emission scanning electron microscopy (FE-SEM) analysis. When compared to the control, silver nanoparticles produced by WRRS5 demonstrated superior germination and plant growth. At 60 ppm concentration, biosynthesized particles demonstrated increased activity in superoxide dismutase (SOD) activity of 24.2 U mg Protein^{-1}, ascorbate peroxidase (APX) activity of 1.90 mg Protein^{-1}, and Catalase (CAT) activity of 297.4 U mg Protein^{-1}.

Key words

Pseudomonas plecoglossicida, silver nanoparticle, nano-biofertilizer, FE-SEM, PGPR

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Introduction

Soil microbial diversity and its activity on plant growth is an inevitable factor for the management of Agricultural soils. Plant Growth Promoting Rhizobacteria (PGPR) are a group of microbes in rhizosphere, exerting favourable effects on various aspects of plant growth, development and indirectly on yield (Kloepper et al., 2004). The plant growth promoting rhizobacterial genera such as *Azospirillum*, *Alcaligens, Arthobacter, Enterobacter, Erwinia, Burkholderia, Bacillus, Flavobacterium, Rhizobium, Pseudomonas,* and *Serratia* represent diverse genera of PGPR, and are known to exert tremendous benefits to the plants (Tilak et al., 2005). The improvement in nutrient uptake and increase in phytohormone levels are due to the direct or indirect influence of PGPR (Chauhan et al., 2015; Glick, 2012). In the direct mechanism, the PGPRs facilitate the uptake of phosphorus and iron, phosphate solubilization, siderophore production and N_2 fixation (Saharan and Nehra, 2011). The indirect mechanism of plant growth is mainly by diminishing the impact of plant growth inhibitors (Glick, 2012).

Nanotechnology deals with various particles, systems and devices having a nanoscale size of 1-100 nm and it is a multidisciplinary sphere combining various aspects of science and engineering in developing technologies for their design, production, characterization and application (Balachander et al., 2019). The availability of larger surface area compared to volume of nanoparticles relative to the preparation of bulk materials from the same source endows these nanoparticles with special characteristics leading to unique physicochemical properties for each type of nanoparticle (Varatharaj et al., 2019). Among nanoparticles, bio-nanomaterials will minimize the nutrition losses in fertilization and enhance the yield without affecting the soil health and fertility (De Freitas et al., 1997; Nair et al., 2010; Ngoma et al., 2013).

Taking this into account, the present study was aimed to develop silver nanoparticle from plant growth promoting rhizobacteria and to screen for their efficiency in terms of plant growth promotion efficiency and antioxidative defence response in *Pisum sativum* L. plants.

Materials and Methods

Isolation of Rhizobacteria

The rhizospheric soil was collected from different fields such as continuously cropping rhizospheric soil (CCRS), wild region rhizospheric soil (WRRS) and chemical fertilizer polluted site rhizospheric soil (CFPSRS), located in and around Kundrathur, a suburban area of Chennai, Tamil Nadu, India. The sampling of soil from those sites was carried out following the protocols of Luster et al. (2009). In brief, the sampling was carried out by shaking the roots of uprooted plants and the leftover soil adhering to roots harvested from the rhizosphere soil. Master dilution for serial decimal dilution was done using 10 g of pooled sample and dilutions were plated by spread plate method using nutrient agar media for isolation. The inoculated plates were kept for incubation at 35 °C for 2 days. The single well-isolated colony was sub-cultured as glycerol stock and stored for further studies.

Screening for PGPR

Phosphorus Solubilization

The p-solubilizing ability of the bacteria isolated from rhizosphere soil was screened by spot inoculating 10 µL of active log phase broth culture on Pikovskyaya's medium and incubating for seven days at 35 ^{0}C. The clear transparent zone of clearance around the bacterial growth was indicative of P-solubilization and the diameter of the zone was taken as measure for P-solubilization capacity. The phosphate solubilization index was calculated and recorded as described by Premeno et al. (1996).

Production of Indole -3 Acetic Acid (IAA)

Salkowski method of estimation for IAA production by isolated bacteria was followed according to Huddedar et al. (2002). In brief, five ml of nutrient broth enriched with tryptophan (1.0%) were used to grow bacteria and the inoculated tubes were incubated without light at 120 rpm for 72 h. One ml of culture supernatant obtained after centrifugation was vortexed with 1.0 ml of Salkowski reagent and the resulting mixture incubated in the dark for 30 min at 30 °C. Pink-red colour in the reaction tubes confirmed the presence of IAA and the A_{536} values used to quantify IAA using the standard chart (Fisher et al., 2007).

Production of Hydrogen Cyanide (HCN)

HCN production by the selected isolates was screened by streaking on King's B medium modified by the addition of 4.4 g glycine / 1000 ml (Bakker and Schippers, 1987). Sterile Whatman filter paper soaked with 2% sodium carbonate in 0.5% picric acid solution was used to streak inoculated plates, which were then sealed with cling film and incubated at 28 °C for 48 h. The colour formation above the growth indicated HCN production and the colour maybe interpreted as: orange colour in the plates indicating the effective production of HCN, dark brown colour showing medium and light brown coloration indicating HCN production by the selected isolates.

Cytology and Biochemical Characteristics of Bacterial Isolates

Bacterial cytology and biochemical characters of the selected isolates were examined (Cappuccino and Sherman, 1992). Colony shape, colour, size, elevation, surface, margin, opacity and motility test of all isolates were recorded. Gram's reaction was performed based on the various biochemical characters such as indole, Methyl Red / Voges – Proskauer test (MR VP), Citrate, Urease, Triple Sugar Iron Test (TSI), Nitrate reductase, and Catalase test.

16s rRNA Sequencing

The PCR amplification was done by utilizing (24F and 1492R) universal primers in Eppendorf Thermocycler 96 and PCR kit (Applied Biosystems). The PCR amplification was done using an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 m, followed by annealing at 55 °C for 1 m, and extension at 72 °C for 2 min, with final extension at 72 °C for 5 m. The amplicons were electrophoresed in 1.25% agarose and visualized by ultraviolet florescence on staining with ethidium bromide. The amplicons were eluted and purified by the method given in Gel elution kit (Applied Biosystems)

and PCR purification kit (Applied Biosystems). Finally, the ABI 3730 DNA analyser (Applied Biosystems) was used to obtain the DNA sequences. 16s rRNA gene sequences were aligned using ClustalX2.1version. Phylip 3.69 (http://evolution.genetics.washington.edu/phylip.html) and MEGA 5.05 was used to carry out the phylogenetic and molecular evolutionary analysis (Tamura et al., 2013). The 16s rRNA sequences were submitted in the Gen Bank nucleotide sequence database and obtained the accession number MK5444830.1.

Biosynthesis of Silver Nanoparticles Using Cell Free Supernatant of *P. plecoglossicida*

P. plecoglossicida was grown in nutrient broth medium until the culture reached its early stationary phase – A_{620} ~0.82 (Musaarat et al., 2010). Supernatant from centrifuged (10,000 rpm; 15 m) culture was transferred to fresh tube and fresh stock of $AgNO_3$ was mixed with culture supernatant at a final concentration of 1.0 mM. The mixture was incubated in the dark and monitored for the colour change from pale yellow to dark brown (colour change indicates AgNP formation).

UV-Visible Spectroscopy Analysis

The optical characteristics of biosynthesized AgNPs were analysed using UV-Vis Spectrophotometer (Shimadzu, Japan) by scanning at wavelengths ranging from 200 nm to 1100 nm. The bio reduced AgNPs in the dark brown mixture (stable nanoparticle) were checked continuously by scanning and the peak values were recorded (Subbaiya et al., 2014).

X-Ray Diffraction Measurement

The structure of synthesized AgNPs was detected using X-ray diffraction (XRD) spectrum analysis. Bio synthesized AgNPs were dried and coated on XRD grid and diffraction was recorded in 2θ ranges from 5 °C to 80 °C. The data were obtained by applying 40 kV and a current of 40 mA (Jancy and Inbathamizh, 2012).

Fourier Transform Infrared Spectroscopy Analysis

Lyophilized AgNPs of *P. plecoglossicida* mixed with pure KBr was used for FTIR analysis. FTIR spectroscope (Shimadzu, Japan) was used between the spectra ranges of 4000 – 400 cm^{-1} and the obtained FTIR spectrum was analysed as described by Ramesh and Rajeshwari (2015).

Characterization Using Dynamic Light Scattering

The particle size analysis was carried out using Dynamic Light Scattering to analyse the range of particle size. The sample was mixed well and kept in polystyrene cuvettes and fixed at the detection angle of 173 °C at the temperature of 25 °C.

FESEM with EDS Spectrum Analysis of AgNPs Synthesized from *P. plecoglossicida*

The samples were placed on a glass slide and allowed to dry, then fixed on copper grid supports. The sample surface was studied using the (Quattro ESEM) field emission scanning electron microscope (FESEM) to examine the morphology of the AgNPs and determine their size. The FESEM was also incorporated with (EDS) energy dispersive spectroscopy used for qualitative and quantitative analysis of element and its mapping for produced nanoparticles (Kumar et al., 2013). The synthesis of *P. plecoglossicida* mediated AgNPs was confirmed by colour change in the solution from transparent yellow to brown and the solution sample became deep brown as it had been kept for 30 days without any disturbance.

Evaluation of Silver Nanoparticles for Plant Growth Promoting Activity and Antioxidative Enzymes *in vivo* (pot experiment) on Vegetable Plants

Plant growth promotion studies were carried out in green peas (*Pisum sativum*); selected vegetable seeds were sterilized by soaking them with 5.0% sodium hypochlorite solution for 5.0 minutes and washed thoroughly with deionized water. The seeds (25 seeds per Petri dish) were allowed to germinate on Petri dishes with the support of water porous paper. The sporulated seeds were picked up and used for further studies. The pots were filled with healthy seedlings and allowed to grow. Every day 15 ml of *P. plecoglossicida* mediated AgNPs were applied for all the test plantlets for 15 days and control plantlets did not receive AgNPs for the study period. The synthesized AgNPs using *P. plecoglossicida* was applied every day for all 15 days of the study in different concentrations of – 20/ 40/ 60/ 80/ 100 ppm: to each chosen test plantlet(s) in the pot. The entire set of tests was done in triplicates for statistical relevance. The shoot and root length of test and control plantlets were measured, and triplicate results averaged and presented. For antioxidative enzyme activity the enzymes extracts were taken and the purification was performed as described earlier by Dey et al. (2007). Catalase (CAT) assay was measured based on the extinction coefficient of 40.0 mM^{-1} cm^{-1} for H_2O_2 at 240 nm as described by Aebi (1974). Superoxide dismutase (SOD) activity was measured as described by Das et al. (2000) based on the inhibition of superoxide nitrite formation from hydroxylamine. Ascorbate peroxidase (APX) activity was determined based on the protocol of Lee and Lee (2000) which was based on the H_2O_2 dependent oxidation of ascorbic acid at 290 nm and at an absorption coefficient of 2.8 mM cm^{-1}.

Results

Screening of PGPR from Different Rhizosphere Soil

In this study, 30 isolates from 15 different rhizosphere soil samples were obtained from the different regions in Kundrathur, Chennai, Tamil Nadu, India. Cultural and morphological characterizations were done on these isolates, in which 20% of isolates demonstrated appreciable PGPR activity such as IAA production, phosphate solubilization and production of HCN (data not shown). These isolates were given strain numbers - CCRS1, CCRS5, CCRS7, WRRS1, WRRS5 and CFPSRS5 and were selected for further studies. All six characterized strains were found to be gram negative motile rods and the biochemical characterization results were presented in Table 1.

Phosphate Solubilization, IAA Production and HCN Production from the Selected PGPR Isolates

In this study out of 30 isolates from CCRS, WRRS, and CFPSRS, isolates showed prominent phosphate solubilization. Two isolates from WRRS, three from CCRS and one from CFPSRS samples were effective solubilizers of phosphate showing results as clear

zone of phosphate solubilization on Pikovskyaya's medium after 6.0 days of incubation at 35 ± 2 °C. Among all the PGPR isolates screened in the presented study *P. plecoglossicida* was identified as the highest P solubilizer with a solubilization index of 3.5 and the most prolific IAA producer with 7.8 ± 0.5 mg IAA L^{-1}, (Table 1). After 72 hrs of incubation the filter paper colour turned from pale yellow to deep brown indicating that the isolates had ability to produce HCN. Six PGPR isolates produced HCN effectively, of which WRRS5 showed a significant result (Table 1).

Table 1. Selected PGPR characteristics of rhizosphere isolates

Selected Isolates	P solubilization index	IAA mg/L	HCN
CCRS1	2.7	3.2 ± 0.5	++
CCRS5	1.6	2.7 ± 0.4	+
CCRS7	2.3	2.9 ± 0.8	++
WRRS1	1.6	2.3 ± 0.6	+
WRRS5	3.5	7.8 ± 0.7	+++
CFPSRS5	2.4	3.1 ± 0.6	++

Note: WRRS5 identified as *Pseudomonas plecoglossicida*
where: + = low production, ++ = moderate production and +++ = strong production

Analysis of PCR Amplicon Product

Universal primers (24F and 1492R) were employed to amplify V1 –V3 region of partial 16s rRNA and the final PCR amplicon product was sequenced and dendogram based on phylogenetic analysis confirmed the PGPR isolate as *P. plecoglossicida* and the sequence were submitted to NCBI with accession no. **MK544830**.

Biosynthesis of AgNPs Using *P. plecoglossicida*

The synthesis of silver nanoparticles with selected bacteria *P. plecoglossicida* WRRS5 was investigated. After 24 h of incubation, the change in colour from yellow to brown in the flask was noted. The test flask showed brown coloration indicating positive and the control flask which consisted only of silver nitrate showed no colour change, indicating negative results.

Characterization of Biosynthesized AgNPs

Fig.1a illustrates the UV-visible spectrum of biosynthesized AgNPs and shows the absorbance peal at 417 nm. The crystalline nature of biosynthesized AgNPs was studied by XRD and the pattern showed three intense peaks in the entire spectrum (Fig.1b). In the XRD spectrum values were ranging from 5° to 70°. The evidence of the peak at 2θ values of 28.04°, 32.45° and 46.44° confirmed the presence of AgNPs mediated selective isolate of PGPR and no impurity peaks were observed. The FTIR spectra revealed several absorbance bands and the peak at 3446.79 cm^{-1}

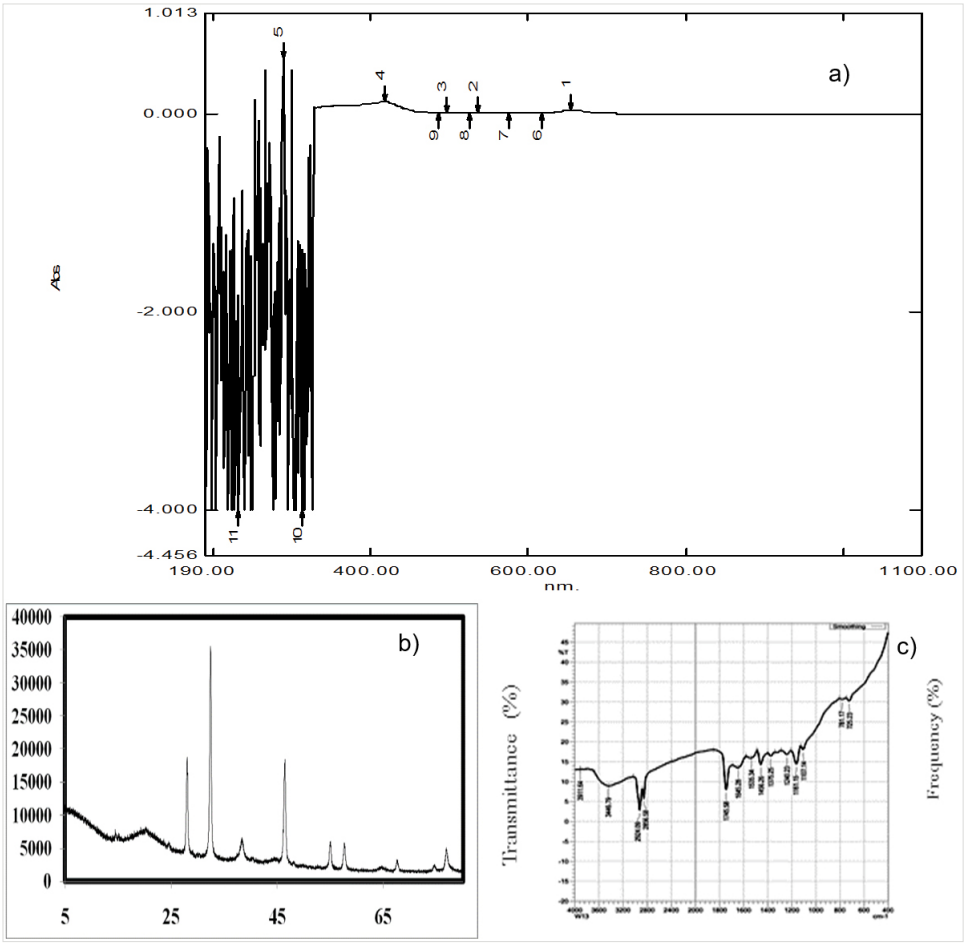

Figure 1. UV-Visible spectrum (a), X-Ray Diffraction pattern (b) and Fourier Transform Infrared spectrum (c) of silver nanoparticles from *P. plecoglossicida* WRRS5

and 2924.09 cm^{-1} is the characteristics of amino acids that contain an amine (NH) functional group (Fig. 1c). The peak at 2856.58 cm^{-1} relates the C-H extension of alkanes. The peaks at 1745.58 cm^{-1} lead to extension of carbonyl stretch and the peak at 1161.15 cm^{-1} refers to C-O extension vibration mode.

The average size of the biosynthesized AgNPs particle was observed by Dynamic Light Scattering particle (Fig. 2a). The peak obtained between 10 to 100 nm revealed that the particle size was in the nanosize. Field emission scanning electron microscopic analysis revealed the morphology of the AgNPs mediated *P. plecoglossicida* as shown in (Fig. 2b). The two-dimensional approach of FESEM provides the details of the morphology of the biosynthesized AgNPs. The results show the spherical shape with the particle size that lies between 30-50 nm. The presence of metallic element was analysed using Energy Dispersive Spectroscopy (EDS) (Fig. 2c). The optical absorption ~3eV confirms the presence of metallic AgNPs in pure form.

Evaluation of Synthesized AgNPs from *P. plecoglossicida* WRRS5 for Plant Growth Promoting Activity *in vivo* (pot experiment) on Vegetable Plants

In this study biosynthesis AgNPs using *P. plecoglossicida* was used as nano biofertilizer and its effect on growth of selected vegetable plants was studied and the results are presented in Fig. 3 a-c. The inoculation of AgNPs with the selected vegetable seeds induced clear changes in germination, plant length and other growth parameters as compared with control plantlets. After 12 days of inoculation the growth parameters such as SL (Shoot length), L (leaf number), LL (leaf length), LW (leaf width), DW (dry weight), and RL (root length) were observed and recorded. When the concentration of AgNPs increased the plant promotion also increased in all the three test plantlets.

The evaluation of biosynthesized AgNPs for enhancing plant growth activity *in vivo* (pot experiment) on vegetable plants was carried out. The increased concentration of biosynthesized AgNPs in ranges 20, 40, 60, 80 and 100 ppm showed better growth when compared to crop treated with distilled water. The highest effects on the plant shoot length, root length and plant dry weight were observed at a concentration of 60 ppm, beyond which the increase was not statistically significant. At a concentration of 60 ppm plant shoot length of 27.2 cm (Fig. 3a), root length of 3.9 cm (Fig. 3b) and dry weight of 0.23 g (Fig. 3c) were observed.

Effect on Antioxidative Enzymes

The effect of synthesized AgNPs on the antioxidative enzyme activity of *P. Sativum* was evaluated under *in vitro* conditions and it was found that AgNPs positively affected the SOD, APX and CAT activity (Fig. 4a-c). It was observed that the highest effect on the antioxidative enzyme activity of *P. Sativum* was seen at a concentration of 60 ppm. Beyond 60 ppm, no significant increase in activity could be observed. At 60 ppm AgNP concentration, 24.2 U mg $protein^{-1}$ of SOD activity (Fig. 4a), 1.9 U mg $protein^{-1}$ of APX (Fig. 4b.) activity and 297.4 U mg $protein^{-1}$ (Fig. 4c) of CAT activity were examined.

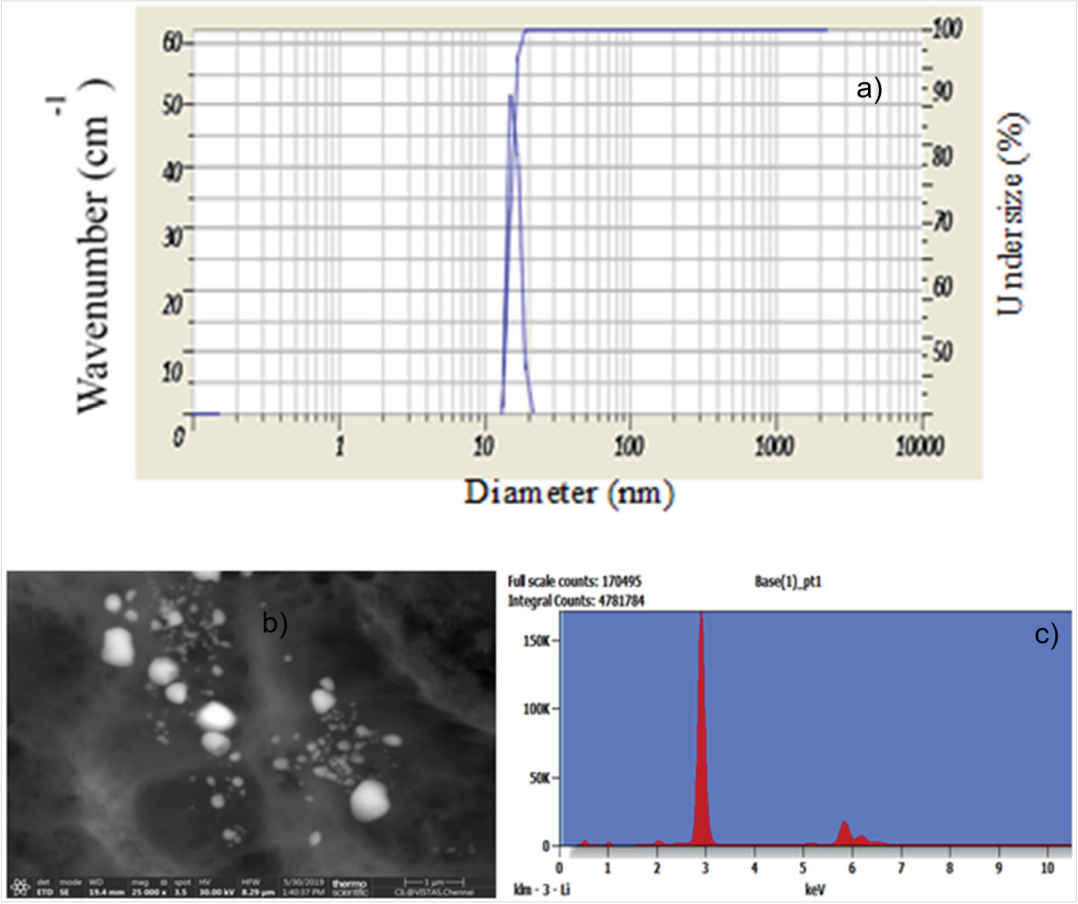

Figure 2. Particle sizing measurements with a) dynamic light scattering (DLS) b) Field Emission Scanning Electron microscope (FESEM) and c) EDS spectrum of synthesized AgNPs from *P. plecoglossicida* WRRS5

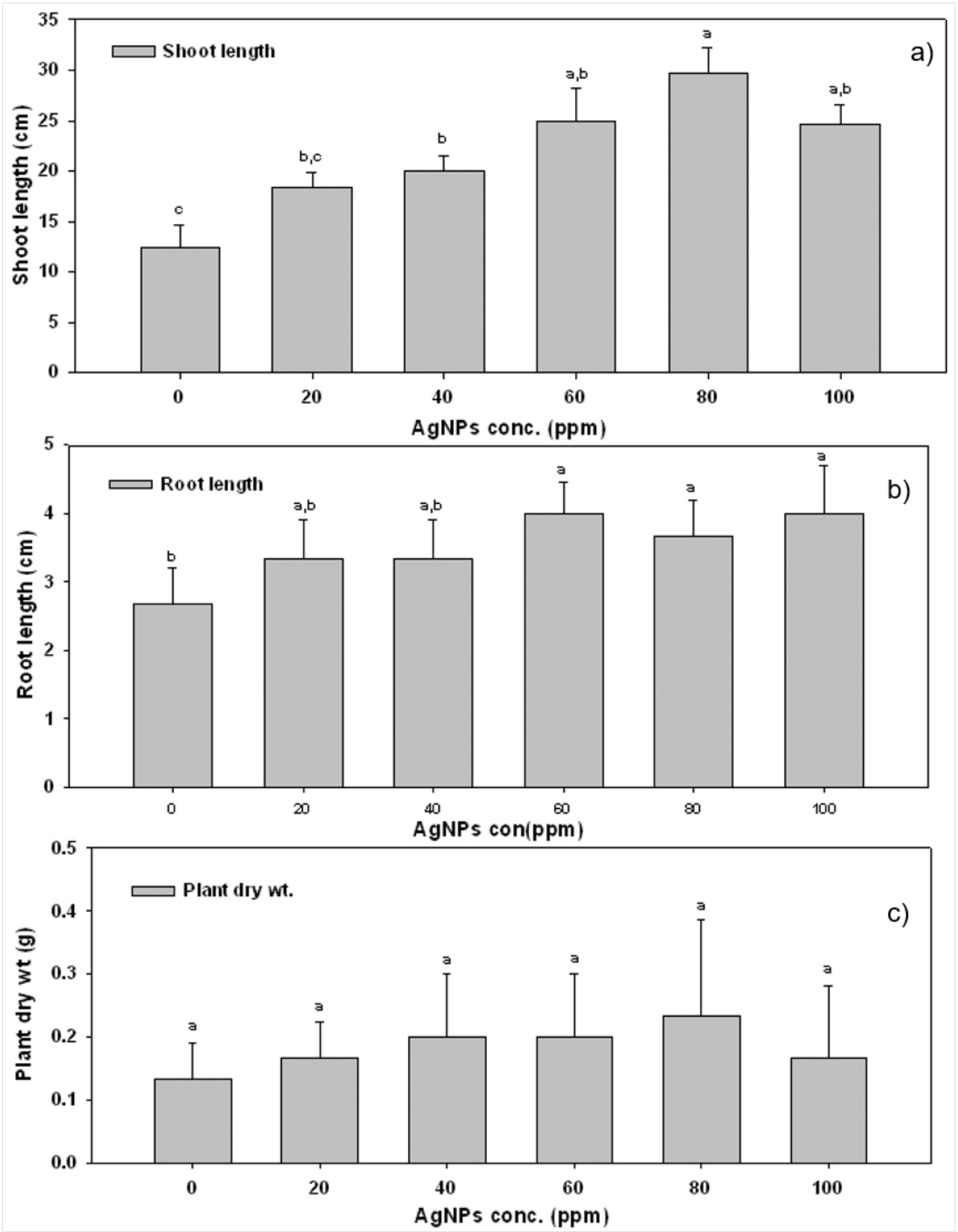

Figure 3. Effect of synthesized AgNPs using *P. plecoglossicida* WRRS5 on growth promoting activity in three different crop plants. Shoot length (a), Root length (b), Plant dry weight (c)

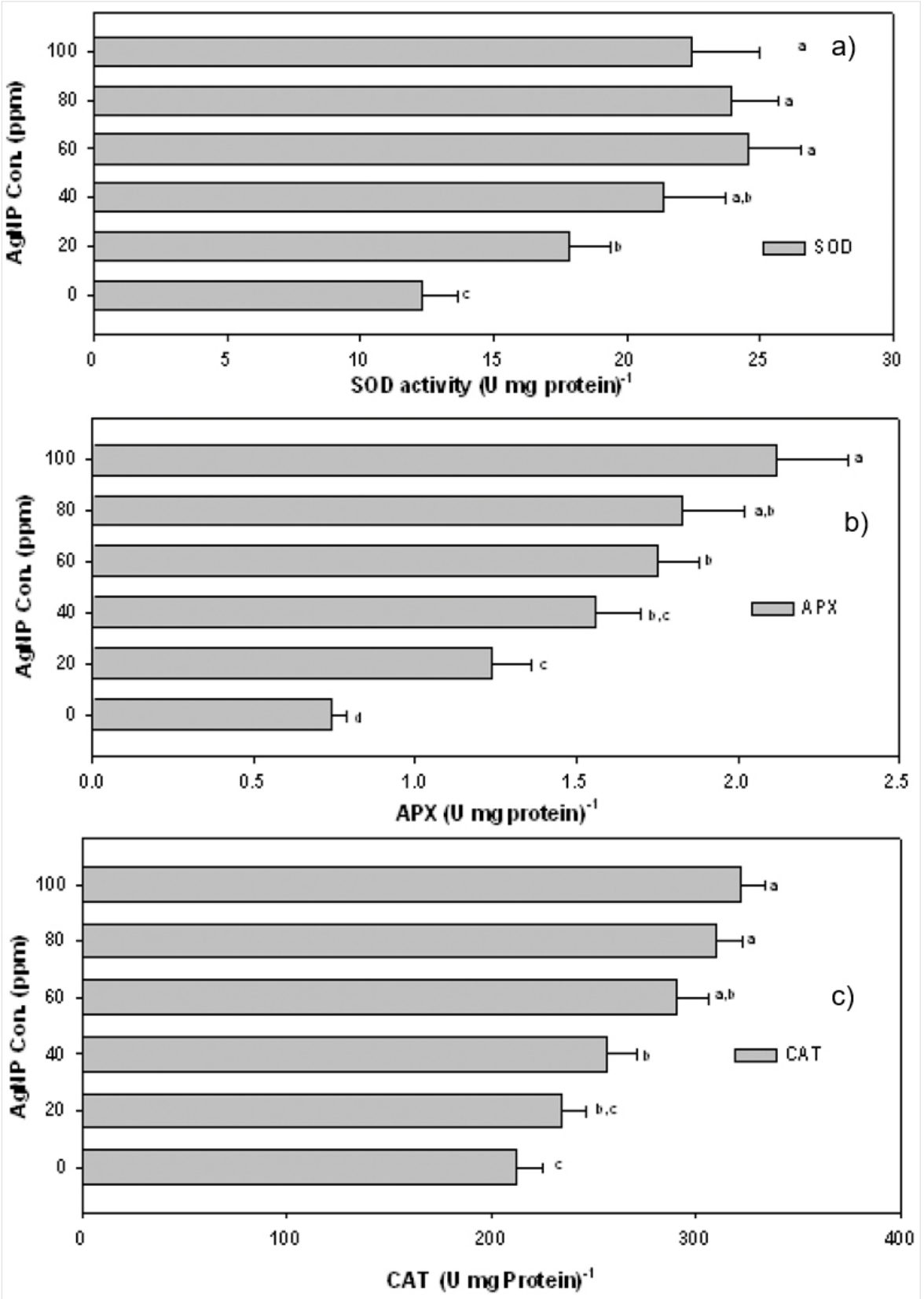

Figure 4. Effect of synthesized AgNPs using *P. plecoglossicida* WRRS5 on the antioxidative enzyme activity of *P. Sativum* grown under pot culture conditions. AgNP concentration in ppm. SOD activity (a), APX activity (b), CAT activity (c)

Discussion

In the present study, among the different isolates, *P. plecoglossicida* has been isolated, identified and screened for PGPR activity. Among different PGPB traits, phosphate solubilization test was carried out on *P. plecoglossicida* and its ability to solubilize phosphate efficiently is in accordance with the previous study by Kaur and Reddy (2014). Apart from P solubilization, Meliani et al. (2017) found that *P. plecoglossicida* was able to produce indole 3 acetic acid effectively. The ability of P solubilizing bacteria to be positive for IAA production goes well in line with the earlier finding of Nacoon et al. (2021), who reported that *Klebsiella variicola* UDJA102x89-9 recorded higher amounts of organic acid production responsible for P solubilization under *in vitro* condition and was also found to be positive for IAA production. Previous research stated that the HCN production was higher in *Pseudomonas* sp. compared to other bacterial species viz. *Bacillus* sp. and *Rhizobium* sp. isolates and this study also confirms the same with (Singh et al., 2013; Ngoma et al., 2013). Rijavec and Lapanje (2016) have reported that HCN, apart from acting as a biocontrol agent, is also involved in biogeochemical process of the substrate, thereby indirectly increasing the phosphate availability.

The biosynthesized AgNPs from *P. plecoglossicida* have been characterized by UV-Spectra, Particle size analysis, FTIR, FE-SEM and XRD analysis. UV- visible spectrum of AgNPs mediated *P. plecoglossicida*, shows absorbance peak at 417 nm, which is similar to that of previous report of Jeevan et al. (2012). The strong, stable peak observed around 417 nm indicates the presence of nanoparticles of the cell free supernatant of the PGPR isolate *P. plecoglossicida*. The previous report about FTIR study affirmed the protein molecule could bind to the nanoparticle with free amine groups (Gol-Dash et al., 2001, Mandal, 2005). The proteins may act as capping agent and provide stabilization for the nanoparticles synthesized using PGPR isolate. The two-dimensional approach of FESEM reveals the AgNPs mediated *P. plecoglossicida* shows spherical shape with the particle size between 30- 50 nm, which is in similarity with the findings of Magudapathy et al. (2001), who reported triangular shape of AgNPs with a size range of 12 to 61nm using *Bacillus flexus*.

The effect of biosynthesized AgNPs on vegetable plant growth (pot experiment) has been evaluated at different concentrations on the growth and the antioxidative activity of green pea plants. The stimulatory effect of AgNPs on the growth of green pea plants goes well with the earlier findings of Salachna et al. (2019), who reported AgNP treatment enhanced the accumulation of leaf and bulb biomass, and also accelerated flowering in the treated *Lilium* cv. Mona Lisa. The reason for a higher growth and yield in plants may be attributed to the improved efficiency of electron transport pathway and the prevention of reactive oxygen species (ROS) generation in AgNPs treated plants (Sharma et al., 2012). Improved antioxidative defence response with respect to CAT, APX and SOD have been observed in the present study. This enhancement in the improved antioxidative defence system of *P. Sativum* is attributed to the botanical changes in response to the AgNP exposure that may affect the generation of ROS, changes in the SOD activity, and H_2O_2 level (Budhani et al., 2019).

Conclusions

The current study was carried out with the objective to exploit the use AgNPs synthesized from cell free supernatant of *P. plecoglossicida* WRRS5 as PGPR in vegetable plants to promote and enhance the growth. The increased concentration of AgNPs was tested in *P. Sativum*. The growth promotion upon inoculation with our biologically mediated AgNPs using *P. plecoglossicida* WRRS5 as nano biofertilizer was demonstrated. The results show that when the concentration of AgNPs increases the plant promotion also increases in all the three test plantlets. Thus, biologically mediated AgNPs using *P. plecoglossicida* WRRS5 have a lot of potential to be used as a nanobiofertilizer.

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CRediT authorship contribution statement

Suresh Dhanaraj: Conceived the project and supervised the work. **Sivaranjani Gopalakrishnan and Somanathan Thirunavukkarasu:** Conceptualization, Investigation, Performed most of the experiments, Data analysis, Original draft preapartion. **Sivaranjani Gopalakrishnan and Manoharan Melvin Joe:** Performed some of the experiments. **Abitha Benson:** Manuscript editing.

Declaration of Competing Interest

The authors declare no known competing financial interests or personal relationships to influence the work reported in this manuscript.

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