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RESEARCH ARTICLE

Synthesis of Biogenic silver nanoparticles using plant growth-promoting bacteria: Potential use as biocontrol agent against phytopathogens

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ABSTRACT

Biogenic nanoparticles (NPs) derived from microbes present an excellent opportunity to deal with various challenges in medicine, diagnosis, environment and agriculture. In the area of agriculture sciences, researchers are facing challenges related to excessive utilization of pesticides which can be answered by utilizing plant growth-promoting (PGP) microbes. Herein, we have employed the culture filtrate of two PBP bacteria strains, *Serratia marcescens* and *Burkholderia cepacia* to prepare biogenic silver NPs. The biogenic silver NPs were characterized by various techniques viz. UV-VIS spectroscopy, SEM, XRD and FTIR. The biogenic AgNPs were able to control the growth of phytopathogenic fungi *Aspergillus niger*, *A. fumigatus*, *Fusarium oxysporum*, *Pythium* sp., and *Rosellinia* sp. by more than 80% as examined by in vitro growth reduction on agar medium. Very significantly, the growth inhibition of seedlings by phytopathogenic fungi was efficiently rescued using biogenic AgNPs derived from PGP bacteria. These results indicate the potential use of biogenic NPs to reduce the burden of chemical-based pesticides.

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Introduction

Nearly 90% of pesticides sprayed in modern agricultural pest and disease management systems are lost to the air and run-off, causing financial and environmental harm to farmers and the environment (Stephenson et al., 2001). Furthermore, pesticides are also used indiscriminately, raising resistance in pathogens, lowering nitrogen fixation, reducing biodiversity of soil, and increasing pesticide accumulation (Tilman et al., 2002). These are important concerns that demand much attention(Rana et al., 2021).

New pesticide concepts based on nanotechnology, collectively known as "nanopesticides," are expected to address these issues. Most of the nanopesticides and nanofertilizers presented so far involve the reformulation of registered active ingredients (AIs) to improve performance over existing AIs and address the major limitations of current agrochemical products (Kah et al., 2013). Nanopesticides could address the major

drawbacks of current pest control strategies (Smith et al., 2008).

Based on the requirement of nutrient different type of nano-fertilizers are required like macro nano-fertilizers, micron nano-fertilizers and nano-particulate fertilizers (Chhipa, 2017). To produce these desirable nutrients, microorganisms are grown on selected media providing all suitable conditions like pH, temperature, and carbon source. Then extracellular proteins (act as a nutrient) are separated using a filtration technique, and size is adjusted according to their need (Patel and Krishnamurthy, 2015). Adapting to environmental conditions nano-fertilizers secret their required compound using slow release and target delivery mechanism (Manjunatha et al., 2016).

The utilisation of bioactive components derived from microorganisms, plants and other biological sources has introduced a new category of nano-bio-pesticides that are more effective and have a lower environmental impact(Abdel-Azeem et al., 2020; Ates et al., 2020). In the present study, culture filtrate (cell-free suspension) of plant growth-promoting rhizobacteria, *Serratia marcescens* (86e-NPs) and *Burkholderia cepacia* (15-AB-NPs) was used for the green production of AgNPs. All the biogenic nanocomposites were further characterized by UV-Visible spectroscopy, EDS, SEM, XRD and FT-IR. Additionally, the bactericidal activity of 86e-NPs and 15-AB-NPs was tested against fungal pathogens. *In* plantae experiment was also done revealed the plant growth promotion after the treatment of gram seeds with green nanoparticles.

Research Methodology

Microbes used in the study

The bacterial strains *Serratia marcescens* and *Burkholderia cepacia* were isolated earlier by our lab (Mittal et al., 2019). As described earlier, the pure cultures were maintained in nutrient broth and on agar medium (HiMedia, Mumbai, India) (Mittal et al., 2019). A total of five fungal pathogens were taken for the study i.e. *Fusarium oxysporum*; *Aspergillus niger*; *Aspergillus fumigatus*; *Pythium* sp. and *Rosellinia* sp.

Preparation of culture filtrate and synthesis of biogenic NPs

Both bacterial strains were inoculated in 300 ml nutrient broth media in flasks and were incubated on an orbital shaker at 30^0C and at 150 rpm for 72 hrs. The culture filtrate (CF) was harvested after 72 hrs of growth by centrifugation. The CF was filtered through 0.45 um filtered and autoclaved at 121^0C. Biogenic NPs were synthesized as described earlier with some modifications (Petatan-Sagahon et al., 2011). Briefly, the sterilized CF was mixed with silver nitrate (1 mM) on a magnetic stirrer, and 1% PEG was added to the reaction mixture as a stabilizing agent. The reaction was carried out at for 90 minutes under stirring conditions in dark conditions.

Further, the mixture was left overnight under dark conditions till the color changes to dark brown. The mixture was centrifuged and washed thrice with distilled water and obtained pellet was further dried at 80 °C.

Physical characterization of biogenic NPs.

The biogenic NPs were characterized by various techniques as described earlier (Raizada et al., 2016). The UV –Vis spectra of the purified NPs suspension in the wavelength range 300 – 800 nm was taken using Shimadzu UV 2600 spectrophotometer. After that, Energy dispersive X-ray chemical (EDS) analysis, scanning electron microscopy (SEM), X-ray diffraction (XRD) spectroscopy and Fourier transform infrared spectroscopy (FTIR) was done.

Biocontrol activity of NPs

The antagonistic activity of biogenic NPs was examined against pathogenic fungi. For this potato dextrose agar media containing NPs was used. The fungal disc was placed in the center and growth in the presence and absence of NPs was compared after incubating the fungal disc *Fusarium oxysporum*; *Aspergillus niger*; *Aspergillus fumigatus*; *Pythium* sp. and *Rosellinia* sp. at 28 °C for 7 days as described earlier (Mittal et al., 2019). For analysing the impact of NPs in controlling the adverse effects of phytopathogenic fungi, the surface-sterilized gram (chick pea) seedlings were grown in dark at 25 °C under moist conditions (Gupta et al., 2016). On the fourth day, the germinated seedlings were incubated with fungal strain ($1.8x\ 10^5$ cells/ml) for 1 hr and grown at 25 °C under moist conditions.

Further on the sixth day the seeds were treated with 100 µl of bio-nano pesticides and grown in dark at 25 °C under moist conditions. Seeds treated with normal water and fungal pathogens were taken as control. After 16 days of treatment, root length and shoot length were measured (Narayan et al., 2017).

Results and discussions

The increasing pressure to improve the agriculture productivity relies on the use of chemical fertilizer to improve nutrients availability and the use of pesticides to inhibit the growth of phytopathogens. Both the methods improve agriculture productivity but cause various harmful effects. Indiscriminate use of pesticides threatens the quality of produce as well as the health of soil. Our lab has shown that these challenges can be answered by using plant growth promoting microbes that act as biofertilizers and biocontrol agents (Devi et al., 2013; Khatri et al., 2013; Gupta et al., 2016; Mittal et al., 2019). Previously, we isolated the plant growth promoting bacterial strains *Serratia marcescens* 86e and *Burkholderia cepacia* 15AB, abbreviated as SM-86e and BC-15AB (Mittal et al., 2019). Herein we further want to utilize both strains for developing biogenic nanoparticles and analyze them for biofertilizer and biocontrol activities.

Culture filtrate of SM-86e and BC-15AB exhibit antifungal activity

The culture filtrate from SM-86e and BC-15AB was tested to exhibit antifungal activity against *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus fumigatus*, *Pythium* sp. and *Rosellinia* sp. Both bacterial strains showed antimicrobial activity against the tested strain . In addition, SM-86e and BC-15AB inhibited the growth of *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus fumigatus*, *Pythium* sp. and *Rosellinia* sp. by more than 80% data not shown. Nanoparticles can affect plants metabolic activities and can mobilise nutrients like phosphorous in the rhizosphere (Zahra et al., 2015). Nano-material like carbon and metal based are used to perform functions like storage, assimilation, transportation of minerals which have role in increasing the crop productivity (Nair et al., 2010).

Thereafter, we tested the impact of the culture filtrate (CF) of SM-86e and BC-15AB for inhibiting the growth of *Fusarium oxysporum*. The autoclaved CF of SM-86e and BC-15AB did not allow the growth of *Fusarium oxysporum* (Figure 1A (b-c)) as compared to the control (Figure 1A (a)). Now we went on to prepare the NPs by using the CF of SM-86e and BC-15AB.

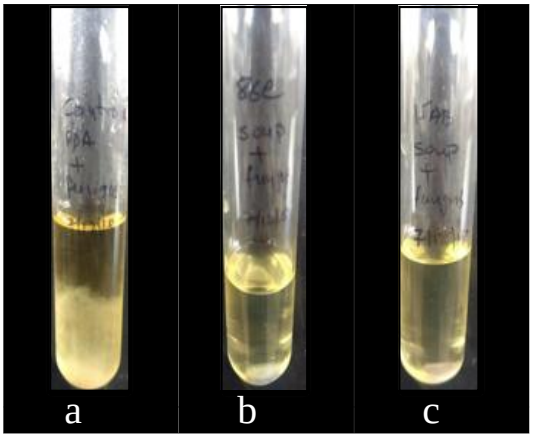

Figure 1A. Antifungal activity of the CF against phytopathogen. The potato dextrose broth (a), CF of SM-86e (b) and BC-15AB (c) were inoculated with Fusarium sp. and growth was visualized. All the experiments are done in triplicates.

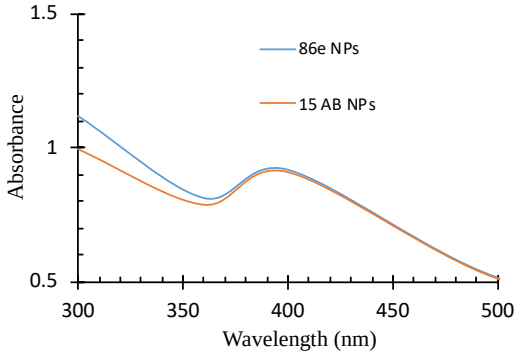

Figure 1B. UV-Vis Spectroscopy for 86e-NPs (blue) and 15-AB-NPs (orange). The λ max was around 438 nm and 432 nm for 86e-NPs and 15-AB-NPs respectively.

Physico-chemical Characterization of biogenic NPs from SM-86e and BC-15AB

Silver NPs were synthesized from CF of SM-86e and BC-15AB (abbreviated as 86e-NPs and 15-AB-NPs). Initially the synthesis of 86e-NPs and 15-AB-NPs was confirmed by UV-VIS spectroscopy. The 86e-NPs and 15-AB-NPs showed peaks at 438 nm and 432 nm, which is surface plasmon resonance of silver nanoparticles (Figure 1B) The UV spectra indicated green synthesis of these nanoparticles by using CF of SM-86e and BC-15AB as silver nanoparticles depict characteristic absorption spectra around 400-500 nm.

FT-IR, SEM-EDS and XRD analysis of NPs

The FT-IR spectra (Figure 2 (a-b)) of the CF of SM-86e and BC-15AB were analyzed to determine the functional groups involved in reducing the silver nanoparticles. The spectrum of 86e-NPs showed a peak at 3252 cm^{-1} depicting

the involvement of hydroxyl functional groups from polyphenols in the biosynthesis of these nanoparticles. The peak at 1647 cm^{-1} in CF of SM-86e shifted to 1634 cm^{-1} in 86e-NPs (Figure 2a), suggesting that ether (C=C) functional group are involved in the synthesis. Similarly, the spectrum of CF of BC-15AB showed a peak at 3297 cm^{-1} that reflects O-H stretching of hydroxyl groups from polyphenols and polysaccharides. This peak shifted to 3320 cm^{-1} in 15-AB-NPs (Figure 2b), suggesting the role of hydroxyl groups in the nanoparticles' bio-reduction. A vibrational peak at 2877 cm^{-1} in 15-AB-NPs showed the presence of CH_3 group. The 15-AB-NPs spectrum exhibited a peak at 1673 cm^{-1} corresponding to the vibrations of the amine groups (NH_2). Peaks at 1066 and 1078 cm^{-1} in CF of SM-86e and BC-15AB shifted to 1033 and 1101 cm^{-1} in 86e-NPs and 15-AB-NPs (Figure 2), respectively, indicating the involvement of C-O-C alkyl-substituted ether stretch. The presence of aliphatic bromo and aliphatic chloro compounds can be estimated at the peak range of 700 - 600 and 800 - 900 cm^{-1}.

SEM images showed the spherical structure of 86e-NPs and 15-AB-NPs (Figure 2c and d). The EDS elemental composition of the nanoparticles showed higher counts of silver at 3 keV, which are characteristic for the absorption of Ag nanocrystallites owing to SPR, thus endorsing the synthesis of these nanoparticles (Figure 3Aa and 3Ba) The presence of other elements like carbon, oxygen, boron, chlorine, and nitrogen etc. were also detected by EDS (Figure 3A and 3B).

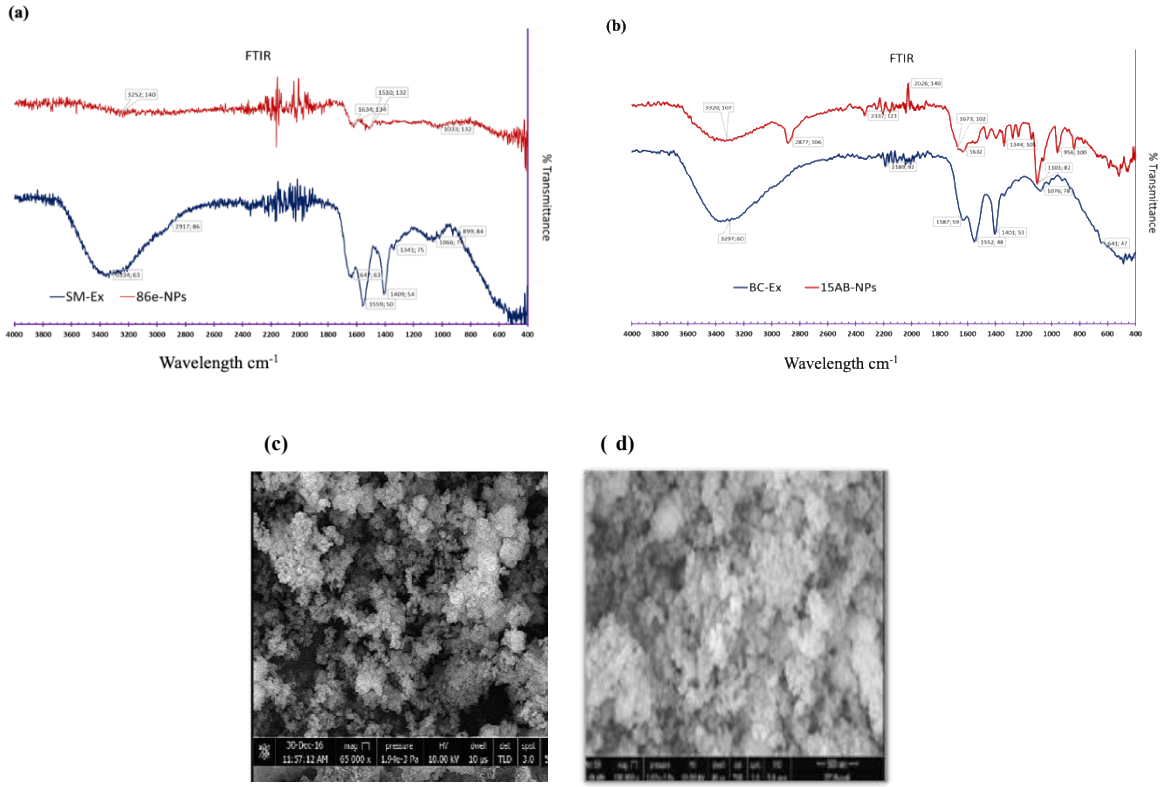

Figure 2. FTIR and SEM Analysis. FTIR spectra of (a) 86e-NPs (red) and CF (blue) and, (b) 15-AB-NPs (red) and CF (blue). SEM images of the biogenic nanoparticles (c) 86e-NPs (d) 15-AB-NP.

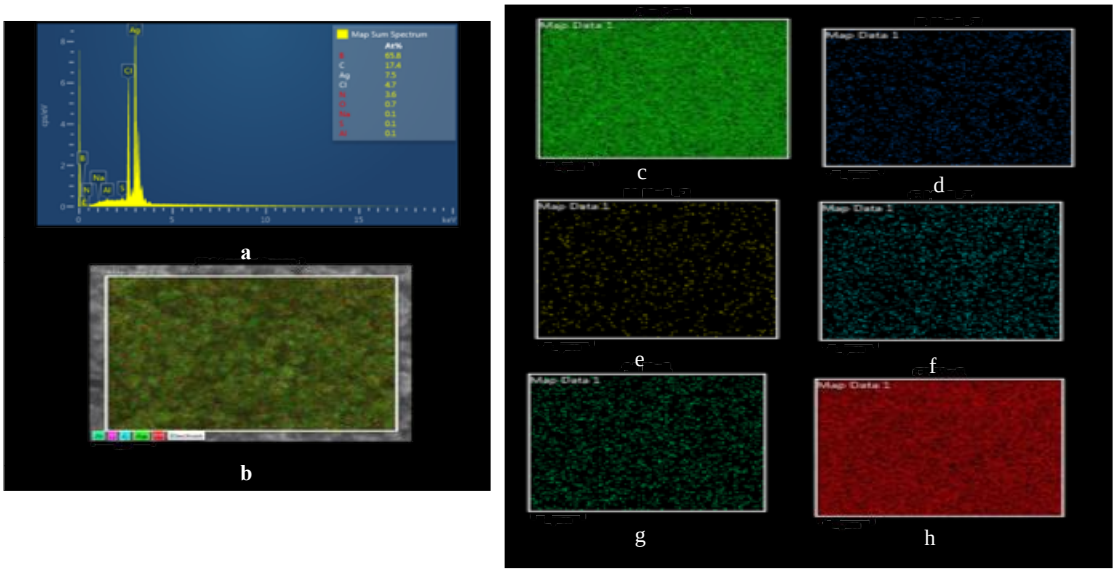

Figure 3A. EDS Analysis of 86e-NPs. (a) Elemental composition of 86e-NPs and, (b) SEM image for 86e-NPs. Different elements present are shown in different panels. (c) Silver, (d) Carbon, (e) Nitrogen, (f) Boron, (g) Oxygen and, (h) Chlorine.

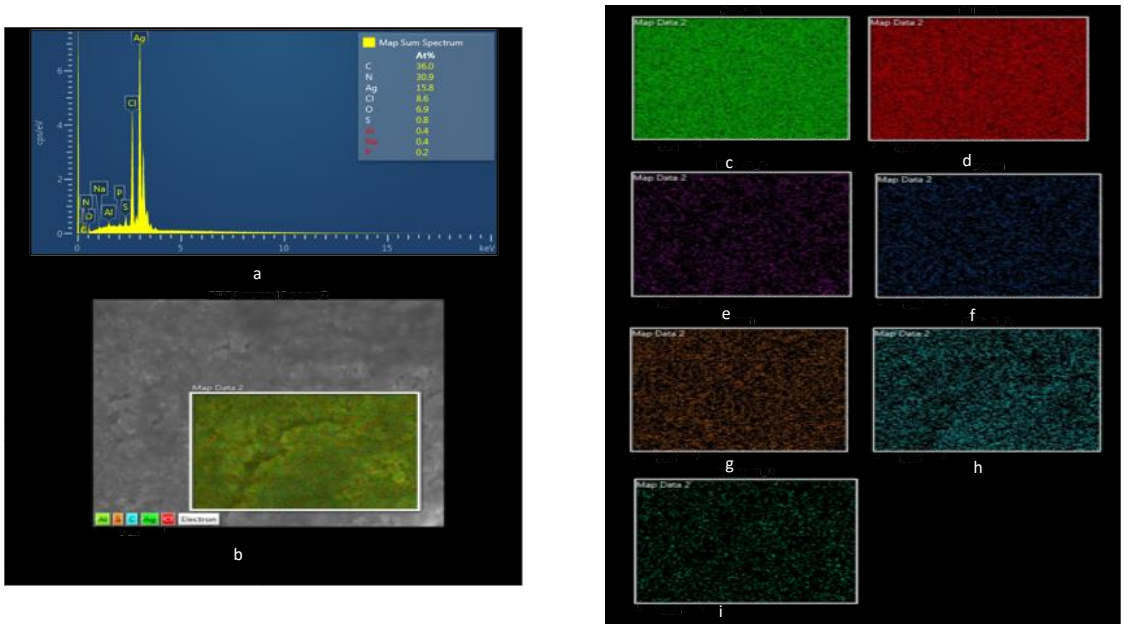

Figure 3B: EDS Analysis of 15-AB-NPs. (a) Elemental composition of 15AB-NPs and, (b) SEM image for 15-AB-NPs. Different elements present are shown in different panels. (c) Ag (Silver), (d) Chlorine, (e) Nitrogen, (f) Phosphorus, (g) Sulphur, (h) Carbon and, (i) Sodium.

The X-Ray diffractogram of 86e-NPs and 15-AB-NPs (Figure 4a and b) showed a crystalline structure. The peaks observed at 32.2°, 46.3°, 55°, 57.6° and 76.8° in the 2θ range, correspond to the (200), (220), (311), (222) and (311) reflection planes, respectively, depicting silver as nanocrystals with face-centered cubic structure of the standard powder diffraction card of Joint Committee on Powder Diffraction Standards (JCPDS), silver file No. 04-0783 (Kumari et al., 2020).

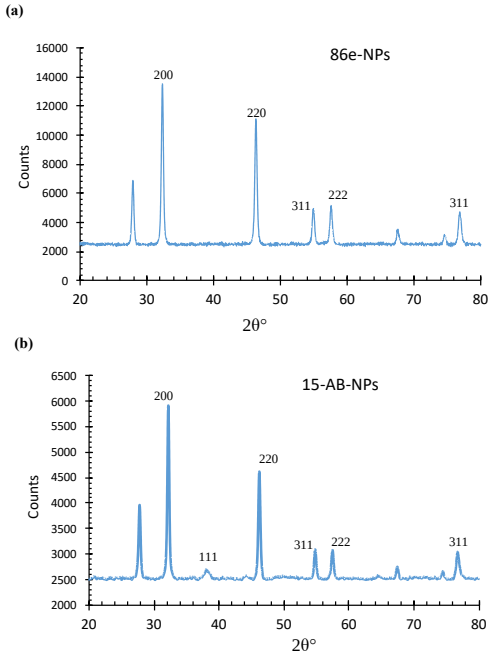

Figure 4. XRD-Analysis of (a) 86e-NPs and, (b) 15-AB-NPs.

86e-NPs and 15-AB-NPs exhibit biocontrol activity

Media containing 86e-NPs and 15-AB-NPs was utilized to analyse the antimicrobial activity against *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus fumigatus*, *Pythium* sp. and *Rosellinia* sp. We found that both 86e-NPs and 15-AB-NPs did not allow the growth of respective fungus on PDA media. The percentage inhibition for both the NPs was almost 80% in which the fungal disc was not able to grow at all as shown in (Figure 5). It is interesting to observe that the CF contents from both the bacterial strains could inhibit the growth in the present formulation with Ag. We also found that AgNPs could inhibit fungal growth, as seen in the AgNPs panel.

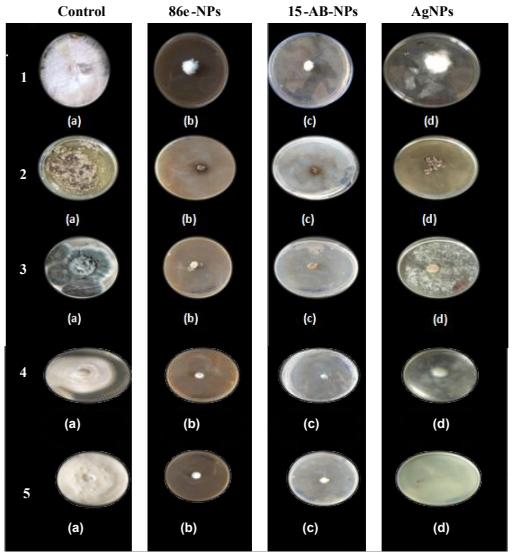

Figure 5. The figure depicts antifungal activity of 86e-NPs (b), 15-AB-NPs (c) and Ag-NPs (d) against different phytopathogens. Row 1 *Fusarium oxysporum*, row 2 *Aspergillus niger* , row 3 *Aspergillus fumigatus*, row 4 *Pythium* sp. and row 5 *Rosellinia* sp. PDA agar plates were supplemented with or without NPs (control) and fungi were allowed to grow. All the experiments are done in triplictaes.

Ghiuta et al., 2018 used *B. amyloliquefaciens* and *B. subtilis*, to synthesize Ag-NPs against *Candida albicans*. In another approach, the bioformulation of Ag-NPs by using the supernatant of *Serratia* sp. BHU-S4 showed antifungal activity against *Bipolaris sorokiniana* which cause foliar spot blotch disease in wheat (Mishra et al., 2014). Mishra et al., 2017 found that *Stenotrophomonas* sp. BHU-S7, could biosynthesize spherical Ag-NPs extracellularly. It was hypothesized that extracellular enzymes (like nitrate reductase) or other proteins present in CF could help in electron transfer to Ag+ ions, yielding Ag-NPs. Another possibility is the involvement of protein based carbonyl groups, -SH, -OH,–NH_2, or –COOH could stabilize the Ag-NPs by binding to their surface. By assisting in providing the binding sites for fixing Ag+ ions. Li et al., 2018 reported the preparation of Au-NPs, Ag-NPs and Au-Ag NPs via protein extracts of *Deinococcus radiodurans* extremophilic bacteria showed low cytotoxicity against non-tumorigenic epithelial cell lines.

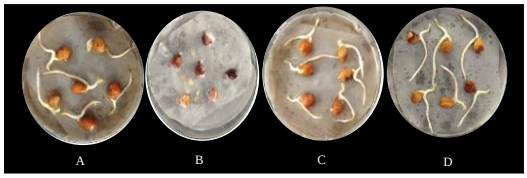

Figure 6A. Effect of Ag-NPs and 86e-NPs on gram seeds A. Normal control seeds, B. Fungus

(*Fusarium oxysporum*) treated seeds, C. Ag-NPs treated seeds and D. 86e-Nps treated seeds.

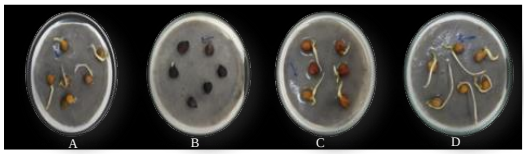

Figure 6B: Effect of Ag-Nps and 15-AB-Nps on gram seeds A. Normal control seeds, B. Fungus (*Fusarium oxysporum*) treated seeds, C. Ag-NPs treated seeds and D. 86e-Nps treated seeds D. 15-AB-Nps treated seeds.

Further, it was found that green silver nanoparticles prepared by *Bacillus* sp. strain GP23 isolated from marine soil inhibit the growth of pathogenic fungi *Fusarium oxysporum*. This fungal strain was responsible for wilting of tobacco, banana, sweet potatoes, legumes, cucurbits and tomatoes (Gopinath and Velusamy, 2013). In another study, graphite and silica NPs were developed using endophytic bacteria Lysinibacillus and plant growth-promoting bacteria *B. subtilis* and *P. fluorescens* reduces the wilting of potato caused by *Ralstonia solanacearum* aerobic non-spore-forming bacteria. Additionally, Lin et al. worked on nanocrystallization in which he made nanocrystals of the cyclic lipopeptides extracted from *Bacillus subtilis* to inhibit the growth of *Aspergillus carbonarius* (Lin et al., 2020). In another study, silver nanoparticles were made by *Bacillus* sp. exhibited antifungal activity against *Colletotrichum falcatum* causal organism of ret rot of sugarcane (Ajaz et al., 2021). Besides, Qin et al. used nanoscale *B. thuringiensis* chitinases to deliver nematicidal material against Caenorhabditis elegans better. Hence, apart from pesticidal activity nanoformulation made from microbes can work as an excellent biopesticidal delivery system (Qin et al., 2020).

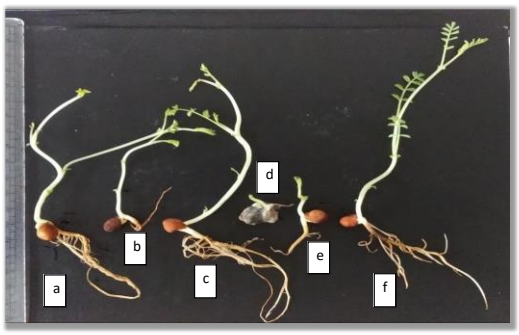

Figure 6c. Growth of seedlings after 16 days as shown in Fig 6A. (a) control seeds, (b) AgNPs treated seeds (c) 86e-NPs treated seeds, (d) fungus treated seeds, (e) AgNPs + fungus treated seeds and (f) 86e-NPs+fungus treated seeds. A representative among 25 seedlings is shown here.

Figure 6d. Growth of seedlings after 16 days as shown in Fig 6B. (a) control seeds, (b) AgNPs treated seeds (c) 15-AB-NPs treated seeds, (d) fungus treated seeds, (e) AgNPs + fungus treated seeds and (f) 15-AB-NPs +fungus treated seeds. A representative among 25 seedlings is shown here.

We previously found that bacterial strains could inhibit the impact of phytopathogens on the growth of seedlings. To provide evidence that the microbial CF-derived AgNPs could inhibit the impact of fungal pathogens, we treated the sterilized seedlings with 86e-NPs and 15-AB-NPs and analyzed the effect of phytopathogens. Seeds treated with normal water or pathogens alone were taken as control. We found that *Fusarium oxysporum* did not allow the seedlings to grow as compared to the water-treated control seedlings or AgNPs treated seedlings (Figure 6 A and B). Interestingly we found that on 8th day the seeds treated with 86e-NPs or 15-AB-NPs (Figure 6 A and B) were able to rescue the impact of the phytopathogens.

When the seedlings were allowed to grow further, we found that there is a remarkable inhibition on decreasing the impact of phytopathogen. The seeds treated with phytopathogen could not grow (Figure 6) compared to uninfected seedlings (Figure 6). Importantly, we found that the seeds treated with 86e-NPs or 15-AB-NPs rescued the growth inhibition by *Fusarium*. We also observed that the AgNPs could rescue the growth inhibition by Fusarium, but the growth of 86e-NPs or 15-AB-NPs treated seeds was better. The average shoot length of untreated seedlings and AgNPs treated seedlings was 11.75 cm and ~7 cm indicating a negative impact of AgNPs. Similarly, the root length of untreated seedlings and AgNPs treated seedlings was 10 cm and ~7 cm. The average root length and shoot length of 86e-NPs or 15-AB-NPs was 9 cm and 10 cm, respectively. Altogether we observed that 86e-NPs or 15-AB-NPs showed an intense biocontrol activity. Surprisingly we found that both the 86e-NPs or 15-AB-NPs dramatically increased the shoot length of seedlings to ~16 cm treated with phytopathogen. Taken together our results

indicate a positive impact of 86e-NPs or 15-AB-NPs on inhibiting the deleterious effects of fungal pathogens.

Conclusions

The application of the nanopesticides on the seeds controls the phytopathogen fungal strains and accelerates their growth, which is a remarkable outcome of the synthesized nanoparticles from the microbes. Generally, these microbes are found in the soil exhibiting biofertilizer activity. The bio-control mechanism is unknown for now, but more research is required to find out the possible mechanism of their activity and the drug delivery system of the nanoparticles to the plants by foliar and root pathways. Also, the impact of these nanoparticles on human health is to be studied. The synthesized nanoparticles using the strains SM-86e and BC-15AB exhibit high biocontrol activity compared to the Ag-NPs. Also, their retention of biofertilizer activity before nanoformulation is also considerable. It directs future studies to confer nanoscale formulations from more bio-fertilizing bacterial species for the pesticidal role to counter the phytopathogen fungal strains that destroy agriculture crops.

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Data statement

Not applicable

Conflict of interest

The authors declare to have no conflict of interest.

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