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Original Research Paper

Dragon fruit peel extract mediated green synthesis of silver nanoparticles and their antifungal activity against *Colletotrichum truncatum* causing anthracnose in chilli

Gowda S.[1,2] and Sriram S.[1*]

[1]Division of Crop Protection, Indian Institute of Horticultural Research,
Hesaraghatta Lake Post, Bengaluru - 560089, Karnataka, India
[2]Department of Biochemistry, School of Sciences, Jain University, Bengaluru - 560027, Karnataka, India
*Corresponding author Email : Subbaraman.Sriram@icar.gov.in

ABSTRACT

Plant extracts have been used as reducing and stabilising agents to synthesise various metal-based nanoparticles due to their cost-effective and eco-friendly nature. In the present work, a green and environment-friendly method is adopted for synthesising silver nanoparticles (Ag NPs) using a biowaste of dragon fruit (*Hylocereus* spp.) peel aqueous extract at 80ºC in an alkaline condition. The Ag NPs were characterised through various analytical and microscopic techniques. The UV-Vis spectra of Ag NPs showed a characteristic peak between 400 - 410 nm. Transmission and scanning electron microscopic studies confirmed spherical monodispersed particles with an average size of 7 nm. Energy-dispersive X-ray spectroscopy (EDX) confirmed the presence of silver and silver chloride among the principal elements. The X-ray powder diffraction (XRD) spectra showed the crystalline nature of synthesised silver and silver chloride nanoparticles. The synthesised nanoparticles showed potential antifungal activity against *Colletotrichum truncatum* spores in both *in vitro* conidial germination and spread plate assays. The efficacy of the synthesised NPs confirmed that these NPs could be used as potential antifungal agents against *C. truncatum* to control anthracnose in chilli.

Keywords : Anthracnose, antifungal activity, chilli, *colletotrichum truncatum,* dragon fruit, green synthesis, silver nanoparticle

INTRODUCTION

Nanotechnology deals with materials in a size range between 1-100 nm. Materials display novel, unique features at this scale. These unusual properties have attracted researchers from different science streams, including agriculture and medicine, to work in the field of nanotechnology (Gowda and Sriram, 2020). Due to their small size, nanoparticles show a larger surface-to-volume ratio and behave differently than bulk materials. Nanoparticles are synthesized by top-down and bottom-up approaches. Metal nanoparticles are commonly synthesized by chemical and physical methods using various toxic, hazardous solvents and chemicals as reducing agents. Moreover, the chemical and physical methods require complex experimental conditions and costly instruments. Green synthesis of nanomaterials is gaining importance due to their nontoxicity, biocompatibility, low cost and eco-friendly synthesis. The plant acts as a potential nano factory since its extracts have been used as a reducing, stabilizing and capping agent for synthesizing nanomaterials. The application of nanotechnology to manage fungal diseases may lead to the development of new nano-based antifungal products, ushering in a new era of nano-fungicide discovery. Nano-based antimicrobial agents have many advantages over conventional agrochemical delivery systems as they possess increased solubility, bioavailability, sustained and targeted delivery, and even give protection from harsh environmental conditions (Chowdappa and Gowda, 2013).

Dragon fruit (*Hylocereus* spp.), well known as Pitaya, is a tropical fruit that belongs to the *Hylocereus* genus and family of cactus, Cactaceae. The fruits are triangular with wide scales. Dagon fruit originated from West Indies and Latin America (Hua *et al.*, 2018). It has gained considerable consumer interest due to its potent nutritional and medicinal benefits. It has been reported that red pitaya fruit is very rich in iron content, and consuming its juice during pregnancy increases hemoglobin and erythrocyte levels and treats anemia in pregnant women (Widyaningsih *et al.*,

2017). The colour of the fruit is due to the presence of Betalains, water-soluble nitrogen-containing pigments. Betalains are plant secondary metabolites that are reported to possess antioxidant, antitumor activities and other health benefits (Tenore *et al.*, 2012). Dragon fruit is usually eaten directly or made into juice. Therefore, fruit peel is the major biowaste. Researchers have explored ways to use dragon fruit peel biowaste as a constituent for microbial growth media (Putri *et al.*, 2017), biochar production (Hu *et al.*, 2020), and biosorbents to remove methylene blue dye from aqueous solution (Jawad *et al.*, 2018).

Researchers have proved that dried pitaya fruit peels are a good source of dietary fiber and pectin (Jiang *et al.*, 2020). The dragon fruit peel is reported to contain higher phenolic and antioxidant capacity than edible portions. It has more flavonoids, total phenols, and antioxidant activities than pulp (Abirami *et al.*, 2021; Nurliyana *et al.*, 2010). The metabolic content of dragon fruits varies based on the fruit's cultivars, colour, size and shape. Nurliyana *et al.* (2010) studied the antioxidant activity of pulps and peels of dragon fruits. They reported that the white dragon fruit peels contained higher phenolic content than the red dragon fruit peel, but the pulp of white dragon fruit had lower Total Phenolic Content (TPC) than the red dragon fruit. They found that peels from both varieties' of dragon fruit contained higher phenolic content and radical scavenging activity than their pulps. This suggests that dragon fruit peel extract (DPE) can be effectively used to synthesize nanoparticles since they are a good source of natural metabolites and antioxidants, which can serve as reducing and capping agents to produce nanomaterials.

The presence of a wide range of reducing phytochemicals in the peel extracts, such as phyllocactin, betanin, betacyanin, hylocerenin, terpenoids, flavonoids, polyphenols, sugars and alkaloids, etc., serve as an excellent source for the production of nanomaterials with various shapes, sizes, compositions, morphology, and crystallinity. Green synthesis of Ag NPs using various plants has been reported in the literature, but only limited studies targeted the biowaste of dragon fruit peel (Aminuzzaman *et al.*, 2019). This study uses an eco-friendly, cost-effective, green synthesis approach to prepare Ag NPs from dragon fruit peel. The synthesized Ag NPs were characterized using various analytical and microscopic tools. The synthesized nanoparticles were tested against *C. truncatum*, a fungus that causes anthracnose disease in chilli.

MATERIALS AND METHODS

Chemicals

Silver nitrate ($AgNO_3$, 99.9%) was purchased from Merck, India. Potato Dextrose Agar (PDA) and Sodium hydroxide were procured from HiMedia Laboratories, India. Whatman No.1 filter paper was purchased from Sigma-Aldrich, India. Deionised water was used throughout the experiment. Dragon fruits were collected from the ICAR-Indian Institute of Horticultural Research (IIHR) experimental farm in Bengaluru, India. All the chemicals were used as received without further purification.

Preparation of dragon fruit peel extract

Dragon fruits were collected from the experimental farm of the ICAR-IIHR, Bengaluru, India. The healthy dragon fruits were hand-picked at a ripe stage, washed once under running tap water, and thrice with deionised water, the peels were separated and oven dried at 60 ºC for two days to completely remove moisture. The dried peel was finely powdered, and the dragon fruit peel powder (1 g) was boiled with distilled water (25 ml) for an hour. The contents were centrifuged for 10 min at 5000 rpm, and the supernatant was collected and filtered using two layers of cheesecloth and Whatman paper (No.1). The extract was carefully collected and used for Ag NP synthesis.

Synthesis of nanoparticles

The Ag NPs were synthesised by mixing 1 ml of peel extract with 100 ml of $AgNO_3$ (1 mM) solution at 80 ±1 °C for 20 min with continuous stirring at 800 rpm in a magnetic stirrer. The pH was altered to 10 with NaOH (0.4 M). The synthesis of Ag NPs was monitored for reaction colour change to yellow. The reaction was continued for 30 min, and the contents were cooled and centrifuged for 30 min at 13000 rpm at 4 ºC. The precipitate was washed with Milli-Q water, re-centrifuged to eliminate any unbound polymers and organic matter and finally dried at 60 °C for 24 h to obtain the Ag NPs, which were used for further characterisation and antifungal studies.

Analytical measurements and characterization

The formation of Ag NPs was monitored by measuring the absorption peak of synthesised NPs in the range of 300-800 nm using a Thermo Scientific UV/VIS

Spectrometer (Genesys 10S UV-Vis). The size, shape and morphology of the nanoparticles were examined by Transmission electron microscopy (TEM) using Hitachi HT7700 (Tokyo, Japan) and field emission scanning electron microscopy (FESEM). For FESEM studies, a drop of the NPs (with an aqueous solution) was placed on an aluminium stub with double-sided copper or carbon tape. Then it was dried under ambient conditions for four hours. The samples were then desiccated for two days. In order to prevent the sample from being charged during the analysis, gold sputtering was done on the sample's surface. The samples were observed using a FESEM (Zeiss-Ultra 55 model; Carl Zeiss; Germany) equipped with an energy-dispersive X-ray (EDX) spectrometer. The XRD patterns of synthesised nanoparticles were recorded on a glass substrate using a Rigaku SmartLab X-ray Diffractometer operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation (λ = 1.5404°A). The XRD pattern of nanoparticles was taken in the range of 5° to 90° in a fixed time mode at room temperature.

Spore germination inhibition assay

The spore germination inhibition assay was done to investigate the antifungal potential of the Ag NPs. Pathogenic *C. truncatum* isolates (NCBI accession number MW677960) from chilli were used for sporulation. *C. truncatum* was maintained on Potato Dextrose Agar (PDA) medium for seven days. The spores were collected by pouring sterile distilled water (5 ml) into the fully grown PDA plate and gently scraping the culture plate with a sterile loop to release the *C. truncatum* spores. The obtained spore suspension was sieved through two layers of sterile cheesecloth to remove any fragments of mycelia. Using a hemocytometer, the spore count of the resulting spore suspension was adjusted to 1.5 x 10^6 conidia/ml.

Aliquots of spore suspension (50 µl) were mixed with NPs (50 µl) of different concentrations (1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.016, 0.008, 0.004, 0.002, 0.001, 0.0005, 0.00025 and 0.000125%) and added to the cavity slide well. The spores were also treated with different concentrations of 50 µl of extracts. Spores mixed with sterile distilled water were placed on the control slide. The slides were incubated for 24 h at 26±1 °C and 95% humidity. Spore germination was measured by counting the spores (germinated and non-germinated) in 10 randomly chosen fields using Zeiss bright field microscope (Axio Scope.A1, Gottingen, Germany) at 200X. When the germ tube length matched or exceeded the length of the conidia, the conidia were considered germinated. The percentage inhibition of spore germination was calculated using the formula, I = (C-T/C) x100, where I is the percentage inhibition of conidial germination in the test, C is the number of germinated conidia in control, and T is the number of conidia germinated in treated samples. The study was done with three replicates (Chowdappa *et al.*, 2014).

In-vitro* antifungal test of silver nanoparticles against *C. truncatum

The *in-vitro* evaluation of synthesised nanocomposites was done against *C. truncatum* on PDA through the spread plate method. Molten PDA medium was poured gently into a 90 mm Petri plate and left for solidification. 200 µl of *C. truncatum* spore suspension containing 2 X 10^6 conidia per ml was mixed with different concentrations of 200 µl of nanomaterials and dragon fruit peel extract (1, 0.5, 0.25, 0.125, 0.062, 0.031 and 0.015%) and incubated for an hour at 26 ºC. The spore suspension treated with sterile water was used as a control. The treated and control spores were then transferred to the Petri plate containing PDA and spread uniformly by gently rotating it with a sterile spreader and incubating at 26 ºC. The assay was carried out in triplicates, and colony growth was observed after 72 h.

RESULTS AND DISCUSSION

In this study, value-addition was done to dragon fruit (DF) peel biowaste by using it as a material for the bio-reduction of Ag NPs. Red dragon fruit peel containing white pulp was used to synthesise Ag NPs. A colour change to yellow confirmed the formation of Ag NPs. The synthesis of nanoparticles was observed by colour change (Kedi *et al.*, 2018). In this study, it was observed that when the $AgNO_3$ solution was mixed with peel extract, the colour of the solution turned brownish yellow, indicating the bio-reduction and formation of silver nanoparticles, as depicted in Fig. 1. There was no nanoparticle formation under ambient conditions and without adding NaOH. The Ag NPs formation was preliminarily observed by the reaction colour change to brownish yellow when the reaction pH was adjusted to 10 at a high temperature (80 ºC). The intensity of the change of reaction colour from light yellow to brownish yellow shows the increased production of Ag NPs by the metabolites of

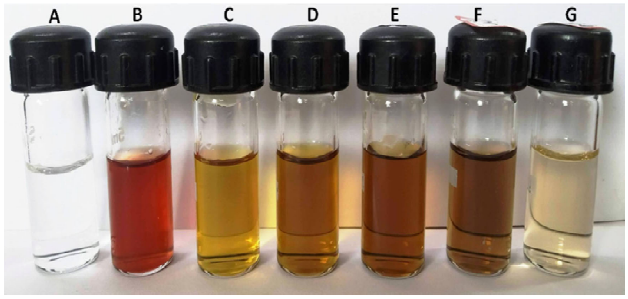

Fig. 1 : Formation of Ag NPs with dragon fruit peel
A) $AgNO_3$, B) Dragon fruit peel extract, C, D, E) Ag NPs synthesized with 0.25, 0.5 and 1 mM $AgNO_3$ at 80 ºC, F) Ag NPs synthesized at ambient temperature, G) Ag NPs synthesized at 80 ºC without NaOH

peel extract. Therefore, the formation of Ag NPs is greatly influenced by elevated temperature and basic pH.

The optical properties of Ag NPs were examined using UV-Vis spectroscopy from 300 to 800 nm. Ag NPs show strong SPR properties because of the collective oscillations of free electrons on the surface of metallic nanoparticles (Haes *et al.*, 2004). These oscillations change with particle size, showing the specific wavelength range in which particles absorb light in the visible spectrum. A larger particle causes a red-shift or a change in the absorption maximum towards higher wavelengths (Politano and Chiarello, 2009). Ag NPs show maximum absorbance at 420 nm (Chowdappa *et al.*, 2014). Fig. 2 depicts the absorption spectra of the synthesized silver nanoparticles. In this work, the maximum absorbance was observed at around 410 nm, which shows the blue

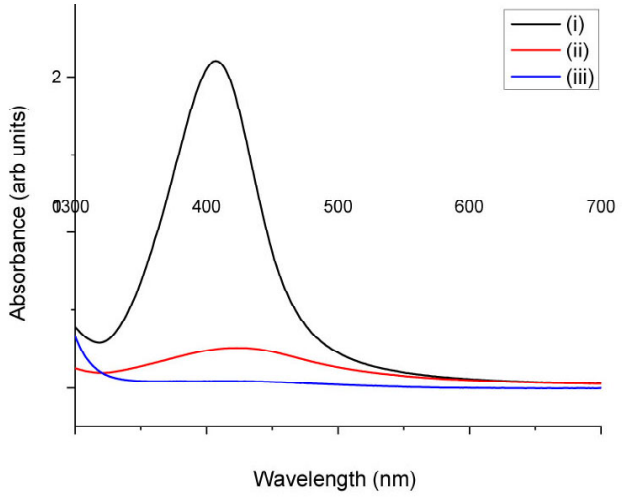

Fig. 2 : UV-VIS Spec observation of DPE-Ag NPs
(i) DPE-Ag NPs synthesised at 80 °C and pH 10, with 1 mM $AgNO_3$, (ii). Ag NPs synthesised at ambient conditions with NaOH, (iii) Ag NPs synthesised at 80 °C without NaOH.

shift and the formation of small-sized Ag NPs. The reaction pH significantly influences nanoparticle size, morphology and formation (Iravani and Zolfaghari, 2013). The synthesis was done under an alkaline condition (pH 10). Previous studies have reported that by changing the reaction mixture's pH, nanoparticle morphology and size could be manipulated (Velgosová *et al.*, 2016). pH alters the electrical charges of biomolecules, thereby influencing the growth of the nanoparticles by affecting their stabilising ability and the amount of nanoparticle synthesis (Mulvaney, 1996).

The morphology and size of the synthesised Ag NPs were examined using TEM and FESEM. The average size of synthesised Ag NPs was 7 nm (Fig. 3). The SEM image of DF peel extract showed a bulky structure, whereas the SEM image of DF peel extract treated with $AgNO_3$ showed spherical Ag nanoparticles formation (Fig. 4). The elemental analysis of Dragon fruit peel extract showed the presence of elements in

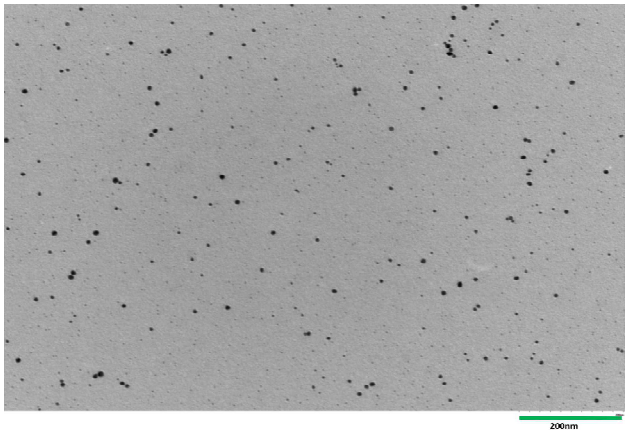

Fig. 3 : TEM of Ag NPs synthesized with dragon fruit peel extract

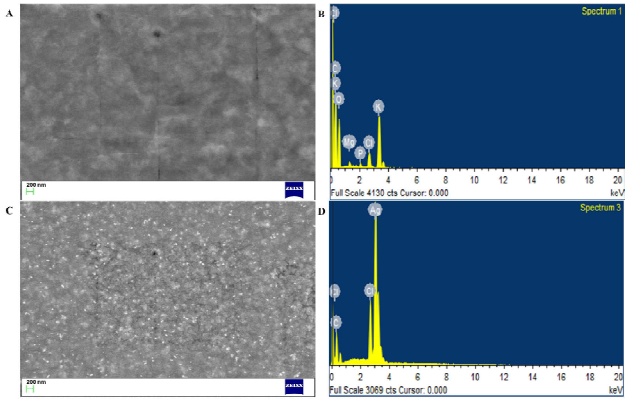

Fig. 4 : SEM micrograph of nano particles
a) Dragon fruit peel extract aggregates, b) EDX of DF peel extract, c) DPE-Ag NPs (1 mM $AgNO_3$), and d) EDX spectra of DPE-Ag NPs.

the peel extract. In contrast, the EDX spectra of Ag nanoparticles synthesised using Dragon fruit peel extract showed a prominent silver and chlorine peak, confirming the formation of silver nanoparticles (Fig. 4). No other peaks have been detected, showing the presence of pure silver in the nano formulation. EDX study shows the presence of chloride ions in the Dragon fruit peel extract, which confirmed the formation of Ag and AgCl nanoparticles. The possible reason for forming AgCl NPs was due to the interaction of the chloride ion in the peel extract and the silver ion from the metal precursor. The EDX of the peel extract confirmed the presence of chlorine. Similar findings were reported by Devi *et al.* (2016). The organic constituents present in the fruit peel extract stabilised AgNP formation.

XRD analysis was done to determine the crystal structure of the Ag NPs. The XRD pattern of dragon fruit peel extract and synthesised nanoparticles were depicted in Fig. 5. The diffraction pattern displayed well-resolved diffraction peaks illustrating crystalline peaks at 2θ values of 38.1°, 44.3°, 64.4°, 77.4°, and 81.5°, which corresponds to the standard face-centred cubic (fcc) crystal lattice planes of Ag (111), (200), (220), (311), and (222) respectively (JCPDS file: 04-0783). Apart from the distinct peaks, the presence of the fcc phase of silver chloride was also observed at 2θ values of 27.8°, 32.2°, 46.2°, 54.8°, 57.4°, 67.4°, 74.4°, 76.7°, and 85.7° that can be assigned respectively to the (fcc) structure planes of AgCl (111), (200), (220), (311), (222), (400), (331), (420) and (422) (JCPDS file: 31-1238). The absence of any other peaks shows the purity of synthesised Ag and AgCl NPs. XRD study of the synthesised Ag NPs showed the formation of sharp and intense diffraction peaks, confirming the crystalline nature of NPs (Awwad *et al.*, 2015). Pattern recognition revealed the production of pure Ag and AgCl crystals. Similar findings were reported in the literature with different plant extracts (Kedi *et al.*, 2018; Siddiqui *et al.*, 2013). The AgCl NPs might have been formed due to the crystallisation of the bioactive components from the DF peel extract (Philip *et al.*, 2011). Ag is the primary material in the composite, as the Ag NP peaks are much stronger than those of the AgCl NPs.

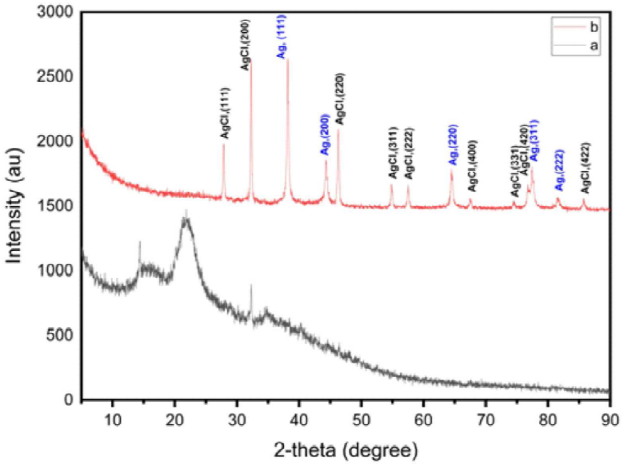

Fig. 5 : XRD pattern of nano formalations
a) Dragon fruit peel extract and b) DPE-Ag NPs

Evaluating the antifungal efficacy of Ag NPs on fungal spore germination is essential because it serves as a primary tool for the preliminary screening of nanomaterials against fungal pathogens (Lopez-Meneses *et al.*, 2018). The antifungal activity of Dragon fruit Peel extract and DPE-Ag NPs (1mm $AgNO_3$) were evaluated by studying its impact on the conidial germination of *C. truncatum* at different concentrations. The Ag NPs effectively reduced the conidial germination of *C. truncatum* more than its counterparts, as shown in Fig. 6. Ag nanoparticles at 1%, 0.5%, 0.25%, 0.125%, 0.062%, 0.031%, 0.015%, 0.008%, 0.004%, 0.002%, and 0.001% concentrations showed 100% inhibition of conidial germination whereas growth was observed below 0.001% concentrations. In addition, conidial germinations were observed in all the concentrations of dragon fruit peel extract (Fig. 7). Normal conidial germination was observed in water. Increased conidia

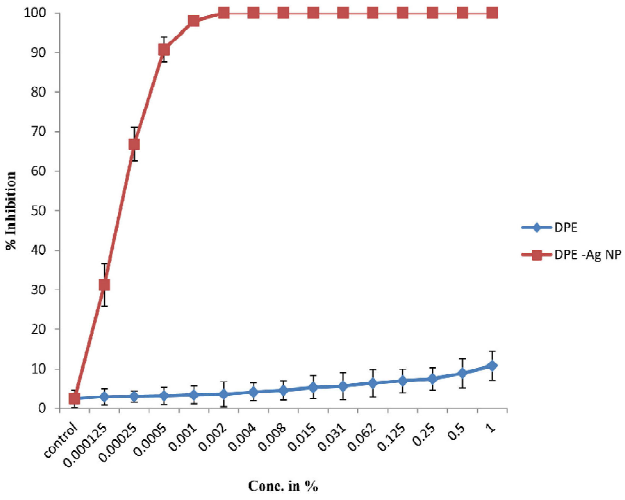

Fig. 6 : Effect of dragon fruit peel extract and DPE-Ag NPs on conidial germination of *C. truncatum* after 24 h of incubation

growth inhibition was observed with the increasing concentration of Ag nanoparticles. Similar inhibitory potential of Ag NPs against *Colletotrichum* was reported by other groups (Chowdappa *et al.*, 2014; Lamsal *et al.*, 2011).

Treating *C. truncatum* conidia with nanoparticles showed structural changes and complete inhibition of conidial germination. Interestingly, nanoparticle treatment also prevented appressoria formation in *C. truncatum,* even at low concentrations. It is apparent from Fig. 7 that normal conidial germination and also appressorial formation is found in conidial spores treated with DF peel extract and control. Melanin production is an important factor in the development of the appressoria structure. The inhibition of appressoria structure in the Ag NP treatment might be due to the reduction of melanin content in *Colletotrichum* by nanoparticles (Wei *et al.*, 2017). Lin *et al.* (2020) reported the inhibition of Anthracnose disease due to reduced melanin synthesis-related genes by Ag nanoparticles. Our previous report also suggested the efficacy of nanoparticles in the management of *Colletotrichum* sp. (Gowda and Sriram, 2020). The inhibition of conidial germination by Ag nanoparticles might be due to damage to cell structure permeability and leaking of the cellular content. Several studies have reported the enhanced efficiency of Ag NPs against *Colletotrichum* species

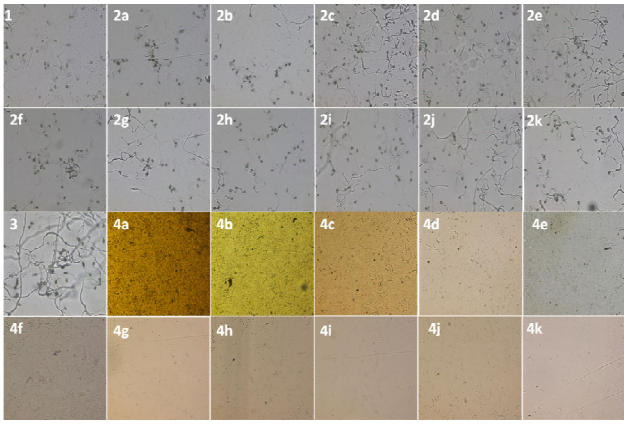

Fig. 7 : Effect of dragon fruit peel extract and DPE-Ag NPs on conidial germination of *C. truncatum*.

1) Spores, 2a-2k) Conidia treated with DF Peel extract 1%, 0.5%, 0.25%, 0.125%, 0.062%, 0.031%, 0.015%, 0.008%, 0.004%, 0.002% and 0.001% concentrations respectively. 3) Conidia treated Water (control), and 4a-k) Conidia treated with DPE-Ag NPs 1%, 0.5%, 0.25%, 0.125%, 0.062%, 0.031%, 0.015%, 0.008%, 0.004%, 0.002% and 0.001% concentrations respectively. Images were recorded at 200 × magnification.

(Aguilar-Mendez *et al.*, 2011; Lamsal *et al.*, 2011). Thus, Ag NPs synthesized by a green, cost-effective approach have a great potential to use as an excellent antifungal agent in controlling spore-forming fungal pathogens.

When evaluating the antifungal efficacy of the synthesised Ag NPs on the colony growth of the *C. truncatum*, it was found that the Dragon fruit peel extract alone did not show an inhibitory effect on *C. truncatum* in a spread plate assay. However, the green synthesised Ag NPs exhibited a potential inhibitory effect on forming fungal colonies compared to the control and DF peel extract. It was evident from Fig. 8 that no colony growth was observed in the PDA

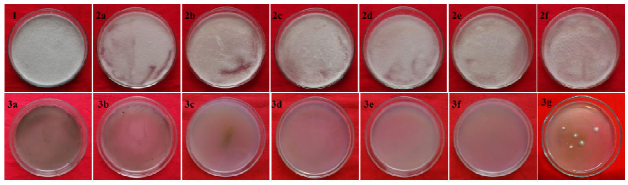

Fig. 8 : Spread plate assay of conidia growth of *C. truncatum* 1. Water, 2a-f-. Dragon fruit peel extract 1, 0.5, 0.25, 0.125, 0.062, and 0.031, respectively. 3a-g. DPE-Ag NP 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015%, respectively.

plates treated with nanoparticles from 1 to 0.031%, whereas growth was seen at 0.015% concentration. In contrast, colony formation was observed in control and dragon fruit peel extract of all the tested concentrations. The fungistatic effect was increased with increasing concentrations of Ag NPs. Due to their small size, high surface area and stability, the synthesised Ag NPs can effectively kill the fungal spores, thereby giving potential antifungal activity. It was also shown that lower concentrations of Ag NPs would be sufficient to kill microbes as they efficiently penetrate microbial cells (Samuel and Guggenbichler, 2004). Ag NPs interact with molecules to disrupt the transport systems and stop cellular functions, including metabolism. Silver ions react with oxygen and form reactive oxygen species, which destroy lipids, nucleic acids and proteins, inhibit ATP production and kill the pathogen (Hwang *et al.*, 2008; Morones *et al.*, 2005). The application of Ag NPs to manage plant fungal pathogens has led to the development of a new class of potential silver-based antifungal agents. With an increase in the application of Ag NPs as a potential antifungal agent, studies need to be done to know their mechanism of action and potential toxicity to animals (Lamsal *et al.*, 2011).

CONCLUSION

The application of Nanotechnology to plant disease management has revolutionised the field of agriculture because of its unique properties. Green and eco-friendly synthesis of Ag NPs offers significant advantages over conventional disease management strategies by being cost-effective and highly efficient at low doses. Silver and silver chloride nanoparticles synthesised in this study exhibited significant inhibition against *C. truncatum* spores even at low doses; hence could be considered as a potential antifungal agent against *Colletotrichum* infections in chilli.

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