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Bioactive bile salt-capped silver nanoparticles activity against destructive plant pathogenic fungi through in vitro system

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Abstract

Colletotrichum gloeosporioides is the most destructive endophytic plant-pathogenic fungi causing Anthracnose disease in a wide number of economically important plants throughout the world. By far there is no existing methods possessing an effective disease management over the outbreak of anthracnose disease and it's precede to huge economic losses for formers and plant tissue culture laboratories. In order to find a new and effective control measure over these endophytes, with aid of nanotechnology, we synthesized bioactive bile salt of sodium deoxycholate (NaDC) capped silver nanoparticles and satisfactorily characterized. Further it was employed to control the endophytic fungus through *in vitro* direct and indirect model system with time dependant/light mediated incubation manner. In our findings we achieved fivefold synergistic effect of NaDC-capped AgNPs with their bioactive capping agent against *Colletotrichum gloeosporioides* upon analyzing different parameters with typical instruments. Moreover, it was evident that NaDC-capped AgNPs did not cause any phytotoxicity to treated plants revealed by molecular markers.

Keywords: silver nanoparticles, sodium deoxycholate, antifungal, plant tissue culture, contamination, endophytic fungus, light irradiation, phytotoxicity

Abbreviations used:

AgNPs — silver nanoparticles

NaDC — sodium deoxycholate

$AgNO_3$ — silver nitrate

MS — Murashige and Skoog medium

RAPD — Random amplified polymorphic DNA

Introduction

In vitro plant tissue culture offers mass cultivation and conservation of endangered and endemic flora, including medicinal and ornamental plants, principally with the production of disease-free planting material in a short time interval. In fact, a successful tissue culture mostly depends on the removal of exogenous and endogenous contaminating microorganisms[6,8], which cause diseases in the host plant. 3–15 % losses due to contamination by bacteria, fungi and viruses at every subculture in the majority of commercial and scientific plant tissue culture laboratories have been reported[18,28,29]. The explants initially undergo surface sterilization for removal of contaminants by chemicals (mercuric chloride, sodium hypochlorite, hydrogen peroxide, fungicides, bactericides and antibiotics) and surfactants, which are fundamentally phytotoxic nature as well as retard or inhibit plant growth[23,53], although using surfactants has only been effective in eliminating all exogenous contaminants of explants but not endogenous contaminants[43]. Endophytic microorganisms are microbes that colonize within plant tissues and may exhibit symptoms in their hosts. The number of endophyic species potential associated with a plant species is often estimated to several hundred[47]. The most frequently isolated endophytes are fungi, and these biotropic contaminants are particularly dangerous when they are identified as plant pathogens[58]. Among these, a fungal phytopathogen named *Colletotrichum gloeosporioides* causes anthracnose disease[17], which has posed serious problems worldwide in the cultivation of economically important plants like coffee[57], olive[32], mango[33], apple[9], citrus[2], strawberry, bean[37], rice[13] and other crop plants. With the aim of developing a model control methodology, we initially guide ourselves to find an alternative to chemically manufactured surfactants efficient against the endophytic fungus. Nanotechnology, the fantasy field has a strong unimaginable virtue on

recreate the older technological aspects to more beneficial of new one. It is emerging scenario at every science laboratories since it is an extremely universal discipline. Nanotechnology literally and physically means to manipulation of various kinds of nano sized particles mostly called nanoparticles. Among the various kinds of nanoparticles, silver has a special attention due its numerous physiochemical properties and mostly it is recognized by for its outstanding antimicrobial activity even on multi drug resistant microbes. At various forms, silver compounds have been exploited as antimicrobial agents since ancient times and are now it's becoming a more commonly used in the medical field[38,45,48.] In fact, both silver ions (Ag^{+}) and silver nanoparticles (AgNPs) display multiple modes of inhibitory action against several pathogenic microorganisms[7], but the mechanism of action of AgNPs (Ag^{o}) differs from Ag^{+} ($AgNO_3$ as a source). The degree of activity depends on concentration[49], sensitivity of the microbial species to silver[14],external factor like irradiation[16], and most significantly, surface physiochemical properties of AgNPs which are given by capping agents that are used to synthesize nanoparticles[19,55.] Therefore, we chose a naturally-occurring mammalian bile salt named sodium deoxycholate (NaDC) as a reducing agent for synthesizing AgNPs. The bile salt has a proven record as a potential bioactive agent against salmonellae[40], enteric bacteria and STD diseases[39,41,46,50,5.] Elsewhere, most of the AgNP-aided antimicrobial activities were carried out by concentration dependant which where undoubtedly gives more toxic to organism and environment too. Though, in a different way, we demonstrate time dependant with light irradiation mediated indirect (tissue culture*)* and direct (agar plate assay) antifungal activity of NaDC-capped AgNPs against *Colletotrichum gloeosporioides*. In support of this experiment, we also studied uptake and movement of nanoparticles into plant. Despite the potential activity against microbes, AgNPs

generating possible cytotoxicity and genotoxicity to treated plants were studied with RAPD molecular markers[35,1]. From this study, we aim to eliminate the *Colletotrichum gloeosporioides* contamination thorough *in vitro* system using NaDC-capped AgNPs as a new strategy of plant disease management for sustainable agriculture productivity with disease free planting materials.

Materials and methods

Chemicals

Silver nitrate (99.99%) and Sodium deoxycholate (~98%) were obtained from Sigma-Aldrich. Sodium hypochlorite (NaOCl), dextrose, agarose, ethidum bromide, MS medium components, hormone kinetin and lactophenol cotton blue were purchased from Himedia laboratories, India. For TAE (tris-acetciacid-EDTA) and Delloporta buffer, all chemicals were purchased from Sisco research laboratories and Loba chemicals. And PCR reaction mixture obtained from Amplicon QIII. All glassware's were thoroughly cleaned with aqua regia (HCl: HNO3=3:1), rinsed with double distilled water and dried in a hot air oven prior to use.

Synthesis of AgNPs

Stock solutions of 4mM $AgNO_3$ and 0.1M sodium deoxycholate (NaDC) were prepared using deionized double distilled water, and the subsequent dilutions were prepared from this stock solution. To study the effect of pH on AgNPs formation, 0.15 mL of $AgNO_3$ stock solution was mixed with 5 mL of 0.05 M NaDC at different pH, and the reaction was carried out under UV light irradiation at 365 nm[24]. To synthesize AgNPs for antifungal activity, about 1.5 mL of $AgNO_3$ stock solution was mixed with 50 mL of 0.05 M NaDC at neutral pH, and the reaction was carried out under UV light

irradiation at 365 nm with constant stirring for 7.5 hours. The formation of AgNPs was observed by the gradual change in color of the solution from colorless to yellow[3]. And the nanoparticles production yield was quantitatively calculated by the help of UV/visible extinction spectra of NADC synthesized silver nanopartilces according to Marquis's statement[34]. Fixed volumes of freshly synthesized AgNPs colloidal solution were subjected to high speed centrifugation (BW-HRC-16M, No.1 angle rotor, Lark Innovative Technologies, India) at 10,976 × g for 10 min in 15˚C to complete the sedimentation of nanoparticles. The pellet of nanoparticles was harvested and dissolved in ultra purified water for further characterization and applications. For investigating the stability of nanoparticles, the synthesized colloidal solutions of aliquots were kept in 15º C to 40 º both in light and dark.

Characterization of AgNPs

UV-visible absorption spectra were measured using a Shimadzu UV-1601 spectrophotometer over the 250–700nm range. High resolution transmission electron microscopy images were obtained using a HRTEM instrument (FEI TECHNAI, G2 MODEL T-30 S-TWIN) at an acceleration voltage of 250 kV. The samples were drop casted onto a carbon-coated copper grid and allowed to dry at room temperature before analysis. The hydrodynamic diameters of the synthesized AgNPs were measured using dynamic light scattering analysis (DLS-Nanotrac Ultra NPA 253 from Microtrac, USA).

Indirect antifungal activity of NaDC Capped AgNPs through tissue culture

Phyllanthus amarus plants were selected and used for this *in vitro* tissue culture study. There are two valuable reasons behind the selection of this well-known medicinal plant[27]as a test plant: it is one among the hosts for *C. gloeosporioides*[51],and

endophytic contamination produces toxic metabolites colletotrichin and ferricrocin[21,22] that may affect the quality of the medicinal herb used as a raw formulation for drugs worldwide. The explants of *Phyllanthus amarus* were collected from the Herbal Garden belonging to Department of Botany, University of Madras, Chennai, India. The explants were kept under running tap water for 30 min, adding two drops of detergent (Tween 20), followed by a thorough rinse with double distilled water 2 times. The nodal explants were 1.5–2cm in length incised aseptically with sterile blades, and they were surface sterilized with 1% sodium hypochlorite (NaOCl) for 4–5 min, after the nodes were thoroughly washed with sterile distilled water 3–4 times. The plant tissue culture-specific MS medium was prepared by the standard procedure outlined by Murashige and Skoog, 1962[30]. The medium was also fortified with kinetin hormone (0.5mg/l) for shoot proliferation. Borosil glass tubes (25 × 150mm) each containing 15ml of the culture medium and capped with plugs of non absorbent cotton were autoclaved at 120˚C for 15 min. Initially, few explants (shows no morphological symptom) were screened for the endophytic contamination by inoculating on the MS medium following the existing standard methodology[52]. After positive confirmation of contamination, emerged fungi were isolated (fungal-contaminated one-week culture) by inoculating them on potato dextrose agar (PDA: peeled potato 250g, dextrose 20g, agar 15g, mixed in 1L distilled water) plates followed by incubation at 37 ºC for seven days under a 12hr photoperiod. Pure isolates were obtained from repeated sub-culturing of the isolates. They were then transferred to a clean glass slide and stained with a drop of lactophenol cotton blue. The stained slide was visualized under the light microscope for the identification of fungal isolates. Fungal isolates were identified using cultural characteristics and morphology and by comparing with standard taxonomic protocols indexed by Barnett

and Hunter, 1972[4]. After that, the NaDC capped AgNPs efficacy to eradicate disease-causing organisms on plants was examined (on the basis of contamination generated) at *in vitro* tissue culture system. The *C. gloeosporioides* infected explants of *P. amarus* were taken into separate 20 ml test tubes and treated (completely submerged) separately with the following three solutions: NaDC capped AgNPs (1mL), 0.1M of NaDC (1mL), and 1mM $AgNO_3$ (1mL), respectively, in the presence/absence of light irradiation at various time intervals (30, 60, 90, 120 and 150 min). Both $AgNO_3$ and NaDC-treated explants were considered as control (C) and reference (R), respectively, for AgNPs-treated explants. For this treatment, molar concentrations of the control (NaDC) and reference ($AgNO_3$) solutions were the same used at AgNPs synthesis. In this experiment, the culture chamber white light with 3,000 lux were used as a source of light irradiation with distance of 0.5 meter to treat the samples. At the completions of every incubation time both at light/dark, the treated explants were washed with ddH_2O at two times and left for drying for a few minutes. Then all the treated explants were taken and vertically implanted in each culture tube containing MS medium. All the treatments were carried out with proper care inside the laminar air-flow chamber to avoid external microbial contamination. Furthermore, cultured tubes were kept inside the tissue culture chamber at a temperature of 25 ± 2˚C for 16/8 hours light/dark cycles per day. At least seven replications with six explants were maintained and evaluated for each treatment. The result was evaluated from the 4^{th} and 5^{th} day of inoculation and the percentage of contamination was calculated by measuring the width and height of the appearance of fungal growth around the explants. In relevance to this anifungal activity, fungal morphology were investigated before and after treatment of AgNPs through simple light microscopy. By our curiosity to understand the possible uptake and presence of AgNPs into the treated

plant, was analyzed by UV-Vis spectra and HRTEM with EDX (Energy-dispersive X-ray spectroscopy). For this UV- Vis spectra analysis, AgNPs colloidal solution alone and AgNPs with explants incubated were used. For HRTEM analysis, through ultramicrotome obtained semithin plant section (1µm) were placed on a copper grid and fixed with 4% paraformaldehyde and 2% glutaraldehyde then postfixed in 1% Osmium oxide, all in cacodylate buffer (0.05M, pH 7.0). Further the samples processed with 2% uranyl acetate, dehydrated in acetone and fixed in Epoxy medium. Finally the sections were used to get HRTEM images and EDX was performed to determine mineral identification, especially here for silver.

Direct antifungal activity of AgNPs by *in vitro* Petri-plate assay

Fungal spores were picked up from 2-week-old growing *C. gloeosporioides* cultures with a sterile inoculation needle and suspended directly into required number of eppendorf tubes each containing 100µl of AgNPs colloidal solution. Further, the tubes were incubated at different time intervals (30, 60, 90, 120 and 150 min) in both dark and light with same condition described in previous section. After incubation, 100µl of conidia was taken from each tube and spread on petri dishes containing PDA agar. All the petri dishes were incubated at 26 ± 1˚C in a light chamber. After the 4^{th} day of incubation, direct antifungal activity of AgNPs was qualitatively assessed (visually) by the number of colonies that appeared in the petri dishes. Subsequently, fungal morphological changes were also investigated with Field Emission Scanning Electron Microscopy (FESEM-Hitachi SU600) using the same silver blend at all respective incubation timings. For FESEM analysis, specimens were prepared by the existing protocol[51].

Phytotoxicity analysis

The study of possible cytotoxicity generation by the silver nanosolution to treated plants was assessed by external morphological changes like growth inhibition, necrotic spots in leaves. The treated plant parts were also subject to a genotoxicity analysis with RAPD molecular markers. A predominant technique, RAPD, has been used to reveal DNA damage or molecular alterations in all the levels of plant gene alterations[56,54,12]. From the collected plant parts, genomic DNA was extracted using the standard procedure outlined in Dellaporta, *et al*.1983[11]. The RAPD decamer, OPK-7 (5′-AGC GAG CAA G-3′), specifically obtained from Operon technologies, Inc., USA, was screened and selected for further analysis based on its ability to detect distinct polymorphic amplified products between treated and non-treated mother plants. The reactions were carried out in a DNA-gradient thermocycler (Peltier thermocycler model L196GGD, Lark Innovative Technologies, India). After the reaction was completed, 20µl of the PCR-amplified products was subjected to electrophoresis in a 2% agarose gel immersed in 1x TAE buffer at 100V for 2.5hr. The gel product was stained with ethidium bromide and analyzed in a UV transilluminator (Lark Innovative Technologies, India).

Results and Discussion

Characteristic features of NaDC capped AgNPs

In order to optimize the reaction condition, 150µL of $AgNO_3$ stock solution was mixed with an aqueous solution of 0.05M NaDC in 5 mL and the reaction was carried out under UV light irradiation at 365nm with different irradiation time. The color of the solution was gradually changed from colorless to intense yellow. The UV-visible spectrum of the prepared AgNPs shows a characteristic SPR peak at 408nm (Fig.1a). Furthermore, sizes of the obtained AgNPs were examined by high resolution

transmission electron microscopy (HR-TEM) and dynamic light scattering (DLS). Fig.1b., HRTEM image showed that the formed particles were nearly monodispersed and spherical in shape with size ~21 ± 2 nm in diameter. The average size of AgNPs was found to be 22 nm in the DLS analysis and their sizes were consistent with the HRTEM observations (Fig.1c). Regarding yield of AgNPs, the AgNPs were quantified to have a concentration of 0.02mg in 1ml (20ppm) of the silver colloidal solution according to Marquis's statement of UV/visible extinction spectra analysis[34]. Time-dependent UV-visible spectra were also performed to evaluate the kinetics of the formation of AgNPs (Fig. 2a). The intensity of the SPR band gradually increased with time and finally reached the saturation level within 7.5 h, after which no significant changes in the absorbance intensity were observed that confirmed the completion of reaction. The effect of pH on AgNPs formation was studied at different pH values, ranging from 7.0 to 11.0, under UV light irradiation at 365nm as shown in Fig.2b. At pH 11.0 maximum intensity of the SPR peak of AgNPs was noted; however, increase in the pH from neutral to alkaline the intensity of the SPR band gradually decreased with a small blue shift. This phenomenon indicated AgNPs were agglomerated in basic pH while stable dispersion was noted in neutral pH. No reaction was observed at pH below 7, this is because in acidic pH sodium deoxycholate was precipitated in the form of deoxycholic acid. The synthesized silver nanoparticles were very much stable and monodispersed condition in 15º C to 40 º at dark kept for four months (UV-Spectrum data not shown).

Indirect antifungal activity of NaDC-capped AgNPs

Endophytic fungus isolated from contaminated *in vitro* cultured plants (5th day of explants inoculation) was grown on fungus-specific medium (PDA) and had a grayish-white appearance. The isolated fungus was further identified through

microscopic observation based on its morphological structure[4]. Light microscopic images show that the conidiogenous cells are acervular, enteroblastic, phialidic, separate, composed of hyaline to dark brown septate hyphae of *Colletotrichum gloeosporioides* . A similar identification of *Colletotrichum gloeosporioides* is seen in two reports[15, 52]. From 4th to 5th day of inoculation of explants, inhibition effect was measured on formation of radial hyphal growth around the treated plants within tube and the data were obtained calculated mean from the seven replicates. The $AgNO_3$ (R) solution treated shows decreased level of contamination, light treated has 94.74%–70.41% and dark has 98.54%–86.81% from 30min to 150 min (Fig.3a). In NaDC (C) with light irradiation, treated explants showed 99% fungal contamination level around the plants at both 30 and 60min, and other tubes showed decreased contamination level which has 94.9% to 90.7%. But dark-treated NaDC (C) exhibited 99% contamination growth in all the tubes (Fig. 3b). Interestingly, NaDC-capped AgNPs treated in the presence of light alone showed a gradual decrease in the contamination level (90.21%-50.52%) from 30–90min; amazingly no fungal colonies were observed remaining 120min &150min treated plants (Fig.3c). We obtain clear evidence for fungal growth inhibition through light microscopic analysis, figure 3 d & f shows fungal morphology before and after AgNPs treatment (150 min incubation) respectively. At dark treated with AgNPs, contamination appeared in all the tubes and the rate of contamination merely decreased (98.5%-92.2) from 30 to 150min, which is clearly represented in bar graph of Fig.3c. From the cumulative results, both well-established antimicrobial agents like NaDC [5, 39] and $AgNO_3$ [20] had not sustained in eliminating the endophytic fungal contaminants which reside inside the plants. The outcome of these experiments clearly revealed that NaDC-capped AgNPs under longer time light exposure had produced a highly affirmative result on fungal

inhibition activity. From the certainty of results, it was confirmed that light irradiation played a crucial role in the activation of AgNPs to inhibit *Colletotrichum gloeosporioides* growth. This phenomenon very well correlates with a previous report[16]: irradiation may induce the photo-ejection of electrons, causing oxidation or ionization at the NP surface and segregation of metal ions. For the authentication of silver dissolution by light and its consequence to uptake into plants were studied through UV-Vis spectral analysis. It was carried out with the AgNPs solution alone and AgNPs with explants, incubated separately at light exposing times (0, 30, 60, 90, 120 and 150 min) were same as previously examined. Obtained spectral data (Fig.4a-b) ensures a fair understanding of the dissolution of ions and the capping agent. The degree of dissolution increases while the time of light incubation increases and AgNPs alone was not much dissolute compared to AgNPs incubated with explants. The dissolute solution has to be named "nanomixture". Since this was preliminary data, we intensively followed the TEM and EDX analysis to know the fact of uptaken of AgNPs into treated plant. For TEM analysis of prepared AgNPs treated plant specimen, we had a track of silver nanoparticles and its dissolution stage in the vascular region (Fig. 4c & d). It seems like an oozing out of silver ions from the AgNPs at vascular tissues. Figure 5e shows, EDX spectrum of particular region of AgNPs dissolution indicated a strong signal that it was silver elements. Therefore, it was confirmed that both light exposure and explants proximity accelerated the dissolution of AgNPs to metal ions along with the capping agent. Meanwhile, the gradual extension of time employed to active adsorption via the stomata was well possible such like active adsorption of nutrient medium into tissue culture grown wounded plants. Once taken up, transport within the plant occurs as a mass flow via the stomata[10]. Hence, there is a rapid uptake of the nanomixture solution into plant

cells leading to internalization and localization of the endophytic environment might disrupt the metabolic activity by the accumulation and interactions with macromolecules found in the fungal body. Long time exposures of the nanomixture solution to microorganisms would increase the surface contact to microorganisms, which has been known to alter a few significant biochemical pathways would proceed to kill the organisms[18, 31].

Direct antifungal activity of NaDC capped AgNPs

In order to reveal the effect of AgNPs directly against endophytes on the 4th day post-inoculation, inhibition levels on colony formation of *C. gloeosporioides* decreased on PDA plates randomly when the incubation time increased (Fig.5a-f). Apparent colony formation is seen at 90min incubation time and none thereafter. Dark incubation showed the highest number of colonies on all the PDA plates (figure not shown). The result demonstrates an excellent AgNP activity only in the presence of light shown in graph (Fig. 5g). These assay results bear much similarity with previous results which were obtained from the explant-treated assay. The FESEM investigation on the same blend at the same incubation times has pictorially listed out in Fig.6a-f. The microscopic images clearly show the cell depletion (shrinking and cracking) of fungal structures at the respective incubation time in a progressed level. AgNPs irradiated by light forming a nanomixture solution have been understood to be the mechanism behind this activity. The nanomixture is subsequently excited by prolonged light irradiation and is more involved in the catalytic activity on the surface contacting endophytes. However, approach on the surface of *C. gloeosporioides* disrupts the cell structures through the generation of reactive oxygen species that react with molecular oxygen present on the surface of endophytes [25].

Phytotoxicity impact of AgNPs

$AgNO_3$ (R)-treated explants left some silver metal-like trace all over the explants which later progressed to a gray black colored stem with reduced growth (figure not shown). Hence, ionic Ag causes discoloration by itself or allows other organic materials to cause undesirable coloration while directly treated. However, in the case of NaDC (C)-treated explants both light/dark, no morphological damages were observed, but the originated contamination was reduced the growth of the explants. The light irradiated AgNPs treated explants showed no undesirable changes on plant growth and no negative morphological changes were observed. After 45 days, AgNPs treated *phyllanthus* plants grown upto 11 cm with no detrimental morphological changes and it were successfully acclimatized on field. According to the result, it was affirmed that the nanomixture-containing bio active capping agent might reduce the toxicity of dissoluted Ag^+ ions in treated plants. With the help RAPD molecular markers, genotoxicity of treated plants was revealed intensively at Figure. 7. The gel image shows a 100 bp marker in the end lane, and the remaining four lanes show PCR-obtained RAPD products which were sourced from all the three treated (lane 2-AgNPs, lane 3-NaDC & lane 4-$AgNO_3$) explants and one untreated mother plant as control (lane 1), respectively. The sizes of the amplified fragments are obtained from the 100 bp to 1kb range. A similarity index was calculated on all four banding patterns which were counted by the presence (indicated as '1'), absence (indicated as '0'), and the presence of faint or unclear bands (which were not considered part of the count). A total of 10 bands of different kb were obtained from the control untreated plant, and same numbers of bands of similar molecular size were found in both NaDC-capped AgNPs and NaDC-treated plant (lane 2&3). Adversely, polymorphic bands were identified only in $AgNO_3$-treated plants (lane 4). According to the

similarity index calculation, the NaDC-capped AgNP-treated plant clearly shows no mutational changes on genomic DNA; genetic stability was retained at 100% in the NaDC-capped AgNPs treated plant as well as in NaDC-treated plant. Therefore, only $AgNO_3$, as ionic in nature, caused cytotoxicity and mutational changes at the gene level and could confirm the genotoxicity to the plant also previously reported [42].

Conclusions

This experimental outcome ensured that the prolonged light exposure of NaDC-capped AgNPs to the close proximity of plant tissues could reason for gradual dissolution of silver ions along with capping agent. However, by HRTEM with EDX analysis pave the way to confirm the dissolution of AgNPs inside the treated plant and UV-vis spectra also confirmed that the explants treated solution contains a blend of Ag ions, AgNPs, and capping agent in different ratio. The mechanism behind the successful antifungal activity of a nanomixture was, it agitated by prolonged irradiation could motivated then actively transported via stomatal opening and localized in the endophytic environment which effectively reduced the *Colletotrichum gloeosporioides* contamination with no toxicity on treated plants. Except our report, there were no reports attained 100% removals of *Colletotrichum gloeosporioides* fungal contamination with aid of fungicides and even by silver nanoparticles[26]. And also bioactive salt NaDC capped AgNPs proves that 100% genetic uniformity and stability retained than the $AgNO_3$ treated plants analyzed by RAPD markers. This *in vitro* model study could offer potential key to use AgNPs as potential antifungal agent to maintain high degree of sterility against endophytes in tissue culture and agricultural crops. Since the world needs more and more novel products for the well-being of humankind, definitely our findings on control of phytopathogens would

create one of the effective and everlasting methods to replace the toxic chemical usage. But in *invivo*, there should be certain parameters must follow to obtain an effective result with no environmental adverse effects. There has been only few studies conducted and reported the possible toxicity of silver nanoparticles and silver ions to plants, here we firmly says through various analysis, the mechanisms of silver nanoparticles generated toxicity was very much relevant to its capping agent and response to plants which employed. Therefore, bioactive salt capped AgNPs may be used with relative safety for control of various non specific plant pathogens compared to highly toxic synthetic fungicides[36,38,44]. The efficacy of the NaDC capped AgNPs provides long-term residual that is perfectly safe and acts quickly compared with other antimicrobial agents. This technology could effectively minimize the expenditure spend for plant disease management and definitely would increase the income where it should be executed.

Acknowledgement

The authors thank Centre for Nanoscience and Nanotechnology, University of Madras for Instrumentation facilities.

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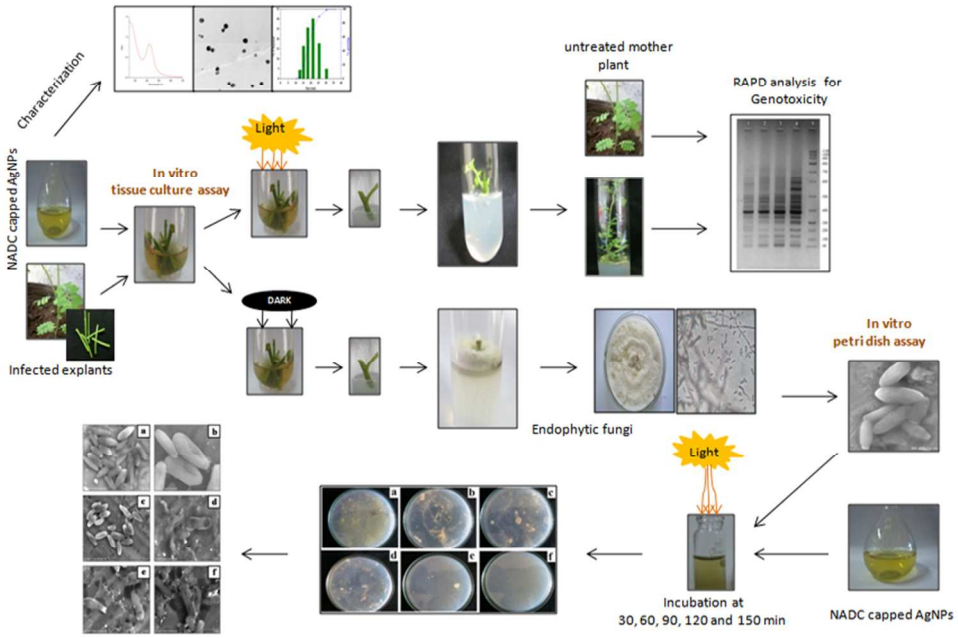

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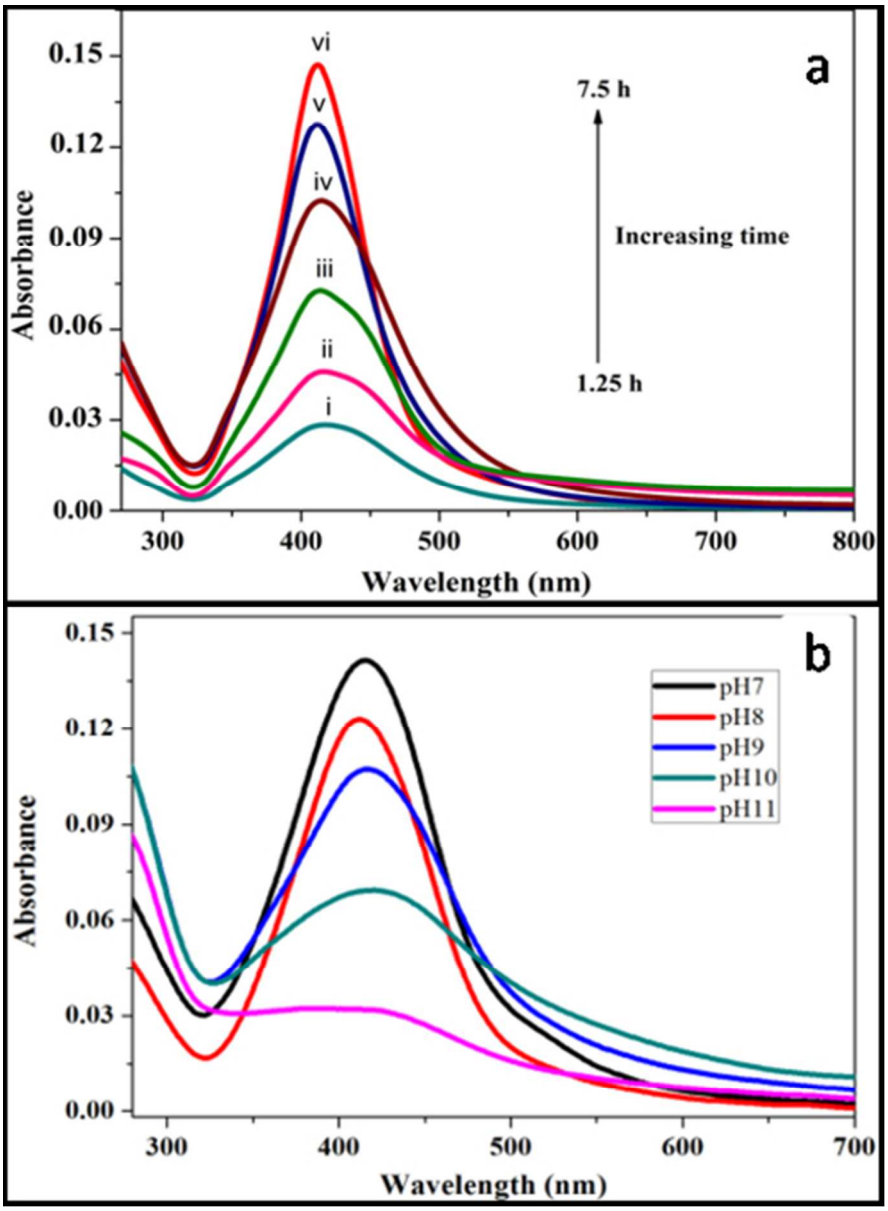

Figure: 2
Kinetics of formation of NaDC capped AgNPs analysis: (a) various time-dependant UV–visible spectrum, (b) various pH-dependant UV–visible spectrum.

45x61mm (300 x 300 DPI)

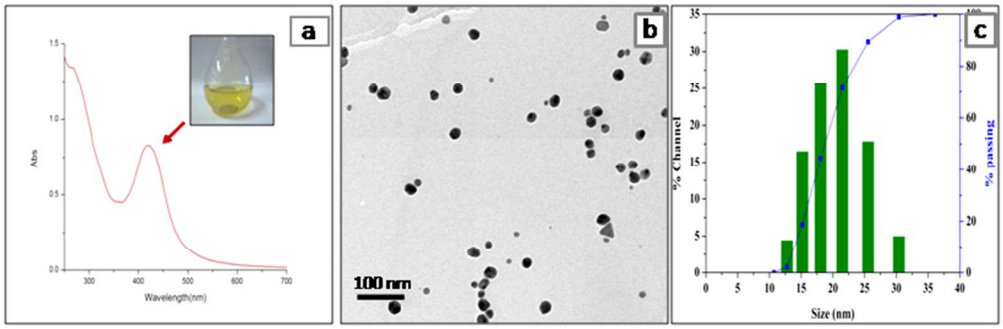

Figure: 1. AgNPs synthesis & Characterization: (a) UV–Vis absorption spectra of NaDC capped AgNPs colloidal solution, (b) HRTEM micrographic image of AgNPs at100nm scale bar, (c) DLS histogram of NaDC capped AgNPs.

207x68mm (100 x 100 DPI)

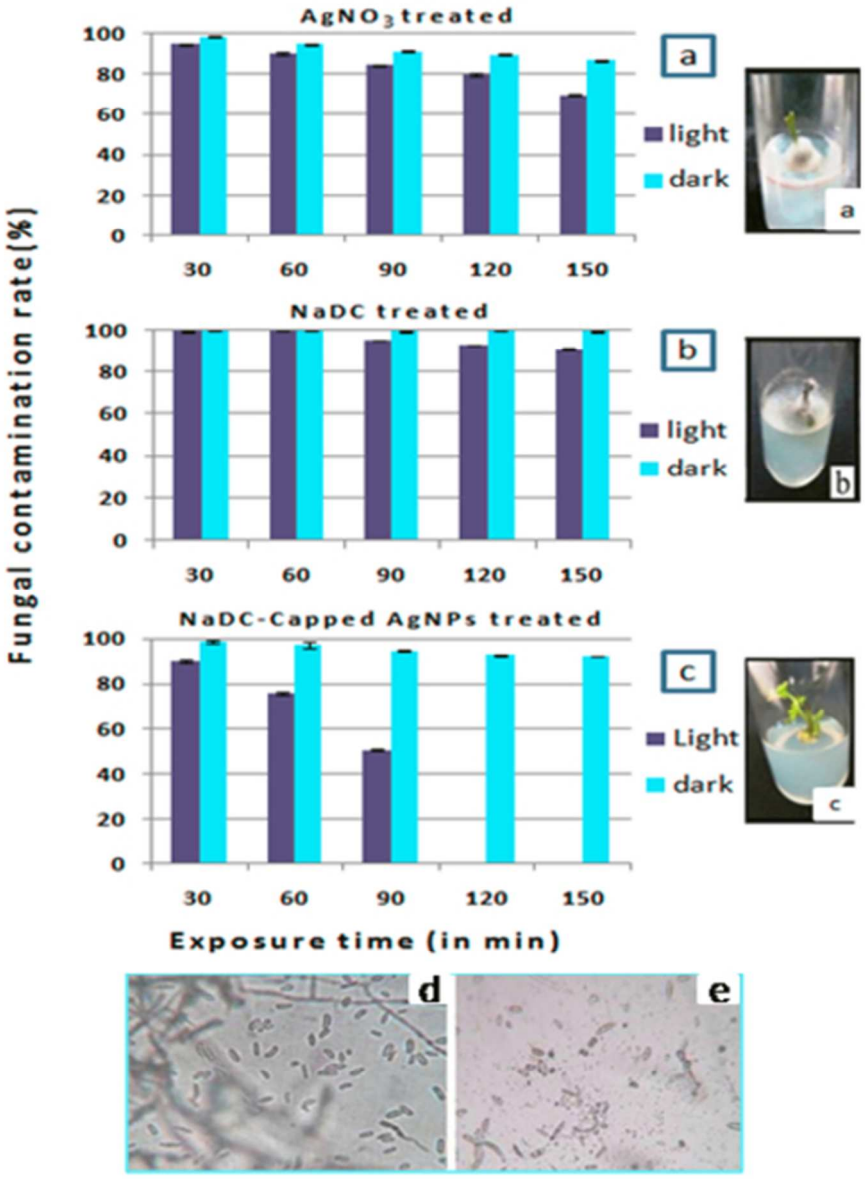

Figure: 3. Histogram along with photographs represents the percentage of contamination rate for in vitro tissue culture treated explants in the presence/absence of light at various time exposures with: (a) AgNO3 (b) NaDC (c) NaDC Capped AgNPs. Each value shown was the mean of seven determinations and error bars represent standard deviation (±SD). (d) & (e)-Images of fungal structures of Colletotrichum gloeosporioides, before and after treatment of AgNPs (150min) respectively.

42x57mm (300 x 300 DPI)

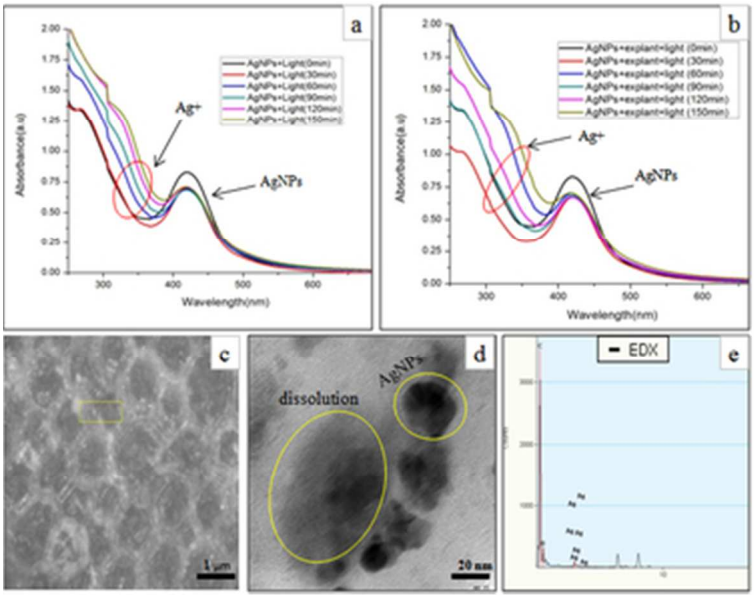

Figure: 4. Assessment of NaDC capped AgNPs uptaken into treated plant: (a) UV-vis spectral data of AgNPs incubated with light at various timings (0,30, 60, 90, 120 and 150 min) and (b) UV–vis spectral data of AgNPs incubated with explants in presence of light at various timing (0,30, 60, 90, 120 and 150 min) (c) TEM image of plant specimen depicts vascular region contains silver metal trace at 1μm bar scale (d) TEM image of plant specimen depicts AgNPs dissolution state projected at20nm bar scale d) EDX spectrum represents silver element signal from vascular region of plant specimen.

31x25mm (300 x 300 DPI)

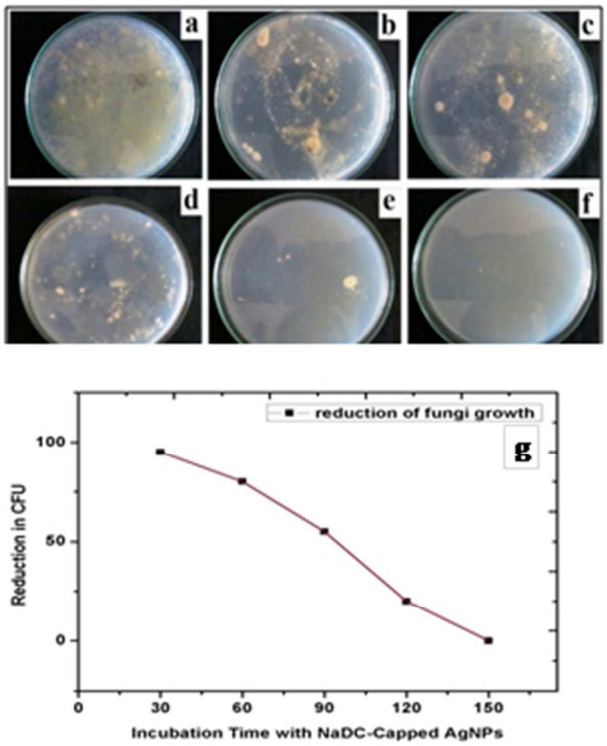

Figure: 5. Direct antifungal activity of NaDC capped AgNPs on Colletotrichum gloeosporioides on PDA plates at; (a) 0 min (b) 30 min (c) 60 min (d) 90 min (e) 120 min (f) 150 min and (g) Data (mean ±SD) expressed over all activity of AgNPs against fungus.

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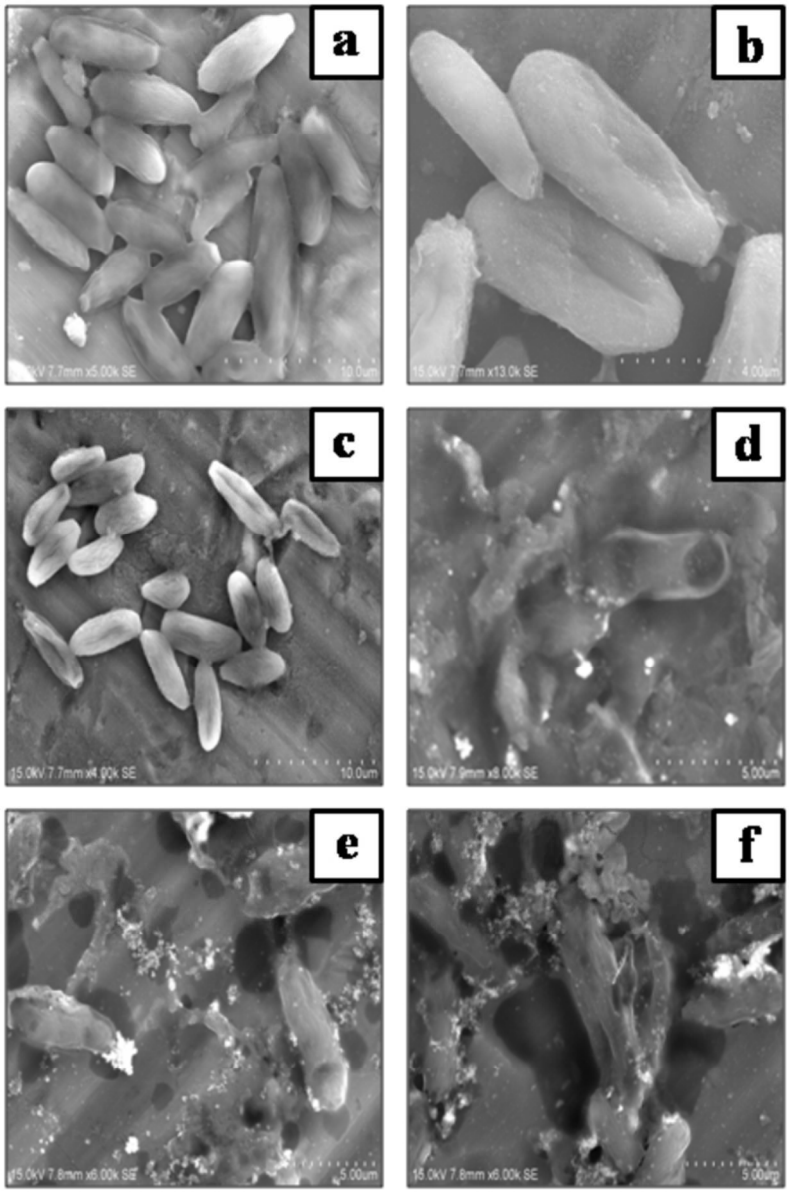

Figure: 6. Direct antifungal activity: FESEM images of Colletotrichum gloeosporioides incubated with NaDC capped AgNPs at various timings shows degrees of cell damage: (a) 0 min (b) 30 min (c) 60 min (d) 90 min (e) 120 min and (f) 150 min.

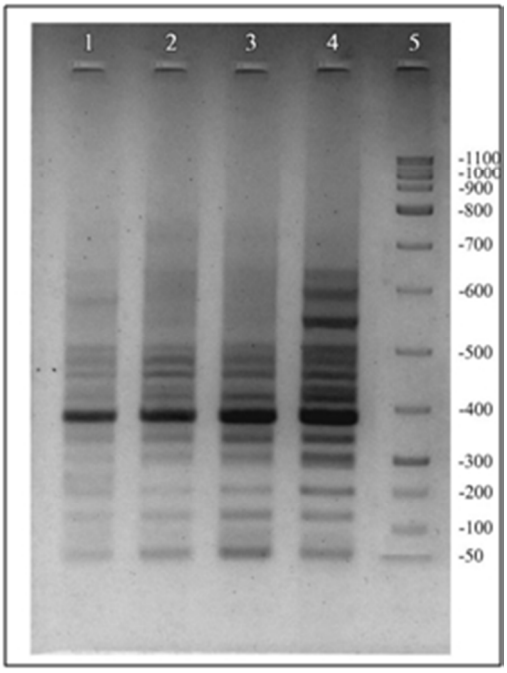

Figure: 7. Genotoxicity analysis by RAPD markers; Fluorogram image shows banding pattern of PCR amplified products. Lane1: Control treated, Lane 2: NaDC capped AgNPs treated, Lane 3: NaDC treated, Lane 4: AgNO3 treated, Lane 5: 100 bp marker.