

In vitro disease burden analysis using green silver nanoparticles

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Abstract

Biologically manufactured silver nanoparticles are increasingly being used for various sterilization purposes because of its broad spectrum antibacterial activity. There have been relatively few studies on the applicability of silver NPs to control plant diseases. The present study was aimed to investigate the potential of green synthesized silver nanoparticles (GAgNPs) to analyze disease burden in poppy plants affected with Downy mildew (DM) disease caused by fungi *Peronospora arborescens* for the first time. The GAgNPs was also assayed to determine its antimicrobial potential against bacterial strains. We found that there were some bacterial strains in addition to the fungus which affected the crop yield, by measuring colony forming unit (CFU), caused disease burden on poppy plants. In *in vitro* examination shows, GAgNPs significantly inhibited bacterial strains even at 10 ppm (least minimum inhibitory concentration (MIC)) then control. Maximum inhibition shows at 100 ppm (most MIC) which is an optimize concentration of GAgNPs. These results suggest that GAgNPs have potential for use as economic, low-dose, potentially non-persistent anti-microbial agents against both DM fungi and the bacterial strains. Copyright © 2017 VBRI Press.

Keywords: Disease burden, green silver nanoparticles, downy mildew, colony forming unit, serial dilution.

Introduction

The novel properties of nanoparticles have been exploited in a wide range of potential applications in medicine, cosmetics, renewable energies, environmental remediation and biomedical device [1-3]. Among them, silver nanoparticles (AgNPs) have attracted increasing interested due to their unique physical, chemical and biological properties compared to their macro-scaled counterparts [4]. AgNPs have distinctive physio-chemical properties, including a high electrical and thermal conductivity, surface enhanced Raman scattering, chemical stability, catalytic activity and nonlinear optical behavior [5]. These properties make them of potential value in microelectronics, and medical imaging [6]. AgNPs exhibits broad spectrum bactericidal and fungicidal activity [7] that has made them extremely popular in cosmetic industry and food and agricultural sector and increasing their market value [8-10].

Metal such as silver are considered safe and are effective antibacterial agents that can kill more than 650 types of microorganisms including bacteria, fungi and virus. By increasing the surface to volume ratio, the nanoscale silver antibacterial properties can be improved [11]. Silver ions are very reactive, they inhibit microbial respiration and metabolism and they cause physical

damage [12, 13] to microbes. Silver has been used to treat medical ailments for over 100 years due to its natural antibacterial and antifungal properties [14]. Recently, nanotechnology practices have amplified the effectiveness of silver particles as antimicrobial agents [15, 16]. AgNPs have extremely large relative surface areas which increases their contact with bacteria and fungi, vastly improving its bactericidal and fungicidal effectiveness. The larger surface-to-volume ratio of AgNPs increases their contact with microbes and their ability to permeate cells. When in contact with bacteria and fungi, they will adversely affect cellular metabolism and inhibit cell growth.

In the present study the antimicrobial potential of AgNPs has been employed for the first time to analyse disease burden in downy mildew (DM) suffered poppy crop. Opium poppy (*Papaver somniferum*) is the most strategic crop for the pharmaceutical industry because it is the only source of morphine, codeine, papaverine, and thebaine alkaloid drugs. More than 80 alkaloids belonging to various tetrahydrobenzyliso-quinoline derived classes extracted from the dried latex [17]. The cause of low seed and alkaloid productivity is plant losses due to the several bacterial, fungal and viral diseases, of which downy mildew (DM) disease caused by the fungus *Peronospora arborescens* (Berk) de Bary is one of the most severe and

widespread diseases causing serious harm to the crop. Pests and diseases affect crop yield, quality and reduce resource-use efficiency [18]. Whenever a plant is infected with any agent like bacteria, virus or fungi, it becomes fragile and susceptible and works as host for different diseases and injuries. Disease burden or microbial load means that total number of bacteria and fungi on the given surface of leaf, and the presence of the bacteria and fungi may not be related to the presence of disease-causing organisms.

The present study was aimed to investigate the potential of green synthesized silver nanoparticles (GAgNPs) to analyze disease burden in poppy plants affected with Downy mildew (DM) disease caused by fungi *Peronospora arborescens* for the first time. The GAgNPs was also assayed to determine its antimicrobial potential against bacterial strains. We found that there were some bacterial strains in addition to the fungus which affected the crop yield, by measuring colony forming unit (CFU), caused disease burden on poppy plants. In *in vitro* examination shows, GAgNPs significantly inhibited bacterial strains even at 0.1 mg/mL then control. Maximum inhibition shows at 1 mg/mL which is an optimize concentration of GAgNPs. In the present work we have found that there were some bacterial strains in addition to DM fungus which affected the yield, hence is a disease burden on DM suffered poppy plants. No pesticides are there which have broad spectrum activity i.e. kill fungi and bacteria together. These results suggest that GAgNPs have potential for use as economic, low-dose, potentially non-persistent anti-microbial agents against both DM fungi and the bacterial strains and can be used as effective growth inhibitors in various microorganisms.

Experimental

Materials

Biologically synthesized silver nanoparticles (GAgNPs), nutritive agar (NA) media purchased from HiMedia Laboratories and all the aqueous solutions during the experiment were prepared using deionized water.

Nanosilver solution

Different working concentrations of GAgNPs (10 ppm, 30 ppm, 50 ppm, 75ppm, 100 ppm, and 110 ppm) were prepared by diluting the AgNPs powder.

In vitro disease burden analysis

Serial dilution method was used to measure disease burden in the downy mildew infected opium poppy plants. Infected leaves were collected aseptically and brought into the laboratory for *in vitro* analysis to identify the number of viable micro-organisms in a fixed amount of a liquid which was present on the infected poppy leaves along with DM fungi. From the collected infected leaves 1 gm of leaf is taken out from each treatment for serial dilution. Serial dilution was performed using

10-fold dilution. Infected opium poppy leaves showing typical symptoms of the DM disease were collected from designed experimental fields. All the infected leaves were rinsed under tap water, small fragments of the infected region were cut off, dried at room temperature and took 1gm out of this. The sample was then surface sterilized with autoclaved distilled water in sterilized condition in laminar air flow. These fragments were then placed in a conical flask and Serial dilution was performed using 10-fold dilution. Spread the 10^{-4} diluted inoculums on nutritive media for the proliferation of other viable microorganism present on the infected leaves which also affect poppy yield, as downy mildews grow only in living plant tissues; they are obligate parasites. They cannot be grown on agar culture plates. Observation has been taken at dilution 10^{-4} for 24 h at 37°C. Reading has been taken after 24hr of spreading on NA media plate. NA plate was used to count CFU for bacteria. Using equation1, CFU can be calculated.

$$(Eq. 1) \quad CFU/ml = \frac{\text{Number of colonies per ml plated}}{\text{Total dilution factor}}$$

Statistical analysis

All determinations were carried out at in triplicate. Values were averaged and described as mean values. All data were analyzed with One- Way ANOVA followed by Post Hoc Test. Possibilities less than 0.05 were considered as statistically significant ($P < 0.05$). All statistical calculations were performed with the Microsoft Office World 2007.

Results and discussion

Serial dilution was used to measure disease burden in downy mildew infected poppy leaves and to observe the effect of doses of silver nanoparticles by counting colony forming unit on NA plate using 1 mL of 10^{-4} diluted microbial stock after 24hr of plating, shown in **Fig. 1** and **Table 1**. The CFU was observed as 186, 144, 132, 110, 88, and 83 bacteria/gm in 10, 30, 50, 75, 100 and 110 ppm concentrations of GAgNPS respectively and for control it was observed as 289 bacteria/gm. **Table 1** clearly represented that there is no much difference in CFU on infected opium poppy leaves treated with 100 and 110 ppm GAgNPs; hence 100 ppm of GAgNPs was optimized concentration. The results of GAgNPs showed that the most Minimum inhibitory concentration (MIC) was 100 ppm concentration and the least MIC was 10 ppm concentration. No significant difference was observed between the inhibition of bacterial growth regarding the GAgNPs concentration ($P < 0.05$). From **Fig. 1** and **Table 1**, it is noticed that disease burden on the plant treated with 100ppm concentration shows minimum colonies of viable microbes in comparison to plants treated with 10 ppm, 30 ppm, 50 ppm and 75 ppm concentration of GAgNPS. It results that the bactericidal effects of GAgNPs were increased by increasing the concentration of the GAgNPs.

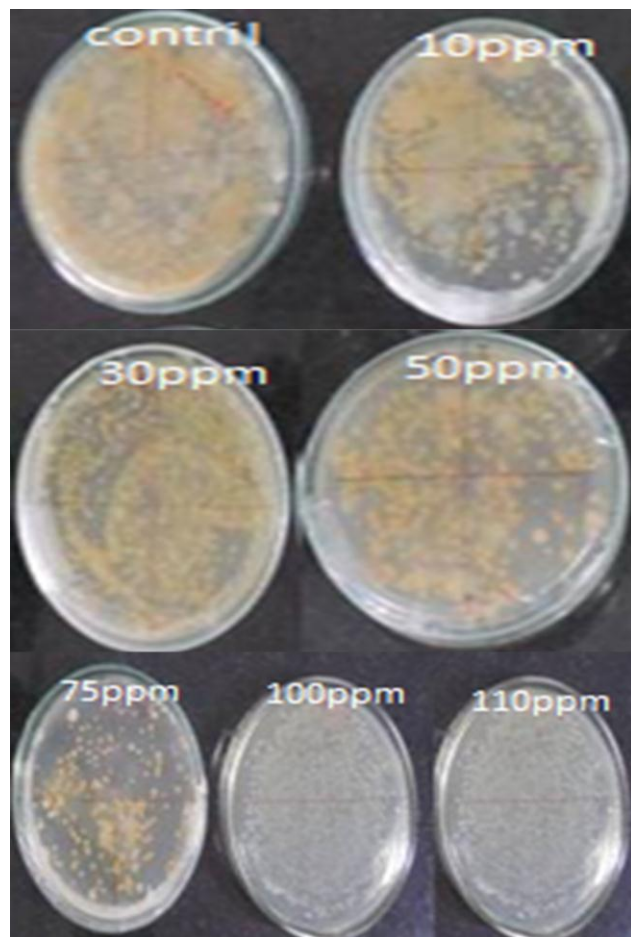


Fig. 1. Showing numbers of viable micro-organisms in NA media plate from 1 mL of 10^{-4} diluted microbial stock after 24hr of plating.

From **Table 1**, it is noticed that there is negligible variation in the number of colonies in plants treated with 100 and 110 ppm concentration due to other environmental factors. Therefore, this study demonstrated that using GAgNPs antimicrobial activity, along with DM fungi growth and colony formation of bacterial strains were also treated. It is not possible with traditional fungicide.

Table 1. Minimum inhibitory concentration (MIC) of Green silver nanoparticles (at different concentration) and control by measuring Colony Forming Unit (CFU).

S.N.	Treatments	CFU Bacteria/gm
1	10 GAgNPs	186
2	30 GAgNPs	144
3	50 GAgNPs	132
4	75 GAgNPs	110
4	100GAgNPs	88
5	110GAgNPs	87
6	Control	289

Conclusion

Antimicrobial activity of ionic or nanoparticle silver has a great potential for use in controlling plant pathogens [19]. In spite of various reports on antibacterial activity of silver nanoparticles, a precise mechanism of such effect has not been reported. We conclude that the GAgNPs treatment is much more effective (both in terms of dose and CFU reduction) in controlling the disease even in small dose. One of the possible explanations is destruction of membrane integrity of bacteria and inhibition of reproduction process. Since the efficacy of silver is greatly influenced by application timings, preventative applications of silver ions and nanoparticles work better before spores penetrate and colonize within the plant tissue. Application of 100 ppm GAgNPs is the optimum concentration for reducing disease burden in downy mildew diseased Poppy plants. Precise action of Silver nanoparticles as broad spectrum antimicrobial agent is under exploration.

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Author's contributions

Conceived the plan: Deepika Gupta, Pratima Chauhan; Performed the experiments: Deepika Gupta; Data analysis: Deepika Gupta; Wrote the paper: Deepika Gupta, Pratima Chauhan. Authors have no competing financial interests.

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