Citrus maxima (Burm.) Merr. fruit juice and peel extract mediated synthesis of silver nanoparticles (AgNPs) and their applications as antimicrobial agents and plant growth enhancers

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Abstract

Green synthesis of metal nanoparticles (NP) has emerged as a recent trend in green chemistry. However, their potential in the field of agriculture is yet underexplored. In this study, silver nanoparticles (Ag NPs) were synthesized by using the fruit juice and peel extract of *Citrus maxima* (Burm.) Merr.as a reducing and capping agent. The nano-primed seeds of *Zea mays*, *Glycine max*, and *Cicerarietinum*showed enhanced germination rate up to 90% as compared to the respective control (30-60%) and also enhanced plant growth. The HRTEM images of fruit-mediated Ag NPs showed well-dispersed particles. The XRD diffraction pattern confirmed the crystalline nature of Ag NPs with fcc lattice points of 101, 111, 200, 220, and 311 with an average crystal size of 19.1275±1.73 nm. On the other hand, the peel-mediated Ag NPs exhibited a size range of 10-20 nm with average crystal size of 19.09±1.71 nm. The FTIR spectra confirmed the presence of $\upsilon_{C=C\text{-}H}$, υ_{CH2}, $\upsilon_{C=O}$ (stretch), $\upsilon_{C=C}$ (stretch), $\upsilon_{C\text{-}H}$ (bend), $\upsilon_{C\text{-}O}$ (bend) in the Ag NPs synthesized by the fruit and peel extract. The Ag NPs also showed potential antimicrobial effects against both Gram-positive (*Staphylococcus aureus*) and negative bacteria (*Klebsiella pneumonia*).

Keywords: Antimicrobial activity; green synthesis; plant growth; seed germination; silver nanoparticles.

1. Introduction

In the last few years, researchers are attracted to the exploration of scopes of nano-materials in agricultural sectors to meet the increasing global food crisis through the enhancement of crop yield and quality (Soliman *et al*., 2020). The metallic nanoparticles have also shown their potential to reduce nutrient loss and improvement of plant augmentation (Dimkpa *et al*. 2017). In order to maintain economic stability and increase the commercial production of agricultural resources, the foremost concern should be on expeditious and consistent germination of seeds (Acharya *et al*., 2020). Hence, it is very crucial to undergo treatment of the seeds before sowing under certain controlled conditions to improve the quality and production of some commercially vital crops. The process of treating the seeds before sowing is called seed priming, which has exhibited the potential to solve diverse agricultural problems and thereby benefit the farmers as such it reduces the time of germination, enhances growth, increases the activity of various enzymes, and acts as biofertilizer (Acharya *et al*., 2020).

The basic reason behind the rapid growth of nano-primed seeds may be enhancing the activity of some vital enzymes such as amylases, proteases, and lipases that are involved in the germination process of seeds (Acharya *et al*., 2020). Moreover, nano-primed seeds are also mitigated from stress, and as a result of which the rate of emergence of seedlings increases (Acharya *et al*., 2020). As a result of which, nano-priming is thought to have the potential to enhance the rate of seedling emergence, yield, and quality of the crop (Mahakham *et al*., 2017; Acharya *et al*., 2020).

Both physical and chemical methods of synthesis have their disadvantages as they are capital intensive, require high energy, produce unstable nanoparticles with reduced target activity, and release toxic substances (Kiran *et al*., 2011). Therefore, researchers are trying to substitute the physical and chemical methods with an eco-friendly, non-toxic, highly stable, and cost-effective mode of nanoparticle synthesis. As a result, research on the biological mode of synthesis is gaining importance in recent times (El-Chaghaby *et al*., 2022).

A series of literature has revealed that plant extracts, fungi, algae, cyanobacteria, bacteria, yeasts, actinomycetes, viruses, standalone biomolecules such as proteins, amino acids, enzymes, glucose, biosurfactants, *etc*. (Jayabalan *et al*., 2019; Selvan *et al*., 2018; Tomer *et al*., 2019; Sowbarnika *et al*., 2018; Nabila & Kannabiran, 2018) have the potential to act as a reducing and stabilizing agent that is essential to synthesize nanoparticles of varied shapes and sizes (Jayabalan *et al*., 2019). Plant extracts of *Tagetes* sp., *Rosa* sp., *Achillea wilhelmsii*, *Piper sarmentosum*, *Mentha piperita*, *Ipomoea digitata*, *Pelargonium graveolens*, *Azadirachta indica*, *Cymbopogon flexuosus*, *Tamarindus indica*, *Aloe vera*, *Coriandrum sativum*, *Cinnamomum camphora*, *Capsicum annuum*, and *Calendula officinalis etc*. have been extensively studied for their potential to reduce various metal salts into the respective metal nanoparticles with excellent antimicrobial activity against several phytopathogens (Hernández-Díaz *et al*., 2021).

Citrus maxima (Burm.) Merr. is a tropical fruiting plant, widely available throughout the sub-Himalayan foothill regions which includes the North-eastern region of India (Ani *et al*., 2020). It is widely known as a rich source of various phytochemicals such as flavonoids, alkaloids, saponins, phenolics, *etc*., and is known to have the potential to act as a reducing agent for the synthesis of metal nanoparticles (Ali *et al*., 2020). Considering the above facts, the potential of *Citrus maxima* (Burm.) Merr. fruit juice and peel extract were explored for the synthesis of Ag NPs as they are known to have unique abilities to fight against pathogenic microbesand some recent studies also reveals their potential in the agricultural sector (Almutairi & Alharbi, 2015).

These bio-inspired Ag NPs were used for enhancing the germination and growth of soybean, maize, and chickpea seed. Further, the antimicrobial effect of Ag NPs was also explored. It may be noted that maize (*Zea mays*), soybean (*Glycine max*), and chickpea (*Cicerarietinum*) are among the most commonly grown and economically important food crops in the world (Afzal, 2021). According to the Food and Agriculture Organization (FAO), 44% of the total soybean produced in the world is used for the production of oil (Radočaj *et* *al.*, 2020). Chickpea is a major leguminous crop that is extensively distributed in various parts of the globe (Afzal, 2021). However, the production of economically important food crops needs to be increased in response to the rising demands of the growing world population and to cope with the challenges faced by drastic climatic changes (Pereira, 2017). Therefore, there is a need to increase the yield of such economically important crops by using some recent smart nano-based agricultural methods.

2. Materials and Methods

2.1 Extraction of juice and peel extract from *C. grandis* (Burm.) Merr. Fruit

The juice was extracted from the fruit and filtered through a strainer to remove the pulp followed by filtration using Whatman no.1 filter paper to remove the unwanted residues. The peel extract was prepared by boiling small pieces of peel (10 g) in distilled water (100 mL) for 20 min followed by filtration through Whatman no.1 filter paper. These were then kept at 4°C for further use.

2.2 Synthesis of Silver nanoparticles

For the synthesis of Ag NPs, 10 mM AgNO3 solution was prepared in distilled water. A hundred milliliters for such solution was reduced to Ag NPs by adding 40 mL of *C. grandis* (Burm.) Merr.fruit juice and 0.3% soluble starch (w/v) with constant stirring followed by autoclaving at 121°C, 15 lbs pressure for 15 min. Similarly, the second set was reduced by 40 mL of peel extract. After this, the solution was brought back to room temperature followed by centrifugation at 10,000 rpm for 10min. The precipitate was dried and kept for further use.

2.3 Characterization of Ag NPs

The synthesized Ag NPs were extensively characterized by UV-Vis spectrophotometry (Shimadzu UV-1800 spectrophotometer, Tokyo, Japan), X-ray Diffraction (Bruker D8 Advance Powder XRD, Karlsruhe, Germany), FTIR (Bruker Corporation, Germany) and HRTEM (JEOL-JEM2100F, Japan).

2.4 Evaluation of antimicrobial activity

The antimicrobial potential of Ag NPs was evaluated against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Klebsiella pneumonia*) bacterial pathogens by the standard agar well diffusion method (Khalid *et al*., 2021). Overnight microbial cultures ($O.D._{600}$=1.0) were inoculated by spread plating method on Mullar-Hinton agar and 50 µL of 1 mg/mL Ag NPs suspension in distilled water was loaded in the respective well taking suitable controls for the experiment. The cultured plates were then incubated for 24 h at 37°C. The diameter of the clear zone around the respective well (zone of inhibition) confirming the antimicrobial activity of Ag NPs was recorded.

2.5 Seed germination assay

Thoroughly cleaned seeds of maize, chickpea, soybean, and wheat were soaked in distilled water (control), 0.3% (w/v) soluble starch, Ag NPs suspension of 10 mg/L and 20 mg/L for

24 h followed by placing the primed seeds on top of the humid cotton bed and allowed to germinate. Weight of the seeds after nano-priming was recorded at the end of day-1 and day-3. The total length of the germinated seeds was also recorded after 5 days of treatment.

2.6 Statistical analysis

The experiments were performed in triplicate for the reliability of the data and the results were expressed in mean ± S.D. Student's *t*-test was performed by using GraphPad^{TM} online statistical tool to analyse the significance of the results.

3. Results

Change in colour of the solution from pale yellow to dark brown confirms the formation Ag NPs after reducing by both fruit juice and peel extract. The UV-Vis spectrophotometric analysis also showed a broad peak at a wavelength of 418 nm also confirms the formation of Ag NPs in both solutions. TEM image shows well-dispersed Ag NPs synthesized by reducing with *C. grandis* (Burm.) Merr.fruit juice (figure 1a and b). The average particle size distribution shows the majority of NPs within the range of ~50-60 nm.

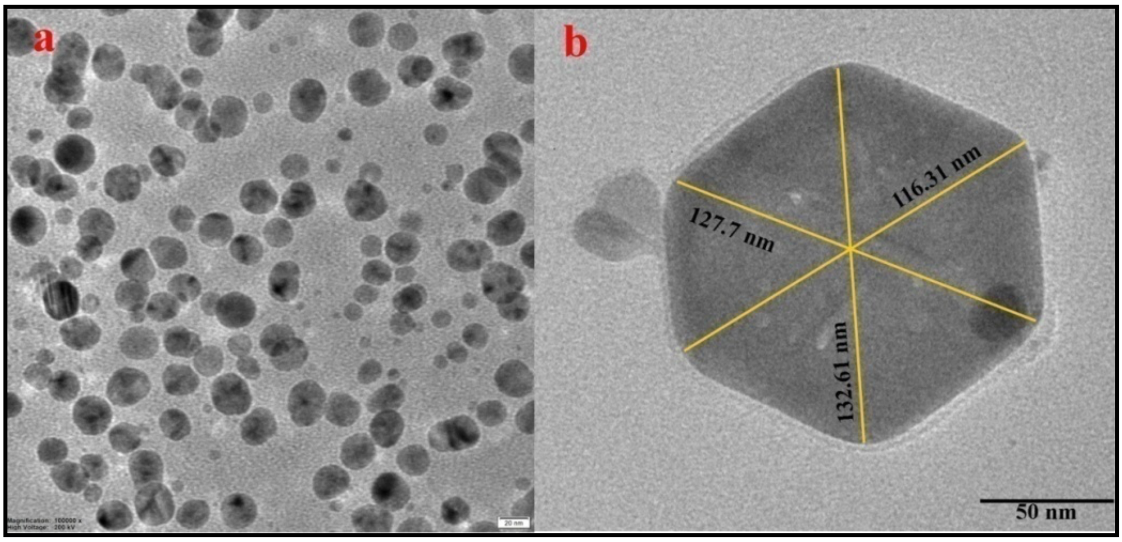


Fig. 1. TEM images of *C. grandis* (Burm.) Merr. fruit juice mediated Ag NPs at (a) low magnification, and (b) high magnification.

The X-ray diffraction analysis of the synthesized Ag NPs has shown distinct peaks attributed 2θ values of approximately 32.5°, 38°, 44.5°, 64.5°, and 77°corresponds to fcc lattice points of 101, 111, 200, 220, and 311 respectively confirm the crystalline structure of the NPs with an average crystal size of 19.1275±1.73 nm based on Scherrer's equation (figure 2). These were further confirmed by selected area diffraction (SAED) analysis using HR-TEM (figure 2). On the other hand, more dispersed Ag NPs with a size range of 10-20 nm were formed when synthesized by using the peel extract of *C. grandis* (Burm.) Merr. as a reducing agent (figure 3). However, the XRD analysis of peel-mediated Ag NPs showed similar lattice fringes when compared to juice-mediated Ag NPs with average crystal size of 19.09±1.71 nm (figure 3).

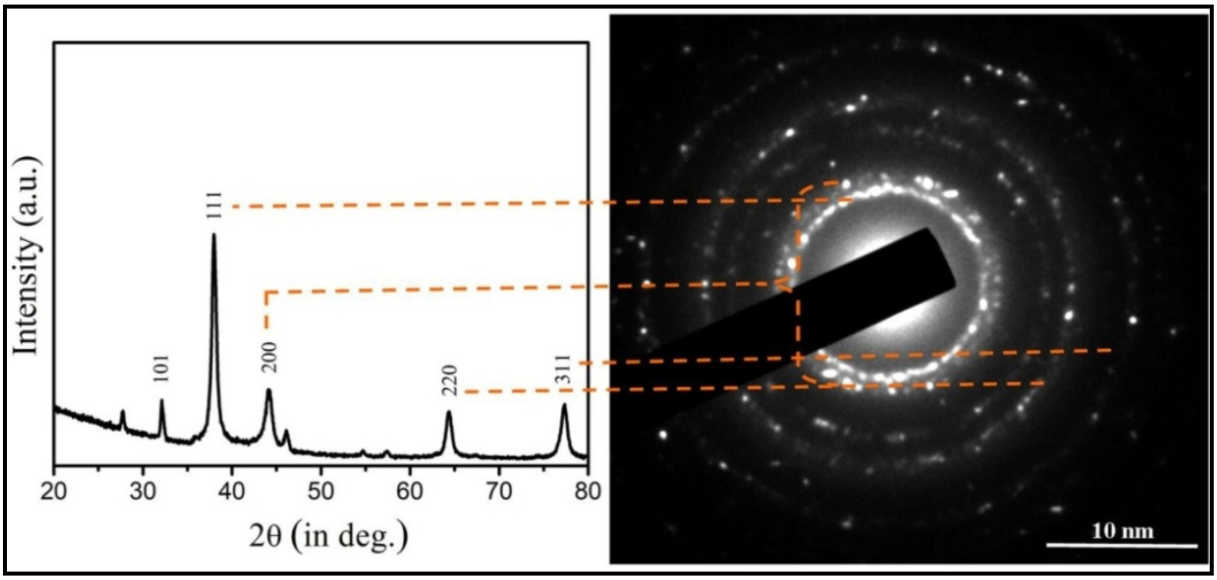


Fig. 2. XRD (left) and SAED (right) images of *C. grandis* (Burm.) Merr. fruit juice mediated Ag NPs.

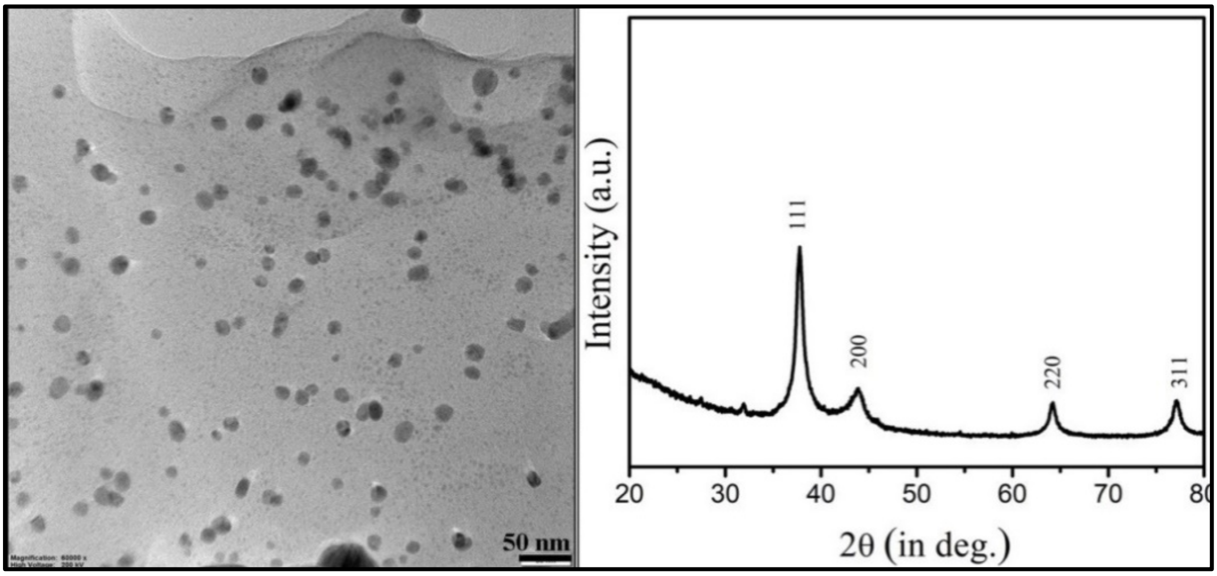
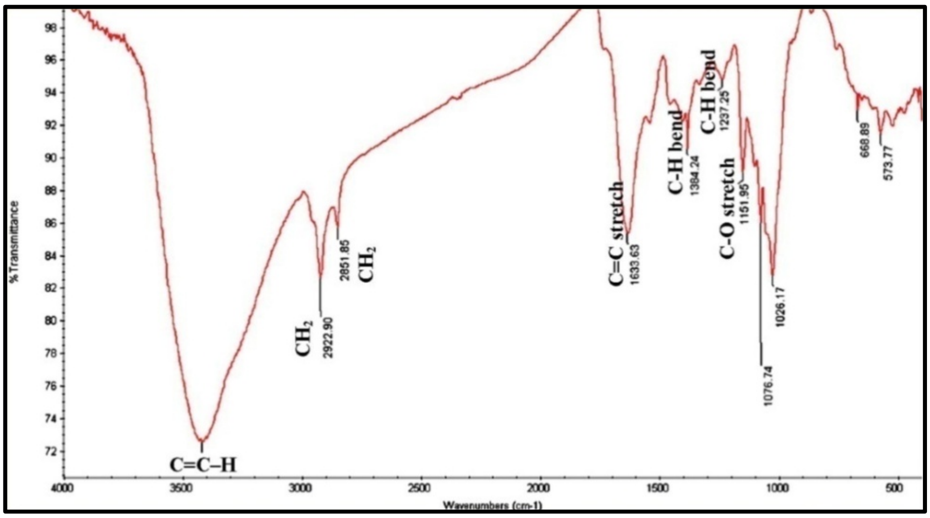


Fig 3. TEM (left) and XRD (right) images of *C. grandis* (Burm.) Merr. peel-mediated Ag NPs.

The FTIR spectra of juice mediated Ag NPs showed distinct peaks in the regions 2922.90, 2851.85 1633.63, 1384.24, 1237.25, 1151.95, 1076.74, 1026.17, 668.89 and 573.77 cm^{-1} (figure 4a). Similarly, peel-mediated Ag NPs showed peaks in the regions 2923.41, 2852.94, 1743.05, 1641.93, 1541.09, 1383.91, 1238.75, 1153.38, 1078.16, 1025.21, 669.19, 576.03, 523.18 cm^{-1} (figure 4b). Both the FTIR spectra have shown the presence of $\upsilon_{C=C-H}$, υ_{CH2}, $\upsilon_{C=O}$ (stretch), $\upsilon_{C=C}$ (stretch), υ_{C-H} (bend), υ_{C-O} (bend) in the synthesized NPs.

(a)



(b)

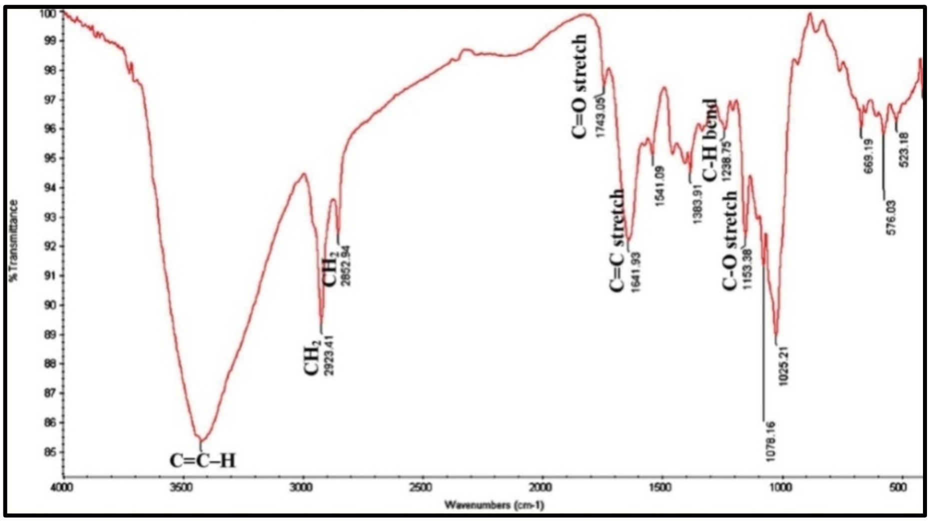


Fig. 4. FTIR spectra of juice mediated (a) and peel extract mediated (b) Ag NPs.

The Ag NPs synthesized by both the methods were tested against Gram-positive (*S. aureus*) and negative bacteria (*K. pneumonia*) by the agar well diffusion method. Both of these NPs exerted similar antimicrobial potential against both the microbes ($p = 0.86$) (table 1 and figure 5).

Table 1. Diameter of zone of inhibition of Ag NPs against test microbes

Test microbes	Zone of inhibition (in mm) exerted by			
	Juice mediated Ag NPs	Peel-mediated Ag NPs	Juice (control)	Peel extract (control)
S. aureus	19.7±2	20±2	-	-
K. pneumonia	15±2	15±2	-	-

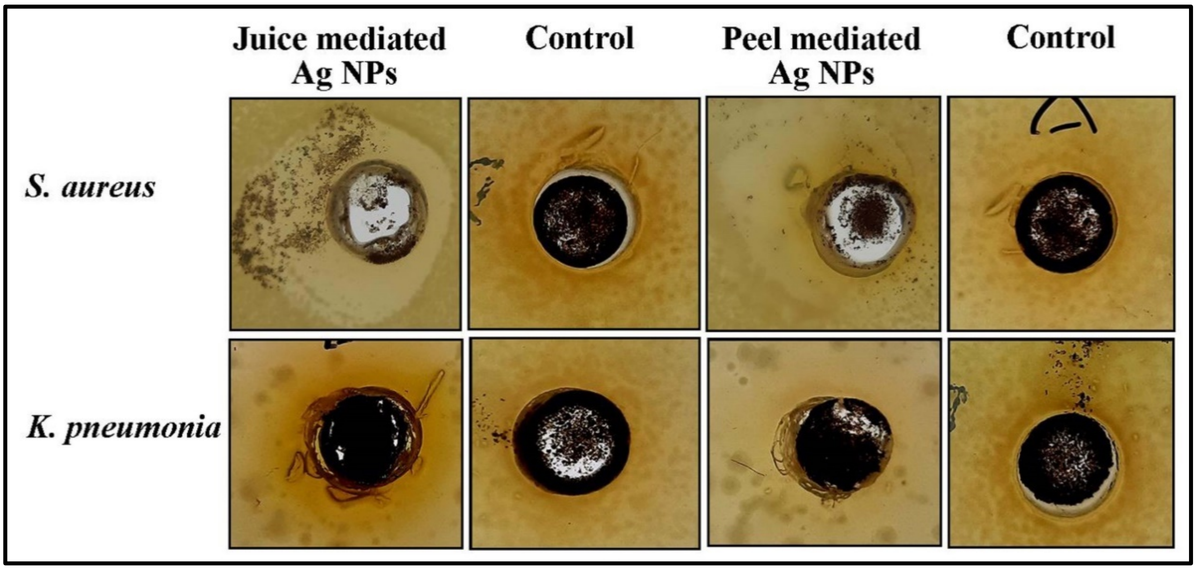


Fig. 5. Zone of inhibition exerted by Ag NPs against bacterial pathogens

Seeds of maize, chickpea, soybean, and wheat were primed with Ag NPs synthesized by both methods. Figure 6 depicts the significant increase in the average weight of each type of seed after the 3rdday of treatment as compared to the end of the 1stday. When chickpeas were primed with starch solution and distilled water considered as positive and negative controls respectively, the germination percentage was 52% and 60% respectively. The rate of germination was increased to 88% and 92% when chickpea was primed with 10 mg/L of fruit-mediated Ag NPs and 10 mg/L of peel-mediated Ag NPs respectively but comparatively decreased to 76% and 84% when primed with 20 mg/L Ag NPs. Similarly, the germination rate of maize seeds increased to 80% and 88% as compared to control (44%) when primed with 10 mg/L of fruit and peel-mediated Ag NPs respectively but decreased to 64% and 76% at a concentration of 20 mg/L (fig 7). The rate of germination of soybean seeds was increased up to 84% and 88% respectively when primedwith10mg/L concentration of fruit and peel-mediated Ag NPs as compared to control (32-52%) but decreased to 72% and 80% when primed with 20 mg/L of Ag NPs (figure 7).

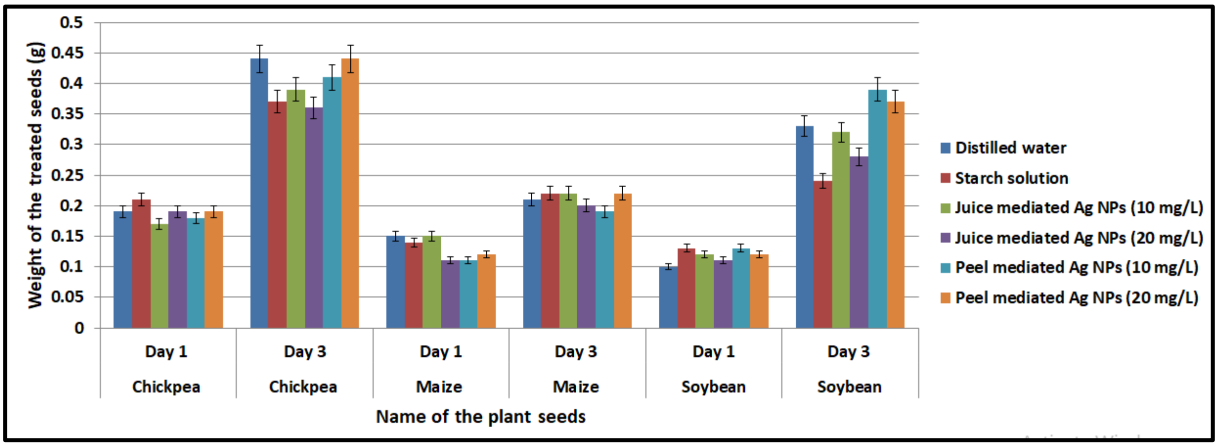


Fig. 6. Weight of the nano-primed seeds on day-1 and 3 before germination

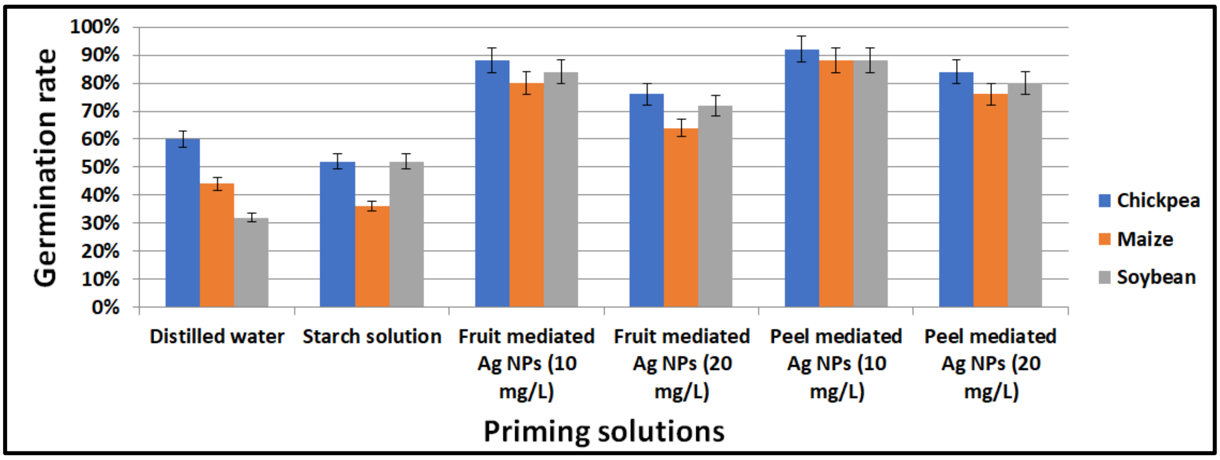


Fig. 7. Germination rates data of at least 25 seeds of each type

Priming of seeds with different solutions also showed variations in the total length of the sprouted seedlings (figure 8). When distilled water and the starch solution were used as priming solution, the total length of chickpea seedlings was 4.5 and 4.8 cm respectively. The length increased to 8.73 and 8.18 cm when primed with 10mg/L of fruit and peel-mediated Ag NPs respectively. On the contrary, the seedling length decreased to 4.43±0.5 and 4.6±0.5 cm when primed with 20mg/L of fruit-mediated and peel-mediated Ag NPs respectively (figure 8). Similarly, the total length of maize seedlings was increased to 2.68 and 4.33 cm but decreased length to 1.78±0.2 and 1.1±0.2 cm respectively when treated with 20 mg/L of fruit and peel-mediated Ag NPs individually. Length of nano-primed soybean seedlings was increased to 3.68±0.4 and 3.88±0.4 when primed with 10 mg/L and decreased to 1.98±0.2 cm and 0.88±0.2 cm when primed with 20 mg/L of fruit and peel-mediated Ag NPs respectively. Fig 9 represents the germinated seed of chickpea, maize, and soybean under the influence of AgNPs.

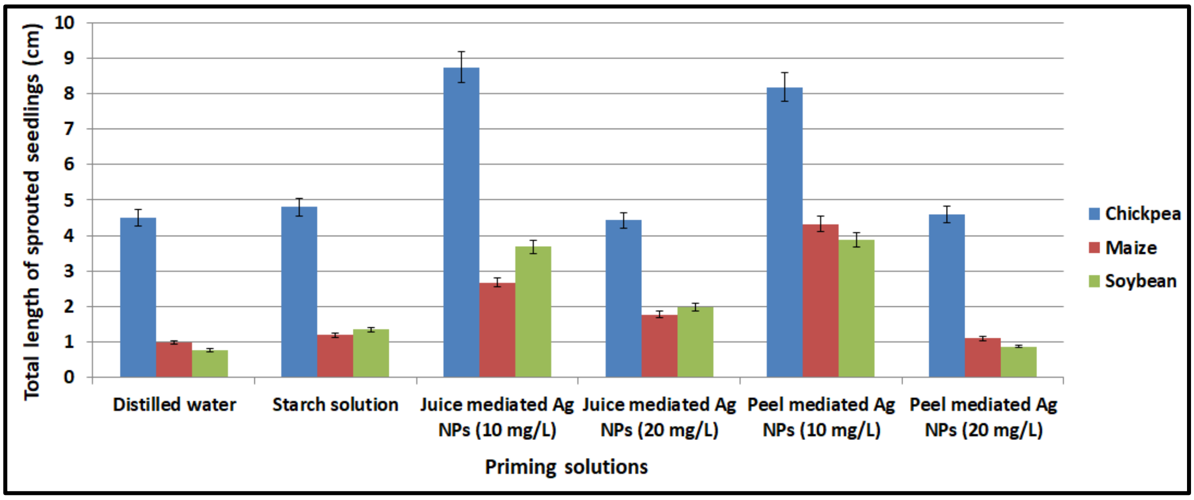


Fig. 8. Total length of the sprouted seedlings after nano-priming.

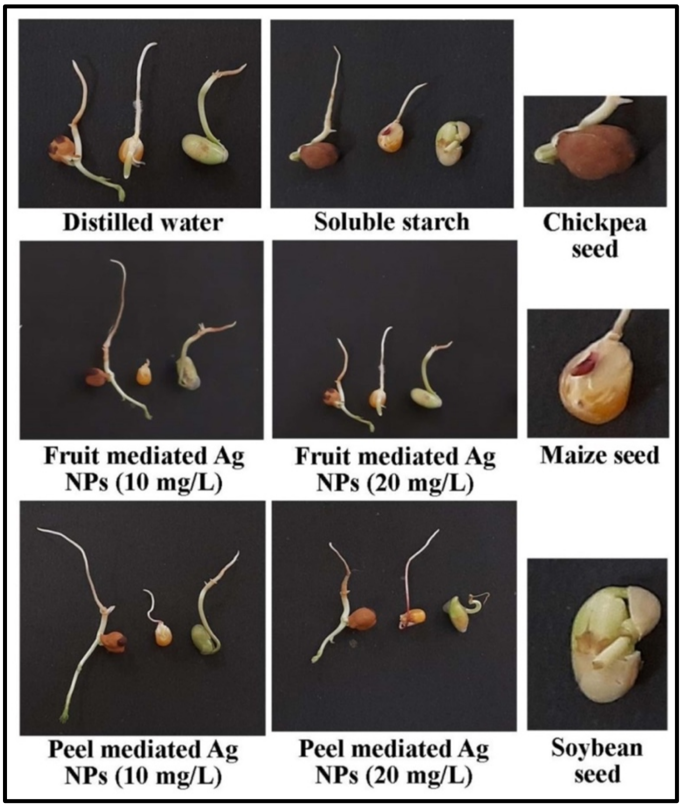


Fig. 9. Effect of fruit and peel-mediated AgNPs on seed germination.

Discussions

Green synthesis of Ag NPs using plant, microbial, or animal extracts has now become a new trend in green chemistry as such mode of synthesis is cost-effective and eco-friendly (Salem & Fouda *et al*., 2021). *Citrus maxima* is a citrus fruiting plant that is rich in citric acid and phytochemicals such as flavonoids, alkaloids, saponins, and phenolics *etc*. and is widely distributed throughout the Himalayan foothill regions which include the North-eastern region of India and south-east Asian countries (Khan *et al*., 2018). These phytochemicals are known to have a reducing potential to reduce metallic salt into metal nanoparticles and also act as capping agent to provide better stability (Muthuvel *et al*., 2020). The Ag NPs synthesized by both methods have shown similar potential in terms of their antimicrobial activity against both Gram-positive and negative test bacterial strains which is following the finding of some concurrent studies (Jomehzadeh *et al*., 2021; Shirzadi-Ahodashti *et al*., 2021; Jyoti *et al*., 2021).Both the Ag NPs exhibited lesser antimicrobial activity against *K. pneumonia* as compared to *S. aureus*. This may be due to the fact that the overall thickness of *K. pneumonia* cell wall is more as compared to that of *S. aureus*. *Klebsiella pneumonia* has an overall cell wall thickness of approximately 160 nm as compared to the cell wall thickness of *S. aureus* which is approximately 20 to 40 nm only (Giesbrecht *et al*., 1998; Amako *et al*., 1988).

Applications of NPs in the field of agriculture as plant growth promoter has been least explored as compared to their pharmaceutical and medical applications (Ahmed *et al*., 2021; Rai-Kalal & Jajoo, 2021; Rafique *et al*., 2022; Ali *et al*., 2021). And also the interactions of metal nanomaterials with plants have still not been fully elucidated. The process of germination starts with the uptake of water by dry seeds through a process called imbibitions

(Mahakham *et al.*, 2017). But the impact of Ag NPs on plant growth largely varies based on several factors *viz.*, species of the plant, size of NPs, the concentration of the NPs, experimental conditions which include temperature, duration of treatment, and method of exposure *etc* (Almutairi & Alharbi, 2015).

One of the vital enzymes involved in the degradation of starch during germination of cereal seeds and the subsequent emergence of the seedling is α-amylase (Mahakham *et al.*, 2017). In addition, metal NPs such as Ag NPs are also known to exhibit catalytic activities hence it may also be assumed that NPs may catalyze the activity of α-amylase by entering through the cellular pores of the seeds by taking the advantage of their nanoscale size and thus enhancing the seed germination rate (Mahakham *et al.*, 2017). It is also hypothesized by many researchers that the treatment of seeds with metal NPs may also boost the expression levels of aquaporin genes which are responsible for the absorption of water in germinating seeds (Mahakham *et al.*, 2017). Literature also shows that nano-priming of seeds may fuel up the superoxide dismutase and other reactive oxygen species scavenging enzyme activity in seeds (Govindaraj *et al.*, 2017). Nano-priming of chickpea, maize, and soybean seeds with 10 and 20 mg/L concentration Ag NPs synthesized by both methods showed variations in terms of seed germination rate and plant growth. However, the germination rate and also the plant growth was found maximum at 10 mg/L concentration of Ag NPs and found to decline at 20 mg/L concentration. On the other hand, the control study showed no significant results when treated with starch solution and distilled water as alone. It can be seen that the average growth rate of nano-primed seeds was faster compared to the control (Zhou *et al.*, 2022). This thereby shows that enhancement of seed germination depends on the dosage of Ag NPs used for seed priming (Almutairi &Alharbi, 2015).The size of Ag NPs also plays an important role in Ag NP effectiveness (Syu *et al.*, 2014). A number of studies have indicated that the cellular uptake of nanoparticles is dependent on their size, charge and surface properties (Syu *et al.*, 2014).It was observed that peel-mediated Ag NPs showed somewhat better activities in terms of seed germination rate which may be due to the fact that peel-mediated NPs were much smaller as compared to that of fruit-mediated counterparts which helps is defying the cellular barrier. Furthermore, this study opens up the possibility for the application of Ag NPs to be used in enhancing crop productivity in the future.

Conclusion

We reported a rapid green method for the synthesis of Ag NPs using fruit juice and peel extract of *Citrus maxima* (Burm.) Merr. The XRD analysis confirmed the crystalline nature of the NPs. The Ag NPs exhibited similar antimicrobial activities against both the bacterial samples irrespective of their mode of synthesis. Recently, it has been explored that, in order to fulfil the high agricultural and food demands, there is a need to improve the quality of seeds. Therefore, it is important to treat the seeds and enhance the rate of seed germination by using recently developed technologies. Among the other methods of seed quality improvement, the most commonly used method is the priming of seeds. The nano-primed test seeds of the selected economically important crop species under this study showed enhanced seed germination rate and plant growth as compared to the respective controls which advocate their future application in the field of agricultural science. However, the mode of action of Ag NPs on seed germination and plant growth *i.e*., the interaction with plants in detail is to be studied.

Conflict of interest

The authors declare that there is no conflict of interest.

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