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ELICITATION OF SECONDARY METABOLITES AND PHYSIOLOGICAL CHANGES IN *IPOMEA TURBINATA* AND *CONVOLVULUS ARVENSIS* PLANTLETS EXPOSED TO GREEN SYNTHESIZED SILVER NANOPARTICLES

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Abstract

The current study focuses on the use of silver nanoparticles (AgNPs) as an elicitor to enhance the secondary metabolite content of *Ipomea turbinata* and *Convulvulus arvensis* plantlets. Six different concentrations of AgNPs were used to evaluate the effect of AgNPs on the phenology and secondary metabolite content of *I. turbinata* and *C. arvensis*. The shoot and root length of *I. turbinata* was inhibited with increasing AgNPs concentration. In case of *C. arvensis,* the root length was increased whereas shoot length was inhibited upon treatment with AgNPs. On the other hand, the biochemical analysis showed significant variation in plant phytochemicals especially the total flavonoid content which were increased two folds as a result of AgNPs treatment compared to the control. With such a promising result, it can be concluded that AgNPs is effective in the enhanced production of commercially important secondary metabolites (such as flavonoids and phenolics contents) compared to production in low amounts under normal growth conditions.

Key words: Green Synthesis, Nanoparticles, Flavonoids, Phenolics, Antioxidant, DPPH.

Introduction

Elicitation - an important tool for enhancing the secondary metabolite content within a plant can be induced by exposing the plant to different stress conditions. The use of NPs as an elicitor to enhance the secondary metabolite content of plants is a modern approach (Anjum *et al*., 2019; Cai *et al*., 2020; Rivero-Montejo *et al*., 2021).

Nanoparticles (NPs) have been found useful in various disciplines including pharmaceutical, medicinal, agricultural, and plant sciences (Ahmad *et al.,* 2022; Capaldi Arruda *et al*., 2015; Dolenc Koce, 2017; Gonzalez-Garcia *et al.,* 2023; Hashmi *et al.,* 2021 Rico *et al*., 2011; Selvakesavan *et al.,* 2023). In earlier studies, NPs have been frequently utilized in the field of plant biotechnology for monitoring their effect on several important factors like seed germination, root length, shoot length and biochemical changes in plants (Logeswari *et al*., 2015; Nadeem *et al.,* 2022; Zaka *et al.,* 2021; Zaka *et al*., 2016). The ultimate effect may range from positive to negative owing to a variety of factors that influences these changes (Zaka *et al*., 2016).

Among the various types of nanomaterials, AgNPs have been abundantly studied by several researchers owing to their potent antioxidant, antifungal, antibacterial and anti-inflammatory potency, improved conductive potential, catalytic potency and much stable chemical nature (Logeswari *et al*., 2015). Previously, Lee et al. (Lee *et al*., 2012) reported in vitro concentration dependent inhibitory effect of AgNPs on growth of *Phaseolus radiatus* and *Sorghum bicolor*. Zuverza-Mena et al. (2016) reported almost similar results for *Raphanus sativus*. On the contrary, Jasim *et al*., (2017) showed that AgNPs positively influenced both physiological and biochemical profile of *Trigonella foenum-graecum* seedlings. AgNPs also showed a dose dependent enhancement in physiological features like seedling vigor index, shoot length, root length and biomass of *Brassica juncea* (Sharma *et al*., 2012).

The present work emphasizes on the study of the effect of AgNPs on phenology and biochemical profile of *I. turbinata* and *C. arvensis* plantlets. The genus *I. turbinata* have long been explored for their nutritional and medicinal value (Bhellum, 2012; Meira *et al*., 2012). Whereas, *C. arvensis* is a drought resistant perennial plant that has the ability to grow in dry soils due to its taproot which has the ability to penetrate deep into the soil (Gardea-Torresdey *et al*., 2004). To the best of our knowledge, no literature is available on *In-vitro* seed germination of *I. turbinata* and *C. arvensis* under the influence of NPs. Herein, we report enhanced production of phenolics and flavonoids and improved DPPH free radical scavenging potential of *I. turbinata* and *C. arvensis* upon exposure to AgNPs.

Material and Methods

Biosynthesis of AgNPs: In order to evaluate the physiological and biochemical changes, the seeds of *I. turbinata* and *C. arvensis* were exposed to AgNPs stress by changing the concentration of AgNPs in MS-Media. The biosynthesis and structural characterization of AgNPs were presented elsewhere (Hashmi *et al*., 2019). Briefly, the AgNPs were prepared by separately adding 5 ml plant extract of *Justicia peploides* to 95 ml of $AgNO_3$ solution of different concentrations, i.e. 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM respectively. Subsequently, the solutions were kept for 24 h at room temperature. The NPs were isolated through centrifugation at 12000 rpm for 12 min. The residue containing AgNPs were washed thoroughly with distilled water for multiple times. The formation of AgNPs (λ_{max} ~ 426-430 nm) were initially confirmed by UV/Vis spectrophotometer. The average crystallite size (~ 40 nm) of the AgNPs were calculated using XRD technique.

Seed collection and sterilization: Seeds of *I. turbinata* and *C. arvensis* were collected from Peshawar and Bannu, Khyber Pakhtunkhwa Province, Pakistan, respectively. The seeds were sterilized by following the protocols with slight modifications (Abbasi *et al*., 2010). The collected seeds were washed with Distilled Water to clean any debris present on the surface. The seeds were then immersed in Mercuric Chloride solution (0.1 % w/v) for 90 seconds followed by rinsing with 70% Ethanol. The seeds were finally washed with Distilled Water and left to dry out on a filter paper before inoculation.

Preparation and supplementation of growth media for seed germination: MS basal media containing 3% (w/v) sucrose and 0.8% (w/v) agar was supplemented with 3 ml of AgNPs colloidal solutions of various concentrations. Each flask contained 27 ml MS basal media and 3 ml AgNPs colloidal solution. Media without any AgNPs solution was considered as control (C) while the media with AgNPs colloidal solution was labelled as T1, T2, T3, T4, T5, and T6 for 25, 50, 75, 100, 150, and 200 mg/l, respectively. Agglomeration of AgNPs in media was minimized through sonication for 25 minutes at 37°C. After sonication, nutrient agar was boiled and autoclaved at 121°C and 15 psi pressure for 15 minutes. Autoclaved media was cooled for a few minutes at room temperature followed by rapid cooling in refrigerator in order to ensure that AgNPs does not settle down in the media.

Inoculation of seeds: Inoculation of seeds upon the media in flasks was carried out inside laminar air flow in a sterile environment. Four seeds were placed into each flask. Three flasks were used for each concentration and the flasks were placed in growth chamber and the seeds were allowed to germinate and grow at 25±2°C and a photoperiod of 16/8 hours.

Estimation of phenotypic variations in *In-vitro* derived plantlets: For the analysis on germinated plantlets, the plantlets were harvested after 12 days. The root and shoot lengths of in-vitro derived plantlets were recorded in centimeters. Fresh weight of the plantlets was recorded using digital balance. Similarly, the dry weight was also recorded after drying the samples in an incubator at 37°C for 3 days. Seed germination frequency (%) and seedling vigor index was calculated using the eqns (1) & (2), respectively.

$$\text{Seed germination frequency (\%)} = \frac{\text{Germinated seeds}}{\text{Total seed}} \times 100 \qquad (1)$$

$$\text{Seedling vigor index} = (a + b) \times \text{Percent germinated} \qquad (2)$$

where, a and b represent mean root length and shoot length in cm respectively.

Estimation of biochemical parameters

Preparation of plant sample: To calculate the total phenolic, flavonoid, and free radical scavenging assay, 200 mg (approx) powder of the dried plantlets was added into 1 ml methanol (absolute). The resulting mixture was vortexed for 2 minutes. The sample vortexed was sonicated for 25 minutes at 37°C. This process was repeated thrice before centrifugation of the sample at 12000 rpm for 3 minutes. The resulting supernatant was collected with a micropipette and introduced into Eppendorf tubes for storage at 4°C until for further analysis.

Estimation of total phenolics, flavonoids and free radical scavenging potential: Protocols of Singleton *et al*. (Singleton & Rossi, 1965) was used for determination of total phenolic content (TPC). Briefly, 20µl from each sample was separately mixed with 90 µl of diluted FC. The mixture was incubated at room temperature for 5 minutes and 90 µl of sodium carbonate (6%) was then added. The absorbance was recorded at 630 nm with the help of microplate reader. Different concentrations (25, 20, 15, 10 and 5 µg/ml) of gallic acid were used as for plotting calibration curve. The TPC was represented by (GAE)/g of DW equivalents of gallic acid.

Similarly, total flavonoid content (TFC) was examined using the protocols (Zia-ul-Haq, 2012). Briefly, 20 µl of extracted sample was mixed with $AlCl_3$ (10 µl) (10%, w/v) and potassium acetate (1 M, 10 µl) followed by distilled water (160 µl) addition. At ambient room temperature, samples mixtures were incubated for 30 min. The absorbance was recorded on a microplate reader at 415 nm. Different concentrations (40, 20, 10, 5 and 2.5 µg ml^{-1}) of quercetin and 20 µl of methanol were used as a positive and negative control, respectively. The TFC value was determined by quercetin equivalents (QE)/g of DW.

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity (FRSA) was evaluated as per the protocols of Amarowicz et al., (2004). Briefly 20 ml plant extract and 180 ml DPP reagent was put into 96 well plates and incubated for 1 hr. The ascorbic acid concentrations (40, 20, 10 and 5 mg ml^{-1}) were used as control. Microplate reader was used to record the absorbance at 517 nm. Percent FRSA was calculated using the following equation (3)

$$\% \text{ FRSA} = (1 - A_s/A_c) \times 100 \qquad (3)$$

where, A_s is the absorbance of the control when the plant extract is added at a specific concentration and A_c is the standard solution absorbance without test samples.

All experiments were performed in triplicates manner. The calibration curves were plotted for determination of TPC, TFC, and DPPH activity using gallic acid, quercetin, and ascorbic acid as the standard, respectively.

Results and Discussions

Phenological variations: Different factors including growth and phenology of *I. turbinata* and *C. arvensis* showed substantial variations under the influence of

various concentrations of AgNPs. This abrupt increase in root length may be due to the defense mechanism found in plants in which they respond to stress conditions in a variety of manners. Plant may follow escape strategy route or quiescence strategy route in response to stress conditions however, this response is not plant specific. In order to escape the heavy AgNPs levels, the plant may have promoted faster root elongation compared to the control (Manzur *et al*., 2009; Yin *et al*., 2012). Features like seed germination, seed vigor, root length, shoot length, fresh weight and dry weight were influenced by the different concentrations of AgNPs present in the growth media (Fig. 1) shows some of these variations in growth and phenology of Plantlets.

Effect of AgNPs on root and shoot length: The root and shoot length showed significant difference in terms of length (Fig. 2). On comparing with control, the shoot length initially showed a positive response to low concentrations of AgNPs while, the shoot length exhibited random behavior; for example, shoot length for T2 (50 mg/l) concentration of AgNPs was less than that for T1 (25 mg/l) however, further increase in AgNPs concentrations displayed shoot length inhibition in plantlets. The shoot length for higher concentrations (T3 ~ 75 to T6 ~ 200 mg/l) of AgNPs showed even less growth that that of the control. Unlike the shoot length, the root length was observed to be completely inhibited for each concentration of AgNPs.

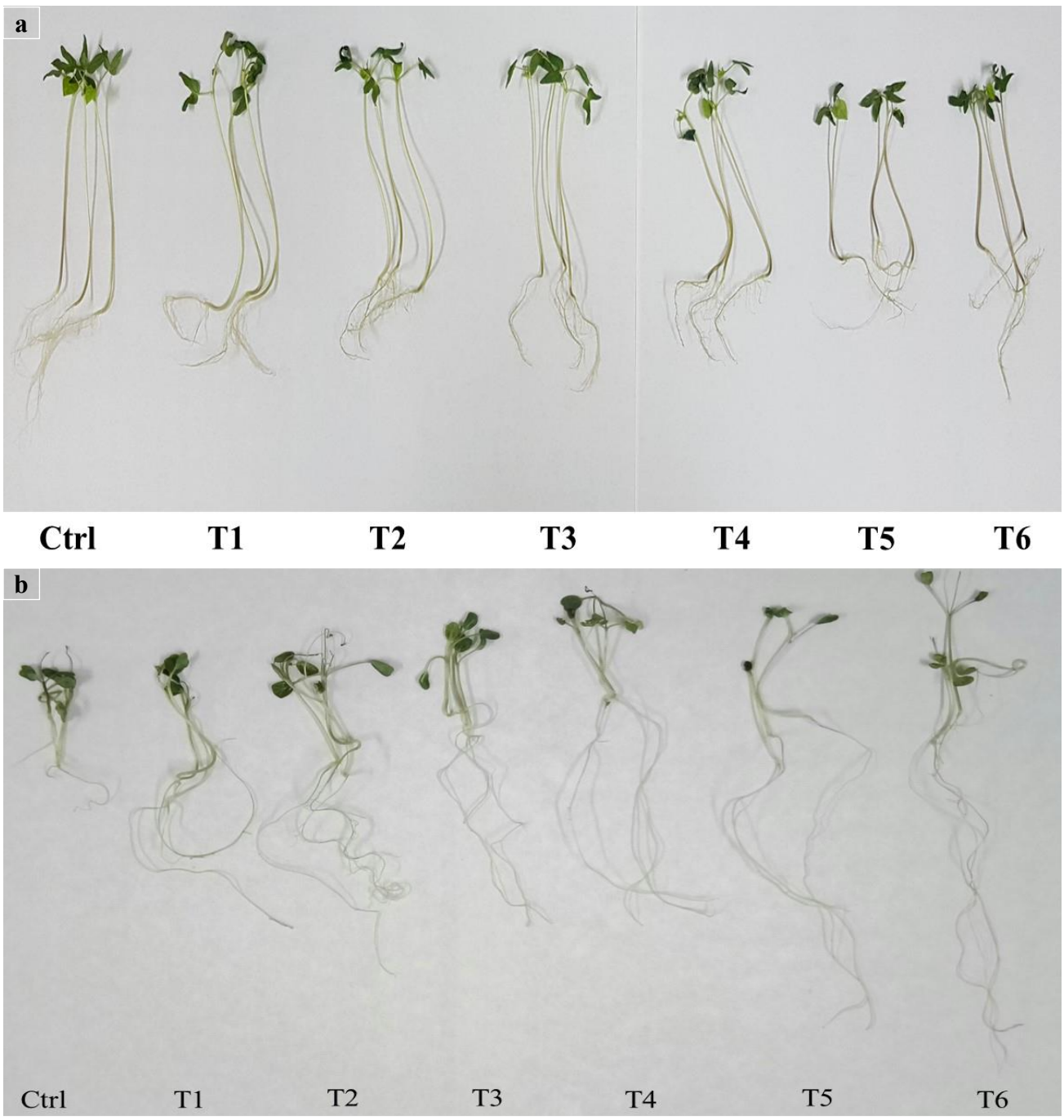

Fig. 1. Differential impact of different concentrations of AgNPs on shoot and root length of 12 days old in-vitro germinated (a) *I. turbinata* seeds and (b) *C. arvensis* seeds.

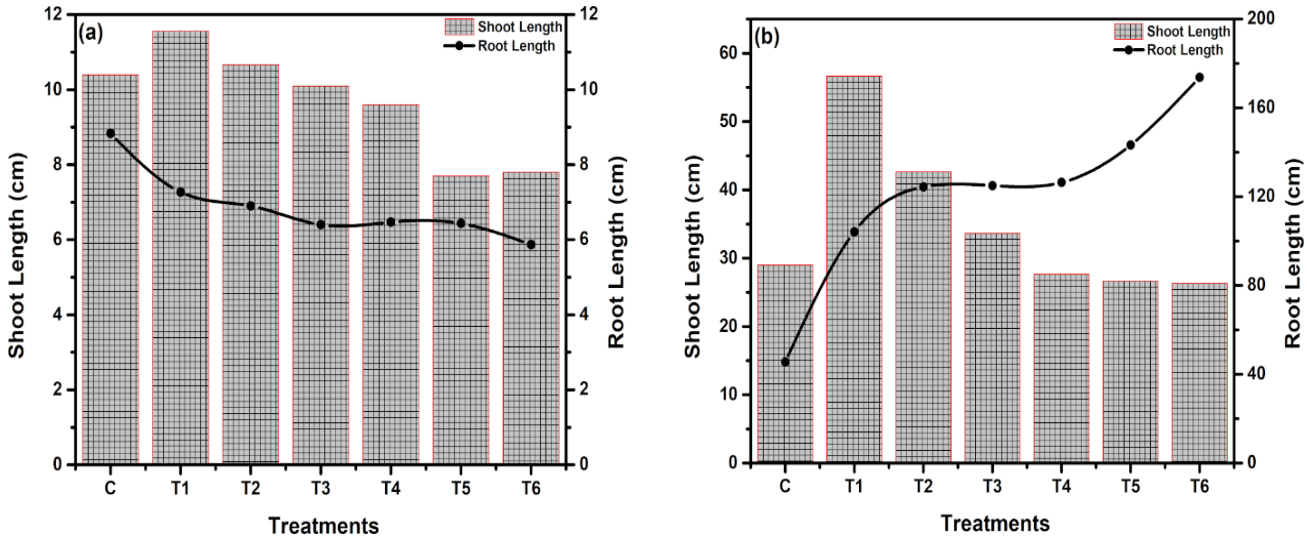

Fig. 2. Variations in the length of roots and shoots in 12 days old (a) *I. turbinata* and (b) *C. arvensis* plantlets grown in-vitro subjected to different AgNPs concentrations.

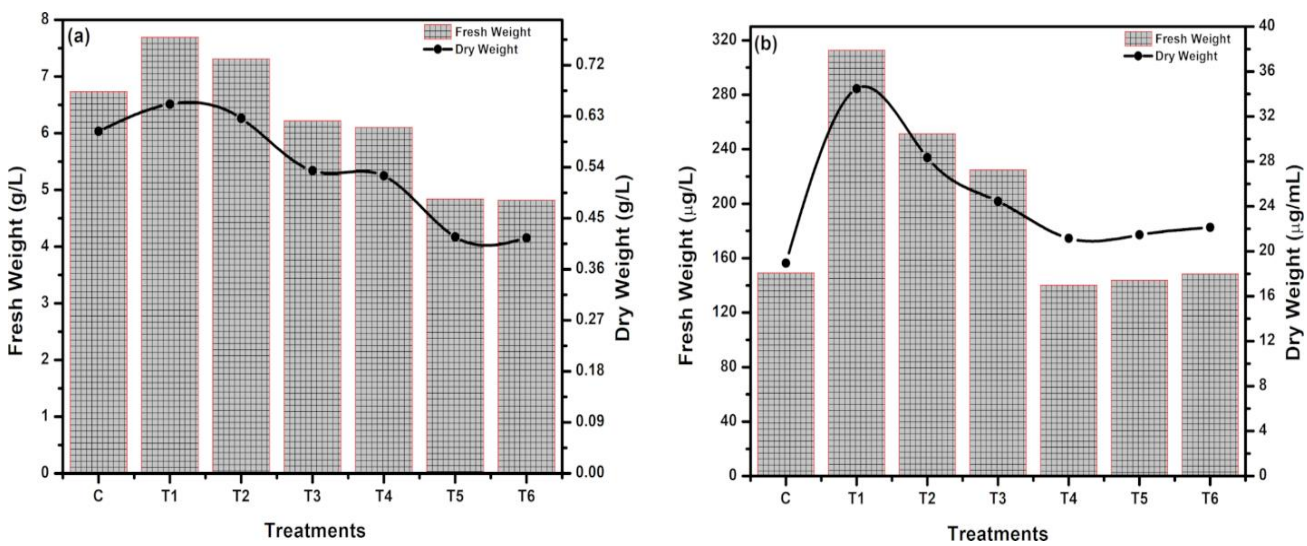

Fig. 3. Variations in the fresh and dry weights of 12 days old (a) *I. turbinata* (b) *C. arvensis* plantlets grown in-vitro subjected to different AgNPs concentrations.

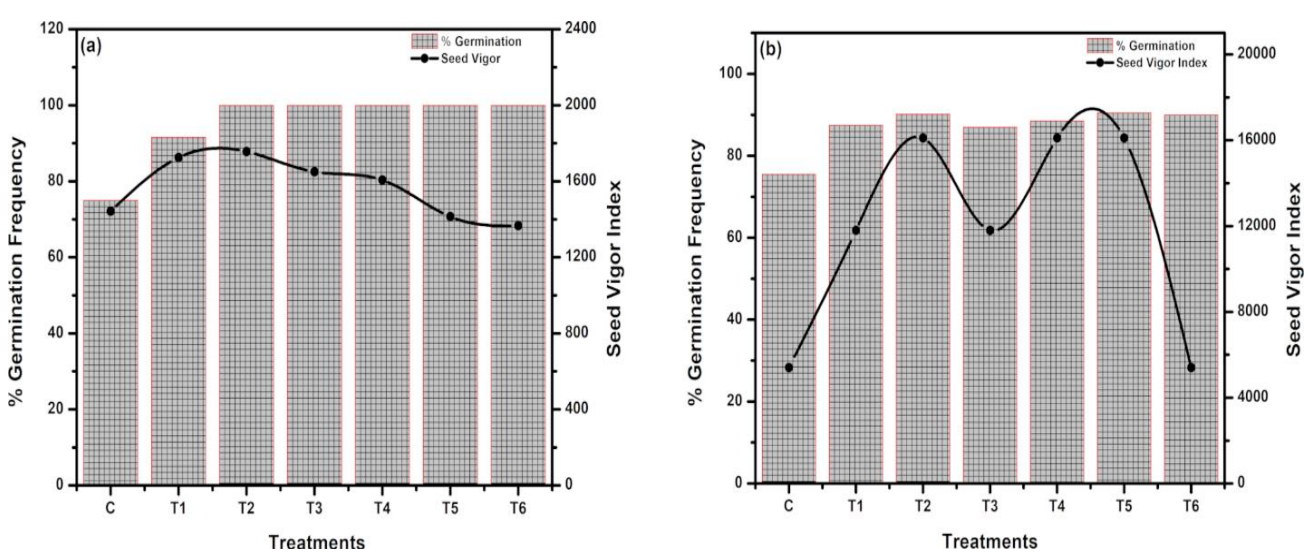

Fig. 4. Variations in germination frequency and seed vigor of (a) *I. turbinata* (b) *C. arvensis* plantlets grown in-vitro subjected to different AgNPs concentrations.

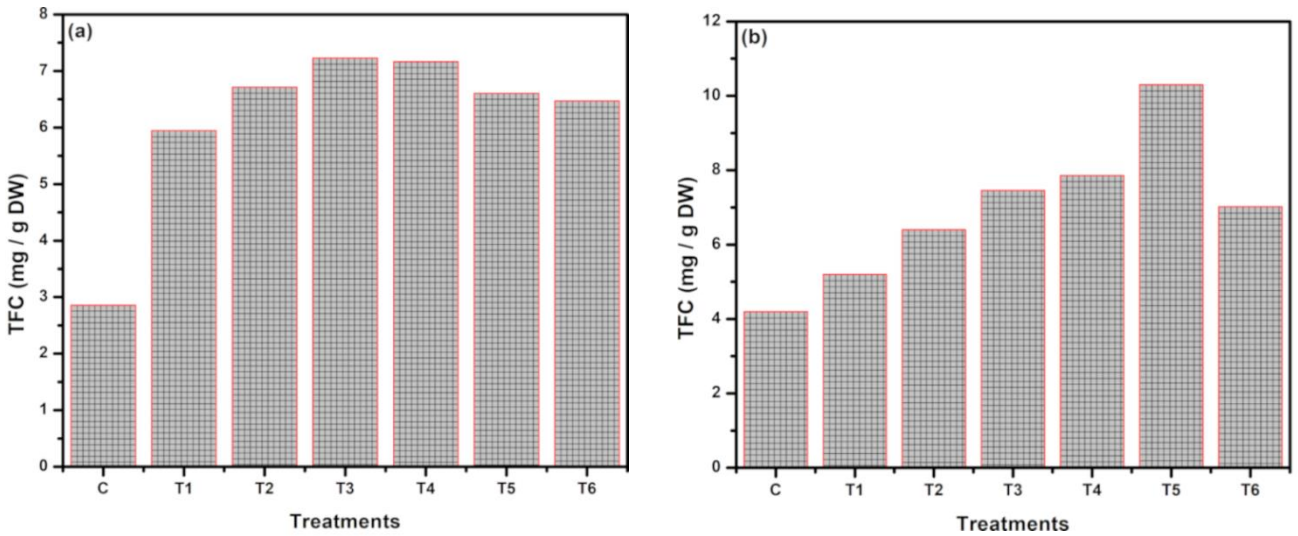

Fig. 5. Variations in the amount of flavonoids in 12 days old (a) *I. turbinata* (b) *C. arvensis* plantlets grown in-vitro subjected to different Ag NPs concentrations.

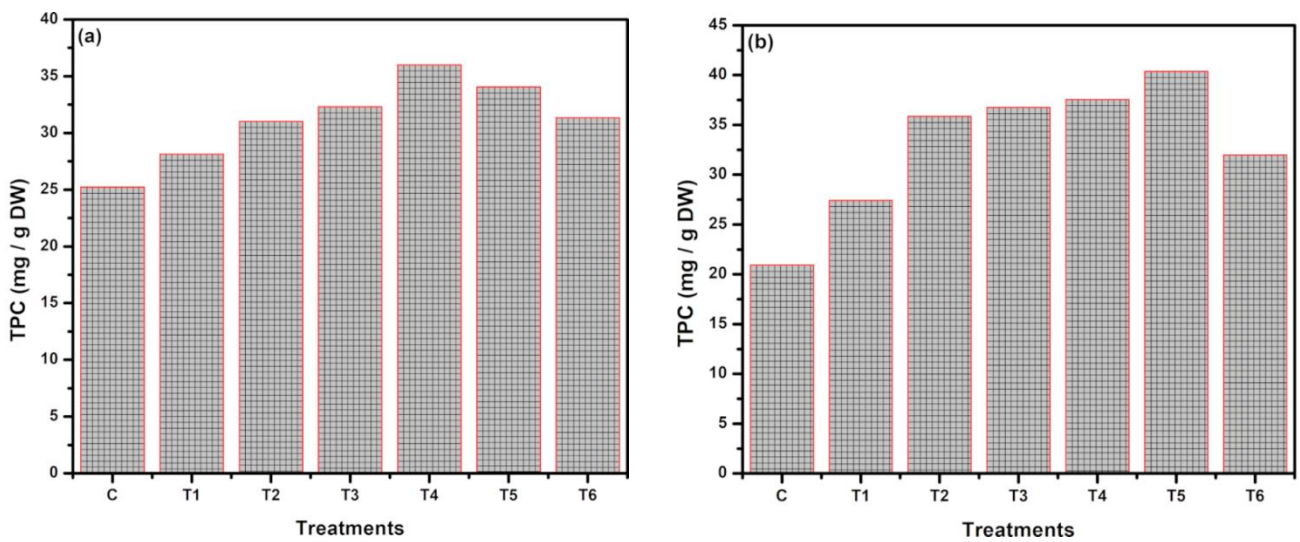

Fig. 6. Variations in the amount of phenolics in 12 days old (a) *I. turbinata* (b) *C. arvensis* plantlets grown in-vitro subjected to different Ag NPs concentrations.

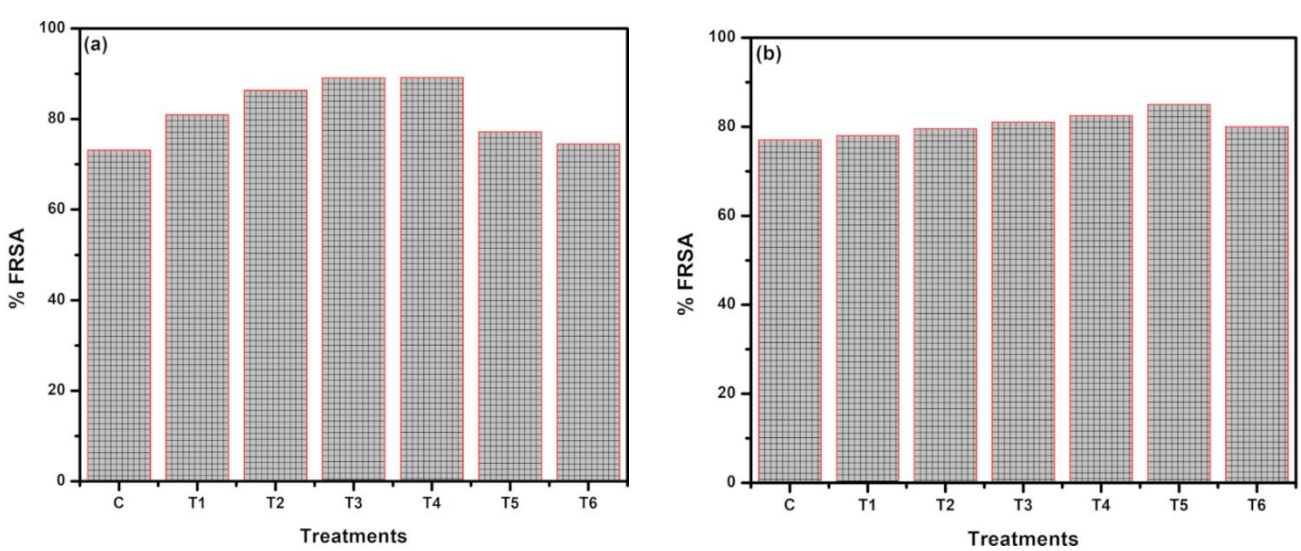

Fig. 7. Variations in scavenging potential of DPPH radicals in 12 days old (a) *I. turbinata* and (b) *C. arvensis* seeds plantlets grown in-vitro subjected to different Ag NPs concentrations.

Effect of AgNPs on fresh and dry weight: AgNPs had a significant effect on both fresh and dry weight of plantlets (Fig. 3). Slight increase in both fresh and dry weight was detected for T1 (25 mg/l of AgNPs) and T2 (50 mg/l of AgNPs) compared to the control. However, a constant diminution in both fresh weight and dry weight was monitored as the concentrations were raised above T1 which showed the negative impact of AgNPs when it came to biomass of plantlets. Lowest fresh weight and dry weight were recorded for highest AgNPs concentration. This decrease in fresh and dry weights was obvious since plantlets showed root and shoot growth inhibition at higher concentrations.

Effect of AgNPs on seed germination frequency and seed vigor: Seed germination frequency was effectively enhanced upon exposure of the seeds to AgNPs (Fig. 4). The seed germination frequency of plantlets (at 75% in control) was increased to 91.6% upon exposure to AgNPs (T1 ~ 25 mg/l). Further increase in concentrations of AgNPs caused 100% seed germination. AgNPs also showed improved seed vigor compared to the control for concentrations ranging from T1-T4 (25 – 100 mg/l). The optimum AgNPs concentration for highest seed vigor *I. turbinata* and *C. arvensis* was found to be T2 (50 mg/l) and T5 (150 mg/l) respectively. T2 (50 mg/l of Ag NPs) showed highest seed vigor (1756.67) for *I. turbinata* while T5 (50 mg/l of Ag NPs) exhibited highest seed vigor (15561.82) for *C. arvensis*. Further increase in concentration lead to reduced seed vigor. For example, in case of *I. turbinata*, at high concentrations (T5 ~ 150 and T6 ~ 200 mg/l), the seed vigor (1413.33 and 1366.67, for T5 and T6, respectively) was found to be even lower than that of the control (1442.5) with T6 showing lowest seed vigor despite improved seed germination frequency which can be attributed to the reduced root and shoot length.

Effect of AgNPs on biochemical parameters: *In-vitro*, AgNPs showed effective elicitation of secondary metabolites in plantlets. In case of *I. turbinata* plantlet, the control produced 2.86 mg/g DW flavonoid content which was increased up to 5.94 mg/g DW in response to T1 (25 mg / l of AgNPs). The concentration of flavonoids was further increased upon increasing AgNPs concentration with T4 (100 mg / l of AgNPs) being the optimum concentration resulting in total production of 7.17 mg/g DW. Further increase in AgNPs concentration was decreased the TFC value (Fig. 5). AgNPs also showed significant improvement in TPC of *I. turbinata* plantlets, though production was increased but it was not as significant as the TFC. The production of phenolics as recorded for the control was 25.23 mg/g DW which was increased to 28.13 mg/g DW for T1 (25 mg/l of AgNPs). Optimum production of phenolics was recorded for T4 (36.00 mg/g DW) and further increase in AgNPs concentration resulted in a decrease in TPC (34.05 and 31.10 mg/g DW for T5 and T6 respectively) (Fig. 6a).

Similarly, in case of *C. arvensis*, the production of flavonoids and phenolics in response to AgNPs was highly increased compared to the control. The control resulted in production of 4.19 mg/g DW flavonoid content which was increased to 5.19 mg/g DW in response to T1. The concentration of flavonoids was further increased (up to 10.3 mg/g DW) upon increasing AgNPs concentration (T5 ~ 150 mg/l of AgNPs). Further increase in AgNPs concentration resulted decrease in TFC (i.e., 7.01 mg/g DW for T6). AgNPs also showed significant improvement in TPC of *C. arvensis* plantlets and the increase in production of phenolics was as significant as that of TFC. The production of phenolics for the control was 20.91 mg/g DW, which was increased to 27.43 mg/g DW for T1 (25 mg/l of AgNPs). Optimum production of phenolics was recorded for T5 (40.37 mg/g DW) and further increase in AgNPs concentration resulted in a decrease in TPC (31.96 mg/g DW for T6) (Fig. 6b).

To study the antioxidant potency of in-vitro derived plantlets, FRSA was performed using DPPH free radical. FRSA was assessed for each AgNPs concentration (Fig. 7). For *I. turbinata* plantlet, T4 (100 mg/l of AgNPs) exhibited maximum FRSA (89.17%) while for *C. arvensis* the maximum FRSA (85%) was shown by T5 (100 mg/l of AgNPs), theses observation endorsed the previous results about TPC and TFC (Abbasi *et al*., 2010) because it is reasonable that the phenolics and flavonoids present in plants are the principal scavengers of free radicals.

In case of *I. turbinata* plantlet, the exponential growth in FRSA was observed for T1 (25 mg/l) to T4 (100 mg/l) concentrations of AgNPs while for *C. arvenssis* the growth was up to T5 (150 mg/l of AGNPs). However, it was observed that further increase of AgNPs concentration reduced the scavenging potential. Actually, during metabolism, plant cells produce certain levels of ROS (Reactive oxygen species) which during stress condition, are raised up to critical levels. These ROS results in oxidative damages in plants however, plants possess antioxidant enzymes in order to cope with ROS. Several phenolic and flavonoids acts as non-enzymatic ROS scavengers in plants (Sharma *et al*., 2012). Since AgNPs acts as stress inducers, ROS production is increased which in turn results in increased production of phenolic and flavonoid contents. However, as mentioned earlier, a decrease in phenolics and flavonoid content upon increase in AgNPs concentration (from T4 upto T6) may be due to the disruption of ROS and antioxidant production equilibrium condition.

Conclusion

The effects of AgNPs on various physiological and particularly biochemical features of *I. turbinata* and *C. arvensis* was evaluated. The germination frequency of the seeds increased with the concentration of AgNPs. The present study also revealed a dose dependent negative effect of AgNPs on the fresh weight and dry weight of plantlets, for instance higher concentrations of AgNPs showed decrease in fresh and dry weight of plantlets. This decrease in the fresh weight and dry weight content can be attributed to the negative effect of AgNPs on *in-vitro* both root and shoot generation. As compared to controlled, rooting in both plantlets were reduced in all the AgNPs treatments. Since, lowest root length was recorded for T6 (200 mg/l of AgNPs), hence it is therefore proposed that that high concentrations of AgNPs inhibited rooting in plantlets. Shoot generation on the other hand was initially enhanced at lowest concentration used. However, shoot generation was also reduced significantly with increase in AgNPs concentration. Significant variations were also observed in phenolic and flavonoid contents. AgNPs significantly enhanced the amount of phenolics and flavonoids in plantlets. Antioxidant activity against DPPH free radicals were also in accordance with amount of phenolics and flavonoids which is understandable as they

are the principal scavengers of free radicals. The study concludes that AgNPs can prove efficient replacement for conventionally used elicitors for improvement in plant phenology and biochemistry.

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