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Seed germination and biochemical profile of *Citrus reticulata* (Kinnow) exposed to green synthesised silver nanoparticles

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Abstract: The biosynthesis of silver nanoparticles (AgNPs) is substantial for its applications in different fields. The *Moringa oleifera* leaves were used as reducing and stabilising agent for the biosynthesis of AgNPs. The synthesised AgNPs were characterised through UV–visible spectroscopy, zeta analyser, scanning electron microscopy (SEM) and energy dispersive Xray (EDX). In this study, effects of the synthesised AgNPs were also evaluated on nucellus tissues germination frequency and biochemical parameters of plant tissues. Nucellus tissues of Citrus reticulata were inoculated on MS medium supplemented with 10, 20, 30 and 40 µg/ml suspension of the synthesised AgNPs. Green synthesised AgNPs enhanced the in vitro germination because of low toxicity and nonfriendly issues. Significant results were obtained for germination parameters i.e. root and shoot length and seedling vigour index in response to 30 µg/ml suspension of green synthesised AgNPs. The 30 µ/ml suspension of AgNPs also enhanced antioxidant activity (41%) and SOD activity (0.36 nM/min/mg FW) while total phenolic content (4.7 µg/mg FW) and total flavonoid content (1.1 µg/mg FW) was significantly high when MS medium was fortified with 40 µg/ml suspension of the synthesised AgNPs. The content of total protein was significant (558 µg/BSA Eq/mg FW) in control plantlets as compared to the other treatments.

1 Introduction

The use of nanotechnology is extensively increasing in the last decade in the different fields like biotechnology, medical and energy sector, information technology, consumer goods, and agriculture. Nanotechnology deals with the production of minute particles having dimension <1 µm [1]. The nanoparticles (NPs) can be synthesised from a wide variety of materials and they can expand their actions, depending on the size, shape, and chemical composition of the particles [2]. NPs are synthesised by microwave radiation [3], UV irradiation [4], γ-ray irradiation [5], electrochemical reduction [6], photochemical reduction [7], and chemical reduction [8]. Among them, chemical synthesis is the most commonly used method having some shortcomings such as high cost, toxicity, and non-friendly issues [9]. Alternatives include the biological synthesis of NPs by different biological agents, i.e. microorganisms, yeast, fungi, plant extracts, and sometimes intact plants [10].

Citrus is one of the most important horticultural and economically important tree fruit crop in the world [11–13]. Citrus belongs to family rutaceae and includes *Citrus reticulata*, *Citrus sinensis*, and *Citrus limon* which comprise 18% of the total fruit production in the world [14]. According to an estimate, ∼95% of the total *C. reticulata* (Kinnow) all over the world is produced in Pakistan [15]. Kinnow is a rich source of phenolics, flavonoids, terpenes, and some other biologically active ingredients. Kinnow is nutritionally very important because of rich source of vitamin C in conjugation with sugar, organic acids, amino acids, and minerals [16].

Plant tissue culture is considered as an alternative tool to the conventional breeding practices. It expands the quality of economically important plants and establishes the high-frequency regeneration protocol. In vitro regeneration is very beneficial for the production of various secondary metabolites and antioxidants under stress because conventional breeding practices are imperilled to land availability, weather, and varieties of pests which adversely affect the qualities of harvested plants [17].

Reactive oxygen species (ROS) are produced under stress condition to tackle the new environment; however, they also interact with biological molecules and inhibit growth and differentiation. Two types of secondary metabolites are produced under unfavourable condition in order to nullify the damaging effect of ROS, i.e. non-enzymatic compounds and anti-oxidative enzymes [18]. Non-enzymatic compounds contribute in quenching toxic-free radicals, apoptosis induction, enzyme activation, gene expression, and immune system stimulation [19]. Similarly, antioxidative enzymes play vital role in morphogenesis, plant development, and conversion of oxygen radical to hydrogen peroxide [20]. Generally, a strong relationship exists between antioxidant activity and non-enzymatic components [21].

So far, few studies have been carried out on the applications of nanomaterials in plant tissue culture. An increase in root and shoot length and seedling vigour index (SVI) was observed in *Artemisia absinthium* seeds exposed to various metallic NPs [22]. Zaka *et al.* [23] reported the seed germination and biochemical profile of *Eruca sativa* exposed to various NPs. The NPs do not directly influence the plants but indirectly alters the mechanism and this change in growth behaviour was observed after a certain time period [24].

The understanding of how NPs are involved in controlling the growth and development in plants is still to be explored and requires more comprehensive research work. The prime objective of this research work is to find the effect of various concentrations of green synthesised silver nanoparticles (AgNPs) on root length, shoot length, SVI, total phenolic content (TPC), total flavonoid content (TFC), superoxide dismutase (SOD) activity, content of total protein (CTP), and antioxidant activity in *C. reticulata*. There are very few reports which are available on the applications of chemically synthesised NPs on the in vitro germination and biochemical profiling of some plant species. To the best of our knowledge, this is the first study on the application of green synthesised AgNPs on in vitro germination and to explore the probable effects on the secondary metabolites production and antioxidant activity of *C. reticulata*.

Table 1 Layout

Treatments	Concentrations
T0	control
T1	AgNPs (10 µg/ml)
T2	AgNPs (20 µg/ml)
T3	AgNPs (30 µg/ml)
T4	AgNPs (40 µg/ml)

2 Materials and methods

2.1 Green synthesis of AgNPs

The green synthesis of AgNPs was done according to the protocol described by Hussain *et al.* [25]. Silver nitrate solution (SNS) was reduced by using *Moringa oleifera* leaves extract as main reducing and stabilising agent. The aqueous extract of *M. oleifera* was prepared by boiling 50 g leaves in 500 ml distilled water for 10 min. The extract was filtered thrice to remove all the remains of contaminants. A solution of silver nitrate (5 mM) was prepared by dissolving 0.85 g/l of distilled water. SNS was then continuously boiled for 5 min and reduced stepwise by the addition of plant extract until the colour of solution turned to dark brown. As soon as colour changed to dark brown, solution was centrifuged four times at 14,000 rpm for 10 min. Pellets were collected and supernatants were discarded. This process was repeated four times to remove the unreacted silver salt and plant extract. The synthesised AgNPs were used for characterisation as well as accessing germination parameters and biochemical profiling in *C. reticulata*.

2.2 Characterisation of the green synthesised AgNPs

2.2.1 UV–Visible spectroscopy: The reduction in silver nitrate ($AgNO_3$) to AgNPs was observed by recording UV–Visible spectrum from National Centre of Physics (NCP), Islamabad. The synthesised AgNPs were added in sterilised water and then subjected to ultra-sonication for 5–10 min. UV–Visible spectrum analysis was done by recording the spectrum from 300 to 700 nm.

2.2.2 Zeta analyser: The size range of the green synthesised AgNPs was determined through zeta analyser from Nuclear Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad. The synthesised NPs were ultra-sonicated for 15 min and then size range was determined through zeta potential.

2.2.3 Scanning electron microscopy: The structural analysis of the synthesised AgNPs was examined by scanning electron microscopy (SEM) by utilising SIGMA model operated at 5 kV, magnification ×10k from the Institute of Space and Technology (IST), Islamabad. Then film of the sample was prepared on a carbon-coated copper grid by just dropping the suspension of AgNPs in water on the grid, extra solution was removed by utilising the blotting paper and then the film on the SEM grid was allowed to dry by putting it under mercury lamp for 5 min. The sample surface images were taken at different magnifications.

2.2.4 Energy dispersive X-ray spectroscopy: The elemental analysis of the green synthesised AgNPs was also done from the Institute of Space and Technology (IST), Islamabad. Energy dispersive X-ray (EDX) detector was utilised for the elemental analysis of the green synthesised AgNPs by dropping the synthesised NPs on carbon film.

2.3 Plant source and surface sterilisation

Immature fruits of *C. reticulata* were collected from the Bhalwal region of district Sargodha. Seeds were harvested from the *C. reticulata* fruits and nucellus tissues were separated and surface sterilised by following the protocol described by Hussain *et al.* [15].

2.4 Nucellus tissues germination protocol

AgNPs were suspended directly in distilled water by sonication. About 10, 20, 30, and 40 µg/ml suspension of AgNPs were separately augmented to the MS medium with the help of micropipette (Table 1). Sterilised nucellus tissues were inoculated on the MS medium under completely sterilised conditions. The germination frequency was recorded after a period of 14 days. The whole experiment was conducted for 42 days and data was recorded at 7 days interval.

2.5 Nucellus tissues germination parameters

Different germination parameters were recorded, i.e. germination frequency, root length, shoot length, and SVI.

2.5.1 Nucellus tissue germination frequency: The germination frequency was recorded after every week. The germination frequency was calculated by utilising the following formula [26] (see equation below).

2.5.2 Root and shoot length: Root and shoot length was recorded in centimetre (cm) after every week and first data was collected after 14 days.

2.5.3 Seedling vigour index: SVI was calculated by following the protocol described by Abdul-Baki and Anderson [27] and expressed as index number as described by Ushahra and Malik [28]

$$\text{SVI} = [\text{root length (cm)} + \text{shoot length (cm)}] \times \text{germination percentage}.$$

2.6 Biochemical parameters

2.6.1 Antioxidant activity: The antioxidant activity of the plant extracts was determined by using the method of Abbasi *et al.* [29] with slight alterations. Approximately 0.5 ml of the DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was mixed with 4 ml of methanol augmented with 10 mg of dried plant tissue. The resulting reaction mixture was vortexed for 15 s followed by incubation at room temperature for a period of 30 min. Finally, the absorbance of samples was recorded at 517 nm by using UV/Visible spectrophotometer. The antioxidant activity was calculated by using the following equation

$$\%\text{age of DPPH discolouration} = 100 \times (1 - \text{As/Ab})$$

where As is the absorbance of solution after the addition of extract and Ab the absorbance of control.

2.6.2 TPC determination: For the quantification of TPC, Folin–Ciocalteu reagent was used according to the protocol described by Velioglu *et al.* [30] with slight alterations. Approximately, 0.75 ml of the Folin–Ciocalteu reagent was mixed with 100 µl of the plant extract followed by incubation for 5 min at 22°C. Thereafter, Na_2CO_3 solution (0.75 ml) was added to the mixture and kept for 90 min at 22°C. Finally, the absorbance of the sample was recorded at 725 nm by using UV–Visible spectrophotometer.

2.6.3 TFC determination: TFCs were determined by using the protocol described by Chang *et al.* [31] with minor modifications. Briefly, quercetin (10 mg) was dissolved in 80% C_2H_5OH and further dilutions were prepared. The resulting standard solution was mixed with 0.1 ml of 1 M potassium acetate, 0.1 ml of 10%

$$\text{Nucellus tissues germination frequency} = \text{number of nucellus tissues germinated} \times 100/\text{total number of nucellus tissues cultured}.$$

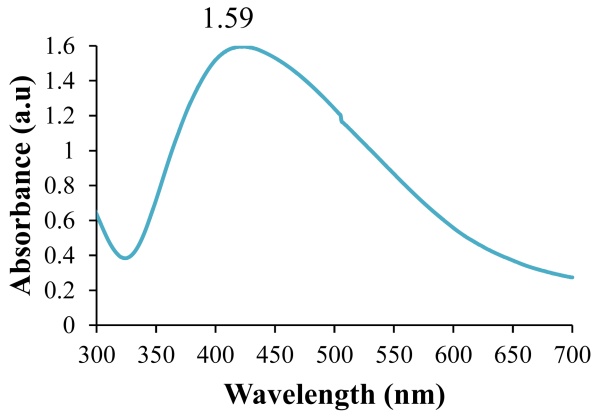

Fig. 1 *UV–Visible spectrum of green synthesised AgNPs*

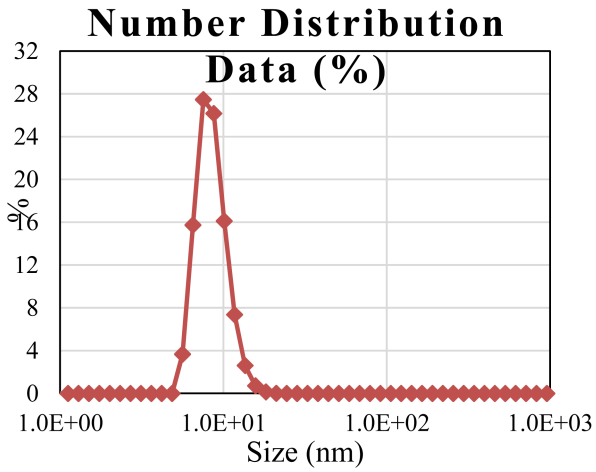

Fig. 2 *Size distribution of green synthesised AgNPs through zeta analyser*

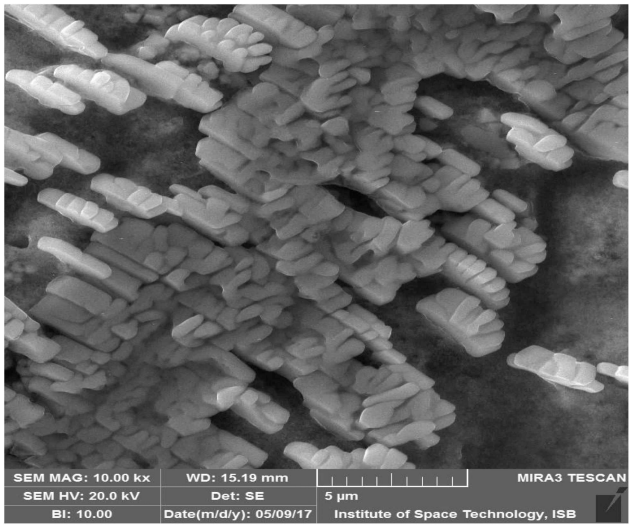

Fig. 3 *SEM micrograph of green synthesised AgNPs*

aluminium chloride, 1.5 ml of 95% ethanol, and 2.8 ml of distilled water followed by incubation for 30 min at room temperature. Finally, the absorbance of mixture was recorded at 415 nm by using UV/Visible spectrophotometer.

2.6.4 Total protein content determination: For the quantification of total protein content, protocol described by Lowry *et al.* [32] was used with some modifications. Enzyme extract was prepared in the same way as for antioxidant assay. The absorbance was then recorded at 650 nm by using spectrophotometer.

2.6.5 SOD activity: SOD assay was performed according to the method of Ullah *et al.* [33] with minor modifications. The mixture

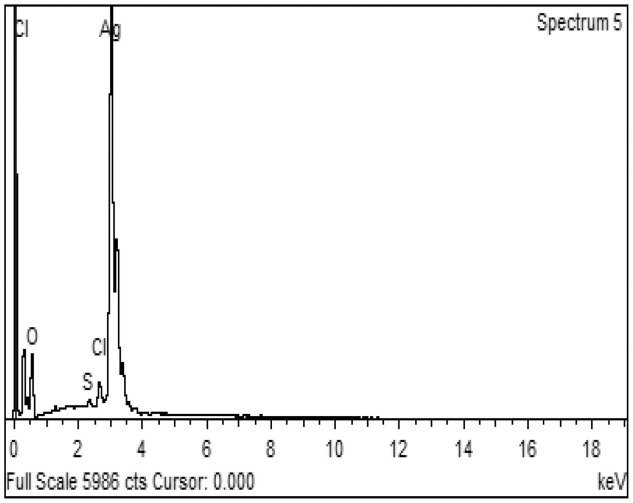

Fig. 4 *EDX spectrum of green synthesised AgNPs*

was prepared using 1 mM EDTA, 0.75 mM NBT, 50 mM phosphate buffer (pH 7), 130 mM methionine, and 0.02 mM riboflavin. The mixture was exposed to fluorescent light for 7 min and then the absorbance was recorded at 560 nm. SOD activity was calculated applying the Lambert–Beer law

$$A = \varepsilon LC$$

where A is the absorbance, ε the extinction coefficient, L the length of each wall, and C the concentration of enzymes.

2.7 Statistical analysis

The experimental design consisted of three replicates and each experiment was repeated twice. The results were interpreted as mean standard deviation. Student's *t*-test was selected for the statistical analysis of experimental data and each experimental value was compared with the corresponding control value.

3 Results and discussion

3.1 Synthesis and characterisation of AgNPs

The addition of *M. oleifera* leaves extract to SNS resulted in the change in colour to dark brown. To study the initial synthesis of AgNPs, the product produced by the reaction of plant extract and SNS is principally analysed through UV–Visible spectrum on HALO DB-20 spectrophotometer. The surface plasmon resonance (SPR) at 423–425 nm indicated the formation of AgNPs. Fig. 1 shows the UV–Visible spectrum of the synthesised AgNPs. Different characterisation peaks usually in the range of 410–480 nm are obvious for AgNPs synthesis [34, 35]. However, various wavelengths may attribute different sizes and shapes of AgNPs [36]. The synthesised AgNPs were also characterised through zeta potential for determining the size range. The zeta analyser elucidated that the size of synthesised AgNPs ranged from 8 to 28 nm (Fig. 2). Structural analysis of the green synthesised AgNPs was done through scanning electron microscope (SEM). The SEM image represents the rectangular segments fused together (Fig. 3). Similar shape of the synthesised AgNPs was also reported by Anjum and Abbasi [37] in an attempt to find the structural analysis by utilising WPE and CE. Elemental analysis of the synthesised AgNPs was done by the EDX detector (Fig. 4). The presence of metallic silver ions in the *M. oleifera* leaves extract mediated AgNPs was confirmed by using EDX. EDX spectrum showed absorption peak of silver in the range of 3–4 keV while silver nanocrystal showed absorption peaks in the range of 2.5–4 keV. The present findings are in line with some other researchers who reported similar results in an attempt to find absorption peaks of metallic silver ions by using the EDX detector [38, 39]. Impurities in the sample are attributed with the organic compounds which are involved in the reduction in $AgNO_3$ to AgNPs

Table 2 Germination frequency of nucellus tissues of *C. reticulata* in response to different treatments of green synthesised AgNPs

Sr. no.	Tr.	Germination frequency				
		After 14 days	After 21 days	After 28 days	After 35 days	After 42 days
01	T0	66.3 ± 3.31	71.4 ± 3.57	78.2 ± 3.91	83.7 ± 4.18	88.6 ± 4.43
02	T1	58.8 ± 2.94	74.1 ± 3.70	83.5 ± 4.17	92.1 ± 4.59	93.4 ± 4.67
03	T2	72.6 ± 3.63	78.3 ± 3.91	88.4 ± 4.42	92.1 ± 4.60	94.5 ± 4.72
04	T3	76.9 ± 3.84	81.7 ± 4.08	90.2 ± 4.51	93.6 ± 4.68	98.4 ± 4.92
05	T4	52.6 ± 2.63	62.1 ± 3.10	71.7 ± 3.58	75.5 ± 3.77	80.8 ± 4.04

Table 3 Effect of different treatments of green synthesised AgNPs on root length, shoot length, and SVI of *C. reticulata*

Germination frequency	Tr.	Root length, cm	Shoot length, cm	SVI
after 14 days	T0	1.1 ± 0.05	2.3 ± 0.11	225.4 ± 11.2
	T1	1.0 ± 0.05	2.1 ± 0.10	182.3 ± 9.1
	T2	1.4 ± 0.07	2.5 ± 0.12	210.5 ± 10.5
	T3	1.9 ± 0.09	2.7 ± 0.13	353.7 ± 17.6
	T4	1.7 ± 0.08	2.2 ± 0.11	205.1 ± 10.2
after 21 days	T0	1.5 ± 0.07	2.8 ± 0.14	307.0 ± 15.3
	T1	1.6 ± 0.08	2.7 ± 0.13	318.6 ± 15.9
	T2	2.1 ± 0.10	3.0 ± 0.15	399.3 ± 19.9
	T3	2.4 ± 0.12	3.5 ± 0.17	482.0 ± 24.1
	T4	1.9 ± 0.09	3.2 ± 0.16	316.7 ± 15.8
after 28 days	T0	1.8 ± 0.09	3.1 ± 0.15	383.2 ± 19.1
	T1	1.9 ± 0.09	3.3 ± 0.16	434.2 ± 21.7
	T2	2.4 ± 0.12	3.8 ± 0.19	548.1 ± 27.4
	T3	2.7 ± 0.13	4.0 ± 0.20	604.3 ± 30.2
	T4	2.2 ± 0.11	3.7 ± 0.18	423.0 ± 21.1
after 35 days	T0	2.4 ± 0.12	3.5 ± 0.17	493.8 ± 24.6
	T1	2.6 ± 0.13	4.0 ± 0.20	606.5 ± 30.3
	T2	2.7 ± 0.13	4.3 ± 0.21	644.7 ± 32.2
	T3	3.3 ± 0.16	4.9 ± 0.24	767.5 ± 38.3
	T4	3.0 ± 0.15	4.4 ± 0.22	558.7 ± 27.9
after 42 days	T0	2.8 ± 0.14	4.2 ± 0.21	620.2 ± 31.0
	T1	3.4 ± 0.17	4.6 ± 0.23	747.2 ± 37.3
	T2	3.5 ± 0.17	5.4 ± 0.27	841.1 ± 42.0
	T3	4.1 ± 0.20	6.1 ± 0.30	1003.7 ± 50.1
	T4	3.7 ± 0.18	5.3 ± 0.26	727.2 ± 36.3

3.2 Effect of green synthesised AgNPs on the nucellus tissue germination

Seed germination is an efficient phytotoxicity test because it is rapid, easy, low cost, and most suitable for unstable samples [40, 41]. Percentage germination of nucellus tissues was recorded at different day intervals i.e. 14, 21, 28, 35, and 42 days and first data was recorded after 2 weeks of incubation on the MS medium. The effect of different treatments of green synthesised AgNPs on the in vitro germination of nucellus tissues is shown in Table 2. The nucellus tissues augmented on the MS medium without the suspension of green synthesised AgNPs were taken as control. The germination frequency was progressively increased in all the applied treatments with the passage of time. The maximum germination percentage was recorded when MS medium was fortified with 30 µg/ml suspension of the green synthesised AgNPs at different day intervals, i.e. 14, 21, 28, 35, and 42 days as compared to the other concentrations of the synthesised AgNPs. It was found that 40 µg/ml suspension of the green synthesised AgNPs show inhibitory effects on nucellus tissues germination at different day intervals. Toxicity of NPs depends on the size, shape, chemical composition, and release of toxic ions from the synthesised NPs [2]. AgNPs can act as Trojan horse because it passes certain obstacles and release silver ions in aqueous state with the passage of time [42].

3.3 Effect of green synthesised AgNPs on root length, shoot length, and SVI

After exposing the nucellus tissues of *C. reticulata* to different treatments of green synthesised AgNPs, significant root and shoot length and SVI were observed at different day intervals such as 14, 21, 28, 35, and 42 days (Table 3). The effect of different treatments of the green synthesised AgNPs on root and shoot length and SVI varied greatly depending on the concentration of the synthesised AgNPs and number of days passed after inoculation. It was observed that 30 µg/ml suspensions of the AgNPs have positive effect on root and shoot length and SVI as compared to the other treatments of the synthesised AgNPs. Root inhibition varied greatly among the NPs, type of plants, and partially correlated to NPs concentration [40]. The maximum root length (4.1 cm), shoot length (6.1 cm), and SVI was observed when MS medium was augmented with 30 µg/ml suspension of the *M. oleifera* leaves extract mediated AgNPs after 42 days of the experiment. The present outcomes are in agreement with Savithramma *et al.* [43] who reported that AgNPs pierced and persuaded pores in seeds resulted in the rapid influx of nutrients. The influx of nutrients induced rapid germination of seed. The 40 µg/ml suspension of the synthesised AgNPs have reduced the root and shoot length and SVI and showed more stress as compared to the other treatments of the synthesised AgNPs. The findings of Lee *et al.* [44] also affirmed our results.

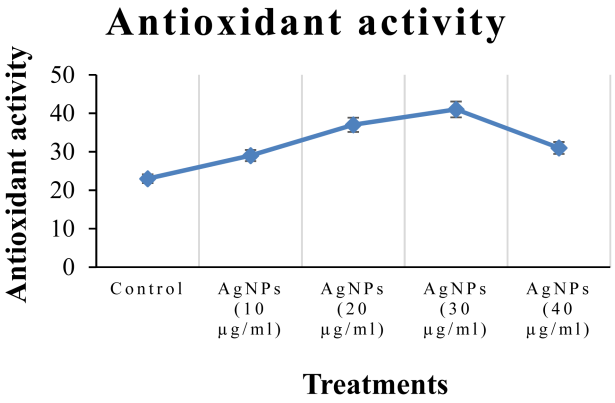

Fig. 5 *Percentage DPPH RSA of C. reticulata against different treatments of green synthesised AgNPs*

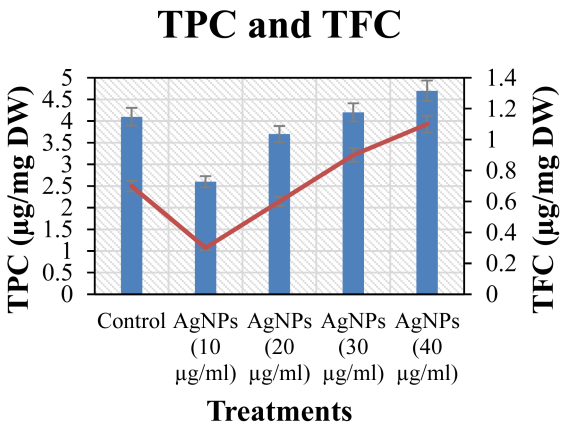

Fig. 6 *TPC and TFC of C. reticulata against different treatments of green synthesised AgNPs*

3.4 Antioxidant activity

Among the different methods used for antioxidants determination, DPPH radical scavenging assay (RSA) is the simplest, rapid, efficient, and inexpensive method for the determination of antioxidant activity in plant cell cultures [45]. In the present study, the effect of green synthesised AgNPs on the DPPH RSA was investigated after 42 days of the experiment (Fig. 5). Different treatments of the synthesised AgNPs significantly affected the antioxidant activity in *C. reticulata*. It was observed that DPPH RSA was significant (41%) when MS medium was augmented with 30 µg/ml suspension of the green synthesised AgNPs as compared to the other treatments. The 10 µg/ml suspension of AgNPs have the reduced antioxidant activity as compared to the other treatments. Similar results were reported by other researchers in an attempt to find the effect of AgNPs on the antioxidant activity in *Silybum marianum*, *A. absinthium*, and *C. reticulata* [22, 46, 47].

3.5 TPC and TFC

In this study, TPC and TFC were also investigated in response to different treatments of the green synthesised AgNPs (Fig. 6). It was observed that TPC (4.7 µg/mg FW) and TFC (1.1 µg/mg FW) in relation to dry mass was significantly high in plantlets treated with 40 µg/ml suspension of the green synthesised AgNPs. TFC show dependency and linear correlation with TPC. However, 10 µg/ml suspension of the green synthesised AgNPs-treated plantlets showed comparatively less production of non-enzymatic components. The elicitors not only affect the organogenic potential but also the synthesis and production of non-enzymatic components and antioxidative enzymes [48]. Furthermore, different researchers have also reported the TPC and TFC in the in vitro cultures of different medicinal plants [25, 49]. Applications of flavonoids-based AgNPs resulted in the enhanced production of non-enzymatic components of defence system [50].

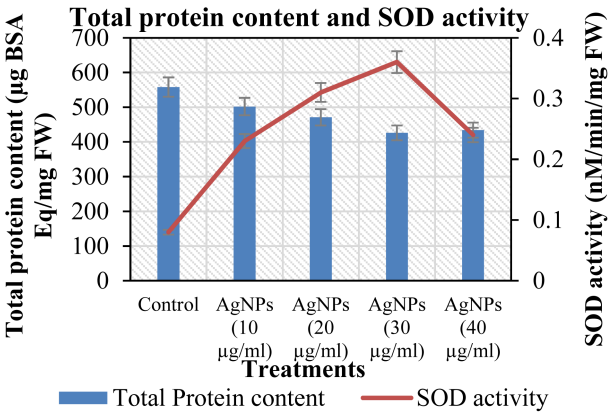

Fig. 7 *Total protein content and SOD activity of C. reticulata against different treatments of green synthesised AgNPs*

3.6 CTP and SOD activity

As shown in Fig. 7, the effect of different treatments of the green synthesised AgNPs on CTP and SOD activity was also evaluated in plantlets after 42 days of experiment. CTP is comparatively lesser in plantlets treated with 30 µg/ml suspension of the synthesised AgNPs as compared to the control and other treatments of the green synthesised AgNPs. Plantlets which were not treated with suspension of AgNPs showed significant CTP (558 µg/BSA Eq/mg FW) as compared to the other treatments. Moreover, the present findings are in agreement with Tariq *et al.* [51] who reported almost similar CTP on in vitro cultures of *A. absinthium*. Unlike the CTP, plantlets treated with the suspension of different suspensions of green synthesised AgNPs showed significant SOD activity as compared to the control. The incorporation of 30 µg/ml suspension of the synthesised AgNPs to the medium enhanced the SOD activity (0.36 nM/min/mg FW) as compared to the other treatments. The differences in pattern are mainly due to size, shape, and composition of NPs. The enzymatic role of SOD is widely reported in different medicinal plants [25, 52, 53].

4 Conclusion

The biosynthesis of AgNPs by utilising *M. oleifera* leaves extract has potent in vitro germination, growth, and biochemical effect on *C. reticulata*. It was found that effects of different treatments of green synthesised AgNPs on germination, growth, and biochemical profile of *C. reticulata* mericlones were significantly positive. Once the NPs get entry into seeds, then they have long-lasting effects on seed germination and growth. Furthermore, it can also considerably alter the biochemical profile. So far few studies have been conducted regarding nanomaterials as elicitors of secondary metabolites and antioxidant activity in the mericlones. These preliminary findings pave the way for more comprehensive study about the ecotoxicity of NPs and understanding the mechanism at molecular level that would provide basis to recognise their chemistry for future challenges.

5 Acknowledgment

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