Research Article

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Green synthesized silver and copper nanoparticles induced changes in biomass parameters, secondary metabolites production, and antioxidant activity in callus cultures of *Artemisia absinthium* L.<https://doi.org/10.1515/gps-2021-0010>

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Abstract: *Artemisia absinthium* L. is a highly medicinal plant with a broad range of biomedical applications. *A. absinthium* callus cultures were established in response to bio-fabricated single NPs (Ag and Cu) or a combination of both NPs (Ag and Cu) in different ratios (1:2, 2:1, 1:3, and 3:1) along with thidiazuron (TDZ) (4 mg/L) to elicit the biomass accumulation, production of non-enzymatic compounds, antioxidative enzymes, and antioxidant activity. Silver and copper nanoparticles (Ag and Cu NPs) were synthesized using the leaves of *Moringa oleifera* as reducing and capping agent and further characterized through UV-Visible spectroscopy and SEM. The 30 µg/L suspension of Ag and Cu NPs (1:2, 2:1) and 4 mg/L TDZ showed 100% biomass accumulation as compared to control (86%). TDZ in combination with Ag NPs enhanced biomass in the log phases of growth kinetics. The Cu NPs alone enhanced the superoxide dismutase activity (0.56 nM/min/mg FW) and peroxidase activity (0.31 nM/min/mg FW) in callus cultures. However, the combination of Ag and Cu NPs with TDZ induced significant total phenolic (7.31 µg/g DW) and flavonoid contents (9.27 µg/g DW). Furthermore, the antioxidant activity was highest (86%) in the Ag and Cu NPs (3:1) augmented media. The present study provides the first evidence of bio-fabricated single NPs

(Ag and Cu) or a combination of both NPs (Ag and Cu) in different ratios (1:2, 2:1, 1:3, and 3:1) along with TDZ (4 mg/L) on the development of callus culture, production of endogenous enzymes, non-enzymatic components, and further antioxidant activity in callus cultures of *A. absinthium*.

Keywords: *Artemisia absinthium*, nanoparticles, callus culturing, biomass accumulation, antioxidants

1 Introduction

Artemisia absinthium is commonly known as “wormwood” belonging to the family Asteraceae and used as herbal medicine in Asia, North Africa, Europe, and Middle East [1]. Traditionally, *A. absinthium* was used because of its diuretic and antispasmodic properties [2], vermifuge, trematocidal [3], bitter, insecticidal properties [4], and against diarrhoea, cough, and common cold [5]. Recently, aerial parts of wormwood are renowned to possess anti-snake venom activity [6]. *A. absinthium* possess a broad range of biological properties including antitumor, neurotoxic, neuroprotective, hepatoprotective, antimalarial, anthelmintic, and antiprotozoal [7]. Currently, the production of *A. absinthium* in natural repositories is less as compared to its requirements as pharmacological implications. However, fewer studies have been reported on *in vitro* production of *A. absinthium*. *In vitro* callus culture techniques will reduce the time required for the mericlones development and production of antioxidative enzymes, non-enzymatic compounds, and antioxidant activity, which are either problematic to synthesize under laboratory conditions or produced in less quantity in parental plants [8].

Reactive oxygen species (ROS) are produced under stress to tackle new environments; however, they also interact with biological molecules to inhibit growth and differentiation. However, in such environments, plant cells use either non-enzymatic components such as

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flavonoids and phenolics or enzymatic components such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) that nullify the damaging effects of ROS [9]. Endogenous enzymes play an important role in the conversion of oxygen radicals to H_2O_2 , mericlones development, direct, and indirect organogenesis [10,11]. Similarly, non-enzymatic components contribute to apoptosis induction, enzyme activation, quenching of toxic-free radicals, and expression of gene immune system stimulation and interaction with cell cycle arrest [12]. Secondary metabolites are the main source of antioxidant activity in plant cells and tissues. This antioxidant activity is associated with flavonoids and phenolics [13].

Nanotechnology is famed as twenty-first-century science, and its applications have been extended in different fields of biology, physics, and chemistry. The science deals with the production of minute particles having dimension between 1 and 100 nm [14]. Nanoparticles (NPs) have been applied in various areas of biotechnology because of their exceptional characteristics. However, in medicinal plant biotechnology, the utilization of NPs is a new area of interest and requires more comprehensive research. The recent studies focused on the role of NPs in seed germination, root/shoot length, seedling vigour index (SVI), and biochemical profiling in different medicinally important plants [15–17]. The enhanced production of enzymatic components (SOD, POD, and CAT) and non-enzymatic components (total phenolic content [TPC] and total flavonoid content [TFC]) was observed in *A. absinthium* plantlets exposed to different NPs [18]. Ag NPs boosted *in vitro* seed germination and growth of seedlings, and enhanced production of antioxidant enzymes [19]. In contrast, zinc oxide NPs suppressed the seed germination in various plants [20].

The mechanism of NPs in regulating the plants growth and development demands more comprehensive research works. The prime objective of the present research work is to check the effect of bio-fabricated silver (Ag) and copper (Cu) NPs with or without application of thidiazuron (TDZ) on the morphogenic variations in callus cultures, proliferation and production of secondary metabolites including TPC, TFC, SOD, POD, and 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay (RSA). Very few studies have been conducted for the applications of chemically synthesized NPs in callus cultures of some plant species. According to our knowledge, this study provides the first evidence on the application of various ratios and combinations of bio-fabricated NPs with or without the application of TDZ on callus

cultures development and to explore the probable effects on the secondary metabolites production and antioxidant activity in callus cultures of *A. absinthium*.

2 Materials and methods

2.1 Synthesis of Ag and Cu NPs

Ag and Cu NPs were synthesized by following the protocol described by Hussain *et al.* [21]. The extract of *Moringa oleifera* leaves was used for the reduction and further capping of corresponding salts. The $AgNO_3$ (0.17 g) and $CuSO_4 \cdot 5H_2O$ (0.25 g) were dissolved, respectively, in 1 L of distilled water to make 1 mM solution of both salts. The solutions were then reduced stepwise by adding the extract of *M. oleifera* with continuous boiling until the solution colour turned to brown and light green for Ag and Cu NPs, respectively. The reaction mixtures were then added in 15 mL falcon tubes and centrifuged for 15 min at 10,000 rpm. The resultant pellets were taken and centrifuged again in double-distilled water. This process continued for three times to get the purity of NPs. The resultant Ag and Cu NPs were used for characterization as well as for accessing the biomass accumulation and secondary metabolite productions in callus cultures of *A. absinthium*.

2.2 Characterization of Ag and Cu NPs

2.2.1 UV-Visible spectroscopy

The green synthesized Ag and Cu NPs were suspended directly in distilled water by sonication. The biosynthesis of Ag and Cu NPs was observed by recording the UV-Visible (UV-Vis) spectra using spectrophotometer.

2.2.2 Scanning electron microscopy (SEM)

The structural analysis of bio-fabricated Ag and Cu NPs was performed using SIGMA model of SEM. For SEM, the samples of bio-fabricated Ag and Cu NPs were prepared by simply dropping a minor amount of samples on Cu grid coated with carbon. The films were then air-dried, and SEM micrographs were taken at different magnifications.

2.3 Surface sterilization of explants

The seeds of *A. absinthium* were surface sterilized in 70% ethanol for 60 s followed by immersion in 0.1% (w/v) mercuric chloride (HgCl_2) solution for 1 min. The seeds were then rinsed with sterilized distilled water and dried on sterilized filter papers [22].

2.4 Callus culture development

To explore the effect of bio-fabricated Ag and Cu NPs and TDZ on callus culture, approximately 1.5 cm leaf sections of *in vitro* derived plantlets were added on MS medium supplemented with various treatments of NPs and TDZ. To explore the effect of bio-fabricated Ag and Cu NPs, NPs were added in the MS medium under sterilized conditions. Cultures were kept at $27 \pm 1^\circ\text{C}$ under a photoperiod of 16 h light and 8 h dark. Callus formation frequency, texture, and proliferation were recorded after 4 weeks of incubation period. The growth curve for biomass accumulation was developed for the rapidly growing friable callus. Analysis of biomass accumulation was performed for 42 days with an interval of 7 days.

2.5 Determination of antioxidative enzymes

For the analysis of antioxidative enzymes, callus cultures were harvested according to the method described by Nayyar and Gupta [23]. About 10 g of fresh sample was extracted in 10 mL of the extraction buffer. The homogenize was centrifuged at 14,000 rpm at 4°C for 30 min. The supernatant was collected and used for antioxidative enzyme assays. SOD activity was analysed by following the protocol of Ahmad et al. [24], and POD activity was analysed by following the protocol of Lagrimini [25].

2.6 Analyses of non-enzymatic compounds

For the analyses of non-enzymatic compounds, the extraction of calli was performed using the protocol described by Giri et al. [26] with minor modifications. About 100 mg dried samples were crushed in 10 mL of 80% (v/v) methanol. The mixture was sonicated three

times and centrifuged at 10,000 rpm for 10 min, and supernatant was stored at 4°C .

2.6.1 Total phenolic content

For the analyses of TPC, Folin–Ciocalteu reagent was used according to the protocol described by Velioglu et al. [27]. The mixture of Folin–Ciocalteu reagent (0.75 mL) and enzyme extract (100 μL) was kept at 22°C for 5 min followed by the addition of 0.75 mL Na_2CO_3 solution and kept at 22°C for 90 min. The absorbance of the sample was recorded at 725 nm using UV/Vis-DAD spectrophotometer.

2.6.2 Total flavonoid content

The TFC assay was performed using colorimetric method described by Chang et al. [28]. About 10 mg quercetin was added in 80% ethanol and then further dilutions were made. Each standard diluted solution was mixed with 1.5 mL ethanol, 0.1 mL Al_2Cl_3 , 0.1 mL of 1M $\text{CH}_3\text{CO}_2\text{K}$, and 2.8 mL of distilled water and incubated for 30 min at 25°C . The absorbance of the sample was recorded at 415 nm using UV/Vis-DAD spectrophotometer.

2.7 Antioxidant activity

For determining the antioxidant activity, DPPH was used by following the protocol described by Abbasi et al. [29]. About 10 mg dried plant material was mixed in 4 mL methanol followed by subsequent mixing in 0.5 mL of 1 mM DPPH solution. The mixture was then vortexed for 15 s and kept at 25°C for 30 min. The absorbance of the sample was then recorded at 517 nm using UV/Vis-DAD spectrophotometer. The antioxidant activity was determined by following the formula:

$$\% \text{ age of DPPH discoloration} = 100 \times (1 - A_s/A_b)$$

where A_s – reaction mixture absorbance after the addition of extract, A_b – control samples absorbance.

2.8 Statistical analysis

The experiment was repeated two times consisting of three replicates. The mean value of all treatments was

analysed through ANOVA, and for mean standard deviation, the statistical 8.1 software was used.

3 Results

3.1 Synthesis and characterization of Ag and Cu NPs

The change in colour of reaction mixtures to brown and light green after mixing corresponding salts and plant extract is a general characteristic of Ag and Cu NPs' bio-synthesis, respectively. The extract of *M. oleifera* leaves acts as a main reducing and capping/stabilizing agent. Hussain *et al.* [30] reported that plant flavonoids are actually involved in the reduction and capping of the bio-fabricated NPs (Figure 1).

Different techniques can be used for the characterization of NPs. Characterization of NPs requires a combination of various techniques because a single technique is unable to fully characterize colloidal NPs. The synthesis of NPs was observed by recording UV-Vis spectra on UV-Vis spectroscopy. To study the initial synthesis of NPs, the product produced by the reaction of plant extract and corresponding salts is monitored by UV-Vis spectroscopy. The mixture of plant extract with corresponding salt showed surface plasmon resonance at 423–425 nm for

Figure 1: Flow chart for the possible synthesis of silver and copper NPs.

Figure 2: UV-Visible spectra of: (a) Ag NPs, (b) Cu NPs.

Ag NPs and 545–554 nm for Cu NPs. Figure 2 illustrates the UV-Vis spectra of Ag NPs (Figure 2a) and Cu NPs (Figure 2b), respectively. The structural analysis of the bio-fabricated NPs was performed using SIGMA model through scanning electron microscopy. SEM elucidated that Ag NPs were rectangular in shape, whereas Cu NPs were spherical in shape (Figure 3).

3.2 Nanoparticles mediated proliferation in callus cultures

The different combinations of Ag NPs and Cu NPs alone and in combination with TDZ were used to check their response on callus proliferation percentage (Table 1). Proliferation of callus was observed in almost all the applied treatments. However, callus proliferation was found maximum (100%) in the MS medium supplemented with 30 µg/L suspension of Ag and Cu NPs (1:2) and Ag and Cu NPs (2:1) in combination with TDZ. Callus proliferation was 45% (Ag NPs), 57% (Cu NPs), and 86% (TDZ) when used alone (Figures 4 and 5). Furthermore, Ag and Cu NPs (1:2) and Ag and Cu NPs (1:3)-augmented media showed optimal callogenic frequency. The results for callus proliferation percentage in response to 4 mg/L TDZ and various combinations of NPs are presented in Table 2.

Figure 3: Scanning electron microscopy (SEM) micrographs of: (a) Ag NPs, (b) Cu NPs.

3.3 Effect of NPs on biomass accumulation in callus cultures

The effect of various combinations of NPs and TDZ on the growth rate of the calli was examined with 7-day intervals for a maximum period of 42 days (Figure 6). Various profiles of biomass accumulation were observed in the lag, log, and decline phases in response to NPs alone or in combination with TDZ. Comparatively, lag and log phases did not change in all the treatments started from 7 to 28 days. However, biomass accumulation in the decline phase was observed for all treatments after 28 days and reached a period of 42 days (Figure 6).

To check the response of NPs on biomass accumulation, Ag and Cu NPs were supplemented on MS medium, growth of callus was determined for a period of 42 days, and data were collected after every week, in comparison with the TDZ supplemented media. The maximum growth

Table 1: Treatment layout

Treatments	NPs (30 µg/L) + TDZ (mg/L)
T1	Murashige and Skoog media (MS) + Ag NPs
T2	MS + Cu NPs
T3	MS + Ag NPs + TDZ (4.0)
T4	MS + Cu NPs + TDZ (4.0)
T5	MS + 1:2 (Ag and Cu NPs)
T6	MS + 1:2 (Ag and Cu NPs) + TDZ (4.0)
T7	MS + 1:3 (Ag and Cu NPs)
T8	MS + 1:3 (Ag and Cu NPs) + TDZ (4.0)
T9	MS + 2:1 (Ag and Cu NPs)
T10	MS + 2:1 (Ag and Cu NPs) + TDZ (4.0)
T11	MS + 3:1 (Ag and Cu NPs)
T12	MS + 3:1 (Ag and Cu NPs) + TDZ (4.0)
T13	TDZ (4.0)

rate (2.2 g) was recorded in response to Ag NPs in the log phase as compared to the TDZ supplemented media (0.38 g) (Figure 6a). However, Cu NPs supplemented media showed twofold less biomass accumulation as compared to Ag NPs, where maximum biomass accumulation (0.75 g) was recorded on 28 days (Figure 6b). Similarly, TDZ in combination with Ag and Cu NPs were also explored for the accumulation of biomass. MS media supplemented with TDZ and Ag NPs, and the total biomass was moderate in the lag as well as in the decline phases. The maximum growth rate (1.46 g) of callus was recorded on the 28th day of the culture (Figure 6c). Maximum biomass (0.98 g) was observed after 35 days, when MS media was supplemented with Cu NPs in combination with TDZ, which is lesser as compared to combination of Ag NPs and TDZ (Figure 6d). Moreover, various ratios of Ag and Cu NPs alone or with the addition of TDZ were also observed for biomass accumulations. The Ag and Cu NPs (1:2) supplemented media elucidated the maximum growth rate (0.62 g) of callus on the log phase of the growth kinetics (Figure 6e) which is less as compared to media supplemented with Ag and Cu NPs in combination with TDZ (0.84 g) after 28 days of the culture (Figure 6f). Similarly, Ag and Cu NPs in (1:3) ratio showed maximum biomass (0.71 g) on the 28th day of the culture (Figure 6g) which is less as compared to media supplemented with Ag and Cu NPs (1:3) in combination with TDZ on the 28th day of culture (Figure 6h). However, Ag and Cu NPs (2:1)-supplemented media exhibited prolonged lag phase of 28 days (Figure 6i). Maximum biomass (1.08 g) was recorded on the 28th day of the lag phase than control (0.62 g). However, onefold reduction (0.75 g) in biomass accumulation was recorded when MS media was supplemented with Ag and Cu NPs (2:1) in combination with TDZ (Figure 6j). Similarly, significant

Figure 4: Effect of different treatments on callus proliferation in *A. absinthium*.

results were obtained in the media augmented with Ag and Cu NPs (3:1) without the application of TDZ (Figure 6k). However, the addition of TDZ to the same ratio of Ag and Cu NPs showed antagonistic effects on biomass accumulation (Figure 6l). The results revealed that the addition of single NPs (Ag and Cu) or combination of both nanoparticles (Ag and Cu) along with TDZ was found to be very effective for biomass accumulation as compared to the control.

3.4 SOD and POD activities

The SOD and POD activities were investigated in response to single NPs (Ag and Cu) or combination of both NPs (Ag and Cu) in different ratios (1:2, 2:1, 1:3, and 3:1) along with TDZ (4 mg/L). The highest POD activity (0.31 nM/min/mg FW) and SOD activity (0.56 nM/min/mg FW) was recorded

in MS medium supplemented with 30 µg/L suspension of Cu NPs. Similarly, significant results for antioxidative enzymes (0.33 and 0.26 nM/min/mg FW) were also observed with Ag and Cu NPs in combination with TDZ for SOD and POD, respectively (Figure 7). It shows that NPs applied alone or in combination with TDZ increased the production of antioxidative enzymes in callus cultures. It was found that production of SOD and POD was not associated with the type of NPs, rather it shows independent behaviour. The increase in the production of SOD leads to a decrease in the POD production and in this way helps in plant production and growth of plants.

3.5 TPC and TFC

Total phenolic and flavonoid contents of non-enzymatic defence system were investigated in callus cultures in

Figure 5: Maximum proliferation in callus cultures of *A. absinthium* in: (a) Ag and Cu NPs (1:2) + TDZ, (b) Ag and Cu NPs (2:1) + TDZ.

Table 2: Impact of different ratios of Ag NPs and Cu NPs alone or in combination with TDZ on callus proliferation and texture in *A. absinthium*

Tr.	Callus induction (day)	Callus proliferation (%)	Callus texture
T1	25	57	F
T2	25	45	F
T3	20	82	C
T4	20	78	C
T5	22	65	C
T6	18	100	C
T7	22	62	C
T8	18	75	C
T9	22	60	C
T10	18	100	C
T11	22	66	C
T12	18	82	C
T13	18	86	C

C – compact; F – friable.

response to NPs alone or in combination with different ratios of NPs along with TDZ (Figure 8). Maximum TPC (7.31 GAE- μ g/mg DW) was recorded in MS medium supplemented with Ag and Cu NPs (3:1) in combination with TDZ. However, TPC production (6.88 GAE- μ g/mg DW) was not changed with Ag and Cu NPs (3:1)-supplemented medium without TDZ. TPC production was moderate when NPs were supplemented alone. Moreover, TFC showed positive correlation with TPC in callus cultures. Maximum production of TFC (9.27 RE- μ g/mg DW) was recorded with Ag and Cu NPs (1:3) in combination with TDZ incorporated media. Furthermore, low quantities of TFC (5.68 RE- μ g/mg DW) and TPC (5.09 GAE- μ g/mg DW) were recorded under control conditions. The results of the present study propose that NPs have potential to improve the morphological and biochemical fluctuations in callus cultures of *A. absinthium*. It means that combinations of NPs and TDZ have synergistic effects on TPC and TFC.

3.6 Antioxidant activity

The antioxidant activity was also explored in response to all the applied treatments. It was found that NPs alone or with the addition of TDZ to different ratios of NPs also affected the antioxidant activity in callus cultures. The highest antioxidant activity (86%) was observed when medium was supplemented with the Ag and Cu NPs (3:1). However, the MS medium supplemented with 4 mg/L TDZ showed higher antioxidant activity in comparison with

most of the applied treatments (Figure 9). The combinations of NPs and TDZ have synergistic effects on anti-oxidant activity.

4 Discussion

The effect of different ratios and combinations of bio-fabricated Ag and Cu NPs applied alone or with the addition of TDZ was explored for biomass accumulation and further quantification of anti-oxidative enzymes and non-enzymatic compounds. Ag and Cu NPs were synthesized using the leaves of *M. oleifera* as a reducing and capping agent. The bio-fabricated Ag and Cu NPs were characterized through UV-Vis spectroscopy and SEM. UV-Vis spectroscopy confirmed the synthesis of the Ag and Cu NPs. The surface plasmon resonances in the range of 410–480 nm are the indicator for the synthesis of Ag NPs [31–33]. However, variation in the wavelengths may attribute to different sizes and shapes of the synthesized Ag NPs [34]. SEM elucidated that the bio-fabricated Ag NPs were rectangular segments fused together in structure, whereas Cu NPs were spherical in shape.

MS media supplemented with the suspension of individual NPs or combination of both NPs significantly induced biomass accumulation in different phases. In the present study, callus proliferation was reported from leaf explants of *A. absinthium* which were grown under *in vitro* conditions. Tariq et al. [22] have also reported the biochemical variations in callus cultures from leaf explants of *A. absinthium* under controlled conditions. The understanding of how NPs control the callus growth and further secondary metabolites production in response to NPs application alone or in combination with TDZ is still to be explored.

There are various conflicting reports on the absorption, translocation, and accumulation of NPs in different plants [35]. The effect of different NPs on the germination parameters depends on physiological state, age, type of tissues, and plant species [36,37]. Previous reports have shown that different NPs showed different responses to the germination parameters in *Eruca sativa* and *A. absinthium* [17,18]. The present study has reported that different ratios and combinations of bio-fabricated NPs applied alone or with TDZ have stimulatory effects on callus biomass and secondary metabolism. MS media supplemented with suspension of Ag and Cu NPs (1:2) and Ag and Cu NPs (2:1) along with TDZ (4 mg/L) showed 100% callus proliferation as compared to the medium supplemented with TDZ alone (86%). The 30 μ g/L suspensions

Figure 6: Effect of Ag NPs and Cu NPs alone and in different combinations with TDZ on biomass accumulation at different day intervals.

Figure 7: Effect of different treatments of NPs alone or in combination with TDZ on SOD and POD activity in callus cultures of *A. absinthium*.

of bio-fabricated Ag and Cu NPs (1:2; and 2:1) along with TDZ (4 mg/L) have stimulatory effect on callus proliferation in *A. absinthium* when we compare it with TDZ alone or chemically synthesized NPs because bio-fabricated NPs are less toxic and more stimulatory effect on callus induction frequency as compared to their chemically synthesized counterparts. Similarly, biomass accumulation was significantly high in the lag and log phases of the cultures.

During callus formation and morphogenesis, both biotic and abiotic stresses delay differentiation and dedifferentiation because of ROS production that damages the cells directly through the synthesis of toxic metabolites [38]. To combat such unfavourable conditions, various

enzymes are produced during the process of cell division [39]. A complex system is formed by the production of these endogenous enzymes which are involved in scavenging both non-radical oxygen species and toxic-free radicals [37]. The applications of bio-fabricated Ag and Cu NPs significantly enhanced the endogenous enzymes production in comparison with control treatment because ROS are produced in response to the application of NPs. However, antioxidative enzymes did not show linear correlation with each other. An analogous pattern of variations in endogenous enzymes production system is extensively reported in *Solanum*, *Prunus*, and *Prunella* [40–42]. Moreover, Martin et al. [43] reported comparatively lesser SOD production

Figure 8: Effect of different treatments of NPs alone or in combination with TDZ on total phenolic content and total flavonoid contents in callus cultures of *A. absinthium*.

Figure 9: Effect of different treatments of NPs alone or in combination with TDZ on antioxidant activity in callus cultures of *A. absinthium*.

during callus differentiation. The antioxidative role of various enzymes (SOD and POD) is extensively reported in different medicinal plants [10,44].

During unfavourable conditions, toxic-free radicals and radical oxygen species are produced in sufficient quantities that initiate series of reactions that ultimately leading to cell or tissue death [45]. To cope up with the unusual conditions, various plants release active class of natural antioxidants such as polyphenols [46]. Hence, plant-based antioxidants are supposed to be scavenger for both the radical oxygen species and toxic-free radicals [47]. Utilization of *in vitro* techniques with fluctuated media additives such as NPs, light, and temperature is potentially beneficial for the enhanced production of non-enzymatic components. There is still no report on the application of bio-fabricated NPs in callus culture to enhance the non-enzymatic compounds in *A. absinthium*. The applications of bio-fabricated individual NPs (Ag and Cu) or combination of both NPs (Ag and Cu) in different ratios (1:2, 2:1, 1:3, and 3:1) along with TDZ (4 mg/L) were found to be effective for the enhanced production of non-enzymatic components because radical oxygen species and toxic-free radicals are produced. Antioxidant efficacy of natural antioxidants is mainly because of the non-enzymatic components of defence system. TPC and TFC have also been reported in the callus cultures of some other medicinal plants [42,48] in response to NPs; however, there are still no reports available on the *in vitro* production of TPC and TFC in *A. absinthium*. The elicitors and PGHs not only affected the organogenesis but also the production and translocation of various metabolites [49,50]. Higher quantity of TPC was also reported in callus cultures as compared to other tissues [51]. The results of the present study propose that NPs have potential to improve the morphological and biochemical

fluctuations in callus cultures of *A. absinthium*. Different treatments of NPs and TDZ have synergistic effects on the production of non-enzymatic components in callus cultures.

Different ratios and combinations of NPs alone or with the addition of TDZ resulted in differential profiles of DPPH RSA. Among the different methods used for antioxidants determination, DPPH RSA is the simplest, rapid, inexpensive, and efficient method for the determination of DPPH RSA in plant cell cultures [52]. Here, we observed higher DPPH radical scavenging activity in the callus cultures of *A. absinthium* after the application of different ratios and combinations of NPs and TDZ because NPs ameliorate the accumulation of MDA content by induction of plant antioxidant systems. Our findings are in agreement with Güllüce *et al.* [53] who reported similar findings in the callus cultures of *Satureja hortensis*.

5 Conclusion

From the present study, it is concluded that the applications of different ratios and combination of NPs along with TDZ have positive influence on the growth kinetics in callus cultures of *A. absinthium*. NPs may be involved in optimizing the different growth parameters in callus cultures to elicit the production of antioxidative enzymes and non-enzymatic compounds especially in the field of plant biotechnology under controlled conditions. However, both enzymatic and non-enzymatic defence components contribute to the growth kinetics of callus cultures in *A. absinthium* up to some extent but the regulating mechanism is yet to be explored. So far, few studies have been conducted regarding nanomaterials as elicitors of endogenous enzymes, non-enzymatic components, and antioxidant activity in callus cultures of *A. absinthium*. These preliminary findings pave the way for a more comprehensive study in understanding the mechanism at the molecular level that would provide basis to recognize their chemistry for future challenges.

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and endorsed the final version of the manuscript for submission.

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