



ALKALOID ACCUMULATION IN *CATHARANTHUS ROSEUS* L. IN VITRO CULTURE ENHANCED VIA Ag AND TiO₂ NANOPARTICLES

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SUMMARY

Catharanthus roseus L. plant is highly beneficial as chemotherapy drugs due to its rich alkaloids. Nanoparticles (NPs) have served as an abiotic elicitor; therefore, these chemical inputs stimulate various secondary metabolites. The present-day study sought to develop a callus culture and its utilization by applying the NPs to enhance the alkaloids in *C. roseus*. For callus induction, *in vivo*, leaves' inoculation on MS medium had different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D), Naphthaleneacetic acid (NAA), and Benzylaminopurine (BAP). After this stage, the induced callus culture gained stimulating by different rates (0, 1, and 2 mg L⁻¹) of silver nanoparticles (Ag-NPs) and titanium dioxide nanoparticles (TiO₂-NPs). The highest fresh and dry weights of calluses resulted in a combination of 0.5 mg L⁻¹ BAP and 0.5 mg L⁻¹ 2,4-D, regarded as the best treatments for callus induction. The study showed no significant effect of NPs on callus growth compared with control. HPLC analysis revealed that field-grown plant leaves had the lowest alkaloid levels compared with elicitor-free callus cultures. However, all NP treatments significantly increased alkaloid contents versus the control. Ag-NPs were more effective than TiO₂-NPs in enhancing alkaloid biosynthesis. The highest range of vincristine and catharanthine (0.736 and 1.378 mg g⁻¹, respectively) emerged with 1 mg L⁻¹ Ag-NPs, while 2 mg L⁻¹ Ag-NPs increased vindoline and vinblastine contents (1.30 and 0.949 mg g⁻¹, respectively). The control exhibited lower alkaloid contents of vindoline, vincristine, catharanthine, and vinblastine (0.891, 0.492, 0.974, and 0.307 mg g⁻¹, respectively).

Keywords: *C. roseus*, nanoparticles, plant growth regulators, vincristine, vinblastine, MS medium, callus culture

Key findings: The presented trial provides a basis for how plant biotechnology contributes to pharmaceutical industry advancement, specifically how to use medicinal plants' compounds for cancer treatment. The study confirmed that low doses of exact nanoparticles can enhance the production of essential alkaloids in the *C. roseus* callus culture.

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INTRODUCTION

Vinca rosea (*Catharanthus roseus* L.) is a herbaceous plant belonging to the family Apocynaceae, also known as Madagascar periwinkle plant (Naeem *et al.*, 2017). Being an effective medicinal plant, it contains two major terpenoid indole alkaloids (TIAs), including vincristine and vinblastine, the vital components medically used for the treatment of leukemia and lymphoma (Zhu *et al.*, 2015). The chemical compounds, viz., Ajmalicine, catharanthine, serpentine, tryptamine, vindoline, and tabersonine, also identified in *C. roseus*, used HPLC (Tikhomiroff and Jolicoeur, 2002). These medicinal compounds frequently thrive in plant tissues *in vivo* with low concentrations (Ataei-Azimi *et al.*, 2008). The challenge for pharmaceutical industry since manufacturing compounds is complex, making it unable to provide the amounts required commercially. Therefore, various studies tried to enhance their biosynthesis in plant tissues using biotechnological strategies *in vitro* cultures (Zhu *et al.*, 2015). In this regard, several alkaloids' extraction from *C. roseus in vitro* served pharmaceutical uses (Naeem *et al.*, 2017; Al-Haidari and Al-Tamimi, 2023; Al-Musawi and Al-Tamimi, 2023).

In *C. roseus*, stimulating callus formation is one of the crucial stages applied through plant tissue culture. However, several researchers have tried to use various combinations and concentrations of plant growth regulators (PGRs) for this purpose (Das *et al.*, 2020). The shoot tips of *C. roseus* produced a higher rate and mass of callus in combination with 1 mg L⁻¹ NAA and BAP (Taha *et al.*, 2008). Leaf segments succeeded in establishing a maximum callus induction with various combinations of Kinetin (Kin) with 2,4-D and BAP with Indole-3-butyric acid (IBA) (Saifullah and Khan, 2011). Furthermore, Kaya and Akı (2013) reported that combining 5 mg

L⁻¹ NAA with 2 mg L⁻¹ BAP resulted in a high callus weight. In the *in vitro* techniques used to develop plant secondary metabolites, elicitation is the best way to enhance metabolite production. It begins with adding biotic and abiotic elicitors to the culture medium during plant cell, tissue, and organ culture, which boosts molecule biosynthesis (Isah, 2019).

Nanoparticles (NPs) have unique physicochemical properties that proved to be significant growth promoters in a wide range of plant species (Verma *et al.*, 2018). Some of these NPs, like silver NPs (Ag-NPs), are proven to be low-toxic and biodegradable, which can also promote the accumulation of various plant secondary metabolites (Mirhadia *et al.*, 2018). Ag-NPs' used *in vitro* cultures of *Chrysanthemum* enhanced the carotenoid compounds (Tymoszuk and Kulus, 2020). Additionally, MgO-NPs and CuO-NPs induced tannin and phenolic components in *Punica granatum* L. culture (Al-Oubaidi and Al-Khafagi, 2018). Using chitosan nanoparticles has also improved the antioxidant activity and alkaloid production in *C. roseus* (Hassan *et al.*, 2021). Furthermore, in *C. roseus* suspension culture, employing cobalt nanoparticles (Co-NPs) has positively correlated with increasing alkaloid accumulation and *CrMPK3* gene expression (Fouad and Hafez, 2018).

As mentioned earlier, several biotic and abiotic elicitors' application has aided in studying their biological effects on the accumulation and biosynthesis of *C. roseus* secondary metabolites (Iskandar and Iriawati, 2016). However, limited scientific reports about using NPs to increase *C. roseus* alkaloids through *in vitro* cultures still exist (Hassan *et al.*, 2021). Relatedly, the presented research aimed to evaluate the effects of low levels of nanoparticles on the callus growth and accumulation of some alkaloids in the *C. roseus* callus culture.

MATERIAL AND METHODS

Plant material and surface sterilization

Laboratory trial on *Catharanthus roseus* L. proceeded at the Biotechnology Research Centre, Al-Nahrain University, Baghdad, Iraq. Collected healthy leaves of *C. roseus* at the flowering stage underwent the following preparatory steps: sterilized with 20% sodium hypochlorite with a few drops of Tween-20 for 10 min, rinsed with 70% ethanol for 30 sec, and then washed 4–5 times with sterilized distilled water (Das *et al.*, 2020), performing all the steps under aseptic conditions.

Callus induction

The sterilized leaf explants, cut into homogenous pieces, continued transplanting on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), provided with various combinations of PGRs, i.e., 0.0, 0.5, 1.0, and 1.5 mg L⁻¹ of 2,4-D/NAA combined with 0.25 and 0.5 mg L⁻¹ of BAP. Then, adding 30 g L⁻¹ sucrose and 7 g L⁻¹ agar to the components of the medium gained mixing well by boiling before being sterilized by the autoclave at 121 °C for 20 min. All the cultures had an incubator for storing (at 25 ± 2 °C and 16 h light/8 h dark, with a light intensity of 3000 lux). Determining the fresh and dry weights of the initiated callus began after eight weeks of culture, with the callus dried at 45 °C for 24 h in a laboratory oven.

Nanoparticles elicitation

In the following investigations, two types of nanoparticle stimuli used comprised silver nanoparticles (Ag-NPs) (50 nm, purchased from Nanjing Nano Technology Co., Ltd., China) and titanium oxides nanoparticles (TiO₂-NPs) (50 nm, purchased from Hongwu Nanometer, China). The addition of various concentrations of each elicitor (0, 1, and 2 mg L⁻¹) went on individually to the selected growth medium (MS medium provided with 0.5 mg L⁻¹ BAP and 0.5 mg L⁻¹ 2,4-D). The callus culture, divided into equal pieces (250 mg), took to transplant onto nanoparticle stimulation

treatments. All the cultures remained incubated under the incubator conditions previously mentioned, then growth mass of callus (fresh and dry weight) measuring ensued 30 days after culture.

Extraction of alkaloids

The extraction principles for terpenoid indole alkaloids (TIAs) preparation followed the method of Tikhomiroff and Jolicoeur (2002). Grinding to a fine powder, approximately 200 mg each of dried callus tissue and dried leaves of the *in vivo* plant underwent extraction for 1 h at room temperature in 5 ml of MeOH in a sonicator bath. The supernatant, filtered at 0.45 µm, used a syringe filter unit.

Measurement of alkaloids by HPLC

All the purchased standards of tested alkaloids came from Sigma-Aldrich, USA. The RP-HPLC analysis ran using liquid chromatography, Shimadzu 10 AV-LC, pump module LC-10A Shimadzu, and a UV-Vis 10 A-SPD detector. A column is C-8 (5 µm, 50 mm × 4.6 mm I.D.). The mobile phase had 0.01 M ammonium acetate with 0.1% trimethylamine, pH 6.2: acetonitrile (75:25, v/v). The detection set for UV was 297 nm, the flow rate was 1 ml/min, and the injection volume was 20 µl (Raorane *et al.*, 2022).

Statistical analysis

The completely randomized design setup served as an experimental layout. Each treatment contained 20 replicates. One-way ANOVA tests used for data analysis ran under the SPSS software, release 23. The data means' further comparison and separation employed Duncan's Multiple Range (DMR) tests at 5% probability level (Midway *et al.*, 2020).

RESULTS

PGRs' effect on callus induction

The data demonstrates that the leaf segments showed no development in the control

Table 1. Effects of various PGRs' combinations on callus induction of *C. roseus* leaf explants after eight weeks of cultivation.

PGRs (mg L ⁻¹)			Growth and morphology of callus			
BAP	2,4-D	NAA	Callusing %	Fresh w. (mg)	Dry w. (mg)	Color and texture
0	0	0	0	0.0 f	0.0 g	-
0	0.5	0	0	0.0 f	0.0 g	-
0	1.0	0	0	0.0 f	0.0 g	-
0	1.5	0	0	0.0 f	0.0 g	-
0.25	0.5	0	80	691.2±71.9 cd	68.5±3.2 cde	Yellow, Friable
0.25	1.0	0	90	732.5±87.1 cd	77.9±3.7 cd	Yellow, Friable
0.25	1.5	0	100	1155.6±103.7 b	120.9±9.2 b	Yellow, Friable
0.5	0.5	0	100	1837.1±106.3 a	187.7±12.2 a	Light green, compact
0.5	1.0	0	100	1784.8±159.4 a	165.8±15.0 a	Light green, compact
0.5	1.5	0	100	1131±81.4 b	121.6±7.7 b	Light green, compact
0	0	0.5	0	0.0 f	0.0 g	-
0	0	1.0	0	0.0 f	0.0 g	-
0	0	1.5	0	0.0 f	0.0 g	-
0.25	0	0.5	60	336.7±36.4 e	40.9±3.7 f	Yellow, compact
0.25	0	1.0	90	815.0±82.9 c	85.5±9.9 c	Yellow, compact
0.25	0	1.5	95	1173.9±119.6 b	123.7±11.9 b	Yellow, compact
0.5	0	0.5	80	583.5±70.8 cde	65.7±5.5 cde	Light green, compact
0.5	0	1.0	80	545.4±49.8 cde	59.9±5.5 cde	Light green, compact
0.5	0	1.5	85	472.4±55.0 de	52.5±7.5 cde	Light green, compact

The values represent mean ± SE (standard error). Means followed by same letters in each column were not significantly different at 5% level of probability using Duncan's multiple range test.

treatment in *C. roseus* (Table 1). The percentage of callogenesis, mass callus weights, and morphological characteristics varied according to various PGRs' combinations. In the two auxins used for callus induction from *C. roseus* leaf explants, the 2,4-D proved more effective for promoting callus tissue than NAA alone or with BAP. The treatment with 0.5 mg L⁻¹ BAP and 0.5 mg L⁻¹ 2,4-D achieved the highest callus formation rate (100%) with a significant maximum value of fresh and dry weight of callus (1837.1 and 187.7 mg, respectively), compared with all other treatments. However, the lowest percent rate (60%), with fresh and dry weights (336.7 and 40.9 mg, respectively), appeared in the treatment with 0.25 mg L⁻¹ BAP + 0.5 mg L⁻¹ NAA.

The callus appearance changed from yellowish (in treatment with a low concentration of BAP - 0.25 mg L⁻¹) to light greenish (with 0.5 mg L⁻¹ BAP) in *C. roseus*. However, a compact callus occurred in most combinations. As a result, the mixture of 0.5

mg L⁻¹ BAP and 0.5 mg L⁻¹ 2,4-D attained selection as a medium for best maintenance and having the ability to induce the appropriate mass of callus with the quality nature of the tissue.

Nanoparticles' role in callus growth

The effect of different concentrations of nanoparticles (Ag-NPs and TiO₂-NPs) on callus growth's evaluation ensued through the fresh and dry callus weights after 30 days of elicitation in *C. roseus* (Table 2). The result revealed no significant differences between the elicitation treatments and the control group. However, the data disclosed that 2 mg L⁻¹ TiO₂-NPs produced the highest fresh and dry weight values (2,454.7 and 227.9 mg, respectively), whereas 2 mg L⁻¹ Ag-NPs exhibited the lowest values (2,231.7 and 209.0 mg, respectively). Furthermore, the callus tissue appearance showed no variation among the treatments.

Table 2. Effects of Ag-NPs and TiO₂-NPs on fresh and dry weight (mg) of *C. roseus* callus after 30 days of elicitation (initial callus weight was 250 mg).

Weight of callus (mg)	Elicitation with NPs				
	Control 0 mg L ⁻¹	Ag-NPs 1 mg L ⁻¹	Ag-NPs 2 mg L ⁻¹	TiO ₂ -NPs 1 mg L ⁻¹	TiO ₂ -NPs 2 mg L ⁻¹
Fresh w.	2358.9±157.8	2270.8±155.2	2231.7±127.6	2431.9±85.8	2454.7±103.6
Dry w.	220.5±13.5	216.1±13.7	209.0±16.8	226.2±8.7	227.9±9.9

The values represent mean ± SE (standard error). Nonsignificant differences at 5% level of probability.

Table 3. Concentrations of some terpenoid indole alkaloids (mg g⁻¹) in the leaves of the *in vivo* plant and callus culture of *C. roseus*.

Compounds (mg g ⁻¹)	<i>In vivo</i> plant	Callus culture (Without elicitation)	% of increase	Fold of increase
Vindoline	0.213 b	0.891 a	318.3 %	4.1
Vincristine	0.130 b	0.492 a	278.4 %	3.7
Catharanthine	0.186 b	0.974 a	423.6 %	5.2
Vinblastine	0.183 b	0.307 a	67.4 %	1.6

The values represent mean ± SE (standard error). Means followed by same letters in each row were not significantly different at 5% level of probability using Duncan's multiple range test.

Alkaloids profile in the *in vivo* plant and callus culture

The concentrations of several terpenoid indole alkaloids (TIAs), estimated in the leaves of *in vivo* plant and callus culture (*in vitro*), ran through HPLC analysis in *C. roseus* (Table 3, Figure 1). The results enunciated that the highest alkaloids content abound in the callus tissue, with significant differences compared with the field plant. The catharanthine compounds presented the highest value increases (423.6%, 5.2-fold), whereas vinblastine showed the lowest rise (67.4%, 1.6-fold), comparing the two groups.

Nanoparticles' effect on alkaloids accumulation

The impact estimation of NPs on alkaloid accumulation started after 30 days of elicitation in *C. roseus*. The quantification of identified alkaloids appears in Figure 2. All the used doses stimulated the biosynthesis of alkaloids by recording significant differences compared with the control group (0 mg L⁻¹). In the treatment with 2 mg L⁻¹ Ag-NPs, the vindoline and vinblastine alkaloids achieved the highest values of their contents (1.300 and

0.949 mg g⁻¹, respectively). In contrast, the callus elicited with 1 mg L⁻¹ Ag-NPs revealed the highest amount of vincristine and catharanthine (0.736, and 1.378 mg g⁻¹, respectively). Briefly, Ag-NPs outperformed the TiO₂-NPs by establishing the highest concentrations of studied alkaloids.

DISCUSSION

In pharmaceutical, cosmetic, and related industries, the callus cultures could benefit the long-term and large-scale production of secondary products (Su et al., 2021). In many plants' *in vitro* cultures, the initiation callus usually correlated with the ratio of auxin to cytokinin applied (Su et al., 2011), and often, the best development showed at modest concentrations of PGRs (Hurný et al., 2020). The successful use of synthetic auxin like 2,4-D with BAP encouraged the development of calluses in the *in vitro* cultures of *C. roseus* (Verma et al., 2012). The presented results were in analogy with the findings of Abdul-Rahman et al. (2019), where auxins alone did not generate callus tissue from leaf explants of *C. roseus*; however, after combination with BAP, the highest callogenesis occurred.

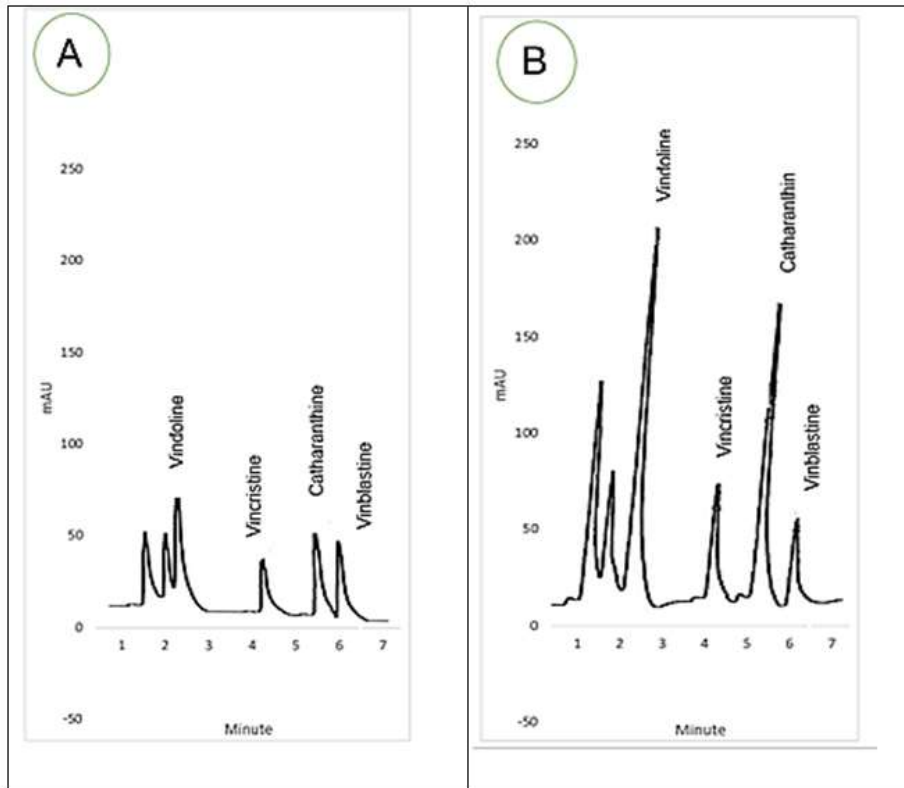


Figure 1. HPLC profile of different alkaloids in *C. roseus*: A) *in vivo* plant, B) control treatment of callus culture.

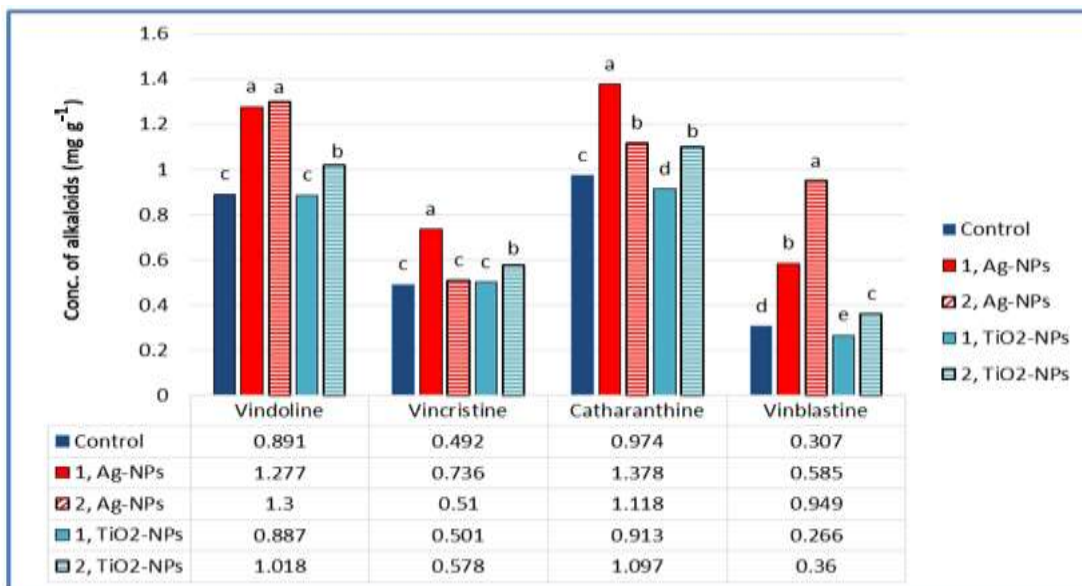


Figure 2. Effects of Ag-NPs and TiO₂-NPs on alkaloid accumulation in callus culture of *C. roseus* after 30 days of elicitation. The same letters in each alkaloid are not significantly different at 5% level of probability using Duncan's multiple range test.

Additionally, the endogenous level of PGRs plays an essential role in the differentiation of tissues *in vitro* cultures (Weyers and Paterson, 2001). In the given results, the callus did not develop from the leaf in the control group, which might be due to low levels of endogenous hormones.

The unique chemical and physical properties of nanoparticles (NPs), primarily attributed to their small size of less than 100 nm, play a crucial role in influencing various aspects, including bio-distribution, release kinetics, and cellular uptake. These characteristics have significant implications for the behavior and interactions of NPs within biological systems (Hu *et al.*, 2020). The effects of NPs on crop plants can vary depending on several factors, including the concentration of NPs, their size, the dosage applied, and the specific plant species involved. It is vital to consider these variables, as they can determine whether NPs have positive or negative impacts on crop plants (Rivero-Montejo *et al.*, 2021). Moreover, Ebadollahi's *et al.* (2019) findings revealed that moderate doses of NPs showed no adverse effects on the growth of *Hypericum perforatum* callus cultures, while a rise surfaced in the growth parameters of *Melissa officinalis* organ cultures (Rezaeia *et al.*, 2019). However, in different growth parameters, a noticeable inhibition has been reported with higher doses of NP compounds in various plant species (Sami *et al.*, 2020).

Excessive concentrations of nanomaterial often cause adverse effects on plant development or may even cause plant death due to the high reactive oxygen species (ROS) stimulation to levels the plant cannot tolerate (Marslin *et al.*, 2017). According to recent research, nanoparticles can boost the antioxidant activity and bioactive chemicals found in certain medicinal plants. This innovative method efficiently increases these plants' general effectiveness and ability as enhancers by acting as a stimulant and abiotic elicitor (Rivero-Montejo *et al.*, 2021). In this matter, many investigators have found that the desired characteristics of therapeutic plants have increased noticeably with nanoparticles' use (Anjum *et al.*, 2021). Achieving this

stimulation results from the activation of ROS molecules and various secondary signaling messengers (Tariverdizadeh *et al.*, 2021) and the development of defensive systems against oxidative stress (Sami *et al.*, 2020). Furthermore, observations on these nanoparticles have revealed a significant impact on the genetic, phytochemical, and phenotypic characteristics of various *in vitro* plant cultures (Tymoszuk and Kulus, 2020).

Several studies showed that NPs could access cells and influence sugars, proteins, and some compounds in DNA, thus, resulting in heterogeneity in various gene expressions responsible for precursors of primary and secondary metabolism (Marslin *et al.*, 2017). The TiO₂-NP also stimulated glutathione and catalase activity in *Hydrilla verticillata* plants (Okupnik and Pflugmacher, 2016). Furthermore, applying Ag-NPs improved the antioxidant activities, which led to enhanced anthocyanin accumulation and a decline in the toxic ROS quantities in the Arabidopsis plant (Syu *et al.*, 2014). Therefore, its external application has enhanced various physiological effects in agriculture and pharmaceutical research (Anjum *et al.*, 2021). Regarding this, the presented results revealed an increase in the values of the DPPH antioxidant enzyme, which also agrees with the findings of Sadak (2019), who confirmed that Ag-NPs treatments showed upsurges in the antioxidant activities in the *Trigonella foenum-graecum* plant.

For amplification of secondary metabolites, Ag-NPs enhanced the biosynthesis of alkaloids, flavonoids, and steroids in *Catharanthus roseus* leaves and roots (Jan and Naskar, 2021), increased capsaicin content in cell cultures of *Capsicum frutescens* and atropine in the hairy roots of *Datura metel* (Anjum *et al.*, 2021), and improved the chlorophyll and carotenoid contents in the leaves of *Cucurbita pepo* plant (Dziwulska-Hunek *et al.*, 2021). Moreover, TiO₂-NPs showed to be better enhancers of chlorophyll content and photosynthetic efficiency with similar physiological effects in the *Vigna radiata* plant (Raliya *et al.*, 2015). Likewise, TiO₂/perlite nanocomposites increased alkaloids, hypericin, and volatile compounds in the *Hypericum perforatum* callus culture

(Ebadollahi *et al.*, 2019). The nanocomposites also increased the levels of rosmarinic acid in *Melissa officinalis* organ culture (Rezaeia *et al.*, 2019).

CONCLUSIONS

The comparative study showed that using the PGRs, 2,4-D, and BAP together brought about good callus formation and high callus masses. Thus, they can be the best medium for inducing and growing callus in *C. roseus*. The low Ag-NPs and TiO₂-NPs doses acted as an abiotic elicitor, significantly enhancing the important alkaloids in callus culture. Research in this area could develop efficient and sustainable methods for producing chemotherapy drugs derived from the *C. roseus* plant.

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