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Original Article

Enhanced Curculigoside and Phenolic Compounds Elevate Antioxidant Activity in *C. orchioides* Gaertn In Vitro Under Exogenous Augmentation with Elicitors Salicylic Acid (SA) and Polyethylene Glycol (PEG) 6000

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Abstract

Introduction *Curculigo orchioides* Gaertn, an endangered medicinal plant, possesses a bioactive compound known as curculigoside (phenolic glycoside) in addition to other phenolic compounds. Curculigoside has multifunctional pharmacological properties including antioxidant and anti-inflammatory. Advances in plant tissue culture provide us with tools for the in vitro propagation of medicinal plants in addition to a well-founded system to enhance pharmacologically bioactive compounds through the use of elicitors.

Objective The study evaluates the effect of exogenous application of salicylic acid (SA) and polyethylene glycol 6000 (PEG 6000) to in vitro cultures of *C. orchioides* for enhanced curculigoside, phenolic compounds production followed by an increase in antioxidant activity (AA).

Method Eight-week-old subcultured plants grown in one-fourth strength Murashige and Skoog media were subjected to elicitors treatment. SA (10, 100, and 1,000 ppm) and a PEG 6000 (1,000, 5,000, and 10,000 ppm) were used as elicitors for a period of 1, 2, and 4 weeks. Plants without treatment were considered as control. The treated plants were subjected to 80% acetone solvent for efficient phenolic extract preparation. High-performance liquid chromatography was used for the analysis of curculigoside content (CC), and total phenolic content (TPC) was determined using the Folin–Ciocalteu colorimetric method. AA was evaluated by 1,1-diphenyl-2-picrylhydrazyl assay.

Results SA treatment increased CC, TPC, and biological activity of AA properties compared to the control, with the maximum value observed at 10 ppm treatment after week 1 elicitation. CC, TPC, and AA gradually decreased after week 2 elicitation, and the lowest values were observed after week 4 elicitation. In the PEG 6000 treatment, 10,000 ppm elicited the highest CC and TPC, with the maximum AA observed after week 2 elicitation. The treatment also elicited higher CC, TPC, and AA in both the 1st and 4th weeks compared to the control.

Keywords

- *C. orchioides*
- curculigoside
- total phenolic content (TPC)
- antioxidant activity (AA)
- HPLC
- polyethylene glycol 6000 (PEG 6000)
- salicylic acid (SA)

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Conclusion SA and PEG 6000 successfully enhanced CC, TPC, and improved AA in *C. orchioides* in vitro. The study also offers insightful information for the neutraceutical, cosmeceutical, and pharmaceutical industries about how to potentially manipulate medicinal plants in vitro to increase the synthesis of bioactive compounds.

Introduction

Curculigo orchioides Gaertn is an endangered seasonal medicinal plant that belongs to the Hypoxidaceae family. This plant is commonly found in Asian countries such as India, China, and Japan. *C. orchioides* is commonly known as golden eye grass in English and Kali Musli in Hindi. It is gaining remarkable significance in pharmacognosy and pharmaceutical research because of the presence of bioactive pharmacological compounds or secondary metabolites. To name a few, the rhizome of the plant possessed phenols and phenolic glycosides,[1] chlorophenolic glucosides,[2] lignans, and lignan glycosides,[3,4] alkaloids,[5] and terpenoids.[6] A phenolic glycoside known as curculigoside ([5-hydroxy-2-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxyphenyl] methyl 2,6-dimethoxy benzoate) present in the rhizome of *C. orchioides* is highly studied because of its multifunctional pharmacological applications in vitro or in vivo models such as antioxidant properties, anti-inflammatory activity, neuroprotective, and antiosteoporotic effects.[7–11]

Endangered and extinction of plants are a major concern for the ecosystem and the environment. *C. orchioides*, a medicinal plant, been listed as an endangered species; an alternative tool is required to conserve the plant. One such effective tool is plant tissue culture. Plant tissue culture not only provides a tool for the conservation, sustenance, and propagation of the plant but also provides a platform to study the effect of elicitor or biotic and abiotic stress in the accumulation or production of secondary metabolites under in vitro conditions. Using the tissue culture technique, the influence of different biotic and abiotic elicitors can be studied on the higher production of secondary metabolites in plants.[12,13]

Elicitors can be biotic or abiotic molecules and they act as signaling molecules. The signals are recognized by the plant cellular membrane-bound receptors and lead to the activation of signal transduction pathways, altering the expression of regulatory factors, and resulting in enhanced synthesis and accumulation of bioactive secondary metabolites.[14,15] Biotic elicitors include defined composition (glycoproteins, chitin, elicitin), nondefined composition (crude extracts of bacteria and fungus), and intercellular signaling molecules (salicylic acid [SA], jasmonic acid, methyl jasmonate). Whereas abiotic elicitors include heavy metals ($CdCl_2$, $CuSO_4$, $AgNO_3$), gaseous substances (nitric oxide, ethylene), drought stress mimickers like polyethylene glycol 6000 (PEG 6000), and a physical stressors (drought, salinity, ultraviolet [UV] radiation).[12,16] The current study attempts to determine the effect of exogenous SA and PEG 6000 in the production and enhancement of curculigoside, phenolic compounds, and antioxidant activity (AA) in *C. orchioides* in vitro.

Materials and Methods

Collection of *C. orchioides* Leaf for In Vitro Culture

Following the institutional guidelines, the leaves of *C. orchioides* were collected from the herbal garden of Nitte University Centre for Science Education and Research, Nitte (Deemed to be University), Mangalore, Karnataka, India (12.800513° N, 74.885447° E). The herbarium specimen of the plant was prepared (voucher number: NU2024:01) and deposited at Nitte University Centre for Science Education and Research, Nitte (Deemed to be University). The plant was identified by the authors and confirmed using digital herbarium of the Botanical Survey of India.

In Vitro Propagation and Subculture of *C. orchioides*

In vitro propagation of *C. orchioides* was established as previously reported.[17] Plant leaves collected from the soil were surface sterilized for 10 minutes using 0.1% mercuric chloride and 0.1% sodium dodecyl sulfate solution. The sterilized leaves were washed four to five times with autoclaved distilled water. The washed explant leaves were sliced and cultured in one-fourth strength Murashige and Skoog (MS) media (HiMedia Laboratories Pvt. Ltd., India) for the mass propagation of the plant. The cultured explants were maintained inside the tissue culture chamber at 22 to 24°C temperature, 3,000 to 4,000 lux light, 50 to 60% humidity, and a photoperiod of 16 hours light and 8 hours darkness.

The stages of the plantlet development were noted and the 12-week-old matured plant was subcultured (►**Fig. 1**). The subcultured plants were grown for 8 weeks, and they were used for mass propagation (►**Supplementary Fig. S1**). The 8-week-old subcultured plants grown in one-fourth strength MS media were subjected to elicitors treatment stated below.

Elicitors Treatment

A stock solution of 10,000 ppm (10 mg/mL) SA was prepared in autoclaved distilled water and filtered using a micron syringe filter of 0.22 µm (HiMedia Laboratories Pvt. Ltd., India). The subcultured plants were treated with a final concentration of 10, 100, and 1,000 ppm, respectively.

PEG 6000 stock containing 50,000 ppm (50 mg/mL) was prepared in autoclaved distilled water. The solution was filter sterilized using a 0.22-µm syringe filter (HiMedia Laboratories Pvt. Ltd., India), and a treatment concentration of 1,000, 5,000, and 10,000 ppm were added to the subcultured plants.

Eight weeks old in vitro subcultures of *C. orchioides* ($n = 10$) were treated with various concentration of elicitors

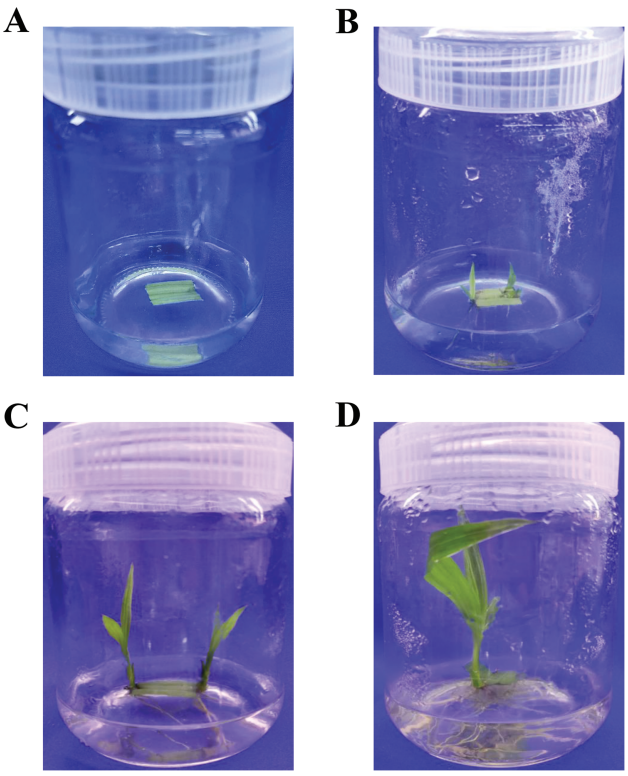

Fig. 1 Development of the somatic embryo of *C. orchioides* in vitro. (**A**) Leaf explant. (**B**) Regeneration of shoots and roots post-4 weeks of subculture. (**C**) Development of whole plant at the end of 8 weeks. (**D**) Twelve-week-old matured whole plant.

SA (HiMedia Laboratories Pvt. Ltd., India) and PEG 6000 (HiMedia Laboratories Pvt. Ltd., India) for 1, 2, and 4 weeks with different concentrations.

Phytochemical Extraction

Posttreatment or elicitation, the roots of the *C. orchioides* were harvested and dried. Note that 2 g each of the dried roots was grounded and subjected to Soxhlet extraction. The powdered root was defatted using *n*-hexane followed by extraction of phytochemicals using 80% acetone. The extraction process was maintained for 6 hours at 50°C. The extracts were concentrated at 42°C, 20 revolutions per minute under a vacuum using a rotary evaporator (IKA-Rotary Evaporator-RV10, IKA, China) and stored at 4°C for further analysis.

Estimation of Curculigoside Content by High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) (CTO-10ASVP SPD-M20A230V, Shimadzu, Japan), Waters Spherisorb ODS1 RP C-18 (250 mm × 4.6 mm, 5 µm) column, and the isocratic mixture of acetonitrile (HPLC grade, Merck) and 0.1% phosphoric acid (HPLC grade, Sigma-Aldrich) in the ratio of 23:77 (v/v) were used for the analysis of curculigoside content (CC) in the plant extract. HPLC grade standard curculigoside (85643-19-2, Shanghai Tauto Biotech, China) was used as a standard and a concentration of 0.1 to 0.4 µg were injected. The flow rate was adjusted to 1 mL per minute. A standard calibration curve was plotted. The area unit of each extract with respect to the standard calibration plot (applying set intercept, $Y = 27048 \times$, $R^2 = 0.9978$) were used in determining the CC expressed as µg per mg extract dry weight (µg/mg EDW). All the extract stocks were prepared by dissolving 1 mg/mL in an isocratic solvent with a final concentration of 20 µg/mL. Note that 10 µL was injected and monitored at 283 nm in HPLC with a photo diode array detector.

Analysis of Total Phenolic Content

Total phenolic content (TPC) was estimated by Folin–Ciocalteu colorimetric method using gallic acid as a standard with a minor modification.[18] Briefly, 1 mg/mL of standard stock solution was prepared. To a working concentration of the standard (20–100 µg/mL), 2 mL of 7% Na_2CO_3 and 0.2 mL of 10% Folin–Ciocalteu reagent was added. The reaction mixture was incubated for 30 minutes in the dark at room temperature. The absorbance was taken at 760 nm against the blank using a UV-Vis Bio-spectrophotometer (MJEA129893, Eppendorf, Germany).

For the extracts, a working solution of 0.1 mg/mL was used, the same procedure as that of the standard was followed. The absorbance value of the samples with respect to the standard calibration plot ($Y = 0.0448 \times$ -0.0418, $R^2 = 0.9987$) was used for determining the TPC of the extracts expressed as gallic acid equivalents of extract dry weight (µg GAE/mg EDW).

Determination of Antioxidant Activity

AA of the *C. orchioides* extract was performed using DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay as described earlier with a slight modification.[19] The DPPH stock solution of 1 mM/mL was freshly prepared in methanol. An aliquot of 100 µL (4 mg/mL) extract solution was added to a 3-mL DPPH solution in a test tube ($n = 3$). The reaction mixture was incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm against the reagent blank. Ascorbic acid was taken as a standard. Percentage inhibition of DPPH was calculated using the following formula:

$$\%\ \text{DPPH reduction} = \%\ \text{scavenging activity} = [(A_{bc} - A_{bs}) / A_{bc}] \times 100$$

where A_{bc} indicates the absorbance of DPPH with control and A_{bs} is the absorbance of DPPH with the sample.

Statistical Analysis

All data are expressed as mean ± standard deviation performed in triplicates ($n = 3$). Statistical analyses were performed by one-way analysis of variance post-Tukey's multiple comparison tests with the aid of statistical software GraphPad Prism (version 8.4, San Diego, California, United States). A p-value of ≤ 0.05 was considered statistically significant.

Results

Curculigoside Content

The HPLC chromatogram of the standard curculigoside is shown in ►**Fig. 2A**. CC was significantly increased in SA treated group in comparison to control post-week 1, week 2, and week 4

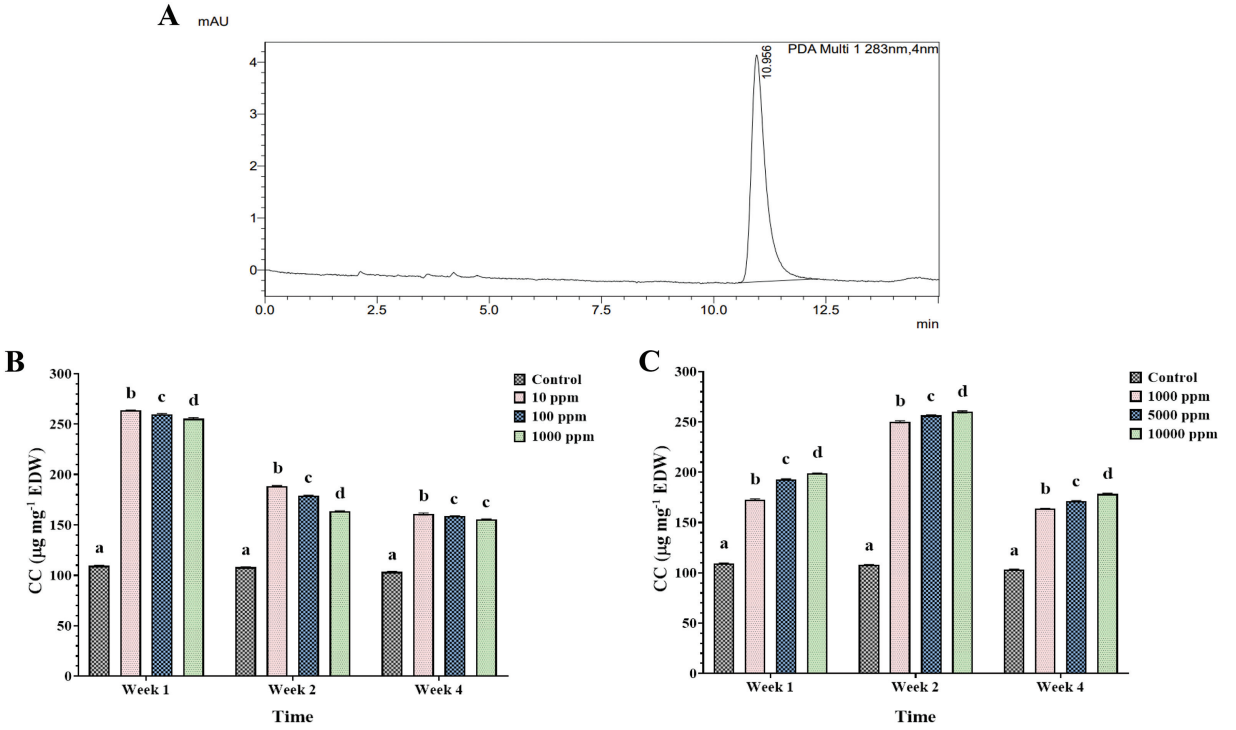

Fig. 2 Curculigoside content (CC) in 80% acetone Soxhlet extracts of dried roots of *C. orchioides* post-week 1, week 2, and week 4 elicitation. (**A**) High-performance liquid chromatography (HPLC) chromatogram of the standard curculigoside. (**B**) Salicylic acid elicitation and (**C**) polyethylene glycol 6000 (PEG 6000) elicitation. Values are mean ± standard deviation (SD) consolidated from triplicates ($n = 3$) and values with different alphabet indicate a significant difference ($p \leq 0.05$) subjected to one-way analysis of variance (ANOVA) post-Tukey's multiple comparisons test.

elicitation treatment (►**Fig. 2B**). The highest CC in SA treatment was observed in post-week 1 elicitation at 10 ppm with a value of 263.75 ± 0.24 µg/mg EDW (2.4-fold increase) and showed a significant increase when compared to the value of control and the treated group of 100 and 1,000 ppm (►**Supplementary Table S1**). Eventually, the value of the CC decreases following post-2nd and 4th week in elicited plants. Among the treated group, the least CC was seen in SA 1,000 ppm (155.54 ± 0.40 µg/mg EDW) post-week 4 elicitation.

In PEG 6000 treatment, CC was increased, and a higher value of CC was observed in post-week 2 elicitation than in the 1st and 4th week (►**Fig. 2C**). The highest CC was observed when treated at 10,000 ppm with a value of 260.05 ± 0.93 µg/mg EDW (2.4-fold increase) and is significantly higher to the control and the corresponding treated group (1,000 and 5,000 ppm) (►**Supplementary Table S1**).

Total Phenolic Content

The highest TPC in SA treatment was observed in the 1st week of elicitation at 10 ppm with a content value of 6.84 ± 0.18 µg GAE/mg EDW (2.0-fold increase) (►**Supplementary Table S2**). The value was observed to be significantly higher when compared to the treated group (10 and 100 ppm) and the control (►**Fig. 3A**). Subsequently, the TPC value started to decline with time but the treated group still showed a significant increase in TPC when compared to control post-week 2 elicitation. The least content was observed in post-week 4 elicitation with no significant difference.

In PEG 6000 treatment, the TPC value increased gradually from the 1st to 2nd week after treatment and decreased post-week 4 elicitation (►**Fig. 3B**). The highest TPC was observed at 10,000 ppm post-week 2 elicitation with a yield of 7.01 ± 0.24 µg GAE/mg EDW (2.1-fold increase) (►**Supplementary Table S2**). The value also shows a significantly higher TPC when compared to the control and the treated value of 1,000 ppm.

Antioxidant Activity

The SA-elicited plants in both 1st and 2nd week showed a significantly higher AA (% of DDPH reduction) when compared to the control (►**Fig. 4A**). The highest activity was observed in the elicited plant at 10 ppm post-week 1 elicitation with 55.87 ± 2.92% DPPH reduction (►**Supplementary Table S3**). It also shows a significant percentage increase in the DPPH reduction when compared to the control and the treated group of 100 and 1,000 ppm, respectively. Gradually, the AA started to decline post-week 2 and week 4 elicitation.

In the case of PEG 6000 treatment, the elicited plants show a significantly higher percentage of DPPH reduction activity in all the weeks including the 4th week (►**Fig. 4B**). However, the maximum AA was observed in the 2nd week at 10,000 ppm treatment with a reduction DPPH percentage value of 70.29 ± 1.10 (►**Supplementary Table S3**). The maximum value with the highest AA also shows a significantly higher percentage of DPPH reduction compared to the control and the treated values of 1,000 and 5,000 ppm, respectively.

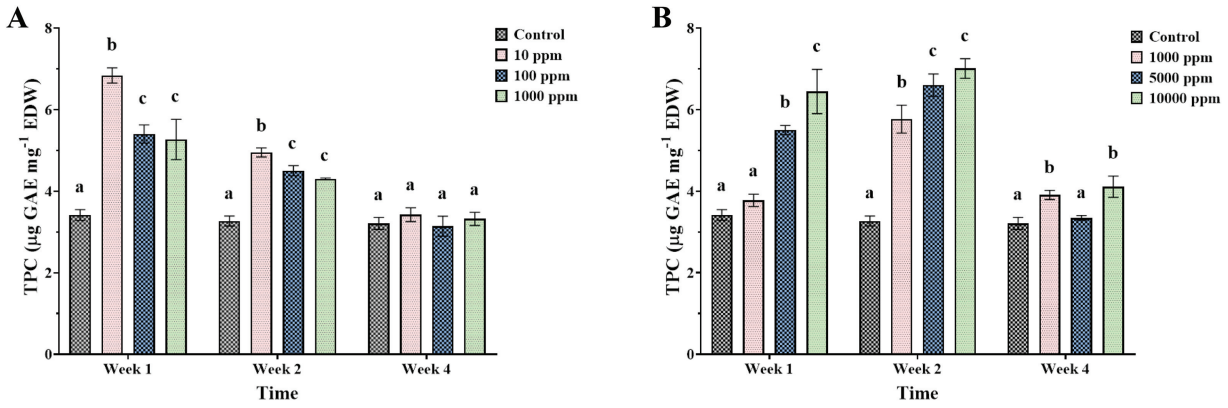

Fig. 3 Total phenolic content (TPC) in 80% acetone Soxhlet extracts of dried roots of *C. orchioides* post-week 1, week 2, and week 4 elicitation. (**A**) Salicylic acid elicitation and (**B**) polyethylene glycol 6000 (PEG 6000) elicitation. Values are mean ± standard deviation (SD) consolidated from triplicates ($n = 3$) and values with different alphabet indicate a significant difference ($p \leq 0.05$) subjected to one-way analysis of variance (ANOVA) post-Tukey's multiple comparisons test.

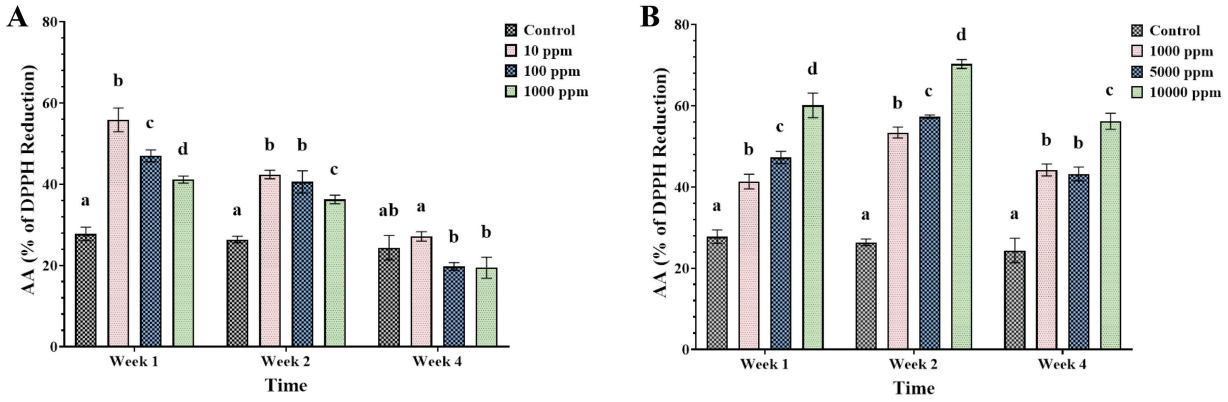

Fig. 4 Antioxidant activity (AA) in 80% acetone Soxhlet extracts of dried roots of *C. orchioides* post-week 1, week 2, and week 4 elicitation. (**A**) Salicylic acid elicitation. (**B**) Polyethylene glycol 6000 (PEG 6000) elicitation. Values are mean ± standard deviation (SD) consolidated from triplicates ($n = 3$) and values with different alphabet indicate a significant difference ($p \leq 0.05$) subjected to one-way analysis of variance (ANOVA) post-Tukey's multiple comparisons test.

Discussion

The utility of plant tissue culture extends beyond propagation of plants. It offers a well-founded biotechnological approach to augment bioactive metabolites or pharmacologically important secondary metabolites through elicitors or stressors.[20] This study corroborates the findings and demonstrates that SA and PEG 6000 successfully elicited higher production of curculigoside and phenolic compounds in *C. orchioides* in vitro. Heavy metals (chromium and nickel) and the use of amino acids (tyrosine and phenylalanine) induce higher production of curculigoside in the static culture of *C. orchioides* in vitro.[21] However, our study is the first to report the effect of SA and PEG 6000 elicitors in the enhancement of curculigoside and phenolic compound in vitro cultures of *C. orchioides* and subsequently elevated antioxidant activity. The study also outlines those variations in CC, TPC, and AA occur at different concentrations of elicitors and duration of treatment.

SA, a phytohormone, acts as a mediator in plants under stress conditions like cold, salinity, and drought, partly by promoting the biosynthesis of secondary metabolites. During stress, SA interacts with diverse metabolic compounds, regulating comprehensive molecular signaling pathways. SA regulates redox homeostasis and activates the function of specific transcription factors.[22] Utilizing exogenous SA may boost agricultural yield and also increase bioactive secondary metabolites in vitro. In a recent study, it was found that the SA-responsive transcription factor positively influences the biosynthesis of taxol in *Taxus chinensis* by increasing the expression of taxol biosynthesis genes.[23] Similarly, in *Vitis vinifera*, SA enhanced the production of phenylalanine ammonia-lyase messenger ribonucleic acid, a crucial enzyme in phenylpropanoid metabolism, leading to a significant increase in phenolic content.[24,25]

It is observed that SA elicited higher CC, TPC, and AA during the first week, with maximum elicitation occurring at the lowest treatment concentration of 10 ppm. This low dose inducing higher elicitation effect could be possibly due to a hormetic response.[26] A similar study was also observed on safflower culture that low SA (50 mg/L) treatment under salinity stress induces a higher content of phenolics and flavonoids with higher DPPH reduction activity (AA)

compared to higher dose of SA (100 mg/L) treatment.[27] Dong et al[28] stated a similar effect on *Salvia miltiorrhiza* cell culture for the phenolic compound accumulation when treated with SA at low dose. It is also possible that after 7 days of treatment, the level of CC and TPC began to decline due to extracellular enzymatic activity.[29] An increase in the curculigoside and phenolic compound in the SA-treated group is an important intervention for significantly increasing the AA bioactivity.[30,31] To summarize, the enhancement of CC, TPC, and AA in SA-elicited plants was observed with low-concentration treatment over a short duration. Thus, the elicitation effect was contingent on both the dosage of SA applied and the duration of exposure.

PEG 6000 is an elicitor found to simulate drought stress, leading to a decrease in the plant's water potential as a result of osmotic stress.[16] There is speculation that drought stress, resulting from critical water deficiency, manipulates both the physiology and biochemistry of plants. This process involves the activation of cellular receptors, initiating a signal transduction cascade that impacts physiological processes. These physiological changes in turn influence primary metabolism, thereby causing signaling for secondary metabolism through the provision of biosynthetic intermediates or precursors. Hence, enhancement of bioactive metabolites.[32]

This study on the PEG 6000 stress response in *C. orchioides* on the enhancement of CC, TPC, and AA occurs under different concentrations of PEG 6000 treatment (1,000, 5,000, and 10,000 ppm) and duration of exposure. It was observed that the second week after exposure at 10,000 ppm elevated the highest level of CC and TPC which subsequently increased the AA bioactivity. Likewise, several reports have stated the enhancement of phenolic compound and AA after exposure to PEG 6000 elicitor. Hossain et al[33] reported that PEG at 30% yielded a higher level of the phenolic compound and flavonoids (polyphenol) compared to control, 10 and 20% PEG exposure in *Fagopyrum tataricum* (Tartary buckwheat) genotype XiNong 9943. The same study observed the higher inhibition of DPPH activity in drought-exposed plants compared to control plants indicating enhanced antioxidant capacity. It was also reported that PEG 6000 induced a marked increase in TPC almost three times greater than the control and the antioxidant enzyme activities including superoxide dismutase, ascorbate peroxidase, catalase, and polyphenol oxidase in *Beta vulgaris* L. cv. Felicita.[34] On the contrary, there is a report that stated prolonged periods of drought stress decreases the phenolic content and the antiradical activity in *V. vinifera L.* which also verified our finding where CC, TPC, and AA declined post-week 4 elicitation.[35] These findings emphasize the impact of the phenolic compounds on the antioxidant efficacy, illustrating the direct correlation between PEG 6000-induced stress response, phenolic compounds, and free radical scavenging activity.[36,37]

Conclusion

The findings of the current investigation revealed that exogenous SA and PEG 6000 elicitors successfully increase the CC, TPC, and AA in *C. orchioides* in vitro. This study is also the first to show how exogenous SA and PEG 6000 elicitors can enhance CC, TPC, and improved AA in *C. orchioides* in vitro. Similar kinds of factors can be applied in tissue culture via elicitation in enhancing other secondary metabolites or compounds of interest. For the neutraceutical, cosmeceutical, and pharmaceutical industries, the study offers an insightful potential of propagating medicinal plants in vitro and increase the synthesis of bioactive metabolites purported to have medical benefits without affecting the plant population and biodiversity in its natural habitat.

Data Availability

The data of the current study will be made available by the corresponding author upon request.

Conflict of Interest

None declared.

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