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Effects of β-cyclodextrin on the Production of Reserpine in *Rauwolfia serpentina* Callus Cultures

SHRADDHA MISHRA, DIVYA YADAV, NEERAJ KHARE[1]* AND SADANAND PANDEY[2,3]

NIAMST, NIMS University Rajasthan, Jaipur-303 121 (Rajasthan), India
**(e-mail: neerajsnkhare@gmail.com; Mobile: 70735 92400)*

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ABSTRACT

Rauwolfia serpentina (Indian snakeroot) is an endangered medicinal plant renowned for producing reserpine, a pharmaceutically valuable alkaloid with antihypertensive and neuroactive properties. Conventional extraction from roots is unsustainable and limited by overharvesting, making *in vitro* culture a promising alternative for controlled metabolite production. This study investigated the effect of β-cyclodextrin – A cyclic oligosaccharide known for its dual elicitor and solubilizing roles – on biomass growth, reserpine accumulation, antioxidant enzyme activity and proline content in callus cultures. Calli were grown on Murashige and Skoog medium supplemented with 1 mg/l NAA and treated with graded concentrations of β-cyclodextrin (25-100 mM). Biomass, alkaloid content and stress-related parameters were evaluated at different culture ages. Increasing β-cyclodextrin concentrations caused a moderate but consistent decline in biomass, while significantly enhancing reserpine accumulation, with the highest yield observed at 75 mM after 10 days of elicitation. Activities of superoxide dismutase and catalase rose markedly between 10 and 20 days, followed by a slight decline at 30 days, indicating activation of antioxidant defenses under elicitor-induced stress. Proline levels displayed a similar trend, suggesting osmoprotective adjustment in the cells. Overall, β-cyclodextrin successfully stimulated secondary metabolite production in *R. serpentina* calli without requiring whole plants, demonstrating its potential as an effective and sustainable elicitor for large-scale reserpine production *in vitro.*

Key words: *Rauwolfia serpentina*, cell cultures, reserpine, elicitation, β-cyclodextrin

INTRODUCTION

Rauwolfia serpentina, commonly known as Indian snakeroot, is a medicinally important plant valued for its wide range of alkaloids. Among these, reserpine is the most significant secondary metabolite due to its antihypertensive, tranquilizing and antipsychotic properties. Traditionally, reserpine has been extracted from the roots of *R. serpentina*, but the natural supply of the plant is limited, and overharvesting has placed it under pressure as an endangered species (Dhyani *et al*., 2022; Zhou and Chen, 2022). To overcome these constrains, plant cell and tissue culture techniques have come out as sustainable approaches for producing pharmaceutically important metabolites without dependence on whole plants. However, *in vitro* cultures often show low or inconsistent levels of secondary metabolite production compared to natural tissues. To overcome this limitation, various strategies such as elicitation, precursor feeding and the use of carrier molecules have been explored. Elicitors are substances that stimulate plant cells to enhance the synthesis and accumulation of secondary metabolites. Among them, β-cyclodextrin (β-CD), a cyclic oligosaccharide derived from starch, has gained attention due to its ability to form inclusion complexes with hydrophobic molecules (Bravo-Vazquez *et al*., 2023). This characteristic not only increases the metabolites' solubility and bioavailability but also induces a stress like environment in plant cells, which sets off defense related pathways and increases the production of metabolites (Rana *et al*., 2024).

[1]P.I.P.M. Innovations LLP, Amer, Jaipur-302 028 (Rajasthan), India
[2]Faculty of Applied Sciences and Biotechnology, Shoolini University, Solan-173 229 (Himachal Pradesh), India.
[3]Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan.

The application of β-CD in plant cell cultures has been reported to increase the production of several bioactive compounds, particularly alkaloids, flavonoids and terpenoids (Halder *et al*., 2021). Its dual role as a solubilizing agent and elicitor makes it a promising tool in metabolic engineering. Despite this, there is limited information on its specific impact on reserpine accumulation in *R. serpentina* cell cultures (Bhagat *et al*., 2019). Investigating this relationship is essential, as improving reserpine yield through cell culture methods could contribute to sustainable and controlled production of this valuable alkaloid for pharmaceutical use (Bhagat *et al*., 2020).

The present study focused on examining the effect of β-cyclodextrin on reserpine production in *R. serpentina* cell cultures. By systematically analyzing changes in biomass growth and alkaloid accumulation, this research aimed at providing insights into how β-CD can be employed as a practical strategy for enhancing secondary metabolite production in medicinal plants.

MATERIALS AND METHODS

All plant tissue culture grade chemicals and phytohormones were procured from HiMedia, India. β-CD used in the experiments was sourced from the same supplier. Chemicals used in High Performance Liquid Chromatography (HPLC), such as the Reserpine standard, were acquired from Sigma-Aldrich, St. Louis, MO, USA.

Cell cultures of *Rauwolfia serpentina* were established using the modified protocols (Verma *et al.,* 2024). Leaves collected from wild populations of *R. serpentina* were thoroughly washed under running tap water for 30 min, followed by surface sterilization with 75% ethanol for 30 sec and 0.1% mercuric chloride ($HgCl_2$) solution for 1 min with intermittent washing with sterilized distilled water (dH_2O). Finally, explants were rinsed with dH_2O containing 150 mg/l citric acid. The sterilized leaf tissues were transferred onto Murashige and Skoog (MS) medium supplemented with 1.0 mg/l β-naphthalene acetic acid (NAA), 30 g/l sucrose and 4 g/l gelrite, with the pH adjusted to 5.8 prior to autoclaving. To maintain callus growth, cultures were sub cultured at two week intervals and incubated at 25 °C under dark conditions. Phytohormone free MS medium served as the control. Each experiment was performed in triplicate using 12 explants per treatment, and observations were recorded after four weeks of culture.

Elicitation experiments were carried out to assess the impact of β-CD on reserpine production in callus cultures of *R. serpentina*. For each treatment, 0.5 g of callus tissue was cultured in MS culture medium corroborated with 1 mg/l NAA, along with different concentrations of β-CD (25, 50, 75 or 100 mM). Cultures were maintained under dark conditions at 25°C. On days 6, 9, or 12, the cultures were subjected to treatment with β-CD, and samples were harvested 24 h post-treatment. Control sets comprised identical media without β-CD. The influence of β-CD elicitation on *R. serpentina* callus cultures was investigated by quantifying reserpine accumulation, biomass yield, antioxidant enzyme activities and proline content. Each experimental group consisted of five independent callus lines, with all treatments conducted in triplicate to ensure statistical robustness.

To evaluate the effect of β-CD on biomass accumulation in *R. serpentina* callus cultures, 1.0 g of callus tissue was inoculated into media supplemented with varying concentrations of β-CD: E1 (25 mM), E2 (50 mM), E3 (75 mM) and E4 (100 mM). Control cultures were maintained on similar culture media without β-CD and incubated at 25°C in darkness. Biomass accumulation was determined by measuring the dry weight (DW) of calli after 45 days. Sub culturing was performed every 15 days.

To investigate the effect of β-CD elicitation on reserpine accumulation in *R. serpentina* callus cultures, 1.0 g of callus tissue was cultured on media containing varying concentrations of β-CD: E1 (25 mM), E2 (50 mM), E3 (75 mM) and E4 (100 mM). Reserpine extraction was carried out following the protocol of Verma *et al*. (2024). Approximately 250 mg of powdered callus was sonicated for 15 min in 1 ml of chloroform:methanol (3:1 v/v) using a CPX 130 sonicator (Cole-Parmer, Illinois, USA) and then incubated at room temperature for 8 h to facilitate extraction. The extracts were subsequently centrifuged at 4°C for 20 min at 12,000 rpm. The pooled supernatants were concentrated using a rotary evaporator at 50°C under reduced pressure and reconstituted in

1 ml of methanol:HCl (98:2 v/v). The solution was filtered through a 0.22 µm nylon membrane and a 20 µl aliquot was injected into the HPLC system for quantification. Reserpine analysis was carried out using a reversed-phase high-performance liquid chromatography (HPLC) system (Shimadzu Scientific Instruments, USA). The mobile phase consisted of methanol and water (70:30 v/v) delivered at a flow rate of 1 ml/min, with detection set at 268 nm. Calibration was performed using standard reserpine stock solution (1 mg/ml; Sigma-Aldrich, USA) prepared in acidic methanol. Each experiment utilized five independent callus lines and all treatments were conducted in triplicate to ensure reproducibility.

To investigate the effect of β-CD on antioxidant enzyme activities in *R. serpentina* callus cultures, 1.0 g samples of calli aged 6, 9 and 12 weeks were treated with four concentrations of β-CD: E1 (25 mM), E2 (50 mM), E3 (75 mM) and E4 (100 mM). Samples were harvested 24 h after treatment. Antioxidant enzymes were extracted following the method of Verma *et al.* (2024). In brief, 0.5 g of callus tissue was ground to a fine powder in liquid nitrogen and homogenized in 10 ml of extraction buffer (50 mM KH_2PO_4, pH 7.8, containing 1% PVPP and 0.1 mM EDTA). The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C, and the resulting supernatant was used for enzyme assays. Total soluble protein content was quantified using the Bradford assay. Activities of superoxide dismutase (SOD) and catalase (CAT) were determined according to the procedures described by Verma *et al*. (2024).

The effect of β-CD elicitation on proline accumulation in *R. serpentina* callus cultures was examined using 1.0 g of callus tissue for 6, 9 and 12 weeks old cultures. Treatments included four β-CD concentrations: E1 (0.5%), E2 (2.5%), E3 (5.0%) and E4 (7.5%). Samples were harvested 24 h after treatment. Free proline levels were quantified using L-proline as the calibration standard. For analysis, 200 mg of callus tissue from each sample was homogenized with 5.0 ml of 3.0% aqueous sulfosalicylic acid and centrifuged at 8000 rpm for 15 min to remove particulate matter. A reaction mixture comprising 1.0 ml of acid ninhydrin, 1.0 ml of glacial acetic acid and 1.0 ml of the resulting supernatant was incubated at 100°C in a boiling water bath for 1 h. The reaction was stopped by rapid cooling in an ice bath. The chromophore was extracted using 2.0 ml of toluene and its absorbance was measured at 520 nm using a UV–Vis spectrophotometer.

All experiments followed a completely randomized design. Results were presented as mean ± standard deviation and subjected to analysis of variance using SPSS version 18 (Chicago, IL, USA). Duncan's multiple range test was applied for post hoc comparisons with untreated controls. Statistical significance was established at $P < 0.05$.

RESULTS AND DISCUSSION

Callus cultures of *R. serpentina* were successfully established, exhibiting healthy and uniform growth under the modified culture conditions. Sterile explants demonstrated high survival and minimal contamination, indicating the effectiveness of the surface sterilization procedure. Callus induction was observed within the initial weeks of culture, with compact, friable and cream-colored calli developing on Murashige and Skoog (MS) medium supplemented with 1.0 mg/l NAA. Consistent biomass accumulation was achieved through regular sub-culturing at two weeks intervals, with calli maintaining vigorous growth throughout the culture period. In contrast, explants maintained on hormone free MS medium exhibited negligible callus formation, confirming the essential role of NAA in initiating and sustaining callogenesis.

Fig. 1. (a) Mature plant of *R. serpentina,* (b) Leaf explants on callusing media, (c) Callus induction from explants and (d) Full grown callus.

Observations recorded after four weeks revealed significant differences in growth patterns between treated and control groups, validating the optimized protocol for reliable callus establishment in *R. serpentina* cultures (Fig. 1). Our results corroborate with the previous findings (Verma *et al*., 2021a, b). These results provide a basis for further studies on metabolite production and pathway regulation.

The impact of varying concentrations of β-CD on callus biomass production was evaluated by supplementing the culture medium with 25-100 mM β-CD. A progressive decline in biomass was observed with increasing elicitor concentration. Specifically, callus biomass recorded at 25, 50, 75 and 100 mM β-CD was 0.497, 0.480, 0.468, and 0.460 mg, respectively (Fig. 2). In contrast, the untreated control culture exhibited the highest biomass value of 0.521 mg, indicating that the addition of β-CD negatively influenced biomass accumulation at all tested concentrations. The reduction in biomass suggests a stress induced response, possibly due to the elicitor's impact on cellular metabolism, nutrient utilization, or growth dynamics. Furthermore, morphological alterations were evident in β-CD-treated calli, which appeared comparatively fragile and developed a brownish pigmentation, in contrast to the healthier appearance of control cultures. These observations implied that while β-CD may play a role in modulating secondary metabolite synthesis, it exerted an inhibitory effect on biomass accumulation beyond certain threshold concentrations. Therefore, optimization of β-CD levels was essential to balance growth and metabolite production in the *in vitro* cultures.

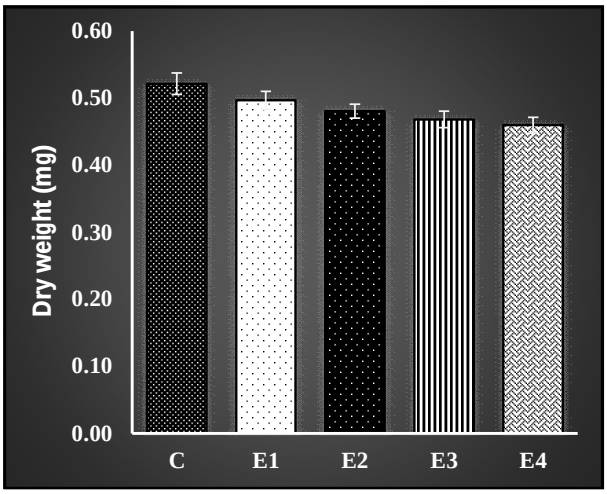

Fig. 2. Effect of elicitation with β-CD (E1: 25 mM, E2: 50 mM, E3: 75 mM and E4: 100 mM) on *R. serpentina* callus culture as compared to unelicited (Control) lines (C–0 mM â-CD). Values are presented as mean ± SD.

Reserpine accumulation in *R. serpentina* callus cultures showed a distinct time-dependent pattern across 7, 10 and 13 days of elicitation with β-CD. This increase was attributed to the metabolic reprogramming of plant cells to cope with β-CD-induced stress, as previously suggested by Zhang *et al*. (2018). Cultures displayed a marked enhancement in reserpine content compared to the untreated control across all exposure periods (Fig. 3). Among the tested concentrations, 25 and 50 mM β-CD significantly promoted metabolite production throughout the experimental duration, with the maximum reserpine yield (0.155 mg/g DW) recorded at 75 mM β-CD after 10 days of elicitation. Beyond this optimal concentration, particularly at 100 mM, a notable decline in reserpine content was observed across all time points. This reduction suggested potential cytotoxic effects of higher β-CD levels, which may impair cellular processes and restrict secondary metabolite biosynthesis. The findings were consistent with earlier reports demonstrating the beneficial role of β-CD as an elicitor in enhancing alkaloid production in plant tissue cultures. However, they also highlight the importance of optimizing elicitor concentration and exposure duration to avoid growth inhibition while maximizing metabolite accumulation. These results provided valuable insights into elicitor-mediated metabolic modulation in *R. serpentina* cultures and its potential for improving reserpine yield.

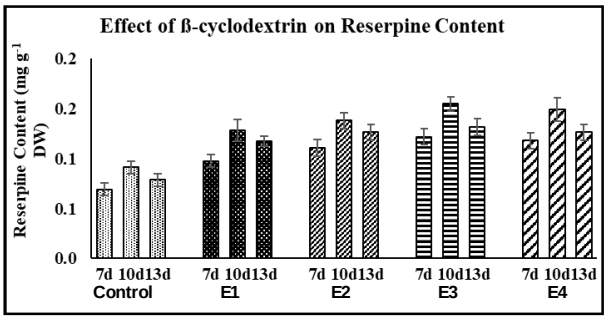

Fig. 3. Effect of β-CD supplementation (C: 0 mM, E1: 25 mM, E2: 50 mM, E3: 75 mM and E4: 100 mM) at different durations (7, 10 and 13 days) on reserpine content in *R. serpentina* callus cultures. Values are presented as mean ± SD.

Elicitation with β-CD induced oxidative stress, resulted in an enhanced generation of reactive oxygen species and prompted cells to activate antioxidant defense mechanisms, notably increasing the activities of enzymes such as catalase and SOD: superoxide dismutase (Tonk *et al*., 2016; Wang *et al*., 2025). In the present study, the activities of these antioxidant enzymes (SOD and catalase) were evaluated in callus cultures of *R. serpentina* after 24 h of exposure to different concentrations of β-CD.

In the control calli, superoxide dismutase (SOD) activity increased progressively with culture age, rising from 2.601 U/mg proteins at 10 days to 3.401 U/mg proteins at 20 days. However, in the 30-day-old calli, SOD activity significantly decreased to 3.070 U/mg proteins. SOD activity exhibited a similar pattern across all β-CD elicitation treatments, showing a notable rise between 10 and 20 days of callus age and a subsequent decrease at 30 days (Fig. 4a).

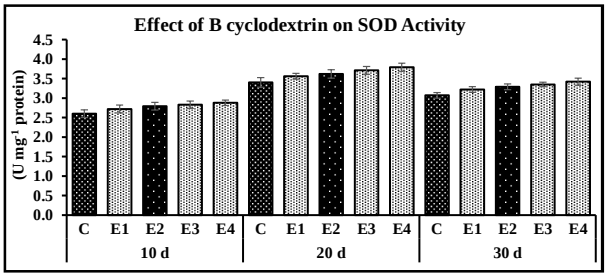

Fig. 4a. Effect of β-CD supplementation (C: 0 mM, E1: 25 mM, E2: 50 mM, E3: 75 mM and E4: 100 mM) at different durations (7, 10 and 13 days) on SOD activity in *R. serpentina* callus cultures. Values are presented as mean±SD.

At all ages, β-CD-elicited calli exhibited consistently and significantly higher catalase activity than their respective controls (Fig. 4b). In control calli, catalase activity increased gradually with age, rising from 2.21 U/mg proteins at 10 days to 2.26 U/mg at 20 days and 2.31 U/mg at 30 days. In contrast, callus cultures subjected to β-CD-elicitation showed a pronounced, cumulative rise in catalase activity as they matured from 10 to 30 days (Fig. 4b).

In control calli, proline content increased progressively with culture age, increasing from at 10 days (15.02 mg/g FW) to 20 days (17.23 mg/g FW), but declined significantly to 16.08 mg/g FW at 30 days. A similar pattern was observed under all β-CD elicitation treatments,

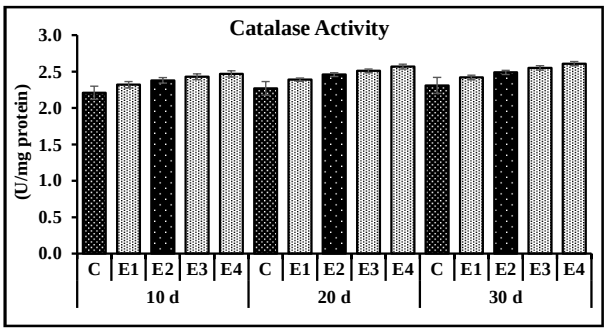

Fig. 4b. Effect of β-CD supplementation (C: 0 mM, E1: 25 mM, E2: 50 mM, E3: 75 mM and E4: 100 mM) at different durations (7, 10 and 13 days) on catalase activity in *R. serpentina* callus cultures. Values are presented as mean±SD.

Table 1. Proline content of callus under β-CD elicitation

Treatment	10 days	20 days	30 days
Control	15.02±0.48	17.23±0.36	16.08±0.32
E_1	17.76±0.51	19.81±0.41	18.79±0.42
E_2	19.08±0.49	21.76±0.39	20.16±0.39
E_3	19.55±0.46	22.14±0.42	21.09±0.29
E_4	20.21±0.39	22.88±0.44	21.88±0.41

with proline levels showing a marked increase between 10 and 20 days of callus age followed by a sharp reduction at 30 days (Table 1).

CONCLUSION

This study demonstrated that β-cyclodextrin effectively modulated growth and secondary metabolism in *Rauvolfia serpentina* callus cultures. While increasing β-cyclodextrin concentrations caused a progressive reduction in biomass, they markedly enhanced reserpine accumulation, antioxidant enzyme activities (SOD and catalase), and proline content in an age-dependent manner. Both SOD and catalase activities rose with culture age up to 20 days, followed by a decline at 30 days, reflecting a typical stress-response pattern. Proline levels also displayed a similar trend, indicating the involvement of osmoprotective mechanisms. These findings highlighted β-CD's dual role as an elicitor and solubilizing agent capable of stimulating defense-related pathways and boosting alkaloid production without relying on whole plants. Optimizing β-CD concentration and exposure time thus offered a promising, sustainable strategy for enhancing reserpine yield and other valuable metabolites from *R. serpentina* under in vitro conditions.

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