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Role of Endophytic Interactions in Enhancing Reserpine Yield in *Rauwolfia serpentina In Vitro* Cultures

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ABSTRACT

Endophytic fungi associated with medicinal plants represent a valuable but under-explored resource for bioactive metabolites. This study examined fungal endophytes isolated from *Rauwolfia serpentina* and evaluated their ability to modulate metabolite production and stress responses *in vitro*. Leaf explants were surface-sterilized and cultured on Murashige and Skoog (MS) medium supplemented with 2,4-D (1.0-3.0 mg/l) and 1 mg/l BAP, with 2.0 mg/l 2,4-D plus 1 mg/l BAP yielding maximal callus induction. Cell-free filtrates prepared from *Aspergillus niger* biomass were applied at varying concentrations (0.5-7.5%, v/v) to established callus cultures for 24 h to assess elicitation effects on growth, reserpine accumulation, antioxidant enzyme activity and proline content. Increasing filtrate concentration progressively reduced biomass yet significantly enhanced reserpine levels, with 5% filtrate producing the highest yield in 20-day-old calli. Elicited calli also exhibited elevated superoxide dismutase and catalase activities, along with higher proline accumulation at 10-20 days compared with controls, indicating activation of oxidative stress defenses. These findings highlighted the dual effect of fungal elicitation; growth suppression coupled with secondary metabolite stimulation, and underscored the potential of non-viable microbial elicitors as a safer strategy to enhance high-value alkaloid production in *R. serpentina* cultures. This work expands understanding of the metabolite-producing capacity of its endophytic community and provides a basis for optimizing elicitation protocols for large-scale applications.

Key words: *Rauwolfia serpentina*, cell cultures, elicitation, endophytic fungi, reserpine

INTRODUCTION

Endophytes are microorganisms that colonize plant tissues without causing visible disease symptoms, establishing a symbiotic relationship that benefits both partners. During this association, these microbes undergo metabolic shifts influenced by the plant's physiological state and environmental conditions, often resulting in the production of diverse bioactive compounds (Ramirez-Estrada *et al.*, 2016; Singh and Kumar, 2023). These metabolites not only enhance the host plant's defence mechanisms but also contribute to the endophyte's survival. Additionally, chemical signalling between endophytes and surrounding organisms can influence the host's overall fitness and adaptive capacity, making them critical players in plant health and secondary metabolism.

Among endophytes, fungi have attracted considerable attention for their ability to produce pharmaceutically significant metabolites. Investigating fungal endophytes of medicinal plants has emerged as a promising approach for the discovery of novel bioactive compounds with therapeutic applications. *Rauwolfia serpentina*, a medicinally valuable species of the Apocynaceae family, is particularly noteworthy in this regard. Native to subtropical regions, the plant is a rich source of secondary metabolites, including more than 130 alkaloids, many of which exhibit pharmacological properties such as anticancer, antidiabetic, antihypertensive, antimicrobial and antioxidant activities. Traditionally, it has been used to treat fever, rheumatism, fatigue and other ailments (Surendran *et al.*, 2021; Rana *et al.* 2024). Notably, its indole alkaloids, such as vincristine and vinblastine, are widely

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used in oncology for treating cancers including Hodgkin's lymphoma and leukemia. Interestingly, earlier studies have suggested that some of these compounds may not be exclusively plant-derived but could also be synthesized by associated endophytic fungi (Paul *et al.*, 2023).

Several reports highlight the potential of endophytic fungi from *R. serpentina* as sources of bioactive metabolites, including compounds with strong cytotoxic and antioxidant properties (Bhagat *et al.*, 2019, 2020; Lata and Gond, 2025). However, studies on the diversity and metabolic potential of its endophytic community remain limited, with only a few investigations documenting these associations (Verma *et al.*, 2021a). Plants growing under unique environmental conditions, such as saline habitats, are often known to harbor endophytes capable of synthesizing specialized metabolites that aid the host in stress adaptation and possess therapeutic value (Verma *et al.*, 2021b).

Elicitation strategies have been successfully employed to enhance metabolite production in plant tissue cultures, with biotic elicitors, derived from microbial sources, showing particular promise (Narayani and Srivastava, 2017). For instance, *Aspergillus flavus* has been shown to improve biomass accumulation and alkaloid yield in *R. serpentina* callus cultures, while *Pythium aphanidermatum* significantly boosted vindoline and serpentina production in shoot cultures (Anjum and Chandra, 2019). Despite these advances, the use of live microbial elicitors poses risks, such as uncontrolled growth disrupting culture stability. As a safer alternative, non-viable powdered microbial elicitors have been proposed, retaining bioactive potential without affecting morphogenesis.

Given the pharmacological significance of *R. serpentine* and its endophytic community, further exploration is essential to understand their metabolite-producing capabilities. This study investigated endophytic fungi from *R. serpentina* cultivated in saline-rich regions and evaluated their potential for producing secondary metabolites with antioxidant and anticancer properties.

MATERIALS AND METHODS

All plant tissue culture-grade phytohormones and related compounds were procured from HiMedia (India). The reserpine standard, along with other reagents used for High Performance Liquid Chromatography (HPLC) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Callus induction in *Rauwolfia serpentina* was carried out following the protocol described by Verma *et al.* (2021a, c). Healthy plants of *R. serpentina* were procured from the Sushila Tiwari Herbal Garden, Rishikesh, Uttarakhand, India, and maintained under controlled conditions in the culture room at NIMS University (Fig. 1 a). Plant materials were thoroughly rinsed under running tap water to remove surface contaminants and then sectioned into small fragments (approximately 4 mm^2). For surface sterilization, the explants were first immersed in 70% ethanol for 2 min, followed by treatment with 0.1% sodium hypochlorite for an additional 2 min. After multiple rinses with sterile double-distilled water to eliminate residual sterilizing agents, the explants were air-dried on sterile filter paper under aseptic conditions. For callus initiation and subsequent proliferation, the sterilized explants were transferred onto Murashige and Skoog (MS) medium supplemented with various concentrations of 2,4-D and 1 mg/l BAP (Fig. 1b). The medium was adjusted to pH 5.8, solidified with 8 g/l gelling agent, and sterilized by autoclaving at 121°C for 20 min. Cultures were maintained at 26 °C under a 16-h light/8-h dark photoperiod (white fluorescent light) and subcultured at two-week intervals to support sustained callus growth.

The biomass of callus cultures, with and without elicitor treatment, was evaluated by measuring their fresh and dry weights. Individual callus masses weighing 0.5 g were inoculated onto Murashige and Skoog (MS) medium supplemented with varying concentrations of 2,4-D and 1 mg/l BAP to assess the influence of these treatments on biomass accumulation. Each experimental set included five replicates and was conducted in triplicate for consistency. For fresh weight estimation, both elicitor-treated calli (exposed to *A. niger* filtrate for 24 h) and untreated controls were harvested from the medium at 10-day intervals (10, 20 and 30 days) and weighed. Dry weight determination was performed after the calli were thoroughly dried prior to measurement.

Aspergillus niger strains were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh. The isolates were initially grown on potato dextrose agar (PDA) plates and incubated at 30°C for seven days. Mycelial biomass from these seven-day-old cultures was transferred to 500 ml Erlenmeyer flasks containing 150 ml of liquid potato dextrose medium and further incubated on a gyratory shaker at 30°C at 150 rpm for nine days. Following incubation, the fungal biomass was harvested, filtered and dried at 65°C for 24 h, then ground into a fine powder using a mortar and pestle. The powdered biomass was suspended in distilled water (10 g L{ ¹) and sterilized by autoclaving at 121 °C for 20 min. The resulting cell-free filtrates (v/v) were subsequently employed as elicitors to enhance reserpine production.

The influence of fungal filtrate on reserpine biosynthesis in *R. serpentina* callus cultures was assessed through a series of experiments. Callus tissues (0.5 g each) were cultured on MS medium supplemented with 2 mg L^{-1} 2,4-D, 1 mg L^{-1} BAP, and varying concentrations of *A. niger* filtrate. The cultures were maintained at 30 °C under dark conditions. After exposure to fungal filtrate at 0.5, 2.5, 5.0, or 7.5% (v/v) for 24 h, samples were harvested. Control cultures were maintained under identical conditions without fungal filtrate. Reserpine accumulation, biomass production, and antioxidant enzyme activities were quantified to determine the elicitation effect. Each treatment included five independent callus lines, and all experiments were performed in triplicate to ensure statistical reliability.

Callus biomass was determined as dry weight (DW) following the method described by Verma *et al.* (2024). To evaluate the effect of fungal filtrate on *R. serpentina* callus growth, 1.0 g of callus tissue was cultured on media supplemented with varying concentrations of *A. niger* filtrate: E_1 (0.5%), E_2 (2.5%), E_3 (5.0%) and E_4 (7.5%). Control cultures were maintained under identical conditions at 30°C in the dark on media without fungal filtrate elicitors. After seven days of incubation, the biomass of *R. serpentina* callus cultures exposed to *A. niger* filtrate was measured.

To evaluate the effect of *A. niger* filtrate elicitation [E_1 (0.5%), E_2 (2.5%), E_3 (5.0%) and E_4 (7.5%)] on reserpine accumulation in *R. serpentina* callus cultures, 1.0 g of callus tissue from 9, 19 and 29-day old cultures was incubated for 24 h on medium containing the respective filtrate concentrations (Verma *et al.* 2024). Briefly, 250 mg of freeze dried and powdered callus was sonicated for 15 min in 1 ml chloroform:methanol (3:1, v/v) using a CPX 130 sonicator (Cole-Parmer, Illinois, USA) and then left at room temperature for 8 h to facilitate metabolite release. The combined supernatants, obtained after centrifugation at 12,000 rpm for 20 min at 4°C, were concentrated under reduced pressure at 50°C using a rotary evaporator. The residues were reconstituted in 1 ml acidic methanol (methanol:HCl, 98:2, v/v), filtered through a 0.22 μm nylon membrane. Twenty μl aliquots were injected into a Shimadzu HPLC system equipped with a UV–Vis detector (SPD-20A) and a C18 Kinetex column. Quantification was performed at 268 nm using a methanol:water (70:30, v/v) mobile phase. Each treatment employed five independent callus lines and was conducted in triplicate to ensure reproducibility.

The effect of *A. niger* filtrate elicitation [E_1 (0.5%), E_2 (2.5%), E_3 (5.0%), and E_4 (7.5%)] on antioxidant enzyme activities in *R. serpentina* callus cultures was assessed by incubating 1.0 g of callus tissue from 9, 19 and 29-day old cultures on filtrate-supplemented medium for 24 h. Antioxidant enzymes were extracted following the procedure of Verma *et al.* (2024). Briefly, 0.5 g of callus tissue was ground to a fine powder in liquid nitrogen, homogenized in 10 ml of extraction buffer (50 mM KH_2PO_4 containing 1% PVPP and 0.1 mM EDTA, pH 7.8), and centrifuged at 12,000 rpm for 15 min at 4°C. The resulting supernatant was used for enzyme assays. Total protein content was determined by the Bradford method, and the activities of superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX) and malondialdehyde (MDA) levels were measured. Free proline content was estimated using L-proline as the calibration standard. Approximately 200 mg of callus tissue from a single test tube was homogenized in 5.0 ml of 3.0% aqueous sulfosalicylic acid and centrifuged at 8000 rpm for 15 min to remove particulate matter. An aliquot of 1.0 ml supernatant was combined with 1.0 ml acid ninhydrin and 1.0 ml glacial acetic acid, and the mixture was incubated in a boiling water bath at 100°C for 1 h. The reaction was

terminated by rapid cooling in an ice bath. The resulting chromophore was extracted with 2.0 ml toluene, and the absorbance of the organic phase was recorded 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

The effectiveness of the surface sterilization protocol was evident from the high survival rate and minimal contamination observed among the aseptic explants. Leaf explants cultured on MS medium supplemented with varying concentrations of 2,4-D (1.0-3.0 mg/l) and a fixed level of BAP (1 mg/l) showed differential responses. The combination of 2.0 mg/l 2,4-D and 1 mg/l BAP produced the highest callus induction (Table 1). In contrast, explants grown on MS medium containing only 2,4-D exhibited slight swelling without noticeable callus formation. The frequency and intensity of callusing were strongly influenced by the concentration of applied phytohormones, while no callus initiation occurred in the hormone-free control medium. Compact, yellowish-white calli appeared within two weeks of culture and expanded vigorously after subculturing at two-week intervals (Fig. 1c, d). After four weeks, marked differences in growth patterns between treated and control groups confirmed the optimized conditions for reliable callus formation in *Rauwolfia serpentina*. These findings provide a robust basis for future investigations on metabolite biosynthesis and pathway regulation and corroborate earlier reports (Verma *et al.*, 2021a, c).

Table 1. Effect of 2, 4-D and BAP on callus induction frequency from leaf explants in *R. serpentine*

Phytohormone (mg/l)		Leaf callus induction (%)
2, 4-D	BAP	
0	0	0 (No callus was observed)
0.5	0	34 ± 1.8
0.5	1	42 ± 1.9
1.0	0	50.4 ± 2.2
1.0	1	56.8 ± 2.9
1.5	0	70.8 ± 1.7
1.5	1	75.4 ± 1.8
2.0	0	88.1 ± 1.9
2.0	1	99.6 ± 2.2
2.5	0	80.3 ± 2.4
2.5	1	86.2 ± 2.3
3.0	0	60.7 ± 2.4
3.0	1	68.3 ± 2.7

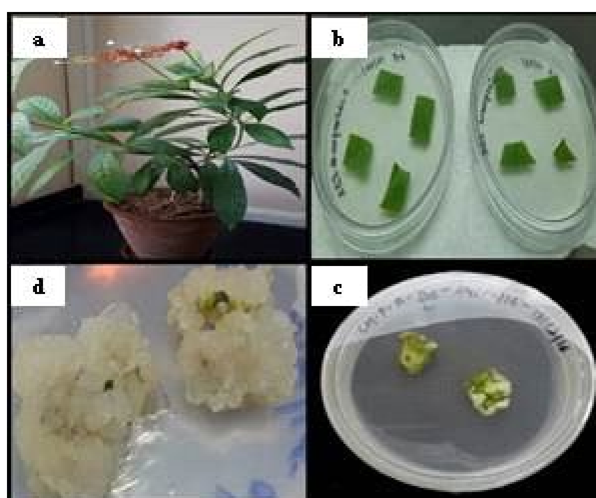

Fig. 1. (a) Fully matured *Rauwolfia serpentine,* (b) Leaf explants cultured on callus-inducing medium, (c) Initiation of callus formation from explants and (d) Development of a fully proliferated callus.

The effect of varying *A. niger* filtrate concentrations (0.5-7.5%) on callus biomass production was evaluated by supplementing the culture medium with the respective elicitor levels. A progressive reduction in biomass was observed with increasing filtrate concentration. Specifically, callus biomass at 0.5% fungal filtrate was 0.513 mg, while at 2.5, 5.0 and 7.5% the values decreased to 0.498, 0.488 and 0.479 mg, respectively (Fig. 2). In contrast, the untreated control cultures recorded the highest biomass (0.521 mg), indicating that fungal filtrate exerted a negative effect on biomass accumulation at all tested concentrations. This decline suggests a stress-induced response, potentially attributable to alterations in growth dynamics, cellular metabolism, or nutrient utilization caused by the elicitor. Furthermore, calli exposed to fungal filtrate exhibited notable morphological changes, becoming more fragile and developing a brownish colouration compared with the healthier appearance of control cultures. These findings indicate that while fungal filtrate may enhance secondary metabolite biosynthesis, it concurrently suppresses biomass production beyond

Table 2. Proline content of callus under *A. niger* elicitation

Treatment	10 days	20 days	30 days
C	15.02±0.48	17.23±0.36	16.08±0.32
E_1	17.05±0.49	19.26±0.37	18.01±0.34
E_2	18.66±0.47	21.07±0.35	19.98±0.29
E_3	19.55±0.41	21.76±0.31	20.56±0.38
E_4	20.21±0.43	22.43±0.34	21.21±0.36

Effect of *A. niger* elicitor supplementation (C–0%, E_1–0.5%, E_2–2.5%, E_3–5.0% and E_4– 7.5%) at different culture age (10, 20 and 30 days) on proline content in *R. serpentina* callus cultures.

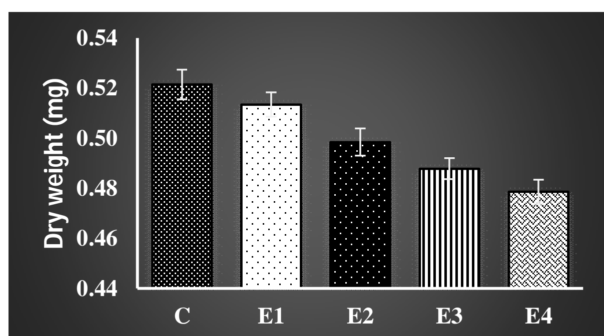

Fig. 2. Effect of elicitation with fungal filtrate (0.5-7.5%) on biomass of *R. serpentina* callus culture as compared to unelicited lines (control).

a threshold level. Therefore, optimizing fungal filtrate concentrations is essential to balance growth and metabolite production in the in vitro cultures.

To evaluate the effect of *A. niger* filtrate on reserpine content, two-week-old callus cultures of *R. serpentina* were established on MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l BAP and subsequently exposed to different filtrate concentrations [E_1 (0.5%), E_2 (2.5%), E_3 (5.0%) and E_4 (7.5%)] for 24 h. Following treatment, callus tissues developed a distinct browning, whereas no such changes were observed in the untreated controls. Elicitation with 10% *A. niger* filtrate for 24 h led to a 2.4 fold increase in reserpine production (0.106 mg/g DW). Notably, callus cultures treated with 5% filtrate produced even higher levels of reserpine (0.126 mg/g DW) compared with those exposed to lower filtrate concentrations (Fig. 3). Among the conditions tested, 20-day old calli elicited for 24 h with 5% *A. niger* filtrate yielded the maximum reserpine content, suggesting that both elicitor concentration and culture age influence metabolite accumulation. Browning of callus cultures after elicitor treatment was consistently observed, which may reflect a

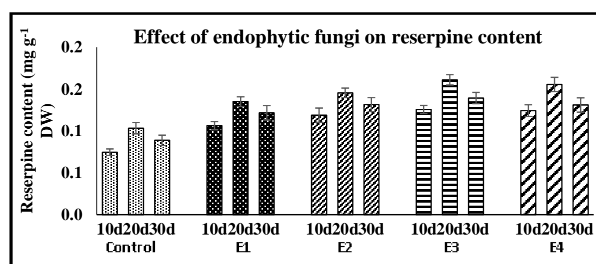

Fig. 3. Effect of fungal elicitor supplementation (C–0%, E_1–0.5%, E_2–2.5%, E_3–5.0% and E_4–7.5%) at different culture age (10, 20 and 30 days) on reserpine content in *R. serpentina* callus cultures.

hypersensitive response, as also reported for *R. serpentina* cell cultures treated with elicitors (Verma *et al.*, 2024).

Elicitation with fungal filtrates induces oxidative stress, leading to enhanced production of reactive oxygen species (ROS), with mitochondria serving as the primary site of ROS generation. To counteract the harmful effects of oxidative stress and free radicals, cells upregulate antioxidant enzymes such as superoxide dismutase (SOD), catalase, etc. (Tonk *et al.*, 2016; Wang *et al.*, 2025). In the present study, the activities of antioxidant enzymes (SOD and catalase) in *R. serpentina* callus cultures were evaluated following 24 h of elicitation with different concentrations of fungal filtrate across various callus ages.

In control calli, superoxide dismutase (SOD) activity increased steadily with culture age, rising from 2.81 U/mg protein at 10 days to 3.29 U/mg protein at 20 days, but declined markedly in older (30-day-old) calli to 2.97 U/mg protein. Across all *A. niger* elicitation treatments, SOD activity showed a significant increase as callus age advanced from 10 to 20 days, followed by a pronounced reduction at 30 days (Fig. 4a).

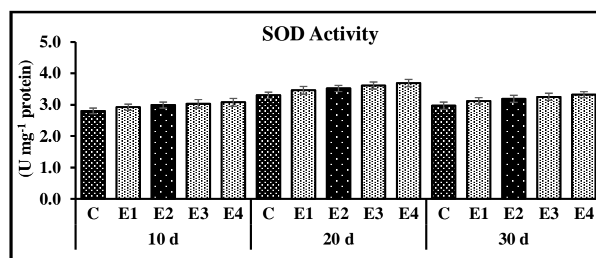

Fig. 4a. Effect of fungal elicitor supplementation (C–0%; E_1–0.5%, E_2–2.5%, E_3–5.0% and E_4–7.5%) at different culture age (10, 20 and 30 days) on SOD enzyme activity in *R. serpentina* callus cultures.

Catalase activity in elicited calli of different ages was consistently and significantly higher than in the corresponding control calli (Fig. 4b). In the controls, catalase activity gradually increased with callus age, from 2.21 at 10 days to 2.26 at 20 days and 2.31 U/mg protein at 30 days. Across all *A. niger* elicitation treatments, catalase activity exhibited a marked and progressive increase as callus cultures matured from 10 to 30 days (Fig. 4 b).

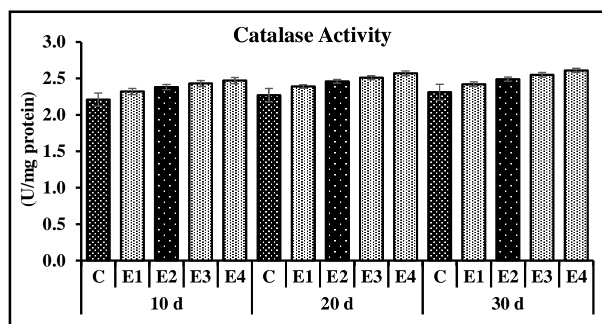

Fig. 4b. Effect of fungal elicitor supplementation (C–0%, E_1–0.5%, E_2–2.5%, E_3–5.0%, and E_4–7.5%) at different culture age (10, 20 and 30 days) on catalase enzyme activity in *R. serpentina* callus cultures.

In control calli, proline content increased steadily with culture age, rising from 15.02 at 10 days to 17.23 at 20 days, but declined markedly in older (30-day old) calli to 16.08 mg/g FW. Across all *A. niger* elicitation treatments, proline content showed a significant increase as callus age advanced from 10 to 20 days, followed by a pronounced reduction at 30 days (Table 2).

CONCLUSION

The study demonstrated that non-viable *Aspergillus niger* filtrate acted as an effective biotic elicitor in *Rauwolfia serpentina* callus cultures, stimulating secondary metabolism while inducing measurable physiological stress. Although callus biomass declined with increasing filtrate concentration, reserpine accumulation, antioxidant enzyme activities (SOD and catalase) and proline content all rose significantly, particularly at 5% filtrate and 20 day culture age, indicating activation of stress-responsive and defense pathways. These findings suggested that carefully optimized elicitation could shift metabolic flux from growth toward high-value alkaloid production without the risks associated with live microbial inoculation. The approach highlightd a scalable, safer strategy for enhancing pharmaceutically important compounds in *R. serpentina* and underscored the broader potential of endophytic fungal metabolites as sustainable tools for improving plant tissue culture yields.

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