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Chitosan induced Enhancement of Ajmalicine Content in *Rauwolfia serpentina* Callus Culture

DIVYA YADAV, NEERAJ KHARE[1], SADANAND PANDEY[2] AND HIMANI GOYAL SHARMA*

NIAMST, NIMS University Rajasthan, Jaipur-303 121 (Rajasthan), India
**(e-mail:himani.goyal@nimsuniversity.org; Mobile: 9676974176)*

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ABSTRACT

The present study is an effort to develop an efficient protocol for the *in vitro* induction of calli from *Rauwolfia serpentina* leaves as explants and evaluation of chitosan as elicitor for enhancement of ajmalicine content in *R. serpentina* calli. Initially MS medium supplemented with various concentrations and combinations of phytohormones (2,4-D, NAA, BAP and Kinetin) were examined for calli development and NAA (2.0 mg/l) in combination with KIN (1.0 mg/l) which recorded maximum of 1.04 gm fresh weight of callus after four weeks of culture period. The established calli were treated with different concentrations of chitosan (25, 50, 75 and 100 mg/l) for seven days to evaluate its elicitor effects on biomass. It was recorded that compared with control cultures, elicited calli on increasing the chitosan concentrations, culture biomass decreased. 25 mg/l chitosan treatment for 24 h on 19 days callus cultures were recorded for highest ajmalicine content. It was recorded that compared with control cultures, elicited callion increasing the chitosan concentrations, proline accumulation and antioxidant enzyme activity increased. Overall, the study demonstrated that chitosan acted as a potent and eco-friendly elicitor capable of stimulating ajmalicine biosynthesis in *R. serpentina* callus cultures, offering valuable insights for optimizing elicitation-based ajmalicine enhancement strategies.

Key words: *Rauvolfia serpentina*, callus cultures, elicitation, chitosan, ajmalicine

INTRODUCTION

Rauvolfia serpentina is an erect, evergreen, woody perennial shrub belonging to the family Apocynaceae and is commonly referred to as Sarpgandha, Chota Chand, Chandrikaetc among other vernacular names. The species is a rich source of pharmaceutically important metabolites, including reserpine, ajmalicine, etc. Among these, ajmalicine (raubasine) is a clinically valuable terpene indole alkaloid widely used in the treatment of hypertension. It functions as a selective á$_1$-adrenergic receptor antagonist and is primarily isolated from the roots of *R. serpentina* (Pathania *et al*., 2017; Bhagat *et al*., 2019, 2020; Verma *et al*., 2021a, b).

These pharmaceutically important metabolites occur in very low amounts in plant cells, making large-scale extraction difficult due to limited plant material and laborious, time-consuming procedures. Moreover, the chemical synthesis of ajmalicine is not feasible because of its complex structure (Ambrin *et al*., 2020; Rana *et al*. 2024). To overcome these constraints, elicitation has become a promising biotechnological strategy for enhancing metabolite production in *in vitro* plant cultures. Elicitors are biotic or abiotic agents that trigger stress responses in plants, leading to enhanced biosynthesis of secondary metabolites (Tiwari *et al*., 2021). Elicitation serves as an efficient approach to boost secondary metabolite content by altering various physiological and biochemical processes within plant cells (Verma *et al*., 2024).

Chitosan is an effective biotic elicitor widely used to enhance secondary metabolite content in plant cultures. Chitosan triggers plant defense responses, including reactive oxygen species generation, activation of MAP kinases and induction of defense-related genes. These signalling events stimulate key enzymes of secondary metabolic pathways, resulting in increased accumulation of alkaloids,

[1]P.I.P.M. Innovations LLP, Amer, Jaipur-302 028 (Rajasthan), India
[2]School of Bioengineering and Food Technology, Faculty of Applied Sciences and Biotechnology, Shoolini University, Solan-173 229 (Himachal Pradesh), India.

phenolics, flavonoids and terpenoids. Owing to its biodegradability, biocompatibility and effectiveness at low concentrations, chitosan is considered an eco-friendly elicitor in plant biotechnology (Chowdhury *et al*., 2025).

The present study investigated the role of chitosan endophytic fungi from *R. serpentina* cultivated in saline-rich regions and evaluated their potential for producing secondary metabolites with antioxidant and anticancer properties.

The present study investigated chitosan elicitation as a biotic strategy to stimulate terpenoid indole alkaloid biosynthetic pathways in *R. serpentina* callus cultures, thereby enhancing in vitro ajmalicine accumulation and providing an efficient, sustainable and controlled approach for improving the production of this pharmaceutically important alkaloid.

MATERIALS AND METHODS

HiMedia (India) supplied all plant tissue culture-grade phytohormones and associated chemicals. Ajmalicine standard and other HPLCgrade chemicals were purchased from Sigma Aldrich (Now Merck, Germany).

Healthy *R. serpentina* plants were obtained from the Sushila Tiwari Herbal Garden in Rishikesh, Uttarakhand, India, and kept in the culture facility at NIMS University, Rajasthan under carefully monitored conditions (Fig. 1a).

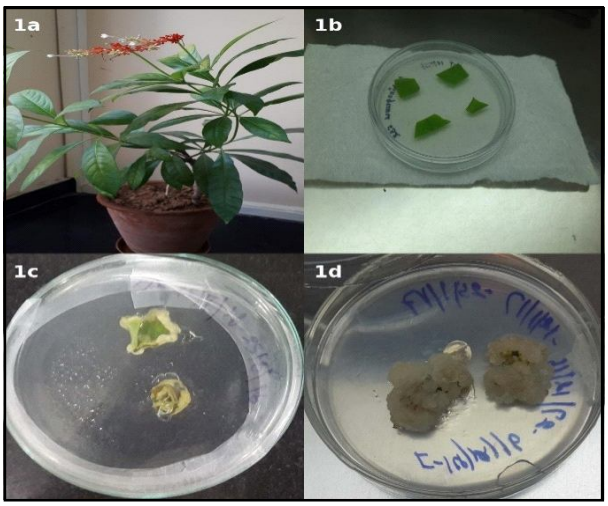

Fig. 1. (a) Fully matured Rauwolfia serpentine, (b) Leaf explants cultured on callus-inducing medium, (c) Initiation of callus formation from explants and (d) Development of a fully proliferated callus.

Plant materials were excised into about 1 cm^2 pieces after being thoroughly cleaned under running tap water to get rid of surface impurities. The explants were surface sterilized by immersing them in 70% ethanol for 2 min, then treating them with 0.1% sodium hypochlorite for 2 more min. The explants were air-dried on sterile filter paper under aseptic circumstances following several rinses with sterile distilled water to exclude any remaining sterilizing agents. The sterilized explants were placed on Murashige and Skoog (MS) media supplemented with a range of dosage levels and combinations of 2,4-D, NAA and Kinetin for callus initiation and subsequent proliferation (Fig. 1b). After adjusting the medium's pH to 5.8 and solidifying it with 8 g/l agar, it was autoclaved for 20 min at 121°C to sanitize it. The cultures were sub-cultured every two weeks and kept at 26°C with a 16-h light/8-h dark photoperiod. The biomass of calli grown on various treatments was evaluated by measuring fresh weight. Data were recorded after 4 weeks. Each treatment consisted of 15 explants and was performed in triplicate to ensure reliability.

Chitosan (Sigma-Aldrich, Germany) was dissolved in 3% (v/v) 0.1 M acetic acid by gentle heating with continuous stirring. The pH of the solution was adjusted to 5.8 and the final concentration was standardized to 10 mg/ml. Prior to use, the solution was sterilized by filtration through a 0.45 ìm microfilter. *R. serpentina* calli of approximately equal weight (0.5 g) were excised and sub cultured to fresh MS media supplemented with different treatments of chitosan: E_1 (25 mg/l), E_2 (50 mg/l), E_3 (75 mg/l), and E_4 (100 mg/l).Control cultures were maintained under identical conditions without chitosan. All samples were placed in an incubator set at 26°C and kept under 24 h darkness. Each treatment consisted of 15 callus and was performed in triplicate to ensure reliability.

To assess the effect of elicitation on the biomass of *R. serpentina* callus, 0.5 g of callus was cultured on MS medium supplemented with 2.5 mg/l 2,4-D and 1.0 mg/l kinetin, and treated with different concentrations of chitosan (E_1, E_2, E_3 and E_4). After one week of culture, both elicited and control calli were harvested from the medium and their dry weights were determined.

The impact of chitosan elicitation on ajmalicine accumulation in *R. serpentina* callus was examined using 0.5 g of callus cultured on MS medium supplemented with 2.5 mg/l 2,4-D and 1.0 mg/l kinetin. Cultures were maintained in darkness at 26°C for 9, 19 and 29 days and subsequently exposed for 24 h to different chitosan concentrations (E_1, E_2, E_3 and E_4).

Ajmalicine was extracted according to Verma *et al.* (2024). Briefly, 250 mg of freeze-dried, powdered callus was sonicated for 15 min in 1 ml of chloroform: methanol (3:1, v/v) and incubated at 25°C for 8 h. After centrifugation at 12,000 rpm for 20 min at 4°C, the supernatant was concentrated under vacuum at 50°C under rotary evaporation. The the residual material was re-solubilized in 1 ml of acidic methanol (methanol: HCl, 98:2, v/v), passed through a 0.22 µm nylon membrane and 20 µl was analyzed using a Shimadzu HPLC system. Ajmalicine was quantified at 254 nm using a with methanol: water (70:30, v/v) mobile phase.

The influence of chitosan elicitation on antioxidant enzyme activities (superoxide dismutase and catalase) in *R. serpentina* callus was evaluated using 0.5 g callus cultured on MS medium supplemented with 2.5 mg/l 2,4-D and 1.0 mg/l kinetin and subsequently treated with chitosan. Cultures were maintained in darkness at 26°C for 9, 19 and 29 days and then exposed to different chitosan concentrations (E_1, E_2, E_3 and E_4) for 24 h.

Antioxidant enzymes were extracted following Verma *et al*. (2024). Briefly, callus was ground in liquid nitrogen, homogenized in 10 ml of extraction buffer (50 mM KH, PO„ 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, and total protein content was determined using the Bradford method.

The effect of chitosan elicitation on proline content in *R. serpentina* callus was studied using 0.5 g of callus grown on MS medium supplemented with 2.5 mg/l 2,4-D and 1.0 mg/l kinetin and subsequently treated with chitosan. Cultures were incubated in the dark at 26°C for 9, 19 and 29 days, followed by a 24 h exposure to different chitosan levels (E_1, E_2, E_3 and E_4).

L-proline was used as the standard to quantify proline. From each sample, 200 mg of callus tissue was ground, extracted with 5.0 ml of 3.0% sulfosalicylic acid, then centrifuged at 8000 rpm for 15 min. The reaction medium was thermally treated to100°C for one hour after one ml of the clear supernatant was combined with one ml of acid ninhydrin and one ml of glacial acetic acid. Following quick cooling in an ice bath, 2.0 ml of toluene was used to extract the coloured product, and a A UV-Vis spectrophotometer was employed to identify the organic phase's absorbance at 520 nm.

Every experiment used a fully randomized design. Statistical analysis of the data was performed using SPSS software (Chicago, IL, USA), and the results are expressed as mean ± standard deviation. At $P < 0.05$, statistical significance was determined.

RESULTS AND DISCUSSION

After 25 days of culture, leaf explants grown on MS medium supplemented with 2.5 mg/l NAA and 0.5 mg/l kinetin showed profuse callus development along with root initiation. Under this treatment, the callus attained a fresh weight of 0.61 g after 30 days (Table 1). An increase in root proliferation was observed at NAA concentrations higher than 2.5 mg/l. Conversely, reduced NAA levels exhibited callus formation with limited root initiation, and the callus exhibited a yellowish-green colouration. Among all treatments, leaf segments cultured on MS medium supplemented with 2.5 mg/l 2,4-D and 1.0 mg/l kinetin yielded the friable, yellowish-green callus, reaching a maximum fresh weight of 1.12 g (Fig. 1c, d). Reducing the 2,4-D concentration suppressed callus proliferation, while levels above 2.5 mg/l led to callus browning and a marked decline in callus growth (Table 1). Necessity of cytokinins

Table 1. Effect of phytohormones on callus growth

Growth hormones (mg/l)			Fresh weight (g) of callus after 30 days
KIN	NAA	2-4,D	
0.50	0.5	-	0.45
0.50	1.5	-	0.53
0.50	2.0	-	0.55
0.50	2.5	-	0.61
0.50	3.0	-	0.55
1.00	-	1.00	0.68
1.00	-	1.50	0.86
1.00	-	2.00	1.01
1.00	-	2.50	1.12
1.00	-	3.00	1.03
1.00	-	3.50	0.85

together with auxins for callus induction was reported in *R. serpentine* (Verma *et al*., 2021a, b).

The influence of chitosan elicitation on callus biomass was assessed by supplementing different concentrations of chitosan (25-100 mg/l) into the culture medium. Callus biomass declined progressively with increasing chitosan levels. The highest fresh weight (0.505 mg) was obtained at 25 mg/l chitosan, whereas further increases to 50, 75 and 100 mg/l resulted in a gradual reduction in fresh weight to 0.489, 0.481 and 0.472 mg, respectively (Fig. 2). In contrast, the control cultures exhibited the highest biomass (0.521 mg), demonstrating that chitosan negatively influenced callus growth across all concentrations tested. The observed reduction in biomass likely reflects a stress-induced response, possibly resulting from elicitor-mediated alterations in growth, cellular metabolism, or nutrient assimilation. Additionally, chitosan-treated calli developed a brownish colouration, whereas control cultures maintained a healthier appearance. Collectively, these results suggest that although chitosan may stimulate secondary metabolite biosynthesis, it simultaneously suppresses biomass accumulation. Therefore, careful optimization of chitosan concentration is necessary to achieve an optimal balance between callus growth and metabolite production.

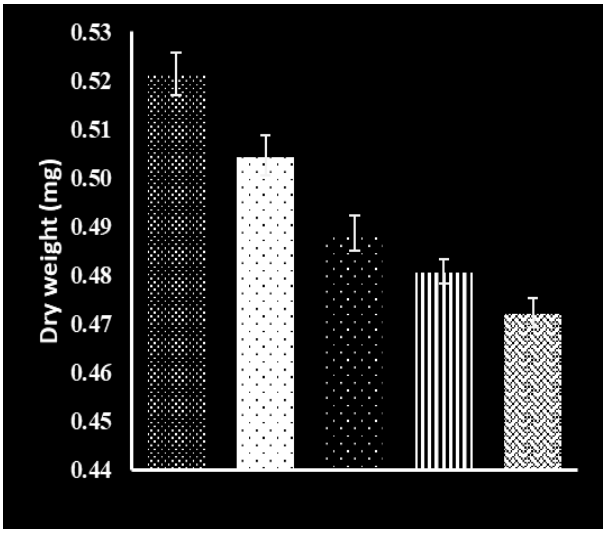

Fig. 2. Effect of elicitation with chitosan [E_1 (25 mg/l), E_2 (50 mg/l), E_3 (75 mg/l) and E_4 (100 mg/l) on biomass of *R. serpentina* callus culture.

The effect of chitosan elicitation on ajmalicine content was evaluated by treating calli of different age (9, 19 and 29 days old) with varying concentrations of chitosan (25-100 mg/l) for a 24-h exposure period in the culture medium. An elicitation with 25 mg/l chitosan applied to 20-day-old calli resulted in the highest ajmalicine accumulation (0.881 mg/g DW). Further increases in either callus age or chitosan concentration led to a decline in ajmalicine content (Fig. 3). These results indicate that both elicitor concentration and culture age significantly influence ajmalicine production. Following elicitor treatment, the calli exhibited noticeable browning, whereas no such changes were observed in the untreated control cultures. The browning observed in chitosan-treated calli may represent a hypersensitive stress response, consistent with earlier reports on elicitor-treated *R. serpentina* cell cultures (Verma *et al*., 2024).

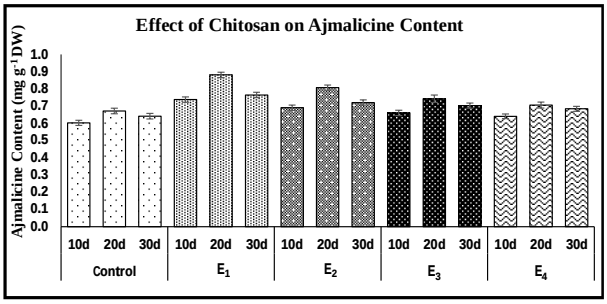

Fig. 3. Effect of chitosan elicitor supplementation [E_1 (25 mg/l), E_2 (50 mg/l), E_3 (75 mg/l) and E_4 (100 mg/l) on ajmalicine content in *Rauvolfia serpentina* callus of different culture age.

Chitosan elicitation induces oxidative stress, leading to elevated accumulation of reactive oxygen species (ROS), with mitochondria representing the major source of ROS production. To alleviate the detrimental effects of excessive ROS and associated free radicals, cells enhance their stress response by upregulating key antioxidant enzymes, including superoxide dismutase (SOD) and catalase (Tonk *et al*., 2016). In the present investigation, the activities of antioxidant enzymes (SOD and catalase) in *R. serpentine* calli were examined after 24 h treatment with varying concentrations of chitosan.

In the control cultures, superoxide dismutase (SOD) activity increased gradually with culture age, rising from 2.81 U/mg protein at 10 days to 3.28 U/mg protein at 20 days, before

declining significantly to 2.95 U/mg protein in 30-day-old calli. A comparable trend was observed under all chitosan elicitation treatments, where SOD activity increased markedly between 10 and 20 days of culture and then decreased sharply at 30 days. Furthermore, within calli of the same culture age, SOD activity showed a concentration-dependent increase with rising chitosan levels (25-100 mg/l) (Fig. 4a).

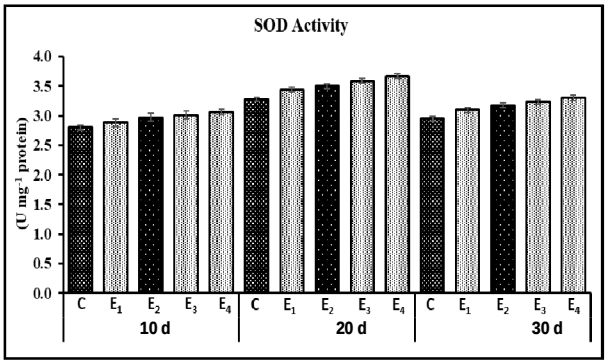

Fig. 4a. Effect of chitosan elicitor supplementation [E_1 (25 mg/l), E_2 (50 mg/l), E_3 (75 mg/l) and E_4 (100 mg/l) on proline content in *Rauvolfia serpentina* callus of different culture age. Values are presented as mean ± SD.

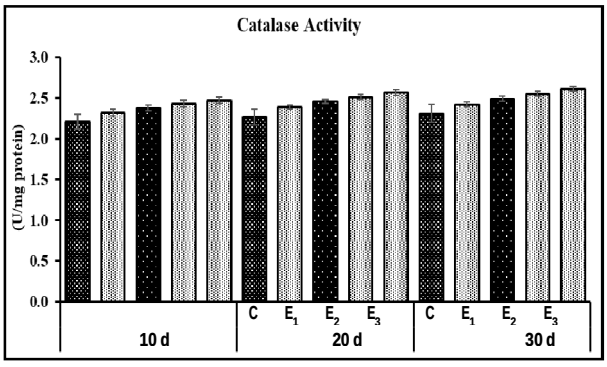

Fig. 4b. Effect of chitosan elicitor supplementation [E_1 (25 mg/l), E_2 (50 mg/l), E_3 (75 mg/l) and E_4 (100 mg/l) on catalase activity in *R. serpentina* callus of different culture age.

Across all culture ages, catalase activity in chitosan-elicited calli increased progressively with rising chitosan concentrations and remained markedly higher than that observed in the corresponding control calli (Fig. 4b). A clear age-dependent enhancement in catalase activity was also evident, with activity levels increasing as the callus matured. In the control cultures, catalase activity showed a gradual increase from 2.21 U/mg protein at 10 days to 2.26 U/mg protein at 20 days and further to 2.31 U/mg protein at 30 days.

In control calli, proline content increased steadily with culture age, rising from 15.02 mg/g FW at 10 days to 17.23 mg/g FW 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).

Table 2. Proline content of callus under chitosan elicitation

Treatment	10 days	20 days	30 days
Control	15.02 ± 0.39	17.23 ± 0.36	15.86 ± 0.32
E_1	16.85 ± 0.42	19.03 ± 0.35	17.82 ± 0.31
E_2	18.42 ± 0.43	20.86 ± 0.29	19.77 ± 0.27
E_3	19.33 ± 0.44	21.54 ± 0.28	20.34 ± 0.33
E_4	19.98 ± 0.39	22.22 ± 0.31	21.01 ± 0.29

CONCLUSION

The present study demonstrated that chitosan elicitation was an effective biotic strategy for enhancing terpenoid indole alkaloid biosynthesis in *Rauvolfia serpentina* callus cultures. Optimal callus induction and proliferation were achieved on MS medium supplemented with 2.5 mg/l 2,4-D and 1.0 mg/l kinetin, yielding friable and actively growing calli suitable for elicitation studies. Chitosan treatment significantly influenced physiological and biochemical responses, resulting in reduced biomass but markedly enhanced ajmalicine accumulation, antioxidant enzyme activities (SOD and catalase), and proline content, reflecting elicitor-induced stress responses. Maximum ajmalicine production was obtained in 20-day-old calli treated with 25 mg/l chitosan, highlighting the importance of optimizing both culture age and elicitor concentration. Overall, this work provides a sustainable and controlled *in vitro* approach for improving ajmalicine production and offers valuable insights for future scale-up and biotechnological exploitation of *R. serpentina.*

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REFERENCES

Ambrin, G., Ali, H. M. and Ahmad, A. (2020). Metabolic regulation analysis of ajmalicine

biosynthesis pathway in *Catharanthus roseus* (L.) G. Don suspension culture using nanosensor. *Processes* **8**: 589.

Bhagat, P., Verma, S. K., Singh, A. K., Aseri, G. K. and Khare, N. (2019). Evaluation of influence of different strains of Agrobacterium rhizogenes on efficiency of hairy root induction *in Rauwolfia serpentina*. *Ind. J. Genet. Plant Breed.* **79**: 760-764

Bhagat, P., Verma, S. K., Yadav, S., Singh, A. K., Aseri, G. K. and Khare, N. (2020). Optimization of nutritive factors in culture medium for the growth of hairy root and ajmalicine content in sarpgandha, *Rauwolfia serpentina*. *J. Environ. Biol.* **41**: 1018-1025

Chowdhury, R., Mehmet, M., Mukherjee, J., Debnath, A. J. and Ražná, K. (2025). Chitosan as an elicitor in plant tissue cultures: Methodological challenges. Molecules **30**: 3476.

Pathania, D., Sharma, S. and Singh, P. (2017). Removal of methylene blue by adsorption onto activated carbon developed from *Ficus carica* bast. *Arab. J. Chem.* **10**: S1445-S1451.

Rana, A., Chatterjee, A., Goyal, M., Kumar, G., Aseri, S. K. and Khare, N. (2024). *Tephrosia purpurea*–An effective anti-bacterial agent against the clinical isolates of methicillin-resistant *Staphylococcus aureus*. *Ann. Biol.* **40**: 164-169.

Tiwari S., Verma S. K., Bhargava A., Kaur R., Sharma R. and Khare, N. (2021). Nanomaterials assisted elicitation of pharmaceutically important secondary metabolites from *in vitro* plant cell cultures. In: Nanotoxicology: *Toxicity Evaluation of Nanomedicine,* H. K. Daima, S. L. Kothari and S. K. Bhargava (eds), pp. 117-132. CRC Press

Tonk, D., Mujib, A., Maqsood, M., Ali, M. and Zafar, N. (2016). *Aspergillus flavus* fungus elicitation improves vincristine and vinblastine yield by augmenting callus biomass growth in *Catharanthus roseus*. *Plant Cell Tiss. Organ Cult.* **126**: 291-303.

Verma S. K., Bhagat, P., Yadav, S., Goyary, D., Aseri, G. K. and Khare, N. (2021 a). The effects of phytohormones and yeast extract on callus development of medicinally important endangered plant, *Rauwolfia serpentina* (L.). *Ann. Biol.* **37**: 23-26.

Verma S. K., Bhagat, P., Bhargava, A., Chatta, A., Goyary, D., Aseri, G. K. and Khare, N. (2021b). Enhanced callus growth of medicinally important *Rauwolfia serpentina* (L.) through supplementation of phytohormones and l-glutamine. *Ann. Agri. Bio Res.* **26**: 25-27.

Verma, S. K., Goyary, D., Singh, A. K., Anandhan, S., Raina, S. N., Pandey, S., Kumar S. and Khare N. (2024). Modulation of terpenoid indole alkaloid pathway via elicitation with phytosynthesized silver nanoparticles for the enhancement of ajmalicine, a pharmaceutically important alkaloid. *Planta* **259**: 30.