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Coimbatore - 641 043, Tamil Nadu, India

(Reg. No. 623/PO/ReBi/S/02/CCSEA)

Certificate

This is to certify that the project proposal no **AIW:IAEC.2023:10** entitled ***In vitro* Plant Regeneration of an endangered medicinal plant of *Rauvolfia tetraphylla* L. and its Biological activities** submitted by **Ms. Lavanya N** has been approved/recommended by the IAEC of **Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore** (Organization) in its meeting held on **14/03/2023** (date) and **50 Nos Zebra fish (*Danio rerio*) and TL/Ek (Fish)** (Number and Species if animals) have been sanctioned under this proposal for a duration of next **12** months.

Authorized by	**Name**	**Signature**	**Date**
Chairman:	Dr. Anitha Subash	[signature]	14/3/2023
Member Secretary:	Dr. R. Nirmaladevi	[signature]	14/3/2023.
Main Nominee of CCSEA:	Dr. V. M. Berlin Grace	[signature]	14-03-2023

Avinashilingam Institute for HomeScience and Higher Education for Women
(Deemed to be University Estd. u/s 3 of UGC Act 1956, Category 'A' by MHRD
Re-accredited with A++ Grade by NAAC. CGPA 3.65/4, Category I by UGC
Coimbatore - 641 043, Tamil Nadu, India

Appendix L2

(Item No 5 of Check List) Details of Research Publications

S.No	Article	Journal	Other Details Vol/No/Page No/ Year	Published in UGC-CARE / Scopus Indexed/ Web of Science
1	Functional characterization of promiscuous tryptophan decarboxylase from indole alkaloids producing Rauvolfia tetraphylla L.	Journal of King Saud University - Science.	36(Vol) 2024 (year)	Scopus
2	Insights of Micropogation, Somatic embryogenesis and Plant Regeneration from an Endangered medicinal Plant Rauvolfia tetraphylla L.	The Journal of plant Science Research	40(1) 1-29 2024	UGC - CARE Group - I

*Proof of list of Journals from Internet to be attached along with copies of reprints.

Scholar :

Supervisor : 01/04/2024 5/4/24

Checked By:

HoD/Dean of Respective School 5/4/2024.

The scholar Miss. Lavanya, N has published her article in the following journals:

1. Journal of King Saud University - ~~university~~ Science - is indexed in Scopus from 2009 to present,
2. The Journal of Plant Science Research - is indexed and active in UGC Care Group I from September 2019 to present. She got acceptance for her paper from this journal. Mails verified.

This may be considered.

J. [signature]
05.04.2024

Jour Pl Sci Res **40 (1)** 1-27 2024

Insights of Micropropagation, Somatic Embryogenesis and Plant Regeneration from an Endangered Medicinal Plant *Rauvolfia tetraphylla* L.

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***Rauvolfia tetraphylla* L. is a medicinal plant renowned for its pharmacological properties, faces threats due to habitat destruction and over-exploitation. This study targeted to explore micropropagation techniques to mitigate these challenges and investigated seed germination, callus formation from various explants, direct organogenesis, and somatic embryogenesis. Surface sterilization methods were optimized to enhance seed germination rates. The seed coat is hard it prevents the seed germination, to overcomethe seed dormancy and the seeds were treated with 4°C for 48 hours. The germination of 83.33% was achieved after the partial seed coat removal. Callus formation was induced from leaf, node, internode, and root explants using different hormone combinations. Maximum callus formation was observed on MS medium amended with 2,4-dichlorophenoxyacetic acid and 6 – benzyl amino- purine (2,4-D+BAP) in all explants. Hormone combinations also influenced direct root and shoot formation, with higher concentrations promoting greater responses. Somatic embryogenesis was induced in leaf and root explants using 2,4-D and BAP. Optimal concentrations (1.5 mg/L each) resulted in the formation of globular stage embryos. Histological analysis revealed distinct cellular processes during somatic embryogenesis, including embryo differentiation. It emphasizes the importance of hormone optimization and provides insights into the stomatic embryogenesis driving the plant regeneration in *R. tetraphylla*, offering valuable contributions to tissue culture protocols and conservation efforts for the endangered medicinal plant.**

Keywords: *Rauvolfia tetraphylla*, Micropropagation, Plant growth regulator, Somatic embryogenesis, Histology.

INTRODUCTION

Plant tissue culture is a method of growing plant cells, tissues or organs in a controlled environment on a nutrient culture medium. It is a widely used technique in plant breeding, genetic engineering and biotechnology Hussain et al, (2012). The significant applications of plant tissue culture is in the micropropagation of plants Caponetti et al, (2018). Micropropagation is the process of producing many genetically identical plants from a small piece of tissue. This technique is especially useful for the propagation of rare and endangered plant species, as well as for the commercial production of ornamental plants and fruits. Chokheli et al, (2020).

It has also been used in the production of secondary metabolites such as alkaloids, flavonoids, terpenoids, and phenolic compounds. These compounds are used in the pharmaceutical and food industries and have a wide range of medicinal properties Bapat et al, (2023); Betül, (2020). There has been an increasing interest in the use of plant tissue culture for the conservation of endangered plant species found in tropical forests, wetlands, and deserts. Coelho et al, (2020) discussed the several techniques used for the conservation of endangered plant species through tissue culture. It has become an essential tool in many areas of plant science, including plant breeding and genetic engineering, enhancement of secondary metabolites and conservation of endangered plant species as well as in the production of genetically modified crops, ornamental plants and fruits Tripathi et al, (2021).

Rauvolfia tetraphylla L. is a medicinal plant belonging to the family Apocynaceae. It is native to India and other parts of Southeast Asia. The plant is widely used in traditional medicine to treat various ailments such as hypertension, mental disorders, fever,

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and malaria. The major bioactive compounds found in *R. tetraphylla* are indole alkaloids, which are known to have various pharmacological properties such as hypotensive, sedative, antipsychotic, antimicrobial, anti-inflammatory, and anti-cancer activities. The plant contains several important alkaloids, including reserpine, ajmaline, and serpentine, which have been extensively studied for their pharmacological properties Mahalakshmi et al, (2019).

The plant faces threats due to habitat destruction and over-exploitation for medicinal purposes. Its shrubby nature and restricted distribution increase its vulnerability to human activities Hoque et al, (2020). The dependence of pharmaceutical companies on resources from wild plants contributes to the rapid decline of this endangered species Nair et al, (2013). The combination of indiscriminate collection and limited cultivation has resulted in its scarcity, leading to its endangered status in India Swarup & Arora, (2000). In Previous study Anitha et al, (2023); Faisal et al, (2006); Faisal & Anis, (2002) *in vitro* plant regeneration of *R. tetraphylla* were evaluated, but there have been no reports of somatic embryo formation from callus culture.

The aim of the study is focusing on the medicinal significance of *R. tetraphylla* and explored micropropagation. The study is not only investigated the germination potential of naturally occurring seeds but also developed an effective protocol for *in vitro* seed germination and regeneration of *R. tetraphylla*. There is a lack of extensive information regarding the optimization of *in vitro* somatic embryogenesis of *R. tetraphylla*. This study represent the first report on the histology of somatic embryogenesis in *R.tetraphylla*.

MATERIALS AND METHODS

Collection of Plant Source

Rauvolfia tetraphylla seeds were collected from Top slip, Pollachi, Western ghats, Coimbatore, Tamil Nadu, India.

Preparation of Tissue Culture Media

To prepare the Murashige and Skoog (MS) (1962) medium for tissue culture, a mixture of six stock solutions was thoroughly combined with sterile distilled water. Sucrose (30 g/L) and 0.1% meso-inositol (100 mg/L) were added, along with the necessary plant growth hormones. The pH of the medium was adjusted to 5.7 using 1N HCl or NaOH before autoclaving. Agar (8 g/L) was added to solidify the medium, which was then poured into culture vials and autoclaved at 15 Ib pressure for 15 minutes at 121 °C.

Auxins - 2,4-D (2,4-dichlorophenoxyacetic acid), IAA-(Indole -3- acetic acid) and IBA (Indole -3-butyric acid) and cytokinin's - BAP (6 – benzyl amino-purine) and KIN –Kinetin (6-furfurylaminopurine) are the plant growth hormone were prepared and stored at 4 °C.

Seed Inoculation

The *in vitro* seed germination begins where the seeds were surface sterilization by adding 2-3 drops of Tween-20 and shaking them continuously for 10 minutes. Then, they were rinsed with sterilized distilled water. Followed by the seeds were immersed in 1% Sodium hypochlorite solution for 10-20 minutes and washed with sterilized distilled water 2-3 times. Subsequently, the seeds were treated with a 0.1% $HgCl_2$ solution by gently shaking them for 1-3 minutes and washed 3-5 times with distilled water. Afterward, the sterilized seeds were placed in a sterilized glass bottle containing autoclaved distilled water and incubated at 4 °C for 2 days. A small incision was made at a side of the seeds, were inoculated on MS basal medium without plant growth regulators (PGRs) and to facilitate seed germination.

Callus Induction and Development

For callus induction, fully expanded leaf, node, internode and root explants of *R. tetraphylla* (6-month-old) were inoculated on MS medium with various PGRs such as auxin (IAA, IBA, 2,4-D), cytokinin's (BAP and KIN) and their concentration (0.5-2.5 mg/L). The cultures were incubated at 25±2 °C and 16/8 hrs. (light/dark) photoperiod of cool white light was provided from 2000 lux fluorescent tubes. The initiation of these inoculated explants was observed after 7th day onwards. The induced calli were sub-cultured at an interval of 21 days on the same medium. Data on callus induction from three replicates were recorded.

Direct Root Induction in Leaves and Node

Leaf and node explants were inoculated on MS medium supplemented with IAA, 2,4-D+BAP, IAA+BAP and IBA+BAP for direct root induction from the explants. Data on direct root induction from leaves and node from three replicates were recorded.

Multiple Shoot Formation

Node explant of *R. tetraphylla* was inoculated on MS medium amended with various concentration and combination of growth hormones such as BAP, 2,4-D+BAP, IAA+BAP and KIN+BAP are used for direct shoot induction without intervening callus. Developed shoot were sub-cultured on fresh MS medium every 28 days with the same growth regulators for further shoot elongation.

Histological Analysis

Histological studies were evaluated on somatic embryogenesis of leaf explant from *R. tetraphylla* at 10th, 20th, 30th, 40th, 50th, 60th and 70th days. The samples were fixed on FAA solution contain 95% ethyl alcohol: glacial acetic acid: formaldehyde: water (10:1:2:7) for 48 hrs (Liao & Wu, 2011). The samples were dehydrated in tertiary-butyl-alcohol and 70% alcohol (60%, 70%, 80%, 90% &100%) for 12hrs each and infiltrated by xylene in the oven at 50 °C. Then the samples were embedded in paraffin wax and blocks were sectioned using microtome. The sections were stained with 1% safranin and observed under light microscope.

Statistical Analysis

All the *in vitro* analysis were done in triplicate and the statistical software SPSS was used for the statistical analysis. P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Surface Sterilization of Seeds

The effect of surface sterilization on *R. tetraphylla* seeds using Sodium hypochlorite ($NaHCl_3$) at a concentration of 1% and Mercuric chloride ($HgCl_2$) at a concentration of 0.1% was investigated. The maximum 98.44% percentage of sterile plantlets were observed when seeds were treated with 20 min $NaHCl_3$ and 3 min of $HgCl_2$ of *R. tetraphylla* (Table 1a). By effectively eliminating contaminants, the sterilization process improves the chances of successful germination and subsequent growth, ensuring the production of healthy and viable plants.

Similarly Bharti et al, (2018) investigated the sterilization of $NaHCl_3$ on tomato plants and maximum was achieved using 10% $NaHCl_3$ for 8 minutes on true leaf (77.08%) and epicotyl (66.66%), 10 minutes on hypocotyl (74.30%), and 6 minutes on cotyledon (61.80%). In the same way, Ramandi et al, (2019) focused on *Catharanthus roseus* (L.) and emphasized the importance of surface sterilization using $NaHCl_3$ and $HgCl_2$. It observed a combination of 5% $NaHCl_3$ for 10 minutes and 0.3% $HgCl_2$ for 6 minutes resulted in significantly lower contamination percentages (14.66%) compared to the control (94.66%).

Darkazanli and Kiseleva (Darkazanli & Kiseleva, 2021) determined sterilization of $NaHCl_3$ on the germination rate of *Phaseolus vulgaris* and *Pisum sativum* seeds. It revealed that increasing the concentration of $NaHCl_3$ and prolonging the immersion times had detrimental effects on the seeds, resulting in a darkened appearance and a decline in the germination rate. Optimal germination, with a rate of 100% was achieved when the seeds were immersed in 20% $NaHCl_3$ for 5 minutes. Surface sterilization reduce microbial contamination, increase the percentage of sterile plant materials, promote successful *in vitro* culture establishment and isolation of endophytic bacteria. Optimization of the sterilization protocols based on specific concentrations and time duration enhances the *in vitro* germination percentage and viability of the seedlings.

Seed Germination

Seed germination of *R. tetraphylla* aimed to improve the seeds germination rate, which have a naturally hard seed coat that hinders germination. A slight cut on a side of sterilized seed and removed the hard seed coat. Uncut seeds were used as a control. Prior to cutting, the seeds endured a cold treatment at 4 °C for 2 days. Cold-treated, pre-cut seeds were inoculated on MS basal medium without any PGRs. Seed germination percentage were observed that 83.33% (Table 1b) at 7th day of inoculation. In contrast, the uncut seeds did not germinate due to the presence of the hard seed coat. (Fig.1) in the study depicts various stages of *in vitro* seed germination and the development of plantlets.

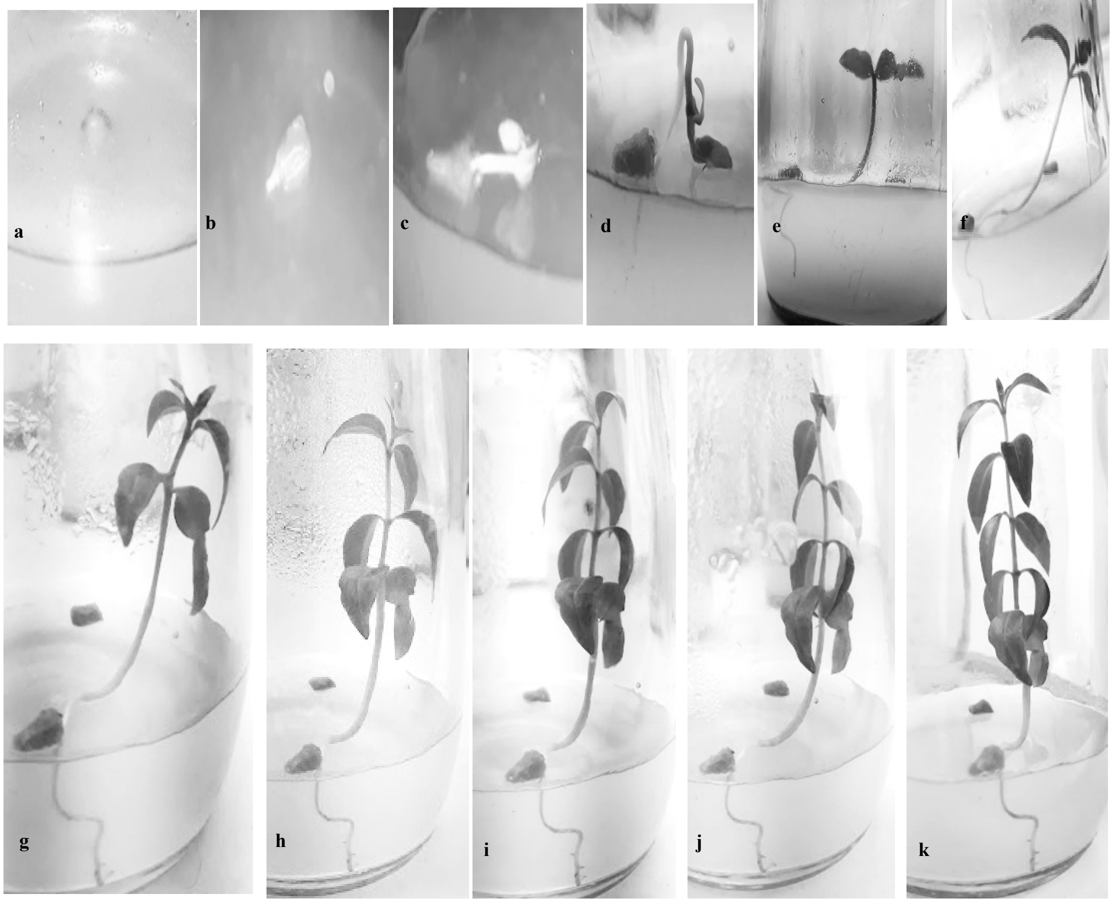


Fig. 1: Seed Culture of *Rauvolfia tetraphylla* (a) On the day; (b) 7th day; (c) 17th day; (d) 27th day; (e) 34th day; (f) 55th day; (g) 77th day; (h) 105th day; (i) 124th day; (j) 152th day; (k) 184th day

The cold treatment played a significant role in break seed dormancy and facilitated germination by seizing the barrier of the seed coat. It concluded that the hard seed coat is indeed a significant obstacle for seed germination in *R. tetraphylla*. In the previous research, Hoque et al, (2020) achieved the *in vitro* seed germination for *R. tetraphylla* and exposed cold-treated for 7 to 10 days, then the pre-cut seeds were inoculated on agar-solidified MS medium with 3% sucrose without any PGRs and noted that 78.00% of the seeds were germinated. Similarly, in this present study, the seeds were exposed only 2 days of cold conditions, maximum germination percentage of 88.00% was achieved.

Callus Induction and Development

In vitro callus were raised from leaf, node, internode and root and inoculated on MS medium supplemented with various growth hormone such as IAA, IBA, 2,4-D, BAP, KIN and combination of these hormones at different concentration and exhibited initial response approximately after 7 days of culture, there was characterized by dedifferentiation and multiplication of parenchymatous cells and formation of mass of undifferentiated cells under the controlled conditions. MS basal medium was used as a control and did not elicit any response from the explants.

Callus Formation from Leaf Explants

The table 2a presents the percentage of callus formation observed in leaf explants when treated with various hormone combinations and concentrations. The results (Fig. 2a) demonstrate the impact of different hormone treatments on callus induction. The maximum callus formation of 88.62% was observed in leaf explants on MS medium with 2,4-D+BAP (2.5 mg/L each). It indicates that the use of specific hormone combinations and concentrations

significantly influenced callus formation in leaf explants. In a related study, Nabi et al, (2018) analysed leaf explants of *Spilanthes acmella* L. are found that a combination of 2,4-D (0.5-2.0 mg/L) with 0.5 mg/L of BAP induced a maximum callus formation (70.5%) on MS medium containing 1.5 mg/L 2,4-D + 0.5 mg/L BAP. In this study, the highest response was observed at a concentration of 2.5 mg/L for both 2,4-D and BAP.

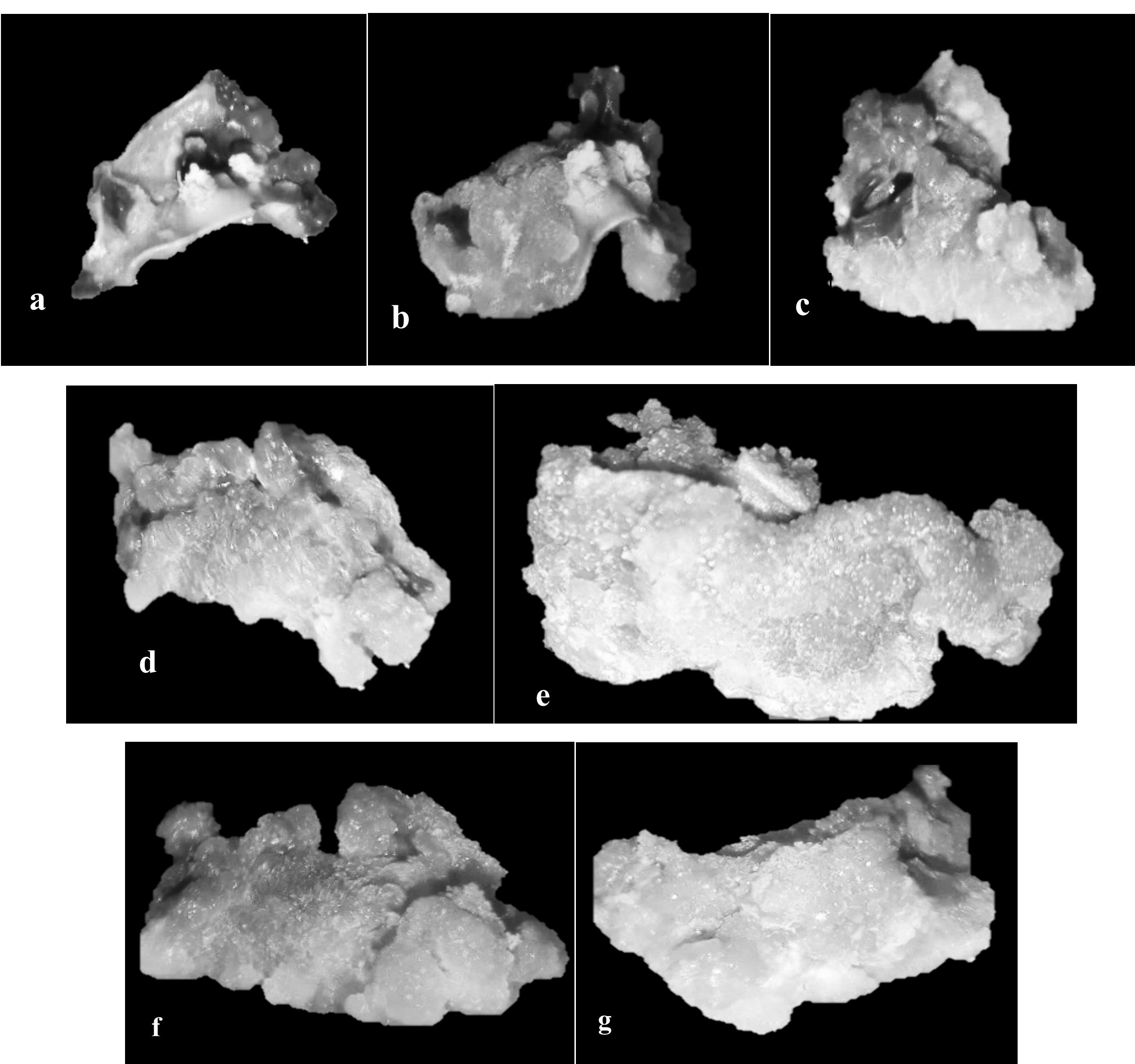


Fig. 2a: Effect of different plant growth hormones on callus formation from leaf of *Rauvolfia tetraphylla* L.

Callus formation (a) 15th day (b) 27th day (c) 45th day (d) 60th day (e) 75th day of inoculation on MS + 2,4D+BAP (f) MS medium with IAA+ 24D after 12 weeks of culture (g) MS medium with IAA+IBA after 12 weeks of culture.

In the same way, Copeland et al, (2020) investigated callus formation in leaf explants of *Crinum americanum* L and highest callogenesis (71.67%) were observed in a MS medium with 2,4-D (2.5 mg/L) and BAP (10 mg/L). The effectiveness of 2,4-D as an auxin in stimulating and improving callogenesis *in vitro* is well-established. Similarly, the results obtained in this study support the stimulatory

effect of 2,4-D on callogenesis induction in leaf explants of *R. tetraphylla*, the maximum percentage of callus induction (67.22%) was observed at 2.5 mg/L 2,4-D. Nurwahyuni et al, (2020) analysed an callus culture in *Styrax benzoin* and maximum callus formation 76.25% was achieved in young leaf explants at 3 mg/L BAP and NAA in MS medium. Furthermore, Yeasmin et al, (2022) demonstrated callus formation in leaf explants of strawberry, with the highest response (77.1%) observed in the MS medium with 2.0 mg/L BAP and 0.5 mg/L IBA.

Callus Formation from Node Explants

The (Table 2b) presents the percentage of callus formation observed in node explants of *R. tetraphylla* when treated with different PGRs at various concentrations. The results (Fig. 2b) demonstrate the effect of these hormones on callus induction in node explants. The combinations of PGRs enhanced callus induction compared to individual hormone treatments. The maximum callus formation of 83.45% was observed in node explants when a combination of 2,4-D+BAP (2.5 mg/L each).

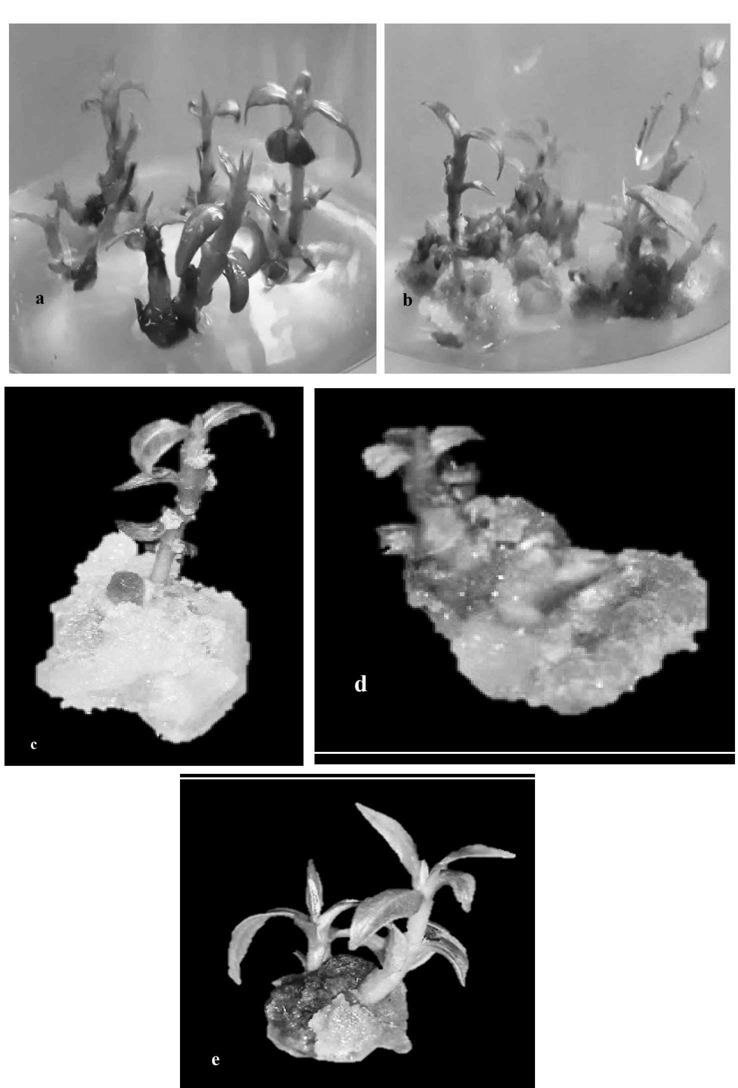


Fig. 2b: Effect of different plant growth hormones on callus formation from node of *Rauvolfia tetraphylla* L.

Callus formation (a) on 7th day of inoculation (b) 4th week of subculture (c) MS medium with 2.5 mg/L of 2,4-D+BAP after 8 week of culture (d) MS medium with 2.5 mg/L of IAA+ 2,4-D after 8 week of culture (e) MS medium supplemented with 2.5 mg/L of IBA+2,4-D after 8 weak of culture

Nurokhman et al, (2019) analysed the impact of PGRs on node explants of *Gynura procumbens* (Lour.) Merr. and maximum callus induction were obtained on MS medium at 2,4-D (0.1 mg/L) and BAP (0.1 mg/L). The combination of 2,4-D and BAP established remarkable callus formation in *R. tetraphylla* compared to other growth hormones. Wani et al, (2018) on *Lavatera cashmeriana* examined the effect of PGRs on callus induction of node explants exhibited 86.5% callus regeneration on 2,4-D (2.0 mg/L). In this study, the application of 2,4-D (2.5 mg/L) resulted in a callus formation percentage of 46.55% in the nodes of *R. tetraphylla*.

Callus Formation in Internode Explants

The (Table 2c) represents the percentage of callus formation observed in internodal explants of *R. tetraphylla* when treated with MS medium at different concentrations of PGRs. The results demonstrate a detailed relationship between the concentrations of PGRs and the formation of callus in internode (Fig. 2c). The maximum callus induction (79.66%) was exhibited on MS medium with 2,4-D and BAP at 2.5+2.5 mg/L in internodal explants of *R. tetraphylla*.

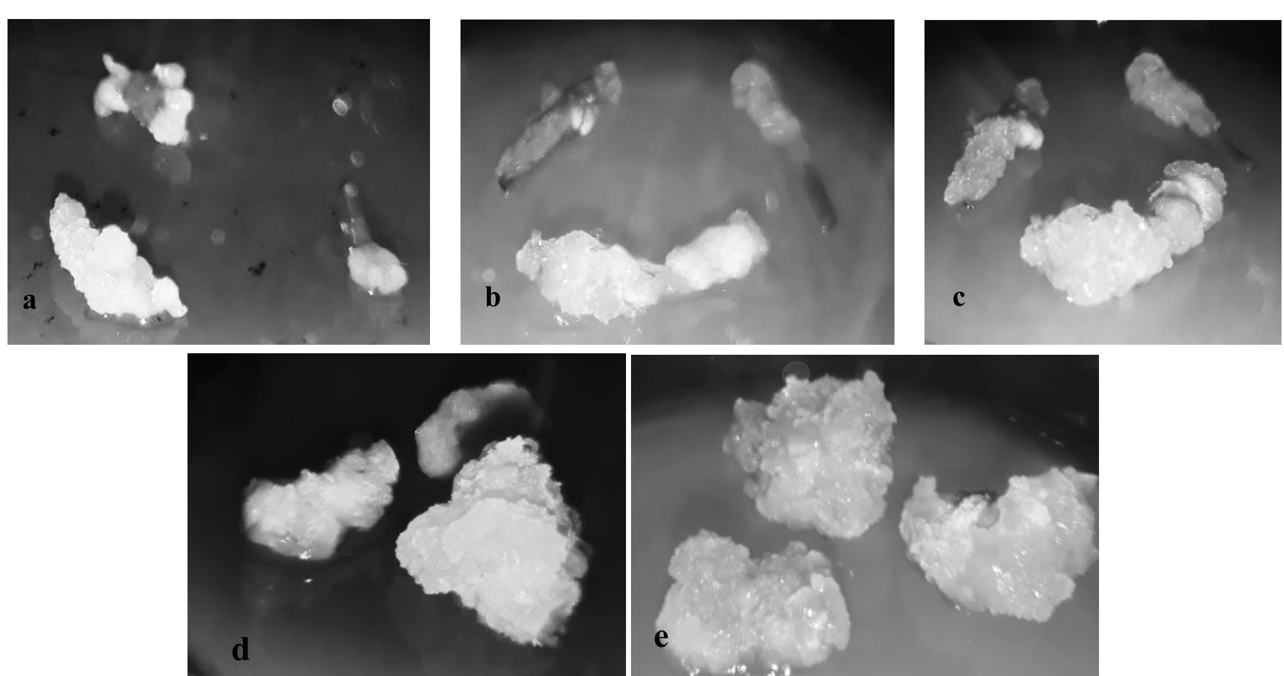


Fig. 2c: Effect of different plant growth hormones on callus formation from internode of *Rauvolfia tetraphylla* L.

Callus formation of internodal explant of *R. tetraphylla* on (a) 15 days of inoculation (b) 4 weeks of culture (c) 6 weeks after culture (d) 8 weeks after inoculation on MS medium with 2.5 mg/L of 2,4-D + BAP (e) 8 weeks after inoculation on MS medium with 2.5 mg/L of IBA +2 ,4-D

Hesami et al, (2018) explored callogenesis in *Ficus religiosa* and investigated the effects of the interaction of PGRs (2,4-D, BAP, IBA, NAA) on MS medium in different explant (leaf, petiole, root, internode). It revealed that a combination of 2,4-D (0.5 mg/L) and BAP (0.05 mg/L) consistently promoted the highest callus across all explants. Nurokhman *et al*. (Nurokhman et al., 2019) investigated callus induction of petiole explants in *Gynura procumbens* at various combinations (2,4-D, IAA, NAA, BAP, KIN) of PGRs and showed maximum callus formation (100%) on MS medium with NAA (0.5 mg/L) and BAP (0.5 mg/L).

Asmono (Asmono, 2020) assessed the internode and leaf explants of *Stevia rebaudiana* Bertoni at different concentrations of BAP (2, 3, and 4 ppm). It revealed that both leaf and internode explants exhibited a remarkable capacity for callus formation,

with a 100% success rate. The synergy between 2,4-D and BAP proved highly effective PGRs in internode of *R. tetraphylla*, enhancing the percentage of callus formation in comparison to alternative combinations.

Callus formation from root explants

The root explants showed best response in MS medium with various concentrations of PGRs. The table 2(d) presents the percentage of callus formation observed in root explants of *R. tetraphylla* when treated with different PGRs. These results shed light on the impact of these hormones on callus induction in root explants (Fig. 2d). When combinations of 2,4-D+BAP (2.5 mg/L) observed maximum 84.11% callus formation compared to other hormones in root explants. Luciani et al, (2006) analysed the effect of PGRs in root-tips of garlic and maximum callus induction (56%) was observed in MS medium with 2,4-D+BAP (0.045 µM). In this study, 84.11% callus formation from root of *R. tetraphylla* were achieved on MS medium with 2,4-D and BAP (2.5mg/L each).

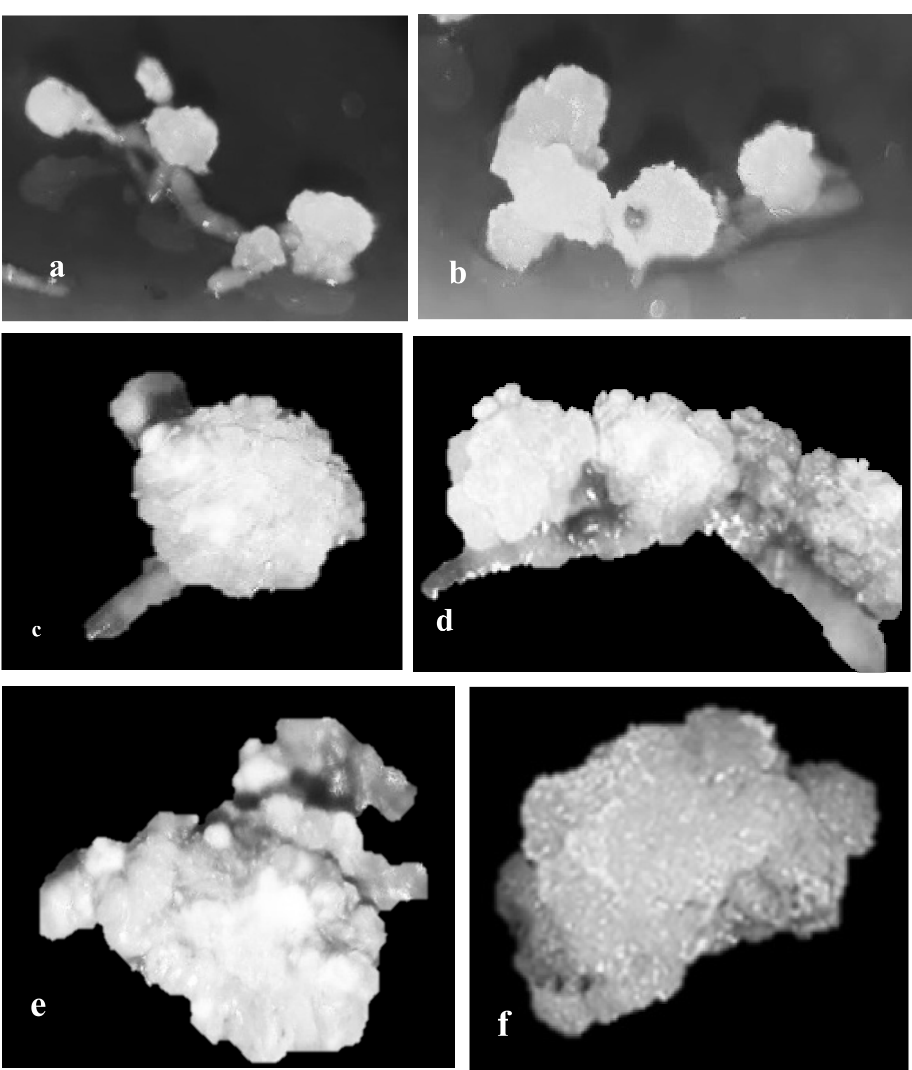


Fig. 2d: Effect of different plant growth hormones on callus formation from Root of *Rauvolfia tetraphylla* L.

Callus formation of root explant on (a) 15 days of inoculation (b) 3 weeks of culture (c) 4 weeks after culture (d) 6 weeks after inoculation (e) 8 weeks after inoculation MS medium with 2.5 mg/L of 2,4-D+BAP (f) 8 weeks after inoculation on MS medium with 2.5 mg/L of BAP

Dar et al, (2021) employed callus cultures from root explants of *Atropa acuminata* at various concentrations of PGRs (BAP, NAA, Kn, and 2,4-D) in MS media and maximum callus development (100%) was achieved in BAP (0.5 mg/L) + NAA (1.0 mg/L). Similarly, Basiri et al, (2022) analysed callus induction of *Eremurus spectabilis* M. Bieb using tuberous root explants. The maximum callus induction 76.67% were achieved on MS medium with BAP (10.0 mg/L). During this study, it found that *R. tetraphylla* root exhibited 51.41% of callus formation when cultured on MS medium with BAP (2.5mg/L).

Direct organogenesis

a. Direct root induction in leaves

The effect of different PGRs on direct root formation in leaf of *R. tetraphylla* were investigated using IAA and a combination of 2,4-D, BAP and IBA (Table 3a and Fig. 3a). The maximum root formation 62.58% was observed in 1.5 mg/L IAA and 0.5 mg/L BAP on MS medium compared to other growth hormone (IAA, 2,4-D+BAP, and IBA+BAP of *R. tetraphylla*.

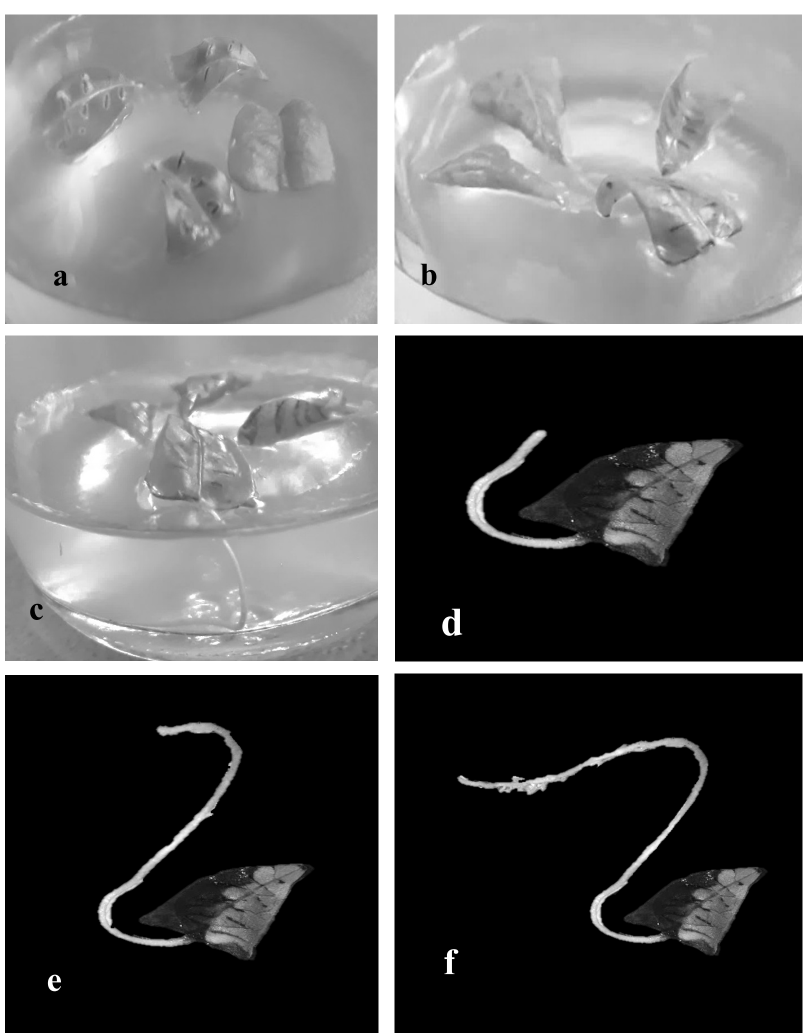


Fig. 3a: Effect of different plant growth hormones on direct root formation from leaf explants of *Rauvolfia tetraphylla* L.

(a) On 7th day of inoculation on leaf explant (b) 15th day after inoculation (c) 3 weeks after culture (d) 4 weeks of inoculation (e) 5 weeks of inoculation (f) 6 weeks of after culture

Similarly, Pandey et al, (2010) evaluated the leaf of *Rauwolfia serpentina* (L.) Benth and inoculated on MS medium supplemented with single and combination PGRs. It revealed that the best combination for inducing roots in *R. serpentina* using the leaf explant was MS media supplemented with para-amino benzoic acid (PABA) 1 mg/L, along with NAA (4 mg/L). However, when the same combination of PABA + NAA and IBA + NAA was used in liquid MS medium observed poor regeneration rate with smaller numbers of roots. In our study, IAA and BAP combinations showed maximum root induction in leaf explant of *R. tetraphylla* compared to other hormones.

Direct root generation in node

The impact of PGRs on direct root formation in node explants of *R. tetraphylla* were analysed using different growth hormone such as IAA, 2,4-D + BAP, IAA+BAP, and IBA + BAP (Table 3b and Fig. 3b). The maximum root formation 83.16% was observed in MS medium with IAA (1.5 mg/L) and BAP (0.5 mg/L) showed promising results of *R. tetraphylla*.

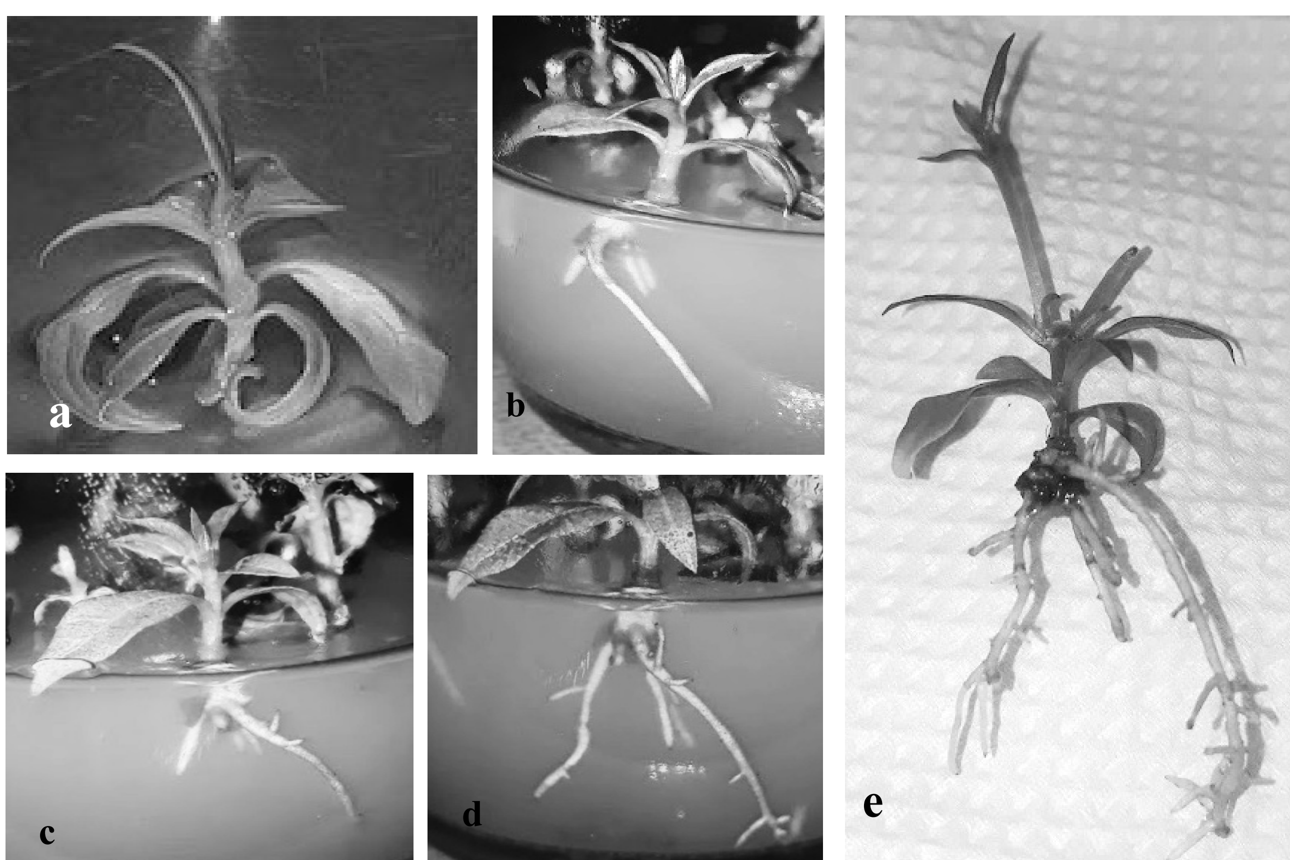


Fig. 3b: Effect of different plant growth hormones on direct root formation from node explants of *Rauvolfia tetraphylla* L.

(a) on the day of inoculation of nodal explant (b) direct root formation in nodal explant after 3 weeks of culture (c) 6 weeks of culture (d) 8 weeks of culture (e) rooting of *in vitro* regenerated shoots in MS medium supplemented with 1.5 mg/L of IBA & 0.5 mg/L of BAP

In the same way (Rout, 2006) focused on induction of rooting from node of *Camellia sinensis* var. TV-20 under optimum condition. The nodal cuttings endured pre-treatment with three different types of auxins (IAA, NAA, and IBA) at various concentration. It was observed that IBA exhibited a positive response of rooting compared to other growth hormone (IAA and NAA). The maximum

root percentage 92.6% was observed at 75 ppm IBA on MS medium after 3 weeks. Furthermore, in our study, a combination of IBA and BAP (2.5 mg/L) demonstrated 63.16% root formation in node explants of *R. tetraphylla*.

Direct shooting and Multiple shoot formation

To investigate the effect of different PGRs on direct shooting and multiple shoot formation in nodal explant of *R. tetraphylla* using BAP in combination with 2,4-D, IAA, and KIN respectively (Table 4 a & b). Results revealed that increasing concentrations of BAP had a significant impact on both direct shooting and multiple shoot formation (Fig. 4 a & b). The combination of IAA and BAP on MS medium established highest shoot formation 83.44% at 1.5 mg/L IAA+BAP. For multiple shoot formation, the maximum percentages 88.44% at 1.5 mg/L IAA+BAP on MS medium of *R. tetraphylla* compared to other plant growth hormone.

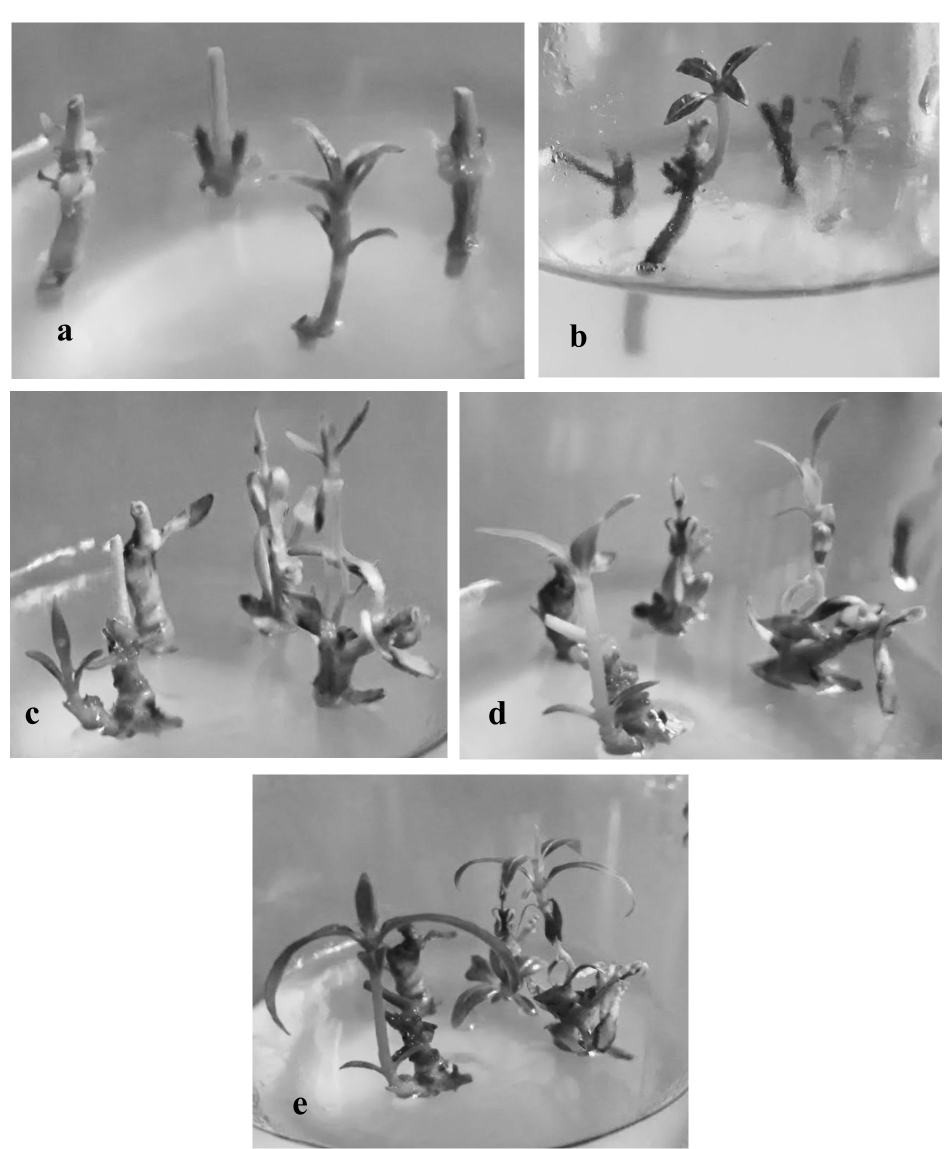
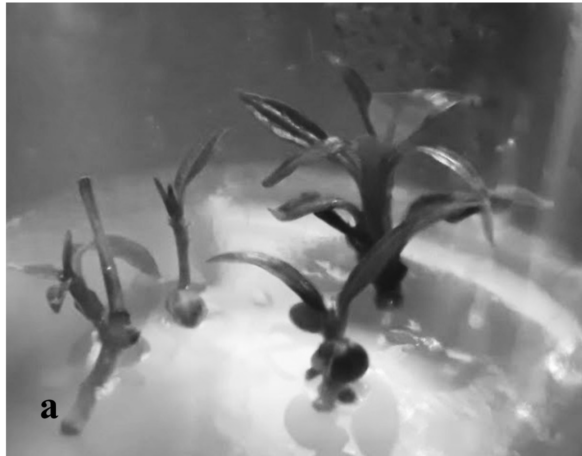


Fig. 4a: Effect of different plant growth hormones on direct shoot induction from nodal explants of *Rauvolfia tetraphylla* L.

Root formation of nodal explant (a) on the of inoculation (b) 3 weeks of culture (c) 4 weeks after culture (d) 5 weeks after inoculation (e) 6 weeks after inoculation



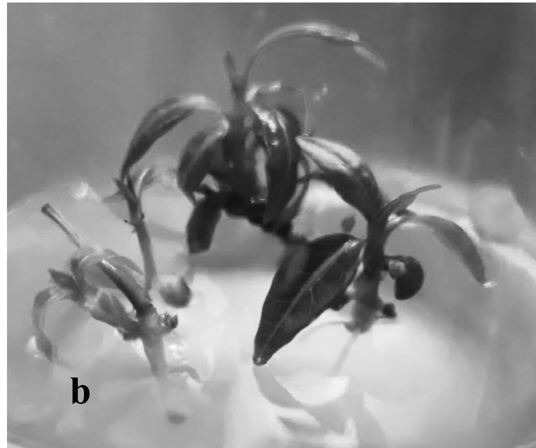


Fig. 4b: Effect of different plant growth hormones on multiple shoot induction from nodal explants of *Rauvolfia tetraphylla* L.

(a) multiple shoot induction from nodal explant cultured on MS medium after 7th day of culture (b) multiple shoot induction from cotyledonary node explant cultured on MS medium supplemented with 2,4-D + BAP after 4 weeks of culture (c) shoot elongation on *in vitro* generated shoot after 6 weeks of culture

Verma et al, (2009) evaluated the regeneration response of cotyledonary node explants obtained from germinating seeds of peanut (*Arachis hypogaea* L.). When MS medium supplemented with BAP at concentrations ranging from 1 to 50 mg/L, multiple shoots were observed to emerge. Among the various concentrations, the highest production of multiple shoots occurred at 15 mg/L in all variety peanut. Specifically, in this study *R. tetraphylla*, 55% of multiple shoots were observed on MS medium with BAP (1.5 mg/L).

Xhulaj and Doriana (Xhulaj, 2019) evaluated multiple shoot regeneration of the *Triticum aestivum* L. from mature embryos without the formation of callus at various combinations of PGRs (2,4-D, IBA, NAA, and BAP). The highest efficiency of direct shoot regeneration was observed on MS medium supplemented with BAP (2 mg/L) and 2,4-D (0.6 mg/L). In this study, *R. tetraphylla* showed the maximum direct shoot (78.44%) and multiple shoot induction (73.44%) was obtained on MS medium combined with BAP+2,4-D (1.5 mg/L).

Somatic Embryogenesis

To assess the impact of PGRs on somatic embryogenesis of *R. tetraphylla*, leaf explants were cultured on MS medium with different PGRs. Preliminary experiments identified 2,4-D and BAP (Fig. 5) as the most effective auxin and cytokinin at an optimal concentration of 2.5 mg/L. Based on this analysis, further experiments were focusing on leaf and root explants, and they were transferred to combination of MS medium containing different concentrations of 2,4-D and BAP (0.5 to 1.5 mg/L).

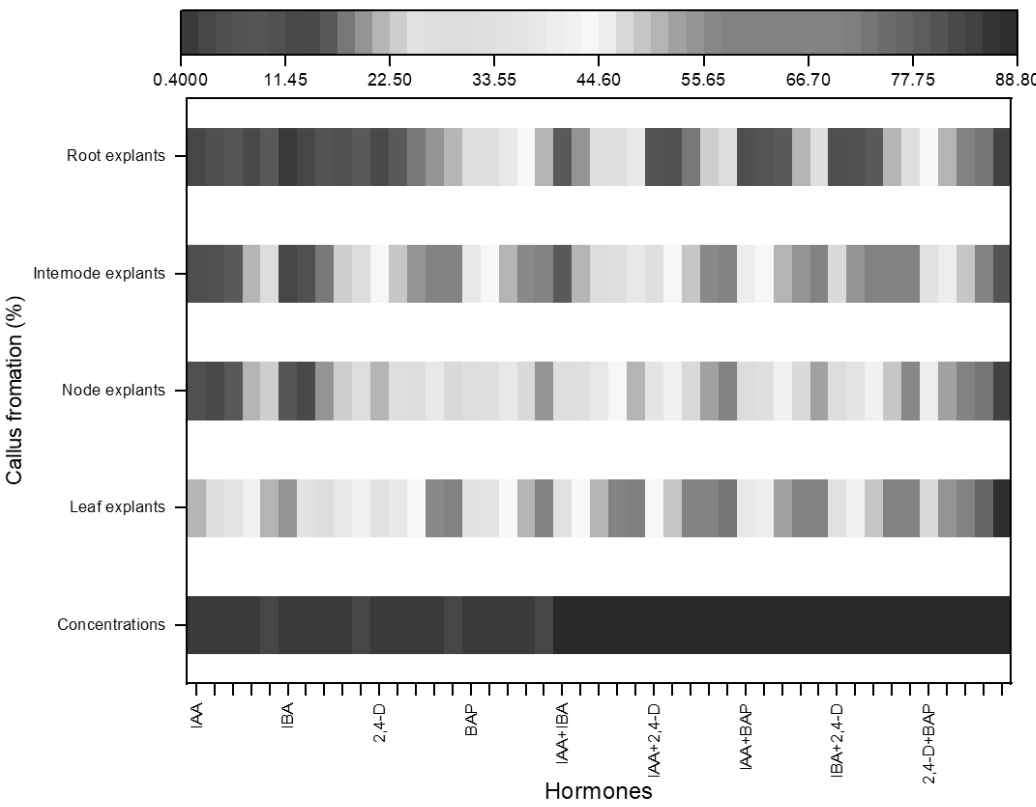
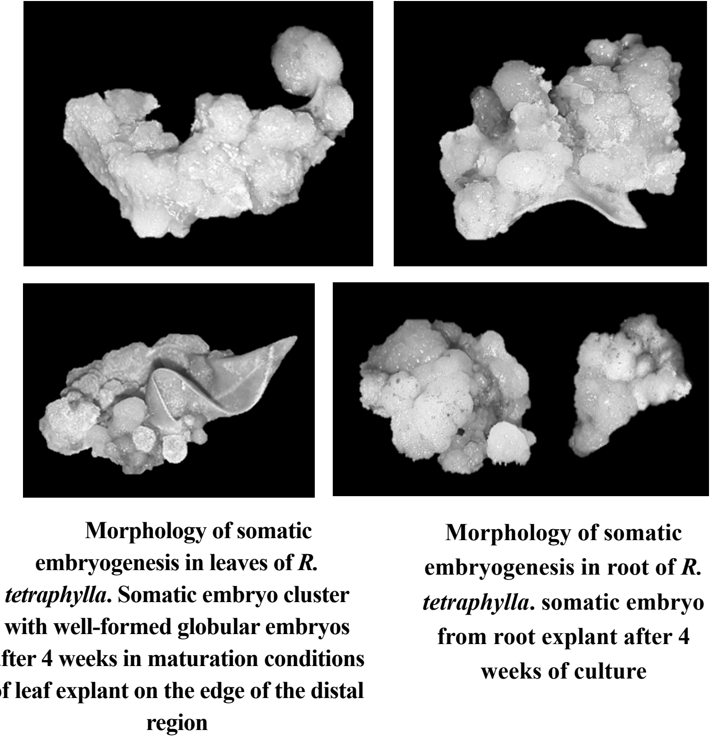


Fig. 5: Heat map of different plant growth hormone on callus formation from leaf, node, internode and root of *Rauvolfia tetraphylla*



Morphology of somatic embryogenesis in leaves of *R. tetraphylla*. Somatic embryo cluster with well-formed globular embryos after 4 weeks in maturation conditions of leaf explant on the edge of the distal region

Morphology of somatic embryogenesis in root of *R. tetraphylla*. somatic embryo from root explant after 4 weeks of culture

Fig. 6: Somatic embryogenesis from leaf and root of *Rauvolfia tetraphylla* L.

After a 4-week culture period, the explants demonstrated the formation of globular stage embryos (Fig. 6). It indicates that the highest embryogenesis was observed on MS medium with BAP+2,4-D (1.5 mg/L). It suggests that these two plant hormones are favourable for the development of somatic embryos through direct somatic embryogenesis. In Gerdakaneh and Mozaffari (Gerdakaneh & Mozaffari, 2021) achieved plant regeneration through direct somatic embryogenesis in strawberry (Fragaria x ananassa Duch.) leaf blades on MS medium supplemented with Thidiazuron (TDZ) at concentrations of 1, 2, 3, and 4 mg/L, both alone and in combination with different concentrations (0, 0.25, 0.5, and 1 mg/L) of 2,4-D. The maximum somatic embryo was obtained on MS medium containing TDZ (3 mg/L) and 2,4-D, (0.25 mg/L) which resulted in the highest induction of embryogenesis.

Chambhare and Nikam (Chambhare & Nikam, 2022) explored the effects of PGRs (BAP, kinetin, IAA, NAA, 2,4-D) on the induction of direct somatic embryogenesis in leaf explant of *Guizotia abyssinica*. It revealed that MS medium containing BAP (1.0 mg/L) along with IAA (0.5 mg/L) observed the maximum somatic embryogenesis. Interestingly, the similar result was observed in this present study, there is no previous report of somatic embryogenesis of *R. tetraphylla*.

Similarly, Pant & Joshi (Pant & Joshi, 2018) analysed somatic embryogenesis of *Rauvolfia serpentina* from nodal explant and maximum organogenesis was obtain on MS medium with NAA and BAP. In the same way, root explant showed maximum embryogenic calli on MS medium with NAA + BAP (4.0 mg/L each) (Afrin et al.). Furthermore, in cotyledon and embryo established embryogenesis on MS medium with 2,4-D (1.0 mg/L) + BAP (0.5 mg/L) (Uikey et al., 2016). The combination of 2,4-D (1.0 mg/L) + BAP (0.5 mg/L) used in *Rauvolfia serpentina* for somatic embryogenesis, the same combination of 2,4-D and BAP exhibited the favourable induction for somatic embryogenesis of *R. tetraphylla*.

Endangered plant species facing threats due to habitat destruction or overexploitation can be rapidly multiplied from a small amount of tissue through micropropagation and preserving their genetic diversity. Similarly, somatic embryogenesis facilitates to propagate large numbers of genetically identical plants from a single parent, aiding in the conservation of unique genotypes and facilitating the propagation of species with low seed viability and producing disease-free plants from sterile tissue cultures. They are essential tools in conserving plant biodiversity and promoting sustainable management of plant resources.

Histology of somatic embryogenesis

Histological analysis revealed the defined cell division process in *R. tetraphylla* leaf explant callus, exhibiting eukaryotic cell division. Initially, cells form with distinct organelles (Fig. 7a) such as the nucleus, nucleolar membrane, and cytoplasm, setting the stage for mitotic division during interphase (Fig. 7b). Early and late prophase follow (Fig. 7c-d), characterized by nucleus replication. In metaphase (Fig. 7e), chromosomes align at the cell's centre, and during anaphase (Fig. 7f), the nucleus divides. Telophase (Fig. 7g) results in the formation of two distinct nucleus envelopes. Cytokinesis (Fig. 7h) completes the process as a cell plate forms in the middle and divides into two daughter cells. The undifferentiated mass of callus cells demonstrates pluripotency and totipotency, potentially developing into various cell types and showing differentiation capabilities. Callus cells, under suitable *in vitro* conditions, contribute to understanding cell differentiation. Subsequent *in vitro* stages reveal different morphogenesis pathways such as organogenesis and somatic embryogenesis, eventually leading to complete plant regeneration in favourable *in vitro* conditions are known as *in vitro* plant regeneration (Kruglova et al., 2023).

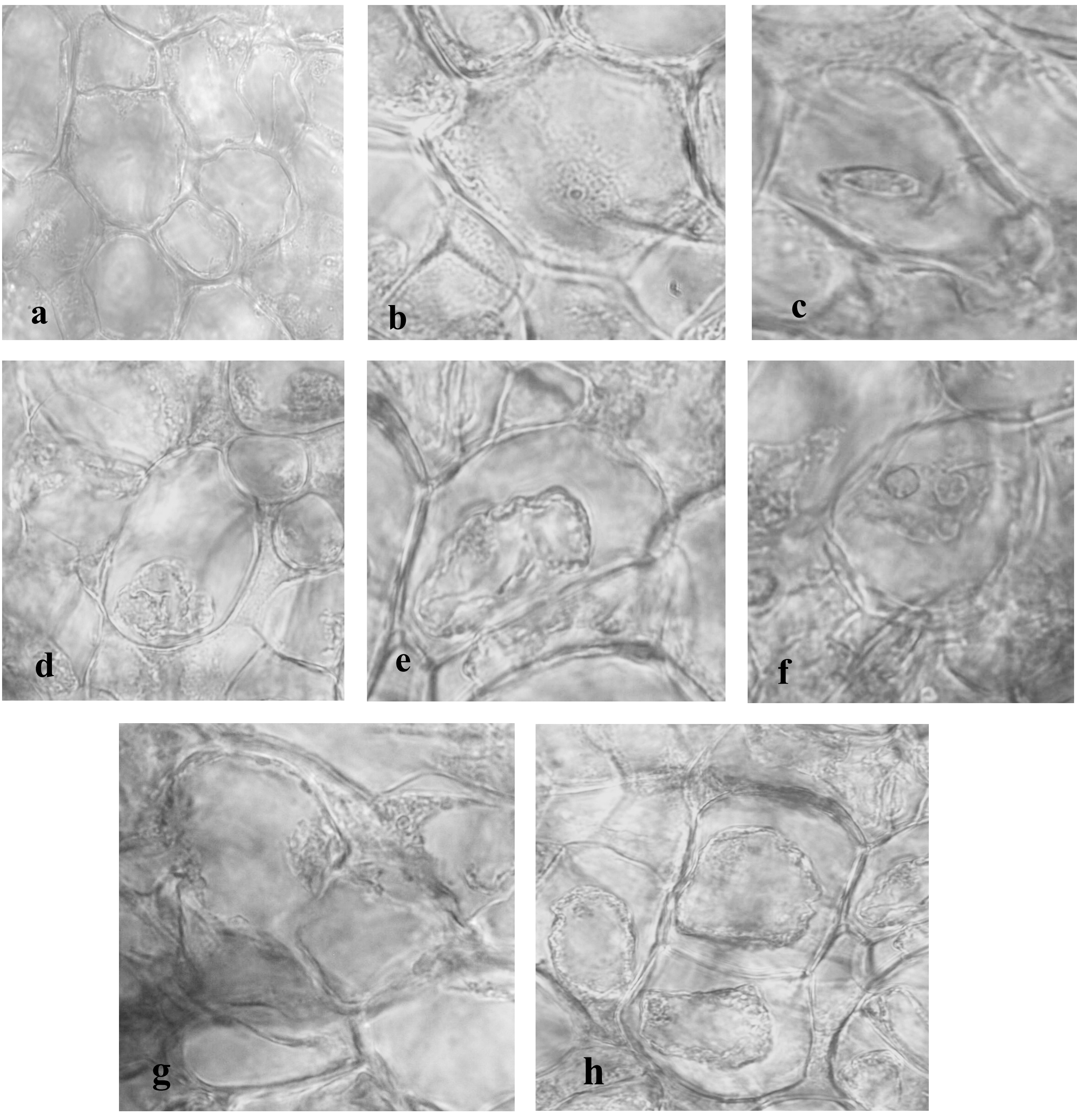


Fig. 7: Histology of cell division

(a) Cell wall; (b) Interphase; (c) Early Prophase; (d) Late Prophase; (e) Metaphase; (f) Anaphase; (g) Telophase; (h) Cytokinesis

Histological analysis during somatic embryogenesis revealed that within four weeks of culturing explants on a callus induction medium, somatic embryo development started at the outer edges of proliferated calli, while non-embryonic cells were found in the inner regions (Fig. 8a). Embryogenic cells at the outer layer were small, isodiametric with dense cytoplasm and a central nucleus while non-embryogenic cells were larger, vacuolated and had nuclei near the cell wall which have no specific structure formation was perceived (Sakr & Sayed, 2018).

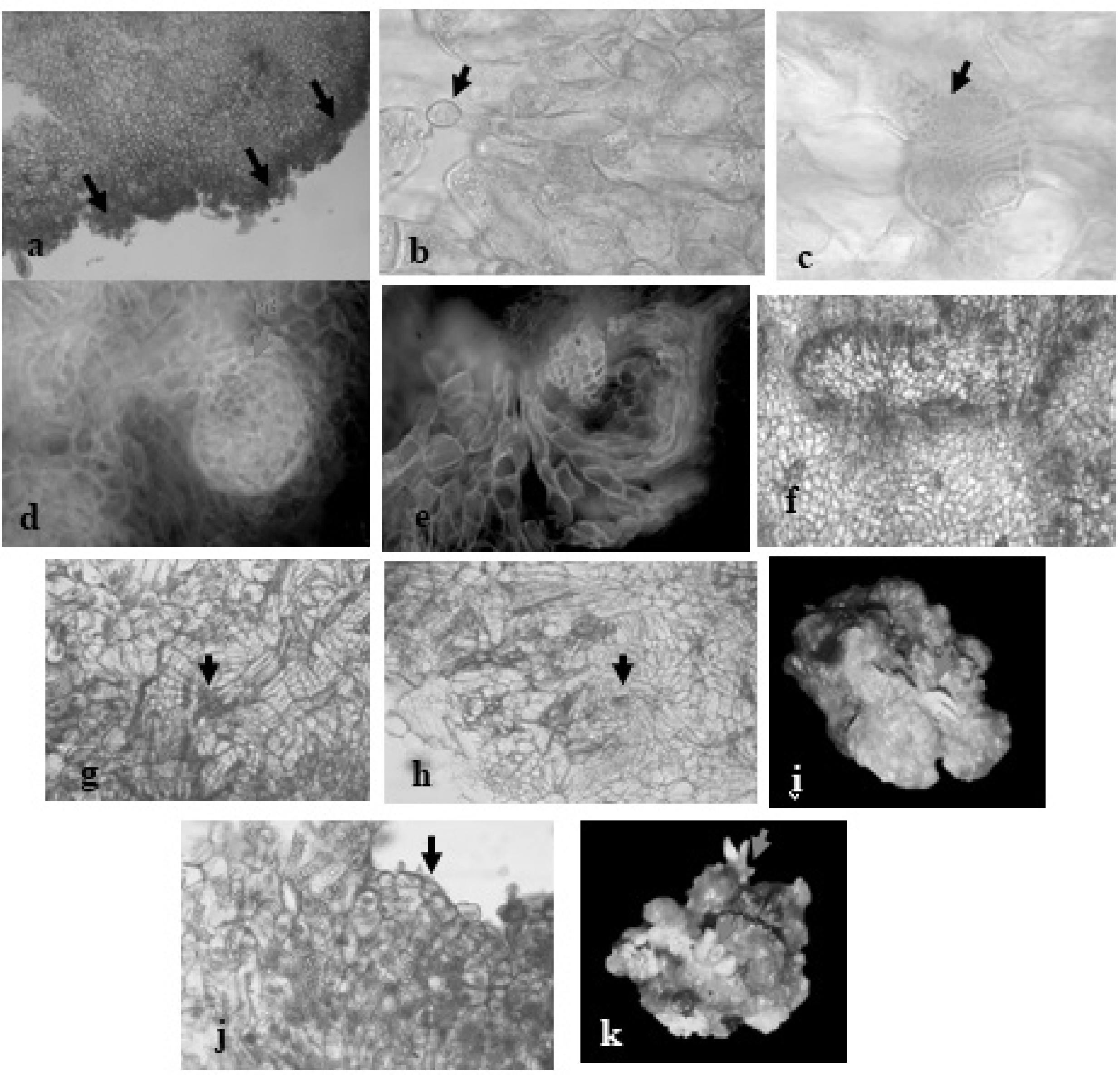


Fig. 8: Histology of somatic embryogenesis of *Rauvolfia tetraphylla* L.

Explant were cultured on MS medium with 2,4-D + BAP and observed histology of somatic embryogenesis for 10, 20, 30, 40, 50, 60 and 75 days (a) culture on 10 days showing Primary callus containing meristematic clusters on the periphery (arrows) (callus distal region) (b) culture of 20 days showing proembryo with suspensor (c) culture on 30 days Proembryo showing multicellular suspensor (d) culture on 40 days showing a well-developed globular somatic embryo in the region of embryogenic callus and protoderm (e) culture on 50 days showing a heart-shaped somatic embryo (f) culture on 60 days showing somatic embryo in torpedo state (g) culture on 75 days showing provascular band (h) root primordia (i) morphology of root organogenesis (j) shoot primordia through meristematic nodules (k) morphology of shoot organogenesis.

Somatic embryo differentiation began with smaller meristematic cells in the primary tissue, characterized by dense cytoplasm and large, round, stained nuclei. Additionally, organized cell divisions led to the presence of a protoderms around the globular embryo (Fig. 8b). In the nodular calli, areas with high mitotic activity and isodiametric cells with prominent nuclei were observed. The

initiation of pro-embryogenesis was linked to the presence of nodular calli, leading to the formation of induced pro-embryogenic masses (PEMs). Globular somatic embryos (gSEs) arising from PEMs showed radial development, with differentiation of primary meristematic tissues including the fundamental meristems and the suspensor (Fig. 8c). Subsequent embryogenic stages saw a reduction in suspensor mass. During the development of embryogenic calli (EC), the transition from globular to torpedo-shaped embryos occurred gradually, featuring a distinct polar structure (Fig. 8d-f). In longitudinal sections of embryos at the early differentiation stage, vascular tissue (vt) (Fig. 8g), root primordia (rp) (Fig. 8h-i) and shoot primordia (sp) (Fig. 8j-k) were clearly visible.

Similarly, Panggabean et al, (2022) found that embryogenic callus retains its nodular structure due to active meristematic cell division. Sample preparation involved dehydration, FAA fixation, paraffin infusion and staining with safranin dye. It developed epidermal tissue from the protoderm, originating through a periclinal cleavage during the proembryonic-to-globular transition. This method supports the proliferation of the original cell mass, giving rise to new calli lineages, especially the embryogenic yellowish nodular lineage, from which somatic embryos develop.

CONCLUSION

The study focused on enhancing germination rates of *R. tetraphylla* seeds by addressing seed coat impermeability through surface sterilization, cold treatment, and partial seed coat removal. It underscored the significance of overcoming this barrier to facilitate seedling development. In tissue culture, various plant growth regulators influenced callus formation on leaf, node, internode and root, with hormone combinations impacting shoot formation, notably BAP, 2,4-D+BAP, IAA+BAP, and KIN+BAP. Optimal hormone concentrations played a crucial role in promoting root and shoot formation. Preliminary experiments identified 2,4-D and BAP as effective auxin and cytokinin at 2.5 mg/L on MS medium. Further experiments revealed a synergistic effect conducive to somatic embryo development on MS medium with BAP (1.5 mg/L) and 2,4-D (1.5 mg/L). Histological analysis provided insights into cellular processes during somatic embryogenesis, emphasise the differentiation of somatic embryos and the development of vascular tissue, root and shoot primordia. Therefore, Plant tissue culture is an advanced technique for *in vitro* plant regeneration from endangered medicinal of *R. tetraphylla*. In future, *Rauvolfia tetraphylla* will be investigated for advanced physiological responses by testing with different plant growth regulators.

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Data Availability

The data used to support the findings of this study are incorporated within the article and can be liberally available to authors with suitable reference in their research work.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding this research study.

Source of Support

There is no funding for this research.

Table 1a: Effect of Surface Sterilization of *Rauvolfia tetraphylla* L. Seeds with Sodium Hypochlorite (1%) and Mercuric Chloride (0.1%)

S. No.	$NaHCl_3$/Min	$HgCl_2$/Min	Percentage of Sterile Plantlets
1	10	1	84.11 ± 0.59
2	15	2	91.55 ± 0.36
3	20	3	98.44 ± 0.37

Table 1b: Effect of germination of *Rauvolfia tetraphylla* L. seeds

S. No	No. of Seed tested	No. of seed germination	Percentage of germination	Mean ± SD
1	control	0	0	0
2	25	22	88	83.33±4.74
3	20	15	75	
4	30	25	83.33	

Table 2a: Effect of Different Plant Growth Hormones on Callus Formation from Leaf of *Rauvolfia tetraphylla* L.

Hormones	Concentrations (mg/L)	Callus formation (%) Leaf explants	SED	CD
IAA	0.5	22.40 ± 0.39[a]	0.4323	CD (P < 0.05) = 0.9633**
	1.0	28.62 ± 0.41[b]		
	1.5	35.51 ± 0.34[c]		
	2.0	42.40 ± 0.39[d]		
	2.5	**51.37** ± 0.42[e]		
IBA	0.5	19.86 ± 0.40[a]	0.4526	CD (P < 0.05) = 1.0084**
	1.0	25.75 ± 0.50[b]		
	1.5	30.58 ± 0.38[c]		
	2.0	37.41 ± 0.38[d]		
	2.5	**41.93** ± 0.25[e]		
2,4-D	0.5	34.48 ± 0.34[a]	0.6055	CD (P < 0.05) = 1.3491**
	1.0	38.62 ± 0.41[b]		
	1.5	44.00 ± 0.66[c]		
	2.0	55.73 ± 0.61[d]		
	2.5	**67.22** ± 0.52[e]		
BAP	0.5	25.51 ± 0.34[a]	0.5028	CD (P < 0.05) = 1.1202**
	1.0	36.83 ± 0.55[b]		
	1.5	43.58 ± 0.38[c]		
	2.0	50.20 ± 0.53[d]		
	2.5	**58.58** ± 0.38[e]		
IAA+IBA	0.5+0.5	35.08 ± 0.44[a]	0.4387	CD (P < 0.05) = 0.9775**
	1.0+1.0	43.44 ± 0.37[b]		
	1.5+1.5	51.55 ± 0.36[c]		
	2.0+2.0	59.58 ± 0.38[d]		
	2.5+2.5	**71.55** ± 0.36[e]		
IAA+2,4-D	0.5+0.5	44.46 ± 0.35[a]	0.4972	CD (P < 0.05) = 1.1079**
	1.0+1.0	50.08 ± 0.61[b]		
	1.5+1.5	61.37 ± 0.42[c]		
	2.0+2.0	66.53 ± 0.35[d]		
	2.5+2.5	**72.40** ± 0.39[e]		
IAA+BAP	0.5+0.5	38.62 ± 0.41[a]	0.4255	CD (P < 0.05) = 0.9481**
	1.0+1.0	45.51 ± 0.34[b]		
	1.5+1.5	52.40 ± 0.39[c]		
	2.0+2.0	57.59 ± 0.39[d]		
	2.5+2.5	**66.55** ± 0.36[e]		

Hormones	Concentrations (mg/L)	Callus formation (%) Leaf explants	SED	CD
IBA+2,4-D	0.5+0.5	33.76 ± 0.82^{a}	1.1452	CD (P < 0.05) = 2.5518**
	1.0+1.0	41.63 ± 1.11^{b}		
	1.5+1.5	49.88 ± 1.25^{c}		
	2.0+2.0	58.77 ± 0.81^{d}		
	2.5+2.5	**66.99** ± 0.88^{e}		
2,4-D+BAP	0.5+0.5	46.55 ± 0.36^{a}	0.4666	CD (P < 0.05) = 1.0396**
	1.0+1.0	55.51 ± 0.34^{b}		
	1.5+1.5	67.59 ± 0.39^{c}		
	2.0+2.0	76.18 ± 0.54^{d}		
	2.5+2.5	**88.62** ± 0.4^{1}e		

Values represent mean ± standard deviation of three replicates per treatment. Group e has the best treatment compared to other treatments. Means with columns with same letter are not significantly (*P* < 0.05) difference according to Duncan's Multiple Range Test

** Significant

Table 2b: Effect of different plant growth hormones on callus formation from node of *Rauvolfia tetraphylla* L.

Hormones	Concentrations (mg/L)	Callus formation (%) Node explants	SED	CD
IAA	0.5	11.37 ± 0.42^{a}	0.4275	CD (P < 0.05) = 0.9526**
	1.0	14.48 ± 0.34^{b}		
	1.5	16.55 ± 0.36^{c}		
	2.0	21.37 ± 0.42^{d}		
	2.5	**23.44** ± 0.37^{e}		
	0.5	08.44 ± 0.37^{a}	0.4663	CD (P < 0.05) = 1.0390**
	1.0	13.45 ± 0.36^{b}		
	1.5	20.16 ± 0.55^{c}		
	2.0	23.44 ± 0.37^{d}		
	2.5	**26.58** ± 0.38^{e}		
2,4-D	0.5	22.40 ± 0.39^{a}	0.4308	CD (P < 0.05) = 0.9599**
	1.0	26.55 ± 0.36^{b}		
	1.5	31.37 ± 0.42^{c}		
	2.0	38.62 ± 0.41^{d}		
	2.5	**46.55** ± 0.36^{e}		
BAP	0.5	26.55 ± 0.36^{a}	0.4186	CD (P < 0.05) = 0.9327**
	1.0	33.44 ± 0.37^{b}		
	1.5	37.59 ± 0.39^{c}		
	2.0	47.58 ± 0.38^{d}		
	2.5	**55.51** ± 0.34^{e}		

Hormones	Concentrations (mg/L)	Callus formation (%) Node explants	SED	CD
IAA+IBA	0.5+0.5	27.59 ± 0.39^{a}	0.4246	CD (P < 0.05) = 0.9460**
	1.0+1.0	33.45 ± 0.36^{b}		
	1.5+1.5	38.62 ± 0.41^{c}		
	2.0+2.0	44.48 ± 0.34^{d}		
	2.5+2.5	**51.41** ± 0.38^{e}		
IAA+2,4-D	0.5+0.5	35.51 ± 0.34^{a}	0.4189	CD (P < 0.05) = 0.9333**
	1.0+1.0	42.40 ± 0.39^{b}		
	1.5+1.5	46.55 ± 0.36^{c}		
	2.0+2.0	53.44 ± 0.37^{d}		
	2.5+2.5	**62.41** ± 0.38^{e}		
IAA+BAP	0.5+0.5	28.62 ± 0.41^{a}	0.4302	CD (P < 0.05) = 0.9586**
	1.0+1.0	33.45 ± 0.36^{b}		
	1.5+1.5	41.38 ± 0.41^{c}		
	2.0+2.0	46.58 ± 0.38^{d}		
	2.5+2.5	**53.41** ± 0.38^{e}		
IBA+2,4-D	0.5+0.5	30.58 ± 0.38^{a}	0.4197	CD (P < 0.05) = 0.9351**
	1.0+1.0	35.51 ± 0.34^{b}		
	1.5+1.5	42.50 ± 0.33^{c}		
	2.0+2.0	48.61 ± 0.41^{d}		
	2.5+2.5	**56.55** ± 0.36^{e}		
2,4-D+BAP	0.5+0.5	45.51 ± 0.34^{a}	0.4234	CD (P < 0.05) = 0.9435**
	1.0+1.0	53.44 ± 0.37^{b}		
	1.5+1.5	61.37 ± 0.42^{c}		
	2.0+2.0	72.40 ± 0.39^{d}		
	2.5+2.5	**83.45** ± 0.36^{e}		

Values represent mean ± standard deviation of three replicates per treatment. Group e has the best treatment compared to other treatments. Means with columns with same letter are not significantly (*P* < 0.05) difference according to Duncan's Multiple Range Test

** Significant

Table 2c: Effect of Different Plant Growth Hormones on Callus Formation from Internode of *Rauvolfia tetraphylla* L.

Hormones	Concentrations (mg/L)	Callus formation (%) Internode explants	SED	CD
IAA	0.5	05.51 ± 0.34^{a}	0.4051	CD (P < 0.05) = 0.9027**
	1.0	11.37 ± 0.42^{b}		
	1.5	16.55 ± 0.36^{c}		
	2.0	22.40 ± 0.39^{d}		
	2.5	**28.08** ± 0.27^{e}		
IBA	0.5	03.94 ± 0.40^{a}	0.4020	CD (P < 0.05) = 0.8957**
	1.0	10.50 ± 0.33^{b}		
	1.5	17.59 ± 0.39^{c}		
	2.0	23.44 ± 0.37^{d}		
	2.5	**30.16** ± 0.22^{e}		

Hormones	Concentrations (mg/L)	Callus formation (%) Internode explants	SED	CD
2,4-D	0.5	43.44 ± 0.37[a]	0.4854	CD (P < 0.05) = 1.0815**
	1.0	48.62 ± 0.41[b]		
	1.5	54.48 ± 0.34[c]		
	2.0	59.33 ± 0.44[d]		
	2.5	**65.14** ± 0.56[e]		
BAP	0.5	38.46 ± 0.30[a]	0.4051	CD (P < 0.05) = 0.9026**
	1.0	44.48 ± 0.34[b]		
	1.5	50.50 ± 0.33[c]		
	2.0	56.55 ± 0.36[d]		
	2.5	**62.40** ± 0.39[e]		
IAA+IBA	0.5+0.5	16.55 ± 0.36[a]	0.4254	CD (P < 0.05) = 0.9478**
	1.0+1.0	22.40 ± 0.39[b]		
	1.5+1.5	28.62 ± 0.41[c]		
	2.0+2.0	32.50 ± 0.33[d]		
	2.5+2.5	**37.59** ± 0.39[e]		
IAA+2,4-D	0.5+0.5	33.44 ± 0.37[a]	0.4206	CD (P < 0.05) = 0.9372**
	1.0+1.0	43.44 ± 0.37[b]		
	1.5+1.5	48.62 ± 0.41[c]		
	2.0+2.0	56.55 ± 0.36[d]		
	2.5+2.5	**66.51** ± 0.34[e]		
IAA+BAP	0.5+0.5	40.70 ± 0.46[a]	0.4547	CD (P < 0.05) = 1.0132**
	1.0+1.0	44.48 ± 0.34[b]		
	1.5+1.5	50.70 ± 0.46[c]		
	2.0+2.0	55.51 ± 0.34[d]		
	2.5+2.5	**61.37** ± 0.42[e]		
IBA+2,4-D	0.5+0.5	47.59 ± 0.39[a]	0.4249	CD (P < 0.05) = 0.9468**
	1.0+1.0	54.48 ± 0.34[b]		
	1.5+1.5	58.62 ± 0.41[c]		
	2.0+2.0	63.44 ± 0.37[d]		
	2.5+2.5	**68.41** ± 0.38[e]		
2,4-D+BAP	0.5+0.5	33.81 ± 0.54[a]	0.5106	CD (P < 0.05) = 1.1378**
	1.0+1.0	40.41 ± 0.38[b]		
	1.5+1.5	49.50 ± 0.33[c]		
	2.0+2.0	61.37 ± 0.42[d]		
	2.5+2.5	**79.66** ± 0.55[e]		

Values represent mean ± standard deviation of three replicates per treatment. Group e has the best treatment compared to other treatments. Means with columns with same letter are not significantly ($P < 0.05$) difference according to Duncan's Multiple Range Test

** Significant

Table 2d: Effect of Different Plant Growth Hormones on Callus Formation from Root of *Rauvolfia tetraphylla* L.

Hormones	Concentrations (mg/L)	Callus formation (%) Root explants	SED	CD
	0.5	02.40 ± 0.39^{a}		
	1.0	04.48 ± 0.34^{b}		
IAA	1.5	08.62 ± 0.41^{c}	0.4230	CD (P < 0.05) = 0.9426**
	2.0	13.44 ± 0.37^{d}		
	2.5	**16.55** ± 0.36^{e}		
	0.5	01.45 ± 0.36^{a}		
	1.0	03.51 ± 0.34^{b}		
IBA	1.5	07.58 ± 0.38^{c}	0.4231	CD (P < 0.05) = 0.9427**
	2.0	11.38 ± 0.41^{d}		
	2.5	**15.58** ± 0.39^{e}		
	0.5	13.44 ± 0.37^{a}		
	1.0	15.51 ± 0.34^{b}		
2,4-D	1.5	17.59 ± 0.39^{c}	0.4172	CD (P < 0.05) = 0.9296**
	2.0	20.50 ± 0.33^{d}		
	2.5	**22.41** ± 0.38^{e}		
	0.5	27.58 ± 0.38^{a}		
	1.0	33.45 ± 0.36^{b}		
BAP	1.5	38.62 ± 0.41^{c}	0.4240	CD (P < 0.05) = 0.9447**
	2.0	44.48 ± 0.34^{d}		
	2.5	**51.41** ± 0.38^{e}		
	0.5+0.5	15.51 ± 0.34^{a}		
	1.0+1.0	20.41 ± 0.38^{b}		
IAA+IBA	1.5+1.5	27.59 ± 0.39^{c}	0.4249	CD (P < 0.05) = 0.9466**
	2.0+2.0	33.44 ± 0.37^{d}		
	2.5+2.5	**38.62** ± 0.41^{e}		
	0.5+0.5	07.22 ± 0.52^{a}		
	1.0+1.0	11.00 ± 0.66^{b}		
IAA+2,4-D	1.5+1.5	17.59 ± 0.39^{c}	0.5500	CD (P < 0.05) = 1.2256**
	2.0+2.0	23.44 ± 0.37^{d}		
	2.5+2.5	**30.50** ± 0.33^{e}		
	0.5+0.5	04.48 ± 0.34^{a}		
	1.0+1.0	08.62 ± 0.41^{b}		
IAA+BAP	1.5+1.5	15.51 ± 0.34^{c}	0.5244	CD (P < 0.05) = 1.1685**
	2.0+2.0	21.03 ± 0.69^{d}		
	2.5+2.5	**26.55** ± 0.36^{e}		
	0.5+0.5	05.51 ± 0.34^{a}		
	1.0+1.0	11.38 ± 0.41^{b}		
IBA+2,4-D	1.5+1.5	16.55 ± 0.36^{c}	0.4985	CD (P < 0.05) = 1.1108**
	2.0+2.0	22.40 ± 0.39^{d}		
	2.5+2.5	**27.88** ±0.88^{e}		

Hormones	Concentrations (mg/L)	Callus formation (%) Root explants	SED	CD
2,4-D+BAP	0.5+0.5	44.48 ± 0.34[a]	0.4906	CD (P < 0.05) = 1.0930**
	1.0+1.0	50.41 ± 0.38[b]		
	1.5+1.5	61.37 ± 0.42[c]		
	2.0+2.0	72.40 ± 0.39[d]		
	2.5+2.5	**84.11** ± 0.59[e]		

Values represent mean ± standard deviation of three replicates per treatment. Group e has the best treatment compared to other treatments. Means with columns with same letter are not significantly ($P < 0.05$) difference according to Duncan's Multiple Range Test

** Significant

Table 3a: Effect of Different Plant Growth Hormones on Direct Root Formation from Leaf Explants of *Rauvolfia tetraphylla* L.

Hormones	Concentrations (mg/L)	Direct Root formation (%)		
		Leaf explants		
			SED	CD
IAA	0.5	03.44 ± 0.37	0.4132	CD (P < 0.05) = 1.0110
	1	09.50 ± 0.33		
	1.5	**16.55** ± 0.36		
2,4-D+BAP	0.5+0.5	13.44 ± 0.37	0.5808	CD (P < 0.05) = 1.4213
	1.0+0.5	20.00 ± 0.66		
	1.5+0.5	**26.55** ± 0.36		
IAA+BAP	0.5+0.5	37.44 ± 0.37	0.4165	CD (P < 0.05) = 1.0191
	1.0+0.5	62.58 ± 0.38		
	1.5+0.5	**87.50** ± 0.33		
IBA+BAP	0.5+0.5	13.16 ± 0.55	0.5092	CD (P < 0.05) = 1.2460
	1.0+0.5	25.66 ± 0.44		
	1.5+0.5	**35.50** ± 0.33		

Values represent mean ± standard deviation of three replicates per treatment. Group e has the best treatment compared to other treatments. Means with columns with same letter are not significantly ($P < 0.05$) difference according to Duncan's Multiple Range Test

** Significant

Table 3b: Effect of Different Plant Growth Hormones on Direct Root Formation from Node Explants of *Rauvolfia tetraphylla* L.

Hormones	Concentrations (mg/L)	Direct Root formation (%)		
		Node explants		
		Mean ± SD	SED	CD
IAA	0.5	02.50 ± 0.33[a]	0.5971	CD (P < 0.05) = 1.4610
	1	12.83 ± 0.77[b]		
	1.5	**37.58** ± 0.38[c]		
2,4-D+BAP	0.5+0.5	33.77 ± 0.81[a]	0.7644	CD (P < 0.05) = 1.8705
	1.0+0.5	50.00 ± 0.66[b]		
	1.5+0.5	**62.77** ± 0.51[c]		

Hormones	Concentrations (mg/L)	Direct Root formation (%)		
		Node explants		
		Mean ± SD	SED	CD
IAA+BAP	0.5+0.5	24.00 ± 0.66[a]	0.6383	CD (P < 0.05) = 1.5619
	1.0+0.5	52.50 ± 0.33[b]		
	1.5+0.5	**83.16** ± 0.55[c]		
IBA+BAP	0.5+0.5	32.43 ± 0.95[a]	0.7504	CD (P < 0.05) = 1.8363
	1.0+0.5	47.50 ± 0.33[b]		
	1.5+0.5	**63.16** ± 0.55[c]		

Values represent mean ± standard deviation of three replicates per treatment. Group c has the best treatment compared to other treatments. Means with columns with same letter are not significantly ($P < 0.05$) difference according to Duncan's Multiple Range Test

** Significant

Table 4a: Effect of Different Plant Growth Hormones on Direct Shoot Induction from Nodal Explants of *Rauvolfia tetraphylla* L.

Hormones	Concentrations (mg/L)	Node		
		Direct shooting (%)		
		Mean ± SD	SED	CD
BAP	0.5	25.50 ± 0.33[a]	0.4132	CD (P < 0.05) =1.0110**
	1	41.55 ± 0.36[b]		
	1.5	**58.44** ± 0.37[c]		
2,4-D+BAP	0.5+0.5	42.40 ± 0.49[a]	0.4703	CD (P < 0.05) = 1.1509**
	1.0+1.0	58.41 ± 0.38[b]		
	1.5+1.5	**78.44** ± 0.37[c]		
IAA+BAP	0.5+0.5	46.55 ± 0.36[a]	0.4735	CD (P < 0.05) = 1.1587**
	1.0+1.0	67.21 ± 0.52[b]		
	1.5+1.5	**83.44** ± 0.37[c]		
KIN+BAP	0.5+0.5	38.44 ± 0.37[a]	0.7165	CD (P < 0.05) = 1.7534**
	1.0+1.0	54.01 ± 0.68[b]		
	1.5+1.5	**75.00** ± 0.66[c]		

Values represent mean ± standard deviation of three replicates per treatment. Group c has the best treatment compared to other treatments. Means with columns with same letter are not significantly ($P < 0.05$) difference according to Duncan's Multiple Range Test

** Significant

Table 4b: Effect of Different Plant Growth Hormones on Multiple Shoot Induction from Nodal Explants of *Rauvolfia tetraphylla* L.

Hormones	Concentrations (mg/L)	Node		
		Multiple shoots (%)		
		Mean ± SD	SED	CD
BAP	0.5	21.55 ± 0.36[a]	0.5808	CD (P < 0.05) = 1.4213**
	1	38.44 ± 0.37[c]		
	1.5	**55.00** ± 0.66[c]		

Hormones	Concentrations (mg/L)	Node		
		Multiple shoots (%)		
		Mean ± SD	SED	CD
2,4-D+BAP	0.5+0.5	41.00 ± 0.66[a]	0.5808	CD (P < 0.05) = 1.4213**
	1.0+1.0	56.55 ± 0.36[b]		
	1.5+1.5	**73.44** ± 0.37[c]		
IAA+BAP	0.5+0.5	55.00 ± 0.66[a]	0.6227	CD (P < 0.05) = 1.5237**
	1.0+1.0	72.22 ± 0.52[b]		
	1.5+1.5	**88.44** ± 0.37[c]		
KIN+BAP	0.5+0.5	43.44 ± 0.37[a]	0.5808	CD (P < 0.05) = 1.4213**
	1.0+1.0	61.00 ± 0.66[b]		
	1.5+1.5	**76.55** ± 0.36[c]		

Values represent mean ± standard deviation of three replicates per treatment. Group c has the best treatment compared to other treatments. Means with columns with same letter are not significantly ($P < 0.05$) difference according to Duncan's Multiple Range Test

** Significant

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Full Length Article

Functional characterization of promiscuous tryptophan decarboxylase from indole alkaloids producing *Rauvolfia tetraphylla* L.

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ABSTRACT

The enzyme Tryptophan decarboxylase (TDC, EC 4.1.1.28) gene facilitates the conversion of tryptophan to tryptamine. A new gene encoding TDC was identified from the alkaloid producing plant *Rauvolfia tetraphylla* by transcriptome analysis, termed as RtTDC. It contains 1,500 base pair which encodes an open reading frame for 499-amino-acid polypeptide with molecular mass of 55729.29 kDa and isoelectric point of 5.37. Multiple sequence alignment and phylogenetic tree analysis showed the closest similarity (95.3 %) with the TDC from the *Rauvolfia verticillata.* This enzyme has property of recombinant tryptophan decarboxylase from *R. tetraphylla* was characterized. The potential activity of tryptophan decarboxylase specific to L-tryptophan may contribute to the biosynthesis of indole alkaloids in *R. tetraphylla*. The finding of tryptophan metabolites in *R. tetraphylla* plants is a novel report, lead to hypothesize the existence of TDC enzymatic activity, from which aromatic amino acid decarboxylases is formed. These results support the *in-silico* annotation of the examined protein sequences of *R. tetraphylla* as TDC and suggest the involvement of TDC enzymatic activity in this plant. Molecular modeling of the TDC gene evidencing the reliability, stability and the structural similarities of the *R. tetraphylla* TDC gene with *R. verticillata* TDC gene. The L-tryptophan used as ligand in docking analysis to verify the TDC gene enzymatic activity for synthesis of Indole alkaloids. High performance liquid chromatography data analyses of RtTDC catalyzed reaction mixture confirmed the catalytically decarboxylative activity of RtTDC.

1. Introduction

The plant *Rauvolfia tetraphylla* L. belongs to the family Apocynaceae, commonly known as 'Devil Pepper' and 'Be Still Tree' has interest because of its capacity to produce compounds with medicinal properties. *R. tetraphylla* has been traditionally used for medicinal purposes in various regions of India and it is sometimes utilized as a replacement for *R. serpentine* (Mahalakshmi et al., 2019). The Malaraya tribes of Tamil Nadu use a decoction of the bark of *R. tetraphylla* as an external application to treat chronic cutaneous diseases and eliminate parasites. Moreover, an extract of this plant is blended with castor oil to form a liniment, which is recommended for specific types of chronic and refractory skin ailments (Vanjari et al., 2022).

A wide range of alkaloids with modern biological effects are still being discovered and studied. Currently, around 60 alkaloids from plants have been approved as drugs in different countries (Cordell et al., 2001). Indole alkaloids are a group of alkaloids that contain an indole structure. Numerous compounds were identified as alkaloids, which contain isoprene groups and known as terpene indole or secologanin tryptamine alkaloid. Indole alkaloids are a major class of alkaloids found in several important plant groups, particularly in the *Catharanthus* and *Rauvolfia* plants from the Apocynaceae family. These plants have been scientifically studied for their pharmacological properties, with some undergoing clinical trials and others already approved for medicinal usage (Murugesan & Kaliappan, 2023).

The biosynthesis of indole alkaloids, which are the secondary plant metabolites primarily accumulated in plants, is mainly regulated by the enzyme tryptophan decarboxylase (Jeevanandham et al., 2022).

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Rauvolfia species are capable of synthesizing secondary metabolites, known as monoterpenoid indole alkaloids (MIAs), which exhibit remarkable biological activities. Indole alkaloids are a diverse class of plant metabolites that have various biological activities, such as anti-cancer, anti-tumour, anti-microbial, anti-inflammatory, and anti-viral properties (Lorensen et al., 2023). Over the past few decades, biosynthesis pathways of terpenoid indole alkaloids (TIAs) have been partially elucidated in *Catharanthus roseus* (L.) G. Don, a medicinal plant that contains various alkaloids (Liu et al., 2021). These mechanisms are believed to be similar in *Rauvolfia* species (Liu et al., 2012). Recently, (Stander et al., 2023) also identified enzymes in *R. tetraphylla* responsible for yohimbane monoterpene indole alkaloid biosynthesis.

R. tetraphylla is known to produce secondary metabolites such as ajmaline, alstonine, aricine, lankanescine reserpine, reserpiline, deserpidine, isoreserpine, sarpagine, rauvotetraphyllines, serpentine, and yohimbine (Lorensen et al., 2023; Mahalakshmi et al., 2019). It remains a fascinating plant species due to its production of unique heterocyclic alkaloids with monoterpene indole skeletons. These compounds have generated significant interest from both biological and therapeutic perspectives. Reserpine is a significant indole alkaloid synthesized by *R. tetraphylla*. It is commonly used to treat hypertension and several psychiatric illnesses by serving as a tranquilizing agent. The biosynthesis of reserpine initiates from tryptophan, which serves as the starting material (Anitha & Ranjitha, 2006). In the quest for an effective strategy to source pharmaceutical indole alkaloids, exploring the molecular techniques of *R. tetraphylla* biosynthetic pathway for indole alkaloids offers a promising avenue to boost their production and meet pharmaceutical demands.

Tryptophan decarboxylase (TDC) is an enzyme involved in the biosynthesis of indole alkaloids (Goddijn et al., 1995). L-tryptophan is the substrate that converted into tryptamine by TDC through a decarboxylation reaction. This study was carried out with a primary objective to explore the potential of *R. tetraphylla* for alkaloid biosynthesis using TDC gene and docking analysis.

2. Materials and methods

2.1. Genomic DNA extraction and PCR amplification

Total genomic DNA of the *R. tetraphylla* was extracted from young leaf tissues by using DNeasy plant Mini Kit (Qiagen, Germany). Purified total DNA was quantified and its quality verified on 0.8 % agarose gel. The gene sequence of *R. verticillata* was retrieve from the NCBI (ABP96805.1) to design the primer for identifying the RtTDC. The target gene was amplified with specific Forward primer 5′-ATGGGCAGCATT-GATTCAACAG-3′ and Reverse primer 5′-TCAAGCTTCCTTGAG-CAAATCA-3′ using PCR amplification (Dharshini et al., 2020). All the PCR products obtained by TDC gene were resolved by electrophoresis on 0.8 % agarose gel in 1x TBE Buffer, stained with ethidium bromide and gel documentation system was used for further calibration.

2.2. DNA elution and ligation

The amplified fragment of TDC was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, Germany) and ligated into pTZ$_{57}$R/T vector using InsTAclone PCR Cloning Kit (Thermo Fisher Scientific, USA). The isolated DNA fragment was cloned into the pTZ$_{57}$R/T vector (Mirahmadi et al., 2015). Following this, the vector containing the inserted DNA was transformed into DH5α *Escherichia coli* strain (Froger & Hall, 2007). A single transformed colony from each ampicillin-containing plate was selected and transferred to separate PCR tubes. Colony PCR was performed and the desired DNA fragment was confirmed by electrophoresis using 1 % agarose gel run at 70 V for 30 min. The transformed bacteria were cultured and the plasmid containing the cloned DNA fragment was isolated by QIAprep Spin Miniprep kit (Qiagen, Germany), following established plasmid isolation procedures (Sambrook et al., 1989).

2.3. Gene sequencing

The isolated plasmid was sequenced using the Sanger sequencing method, which is a widely used DNA sequencing technique. Sequencing was performed using the BigDye Terminator kit v. 3.1 and cleaned up with BigDye XTerminator v. 3.1 (Applied Biosystems, Foster City, CA). The DNA fragment is sequenced by adding a mixture of dideoxy nucleotides (ddNTPs) to the reaction, which terminate the DNA synthesis at each nucleotide. The resulting fragments are separated by size and analysed to determine the sequence of the DNA fragment (Crossley et al., 2020). The M13 primer is a commonly used primer in Sanger sequencing that anneals to the pTZ$_{57}$R/T vector and sequence the cloned DNA fragment. After obtaining the sequencing results, Snap gene viewer was used to analyse the sequence and obtained the complete sequence of TDC.

2.4. Computational analysis of the gene sequence

Sequence similarity analysis between RtTDC and other TDCs from plants was performed using the basic local alignment search tool (BLAST) provided by the National Centre for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast). Nucleotide and amino acid sequences were aligned using CLUSTALW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2). To identify the coding sequence, the open reading frame (ORF) finder graphical analysis by NCBI (https://www.ncbi.nlm.nih.gov/projects/gorf) and ExPASy Bioinformatics resource portal of the Swiss Institute of Bioinformatics (https://www.expasy.org/proteomics/) are used.

2.5. Protein sequence and structure prediction

The protein sequence of the TDC gene was determined using the Translate tool. The homology-based 3D model for the RtTDC protein was generated through the SWISS-MODEL (https://swissmodel.expasy.org), utilizing the 6eew.1.A structure as a template. The conserved regions and multiple sequence were identify using MUSCLE (Sievers & Higgins, 2018). The phylogenetic tree was built using Mega11 software (Kumar et al., 2018). The Neighbour-Joining (NJ) method with 1000 bootstrap pseudo replicates was used to carry out the phylogenetic analysis. The physiochemical properties were assessed using Expasy's ProtParam server (https://us.expasy.org/tools/protparam.html). Secondary structure of TDC were analysed by HNN (https://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html) (Hierarchical Neural Network) (Kashani-Amin et al., 2019). Using the ProtScale program (https://web.expasy.org/protscale/), a Kyte and Doolittle hydropathy plot was generated (Kyte & Doolittle, 1982).

2.6. Ligand-receptor interactions through molecular docking and visualization tools

To investigate the interaction between a gene's protein and a ligand, it is essential to obtain the receptor (TDC) through a 3D model and the ligand (L-Tryptophan) from the pubchem database (https://pubchem.ncbi.nlm.nih.gov/). Docking has been carried out by Autodock vina following the protocol described by Morris et al., (Morris et al., 2008). Visualization of the docking results is achieved using PyMOL software, allowing them to explore the complex 3D structures and interactions between the ligand and receptor (Rauf et al., 2015). To evaluate the interaction between the substrate Trp and the enzyme binding site, docking simulations were conducted using Ligplot + with the best 3D model of TDC (Laskowski & Swindells, 2011).

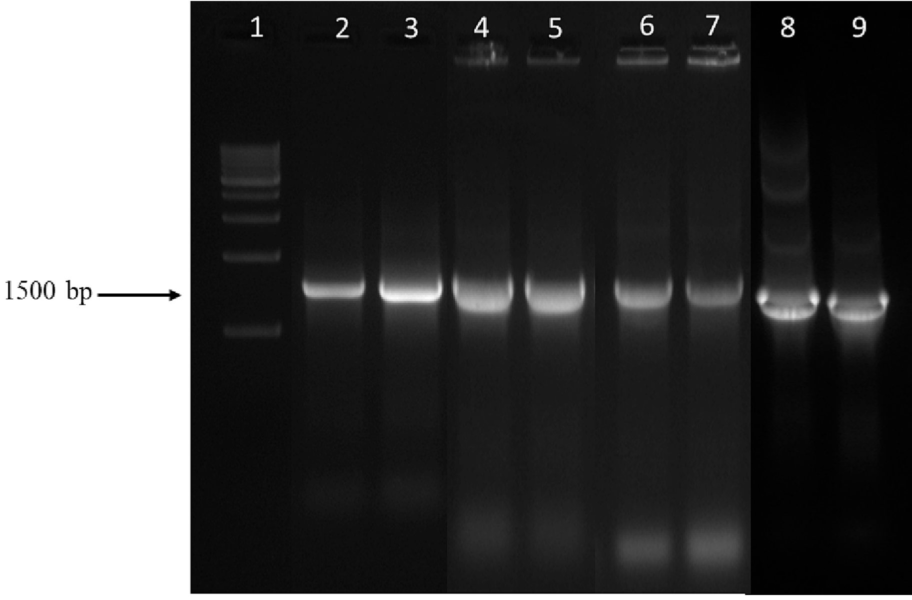


Fig. 1. Electrophoresis of PCR products from genomic DNA on 1 % agarose gel of known molecular weight. Lane 1: DNA molecular weight marker (DNA ladder mix – 1 kb ladder, Gene Ruler); Lane 2&3: A total of 20 μL of PCR product amplification of TDC gene stained with ethidium bromide; Lane 4–7: Colony PCR for the confirmation of transformation; Lane 8&9: Isolation of Plasmid after plasmid extraction process by QIAprep Spin Miniprep kit.

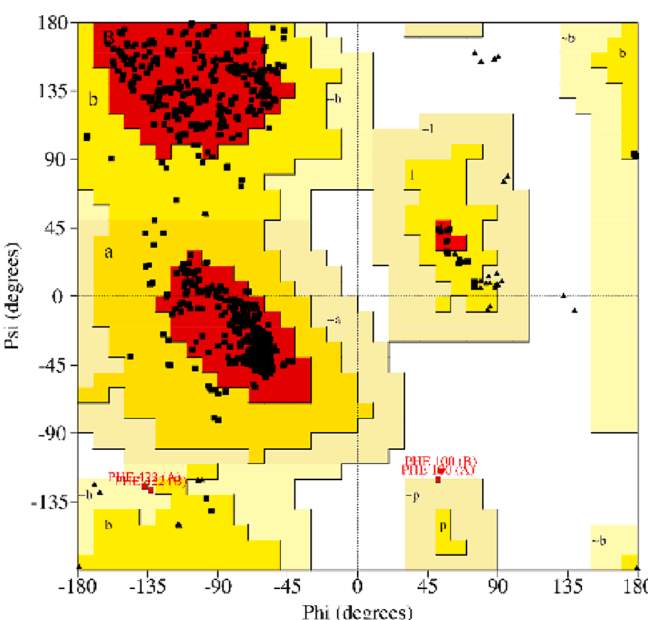


Fig. 2. Ramachandran Plot analysis for structural 3D model protein of TDC gene.

2.7. TDC enzymatic assay

The catalytic activity of TDC from *R. tetraphylla* was evaluated using the established procedures (Islas et al., 1994; Jadaun et al., 2017). Frozen leaves (2 g) were mechanically pulverized in a cold mortar and pestle, followed by homogenized with 1.25 mL of 0.1 M HEPES buffer at pH 7.5. The homogenate was centrifugated at 12,000 rpm for 30 min and the supernatant was immediately utilized for the enzyme source. The TDC assay mixture (100 μL) comprised enzyme extract (25 μL), 4 μM Pyridoxal 5-phosphate (PLP) (25 μL), 5 mM L-tryptophan (25 μL) and 50 mM HEPES buffer (25 μL). The reaction mixtures were incubated at 37 °C for 30 min. Then the reaction was stopped by adding 100 μL of pre-chilled methanol to precipitate proteins. Followed by centrifugation at 12,000 rpm for 10 min, the supernatant was used for further High-Performance Liquid Chromatography (HPLC) analysis to confirm the tryptophan converted into tryptamine in the presence of TDC enzyme. The HPLC system employed a C_{18} column and the mobile phase, composed of solvent A (Acetonitrile) and solvent B (H_2O containing phosphoric acid) at 280 nm. All enzymatic reactions were analysed in triplicate and Tryptamine served as the standard for comparative analysis.

3. Results and discussion

3.1. Isolation of TDC gene

TDC is a key enzyme in the biosynthesis of monoterpenoid indole alkaloids (MIAs) (Liu et al., 2012) and plays a crucial role in the medicinal properties of *R. tetraphylla*. TDC genes have been identified in several plant species, including *R. verticillata* (ABP96805.1). In our study, TDC sequence from *R. verticillata* was used to design primers for PCR amplification of TDC from *R. tetraphylla*. PCR amplification was performed using specific primers designed to target the TDC gene (Fig. 1). PCR product was sequenced and confirmed as the TDC gene as well as submitted to NCBI (Accession No: OR105870). It showed a high degree of sequence similarity 95.39 % between the TDC sequences of *R. verticillata* and *R. tetraphylla*, suggesting that the TDC gene is conserved across different species within the genus *Rauvolfia*.

TDC are a class of PLP-dependent enzymes responsible for catalysing the conversion of tryptophan into tryptamine (O'Connor & Maresh, 2006). The TDC-encoding gene was first isolated from *Catharanthus roseus*, a plant known for producing the antitumor monoterpene indole alkaloid vincristine (De Luca et al., 1989). Several TDC genes have been cloned from various plants, such as *Capsicum annuum*, *Ophiorrhiza pumila*, *Oryza sativa*, and *R. verticillata*, using a homology cloning strategy (Park et al., 2009).

TDC-encoding gene from *R. verticillata* was cloned and exhibit catalytic activity (Liu et al., 2012). Pepper fruits (*Capsicum annuum*) were found to contain TDC-encoding genes, namely CanTDC1 and CanTDC2. CanTDC1 gene showed three times higher specific catalytic activity compared to CanTDC2 (Park et al., 2009). The genome of *Oryza sativa* holds a sum of seven TDC genes, with only the genes OsTDCAK31 and

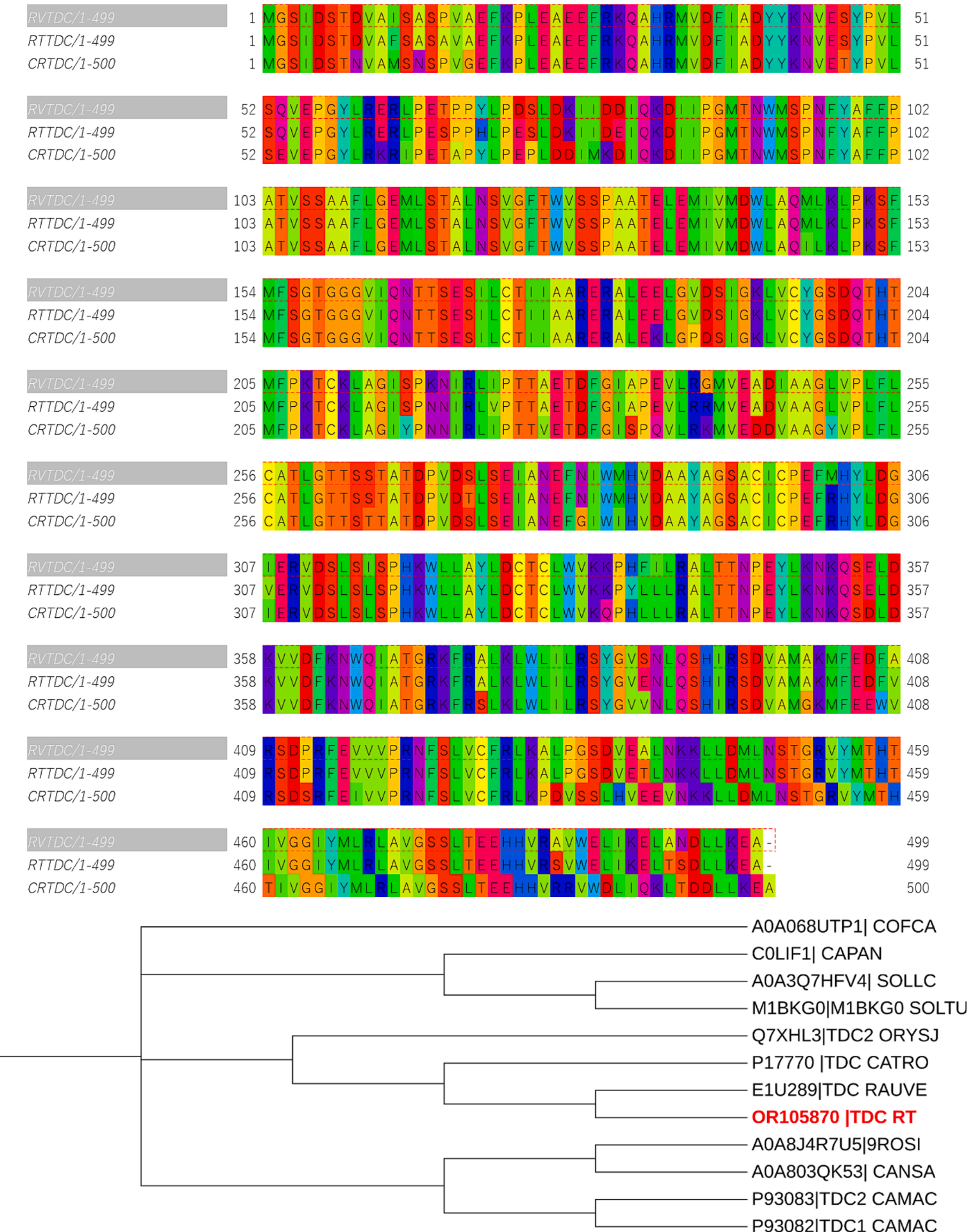


Fig. 3. Multiple amino acid residue sequences alignment of biochemically characterized plant TDCs. (a) TDCs are from *Catharanthus roseus*, *Rauvolfia verticillata*, and *Rauvolfia tetraphylla.* (b) Phylogenetic tree showed an occurrence and evolutionary relationship between TDC from different plant species. The tree was built using Mega11 software. Bootstrap values are all above 95%. COFCA- *Coffea canephora,* CAPAN - *Capsicum annuum*, SOLLC - *Solanum lycopersicum*, SOLTU- *Solanum tuberosum,* ORYSJ- *Oryza sativa,* CATRO–*Catharanthus roseus*, RAUVE- *Rauvolfia verticilla*, TDC RT – *Rauvolfia tetraphylla,* 9ROSI - *Castanea mollissima*, CANSA - *Cannabis sativa*, CAMAC - *Camptotheca acuminata.*

Table 1
Physiochemical characters as predicted by Expasy's Prot-param program.

Protein	Tryptophan decarboxylase
Sequence Length	499 amino acids
Molecular weight	55729.29 kDa
Theoretical Pi	5.37
Total number of negatively charged residues (Asp + Glu)	61
Total number of positively charged residues (Arg + Lys)	48
Extinction Coefficient	70860 $M^{-1}cm^{-1}$
Instability Index	47.92
Aliphatic Index	94.19
Grand average of hydropathicity (GRAVY)	0.029

OsTDCAK53 exhibited effective expression in *Escherichia coli*. It was confirmed that these genes exhibited catalytic decarboxylation activities towards L-tryptophan, leads to one or more TDC-encoding genes in a specific plant species (Kang et al., 2007).

Camptotheca acuminata Decne. is known for producing the renowned antitumor compound camptothecin, has been found to possess TDC genes such as CaTDC1 and CaTDC2. The decarboxylation activity of CaTDC1 and CaTDC2 towards L-tryptophan has been substantiated, resulting in the integration of the decarboxylation product camptothecin (López-Meyer & Nessler, 1997). CaTDC3 from *C. acuminata* was cloned through heterologous overexpression and functional characterization, it was confirmed that CaTDC3 exhibits catalytic decarboxylation activity toward L-tryptophan (Qiao et al., 2022).

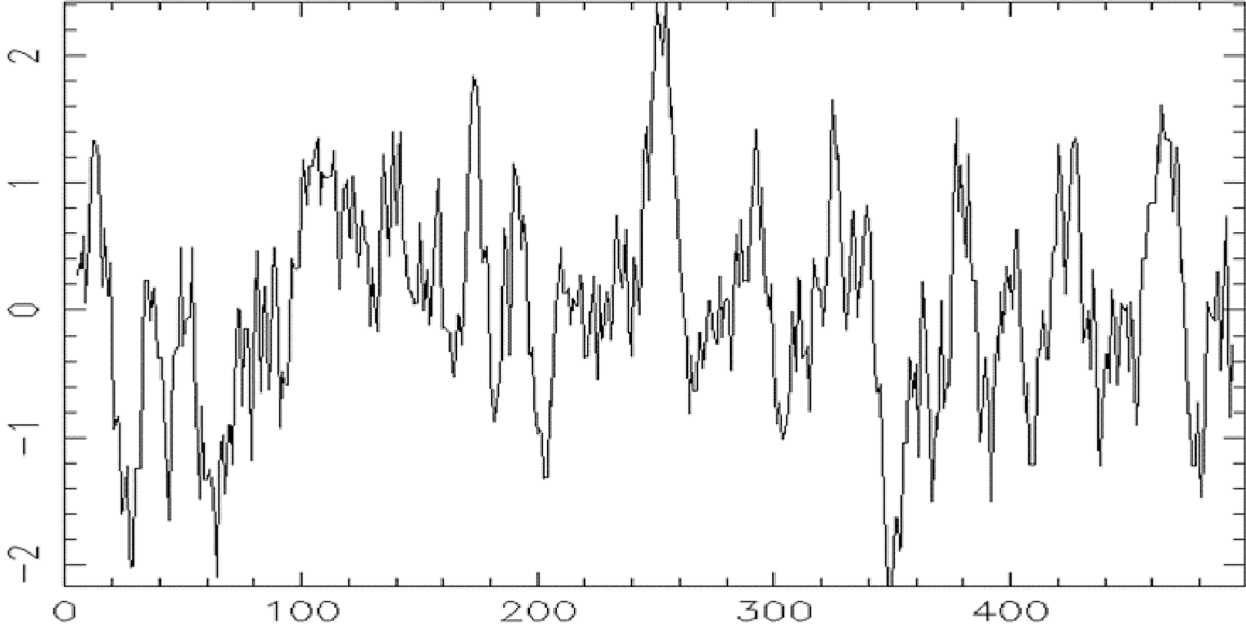


Fig. 4. Hydropathy plot of residues 1 to 499 of sequence Emboss.001 using Kyte and Doolittle hydropathy parameter.

Table 2
The value of amino acid composition in protein along with absolute counts of residues and their percentage and it composed of 20 amino acids.

S.No	Composition of amino acids	Percentage
1	Ala (A) 40	8.0
2	Arg (R) 23	4.6
3	Asn (N) 16	3.2
4	Asp (D) 26	5.2
5	Cys (C) 9	1.8
6	Gln (Q) 9	1.8
7	Glu (E) 35	7.0
8	Gly (G) 25	5.0
9	His (H) 10	2.0
10	Ile (I) 25	5.0
11	Leu (L) 57	11.4
12	Lys (K) 25	5.0
13	Met (M) 17	3.4
14	Phe (F) 23	4.6
15	Pro (P) 25	5.0
16	Ser (S) 41	8.2
17	Thr (T) 32	6.4
18	Trp (W) 9	1.8
19	Tyr (Y) 14	2.8
20	Val (V) 38	7.6

3.2. Model building and evaluation

The unknown 3D structure of *R. tetraphylla* TDC monomer was modelled using comparative protein modelling, with the crystallographically solved structure of *C. roseus* as the template. It is valuable when experimental structural data for the target protein is unavailable, providing valuable insights into protein structure and function in the field of structural biology (Facchiano, 2017).

The amino acid sequences of TDC from *R. tetraphylla* that currently lack structural data in the RCSB Protein Databank (PDB) were obtained using SWISS-MODEL. The best template was selected based on high score, lower e-value, and maximum sequence identity, and utilized as a reference structure to construct a 3D model (Waterhouse et al., 2018). The target protein under consideration is Aromatic-L-amino-acid decarboxylase, with a Uniport ID of P17770 and consists of 500 amino acids. For structural modelling purposes, the crystal structure of *C. roseus* TDC is being utilized as a template and is represented by the PDB ID 6EEW.

The Global Model Quality Estimate (GMQE) for the template stands at an impressive 0.97, indicating high confidence in its overall quality. The Template-Target Sequence-Overlap Quality (QSQE) score is calculated to be 0.88, suggesting significant structural similarities between the target protein and the template. Furthermore, when comparing the sequence of the target protein to that of the template, there is a substantial sequence identity of 88.96 % and indicates a strong relationship between the target and template.

The quality of the homology models was evaluated using the Saves server, with a focus on the Ramachandran plot (Fig. 2) and is a valuable tool for determining the stereochemical quality of protein structures by analysing the distribution of phi (ϕ) and psi (ψ) angles of the protein backbone (Sheik et al., 2002). It provides insights into the conformational stability and overall reliability of the modelled protein structures (LASKOWSKI et al., 2013). The Ramachandran plot demonstrated that over 90 % of the residues in the modelled proteins occupied the “favoured” and “allowed” regions of the plot, indicating their reliability and structural accuracy. To further validate the stability of the modelled proteins, the Deep View Swiss PDB viewer (Guex et al., 2009) was analysed for steric hindrances between residues in the models. The absence of steric hindrances suggests that the modelled protein structures are free from clashes representing their stability and suitability.

3.3. Multiple sequence alignment of RT TDC were performed with RvTDC and CrTDC

The cloned gene of RtTDC encompasses 1,500 bp and encodes a TDC containing 499 amino acid residues with high similarity to those of RvTDC. In the multiple sequence alignment of TDC sequences from *R. verticillata* (Rv), *R. tetraphylla* (Rt), and *C. roseus* (Cr), a functional and highly conserved region was identified (Fig. 3a). Conserved regions in the alignment are areas where the amino acid residues are highly similar across the different sequences, suggesting that these regions have important functional or structural roles that are conserved throughout evolution. The identification of such a conserved region in the TDC sequences is significant because it indicates that this region plays a critical role in the function of TDC. The conserved region is involved in the catalytic function of the enzyme, which is the conversion of L-tryptophan to tryptamine is a key step in the biosynthesis alkaloids.

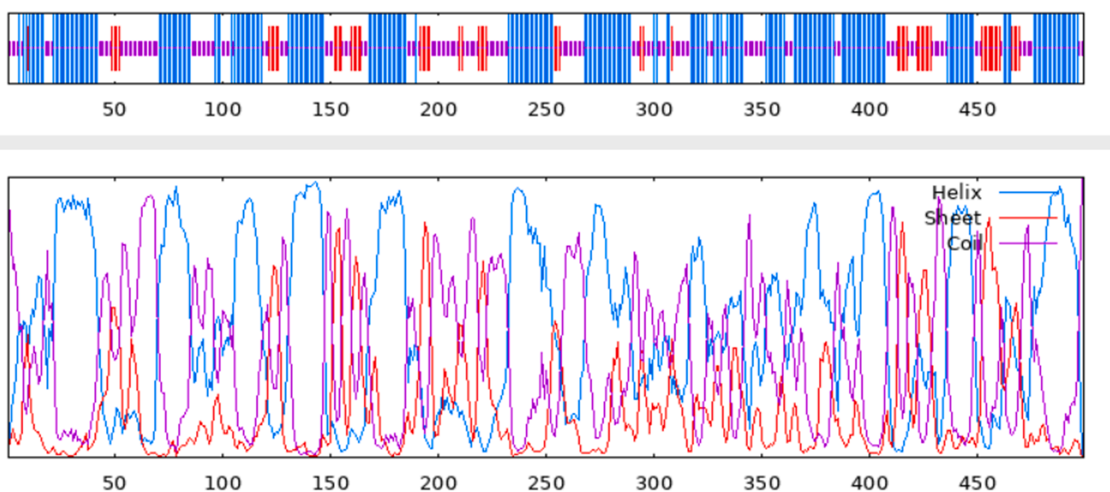


Fig. 5. The HNN software was used to predict the secondary structure of the TDC gene. (a) Structural codes in blue (h-Helix), orange (c-coil), red (e-sheet). (b) Secondary Structure prediction of TDC gene sequence of 499 amino acids in *Rauvolfiate traphylla*. Lines in different colours represent different secondary structures. Blue line indicates α helix; red line indicates extended strand and purple line indicates random coil. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The similar pursuit unveiled that RtTDC show resemblances in amino acid residues with plant TDCs. Among the enzymatic mechanisms of TDCs from plant (Liu et al., 2012; López-Meyer & Nessler, 1997), it is predictable that Lys319 plays a key role in RtTDC as the catalytic amino acid. RtTDC is grouped in the same clade as functionally characterized TDCs from *C. roseus* (CrTDC) (De Luca et al., 1989), *Ophiorrhiza pumila* (OpTDC) (Yamazaki et al., 2003), *R. verticillata* (RvTDC) (Liu et al., 2012) and *Gelsemium sempervirens* (GsTDC) (Franke et al., 2019). These TDCs have been known to participate in the biosynthesis of indole alkaloids. It suggests that RtTDC likely shares a similar catalytic function with these characterized TDCs.

3.4. Phylogenetic tree with related sequences

Phylogenetic tree showed three clades (Fig. 3b), in which one of the branches show common ancestors for *R. tetraphylla* (RtTDC), *C. roseus* (TDC CATRO), *R. verticillata* (RAUVE) and *Oryza sativa* (ORYSJ). Among them RtTDC exhibited close relationship with RAUVE. The results from the neighbour-joining phylogenetic analysis of 12 demonstrative species within flowering plants suggest that *R. tetraphylla* is closely related to *R. verticillata*. *C. roseus*, *R. verticillata* (Liu et al., 2012), *Oryza sativa* evaluated the TDC catalyses to produce Indole alkaloids.

3.5. Physicochemical characterization of TDC

The physicochemical properties of TDC were predicted using

Table 3
Binding energy and RMSD (in Å) value of docking obtained from crystal structure of 3D model protein and substrate (L- tryptophan as ligand).

Rank	Sub-Rank	Run	Binding Energy	Cluster RMSD	Reference RMSD	Grep Pattern
1	1	14	−4.69	0.00	28.56	RANKING
2	1	27	−4.58	0.00	61.50	RANKING
3	1	4	−4.49	0.00	40.28	RANKING
3	2	19	−4.25	0.35	40.19	RANKING
3	3	47	−3.58	1.78	40.42	RANKING
4	1	16	−4.49	0.00	54.44	RANKING
5	1	39	−4.48	0.00	60.37	RANKING
5	2	8	−4.39	1.39	59.42	RANKING
6	1	45	−4.47	0.00	49.99	RANKING
7	1	2	−4.40	0.00	49.03	RANKING
8	1	18	−4.15	0.00	48.81	RANKING
9	1	40	−4.11	0.00	61.04	RANKING
10	1	23	−4.09	0.00	36.98	RANKING

Expasy's Prot-param program (Table 1). The protein sequence length is 499 amino acids and molecular weight is 55,729.29 Daltons. The theoretical isoelectric point (pI) is 5.37, indicating that protein may be acidic. TDC contains a total of 61 negatively charged residues and 48 positively charged residues. The balance between positively and negatively charged residues contributes to the overall charge of the protein under specific conditions. The extinction coefficient of the protein is determined to be 70,860 M^{-1} cm^{-1}, a value often utilized for quantifying the protein concentration in UV absorption measurements. The instability index is 47.92 indicates a moderate propensity for protein instability, suggesting potential sensitivity to environmental factors.

The aliphatic index is computed to be 94.19, characterizes the protein thermostability, as it represents the relative volume of aliphatic side chains. The Grand Average of Hydropathicity (GRAVY) is 0.029, reflecting the overall hydrophobicity. The Kyte and Doolittle hydrophobicity plot of RtTDC protein showed a pattern of high hydrophobicity with the GRAVY value and hydrophobic residues (Fig. 4). These parameters provide valuable information about the protein biophysical properties, stability, and solubility.

The amino acid composition of the TDC protein is presented in Table 2. It provides valuable insights into the protein primary structure, shedding light on the relative abundance of each amino acid residue present. Leucine (L), alanine (A), serine (S), valine (V), glutamic acid (E), threonine (T), glycine (G), aspartic acid (D), proline (P), isoleucine (I), lysine (K), and methionine (M), phenylalanine (F), arginine (R), asparagine (N), tyrosine (Y), cysteine (C), glutamine (Q), histidine (H), and tryptophan (W) are the 20 amino acids, among them leucine is the most abundant, with a total count of 57 residues constituting 11.4 % of the protein.

The hydropathy profiles of *C. roseus* TDC and *D. melanogaster* DDC1 proteins showed 39 % structural similarity. The deduced amino acid sequence of *C. roseus* TDC revealed an unexpected similarity to animal DDC in the secondary structure of conserved domains (De Luca et al., 1989; Goddijn et al., 1994). The comparison of *Campotheca* TDC1 and *Catharanthus* TDC revealed a significant 69 % identity between the two

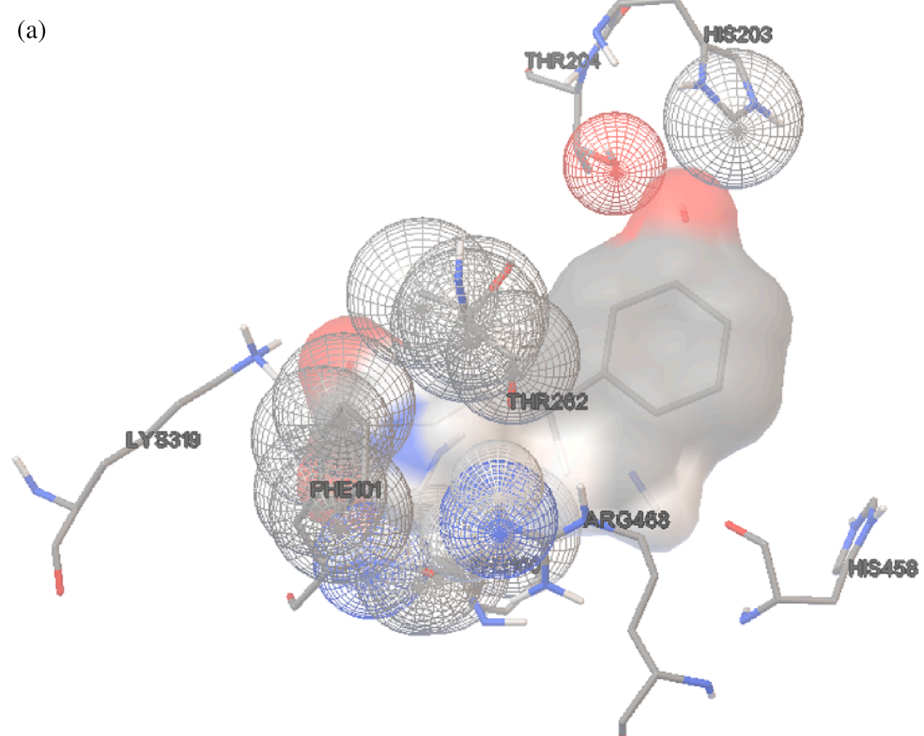
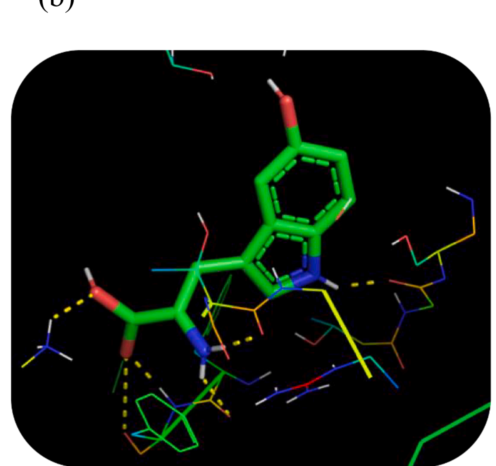


Fig. 6. Pharmacophore model of TDC gene derived from 3D model and its mapping with L- tryptophan ligand. (a) The active amino acids were mapped to the best pharmacophore model. The pharmacophore model was illustrated using Catalyst notation, which encompassed of hydrogen bond acceptor, hydrogen bond donor and hydrophobic region. (b) Hydrogen Bond Interaction of RtTDC protein (Receptor) and L-tryptophan (Ligand); hydrogen bond is showed in yellow dotted line; Hydrogen bonds are observed in Lys319, Phe101, His458 and Thr204 amino acids residues. (c) Ligplot diagram using the model RtTDC and highlights the interaction with Trp Ligand. The ligand is depicted with bold (blue-coloured) bonds, while the hydrogen-bonded residues from the protein are represented with thin (brown-coloured) bonds. Dashed lines illustrate the hydrogen bonds formed between the ligand and the protein. Additionally, hydrophobic contacts made with the protein are indicated by spoked arcs pointing towards the ligand, and corresponding spokes on the ligand atoms reveal which atoms are engaged in these contacts. Likewise, spokes pointing towards the contact atoms mark the atoms in the hydrogen-bonded groups engaged in hydrophobic contacts. The residue names are annotated with letters in parentheses, indicating the corresponding chain identifiers. The diagram showcases the catalytic triad comprising Lys319, Phe101, His458, and Thr204, along with the ligand's TRP residue snuggled within the enzyme's highly hydrophobic specificity pocket in the active site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(b)



(c)

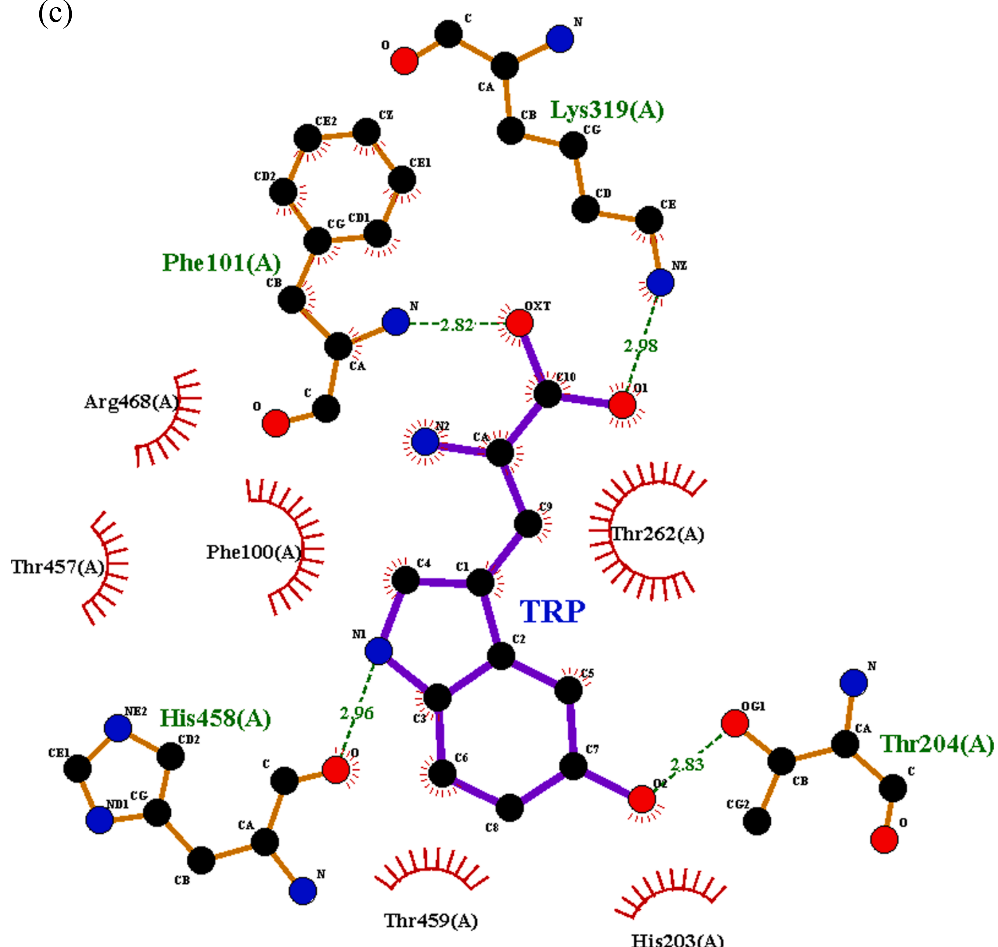


Fig. 6. (*continued*).

sequences. This high level of sequence conservation highlights the potential functional importance of this region in the catalytic activity and stability of TDC enzymes (López-Meyer & Nessler, 1997). It showed that amino acid composition provides information for understanding its chemical nature and potential implications for its function and structure.

3.6. Secondary structure prediction of TDC

The secondary structure of TDC gene *R. tetraphylla* protein sequences were calculated using HNN (Fig. 5 a and b) and reveals the distribution of various structural elements. The most predominant secondary structure is alpha helix (Hh), which comprises 253 residues, accounting for 50.70 % of the protein. The protein contains 61 residues in the extended strand (Ee) conformation representing 12.22 %. The majority of the

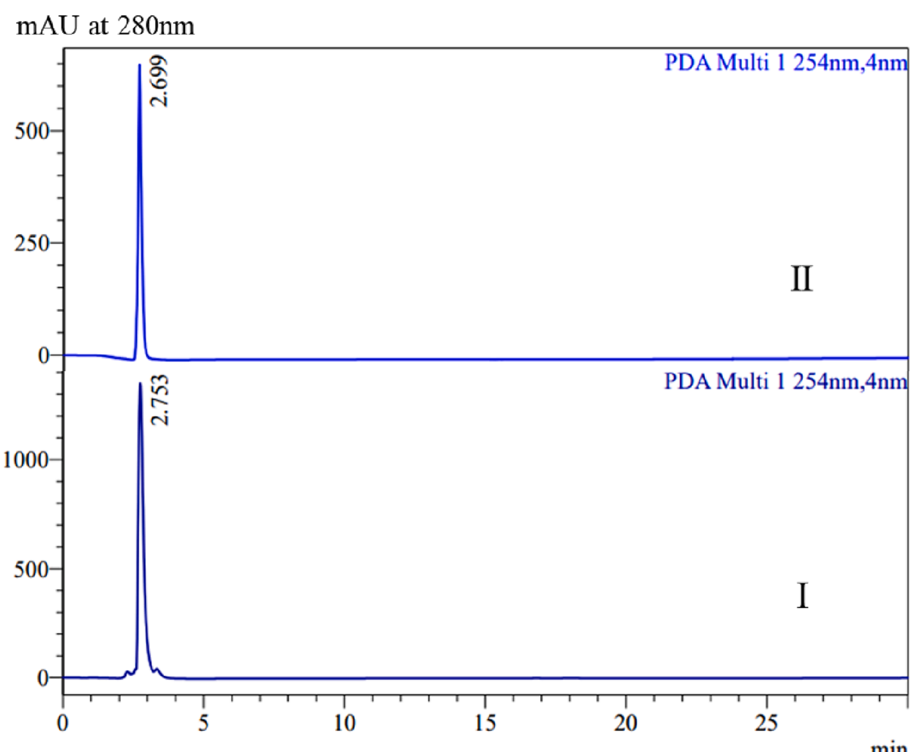


Fig. 7. *In vitro* decarboxylation of tryptophan to tryptamine. HPLC traces of decarboxylation reaction mixture catalysed by RtTDC (panel I) and panel II represent standard tryptamine.

protein, consisting of 185 residues (37.07 %), is categorized as random coil (Cc).

In this study, isolated and characterized a full-length cDNA encoding TDC from *R. tetraphylla*, marking the first time this gene has been studied in this species. The deduced amino acid sequence of RtTDC exhibited significant similarity to TDC counterparts found in other species. Prior research on RvTDC (Liu et al., 2012) has shown that the Lys319 residue directly participates in binding to PLP-binding site. It is conserved in other decarboxylases, including 3,4-dihydroxyphenylalanine (DOPA) decarboxylases from *C. acuminata* (López-Meyer & Nessler, 1997), *D. melanogaster* DDC (Clark et al., 1978), feline glutamate decarboxylase (Kobayashi et al., 1987) and periwinkle TDC (De Luca et al., 1989), strongly suggests that lysine-319 of TDC is responsible for binding to PLP. The conservation of lysine-319 in the PLP binding site emphasizes the importance of conserved domains in facilitating subunit assembly, catalytic function, and substrate specificity of TDC enzymes.

3.7. Ligand binding to TDCs from R. tetraphylla by molecular docking

Molecular docking was analysed by utilizing the predicted three-dimensional structure of RtTDC and L-tryptophan as the substrate. The results of the highest scoring ligand are summarized in Table 3. The table contains rankings and properties of substructures based on Binding Energy, Cluster RMSD (Root Mean Square Deviation), and Reference RMSD. The top-ranked substructure is listed with Sub-Rank 1, Run-Rank 14, a Binding Energy of −4.69, and Cluster RMSD and Reference RMSD values of 0.00, 28.56, respectively. Pharmacophore is a critical concept in docking studies as it refers to the set of molecular features that are essential for a ligand to bind to a receptor (Fig. 6a). Understanding the pharmacophore enabled to design and modify ligand to improve binding affinity and selectivity for the target receptor, leading to more effective and safer drugs.

The aromatic amines are produced through the catalytic action of ubiquitous aromatic amino acid decarboxylases (AADCs), a group of PLP-dependent enzymes. Despite having similarities in terms of extensive amino acid residue, subunit structures, and kinetic properties, AADCs demonstrate significant variations in their substrate specificities. (Facchini et al., 2000). In the case of AADC in plant, they have developed with changes in function and substrate preference. (Günther et al., 2019). The naturally occurring amines obtained from plants are known for wide range of metabolic pathways, mostly contributing to the synthesis of alkaloids (Torrens-Spence et al., 2020). The identified and functionally characterized one or more AADC-encoding genes from a single plant, suggesting the increase in gene families of whole-genome duplication in the process of evolution (Kang et al., 2021).

The protein–ligand docking is to predict the orientation and positioning of a ligand in the binding pocket of a particular receptor. It involves the formation of hydrogen bond interactions between the protein and the ligand (Fig. 6b). The docking simulations with Trp revealed a complex network of interactions involving multiple amino acids besides the binding site residues. Fig. 6c showed the amino acid residues of the RtTDC binding pocket involved in the interaction with the substrate Trp according to the results of molecular docking simulations. The analysis of *C. roseus* TDC binding to L-tryptophan was also performed (Facchiano et al., 2019), showing the same amino acid residues (Lys319) as those of RtTDC interacting with Trp in the docking simulations.

3.8. TDC enzymatic assay

The enzymatic decarboxylation activity of *R. tetraphylla* were analysed to investigate the role of TDC in the terpenoid indole alkaloid biosynthetic pathway. The assays involved the incubation of L-tryptophan, PLP and enzyme extract. HPLC analyses revealed the presence of an enzymatic reaction product in the RtTDC catalyzed reaction mixture (Fig. 7). The HPLC retention time of the enzymatic reaction product was found to be identical to that of authentic tryptamine. It demonstrates that the enzymatic product generated by RtTDC is indeed tryptamine, confirming the enzyme's role in the decarboxylation of L-tryptophan, a

crucial step in the biosynthesis of alkaloids in *R. tetraphylla*.

The investigations into TDC activity in various plant systems reveal its pivotal role in the biosynthesis of indole alkaloids. In *Catharanthus roseus* transformed root culture, TDC demonstrates a coordinated increase in activity with the accumulation of alkaloids ajmalicine and catharanthine, emphasizing its regulatory function in the indole alkaloid pathway (Islas et al., 1994). Additionally, TDC activity in *Citrus x limon* seedlings, evidenced by the conversion of deuterium-labelled tryptophan to tryptamine and subsequent methylation, expands our understanding of TDC across plant genera (De Masi et al., 2017). The CaTDC3 in *C. acuminata* further emphasizes the enzyme's versatility, showing strict stereoselectivity for L-tryptophan and catalytic promiscuity towards various tryptophan derivatives (Qiao et al., 2022). These inferences shed light on the multifaceted nature of TDC, providing insights into its catalytic capabilities and broadening its applications in plant alkaloid metabolism.

4. Conclusion

The wide range of tryptophan-indole metabolism in plants leads to the production of indole phytochemicals in different plant genera. Specifically, within the Apocynaceae family, *Rauvolfia* species such as *R. tetraphylla, R. serpentina*, and *R. verticillata* are known for producing indole alkaloids, with mechanisms involved in their synthesis. TDC is catalytic enzyme plays a crucial role in conversion of tryptophan to tryptamine tailored to the biosynthetic pathway of different plant species for production of various indole alkaloids. Understanding the catalytic properties of TDC from *R. tetraphylla* is essential in elucidating these processes. This study represents a total genomic DNA of TDC gene from *R. tetraphylla* with 1500 base pair. The potential activity of TDC were analysed by molecular docking and enzymatic assay to conform the alkaloid biosynthesis of *R. tetraphylla*. Based on this new study, it's conceivable that RtTDC could play a significant role in the biosynthesis of indole alkaloids, paving the way for novel approaches in the drug development of fast tissue regeneration. It facilitates an alternative means of producing valuable indole alkaloids for wound healing drugs.

CRediT authorship contribution statement

Lavanya Nallasamy: Investigation, Methodology, Writing – original draft. **Najat A. Bukhari:** Data curation, Project administration, Visualization, Writing – review & editing. **Girija Sangari Murugavelu:** Data curation, Writing – review & editing. **Deepika Krishnamoorthy:** Data curation, Writing – review & editing. **S. Mahalakshmi:** Methodology, Visualization. **Amutha Swaminathan:** Conceptualization, Investigation, Supervision, Validation, Writing – review & editing. **Appunu Chinnaswamy:** Conceptualization, Methodology, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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