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Production of secondary metabolites from *invitro* cultures of *Rauwolfia serpentina (L.) Benth*

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ABSTRACT

An efficient and reproducible method for the induction of callus and hairy roots from invitro and invivo explants of *Rauwolfia serpentina* was standardized. Further, secondary metabolites were extracted and analysed by thin layer chromatography (TLC). There are several factors influencing *invitro* induction of callus and hairy roots which include media composition, genotype, and culture conditions. The mature and immature embryos were inoculated on Murashige and Skoog's (MS) medium supplemented with 2,4-Dichlorophenoxy acetic acid (2,4–D; 0.5, 1, 2 or 5 µM concentrations) and 6-Benzylaminopurine (BAP; 1, 2 or 5 µM concentrations) at various combinations to get axenic seedlings. Media supplemented with 2µM BAP and 0.5µM 2,4-D showed better response compared to others. The leaf and seedling explants from *in vivo* and *in vitro* grown plants respectively were cultured on MS media supplemented with 2,4-D at various concentrations (2, 5, 10 or 20 µM). Media supplemented with 5 or 10 µM 2,4-D induced callus, and lower as well as higher concentrations of 2,4-D were not effective for callus induction. The alkaloids were extracted from both callus and leaf and analysed by TLC. The cultures accumulated the same major alkaloids as in the leaves of the parent plant. The leaf explants were infected with *Agrobacterium rhizogenes* to induce hairy roots for the production of secondary metabolites in large scale. Profusely branched hairy roots were observed in one-month-old leaf cultures.

Keywords – *Rauwolfia serpentina, Murashige and Skoog's (MS) medium, 6-Benzylaminopurine, 2,4-Dichlorophenoxy acetic acid, callus, hairy roots, Agrobacterium rhizogenes*

I. INTRODUCTION

Rauwolfia serpentina is a small, woody shrub that originated in South Asia. It is an important endangered medicinal plant belonging to Apocynaceae family. *R. serpentina* is an extraordinarily large and diverse new world genus with an equally diverse number of habitats [1]. *Rauwolfia sp.* is in threatened status in India due to over exploitation for commercial purposes have been increasing exponentially to meet the requirements of pharmaceutical industry. Since seed germination in *Rauwolfia sp*. is highly variable, and it is reported to vary from 5 to 30 percent even when only heavy seeds are chosen for sowing purpose. The problem of poor germination is forcing the farmers to use cuttings for propagation. The supply of the alkaloids does not meet its demand since the plant takes a long time to grow via conventional methods, so to eradicate this problem, production of alkaloids must couple with some modern technique. To satisfy the growing commercial demand of alkaloids, *in vitro* production of such alkaloids is essential and would be economical [3]. It also helps in conservation of this valuable endangered plant itself. Hence improvements in plant tissue culture techniques for the production of alkaloids in *R. serpentina* are highly desirable. The natural reserves of this plant are declining, especially after reports of its medicinal properties appeared in literatures. International Union for the Conservation of Nature and Natural Resources (IUCN) has kept this plant under endangered status [2].

Fig1.1: *Rauwolfia serpentina (*L.) Benth

The roots of *R. serpentina* contain the alkaloid reserpine. This alkaloid was isolated for the first time by Muller et al.

Bein demonstrated that reserpine as sedative and hypotensive action one year later [4]. According to Besset the roots of *R. sepentina* contain not less than 21 kinds of alkaloids [5]. Around the same time, Gupta et al reported the application of *Rauwolfia sp.* in mental disorder [4]. Bhatia and Kapur reported after the administration in animals of the 2 alkaloids isoajamaline and neoajamaline, stimulation followed by depression of central nervous system and lowering of blood pressure(1944) [2]. Bhatt *et al* (2008) devised protocol optimization for invitro propagation. Singh et al, Somatic embryogenesis and *invitro* regeneration of medicinal plant Sarpgandha (2009) [7]. Harisaranraj et al., evaluated the chemical composition *R. serpentina* and *Ephedra vulgaris* [12] and also worked on production of reserpine in somatic embryos of *Rauwolfia serpentina* cultured in bioreactors by the induction of elicitor (methyl jasmonate) (2009) [13]. Panwar and Guru studied alkaloid profiling and estimation of resperine in *R. serpentina* plant by TLC, HP-TLC and HPLC (2011) [14]. In this study, we extend the previous research by demonstrating induction of callus from various explants of *R. serpentina*, extract and analyse the alkaloids present in *invivo* and *in vitro* through TLC and induced hairy roots.

II. MATERIALS AND METHODS

2.1 Plant material and chemicals

R. serpentina plants were generated through vegetative propagation from seed. These plants were kept in a climatic controlled indoor cultivation facility housed at Sangeevini Vana of R. V. College of Engineering, Bangalore. The mature and immature seeds were used as an explant for initiation of shoot cultures and leaf explants were used for callus induction. Chemicals used in present study were procured from Himedia, Mumbai, India and Glassware from Borosil, India. Standard protocol was followed for preparation of MS media [18].

2.2 Preparation and Inoculation of explant

Explants were washed with 0.1% of tween20 and washed with double distilled water. Then washed in sodium hypochlorite solution (0.5-1.0%) for 20 minutes and washed again in double distilled water and taken in Laminar Air Flow (LAF) [20]. Individual seeds and leaf were rinsed in 70% alcohol for 1-2 minutes and then washed in autoclaved water and placed on a sterile Petri plate. These explants were then individually inoculated into different culture bottles containing the autoclaved MS media, supplemented with different concentrations of growth regulators[19].The bottles were sealed with surgical tape and incubated in controlled conditions (24 ± 3^0C and 16/8hr photoperiod) and for callus induction it was kept in dark [22].

2.3 In Vitro Germination of seeds/embryos and Callus Induction:

Seeds, mature and immature embryos were inoculated in media containing 2µM BAP + 0.5µM 2,4-D, 5µM BAP + 1µM 2,4-D, 2µM 2,4-D, 5µM 2,4-D. After germination, hypocotyl were separated from seedling and used for callus induction. For callus induction from leaf and invitro germinated seed, MS media with 2µM, 5µM, 10µM, 20µM concentrations of 2, 4-D were used.

2.4 Data Analysis:

A complete random method was used in all experiments. In case of hypocotyls, each experiment was replicated thrice and with two explants per treatment. In case of leaf explants, each experiment was replicated four times and with three explants per treatment. The number of explants responded were observed and recorded. All data were analysed by SPSS software. Analysis of Variance (ANOVA) was used to test the statistical significance [23] and based on Duncun's multiple range test (DMRT).

2.5 Thin Layer Chromatography

Callus obtained from various explant of *R. serpentina* were incubated in 25ºC, 1mg of callus were taken and grinded with methanol until the residue were colorless, the suspension were filtered through Whatman's filter paper and collected in Petri plates. These were allowed to dry completely in water bath set at 40ºC for 30 min. Dried extracts were scraped out by using scalpels and was collected in pre weighed vials separately. Extracted powders were made available to use as per requirements by resuspended in methanol every time [24]. The qualitative analysis of major groups of indole alkaloid derivatives of *R. serpentina* was initially done by thin layer chromatography (TLC) technique on preparative silica gel (Silica gel 60F plate). Mobile phase or solvent system used for alkaloid estimation was chloroform: methanol (97:3). Bands were visualized by observing the plates under UV- light. Identification was done on the basis of colour of bands and their Rf values under UV light [25].

2.6 Hairy root induction

The culture of *A. rhizogenes* was initiated from glycerol stock and grown overnight at 280C with shaking (180 rpm) in liquid Luria-Bertani medium containing 50mg/L kanamycin, to mid-log phase (OD600 = 0.5) (fig 2.1 and 2.2). The *A. rhizogenes cells* were collected by centrifugation for 10min at 2500rpm and resuspended in liquid inoculation medium (half strength MS salts and vitamins containing 15g/L sucrose). The *A. rhizogenes* cell

density was adjusted to A600 of 1.0 for inoculation **[**26]. Excised leaves of *R. serpentina* is used as explant material for co-cultivation with *A. rhizogenes*. Surface sterilization was carried out with sodium hypochlorite (0.5%) for 20 min and mercuric chloride (0.1%) for 1min. The sterilized leaves were dipped into the *A. rhizogenes* culture in liquid inoculation medium for 1hour in continues stirrer in low rpm. For co-cultivation the infected explant were inoculated in solidified half strength MS media and incubated in the dark at 25°C [27**]**. After two days of co-cultivation(refer figure 2.3), the explant tissues were washed thoroughly with sterilized water and cefatoxamine [10**]** and transferred to a hormone-free half strength MS medium containing salts and vitamins(half strength), 15 g/L sucrose, 50 mg/L cefatoxamine and 8 g/L agar.

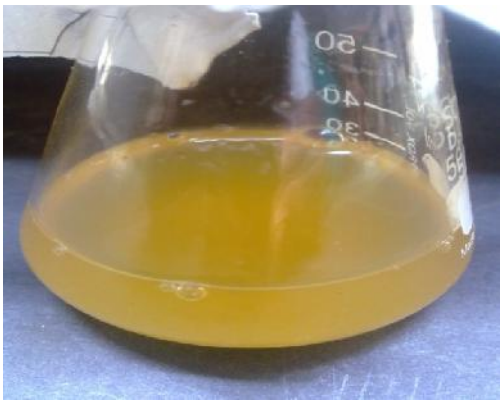
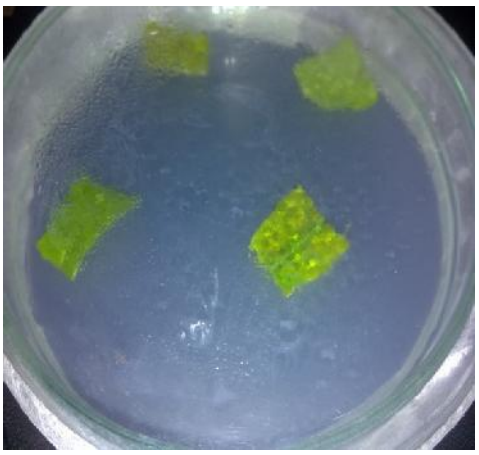
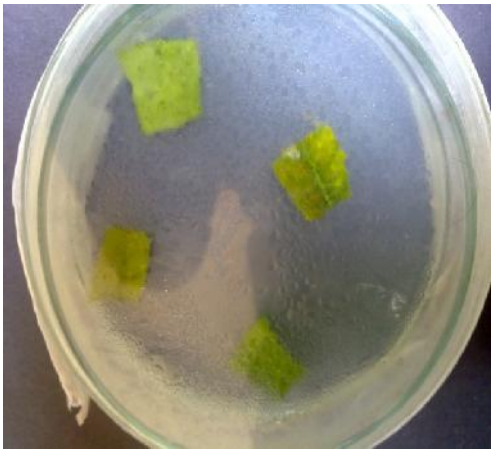


Fig 2.1: Activated culture o*f Agrobacterium rhizogenes*

Fig 2.2: *Agrobacterium rhizogenes* culture plate



(a)



(b)

Fig 2.3: (a & b)Co cultivation of leaf explant infected by *Agrobacterium rhizogenes*

III. RESULTS

***3.1 Invitro* Germination of seeds**:

The cultured embryos were germinated on MS supplemented with 0.5 µM 2,4-D and 2µM BAP. Seeds inoculated in media supplemented with 2µM BAP + 0.5µM 2, 4-D responded very well .Developments such as, swelling of seeds, germination of seeds, appearance of cotyledons and hypocotyl were observed. Regular and constant monitoring of changes in the cultures led to the observation that the seeds which inoculated onto the media supplemented with 2µM BAP + 0.5µM 2, 4-D showed swelling earlier than other combinations. In subsequent weeks faster germination was recorded (fig 3.1, 3.2, 3.3 and 3.4).

Fig 3.1: Germination of seed on MS media supplemented with 2µM BAP + 0.5µM 2, 4-D (6-week old culture)

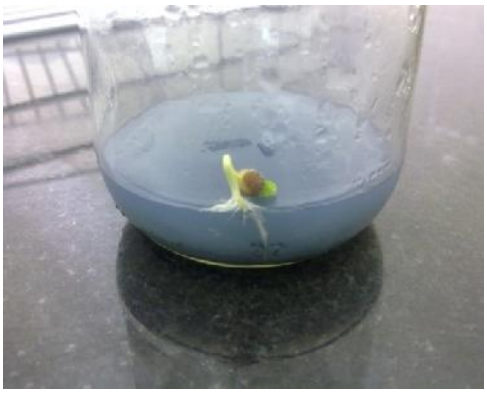


Fig 3.2: Germination of seed on MS media supplemented with 2µM BAP + 0.5µM 2,4-D (8-week old culture)

Fig 3.3: Development of seedling on MS media supplemented with 2µM BAP + 0.5µM 2,4-D (10-week old culture)

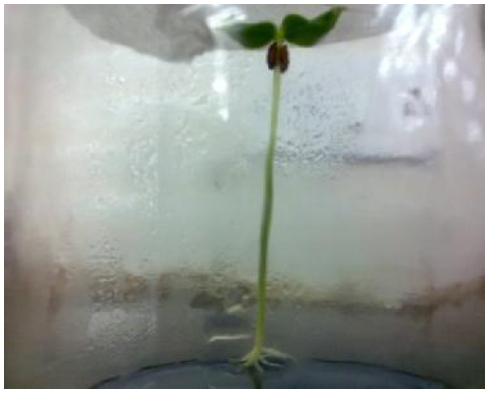


Fig 3.4: *In vitro* Developed Seedling on MS media supplemented with 2µM BAP + 0.5µM 2,4-D (12-week old culture)

3.2 Callus Induction:

In vitro germinated hypocotyls was cut and were sub-cultured onto MS media supplemented with different concentrations of 2, 4-D (2µM, 5µM, 10µM, 20µM)

Fig 3.5: Initiation of callus from hypocotyl on MS media supplemented with 5µM 2, 4-D (1 week after subculture)

Fig 3.6: Initiation of callus from hypocotyl on MS medium supplemented with 5µM 2, 4-D (2 weeks after subculture)

Fig 3.7: Initiation of callus from hypocotyl on MS medium supplemented with 5µM 2,4-D (2 weeks after subculture)

Fig 3.8: Callus from hypocotyl explant on MS medium supplemented with 5µM 2,4-D (4 weeks after subculture)

Initiation of callus was observed in *in vitro* germinated explants (hypocotyl) after 2-3 weeks of inoculation at 5µM concentration of 2, 4-D and after 4-5 weeks of inoculation at 10µM concentration of 2, 4-D. There was no development in the culture bottles containing 2µM and 20µM 2, 4-D (fig 3.5, 3.6, 3.7 and 3.8). And the apical shoot buds were cultured into high concentration of cytokinin and low concentration of auxins to continue its shoot elongation (fig 3.9 and 3.10).

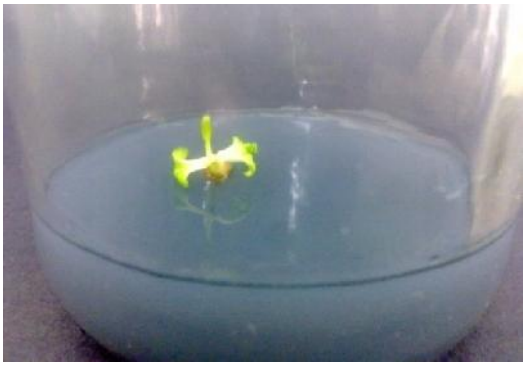


Fig 3.9: Shoot bud subcultured onto MS media with BAP and 2,4-D

Fig 3.10: Shoot bud subcultured onto MS media with BAP and 2,4-D

Leaves of *R. serpentina* were inoculated into MS media supplemented with different concentrations of auxin (2µM, 5µM, 10µM, 20µM). Leaf explants showed better response compared to hypocotyls. Initiation of callus was seen after 2-3weeks of inoculation in 5µM and 10µM (fig 3.11, 3.12, 3.13 and 3.14)

Fig 3.11: Initiation of callus from leaf explants on MS supplemented with 5µM 2,4-D (2 weeks after subculture)

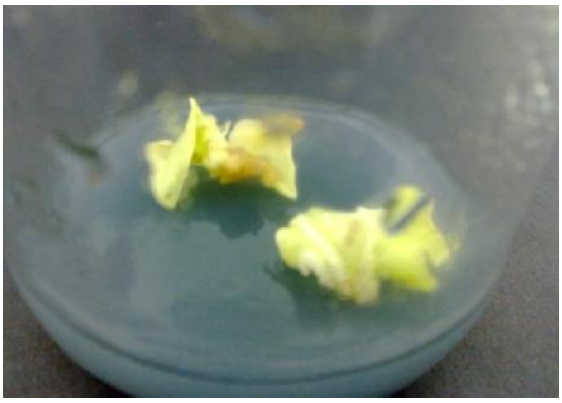


Fig 3.12: Callus produced from the leaf explant (4-5 week old cultures)

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Fig 3.13: Callus produced from the leaf explant (4-5 week old cultures)


Fig 3.14: Callus produced from leaf explant after 5 weeks after subculture.

From the observation, callus induction in both explants (*invitro* and leaf explants) was better in 5µM and 10µM concentration of 2, 4-D. Overall 10µM 2, 4-D showed faster response than other combinations of 2, 4-D in initiation of callus. And data analysis of this study shows that 10µM 2, 4-D has more significance compared to other combinations of 2, 4-D. (table 3.1 and 3.2)

Table 3.1: Effect of 2,4-D on callus induction in *R. serpentina* (L.) Benth, hypocotyl as explant

	Concentration (µM)	No. of explants (hypocotyl)	No. of explants responded	Mean no. of explant induced callus*
Control	0.0	6	0	0.00 c
2,4-D	2.0	6	0	0.00 c
	5.0	6	3	1.00 b
	10.0	6	5	1.66 a
	20.0	6	0	0.00 c

(* In each column, means followed by the same letter are not significantly different according to Duncan's multiple range test at P=0.05**.)**

Table 3.2: Effect of 2,4-D on callus induction in *R. serpentina* (L.) Benth, leaf as explant

	Concentration (µM)	No. of explants (leaf)	No. of explants responded	Mean no. of explant induced callus*
Control	0.0	12	0	0.00 c
2,4-D	2.0	12	0	0.00 c
	5.0	12	5	1.25 b
	10.0	12	11	2.75 a
	20.0	12	0	0.00 c

(* In each column, means followed by the same letter are not significantly different according to Duncan's multiple range test at P=0.05.)

3.3 Thin layer chromatography

The methanolic extracts from callus obtained from leaf, invitro explant and also from *in vivo* root were spotted on TLC Silica gel 60F plate. They spots were identified under UV light.

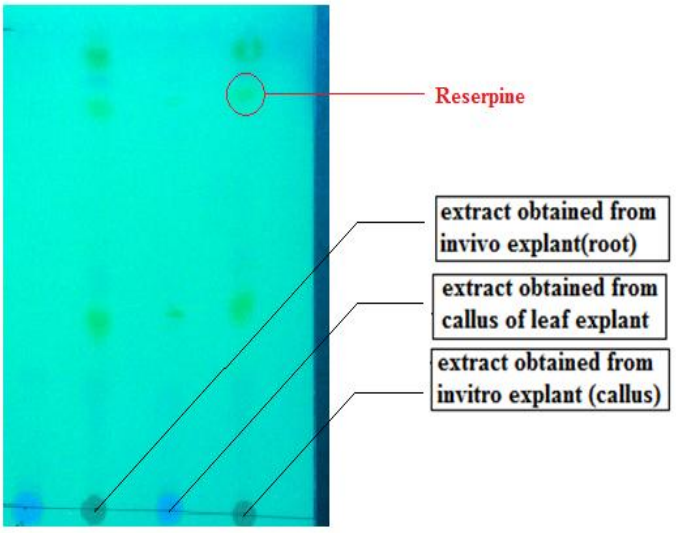

Fig 3.15: Thin layer chromatography of different extracts

The spot in the figure 3.15 had a RF value of 0.95. The standard RF value of Reserpine is 0.96 for the same mobile phase [14].this reveals the presence of Reserpine in all extracts we chose. And intensity of the spot shows

extract obtained from callus of invitro explant has higher concentration of reserpine (fig 3.15).

3.4 Hairy root initiation

Initiation of hairy roots was observed in the leaf explants that were infected with *A. rhizogenes*. Fig 3.16 shows the hairy root initiated after infection of *A.rhizogenes, this* was observed after 4th week of co-cultivation.

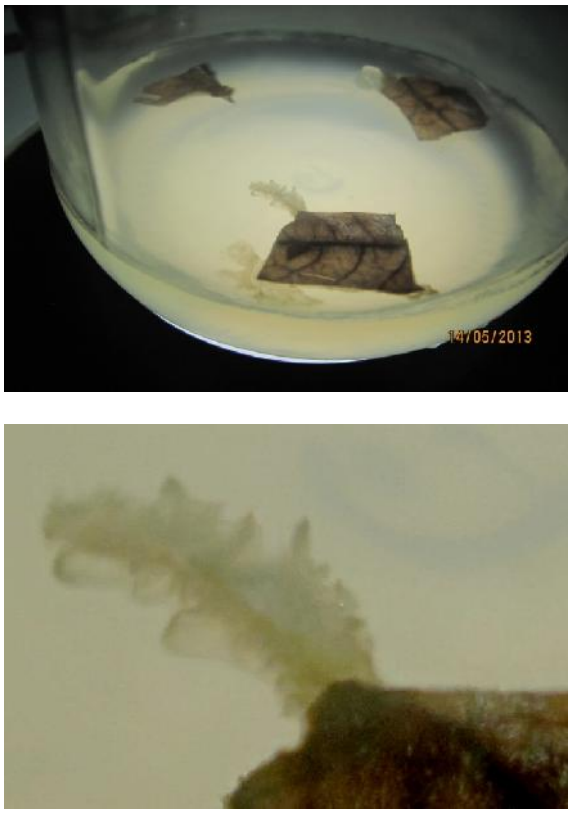

Fig 3.16: Hairy root induced from leaf explants

IV. DISCUSSION

The above studies showed that the seed germination potency is been greatly affected by concentrations of 2, 4-D and BAP in the medium. Out of four combinations tried, only 2µM BAP + 0.5µM 2, 4-D promoted embryo germination and seedling which was then sub cultured to induce callus. The induction of callus primarily focuses on two influential factors - the type of explants used and the combinations of supplements that are been used for the cultures. Callus induction in various species have shown that the different concentrations of auxins and cytokinins influence the callus induction [15], and results suggested that 2, 4-D will show better results in inducing callus compared to other auxins[16]. Based on the results, 5 or 10 µM 2, 4-D showed higher rate of response in both invitro explant and leaf explants.

Optimum concentration of 2,4-D that influence callus induction from the above studies is 5µM 2,4-D or 10µM 2,4-D, and also leaf Explants showed good response compared to hypocotyls. The protocol used in present study is simple, easy to carry out and can provide optimum conditions for callus induction. This finding opens up new prospects for using 2, 4-D as an inducer for callus in *R. serpentina* [9]. TLC being an effective method to identify the presence of many metabolites, the presence of Reserpine was identified by the standardized method [14]. From the observations made, darker spots indicating the high concentration of Reserpine, this enables us to roughly see which extract has higher concentration of Reserpine.

The hairy roots by *Agrobacterium rhizogenes* infection is known for its Ri plasmid and its capability for transferring specific genes. But individually without carrying any foreign gene, Ri plasmid induces the special kind of root structures. On the other hand it also can be used for secondary metabolites production. In present study, initiation of hairy root in leaf explants have showed successful transfer of Ri plasmid, and that can be utilized for the production of secondary metabolites.

V. CONCLUSION

In present study, optimization of media was standardized for callus induction and the efficiency of callus induction of *R. serpentina (*L.) benth was based on the concentration of 2,4-D. The Thin layer Chromatography results revealed that the reserpine was present *in vivo, in vitro* explants and callus, which can be enhanced by using various methods. Further, hairy roots were induced from leaf explants and these hairy roots would be utilized for large scale production of secondary metabolites.

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