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In vitro Callus Culture in *Rauvolfia tetraphylla* L.: Indole Alkaloid Production

[1]S. Anitha and [2]B.D. Ranjitha Kumari
[1]Department of Botany, Government Arts College (Autonomous), Salem-636 007, Tamil Nadu, India
[2]Department of Plant Science, Bharathidasan University, Tiruchirappalli-620 024, Tamil Nadu, India

Abstract: Reserpine is an indole alkaloid reported to possess various biological activities and is chiefly used in chronic hypertension treatment for its hypotensive property and in the treatment of various psychological disorders for its tranquilizing effect. Reserpine is in great demand in the pharmaceutical industries for drug preparation and it has good export value. The quantity of this compound in intact plant varies with the source plant, place of its cultivation and the time of harvest. Hence the present study is undertaken with an objective of producing reserpine under controlled conditions *in vitro* by adjusting phytohormones concentration, sucrose concentration and light illumination duration. Various explants of *Rauvolfia tetraphylla* L. were tested for callusing and the reserpine content was analysed in the calli and the source plant. In the source plant, maximum amount of reserpine was found to present in the root (0.38 mg g^{-1} DW). Leaf derived callus was found to produce more amount of reserpine (0.9 mg g^{-1} DW) than calli derived from other explants, cultured on MS+B_5 medium fortified with 9.04 μM 2,4-D and 4% sucrose with 16/8 hr light/dark illumination duration. This protocol can be used for commercial production of reserpine.

Key words: *Rauvolfia tetraphylla*, *In vitro* production, light, reserpine, root, sucrose

INTRODUCTION

Rauvolfia Plummer is a genus comprises around 1500 species distributed in tropical Africa, America and Asia (Bhattacharjee, 2004). *Rauvolfia tetraphylla* L. (Four-leaf devilpepper) is a woody shrub that belongs to the family Apocynaceae. The root of this plant is widely used as hypotensive in high blood pressure treatment and tranquilizer in various psychiatric diseases (Fasil and Anis, 2002; Pullaiah, 2002). This plant roots contain various monoterpene indole alkaloids and reserpine is reported to present in more than 50% of total alkaloids. Reserpine is reported to possess various biological activities such as antiadrenergic, anticonvulsant, antidyskinetic, antileukemic, antimigraine, antipsychotic, antipyretic, antischizopherenic, antistress, antitumor, bradycardic, CNS-depressant, Calcium-antagonist, catechoaminolytic, hepatoprotective, hypotensive, hypothermic, MDR-inhibitor, peristaltic, rodenticide, sedative, serotoninolytic, teratogenic, tranquilizer, uterotonic and vasoconstrictor (Duke's 2005 Phytochemical and Ethnobotanical Databases, visited on December 2005). Though reserpine has many biological activities, it is chiefly used in chronic hypertension treatment for its hypotensive property and in the treatment of various psychological disorders for its tranquilizing effect (Pullaiah, 2002). Reserpine is in great demand in the pharmaceutical industries for drug preparation and it has good export value (Hoareau and DaSilva, 1999). From the field cultivated plants, the total yield of the alkaloids was reported as 0.7-1.2% of the total root dry mass. The roots of *R. tetraphylla* are often used as a substitute to the roots of *R. serpentina*, a widely exploited plant for indole alkaloids extraction (Sharma, 2004). The indiscriminate collection and limited cultivation of both *R. tetraphylla* and *R. serpentina* made these plants unavailable normally and they are listed as endangered (Swarup and Arora, 2000). The production of alkaloids from the field cultivated *Rauvolfia* plants is not stable and it varies depending upon the plant species and duration of cultivation (Kokate *et al*., 1998), place of the plant growth (Libot *et al*., 1986), agronomic practice like intercropping with other plants (Maheshwari *et al*., 1985) and the time of harvest (Bhattacharjee, 2004). Hence, the present study conducted with an objective to optimize tissue culture conditions for stable production of reserpine from callus culture of *R. tetraphylla*.

MATERIALS AND METHODS

Surface sterilization and explant preparation for callus induction: The experiment was conducted in the

Corresponding Author: S. Anitha, Department of Botany, Government Arts College (Autonomous), Salem-636 007, Tamil Nadu, India

Department of Plant Science, Bharathidasan University from 2003 to 2007. Leaf explants were collected from the field grown plants of the medicinal plants garden of Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The explants were thoroughly washed in running tap water to remove the dirt particles from the surface of the explants. The explants were taken to the laminar airflow chamber and surface sterilized with 70% (v/v) ethyl alcohol for 45 sec followed by 0.1% (w/v) mercuric chloride for four min. The explants were finally washed with sterile distilled water for 4-5 times. Dead portions from the surface sterilized leaf explants were removed in all the margins and the leaves were cut into approximately 0.5 cm lengthy segments. Similarly, the surface sterilized node and internode explants of 0.5 cm segments were used for callus induction. All the explants were slightly wounded on the surface to enhance the production of callus.

Seed source: Seeds from the fully ripened fruits (purple to black coloured) were collected in the month of December, from three years old garden plants and washed thoroughly in tap water to remove the adhering pulp. Seeds of two different sizes produced in the plants , only the fully developed seeds were collected and soaked in water, the seeds whichever sink immediately were separated from the rest. The isolated seeds were again washed in tap water and completely dried. The dried seeds were stored in air tight container at 4°C. The seeds were used upto four months from the date of collection.

Surface sterilization of seeds: The seeds were soaked in tap water for one to two hours and then treated with 1% Teepol solution for 20 min. The seeds were washed in distilled water for two times and taken into the laminar air flow chamber. Then the seeds were treated with 70% ethanol for 1 min and then washed in sterile distilled water for two times and 0.2 % mercuric chloride treatment was given for 7 min. The seeds were thoroughly washed in autoclaved distilled water for 4-5 times to remove the traces of sterilizing agents.

Seeds treatments: The seeds were treated with 85% sulphuric acid for 20 min (scarification) and kept under running tap water for 1 h. Surface sterilization was carried out as explained earlier and the seeds were inoculated for germination. After surface sterilization, the hard seed coat was split without damaging the embryo, introduced into filter sterilized (0.22 μM filter) 0.001% GA_3 solution and allowed to rinse for 30 min. Then the seeds were inoculated for germination in both moist cotton and soil mixture.

***In vitro* seed germination:** The untreated, sulphuric acid and GA_3 treated seeds were introduced onto sterile moist cotton filled in bottles. The seeds also germinated in sterile soil mixture consist of sand, garden soil and farmyard manure in the ratio 1:2:1. The seeds were initially kept under dark for 2-7 days and after the bottles were transferred to 16/8 h light/dark condition. Uniform temperature was maintained (25±1°C) during both light and dark conditions. After 20-25 days of inoculation, the germinated seedlings were used to collect cotyledonary leaf, hypocotyl and root explants (0.5 - 0.7 cm length) and the seedling derived explants also wounded on the surface to enhance the callusing response.

Callus induction: The callus induction medium was prepared by using MS+B_5 medium (MS salts+B_5 vitamins), sucrose (4%), agar (0.8%) and phytohormones such as 2,4-D, NAA, IAA, IBA, BA and KN. The phytohormones with the following concentrations were used in the callus induction medium; 2,4-D at 2.26-11.3 μM, NAA at 0.537-11.04 μM, IAA at 0.57-11.93 μM, IBA at 0.49-4.92 μM, BA at 2.22-8.88 μM and KN at 2.32-9.28 μM. The pH of the medium was adjusted to 5.68 to 5.72 and the medium was sterilized at 15 lb for 15 min. The cultures were exposed to 25±1°C and 16/8 hr light/ dark conditions by using cool white fluorescent tubes (40 μM m^{-2}/sec). The explants were transferred to fresh medium for every 25 days and the callusing response was recorded periodically. For each concentration of hormones tested, 15 explants were used and the all the experiments were repeated five times for concluding the results.

Light illumination period and sucrose treatments: The callus cultures grown in standardized phytohormone concentrations and combinations were exposed to 16, 24 h light or total darkness to test the effect of illumination duration on total alkaloid synthesis. Sucrose at varying concentrations (1-5%) was added in the callus induction medium to test the effect of sucrose on biomass accumulation.

Analysis of biomass: The callus obtained in each treatment was cut into approximately 50 mg pieces and inoculated on fresh medium with the same composition used for callus induction. After 25 days of culture, the calli were collected, washed with distilled water to remove the medium sticking on the surface of the callus. The calli were blotted in tissue paper and allowed to dry under room temperature and FW (fresh weight) was measured. The callus was dried under 50°C for 24 h and the DW (dry weight) of the callus was calculated. From the fresh and dry weights, the moisture content of the callus was calculated by using the following formula:

$$\text{Moisture content (\%)} = \frac{[\text{Fresh weight} - \text{Dry weight}]}{\text{Fresh weight}} \times 100$$

In the similar way leaf, stem and root collected from *in vivo* sources (2 year old plant) were analyzed for the FW and DW. The dried powder of the samples stored in air tight container at 4°C and used for the alkaloid analysis.

Extraction and quantification of alkaloids: Sheludko *et al.* (1999) method was followed with some modifications for the extraction and analysis of alkaloids. One gram callus was powdered and extracted with 10 mL of 80% methanol. The extracted sample was acidified with 0.1 N HCl and then neutralized with NH_4OH. The calli were re-extracted twice with 10 mL of 80% methanol, 5 mL in each time. The supernatant was evaporated under vacuum and the weight was recorded as crude alkaloid content. Crude alkaloid was dissolved in a few drops of methanol and subjected to TLC and reverse phase HPLC (Shimadzu, Japan) analysis by using reserpine (SRL products, India) as standard. By using the peak height and peak area of the standard, reserpine content in the crude alkaloids was calculated and tabulated.

The HPLC conditions were; Octa decyl silane (ODS-C18) column (250×4.6 mm, Shim pack), UV-Spectrophotometric detector monitoring at 254 nm, guard column-G-ODS (10×4 mm). Column head pressure was maintained at 125 kgf cm^{-2}. Sample (20 μL) was eluted with a mobile phase of acetonitrile at a flow rate of 1 mL per minute.

Statistical analysis: The experiments were conducted in completely randomized design, fifteen replicates were used in each treatment and each experiment was repeated at least three times. The alkaloid content was analyzed in the calli subjected to different treatments up to five subculture periods to check the stability of the cultures. Data were subjected to one way ANOVA and comparison of means were carried out with DMRT at 0.05% significance level using SPSS software version 10.0.

RESULTS AND DISCUSSION

***In vitro* seed germination:** The seed germination was achieved in both sterile soil mixture and cotton. The germination was increased when the hard seed coat was split after surface sterilization. GA_3 treated, seed coat split seeds (S1) showed an increase in germination rate to 90% in the moist cotton. When the seeds were germinated in soil mixture, comparatively high per cent seed germination was observed in S1+GA_3 treated seeds as 96% (Table 1).

Table 1: *In vitro* seed germination on sterile moist cotton and sterile soil mixture in *Rauvolfia tetraphylla* L.

	Germination (%)		
	Days after seed inoculation		
Treatments	9	13	17
Germination in moist cotton			
-	12^{ef}	15^{g}	31^{i}
Splitting of seed coat -S1	23^{c}	38^{d}	67^{cd}
0.001% GA_3	18^{d}	30^{e}	43^{f}
S1+0.001% GA_3	40^{a}	69^{ab}	90^{ab}
85% Sulphuric acid	12^{ef}	21^{fg}	35^{hi}
Germination in soil mixture			
-	15^{e}	23^{f}	42^{fg}
Splitting of seed coat –S1	30^{b}	45^{c}	74^{c}
0.001% GA_3	21^{cd}	36^{de}	51^{e}
S1+0.001% GA_3	40^{a}	74^{a}	96^{a}
85% Sulphuric acid	16^{de}	23^{f}	40^{h}

Treatment means followed by different letters are significantly different from each other at $p \leq 0.05$

Callus induction from different explants: The response of different explants varied with the type and concentration of phytohormones used. The auxins such as IAA and IBA were ineffective for callus induction in all the explants tested. Individual treatment of BA or KN also not induced callus from any explant. 2,4-D was effective for callus induction from petiole, leaf, cotyledonary leaf and hypocotyl explants compared to NAA. The callus induced by 2,4-D was compact and the colour varies from light green to dark green in young leaf and cotyledonary leaf explants, respectively. At higher concentration of 2,4-D (11.3 μM), the callus induced from leaf explants were white in colour. In the petiole explants, yellowish callus was produced and in the hypocotyl and root explants the callus was loosely arranged and brown in colour. Among all the explants, the leaf explants showed maximum callus induction (90%) response. The next best responded explant was cotyledonary leaf with 64% callus induction. In hypocotyl explants the maximum response was observed as 60% at 2.26 μM concentration of 2,4-D. The node and internode explants responded well to NAA at 10.74 and 5.37 μM concentrations respectively. The callus induced by NAA was green, compact and nodular in nature. Both the explants showed about 50% callusing response at the above mentioned NAA concentrations (Table 2).

The moisture content also varied in the calli derived from different explants. Leaf derived callus had high dry weight than the calli derived from other explants (Table 4).

In the present study, 2,4-D was proved as the best auxin for callus induction from leaf, cotyledonary leaf, petiole, root and hypocotyl explants. 2,4-D was also used for callus induction in *R. serpentina* (Ilahi and Khanm, 1987; Parveen and Ilahi, 1987; Reddy *et al.*, 2005). The superiority of 2,4-D on callus induction of different

Table 2: Effect of 2,4-D and NAA on callus induction from different explants of *Rauvolfia tetraphylla* L. cultured on MS+B_5 medium

	Callusing response (%)						
Phytohormone concentration (µM)	Petiole	Leaf	Internode	Node	Cotyledon leaf	Hypocotyl	Root
2,4-D							
2.26	25^{fg}	50^{e}	20^{f}	35^{d}	30^{e}	60^{a}	-
4.52	52^{a}	67^{cd}	25^{e}	45^{b}	35^{d}	50^{b}	20^{b}
6.78	46^{b}	73^{c}	25^{e}	49^{ab}	46^{c}	42^{bc}	33^{a}
9.04	32^{de}	90^{a}	30^{d}	40^{c}	64^{a}	23^{d}	15^{bc}
11.3	22^{g}	81^{b}	20^{f}	23^{f}	64^{a}	15^{de}	-
NAA							
0.537	28^{f}	20^{h}	45^{bc}	-	25^{f}	-	-
2.69	35^{d}	31^{g}	48^{b}	-	33^{de}	-	-
5.37	40^{c}	45^{ef}	56^{a}	20^{fg}	35^{d}	15^{de}	-
8.06	25^{fg}	40^{f}	45^{bc}	31^{de}	30^{e}	-	10^{c}
10.74	-	20^{h}	30^{d}	53^{a}	21^{fg}	-	20^{b}

Treatment means followed by different letters are significantly different from each other at $p \leq 0.05$

Table 3: Moisture content, total alkaloid and reserpine content in *in vivo* plant parts of *Rauvolfia tetraphylla* L.

Source	Moisture content (%)	Total alkaloids (mg g^{-1} DW)	Reserpine (mg g^{-1} DW)
Leaf	69.85^{a}	3.8^{ab}	0.18^{b}
Stem	65.00^{ab}	0.5^{c}	0.01^{bc}
Root	43.00^{c}	6.0^{a}	0.38^{a}

Treatment means followed by different letters are significantly different from each other at $p \leq 0.05$, DW: Dry Weight

Table 4: Biomass and reserpine content in the callus derived from different explants of *Rauvolfia tetraphylla* L. cultured on MS+B_5 medium

Callus source	Phytohormone concentration (µM)	Fresh weight (g)	Dry weight (g)	Moisture content (%)	Reserpine (mgg^{-1}DW)
Petiole	4.54 µ (2,4-D)	0.1100^{d}	0.0250^{ef}	77.27	ND
Leaf	9.04 µ (2,4-D)	0.3354^{a}	0.0826^{a}	75.37	0.9^{a}
Inter node	5.37 µ (NAA)	0.1250^{cd}	0.0310^{de}	75.20	Traces
Node	10.74 µ (NAA)	0.1050^{d}	0.0270^{e}	74.29	0.01^{cd}
Cotyledonary leaf	9.04 µ (2,4-D)	0.2100^{b}	0.0530^{b}	74.76	0.3^{c}
Hypocotyl	2.26 µ (2,4-D)	0.160^{c}	0.0380^{d}	76.25	ND
Root	6.78 µ (2,4-D)	0.185^{bc}	0.0500^{bc}	72.97	0.6^{b}

ND: Not detected, Treatment means followed by different letters are significantly different from each other at $p \leq 0.05$, DW: Dry Weight

plant species was reported in various studies (Ramgopal, 1986; Galibo and Yamada, 1988; Thomas *et al*., 1992; Harikrishnan and Hariharan, 1996; Iyer *et al*., 1998).

Reserpine accumulation in *in vivo* sources: Reserpine accumulation was found high in the root (0.38 mg g^{-1} DW), followed by leaf (0.18 mg g^{-1}DW). Reserpine accumulation was very low in the stem as 0.01 mg g^{-1} dry weight (Table 3).

Reserpine accumulation in the callus derived from various explants: Reserpine accumulation was varied in the callus derived from various explants after 25 days of culture. Reserpine accumulation was high in the leaf explants cultured on MS+B_5 medium supplemented with 9.04 µM 2,4-D (0.9 mg g^{-1} DW) followed by root derived callus at 6.78 µM 2,4-D (0.6 mg g^{-1} DW). In the calli induced from petiole and hypocotyl explants, reserpine was not detected. Callus induced from internode explants were also accumulated very less amount of reserpine (Table 4).

Table 5: Effect of sucrose on biomass accumulation in the compact calli of *Rauvolfia tetraphylla* L. cultured on MS+B_5 medium

Sucrose concentrations (%)	Fresh biomass (g)	Dry biomass (g)	Moisture content (%)	Reserpine content (mg g^{-1} DW)
1	0.2300	0.0400^{e}	82.60^{a}	0.20^{d}
2	0.2800	0.0554^{d}	80.20^{b}	0.30^{cd}
3	0.2900	0.0630^{b}	78.27^{c}	0.33^{c}
4	0.3354	0.0826^{a}	75.37^{de}	0.90^{a}
5	0.2900	0.0650^{bc}	77.00^{d}	0.68^{b}

Treatment means followed by different letters are significantly different from each other at $p \leq 0.05$, DW: Dry Weight

Effects of sucrose on reserpine accumulation: Biomass increase was observed with increase with the sucrose concentration in the culture medium up to 4%. At low concentrations of sucrose (1, 2 and 3%), the moisture content of the callus was found high. At 4% sucrose concentration, the biomass accumulation was high than the other concentrations of sucrose. When the sucrose concentration increased to 5%, biomass accumulation was reduced in terms of both FW and DW (Table 5).

In the present study, 4% sucrose was proved effective for more biomass production as well as for reserpine accumulation than other concentrations (Table 5). Chattopadhyay *et al.* (2002) reported that podophyllotoxin production was induced with 3% sucrose than other carbohydrate sources in *Podophyllum hexandrum.* Increased sucrose concentration usually results in increased biomass and secondary metabolite production of plant cell cultures, as well as some nodule cultures and compact callus aggregates (Ellis *et al*., 1996; Xu *et al*., 1998). The concentrations of sucrose and auxins or the ratio of them are supposed to influence the differentiation of phloem or xylem in plant cultures (Bolwell, 1985) besides acting as a carbon source or an osmotic stress factor (Zhao *et al*., 2001).

Callus induction at different light exposure duration: Callus initiation from edges and wounded regions was observed after three weeks of inoculation under

Table 6: Effect of light illumination period on growth and alkaloid production in compact calli of *Rauvolfia tetraphylla* L. cultured on MS+B_5 medium

Light exposure duration (h)	Dry biomass (g)	Moisture content (%)	Reserpine (mg g^{-1} DW)
0	0.0950^{a}	93.00^{a}	0.1^{bc}
16	0.0826^{ab}	75.37^{b}	0.9^{a}
24	0.0700^{c}	72.24^{bc}	0.45^{b}

Treatment means followed by different letters are significantly different from each other at $p \leq 0.05$, DW: Dry Weight

16/8 h light/ dark condition, whereas, this is delayed in complete exposure to light. Complete darkness favored the callus initiation and growth response. High moisture content was observed in the callus obtained in complete darkness and the moisture content reduced with increase in light exposure duration (Table 6).

Reserpine accumulation was high in the leaf derived callus cultured at 16 h photoperiod than 24 h light or complete dark conditions (Table 6). Light increased the alkaloid biosynthesis in 16 h exposure duration than the dark and 24 h illumination conditions. Although, the alkaloid content was increased in 16 h light exposure, continuous light illumination hindered the callus growth and the alkaloid production (Table 6). It is well known that light controls the development of plant chloroplasts and in the developed chloroplasts many important precursors for indole alkaloid biosynthesis are synthesized. There is a possibility that light induces differentiation processes including the activation of genes, which are not expressed in the dark (De Luca *et al*., 1988; Vazquez-Flota and De Luca, 1998; Schroder *et al*., 1999; Zhao *et al*., 2001).

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