In vitro regeneration technique in Rauwolfia serpentina and quantification of reserpine

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Abstract

The reliable technique for the indirect regeneration of R. serpentina was standardised in the present investigation. The secondary metabolites extracted from callus using methanol and analysed using Thin Layer Chromatography (TLC) and UV-Vis Spectrophotometer. The in vitro induction of callus influenced by several factors such as media composition and explants quality. From the various media combinations of 2.4-D and BAP the frequency of highest callus induction (94.67%) was observed on MS + 2.0 mgL⁻¹ 2.4-D + 1.0 mgL⁻¹ BAP when leaf disc used as explant. Throughout the organogenic callus induction, different calli were observed having variation in colour and texture. The shoot regeneration frequency was observed highest (98.33%) in 4.5 mgL⁻¹ BAP + 0.5 mgL⁻¹ NAA. Maximum shoots (15.33 per callus) were observed in leaf callus. The rooting was induced (95.33%) in in vitro regenerated shoots in Murashige and Skoog (MS) medium which contain 1.0 mgL⁻¹ NAA + 0.1 mgL⁻¹ BAP + 1 gl⁻¹ activated charcoal (AC). The in vitro regenerated plantlets through callus culture were hardened in the greenhouse with 75 per cent establishment. The quantity and quality of reserpine was measured in callus and root of R. serpentina. The TLC reveals the accumulation of reserpine at callus as well as roots of cultivated plant. Spectrophotometric estimation showed that concentration of reserpine observed in callus extract was higher (6.8 µgml⁻¹) than the root extract (6.4 µgml⁻¹).

Keywords: Rauwolfia serpentina, callus, reserpine, secondary metabolite

Introduction

Rauwolfia serpentina (L.) family Apocynaceae is a medicinal shrub which is commonly known as sarpagandha. In case of R. serpentina roots are rich source of Indole alkaloids such as, serpentine, recimamine, reserpine ajmalicine, ajmaline, etc. According to ayurveda, whole plant of R. serpentine, specially roots are used for the treatment of various diseases, such as snake bite, rheumatism, cardiovascular disorder, hypotension, insanity, epilepsy, eczema (Joshi et al., 2010). R. serpentina endanger in India because of non-selective plantation and overexploitation of naturally available sarpagandha to fulfill the requirement of industry (Nayar and Sastry, 1987) [9]. IUCN has declare the sarpagandha plant is under endangered condition. The endangered status of sarpagandha is due to poor seed viability and low seed germination rate (Chaudhari et al., 2015) [4]. To satisfy the increasing commercial demand of alkaloids and conservation of this important endangered plant there is a need to produce R. serpentina under in vitro condition (Bhatt et al., 2008) [2]. The quality of alkaloids extracted for in vitro callus culture is almost same as to that of natural sources of alkaloids. (Yoshimatsu and Shimomura, 1991) [18]. Sometimes, higher production capacity of producing secondary metabolites observed in callus culture (Benavides and Caso, 1993) [1] and the callus culture can also efficient and reliable to produce alkaloids at high mass (Yamamoto and Yamada, 1986). The methanolic extraction of alkaloids is also reliable in case of callus culture (Kirillova et al., 2001) [6]. After understanding this problems, the present study deals with standardization technique for in vitro regeneration through callus of leaf, leaf node and stem, and analysis of estimated of secondary metabolite.

Materials and Methods

1. Collection of explant

The experimental material of the present investigation was collected from the Charak nursery, College of Forestry, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist.- Ratnagiri. The seedlings used as source of explants were potted and maintained in the greenhouse.
2. Surface sterilisation
Different explants such as leaf disc, leaf node and meristem were washed with running tap water for 5 minutes and subsequently with distilled water. Explants were treated with carbendazim ethanol, mercuric chloride and subsequently with distilled water in laminar airflow cabinet. Sterilised filter paper used to blot the explants before inoculating on Murashige and Skoog (MS) media. The MS media prepared as per the standard procedure with addition of heat resistant growth regulators and pH was adjusted to 5.8.

3. Callus induction
For callus induction juvenile leaf disc, leaf node and meristem of 5 mm length were cut with sterile scalp and inoculated on MS medium having appropriate concentrations of growth hormones which enhance callus induction. The inoculated cultures were kept in incubation room up to 21 days by adjusting 16 hrs. light and 8 hrs. dark period.

4. Shoot regeneration from callus
The callus growing on proliferation nutrient media was cut with sterile scalp into 0.5 mm size and inoculated on shooting media for shoot initiation and multiplication. To obtain the more number of shoots the MS media supplemented with various concentration of BAP and NAA. After that culture was incubated at 16 hrs. light and 8 hrs. dark period on a constant temperature (25 °C) in incubation room. The sub culturing was continued every after 21 days to get more number of shoots.

5. Rooting and hardening
After development of multiple shoots, the well grown elongated shoots were separated from each other and transferred on media which is favorable for rooting. The MS media was prepared with different concentration of auxin (NAA) and cytokinin (BAP) and activated charcoal for further root development. Hardening is a critical step, the tissue culture plants are taken out from MS media, carefully washed roots with water The potting mixture (coco peat + vermiculite) was used for primary hardening and incubated plants in growth chamber for 15 to 20 days for acclimatisation by maintaining humidity temperature and light. Then the plants are transferred for secondary hardening on another potting mixture (soil, sand and vermiculite; 1:1:1) and kept under natural condition.

6. Extract preparation
The methanolic extraction of R. serpentine was done using 100 g callus which is soaked in 10 mL methanol and kept for 30 min. at room temperature. The extract was filtered using Whatman filter paper No.1, and residue obtained after filtration again dissolved 5 mL of methanol and kept for 10 min. at room temperature. Repeated this process to get 50 mL volume of total filtrate. The filtrate was evaporated by keeping the extract in beaker (without lid) for 20 to 24 hrs. After that the crude extract was dissolved properly in 100 mL HCL (0.001M) and pH was adjusted to 6.00 using NaOH (0.001M). The obtained extract was used for the TLC as well as spectrophotometric analysis.

7. Qualitative analysis of secondary metabolites by Thin Layer Chromatography (TLC)
The TLC was used to check the quality of major groups of alkaloid derivatives present in extract obtained from callus of R. serpentina. The chloroform and methanol (97:3) were used to prepare solvent system for TLC. After completion of the TLC, the Dragendorff’s reagent used to visualise the spots which develop orange s.pots. The spots intensified by using HCl or 50 per cent water-phosphoric acid and then Rf value was calculated for the extract.

8. Quantitative estimation of secondary metabolites using UV-Vis Spectrophotometer:
One milligram of reserpine was taken and dissolved in 10 ml of methanol and various dilutions are made from it having concentration (2 μg/mL-1-10μg/mL-1). All the various dilutions of reserpine were observed under UV spectrophotometer using λmax 268 nm. Absorbance of all the samples and standard was calculated. The experiment was done in triplicate. Calibration curves series of standard curves were prepared with a concentration range 2-10 μg (n=3, five standards). The data of concentration versus absorbance was analyzed by linear test square regression analysis.

Result and Discussion
1. Surface sterilisation
Explants (leaf disc, leaf node and meristem) washed thoroughly with running tap water for 5 min. and then with distilled water. After that explants were treated with 0.1% carbendazim for 10 min. and then rinsed thoroughly with sterile distilled water. The leaves were treated with 70 per cent ethanol for 30 sec., washed with distilled water and then treated with 0.1 per cent mercuric chloride HgCl2 for 5 min. and again washed with distilled water, showed the maximum frequency (95.33%) of aseptic callus (Data not presented).

2. Callus induction
Murashige and skoog media in the absence of growth hormone was not efficient to induce callus (Shah et al., 2003). All the three explants (Leaf, leaf node and meristem) produced callus in MS medium prepared by using 2,4-D (0.5-5.0 mgL-1) and BAP (1.0 mgL-1). From all growth hormones, 2,4-D was more efficient in callus induction. The best response was obtained in MS medium, prepared by using 2,4-D (2.0 mgL-1) in all three explants. Among the three explants the maximum callus induction frequency was noticed in leaf disc (94.67%) followed by leaf node (91.33%) and meristem (80.67%) (Table 1). Sarkar et al. (1996) reported that MS medium prepared by using auxins and cytokinins specifically 2,4-D and BAP having more efficacy of callus induction. Callus cultures initiated through explants taken from any plant parts and the percent callus induction varies according to explant. The type of explant used for the callus induction have efficient role in healthy growth of callus. Among various explants leaf disc explants gave good response for callus induction have been recorded in R. serpentina by Roja et al. (1987), Upadhyay et al. (1992) and Panwar et al. (2011).

3. Proliferation of callus
One-month-old callus was sub-cultured in the medium which is used for callus induction for proliferation of healthy callus. Green-friable callus was obtained in leaf disc and leaf node explants while yellowish-friable callus was obtained when meristem used as explants after 25 to 30 days of subculture (Figure 2). Leaf explant produced loose callus while meristem explant produces tough and compact callus. Growth regulators and its concentration in the medium also plays important role in dedifferentiation of tissues. Proper combination of auxins and cytokinins resulted in efficient
callus proliferation. Pant and Joshi (2008) \[10\] reported that after 10 to 14 days, all sources of the explants (leaf disc, leaf node and meristem) were swelled and callus initiation stared from only cut surface of explants because of injury.

4. Shoot induction
The callus obtained from leaf disc, leaf node and meristem explants was failed to generate shoots in MS medium (without growth regulators). The medium prepared by using BAP (1.5-5.5 mgL\(^{-1}\)) generate multiple shoots in all explants and the shoot induction percentage was calculated which lies between 24.33-98.33 per cent. Table-2 shows that the medium containing 4.0 mgL\(^{-1}\) BAP + 0.5 mgL\(^{-1}\) NAA was observed as the most effective combination for multiple shoot induction (98.33\%) when the callus derived from leaf disc. Leaf node and meristem derived callus showed maximum per cent shooting 67.33 per cent and 97.17 per cent, respectively on MS medium supplemented with 4.5 mgL\(^{-1}\) BAP + 0.5 mgL\(^{-1}\) NAA. Medium containing 4.5 mgL\(^{-1}\) BAP + 0.5 mgL\(^{-1}\) NAA was observed as the most efficient combination for getting multiple shoot induction, about 25 shoots per culture were observed (Figure 3). These results are showing similarity with Pant and Joshi (2008)\[11\] that the high concentration of cytokinin in combination with auxin have the most effective combination for getting multiple shoots. Pant and Joshi (2008)\[11\] reported that the high concentration of cytokinin in combination with auxin have the most effective combination for getting multiple shoots. The multiple shoot induction can be also influenced by physiological status and level of endogenous hormones.

5. Rooting and hardening
The in vitro produced multiple shoots were successfully transferred on rooting medium supplemented with NAA and BAP. However, when the medium was prepared by using NAA 1.0 mgL\(^{-1}\) + BAP 0.1 mgL\(^{-1}\) 95.33 per cent rooting was achieved (Figure 4, Table 3). The plantlets having healthy well developed roots were hardened in green house condition and slowly acclimatized with environment with a success rate of 75 per cent (Figure 5). Roy et al. (1995)\[14\] reported that the overall success of organogenesis depends on the efficiency of transferred plants to survive in environmental condition. The plants produced through in vitro technique are acclimatized to unique set of growth conditions which may increase rapid growth and multiplication in the field.

6. Qualitative analysis of secondary metabolites by Thin Layer Chromatography
The methanolic extract obtained from leaf callus, in vivo root and standard were spotted on TLC Silica gel 60F plate (Figure 6). The spot of callus in the figure shown R\(_t\) value of 0.96. The standard R\(_t\) value of reserpine is 0.96 and spot of root extract shown a R\(_t\) value 0.957 (Table-4). These observations clearly revealed the presence of reserpine in callus and root extracts, and the intensity of the spot showed extract obtained from callus had higher concentration of reserpine. Callus culture contains more or less homogenous clumps of dedifferentiated cells are used for secondary metabolite production. Similarly, alkaloids from Bael (Aegle marmelos) were extracted by Borde et al. (2011)\[13\] in 95 per cent ethanol and separated with chloroform by using synthetic antioxidant as a standard.

7. Quantitative estimation of secondary metabolites by using spectrophotometer
One milligram of reserpine dissolved in 10 ml of methanol and various dilutions are made from it having concentration (2 μgml\(^{-1}\)-10 μgml\(^{-1}\)). Absorbance of standard was recorded (Table-5), with respect to absorbance of standard the unknown concentration of reserpine present in extract was determined by extrapolation (Figure 7). The concentration of reserpine observed in callus extract was 6.8 μgml\(^{-1}\) whereas the concentration of reserpine observed in root extract was 6.4 μgml\(^{-1}\) (Table 6). In the present study, it was observed that the concentration of reserpine observed in callus extract was 6.8 μgml\(^{-1}\) whereas the concentration of reserpine observed in root extract was 6.4 μgml\(^{-1}\). Callus contain higher amount of reserpine than the roots of cultivated plant, some compounds which are infecting the reserpine in the field should be avoided during callus culture (Panwar and Guru, 2011; Mallick et al., 2012)\[11, 12, 7\].

| Sr. No. | Combination details | Explants | Mean (%) |
|---------|---------------------|----------|----------|
| C1M1    | MS medium (control) |          |          |
| C1M2    | MS + 0.5 mgL\(^{-1}\) BAP |          |          |
| C1M3    | MS + 1.0 mgL\(^{-1}\) BAP |          |          |
| C1M4    | MS + 1.5 mgL\(^{-1}\) BAP |          |          |
| C1M5    | MS + 2.0 mgL\(^{-1}\) BAP |          |          |
| C1M6    | MS + 2.5 mgL\(^{-1}\) BAP |          |          |
| C1M7    | MS + 3.0 mgL\(^{-1}\) BAP |          |          |
| C1M8    | MS + 3.5 mgL\(^{-1}\) BAP |          |          |
| C1M9    | MS + 4.0 mgL\(^{-1}\) BAP |          |          |
| C1M10   | MS + 4.5 mgL\(^{-1}\) BAP |          |          |
| C1M11   | MS + 5.0 mgL\(^{-1}\) BAP |          |          |

| Mean (%) |          |          |
|----------|----------|----------|
|          | 53.39(46.94) | 49.52(44.72) | 40.00(39.23) | 47.64(43.64) |

Table 2: Per cent shooting

| Sr. No. | Combination details | Callus | Mean (%) |
|---------|---------------------|--------|----------|
| SIM1    | MS medium (control) |        |          |
| SIM2    | MS + 1.0 mgL\(^{-1}\) BAP + 0.5 mgL\(^{-1}\) NAA |        |          |

| Mean (%) |          |          |          |
|----------|----------|----------|----------|
|          | 0.00(0.00) | 0.00(0.00) | 0.00(0.00) | 0.00(0.00) |

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~ 522 ~
**Table 3**: Per cent rooting

| Sr. No. | Combination details | Explants | Mean (%) |
|---------|---------------------|----------|----------|
|         |                     | Leaf disc (%) | Leaf node (%) | Meristem (%) | Mean (%) |
| RIM1    | MS medium (control) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| RIM2    | MS + 1.0 mgL⁻¹ NAA + 1.0 mgL⁻¹ BAP | 24.67 (29.77) | 20.67 (27.03) | 16.67 (24.09) | 20.67 (27.03) |
| RIM3    | MS + 0.5 mgL⁻¹ NAA + 1.5 mgL⁻¹ BAP | 56.00 (48.44) | 49.33 (44.61) | 46.67 (43.08) | 50.67 (45.38) |
| RIM4    | MS + 0.1 mgL⁻¹ NAA + 2.0 mgL⁻¹ BAP + 1 gl⁻¹ Activated charcoal | 77.33 (61.56) | 72.67 (58.47) | 68.67 (55.96) | 72.89 (58.62) |
| RIM5    | MS + 1.0 mgL⁻¹ NAA + 0.1 mgL⁻¹ BAP + 1 gl⁻¹ Activated charcoal | 95.33 (77.52) | 91.33 (72.87) | 87.33 (69.15) | 91.33 (72.87) |
| RIM6    | MS + 1.0 mgL⁻¹ NAA + 0.5 mgL⁻¹ BAP + 1 gl⁻¹ Activated charcoal | 87.33 (69.15) | 83.33 (65.90) | 79.33 (62.96) | 83.33 (65.90) |
| RIM7    | MS + 1.0 mgL⁻¹ NAA + 0.5 mgL⁻¹ BAP + 1 gl⁻¹ Activated charcoal | 46.67 (43.08) | 40.67 (39.62) | 37.33 (37.66) | 41.56 (40.13) |
| RIM8    | MS + 1.0 mgL⁻¹ NAA + 1.5 mgL⁻¹ BAP | 21.33 (27.50) | 16.67 (24.09) | 13.33 (21.41) | 17.11 (24.43) |
| RIM9    | MS + 1.0 mgL⁻¹ NAA + 0.1 mgL⁻¹ BAP + 1 gl⁻¹ Activated charcoal | 85.33 (67.48) | 76.67 (61.11) | 73.33 (58.90) | 78.44 (62.33) |
| RIM10   | MS + 2.0 mgL⁻¹ NAA + 1.0 mgL⁻¹ BAP + 1 gl⁻¹ Activated charcoal | 92.67 (74.28) | 86.67 (68.58) | 82.00 (64.89) | 87.11 (68.96) |
| RIM11   | MS + 2.0 mgL⁻¹ NAA + 3 mgL⁻¹ BAP | 12.67 (20.85) | 10.00 (18.43) | 8.67 (17.12) | 10.44 (18.85) |

**Table 4**: Thin layer chromatography

| Sr. No. | Sample | Distance travelled by solute | Distance travelled by solvent | R Value |
|---------|--------|------------------------------|------------------------------|---------|
| 1       | Standard | 12.4                         | 12.8                         | 0.96    |
| 2       | Callus   | 12.3                         | 12.8                         | 0.96    |
| 3       | Root     | 12.2                         | 12.8                         | 0.957   |

**Table 5**: Absorbance of reserpine at various concentration

| Sr. No. | Concentration (µgml⁻¹) | Absorbance ± SD |
|---------|------------------------|------------------|
| 1       | 2                      | 0.039 ± 0.000704 |
| 2       | 4                      | 0.081 ± 0.002121 |
| 3       | 6                      | 0.120 ± 0.000704 |
| 4       | 8                      | 0.171 ± 0.000704 |
| 5       | 10                     | 0.208 ± 0.001414 |

**Table 6**: Absorbance and content of reserpine in *R. serpentina*

| Methanolic extract | Absorbance | Conc. of reserpine (µgml⁻¹) |
|--------------------|------------|-----------------------------|
| Callus             | 0.139      | 6.8                         |
| Root               | 0.133      | 6.4                         |

**Fig 1**: Callus Induction. MS + 2 mgL⁻¹ 2, 4-D + 1.0 mgL⁻¹ BAP for Leaf and leaf node MS + 2.5 mgL⁻¹ 2,4-D + 1.0 mgL⁻¹ BAP for Meristem

~ 523 ~
Fig 2: Callus proliferation after 21 days. MS + 2 mgL$^{-1}$ 2,4-D + 1.0 mgL$^{-1}$ BAP for Leaf and leaf node MS + 2.5 mgL$^{-1}$ 2,4-D + 1.0 mgL$^{-1}$ BAP for Meristem

Fig 3: Shoot multiplication MS + 4.5 mgL$^{-1}$ BAP + 0.5 mgL$^{-1}$ NAA

Fig 4: Rooting MS + 1.0 mgL$^{-1}$ NAA + 0.1 mgL$^{-1}$ BAP + 1.0 gL$^{-1}$ activated Charcol

Fig 5: Hardening Soil + Soilrite + Sand = 1:1:1

Fig 6: Qualitative analysis by Thin Layer Chromatography (A) Standard (Reserpine) (B) Extract From Callus (C) Extract From Root
Conclusion
The callus culture is a good alternative for production of secondary metabolites. The quality and quantity of reserpine is better in callus as compare to the root extract. The better quantification can be done using HPLC and LCMS technique and obtained compounds can be docked using molecular docking, which can be found as target against diseases. For increasing quantity of the reserpine the elicitors or precursor feeding is better option. Standardised regeneration protocol can be used in gene editing approaches.

References
1. Benavides MP, Caso OH. Plant regeneration and thiophin formation in tissue cultures of Tagetes mendocina. J. Plant Cell Tissue Organ Cult 1993;35:211-215.
2. Bhatt R, Mohd A, Gaur AK, Rao PB. Rauwolfia serpentina: Protocol optimization for in vitro propagation. Afr. J. Biotechnol 2008;7(23):4265-4268.
3. Borde VU, Pangrikar PP, Wadikar MS, Tekale SU. Extraction and thin layer chromatography of alkaloids from Bael (Aegle marmelos) leaves. J. Ecobiotechnol 2011;3(3):1-4.
4. Chaudhary V, Singh S, Sandhya V, Sharma N. In vitro regeneration of Rauwolfia serpentina through anther culture studies. Biotechnol. Int. 2015;8(3):93-100.
5. Joshi N, Kumar N. Aromatic and medicinal plants a in central Himalayas. In: Rauwolfia. Published by defense Agricultural Research Laboratory 2000, 94-95.
6. Kirillova NV, Smirnova MG, Komov, VP. Sequential isolation of superoxide dismutase and ajmaline from tissue culture of Rauwolfia serpentine Benth. Pricl. Biochim. J. Microbiol 2001;37:181-185.
7. Mallick SR, Jena RC, Samal KC. Rapid in vitro multiplication of an endangered medicinal plant sarpagandha (Rauwolfia serpentina). Am. J Plant Sci 2012;3:437-442.
8. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962;15:473-479.
9. Nayar MP, Sastry ARK. Red data book of Indian plants, Botanical Survey of India, Calcutta. India 1987, 1
10. Pant KK, Joshi SD. Rapid multiplication of Rauwolfia serpentine Benth. Ex. Kurz through tissue culture. Sci. World 2008;6:58-62.
11. Panwar GS, Attitalla IH, Guru SK. An efficient in vitro clonal propagation and estimation of reserpine content in different plant parts of Rauwolfia serpentina L. Am.- Eurasian J. Sci. Res 2011;6(4):217-222.
12. Panwar GS, Guru SK. Alkaloid profiling and estimation of reserpine in Rauwolfia serpentina plant by TLC, HP-TLC and HPLC. Asian J. Plant Sci 2011;10:393-400.
13. Roja PC, Sipahimalani AT, Heble MR, Chadha MS. Multiple shoot cultures of Rauwolfia serpentina growth and alkaloid production. J Nat. Prod 1987;50(5):872-875.
14. Roy SK, Roy PK, Rahman M, Hossain T, Svoboda KP, Laughlin JC, et al. Clonal propagation of Rauwolfia serpentina through in vitro culture. Acta Hortic 1995;390:141-146.
15. Sarkar KP, Islam A, Islam R, Hoque A, Joarder OI. In vitro propagation of Rauwolfia serpentina through tissue culture. Planta Medica 1996;62:358-359.
16. Sushila TG, Reddy S, Jyothsna D. Standardization of protocol for in vitro propagation of an endangered medicinal plant Rauwolfia serpentina Benth. J Med. plant res 2013;7(29):2150-2153.
17. Upadhyay N, Mukoveychuk AY, Nikolaeva LA, Battygina TB. Organogenesis and somatic embryogenesis in leaf callus culture of Rauwolfia caffra Sond. J. Plant Physiol 1992;140(2):218-222.
18. Yoshimatsu K, Shimomura K. Efficient shoot formation on inter-nodal segments and alkaloid formation in the regenerates of Cephaeli sipeccacuanha A. Richard. J. Plant Cell Rep 1991;9:567-570.