Research Article

Influence of Hormones and Explants Towards *In vitro* Callusing and Shoot Organogenesis in a Commercially Important Medicinal Plant

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ABSTRACT

The present study evaluates the effect of plant growth regulators (2, 4-D and Kinetin) and on two types of explants (leaf and node) on induction and growth of friable callus in *Rauvolfia serpentina,* a commercially important medicinal plant. Friable callus are the most suitable source for cell suspension culture that would enable the production of active principles at the *in vitro* level. For callusing, explants were obtained from shoots raised *in vitro*. Percentage response from nodal explant with respect to callus induction was comparatively higher (89.7 %) than that of leaf. While 2, 4-D (2 mg L^{-1}) alone appeared to be effective but with Kinetin (1.6 mg L^{-1}) proved to be the best PGR combination. Callus obtained were whitish to whitish green in color and friable in texture. Proliferation of callus significantly increased at 0.5 mg L^{-1} followed by 0.25 mg L^{-1} of 2, 4-D concentration alone. For shoot regeneration, calli were cultured in MS basal media supplemented with 2 mg L^{-1} BAP. Histological evaluation of paraffin sections of various stages starting from the callus initiation up to shoot regeneration were prepared, to study the developmental pattern of callus formation and shoot organogenesis in *R. serpentina*.

Keywords: Callus, Histology, Leaf explants, Node explants, *Rauvolfia serpentine.*

INTRODUCTION

Rauvolfia serpentina (commonly known as Sarpagandha) is a perennial medicinal shrub[1] belonging to the family Apocyanaceae. Globally, it has drawn exceptional attention in the pharmaceutical industry for its bioactive compounds such as indole alkaloids and other related constituents[2] that are of immense therapeutic importance. The drug obtained from this plant is useful for the treatment of mental diseases, epilepsy, sleeplessness and several other ailments.[3] The increasing demand for *R. serpentina* in national and international markets has encouraged many farmers to cultivate this medicinal plant. However over exploitation for its active content may develop an increased pressure on the plant sustenance. In this context, *in vitro* culture of friable callus can be a fruitful approach paving a pioneer way for large scale harvesting of natural products for future research.[4, 5] In addition to this, an important aspect in plant tissue culture is to study developmental pattern of dedifferentiation and redifferentiation, which can be accomplished by histological studies. This method has significant importance in understanding *in vitro* culture system[6] and therefore had encouraged us to study the histology of various developmental stages of *in vitro* callusing and shoot organogenesis in *R. serpentina*.

The present study was carried out with the objectives of induction and proliferation of friable callus, evaluation of the performance of leaf and nodal explants on friable callus induction, assessment of the differential role of 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 6-furfurylaminopurine (Kn) on friable callus culture and histological study of callusing and shoot organogenesis in *R. serpentina.*

MATERIALS AND METHODS

Explant Source

Thirty days old nodes were collected from the actively growing plant of *R. serpentina.* The collected material were thoroughly washed under running tap water followed by treatment in 10 % Savlon (*v/v*) for 5 min and 10 % Bavistin for 15 min respectively. Finally, the explants were treated with 0.1% (*w/v*) $HgCl_2$ with occasional shaking for 8 min under laminar air flow cabinet. Explants were then thoroughly rinsed with sterile water for 2 min to remove the traces of $HgCl_2$ and then both ends of the explant were trimmed to 2-2.5 cm. The nodes were then aseptically transferred in MS medium containing BAP (2 mg L^{-1}) to obtain multiple shoots. The nodes and leaves obtained from 8 week old *in vitro* raised shoots of *R. serpentina* were used as secondary source of explant to carry out the present investigation.

Culture condition

The basal medium was composed of MS salts and vitamins[7] with 3 % (*w/v*) sucrose. The media was supplemented with PGR *i.e.* 2, 4-D alone or in combination with Kn supporting callus induction and their proliferation. The pH of the media was adjusted to 5.8 before adding agar (0.8 %) and was autoclaved at 121°C for 20 min. Cultures were incubated under artificial condition of 16 h photoperiod (using cool white fluorescent light), 60% RH and 25 ± 1°C.

Callus induction, proliferation and shoot regeneration

For callus induction MS basal media with different combinations of 2, 4-D and/or Kn were used considering MS basal media as control. Nodes and leaf explant from *in vitro* raised shoot of *R. serpentina* were inoculated in the culture media. The cultures were incubated under standard artificial growth conditions as mentioned earlier. Best resulting media formulation was selected based on the days taken for initiation of response, number of explants producing callus (in percentage) and morphology of the callus. Induced callus were then sub cultured for proliferation. During callus induction and its proliferation the cultures were incubated for 4-5 weeks depending on the type of explant (nodal and leaf). Callus subcultures were performed at a specific interval if necessary. Calli were subcultured in fresh media after measuring the initial weight and were allowed to grow for four weeks. The increase in fresh weight under aseptic culture condition was calculated by subtracting initial from final weight. Greenish calli were placed in MS basal media amended with 2 mg L^{-1} BAP[1] to induces micro shoots.

Histological analysis

Samples were fixed in FAA solution (Formalin: Acetic acid: Absolute ethanol, 1:1:18 (*v*/*v*/*v*)) for 48 h at room temperature. This was followed by progressive dehydration of the fixed tissue and finally embedding in paraffin according to the method of Johansen (1940)[8] with some modifications. Serial sections (4-10 µm) were stained with Haematoxylin and Eosin. Sections were observed under light microscope and the photographs were taken using canon digital camera.

Statistical analysis

Each explant was considered as an experimental unit. All the above mentioned experiments were repeated three times using 20 explants in each replication. The data on callus induction and proliferation were taken twice a week after inoculation respectively. The collected percent data were subjected to one way analysis of variance (ANOVA) test and significant means were separated by Duncan's multiple range test (DMRT) at 5% level of significance (P<0.05) using SPSS software version 17.0.

RESULTS AND DISCUSSION

Effect of explants type on callus induction

One of the important factors that regulate callus induction is the explant type. In the present study, leaves and nodes obtained from the *in vitro* raised shoots of *R. serpentina* were used as sources of explant. Friable callus induced was documented within four weeks of culture. Among twelve treatments used in this experiment, the control medium failed to induce any callus.[9] The combination of 0.125 mg L^{-1} 2, 4-D with 0.1mg L^{-1} Kn and 0.25 mg L^{-1} 2, 4-D with 0.2 Kn didn't induce to callus formation, showing curling in case of leaf explant and slight swelling in node segment followed by browning in both the explants (Figure 1 A, B). Callusing was initiated within 5-14 days in leaf segment (Figure 1C-E) and within 5-10 days in case of nodal explant. Furthermore the percent of callus induction was higher in node (Table 1) compared to that of leaf (Table 2). Hence, between the two explants node explant was found to be best for friable callus induction. So for further study nodes were used as a source of explant for callus induction. The present result was in agreement with Palawat and Lodha (2014)[10] where nodes were proved to be the best explant for callus induction in *Ceropegia*.

Figure 1: (A, B) Initiation of swelling in leaf and nodal explant followed by browning; (C, D) curling of leaf; (E) Callus initiation in the margins of leaf explant (F) Profuse callus growth in leaf explant within four weeks of culture (G) Whitish brown friable callus (nodal explant); (H) Whitish friable callus from nodal explant; (I) Whitish green, friable callus from nodal explant; (J) callus turned brownish with necrotic in appearance after subculture.

Influence of Plant growth regulator on callogenesis

Plant growth regulators, mainly auxins are known to play an essential role in callus induction and proliferation.[11] However in combination with cytokinin it has shown significant effects on growth and differentiation in cultured cells.[12] In the present report based on the concentration and combination of PGR there was wide range of variation in percent response to callus induction. Six concentrations of 2, 4-D alone and five concentration of 2, 4-D in combination with Kn were tested respectively to assess and compare their effectiveness. Percent response of callus induction was recorded markedly higher when 2, 4-D alone was supplemented in MS. At lower level of 2, 4-D (0.125-0.5 mg L^{-1}) the callus were whitish in color with dull brown surface, whereas, at higher level (1-2 mg L^{-1}) whitish green calli were obtained. This result supports the earlier study of Prakash and Gurumurthi (2010)[13] who reported the impact of different concentration of 2, 4-D on callus culture in Eucalyptus. There was a gradual increase in response with the increase in concentration of 2, 4-D alone (except 2, 4-D at 0.5 mg L^{-1} concentration in leaf explant and in 1.0 mg L^{-1} 2, 4-D in nodal explant). Furthermore the percent response to callus induction in 2, 4-D and Kn combination was directly proportional with the increasing concentration of PGR showing a 89.7 % response in 2 mg L^{-1} 2, 4-D and 1.6 mg L^{-1} Kn followed by 1 mg L^{-1} 2, 4-D

plus 0.8 mg L^{-1} Kn combination and 0.5 mg L^{-1} 2, 4-D with 0.4 mg L^{-1} Kn in case of nodal explant (Table 1). On the contrary, Rakshit et al (2010)[14] observed a decrease in callus induction and browning of callus with the gradual increase of 2, 4-D in *Zea mays*. The current outcome for callus induction from nodal explant in 2, 4-D and Kn combination is in agreement with the results, reported by Aryal and Joshi (2009)[15] where 2, 4-D and Kn combination was found to be effective in inducing callus from stem explant.

Table 1: Effect of 2, 4-D and Kinetin for the induction of callus from nodal explant of *Rauvolfia serpentina*.

PGRs (mg L^{-1})		Response Initiation (Days)	% Callus induction (four weeks)	Morphology of callus
2,4-D	Kn			
0	0	0	0.0 ± 0.0^{g}	Browning of the base; no callusing
0.125	0	7	26.60 ± 2.8^{ef}	Whitish with dull brown surface; friable; slight callusing
0.25	0	5	35.93 ± 6.8^{d}	Whitish with dull brown surface; friable; moderate callusing
0.5	0	10	48.10 ± 7.1^{c}	Whitish with dull brown surface ; friable; moderate callusing
1.0	0	7	33.66 ± 7.5^{de}	Whitish green; friable; moderate callusing
1.5	0	7	54.33 ± 7.5^{bc}	Whitish green; friable; profuse callusing
2.0	0	7	87.50 ± 0.5^{a}	Whitish green; friable; profuse callusing
0.125	0.1	7	0.00 ± 0.0^{g}	Swelling at the base
0.25	0.2	7	0.00 ± 0.0^{g}	Swelling at the base
0.5	0.4	7	19.0 ± 6.0^{f}	Whitish; friable; moderate callusing
1.0	0.8	5	58.16 ± 3.7^{b}	Whitish green; friable; moderate callusing
2.0	1.6	7	89.76 ± 3.5^{a}	Whitish green; friable; profuse callusing

**The percent data were subjected to One- way ANOVA and Means were separated by DMRT, P = 0.05

Table 2: Effect of 2, 4-D and Kinetin for the induction of callus from leaf explant of *Rauvolfia serpentina*.

PGRs (mg L^{-1})		Response initiation (Days)	% callus induction (four weeks)	Morphology of callus
2,4-D	Kn			
0	0	0	0.0 ± 0.0^{f}	Curling of leaf; no callusing
0.125	0	10	28.3 ± 2.8^{e}	Whitish; friable; slight callusing
0.25	0	7	36.5 ± 1.3^{d}	Whitish; friable ; moderate callusing
0.5	0	7	29.3 ± 7.5^{e}	Whitish; friable; moderate callusing
1.0	0	14	47.2 ± 4.8^{c}	Whitish; friable; Profuse callusing
1.5	0	7	83.2 ± 7.1^{a}	Whitish; friable; Profuse callusing
2.0	0	7	84.5 ± 0.8^{a}	Whitish; friable; Profuse callusing
0.125	0.1	10	0.0 ± 0.0^{f}	Curling with slight swelling
0.25	0.2	5	0.0 ± 0.0^{f}	Curling with slight swelling followed by browning
0.5	0.4	5	37.0 ± 1.7^{d}	Whitish; friable; Profuse callusing
1.0	0.8	10	58.4 ± 4.0^{b}	Whitish; friable; Profuse callusing
2.0	1.6	10	79.1 ± 3.6^{a}	Whitish; friable; profuse callusing

**The percent data were subjected to One- way ANOVA and Means were separated by DMRT, P = 0.05

In case of leaf explant highest response was recorded in 2, 4-D alone with 84.5 % explant showing callus induction (Table 2), this was followed by 83.2 % response in 1.5 mg L^{-1} 2,4-D alone which was not significantly different from the response as observed in 2 mg L^{-1} 2, 4-D. However nearly 80 % response was recorded in 2 mg L^{-1} 2, 4-D plus1.6 mg L^{-1} Kn followed by 1 mg L^{-1} 2, 4-D in combination with 0.8 mg L^{-1} Kn, and least response was observed in 0.5 mg L^{-1} 2, 4-D plus 0.4 mg L^{-1} Kn. No calli were induced in MS basal medium alone which reflects the essential role of PGR in callus induction.[16] Regarding the morphology, the calli obtained in the present experiment from the nodal explant were whitish, whitish with dull brown surface to whitish green in color and friable in texture whereas from leaf explant only whitish friable calli were obtained (Figure 1 F-I). This reflects that color of the callus also depends on the type of the explant. Regarding the texture, friability was observed in all calli, the difference being only in the percentage of response obtained from the two explants. This indicates the effective role of 2, 4-D, alone or in combination with Kinetin. The result supports the findings of Kim et al (2005)[17] where friable calli were obtained in *Catharanthus roseus* at various concentrations of 2, 4-D. Whereas, Raha

and Roy (2003)[18] reported induction of whitish friable callus in 2, 4-D and Kn in *Holarrhena antidysentrica.* Rech et al (1998)[19] also showed induction of callus suitable for cell suspension in 2, 4-D, Kn in *R. sellowii*.

Callus proliferation

Callus obtained from the leaf and nodal explant were selected and sub cultured in 2, 4-D and/or Kn to study their proliferation. From the data (Table 3) it was evident that the best PGR formulation, in which the proliferation of callus increased, was 0.5 mg L^{-1} and 0.25 mg L^{-1} 2, 4-D (Table 3). But in contrary, these two concentrations showed very poor response in terms of callus initiation. The other combination, of PGR was found to be less suitable for proliferation as compared to the above concentration, as calli turned brownish and necrotic in appearance after few subcultures (Figure 1J). In addition the proliferation gradually decreased with the increasing concentration of 2, 4-D.

Table 3: Effect of 2, 4-D and Kinetin for the proliferation of callus induced from nodal and leaf explant of *Rauvolfia serpentina*

PGRs (mg L^{-1})		Callus weight (mg)	
2,4-D	Kn	Node	Leaf
0	0	0.0 ± 0.0^{j}	0.0 ± 0.0^{k}
0.125	0	323.0 ± 2.6^{h}	247.0 ± 13.6^{h}
0.25	0	637.3 ± 5.7^{b}	528.0 ± 5.5^{b}
0.5	0	736.0 ± 13.1^{a}	592.6 ± 4.9^{a}
1.0	0	571.6 ± 4.9^{c}	463.0 ± 7.2^{c}
1.5	0	526.3 ± 4.2^{d}	422.0 ± 7.8^{d}
2.0	0	469.6 ± 4.9^{e}	384.0 ± 4.1^{e}
0.125	0.1	222.6 ± 7.3^{i}	183.0 ± 5.8^{j}
0.25	0.2	221.3 ± 6.2^{i}	214.0 ± 6.0^{i}
0.5	0.4	328.3 ± 6.4^{h}	260.6 ± 7.2^{h}
1.0	0.8	448.0 ± 6.0^{f}	353.0 ± 8.3^{f}
2.0	1.6	410.0 ± 6.0^{g}	329.6 ± 6.6^{g}

** The percent data were subjected to One- way ANOVA and Means were separated by DMRT, P = 0.05

Morphological changes and their histological analysis

Among two explants the performance of nodes was better than the leaf explant. So nodes were implemented for callus induction and shoot regeneration for morphological and histological analysis The nodal explants cultured on callus inducing media showed swelling at the base of the node (Figure 2A) followed by callus growth initiation (Figure 2B) at its basal portion. Callusing further progressed towards the apex of the node followed by profuse growth within 25 days of culture (Figure 2C). For shoot regeneration, the calli (Figure 2D) were transferred in 2 mg L^{-1} BAP. The emergence of shoot buds were observed in the third week while these further elongated into shoots in the same media within 8 weeks of culture (Figure 2E - G).

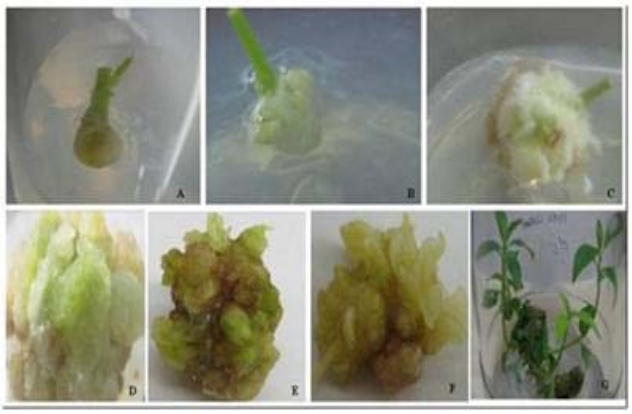

Figure 2: Morphology of developmental stages involved during Callusing and shoot regeneration from callus of *Rauvolfia serpentina.* (A) Swelling at the base of node; (B) Callus initiation; (C) Profuse callusing in the nodal explant; (D) Morphology of the callus after second subculture; (E) Shoot bud development from callus tissue (arrows); (F) Advanced stage of shoot bud differentiation from callus; (G) Callus containing multiple shoot.

The histological analysis of developmental events leading to callus formation and shoot regeneration from organogenic callus of *R. serpentina* included the following stages.

In the initial stage of developmental response, swelling was induced from the basal portion of the node. The histological section of this stage showed a bulged structure of the swollen base of the explant along with the partial rupture of the epidermal layer followed by the induction of active cell division (Figure 3A). In addition, changes along the epidermal layer slightly above the bulged region of the node was also observed indicating their future participation and transformation into callus (Figure 3B). The histological sections of the second and third stage of response which involves callus initiation followed by profuse callusing revealed the random location of cells from their regular arrangement, resulting into an unorganized mass. These cells were loosely arranged, showing intercellular spaces giving the callus, a friable appearance (Figure 3C - D). The histological section of the fourth and fifth stage of developmental response involves the formation of meristemoid followed by shoot bud formation from the organogenic callus. The meristemoids developed from the sub epidermal cells of the callus (Figure 3E). These meristemoids contains a group of meristematic cells, darkly stained present within the inner layer of the callus mass and their appearance indicates the initiation of organogenesis. This stage is followed by the development of shoot buds, the section (Figure 3F-G), shows that the shoot bud is partially flanked by two emerging leaf primordia. Similar result was shown by Chandra et al (2002)[18] in *Flacourtia jangomas* in which the histological section of the shoot differentiated from the callus showed a shoot apex which was partially covered with two leaf primordia. The meristimatic zone of the shoot apex was slightly dome

shaped, darkly stained and very distinct. The section through the portion of the callus containing more than one shoot bud revealed three shoot primordia partially enclosed by a pair of leaf primordia (Figure 3H). In the advanced stage of shoot organogenesis the section showed that the leaf primordia, which initially covered the shoot apex (Figure 3I), were getting elongated thereby exposing the shoot apex, followed by slight elongation of the latter.

Histological methods have significant contribution for understanding callusing and shoot regeneration in *in-vitro* culture system.[6] The histological study of regenerating calli (Shoot - derived) was reported in *Kigella pinnata* (Bignoniaceae)[21], *Flacourtia jangomas* (Flacourtiaceae)[20], *Arachis stenosperma, Arachis villosa.*[22]

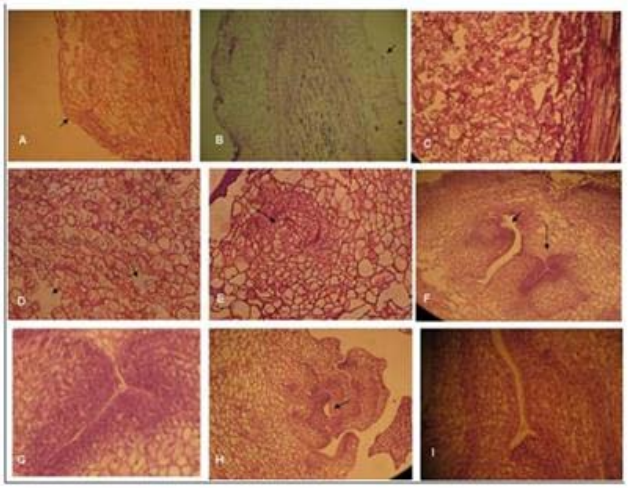

Figure 3: Histological sections of developmental stages involved during Callusing and plant regeneration in *Rauvolfia serpentina.* (A) Histological section of the bulged portion of the node (arrows); (B) Upper portion of the node showing changes in the epidermal layer (arrows); (C) Loose arrangement of cells in developing callus; (D) Structure of friable callus with intercellular spaces after the first subculture (arrows); (E) Meristemoid within the callus mass (arrows); (F) Shoot bud with leaf primordia (arrows); (G) Close view of the shoot bud; (H) Development of three shoot primordia flanked by leaf primordia (arrow); (I) Advanced stage of the shoot showing shoot bud differentiation with elongated leaf primordia.

CONCLUSION

In conclusion, friable callus induction and proliferation depends on the type of growth regulators and source of explants. Among two type of explant, nodes proved to be the best in terms of callus formation. Simultaneously 2, 4-D in combination with Kn (node) appeared to be much effective in callus induction followed by 2, 4-D alone (leaf). In the present investigation, the calli obtained were whitish to whitish green in color, friable in texture and were most suitable for establishment of cell suspension culture, thereby opening new vista that could assist production and extraction of phytochemicals from callus without exploiting the whole plant. Furthermore, the present analysis made an attempt to include maximum developmental stages of callusing and shoot organogenesis. This analysis may play an important role in understanding the developmental pattern and anatomical changes during *in vitro* callusing and shoot organogenesis which can contribute to future research.

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