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In vitro regeneration of medicinal plant Sarpagandha (*Rauwolfia serpentina* L.)

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Abstract
Sarpagandha is a medicinal shrub. It has been used in Ayurveda from ancient times. Nodal segment used on Murashige and Skoog medium. Sterilization of explants done with bavistin, streptomycin, NaOCl and HgCl2. MS+ BAP and IBA proved more responsive and effective for proliferation than BAP alone. MS+ 5 mg/l BAP+ 0.5 mg/l IBA found significantly better with 5.52 shoots and 81.30% shoot proliferation. Explants were slightly more responsive in the media combination MS+ 5 mg/l BAP+ 1.0 mg/l IBA with ±6.53 days and maximum length of shoots (6.93 cm). Root induction found best in ½ MS media+ NAA+ IAA, responded in ±29.3 days showing maximum root length. 1:3 soilrite and cocopeat hardening media with watering after 1 day of planting gave maximum survival in primary hardening. Present investigation indicates that, *in-vitro* regeneration protocol will provide techniques that can be successfully used for mass *in-vitro* propagation of species, *Rauwolfia serpentina* L.

Keywords: Hardening, *In vitro*, Medicinal Shrub, Nodal segment, *Rauwolfia serpentina*, Sterilization

1. Introduction

Rauwolfia serpentina L. is a woody perennial medicinal shrub. In India it is commonly known as sarpagandha or Indian snake-root. The plant belongs to the family Apocynaceae. The shrub grows in moist forests and shady places near rain-forest and has a long, nodular, yellowish root stock. The plant grows well in tropical to subtropical climate and favors deep fertile soil, rich in nitrogen and organic matter with good drainage. The snake-weed genus includes about 50 species, this has a wide area of distribution, including the tropical part of the Himalayas, the Indian peninsula, Sri Lanka, Burma, and Indonesia. The roots of the plant have been used in Ayurveda and medicines from ancient times of Indian medical therapy [1]. The plant is highly effective against high blood pressure. The medicines prepared from sarpagandha also used for diseases related to mental health [2, 3]. Rapid growth of world population, increasing anthropogenic activities, rapidly erosion of natural ecosystems resulted in decreasing the availability of the plant. The plant is an endangered species in India because of the over the limit consumption of plant for developing medicines. The natural reserves of this plant are declining, especially after reports of its medicinal properties appeared in literature. The International Union for the Conservation of Nature and Natural Resources (IUCN) has marked the plant under endangered status [4]. The IUCN Red List Categories are intended to be an easily and widely understood system for classifying species at high risk of regional to global extinction and to provide an explicit and objective framework for the classification of species according to their extinction risk. There is an urgent need to conserve the plant with great medicinal importance [5]. It has been earlier reported that the natural habitat of the plant is diminishing and conservation of the plant is essential [6].

Conventionally, *Rauwolfia serpentina* reproduces via viable seeds, but low percentage of seed germination, short viability of seed, scanty and delayed rooting of seedlings limit its natural propagation. Propagation with sowing seeds has been resulted in the low rate of germination and the seeds are not viable which are longer than 7-8 months [7]. Plant tissue culture is a biotechnological tool for the mass propagation of rare, endangered and threatened medicinal plants which are facing the danger of extinction. *In vitro* propagation is also a powerful tool for the production of medicinal secondary metabolites as well as for the purpose of conservation and commercialization. The technique of *in vitro* cultivation of plant cells or organs is primarily devoted to solve two basic problems; first, to keep the plant cell or organ free from microbes, i.e., bacteria and fungi. Second, to ensure the desired development in the

cell and organs by providing suitable nutrient media and other environmental conditions. *Rauwolfia serpentina* holds an important position in the pharmaceutical world because of its immense anti-hypertensive properties resulting from the presence of reserpine in the roots. Poor seed viability, low seed germination rate, and enormous genetic variability are the major constraints for the commercial cultivation of *Rauwolfia serpentina* through conventional mode. Biotechnological techniques like tissue culture are useful for commercial cultivation [8].

2. Materials and Method

Basal culture medium is comprised of MS salts [9], 100 mg/l inositol, 30 g/l sucrose and imparted with 1-5 mg/l BAP with combination of 0.5 and 1.0 IBA for culture growth of nodal segments and shoot tips explants. MS medium used as basal media was imparted with various levels of IAA and NAA in combination with BAP at minimum quantity for root induction studies. The pH of media was adjusted to 5.8± 0.1 using HCL/NaOH. The media was gelled with 8 g/l agar and poured in pre-sterilized glass culture bottles. The culture bottles were autoclaved in steam sterilizer at 121℃ and 15 lbs for 20 minutes.

The sterilized media was stored in the media storage room for 5-6 days before use. All cultures were incubated in the culture growth room under 16/8 light/dark regime at temperature 25± 2 ℃.

2.1 Plant material

Rauwolfia serpentina L. plant explants were obtained from the field at Nagarjuna Medicinal plant garden of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. Nodal segments were collected from healthy pest and disease-free plants in the field. The smaller size of explants was chosen due to the fact that smaller size of explants provides less chance of contamination [1]. Explants were washed with running tap water for 5 minutes immediately after being brought to the laboratory. Sterilized polythene bag was used to carry explants from the field to the laboratory. The explants were immersed in ethanol for 30 sec and washed with 2 drops of tween-20. Treatment of 100 mg/l Bavistin was given to explants on orbital rotary shaker for 30 min, followed by 100 mg/l streptomycin for 15 min. Samples were taken into the laminar air flow chamber and treated with 1% sodium hypochlorite for 10 min and 0.1% Mercuric chloride for 10 min with gentle shaking. After 3-4 times washing with distilled water has been followed to completely sweep away sterilants from the explants. The sterilized explants were inoculated on culture medium.

Table 1: Sterilants used for sterilization

Sr. no	Time duration	Sterilants
1	30 sec	Ethanol (70%)
2	30 min	Bavistin (0.1%)
3	15 min	Streptomycin (1.0%)
4	10 min	Sodium hypochlorite (1.0%)
5	10 min	Mercuric chloride (0.1%)

2.2 Effect of growth regulators on *in vitro* shoots

For initiation of shoots, nodal segments (3-5 mm in length) with a slant cut the end which was supposed to be in contact with the surface of culture medium were inoculated on MS medium add on with different concentration of BAP (1-5 mg/l) and IBA (0.5 mg/l and 1.0 mg/l). After 20 days of culture, when explants were observed with shooting the explants were transferred in fresh media to avoid any contamination caused by media. The explants were subcultured after 30 days of inoculation. Subculturing of the shoots were done after every 3-4 weeks of inoculation to avoid crowding of shoots and lack/loss of nutrient ability in media. Shoots were regenerated on MS medium supplemented with different concentrations of sucrose (3%, 4.5% and 6%) to test the better proliferation based on the concentration of carbon source availability in the media.

2.3 Effect of growth regulators on *in vitro* root induction

For the root induction the 6-8 weeks old shoots were cultured on half strength MS medium imparted with IAA and NAA 3% sucrose and varying agar concentration (0.7% and 0.8%). The roots were observed after 29-32 days of culturing of shoots for rooting. As sarpagandha is a woody shrub the tap root system was observed in the plantlets. The plantlets with roots were transferred for primary hardening after 5-6 weeks from root induction. After a month of acclimatization secondary hardening is performed.

2.4 Effect of media and watering on hardening

The plantlets with roots were washed with 0.1% bavistin for 5-10 min to remove media containing agar from the roots and prevent fungal contamination during primary hardening. The plantlets were rooted in pro trays filled with soilrite and cocopeat with 1:1 ratio and with 1:3 ratio with combination respectively. The plantlets were acclimatized at 25± 2℃ and some were watered immediately after planting and others were watered 1 day after planting. After a month of acclimatization secondary hardening is performed. The plantlets were transferred in the pro trays filled with soil enriched with nutrients and organic matters and observed in the greenhouse

2.5 Data collection and analysis

Each treatment in the given experiment was replicated three times with 30 explants used per treatment. The shoot formation frequency per explant was estimated after 50 days of inoculation of explants on basal culture media. All data were analyzed by a completely randomized design (CRD) method of statistics. The data was analyzed using ANOVA for finding out significance of the treatments and means were tested against critical difference (CD) at 1%.

3. Result and Discussion

3.1 Explant establishment

Aseptic nodal segment and shoot tip explants of *Rauwolfia serpentina* were cultured on the MS media imparted with 3.0% sucrose (w/v) and plant growth regulators BAP with different concentrations under *in vitro* conditions aseptically. The cultured explants were tested for explant establishment for the regeneration of sarpagandha for mass propagation (Table 2). Shoot initiation of explants was recorded in percentage. The ratio of shoot initiated in number of explants per culture treatment and days required for initiation of shoots in the explants was calculated as shoot initiation percentage. Maximum shoot initiation was observed in MS media containing 5mg/l BAP cultured with shoot tip explants (81.00%) higher than shoot initiation in nodal segment (75.33%). Total number of shoots regenerated from explants

were recorded with both the explants which showed that nodal segments with MS+ 5 mg/l BAP regenerated maximum number of shoots (5.17) than shoot tip explants (3.63) could regenerate. Even the multiplication rate of the regenerated shoots was higher in the culture with nodal segments (43.3%) whereas, shoot tip showed a very low rate (20.67%). Due to the fact that shoot tips are active regions of synthesis of auxin it is responsible for apical bud dominance and buds at the nodal segments are active regions for cell division so formation of shoots speedily is expected. Therefore, Nodal segments were found as the better explant for mass *in vitro* shoot multiplication and the experiment is further carried out. Singh and Guru (2007) inoculated shoot tip and nodal segment on MS as basal medium with BAP. Shoot tip and nodal segment produced showed similar results with multiple shoots [10]. Sarker *et al*., (1996) reported the similar results with 1.0mg IBA and 1.0 mg NAA on MS medium using shoot apices [11].

3.2 *In vitro* shoot multiplication

Effect of combination of growth regulators cytokinin and auxin on shoot multiplication was observed on MS medium fortified with combination of BAP and IBA with various levels of concentrations (Table 3). The detailed results were analyzed when the nodal segment as an explant from sarpagandha plant was inoculated. Shoot initiation was recorded per treatment in the number of days required to initiate. Treatment with MS media supplemented with 5 mg/l BAP+ 1.0 mg/l IBA showed minimum (6.53) number of days on an average for shoot initiation. Higher number of multiples was observed with 5 mg/l BAP+1.0 mg/l IBA regenerating (5.52) number of shoot multiples. When the response of the treatments for length of shoots were recorded the treatment with MS containing 5 mg/l BAP+ 0.5 mg/l IBA showed maximum response (6.93 cm) of length of shoots. The culture of sarpagandha was transferred on fresh medium after 4 weeks of inoculation to maintain the culture and nutrients provided through MS media for the shoot proliferation. The rate of shoot multiplication was recorded by calculating the number of shoots regenerated after subculture in the total number of days. The highest percentage of shoots multiplication (81.30%) was observed in treatment MS containing 5 mg/l BAP+ 0.5 mg/l IBA. Survival of the explants from inoculation up until to the shoot multiplication was highest survival (91.70%) was given by the same treatment. Jain *et al*. (2003) [12] and Alatar *et al*. (2012) [13] reported that, proliferation from nodal explant inoculated on MS media with BAP 5 mg/l and 0.5 mg/l IBA gave the maximum number of shoots and multiplication. The finding of these observations was at par with earlier results reported by Shekhawat *et al*. (2016) they reported that when MS media in combination with 1 mg/l BAP, shoots developed from nodal segments achieved the height on an average of 4.3 cm [14]. Mallick *et al*. (2012) where MS media supplemented with 2.5 mg/l BAP developed shoots with average 4.2 cm length per treatment [7].

3.3 Effect of sucrose on explant

For the *in vitro* multiplication of shoots, carbon source in the form of sugars has to be added to the nutrient medium for growth and proliferation of shoots as the photosynthetic capability of *in vitro* shoots are limited. In the present case, different concentrations of sucrose (3%, 4.5% and 6%) in the MS medium were tested for the growth and development of *in vitro* shoots. Sucrose at 3% in the MS medium gave the best results for *in vitro* shoot multiplication. The present results are in accordance with result findings by Banerjee and Modi (2010) that multiplication of shoots MS media supplemented with BAP (0.1 mg/l) and different concentration (0.50 g/l) of sucrose [15]. Use of sucrose, especially the higher concentration, has never been attempted before and gave very positive results. Arya and Arya (2015) reported that sucrose at 3% in the MS medium gave best results for *in vitro* shoot multiplication of bamboo [16].

3.4 Root regeneration

Regenerated shoots were transferred after 4- 5 subcultures in basal media containing auxin for root induction. For the rooting, different media compositions were attempted for root induction (Table 4). Basal culture media used was half strength MS media with different concentrations of NAA and IAA and other supplements such as sucrose and agar. ½ MS media imparted with 2.5 NAA+ 2.0 IAA+ 0.7% Agar+ 3% sucrose induced roots in minimum number days (29.3 days) than the other combinations. Significantly higher rooting frequency (51.28%) and average root length (2.9 cm). Root induction was not observed on full strength MS media. The present results were in accordance with results reported by Prakasha *et al.* (2017) that, ½ MS media imparted with 1 mg/l IAA showed early root induction and significantly, highest rooting frequency 76.48%, number of roots 7.87 and average length of roots 5.53 cm [17]. Other treatments with BAP and IBA combinations do not induce roots, instead callusing was observed. Susila *et al*. (2013) got 100% rooting when transferred *in vitro* regenerated multiple shoots of *R. serpentina* on a half- strength MS media supplemented with NAA and IBA [18]. Further, Rani *et al*. (2014) has reported 88% *in vitro* root induction on medium containing 3 mg/l of IBA [19]. Rahman *et al*. (1992) established similar results showing the best root formation happened on half strength MS medium imparted with IBA and IAA [20]. The results also exhibit that rooting of the young shoots was obtained with 1.0, 2.0 or 4.0 mg/l of IBA to Kumar *et al*. (1993) [21]. Basal media imparted with BAP, IAA, NAA, induce rooting in slightly lower percentages. Basal media supplemented with NAA was found to be better for root regeneration

Table 2: Effect of different type of explant on shoot multiplication

Treatments	Hormonal composition (BAP mg/l)		Shoot Tip			Nodal segment	
	Shoot initiation (%)		No. o shoots	Shoot multiplication (%)	Shoot initiation (%)	No. of shoots	Shoot multiplication (%)
T1	3.0	76.00	3.77	20.67	77.33	4.37	31.33
T2	4.0	79.67	3.47	27.67	75.33	4.63	39.67
T3	5.0	81.00	3.63	27.00	72.33	5.17	43.33
SE(M) ±		0.13	0.21	0.11	0.14	0.22	0.12
CD @1%		0.41	0.65	0.34	0.42	0.66	0.35

Table 3: Effect of Hormones on *in vitro* shoot multiplication

Treatment No.	Hormonal combination (mg/l)		No. of days for initiation	No. of shoots	Length of shoots (cm)	Shoot multiplication after subculture (%)	Survival of explants (%)
	BAP	IBA					
T1	-	-	12.07	1.87	1.73	31.30	42.70
T2	1.0	0.5	10.97	2.33	2.36	38.30	58.30
T3	1.0	1.0	11.10	2.60	2.73	45.00	64.00
T4	2.0	0.5	9.37	2.20	3.26	47.30	63.30
T5	2.0	1.0	9.10	3.40	3.63	53.70	68.30
T6	3.0	0.5	8.13	3.73	4.63	62.70	73.00
T7	3.0	1.0	8.37	3.97	5.46	60.30	77.30
T8	4.0	0.5	7.23	4.74	5.06	72.00	90.30
T9	4.0	1.0	7.43	4.36	5.23	68.70	87.00
T10	5.0	0.5	6.97	5.52	6.93	81.30	91.70
T11	5.0	1.0	6.53	5.18	6.10	77.00	82.70
SE (M) ±			0.18	0.13	0.16	0.21	0.24
CD @ 1%			0.55	0.39	0.48	0.63	0.72

Table 4: Effect of media composition on rooting

Treatment	Hormonal combinations (mg/l)		Sucrose (g/l)	No. of explants	Response of shoots	No. of days for root induction	Rooting frequency (%)	Average length of roots (cm)
	NAA	IAA						
½ MS	2.5	2.0	3.0	20	11	29.3	37.54	2.9
½ MS	1.5	1.0	2.0	20	14	27.3	51.28	2.5

Table 5: Effect of different hardening media on plants survival

Treatment no.	Media combination	No. of plantlets	Plants survived	Survived plants (%)
T1	1:1 soilrite and cocopeat with immediate watering	20	9	45.00
T2	1:1 soilrite and cocopeat with watering after 1 day	20	13	65.00
T3	1:3 soilrite and cocopeat with immediate watering	20	13	65.00
T4	1:3 soilrite and cocopeat with watering after 1 day	20	17	85.00

3.5 Effect of media and watering on hardening

Plantlets were rooted in hardening media soilrite and cocopeat at different combination and with watering practice, such as 1:1 soilrite and cocopeat with watering immediately after planting plantelets, 1:1 soilrite and cocopeat with watering after 1 day, 1:3 soilrite and cocopeat with immediate watering and 1:3 soilrite and cocopeat with watering after 1 day (Table 5).

Plantlets rooted in the media with 1:3 soilrite and cocopeat with watering after 1 day of planting showed significantly higher survival of the plants (85.00%). Plantlets showed very less acclimatization and survival in the media (45.00%) with 1:1 soilrite and cocopeat and watering immediately after planting. Similar results were noticed in Prakasha *et al*. (2017) in the study, 1:3 soilrite and cocopeat mixture along with one week after planting are found to be the best in response [17]. Pant and Joshi (2008), Hardening of *ex vitro* rooted *Rauwolfia serpentina* plantlets was successful [22]. However, Singh *et al*., (2009) reported hardening of *in vitro* rooted plantlets were not that successful [1]. No reports are available on data of survival of *in vitro* plantlets in hardening. Rani *et al*. (2014) has reported 70% survival of plantlets after hardening and Rashmi and Trivedi, (2016) has reported 77% of rooted plants and survived [19, 24].

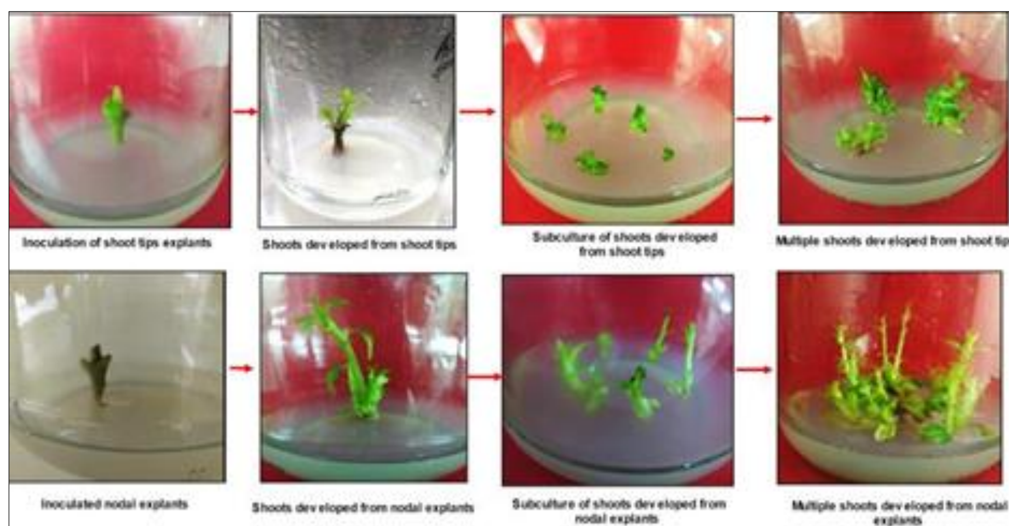

Plate 1: Effect of different type of explants on shoot multiplication

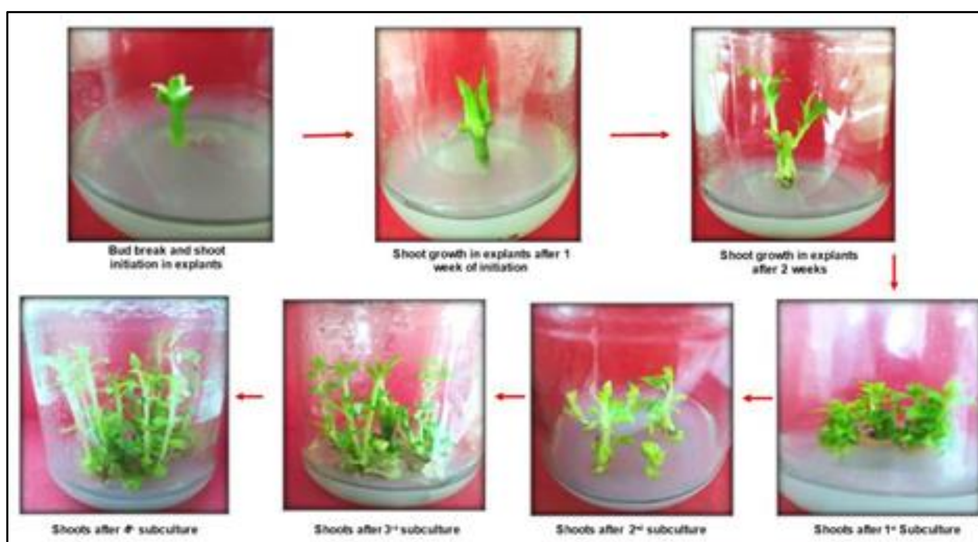

Plate 2: Shoot growth in explants in MS media

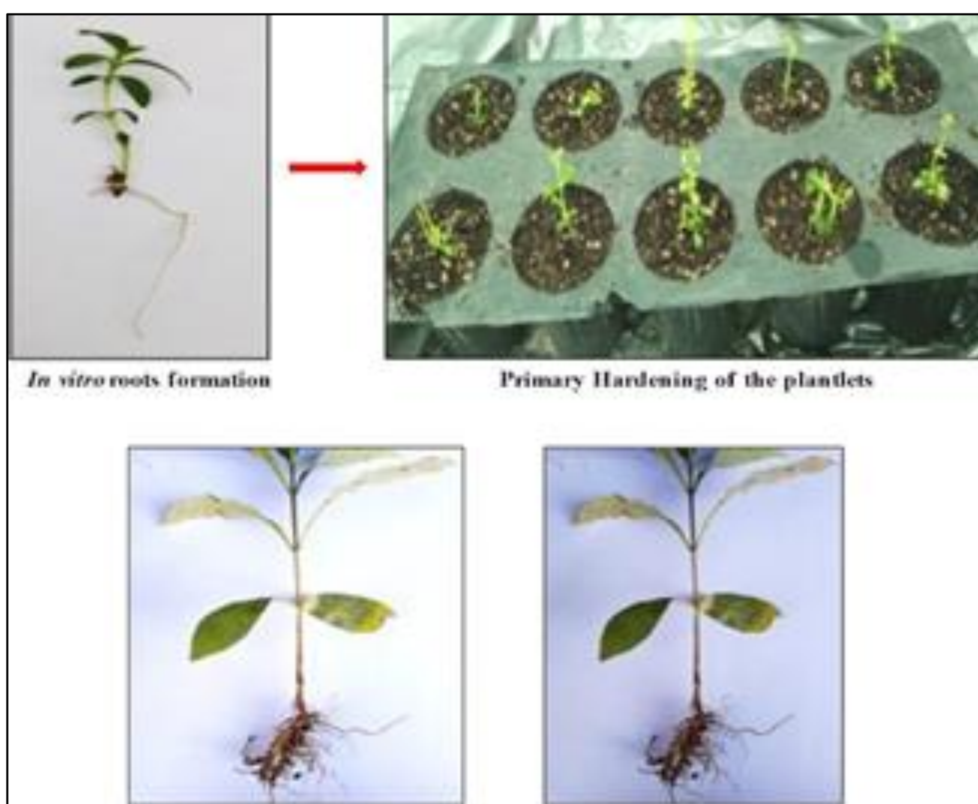

Plate 3: Roots of sarpag and after secondary llardcaing

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