In vitro shoot proliferation in Rauwolfia serpentina. Benth, an endangered medicinal plant.

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Abstract : Protocol for rapid *in vitro* propagation of *Rauwolfia serpentina*. Benth through shoot tip and nodal segments as explants was established. Among the different cytokinins used, MS medium augmented with 0.3 mg/l TDZ yielded a maximum of 10 shoot buds after 30 days. Combined effect of BA 3 mg/l along with 1.0 mg/l KIN resulted a maximum of 6 shoots. When auxin viz. NAA (1.5 mg/l) was used in combination with 2.0 mg/l BA produced a maximum of 15 shoots after 30 days of culture. Presence of AdS (100 mg/l) along with NAA (0.5mg/l) and KIN (2 mg/l) resulted 7 shoot buds after 30 days of culture. The shoots formed through various combinations were rooted in half strength MS medium supplemented with 1.5 mg/l IBA .The rooted plantlets were acclimatized and transferred to field.

Key words : *Rauwolfia serpentina* Benth., Shoot tip and nodal segments, *In vitro* propagation, MS medium, Growth regulators.

INTRODUCTION

Rauwolfia serpentina. Benth., commonly known as sarpagandha is an endangered medicinal shrub belonging to the family Apocynaceae. The genus comprises about 50 species. This is a traditional medicinal plant native to Indian subcontinent and East Asian countries. The plant is enriched with secondary metabolites such as alkaloids, flavanoids, phenols, glycosides, tannins etc. The roots of *Rauwolfia serpentina* is a rich source of indole alkaloids such as ajmalicine, serpentine, reserpine, rescinnamine, ajmaline and yohimbine. The alkaloids of *Rauwolfia* belonging to the general class of medicines called antihypertensive and commonly used to treat cardiovascular diseases(Anitha and Kumari, 2006) , arrhythmia (Killiora et al. 2001) and hypertension (Von poser et al.1990). The plant is also well known for its antimicrobial antifungal, anti-inflammatory, antiproliferative, antidiuretic and anti cholinergic activities. The plant is now facing an endangered situation due to its indiscriminate collection and over exploitation of natural resources for commercial purposes to meet the requirements of pharmaceutical industry. The International Union for the Conservation of nature and natural resources has kept this plant under endangered status(Jain et al. 2003). Conventionally, the plant reproduces via. viable seeds. But low percentage of seed germination, short viability of the seeds ,scanty and delayed rooting of seedlings limit its natural propagation. Large scale propagation is a prerequisite to meet the pharmaceutical needs and also for effective conservation of this valuable medicinal plant. Hence there is an urgent need to apply nonconventional propagation methods for conservation and future commercial delivery of *Rauwolfia serpentina*(Upadhyaya et al. 1992). Plant tissue culture is a powerful tool for the mass propagation of rare and endangered medicinal plants. *Invitro*

propagation system is also used as a tool for the production of secondary metabolites(Islam et al. 2010, Nadeem et al.2000). Therefore the present study was undertaken for the large scale cultivation of this plant through *invitro* shoot tip or nodal segment culture.

MATERIALS AND METHODS

The plantlets of *Rauwolfia serpentina*. Benth were collected from different districts of Kerala. These plants were maintained in the green house of the Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram. Shoot tip and nodal segments collected from 2—4 month old green house plants were used as explants for the present study. The explants were washed thoroughly under running tap water for one hour and subsequently washed with 10 % Labolene (Glaxo, Mumbai) for 7 minutes. The flask containing the explants was shaken continuously so as to get uniform contact with the detergent to all parts of the explant. Then the flask with the explants was again washed well in running tap water for another 30 minutes and subsequently with double distilled water 2--3 times. Explants were then taken to the laminar air flow cabinet. Final sterilization was done using 0.1 % mercuric chloride solution for 8 minutes. The explants were again washed with sterile double distilled water 3—4 times to remove the traces of mercuric chloride. The sterilized explants were excised to remove the sterilant exposed regions and inoculated on to MS medium supplemented with cytokinins viz. BA / KIN (0.1—5.0 mg/l) or TDZ(0.1—0.5mg/l) alone or in combination with auxins viz. NAA,IAA or IBA (0.1—2.0 mg/l). Additives such as Adenine sulphate (50—150 mg/l), L-glutamine (50—200mg/l)and Polyvinyl pyrrolidone(50—250 mg/l) were also incorporated in MS medium to find out their effect on shoot multiplication. The shoots formed from shoot cultures were subcultured on to fresh medium every 30 days for optimum rate of multiplication was calculated as the number of multiple shoots/ single nodes per shoot (for single node culture). Finally the multiplied shoots of appropriate lengths were rooted in the rooting medium. The rooted plantlets were then cultured on liquid MS medium with filter paper bridges. After 2—3 weeks these plantlets were transferred to plastic cups filled with sterile vermiculite. The plants were covered with moist plastic bags punctured at places to facilitate aeration in order to maintain ambient humidity. The plants were nourished with 1/10 strength Hoaglands solution (Epstein, 1972) for one or two weeks. These were then transferred to pots containing soil and sand mixture in the ratio 1 : 3 and maintained in the green house with uniform atmospheric conditions.

RESULTS AND DISCUSSIONS

The shoot tips and nodal segments from green house grown plants were inoculated on to MS medium containing different concentrations and combination of growth regulators.

Effect of cytokinins

Cytokinins viz. BA(0.5—5.0 mg/l), KIN (0.5—5.0 mg/l) and TDZ (0.1—0.5 mg/l) were supplemented to MS medium to find their effect on shoot multiplication. A maximum of 7 shoot bud was initiated from the base of the node when the MS medium was supplemented with 3.0 mg/l BA after 30 days.(Table. 1).(Plate

1.1) Lower or higher concentrations of BA resulted in the decrease in the number of shoot buds.(Plate 1.2and 1.3). The shoots formed were strong and healthier with short internodes. Meager callus formation was noticed from the base of the explants on MS medium fortified with 4 mg/l BA. The superiority of BA over other cytokinins for shoot proliferation has been established for plants such as *Malus sylvestris* (Hutchinson, 1982; Lundergan and Janick, 1980)and mulberry, *Morus nigra*(Yadav et al.,1990).

When the MS medium was supplemented with KIN, the shoots formed were weak with long internodes . Five shoots were initiated in the medium augmented with 3.0 mg/l KIN after 30 days.(Table.1).(Plate 1.5). Lower or higher concentrations of KIN resulted in a decrease in the number of shoot buds.(Plate 1.4 and 1.6). Effectiveness of KIN for elongation of shoot tips was pointed out by Compton et al.. (1993) in watermelon shoot cultures.

The number of shoot buds increased when the MS medium was enriched with 0.1—0.5mg/l TDZ. A maximum of 10 shoot buds were initiated from the base of the shoot tip when the MS medium was supplemented with 0.3 mg/l TDZ.(Table.1).(Plate 1.8). A corresponding decrease in the number of shoot buds was noticed at lower or higher concentrations of TDZ.(Plate 1.7 and 1.9). Thidiazuron is a substituted phenyl urea that exhibits cytokinin like activity (Mok et al; 1982, Thomas and Katterman 1986). TDZ induced high frequency regeneration in bean and peanut which are two recalcitrant legumes(Malik and Saxena,1992, Gill and Saxena 1992.).

A maximum of 6 shoots were developed on MS medium augmented with 3.0 mg/l BA and 1 mg/l KIN used in combination.(Table.1).(Plate 1.10). The newly formed shoots were covered with small scale leaves like structures with short internodes. When the above concentration and combination of hormones were altered, a corresponding decrease in the number of shoot buds was observed. George and Sherrington(1984) were of the opinion that adding more than one cytokinin to the medium can result in improved shoot production or shoots of better quality. The results of the present study also emphasizes the above view.

Combined effect of auxins and cytokinins

A gradual increase in the number of shoots was observed when MS medium was augmented with auxins viz. NAA/IAA/IBA used along with BA/KIN/TDZ. 15 shoots were originated from the node in MS medium augmented with NAA 1.5 mg/l and BA 2.0 mg/l after 30 days of culture.(Table.2).(Plate 1.11). The shoots formed were with short internodes. Similar result was obtained earlier from nodal segments of *R.serpentina* (Roy et al; 1995) The superiority of NAA to IAA/ IBA was also noticed in *Prunas avium* by Snir.(1982). 4 shoots were initiated in MS medium enriched with 1.0 mg/l IAA and 3.0mg/l BA. A single root was also formed along with the development of shoot. (Table.2). (Plate 2.12). IBA (1.0 mg/l) when supplied along with 3.5 mg/l BA in MS medium resulted in the formation of 8 shoot buds. Profuse root formation from the base of the node after 30 days of culture was also observed. (Table.2).(Plate 2.13).

Nodal segments when inoculated on MS medium containing 1 mg/l NAA and 3.0 mg/l KIN resulted in the formation of 2 shoots after 30 days. The shoots were fragile and having long internodes . Meager callusing

along with the development of numerous roots was also noticed from the base of the node. (Table.2).(Plate 2.14). Use of auxins in combination with cytokinins favoured an enhancement in the production of multiple shoot. This was supported by earlier reports in ornamental species of plants (Nugent et al,1992) , Nikam and Shitole (1999).

MS medium supplemented with IAA(1.0 mg/l) and KIN (2.5 mg/l) resulted in the formation of a single shoot along with the formation of very short and thick roots. The leaves of the shoots were broader with pale green colour. (Table.2).(Plate 2.15)

Nodal segments inoculated on MS medium augmented with 1.5 mg/l IBA and 3.5 mg/l KIN developed a single shoot with numerous nodes. Meager callusing was noticed at the base of the node along with the development of numerous roots. (Table.2).(Plate 2.16).

Profound increase in the number of shoots was observed in MS medium containing auxins and TDZ. A maximum of 11 shoot buds was initiated from the base of the node along with the development of meager callusing when the medium was supplemented with 0.1 mg/l NAA and 0.2 mg/l TDZ. (Table.2).(Plate 2.17). In the present study TDZ initiated maximum number of shoots from shoot tips or nodal explants either alone or in combination with auxins. The results are in agreement with the result of adventitious shoot regeneration from cotyledons of white ash(Bates et al;1992).

When IAA (0.1mg/l) was used along with TDZ (0.2 mg/l), 4 shoots were originated from the base of the stem along with the formation of meagre callusing. (Table.2).(Plate 2.18).

A profound increase in the number of shoot buds was observed in MS medium containing IBA and TDZ. A maximum of 10 shoot buds was initiated from the base of the node in MS medium augmented with 0.1 mg/l IBA and 0.2mg/l TDZ after 30 days. (Table.2).(Plate 2.19).

Effect of additives

The presence of additives viz. Adenine sulphate(AdS), L- glutamine and Polyvinyl pyrrolidone (PVP) along with growth regulators has prominently influenced the development of multiple shoots. When nodal explants were inoculated on MS medium containing 0.5 mg/l NAA, 2 mg/l KIN and 100 mg/l AdS resulted in the formation of 7 shoot buds after 30 days of culture. (Table.3).(Plate 2.20). Similar result of increase in the number of shoots in AdS containing medium was reported in Neem by Ramesh and Padhya(1990) and in cow pea by Muthukumar et al. (1995).

0.5 mg/l IBA in combination with 2.0 mg/l KIN and 100 mg/l AdS resulted in the formation of 5 shoots from the base of the node after 30 days of culture. Callus formation was also noticed from the base of the node along with the formation of shoots.(Plate 2.22). When the concentration of the AdS was decreased to 50 mg/l the number of shoots was decreased. (Table.3).(Plate. 2.23).

A negative impact on the development of multiple shoots was noticed in MS medium containing glutamine and growth regulators. 5 shoot buds was resulted in MS medium containing 0.5mg/l NAA, 2.0

mg/l KIN and 100 mg/l glutamine.(Plate. 2.24). Alteration in the concentration of glutamine adversely affected the formation of shoots. A single shoot was noticed in MS medium containing 0.5mg/l NAA, 1.0 mg/l KIN and 50 mg/l glutamine. (Plate 3.25). A maximum of 4—5 shoots were resulted in MS medium augmented with 0.5 mg/l IBA, 2.0 mg/l KIN and 100 mg/l glutamine. (Table.3).(Plate 3.26). A similar effect of reduction in the number of shoots in presence of glutamine was noticed in *Coleus bluemi* shoots by Smith (1981a).

MS medium augmented with NAA (0.5mg/l) , KIN (2.0mg/l) and PVP 100 mg/l resulted in the formation of a single shoot along with basal callusing from the node.(Plate3.27). The shoots were stunted in nature when the concentration of PVP was reduced to 50 mg/l. (Table.3).(Plate 3.28). The impact of PVP in enhancement of shoots was noticed in Datura sps. by Babbar and Gupta (1982) and in neem by Gautham etal. (1993).

When 0.5 mg/l IBA and 2.0 mg/l KIN was used along with 100 mg/l PVP resulted in the formation of 5 shoots.(Plate 3.29). A decrease in the concentration of PVP to 50 mg/l along with 0.5 mg/l IBA and 1.0 mg/l KIN resulted in the formation of a single shoot. Profuse root development was also noticed. (Table.3).(Plate. 3.30)

Table : 1. Effect of cytokinins on shoot multiplication

Cytokinins(mg/l)			Mean no. of shoots after 30 days	Mean no. of shoots after 60 days
BA	KIN	TDZ		
0.5	--	--	1.14 ± 0.46	1.0 ± 0.44
1.5	--	--	2.0 ± 0.62	5.0 ± 0.54
2.5	--	--	4.86 ± 0.74	10.71 ± 0.84
3.0	--	--	7.0 ± 0.72	23.86 ± 1.57
4.0	--	--	5.71 ± 1.49	14.29 ± 1.46
--	0.5	--	1.43 ± 0.26	0.57 ± 0.27
--	1.5	--	0.71 ± 0.29	0.57 ± 0.29
--	2.5	--	4.0 ± 0.69	12.2 ± 0.89
--	3.0	--	5.29 ± 0.87	19.29 ± 1.02
--	4.0	--	4.29 ± 0.75	17.43 ± 1.90
--	5.0	--	3.43 ± 0.65	11.14 ± 1.08
--	--	0.2	7.71 ± 0.81	29.86 ± 1.22
--	--	0.3	10.59 ± 0.92	48.0 ± 3.15
--	--	0.4	7.4 ± 0.48	29.14 ± 1.47
--	--	0.5	7.86 ± 0.60	21.57 ± 0.87
0.5	0.5	--	0.43 ± 0.20	0.57 ± 0.20
1.0	0.5	--	0.42 ± 0.22	0.71 ± 0.29
2.0	0.5	--	1.14 ± 0.40	5.0 ± 0.53
3.0	1.0	--	6.57 ± 0.75	18.86 ± 0.51
4.0	1.0	--	3.14 ± 0.63	7.29 ± 0.61

Table :2. Combined effect of auxins and cytokinins on shoot multiplication (mg/l)

NAA	IAA	IBA	KIN	BA	TDZ	Mean no. of shoots after 30 days	Mean no. of shoots after 60 days
0.1	--	--	--	--	0.1	5.0 ± 0.38	11.0 ± 0.54
0.1	--	--	--	--	0.2	11.14 ± 0.59	24.29 ± 0.42
0.5	--	--	--	1.0	--	8.83 ± 0.84	21 ± 0.54
1.0	--	--	2.0	--	--	1.29 ± 0.57	1.14 ± 0.46
1.0	--	--	3.0	--	--	2.14 ± 0.88	7.0 ± 0.53
1.5	--	--	--	2.0	--	14.71 ± 0.68	30.14 ± 0.88
--	0.1	--	--	--	0.1	2.0 ± 0.53	2.86 ± 0.46
--	0.1	--	--	--	0.2	4.24 ± 0.68	10.0 ± 0.54
--	0.5	--	--	1.0	--	2.14 ± 0.63	6.29 ± 0.68
--	1.0	--	--	3.0	--	4.29 ± 0.75	17.0 ± 0.38
--	1.0	--	1.5	--	--	0.43 ± 0.2	0.71 ± 0.36
--	1.0	--	2.5	--	--	1.86 ± 0.59	3.0 ± 0.54
--		0.1	--	--	0.1	5.0 ± 0.65	8.86 ± 0.74
--		0.1	--	--	0.2	10.14 ± 0.63	20.86 ± 0.59
--		1.0	--	3.0	--	3.0 ± 0.65	6.43 ± 0.61
--		1.0	--	3.5	--	8.14 ± 0.86	17.0 ± 0.81
--		1.5	2.5	--	--	0.43 ± 0.2	0.57 ± 0.29
--		1.5	3.5	--	--	1.29 ± 0.52	1.29 ± 0.52

Table :3. Effect of additives on shoot multiplication(mg/l)

NAA	IBA	KIN	AdS	PVP	Glu.	Mean no. of shoots after 30 days	Mean no. of shoots after 60 days
0.5	--	1.0	50	--	--	1.86 ± 0.59	3.0 ± 0.54
0.5	--	2.0	100	--	--	7.29 ± 0.68	27.0 ± 0.54
0.5	--	1.0	--	50	--	1.14 ± 0.46	1.0 ± 0.38
0.5	--	2.0	--	100	--	1.43 ± 0.61	1.3 ± 0.52
0.5	--	1.0	--	--	50	4.14 ± 0.59	10.0 ± 0.82
0.5	--	2.0	--	--	100	5.14 ± 0.59	20.86 ± 0.59
--	0.5	1.0	50	--	--	4.0 ± 0.53	12.0 ± 0.54
--	0.5	2.0	100	--	--	4.86 ± 0.46	23.86 ± 0.86
--	0.5	1.0	--	50	--	1.14 ± 0.46	1.14 ± 0.46
--	0.5	2.0	--	100	--	4.86 ± 0.59	20.0 ± 0.82
--	0.5	1.0	--	--	50	1.86 ± 0.59	3.0 ± 0.54
--	0.5	2.0	--	--	100	4.86 ± 0.74	22.0 ± 0.66

Plate 1: 1. 7 Shoots from MS medium having 3 mg/l BA.

Plate 1: 5. 5 Shoots from MS medium having 3 mg/l KIN.

Plate 1: 8. 11 Shoots from MS medium having 0.3 mg/l TDZ.

Plate 1: 10. Synergestic effect of 3 mg/l BA and 1 mg/l KIN.

Plate 1: 11. Combined effect of 1.5 mg/l NAA and 2 mg/l BA.

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Plate. 2.12. Shoots in IAA 1mg/l and BA 3 mg/l MS

.Plate 2.13. IBA 1mg/l & BA 3mg/l MS medium

Plate2.14. NAA 1mg/l & KIN 3mg/l medium

Plate 2. 15 IAA 1 mg/l and KIN 2. 5mg/l MS.

Plate 2. 17 NAA 0.1 mg/l and TDZ 0. 2 mg/l MS.

Plate 2. 19 IBA 0.1 mg/l and TDZ 0.2 mg/l MS.

Plate 2. 20 NAA 0.5 mg/l , KIN 2 mg/l and AdS 100 mg/l MS medium resulted 7 shoot buds.

Plate 2. 22 IBA 0.5 mg/l , KIN 2 mg/l and PVP 100 mg/l MS medium .

Plate 2. 24 NAA 0.5 mg/l KIN 2 mg/l and Glutamine 100 mg/l MS medium

Plate 3.27 : NAA 0.5mg/l, KIN 2mg/l and PVP 100 mg/l in MS medium .

Plate 3. 26. IBA 0.5mg/l, KIN 2mg/l and Glutamine 100 mg/l in MS medium

Plate 3. 29. IBA 0.5mg/l, KIN 2mg/l and PVP 100 mg/l in MS medium

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