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High frequency of multiple shoots induction in *Paederia foetida* (L.)- A rare medicinal plant

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Abstract: Plants have been the eternal source of food and medicine since antiquity in all traditions and cultures. The medicinal plants are considered as one of the most vital components of ecosystem and biodiversity. Tissue culture techniques are usually used for the propagation as well as conservation of medicinal plants. *In vitro* multiplication of shoot induction and other tissue culture studies is hither unknown in this species. In this study, experiments were designed to develop regeneration protocols for the propagation as well as conservation of a rare medicinal plant, *Paederia foetida* (L.) The high frequency of multiple shoots was recorded from direct organogenesis of nodal explant. MS medium supplemented with benzyl adenine (4.4µM), kinetin (4.6µM), and 3% sucrose promoted the maximum number of shoots as well as beneficial shoot length. The direct organogenesis in TDZ (2.2µM) containing medium gave maximum percentage of shoots in MS medium from nodal segments compared with that of the other growth regulators. These plants grew normally without showing any morphological variations from the mother plant.

Keywords: Direct Organogenesis, Multiple Shoots, *Paederia foetida*, Rare Medicinal Plant, Regeneration

1. Introduction

Medicinal plants are most exclusive source of life saving drugs for the majority of population in the world. India has a rich heritage of traditional systems of medicines viz. Ayurveda, Sidha, Unanai which are mostly based on botanical formulations. *Paederia foetida* is native to both temperate and tropical Asia, from India to Japan and South East Asia. It has bitter taste with foul smell. The genus *Paederia* is generally distributed in Asia and consists of 20-30 species worldwide. *P. foetida* commonly known as skunk vine is a shrub or a perennial climbing herb found in Himalayas from Dehradun eastwards upto an altitude of 1800 mt and also found in Assam, Bihar, Bengal, Orissa and Andhra Pradesh [1].

Paederia foetida is a potential medicinal plant [2, 3] *P. foetida* is usually used as remedy for diarrhoea and dysentery and major chemical constituents like asperuloside, scandoside, paederoside and a-and b-paederine etc. are present in this plant [4, 5, 6]. *P foetida* is also reported to have ethno medicinal uses both in Bangladesh [7] and India [8]. The leaf extract exhibits the antioxidant activity [9], antitussive activity [10] and antinociceptive activity [11]. Further, methanolic leaf extract was screened for its anthelmintic activity against *Pheretima posthuma* and *Tubiex tubifex* [12]. The protocol developed in the present study for shoot regeneration from direct organogenesis is a simple, economical and effective in induction of multiple shoots by using nodal explants for potential application in large scale propagation and conservation.

2. Materials and Methods

2.1. Plant Material

The plant material of *Paederia foetida* was collected from Mallur hills, Warangal District, Andhra Pradesh, India, in November, 2009. It was systematically identified and authenticated in the plant systematic laboratory, Department of Botany, Kakatiya University, Warangal. The tender twig cuttings were excised from 1year aged young plant growing in the medicinal garden, University campus. The plant samples were treated with 2% fungicide (bavistin) for 20 min. The plant material was kept under running tap water for 30 min, and subsequently was washed with distilled water. The explants were washed with the series of

various sterilents like Tween 20 solution for 10 min and surface sterilized with 0.1% $HgCl_2$ (2 min), 5% Sodium hypochlorite and 0.1% cetrimide. Contrary to this Amin [13] reported the use of $HgCl_2$ (0.1%) for longer duration (10min) for sterilizing the twigs collected from the field grown plants of *P. foetida*. Excised explants for 1.0-1.5 cm were inoculated on MS medium containing various growth regulators.

2.2. Culture Media and Culture Conditions

Analytical grade chemicals and hormones obtained from Himedia laboratories were used for preparing the stock solutions and subsequent media preparation. The basal medium consisted of MS salts [14] supplemented with B - complex vitamins (0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 1mg/l thiamine HCl and 100 mg/l myoinsosital [15], 30 g/l sucrose and 0.8 % agar for gel rite were used for the study. The pH of the medium was adjusted to 5.8 prior to autoclaving, and molten medium was dispensed into test tubes (borosil) at 20 ml per tube and plugged with non- absorbent cotton. Culture tubes containing the media were autoclaved at 121ºC for 20 min. Surface sterilized explants were inoculated vertically and horizontally onto the culture medium with one or two explants per test tube. All the cultures were maintained at 25 ± 2ºC under 16 h photoperiod at photosynthetic flux of 35-50 µmol $m^2 5^1$ provided by 100/daylight florescent lamps.

2.3. Shoot Multiplication

The multiple shoots were developed from nodal explants. Excised nodal explants were cultured on MS medium supplemented with 0.4, 2.2, 4.4, 6.6, 8.8, 11.1µM of BA (Benzyl Amino Purine), 2.3, 4.6, 6.9, 9.2, 11.6µM of Kn (Kinetin) and 0.4, 0.9, 2.2, 4.5, 6.8µM of TDZ (Thidiazuron) either alone or in combination with NAA (for further multiplication of shoots). Subculture was done once in four weeks by transferring explants to fresh medium of same composition for further shoot proliferation. Further experiment was emarginated for observations of shoot generation capacity; their length and morphology and the data was recorded.

2.4. In Vitro Rooting, Acclimatization of Plantlets

Rooting was done by placing the isolated shoots into 25x150 m borosil culture tubes containing half strength solid MS medium along with different levels of various concentrations of auxins to encourage shoot elongation as well as spontaneous rooting. The present practical manure was carried out completely in randomized design with 5 replications. Data was collected after 8 weeks of cultures for number of roots and root length. Afterwards, the developed plantlets cultures were kept out side of the culture room one day advance for acclimatization (Fig. I-d). *In vitro* developed plants were gently separated from the adherent agar and individually transferred to polythene cups. The polythene cups were filled with equal proportion of sand, soil and vermicompost in 1:1:1 ratio. Then they were transferred into polyhouse and irrigated regularly for 3-4 weeks till they were successfully established in pots with around 78% success rate (Fig. e -f). The monitoring of the plant growth and development confirmed their successful restoration, paving a way for a large scale transplantation of tissue culture - raised plants.

2.5. Statistical Analysis

Experiments were set up in a randomized block design (RBD) and each experiment usually had 5 replications and repeated at least three times. 10 to 25 explants were used per treatment in each replication. Observations were recorded on the frequency (number of cultures responding for shoot proliferation and root evaluation) and the number of shoots per explant, shoots length, roots per shoot, and root length. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's [16] Multiple Range Tests at a 5% probability level according to Gomez and Gomez [17].

3. Results and Discussion

The lack of published methods for *in vitro* propagation of related taxa and limited amount of experimental plant material make the choice and development of initial culture medium for threatened plants is somewhat arbitrary. Initially apical node explants were cultured on MS medium supplemented with different concentrations of BA, Kn and TDZ alone or in combination with NAA for high frequency of shoot multiplication. Shoots were regenerated from the nodal region in all the concentrations of cytokinins (BA, Kn and TDZ). The effect and efficacy of different cytokinins viz., BA, Kn and TDZ individually and *in* combination on multiple shoot induction from nodal explants, percentage of response, mean number of shoots and average shoot length were studied and the results were statistically analyzed and tabulated in (Table-1). MS medium was fortified with different concentration of BA (0.4, 2.2, 4.4, 6.6, 8.8, 11.1µM), Kn (2.3, 4.6, 6.9, 9.2, 11.6 µM) and TDZ (0.4, 0.9, 2.2, 4.5, 6.8 µM). The optimal concentration of BA (4.4µM) showed maximum shoot response of 89 % with mean of 15.41 and attained their shoot length of 4.11 ± 0.35 after 6 weeks of culture. Remarkable decrease in % response was observed at lower and higher level of BA. The lower concentration of BA (0.4µM) contributed to 35% of response with mean of 7.28±1.37 and mean length of 2.42±0.36. At higher concentration (11.1µM) showed 75% of shoot response with mean of 9.40±2.23 and average shoot length of 2.68±0.22. The % of response was more at higher level than at lower concentration of BA. Similar tendency were clearly noticed in *Vitex negundo* [18]. Optimal concentration of Kn (4.6µM) contributed 74% response

with mean number of 13.33±0.81 and attained the shoot length of 2.94 ± 0.26 after 4 weeks of culture. Concentration dependent gradual decline in % of response was observed Kn (Table-1). At optimal level of TDZ (2.2μM), induction of maximum number of shoots per explant with mean number of 12.92±1.87 and average shoot length 3.30±0.36 were exhibited. At below and above optimal concentrations, the % of response of mean of shoots and average shoot length was gradually decreased, however TDZ at lower concentration showed better results than at higher level. Among the three cytokinins, BA was more effective in favor of multiple shoot induction than TDZ and KN [19].

With the addition of the NAA (0.5, 2.6, 5.3μM) to BA (2.2, 4.4μM) to MS medium induced less number of shoots (Plate-1) per explant in comparison to BA alone. 40% of responding cultures were noticed at 2.4μM BA+2.6μM NAA. The increasing concentration of NAA and BA in combination did not show any shoot induction or percentage of regeneration response. (Table-1).

The combined contribution of both Kn and NAA on multiple shoot induction from nodal explant with regard to the percentage of response, mean number of shoots, average shoot length was examined and the results are furnished statistically in Table-1. MS medium supplemented with Kn (4.6, 9.2μM) + NAA (0.5, 2.6, 5.3μM) at optimal level of Kn (9.2, 4.6) + NAA (2.6, 5.3) showed maximum response of 35% and 30% with mean number of 10.28±1.42 and 3.16±0.60 with average shoot length of 2.09±0.46 and 1.16±0.23 respectively. Thus, it can be concluded that higher level of Kn (9.2μM) initiates the shoot induction from nodal segments. However the frequency of number of shoots per explant was less in comparison to Kn alone.

Shoot proliferation was also noticed from the medium containing Kn (2.3 - 11.6μM) but the rate of frequency of shoot multiplication was low over the incubation period compared with other cytokinin, BA. Apart from optimal supplement of the medium towards production of healthy shoots, regeneration capacity of the *in vitro* shoot was found to be dependent upon the size and number of shoots per clump and also the time of sub culturing [20].

In micro propagation, resource of *P. foetida* explant in regeneration varied greatly from other plants depending upon the selection of explant age. The shoot elongation (5-9 cm) was noticed at the same concentrations of the medium within 3-4 weeks (Figs. e -f).

The influence of auxins IBA, IAA and NAA on rhizogenesis from the excised shoots was studied employing ½ strength MS medium supplemented with various concentrations of IBA (0.04, 0.09, 0.4, 2.4, 4.9μM), IAA (0.05, 0.1, 0.5, 2.8, 5.7, 11.4μM) and NAA (0.05, 0.1, 0.5, 2.6, 5.3, 10.7μM). Rooting occurred in all concentrations but with different rooting percentages and the optimal response (84%) was observed on ½ MS+0.04μM IBA in terms of average number of roots 6.41+0.94 with mean number of root length of 4.28±0.27 per shoot after 45 days of culture. By increasing the concentration of IBA up to 4.9μM, declined the percentage of response gradually. Besides mean number and mean length, higher concentrations of IBA (2.4, 4.9μM) showed callus formation at basal end of micro-shoot accompanied with rooting, because callus tissue inhibited the vascular supply to rooting so the mean number and mean length was also declined as compared to lower concentration of IBA including *Ocimum basilicum* L. [21], *Mentha piperita* L. [22], *Tylophora in* [23].

It is evident from this investigation that, IBA was most effective in inducing rooting. Similar results were noticed in *Vitex aganuscastus* [24]. Optimal concentration of IAA (0.5μM) favored to initiate maximum response (70%) with mean number of 4.45±0.94 and average root length of 3.07±0.33 per shoot after 8 weeks of culture. Increase in the concentration of IAA (5.7, 11.4μM) resulted in rooting accompanied with callus at cut ends of micro-shoots. In these concentrations of IAA, the induction of rooting with callusing caused to reduced formation of rooting when compared with rooting devoid of calli [25]. In an earlier study, the addition of IAA has also reduced root formation in Costa Rican melon genotypes [26]. NAA was quite antagonized by both IAA and IBA in rhizogenesis from cut ends of micro-shoots of *P. foetida*. Rooting is failed at lower level (0.05, 0.1 and 0.5μM) in NAA. NAA at 2.6 μM showed 30% of response in terms of mean number of roots 4.50+0.42 with mean length of 3.80. Similar results were noticed in *Hydrocotyl confera* [27]. Higher level of NAA (10.7μM) initiated maximum number of root response (55.40) accompanied with callus formation at basal end of micro-shoots. Effectiveness of IBA in regard to rooting was found to be more than IAA and NAA.

Hardening of regenerates and examination of morphological characters:

The well developed plantlets were successfully transferred into the polythene cups. The polythene cups were filled with sand, soil and vermicompost in 1:1:1 ratio. Subsequently they were transferred in to polyhouse and irrigated regularly for 3-4 weeks and successfully established in pots with (78%) success rate (Figs. i -f). Morphological characteristics of the plantlets established in field conditions were identical to that of mother plants. The developed protocol will be useful for the induction of true type plants round the year irrespective of the seasons for the exploitation at industrial level for medicinal purposes at one hand and propagation and conservation of the species on the other hand. *In vitro* propagation could be used to develop the potential plants with more medicinal value by following biotechnological means.

in vitro **conservation of** *Paederia foetida* **a rare medicinal plant**

a,b): Sprouting shoots of the nodal explants. c,d): Induction and elongation of multiple shoots from the mother explant on the basal MS medium supplemented with BA. e): Induction of roots on shoots. f,g): Hardened tissue culture plants established in sand; soil mixture in earthen pots.

Table 1. *Effect of different growth regulators (BA, Kn and TDZ) on high frequency of multiple shoot induction from nodal explants in Paederia foetida (L).*

Growth regulators	(Conc. in µM)	% of Response	Number of Shoots Mean ± S.E	Shoot length Mean ± S.E
BA	0.4	35	7.28±1.37 [b]	2.42±0.36 [b]
	2.2	55	7.36±1.23 [b]	2.34±0.24 [b]
	4.4	89	15.41±2.30 [a]	4.11±0.35 [a]
	6.6	80	11.18±1.53 [ab]	2.73±0.25 [b]
	8.8	80	11.1±1.39 [ab]	2.80±0.29 [b]
	11.1	75	9.4±2.23 [ab]	2.68±0.22 [b]

Growth regulators (Conc. in µM)		% of Response	Number of Shoots Mean ± S.E	Shoot length Mean ± S.E
Kn	2.3	55	8.90±1.15 [b]	2.92±0.27 [a]
	4.6	74	13.33±0.81 [a]	2.94±0.26 [a]
	6.9	60	8.41±1.74 [b]	2.24±0.27 [ab]
	9.2	50	10.00±1.93 [b]	2.06±0.18 [b]
	11.6	45	6.00±1.54 [b]	1.97±0.31 [b]
TDZ	0.4	45	7.22 ±2.06 [b]	2.05±0.22 [b]
	0.9	50	7.20±1.54 [b]	2.24±0.33 [b]
	2.2	86	12.92±1.87 [a]	3.30±0.36 [a]
	4.5	45	7.55±1.79 [b]	2.35±0.22 [b]
	6.8	40	6.37±1.54 [b]	2.08±0.23 [b]
BA+NAA	2.2+0.5	-	-	-
	2.2+2.6	30	5.66±0.71 [a]	1.51±0.37 [c]
	2.2+5.3	30	11.33±1.40 [ab]	1.93±0.48 [bc]
	4.4+0.5	35	14.71±2.24 [a]	3.48±0.34 [a]
	4.4+2.6	40	11.37±3.67 [ab]	3.15±0.49 [ab]
	4.4+5.3	30	9.50±1.97 [ab]	1.71±0.38 [c]
Kn+NAA	4.6+0.5	-	-	-
	4.6+2.6	-	-	-
	4.6+5.3	30	3.16±0.60 [b]	1.16±0.23 [a]
	9.2+0.5	25	3.20±1.31 [b]	1.74±0.35 [a]
	9.2+2.6	35	10.28±1.42 [a]	2.01±0.46 [a]
	9.2+5.3	20	3.25±0.47 [b]	1.67±0.21 [a]

Data were recorded after 45 days of culture,
Treatment means followed by different alphabets within PGR and response variable combinations are significantly different from each other ($p<0.05$); comparison by Duncan's Multiple Range test

Table 2. *Effect of different growth regulators (IBA, IAA and NAA) on root induction from in vitro established shoots after 4 weeks of Paederia foetida cultured on half strength MS medium.*

Growth regulators (Conc. in µM)		Response	Rooting % frequency	Number of Roots Mean ± S.E	Root length Mean ± S.E
IBA	0.04	R	84	6.41±0.94[a]	4.28±0.27[a]
	0.09	R	73	5.00±0.78[a]	3.41±0.27[ab]
	0.4	R	60	4.11±0.93[a]	2.48±0.36[bc]
	2.4	C+R	40	5.83±0.87[a]	2.38±0.26[c]
	4.9	C+R	30	4.16±0.98[a]	2.55±0.21[bc]
IAA	0.05	-	-	0±0.00	0±0.00
	0.1	R	50	3.90±0.54[a]	3.62±0.29[a]
	0.5	R	70	4.45±0.94[a]	3.07±0.33[ab]
	2.8	R	53	3.75±0.61[a]	2.55±0.29[bc]
	5.7	C+R	30	3.50±0.76[a]	2.45±0.28[bc]
	11.4	C+R	30	3.00±0.73[a]	1.70±0.33[c]
NAA	0.05	-	-	0±0.00	0±0.00
	0.1	-	-	0±0.00	0±0.00
	0.5	-	-	0±0.00	0±0.00
	2.6	R	30	4.5±0.42[a]	3.80±0.13[a]
	5.3	C+R	55	3.66±0.37[a]	2.50±0.20[b]
	10.7	C+R	40	3.50±0.73[a]	2.36±0.02[bc]

Data were recorded after 45 days of culture,
Treatment means followed by different alphabets within PGR and response variable combinations are significantly different from each other ($p<0.05$); comparison by Duncan's Multiple Range test.

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