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Research Article

INDIRECT ORGANOGENESIS OF THE MEDICINAL PLANT SPECIES, *CRYPTOLEPIS GRANDIFLORA* WIGHT (APOCYNACEAE) BY TISSUE CULTURE TECHNIQUE

R. Prema[1], S. Paulsamy[2]*, J. Thambiraj[2] and M. Saradha[2]

[1]Department of Botany, Arulmigu Palniandavar Arts College for Women, Palani, Tamil Nadu, India.

[2]Department of Botany, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India.

ABSTRACT

In the present study, protocol for callus induction and regeneration for the medicinal plant species, *Cryptolepis grandiflora* has been developed by employing tissue culture technology. Young leaf explant inoculated on MS medium containing the growth regulators, BAP and NAA at 2.0 and 1.2 mgl^{-1} respectively showed higher callus induction (81%). The amount of callus responded for shoot formation greater was (78%) when subcultured on to the MS medium containing BAP (2.0 mg/L) and GA_3 (0.5 mg/L). The elongated shoots were rooted well on half strength MS medium supplemented with NAA at 1.5 mg/L and IBA at 0.2 mg/L. Regenerated plantlet were successfully acclimatized and hardened off in green house condition with better survival rate of 70%.

Keywords: *Cryptolepis grandiflora*, medicinal plant, *in vitro* indirect organogenesis.

INTRODUCTION

Tissue culture technique is being increasingly exploited for clonal multiplication and *in vitro* conservation of valuable indigenous germplasm that are threatened with extinction (Boro *et al*., 1998). Micropropagation method is specifically applicable to species in which clonal propagation is needed (Gamborg and Phillips, 1995). *Cryptolepis grandiflora* is a climber belongs to the family, Apocynaceae, found in deciduous and moist deciduous forests and endemic to peninsular India (Henry *et al.*, 1987). Its roots, stems and leaves are used for the treatment of bone fracture. It is used for diabetes also. Due to poor availability possibilities of facing threats by this species may be serve in course of time due to its medicinal importance and hence the demand. Therefore increasing the population size and ensuring the greater biomass availability are more essential to meet the demand and conserve the species as well. This paper describes the indirect organogenesis strategies developed by using tissue culture technology for this species under the influence of different combinations and concentrations of certain growth regulators.

MATERIALS AND METHODS

Leaf segments from young and healthy branches of *C.grandiflora* were used as explants. They were collected from pot cultured individuals maintained in a mist chamber. For surface sterilization, the collected immature leaves were washed with tap water twice and then treated with 5 % tween–20 solutions for 5 min followed by rinsing in tap water. To eliminate fungal contamination, explants were further treated with 5 % antibiotics (Amphicillin and Rifampicin) for 30 min followed by 3 rinses in sterile double distilled water. Further, surface sterilization was carried out by dipping the explants in 0.1% $HgCl_2$ for 3 min followed by 3-4 rinses in sterile double distilled water.

Media and culture condition

Murashige and Skoog (MS) (1962) medium containing 3 % sucrose solidified with 1 % agar (tissue culture grade, Himedia, India) was used. The pH of the medium was adjusted to 5.6-5.8

prior to the addition of agar before autoclaving at 121^0 C for 15 min. All the culture bottles were kept in culture chamber at 25± 2^0 C under 16/8 hr (light/dark) photoperiod with a light intensity of 2000 lux supplied by cool white fluorescent tubes and with 60-65% relative humidity.

Callus induction medium

The explants were transferred to culture bottles containing 25 ml MS medium supplemented with different concentrations and combinations of BAP and NAA for callus induction.

Shoot induction medium

MS medium containing different combinations and concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) and GA^3 at 0.5 mg/L was used for shooting.

Rooting of elongated shoots and acclimatization

After proper shoot induction, the plantlets were carefully removed from the medium and washed with sterile double distilled water properly, so as to avoid any trace of medium on roots. *In vitro* regenerated shoots (5-6 cm long) were excised and transferred onto the half strength MS medium supplemented with the auxins, NAA and IAA for rooting. After proper root formation, these rooted plantlets were transferred to hardening medium composed by garden soil, sand and vermicompost in different proportions and maintained in greenhouse condition to know the survivability rate.

Statistical Analysis

All the experiments were performed at least twice using triplicate. The data was statistically processed and means were compared using Duncan's Multiple Range Test (P<0.05).

RESULTS AND DISCUSSION

Calli formation was observed from leaf explants after 24 days of inoculation in MS medium with different combination and concentrations of BAP and NAA (Table 1). The better response of explants for callus induction (81%) was observed in the MS medium supplemented with the cytokinin, BAP (2.0 mg/L) and auxin, NAA (1.2 mg/L). Thambiraj and Paulsamy (2012) also observed similar trend of results for the plant species, *Acacia caecia.* Arumugam *et al.* (2009) explained that the callus formation is depending upon the several factors including the culture, environment, nature of explant and hormonal and non hormonal regulators which may act synergistically in determining the proper induction, proliferation of calli and regeneration into plants.

Shoot initiation was more pronounced by the subculturing of callus on MS medium containing BAP with GA_3. The higher amount of 78% of callus derived shoots on to the MS medium fortified with BAP and GA_3 at 2.0 and 0.5 mg/L respectively. Greater number of multiple shoots (12.77 shoots/callus) and higher shoot length (6.7 cm) were produced in the same combinations and concentrations of growth regulators as observed for shoot initiation (Table 2). The superiority of the cytokinin, BAP over the other growth regulators on shoot bud production and proliferation of shoots has been reported for several medicinal and aromatic plant species (Jebakumar and Jayabalan, 2000; Faisal and Anis, 2003; Hussain and Anis, 2006; David Raja *et al*., 2008). Induction of roots is an important step in *in vitro* plant propagation and for that the excised shoots were inoculated on MS medium containing the auxins, NAA and IBA for effective root development (Table 3). Higher amount of 74% of shoots produced roots on the MS medium supplemented with NAA and IBA 1.5 and 0.2 mg/L respectively. Higher root numbers (9.81 roots/ shoot) and root length (6.5 cm) were observed on the MS medium supplemented with NAA and IBA at 1.5 and 0.2 mg/L respectively (Table 3). These findings are in agreement with that reported by Sujatha and Reddy (1998); Ahn *et al*. (2007); Alam *et al*. (2010) and Ramanathan *et al*. (2011). All these facts indicate that the cytokinin, BAP is the most essential growth regulator for effective shooting of the study species, *C. grandiflora.*

After the development of roots, the plantlets were taken out from the culture bottles and washed with sterilized distilled water to remove adhering agar medium, so that the chance of contamination could be stopped. Then these juvenile plantlets were transferred to the hardening medium containing garden soil, sand and vermicompost (1:1:1 ratio by volume) in that the leaf callus derived plantlets survivability rate was higher (70%) (Table 4). Admixture of all these three components may offer condusive environment by providing proper nutrients, adequate aeration and required minerals respectively to the plantlets. Generally, it is reported that application of the auxin, IBA with another auxin, NAA results in increased rooting in many plants as the physiological stages of rooting are correlated with the changes more effectively by these auxins (Anantha Padmanabha and Vijayalakshmi, 1994; Singh *et al.,* 2005; Torres, 2004; Dhillon *et al.,* 2009). Similar kinds of findings of effective root formation by the

influence of various types of auxins in many plant species have been reported elsewhere (Mallikadevi and Paulsamy, 2009; Mahesh *et al.,* 2010; Loc *et al.,* 2011; Mungole *et al.,* 2011; Rajput *et al.,* 2011).

It is concluded that the multiple shoot formation and elite plantlet production protocol established for the medicinal plant species, *Cryptolepis grandiflora* from leaf explants by employing tissue culture technology is more useful to enhance the population and hence the biomass of this species.

Table 1: Effect of growth regulators on callus induction from leaf explants of the species, *Cryptolepis grandiflora*

Growth regulators (mg/L)				Days required for callus formation after inoculation	Callus formation (%)
BAP	2, 4-D	NAA	Kn	Leaf explant	Leaf explant
0.5	0.0	0.0	0.0	16	$35.25^{a} \pm 1.63$
1.0	0.0	0.0	0.0	19	$47.76^{c} \pm 0.82$
1.5	0.0	0.0	0.0	17	$56.86^{e} \pm 1.63$
2.0	0.0	0.0	0.0	20	$64.35^{g} \pm 2.45$
2.5	0.0	0.0	0.0	21	$78.53^{i} \pm 0.82$
3.0	0.0	0.0	0.0	19	$60.84^{f} \pm 0.82$
0.0	0.5	0.4	0.0	16	$46.57^{c} \pm 1.63$
0.0	1.0	0.4	0.0	15	$54.37^{e} \pm 1.63$
0.0	1.5	0.4	0.0	20	$61.36^{f} \pm 0.82$
0.0	2.0	0.4	0.0	23	$72.58^{i} \pm 1.63$
0.0	2.5	0.4	0.0	16	$57.97^{a} \pm 1.63$
0.5	0.0	0.3	0.0	15	$49.00^{cd} \pm 0.82$
1.0	0.0	0.6	0.0	16	$57.35^{e} \pm 1.63$
1.5	0.0	0.9	0.0	21	$81.35^{i} \pm 0.82$
2.0	0.0	1.2	0.0	24	$77.13^{j} \pm 1.63$
2.5	0.0	1.5	0.0	21	$72.25^{h} \pm 1.63$
3.0	0.0	1.8	0.0	18	$52.87^{b} \pm 1.63$
0.0	0.3	0.0	0.2	15	$46.48^{c} \pm 1.63$
0.0	0.6	0.0	0.4	17	$51.59^{d} \pm 0.82$
0.0	0.9	0.0	0.6	16	$55.32^{e} \pm 1.63$
0.0	1.2	0.0	0.8	18	$60.21^{f} \pm 0.82$
0.0	1.5	0.0	1.0	14	$49.00^{cd} \pm 0.82$

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT.

Table 2: Effect of different concentrations of growth regulators on shoot initiation, shoot number and shoot length after the subculturing of leaf derived callus of the species, *Cryptolepis grandiflora*

Growth regulators (mg/L)				Culture response (%)	No. of shoots/callus	Shoot length (cm)
BAP	NAA	GA_3	IAA			
0.3	0.0	0.0	0.0	$49.23^{d} \pm 0.82$	$4.14^{ab} \pm 0.82$	$2.9^{ab} \pm 0.82$
0.3	0.0	0.0	0.0	$62.64^{hi} \pm 0.82$	$5.74^{bcd} \pm 0.82$	$3.8^{abc} \pm 1.63$
0.3	0.0	0.0	0.0	$65.78^{j} \pm 1.63$	$7.98^{def} \pm 1.63$	$4.0^{abc} \pm 1.63$
0.3	0.0	0.0	0.0	$58.37^{fg} \pm 0.82$	$6.00^{abc} \pm 1.63$	$5.2^{bc} \pm 0.82$
0.3	0.0	0.0	0.0	$52.64^{gh} \pm 1.63$	$5.38^{fgh} \pm 1.63$	$4.6^{abc} \pm 0.82$
0.3	0.0	0.0	0.0	$47.53^{kl} \pm 1.63$	$4.64^{fgh} \pm 0.82$	$5.7^{c} \pm 1.63$
0.5	0.0	0.3	0.0	$69.27^{k} \pm 0.82$	$11.75^{hi} \pm 0.16$	$4.3^{abc} \pm 0.82$
1.0	0.0	0.3	0.0	$71.00^{m} \pm 1.63$	$10.35^{ghi} \pm 0.82$	$3.9^{abc} \pm 0.82$
1.5	0.0	0.3	0.0	$74.58^{n} \pm 0.82$	$11.17^{i} \pm 0.82$	$5.0^{c} \pm 1.63$
2.0	0.0	0.3	0.0	$76.18^{n} \pm 0.82$	$11.82^{i} \pm 0.82$	$5.7^{c} \pm 1.63$
2.5	0.0	0.3	0.0	$78.58^{b} \pm 1.63$	$12.77^{efg} \pm 0.82$	$6.7^{abc} \pm 0.82$
3.0	0.0	0.3	0.0	$50.36^{a} \pm 1.63$	$5.32^{bcd} \pm 0.82$	$2.5^{ab} \pm 0.82$
0.0	0.5	0.0	0.0	$45.26^{de} \pm 0.82$	$7.41^{ghi} \pm 0.82$	$3.5^{abc} \pm 1.63$
0.0	0.5	0.0	0.0	$41.12^{a} \pm 0.82$	$6.67^{fgh} \pm 0.82$	$4.9^{bc} \pm 0.82$

0.0	0.5	0.0	0.0	49.11^{c} ± 0.82	6.52^{cde} ± 1.63	5.3^{bc} ± 1.63
0.0	0.5	0.0	0.0	51.85^{fg} ± 1.63	3.43^{ab} ± 1.63	4.7^{abc} ± 1.63
0.0	0.5	0.0	0.0	54.26^{ij} ± 0.82	5.28^{bcd} ± 0.82	3.3^{abc} ± 0.82
0.0	0.5	0.0	0.0	50.16 ^{l} ± 1.63	8.47^{efg} ± 1.63	2.8^{ab} ± 0.82
0.5	0.0	0.0	0.2	25.49^{d} ± 0.82	1.90^{def} ± 0.82	1.7^{abc} ± 0.82
1.0	0.0	0.0	0.2	49.00^{k} ± 1.63	2.38^{ghi} ± 0.82	2.9^{a} ± 0.82
1.5	0.0	0.0	0.2	59.45^{ef} ± 1.63	4.15^{abc} ± 1.63	3.4^{abc} ± 0.82
2.0	0.0	0.0	0.2	62.75^{hi} ± 0.82	2.47^{a} ± 0.82	3.5^{abc} ± 1.63
2.5	0.0	0.0	0.2	63.32 ^{fg} ± 0.82	5.74^{ab} ± 1.63	2.0^{a} ± 0.82
3.0	0.0	0.0	0.2	64.21 ^{ij} ± 1.63	6.00^{fgh} ± 0.82	2.5^{ab} ± 0.82

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT

Table 3: Effect of different concentrations of growth regulators on root number, rooting percentage and root length after the subculturing of leaf callus derived shoots of the species, *Cryptolepis grandiflora*

Growth regulators (mg/l)			Shoots rooted (%)	No. of roots/shoot	Root length (cm)
IBA	IAA	NAA			
0.5	0.2	0.0	60.30^{j} ± 0.41	6.67^{abc} ± 0.82	5.4^{a-d} ± 0.82
1.0	0.2	0.0	68.25 ^{k} ± 0.82	7.39^{def} ± 1.63	5.9^{ef} ± 0.82
1.5	0.2	0.0	74.29^{m} ± 0.41	9.81^{f} ± 1.63	6.5^{f} ± 1.63
2.0	0.2	0.0	70.65^{l} ± 0.82	8.78^{ef} ± 0.41	6.2^{ef} ± 1.63
2.5	0.2	0.0	57.54^{h} ± 1.63	4.98^{abc} ± 0.82	4.8^{de} ± 0.41
3.0	0.2	0.0	51.45^{g} ± 0.82	3.00^{ab} ± 0.16	3.2^{a-d} ± 0.16
0.5	0.0	0.3	42.65^{e} ± 0.41	2.26^{bcd} ± 1.63	1.1^{bcd} ± 0.82
1.0	0.0	0.3	39.32^{d} ± 0.82	3.19^{ab} ± 1.63	3.9^{a-d} ± 0.33
1.5	0.0	0.3	35.54 ^{c} ± 1.63	4.64^{abc} ± 0.82	2.8^{abc} ± 0.49
2.0	0.0	0.3	31.24^{b} ± 0.82	4.38^{abc} ± 0.33	3.5^{a-d} ± 0.41
2.5	0.0	0.3	27.17a ± 0.41	2.59a ± 0.82	2.6ab ± 0.82
3.0	0.0	0.3	17.98 a ± 0.82	3.38ab ± 0.82	2.2a ± 0.16
0.5	0.0	0.0	34.87c ± 0.82	3.29ab ± 1.63	3.8a-d ± 0.82
1.0	0.0	0.0	45.67 f ± 0.82	5.48bcd ± 0.49	2.9abc ± 0.82
1.5	0.0	0.0	52.54h ± 1.63	6.76cde ± 1.63	4.1bcd ± 0.82
2.0	0.0	0.0	51.87h ± 0.82	5.53bcd ± 0.82	4.5cde ± 0.41
2.5	0.0	0.0	48.88g ± 0.41	4.68abc ± 0.82	3.2a-d ± 0.33
3.0	0.0	0.0	43.34i ± 0.82	5.58bcd ± 0.82	4.8de ± 0.24

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT

Table 4: Effect of different composition of hardening medium on survivability rate of leaf callus derived *in vitro* rooted plantlets of the species, *Cryptolepis grandiflora*

Hardening medium composition (V/V)	No. of plantlets under hardening	No. of plantlets survived	Survivability (%)
Red soil + sand (1:1)	50	24	49^{a} ± 0.82
Garden soil + sand + vermicompost (1:1:1)	50	44	70^{d} ± 0.41
Decomposed coir waste + perlite + compost (1:1:1)	50	38	65^{c} ± 1.63
Vermicompost + soil (1:1)	50	32	62^{b} ± 0.65
Red soil + sand + vermicompost (1:1:1)	50	25	50^{a} ± 0.82

Means in column followed by different letter are significant to each other at 5% level according to DMRT

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