Plant Tissue Cult. & Biotech. **19**(2): 143-150, 2009 (December)

PTC&B

In vitro Propagation of *Ionidium suffruticosum* Ging. – A Seasonal Multipotent Medicinal Herb.

Arunkumar B. Sonappanavar*, M. Jayaraj[1], Asha N. Bagadekar and Anant V. Bhandarkar[2]

Department of Botany, P. C. Jabin Science College, Autonomous College, Hubli, Karnataka, India

Key words: Ionidium suffruticosum, Micropropagation, Medicinal herb

Abstract

Indirect regeneration of plant was obtained through organogenesis in leaf callus cultures of *Ionidium suffruticosum*. Leaf explants were found to be best suited for callus induction on MS with 2, 4-D (0.5 and 1.0 mg/l). Maximum shoot regeneration was obtained in MS supplemented with Kn (4.0 mg/l) alone and NAA (0.4 mg/l) with Kn (2.0 m/l). The *in vitro* shoots thus obtained were successfully rooted in MS supplemented with Kn (4.0 mg/l) alone and with NAA (2.0 mg/l) and Kn (0.2 mg/l). Seventy per cent of the rooted plants survived and they were successfully acclimated in soil.

Introduction

Ionidium suffruticosum, Ging. Syn. *Hybanthus enneaspermus* (L.). F. Muell. is a rare ethno medicinal ephemeral herb (Deshpande 2006) belongs to Violaceae. The plant is widely distributed in Africa, Madagascar, Srilanka, China, New Guinea, tropical Australia and India. The plant is moderately heightening herb of about 10 to 20 cms tall with a long slender tap root. In nature *Ionidium suffruticosum* plants are seasonal and appear only for a few months. Soon after the rainy season the aerial parts dry up and disappear. The roots and a few basal stem stocks retain in the soil and regenerate during rainy season.

The plant is considered to have highest medicinal value which is widely used by the traditional healers for the treatment of diseases like diabetes (Das et al. 2004), malaria (antiplasmodial activities) (Bernard 2004), male sterility (Kheraro and Bouquet 1950), urinary tract infections and water retention (Puspangadan and Atal 1950). The tender leaf stalks are used as demulcent; the

*Author for correspondence. <arun_bso@yahoo.co.in> [1]P.G. Department of Botany, Karnatak University, Dharwad, Karnataka, India. [2]K.L.E. Society's College of Pharmacy, Hubli, Karnatak, India.

roots are antigonorrhoeic, diuretic, bowel complaints and urinary problems (Deshpande 2006). There are some limitations in the propagation of this species. Propagation of the species by seeds or any other conventional methods are not reliable because the plants are seasonally available for a short duration in nature and moreover the seeds loose their viability within a few weeks. The species are under threat due to their exploitation from their natural habitat by traditional healers, over grazing by animals, seasonal habitat and their short seed dormancy. At the same time, a few reports are available on its micropropagation (Prakash et al. 1999) and no data are available for its biological activities (Saxena 1975, Majumadar et al. 1979). Since tissue culture methods have been successfully employed for large scale multiplication of a number of medicinal herbs and sub shrubs (Bidwell et al. 2001) for rapid *in vitro* micropropagation and genetic improvement was considered for this important plant species.

Materials and Methods

The smaller-, medium size healthy leaves were excised from one-month-old plants of *Ionidium suffruticosum* grown in the Botanical garden of the P. C. Jabin Science College, Hubli, Karnatak, India. The smaller size explants provided less chance of contamination and the longer leaves showed total loss of morphogenetic potential (Mujib et al. 2001). The explants were washed thoroughly under running tap water for 45 minutes. Leaves were surface sterilized with a mild phototoxic liquid detergent (2% labolene, Qualigens, India) stirred for 2 min and then washed with tap water. Leaves were dipped in 1% bavistin (Carbendezim 50% WP) for 2 min. Further they were rinsed in distilled and sterilized water for 5 minutes, then immersed in 70% ethanol for 1 min and rinsed in distilled and sterilized water for 2 min. Leaves were then taken in Petri plate into laminar air flow chamber and dipped in 2% sodium hypochlorite solution for 1 min and rinsed in distilled and sterilized water for 2 min twice. Finally leaves were cut into small pieces of 0.5 to 1.0 cm long and trimmed the margin of leaves. The leaf explants were cultured on MS by touching the upper surface of the semisolid medium. MS was supplemented with 2, 4-D (0.1 to 8.0 mg/l) alone or in combinations with cytokinins for callus induction. The MS was comprised of macro- and microelements and congealed with agar (0.8%), myoinositol (100 mg/l) and sucrose (3.0%). The pH of the medium was adjusted to 5.7 before autoclaving at 121ºC for 15 min. The sterilized explants were inoculated in the laminar air flow chamber. All the cultures were incubated at 25 ± 2ºC under a 16 hours photoperiod by cool white fluorescent light (50 μmol/m²/S) and 70 ± 5% relative humidity. All the experiments were repeated thrice and had ten replicates with single explant.

The callus was obtained on MS supplemented with 2, 4-D (0.5 mg/l) and maintained on the same medium with 2, 4-D (0.5 mg/l) by subsequent subculture of leaf callus. About 200 to 250 mg of the leaf callus was subcultured on fresh MS at 30 days interval for the future use. The callus was tested for shoot regeneration potential on MS supplemented with various concentrations of Kn, BAP, zeatin (0.1 - 12.0 mg/l) alone or in combination with IAA and NAA at different concentrations (Table 1). Regenerated shoots were excised and cultured on MS fortified with IAA, NAA (0.1 - 12.0 mg/l) alone or in combinations with cytokinins. Rooted plantlets were taken out from culture vessels. The roots were gently washed with slightly warmed sterile distilled water to remove the adhering agar. The regenerated plantlets were transferred to plastic cups containing mixtures of sterile vermiculate and sand (3 : 1). Initially high humidity was maintained by covering the cups with punctured polythene bags and during the first seven days plantlets were irrigated three times with half-strength of MS inorganic salts and subsequently with sterilized distilled water. The polythene bags were removed and plants were acclimatized for two weeks in aseptic culture room with the same illumination but at higher temperature (16 hr photoperiod, 28 ± 2º day, 25 ± 2ºC night). Further the plantlets were exposed gradually to sunlight for acclimatization and maintained in College garden.

Results and Discussion

Callus formation was observed within 28 days on leaf explants cultured in MS supplemented with different concentrations of plant growth regulators. Callus was initiated after eight - ten days of inoculation at the cut end of the leaf explant in 2, 4-D alone or IAA and NAA along with zeatin and BAP (0.1 to 8.0 mg/l) to the MS basal medium (Fig. 1A). It has been demonstrated in many cases that 2, 4-D is usually the choice of auxin for callus induction and subculture of grasses and herbs (Bhaskaran and Smith 1990). Callus produced on MS supplemented with IAA were yellowish, pale green in colour and showed the limited growth. Although IAA (2 mg/l) with Kn (1.0 mg/l) induced small amount of yellowish creamy callus but failed to grow further. 2, 4-D was supplemented to MS in different concentrations to obtain better responses. It was observed that 2, 4-D at 0.5 mg/l was best for rapid callus induction and subsequent growth. The callus so produced was translucent, crystallized mass turned to creamy to pale green and then turned into hard, compact green solid mass of callus grown even up to 60 to 80-folds in 45 days. Callus grown on MS with 2, 4-D (0.5 mg/l) grew profusely and possessed high regenerative potential. This callus was referred to as “stock callus”. The stock callus was subcultured on MS containing various concentrations of auxins alone or in combination with cytokinins showed different responses with respect to the number of shoot bud formation (Table 1).

However, best results were obtained on MS supplemented with Kn (4.0 mg/l) alone. More and more results indicated that the addition of low concentration of cytokinins in callus culture often enhances callus regeneration (Alpeter and Fosselt 2000).

Reports are available for the seed-derived callus induction on MS supplemented with NAA (2.6 WPM) and BA (2.2 WPM) (Prakash et al. 1999). Bidwell et al. (2001) reported the necessity of medium containing half-strength of MS with 5 μM *N*-BA and 0.5 μM NAA for callus and shoot initiation in *Hybanthus floribundus* and shoot differentiation was achieved from light green compact callus obtained after one year of subculture of seed callus (Bidwell et al. 2001). MS with Kn at 4.0 mg/l was most effective for shoot bud initiation which developed a mean of 15 shoot per leaf callus subculture after five - six weeks of inoculation (Fig. 1B). Shoot bud initiation on leaf callus has been reported in *Justica genderussa* (Mujib et al. 1997), *Ruta graveolens* (Agastian et al. 2006). MS supplemented with Kn (4.0 mg/l) induced more number of shoots at high frequency (95%) compared to MS with NAA + Kn, NAA + BAP, NAA + zeatin and 2, 4-D with Kn (Table 1). Minimal cytokinins and auxins in culture media avoided somaclonal variations and efficiently produced true type plantlets (Edson et al. 1996).

The healthy and sturdy shoots were separated and transferred on to rooting medium (Fig. 1C,D). Rooting was best on MS supplemented with NAA 2.0 mg/l and Kn 0.2 mg/l developed a mean of 68 roots/subculture of leaf callus after 40 - 45 days (Table 2). Basal media supplemented with NAA was found to be better for regeneration according to the Kumar et al. (1993) in *Clitoria ternata.* However, in the present study the thick and long roots with numerous root hairs (Figs. 1E,F) developed on the same shoot initiation medium (MS with Kn 4.0 mg/l) after 35 - 45 days of subculture of leaf callus. The roots developed from the base of the shoots with intervening callus. Rooting was always accompanied by some amount of callus with the shoots. Rooting response was poor on NAA, IAA (Table 2) and least or almost absent in 2, 4-D.

After 35 - 45 days of culture of shoots on rooting medium, the rooted plantlets were transferred to plastic cups (Fig. 1G) containing vermiculate and sand (3 : 1). After 20 - 25 days the plantlets are transferred to pots containing garden soil, vermiculate and sand (3 : 2 : 1) for hardening and acclimatization (Fig. 1 I). To check fungal growth, plants were sprayed with 0.1% bavistin once a week. Humidity was maintained by covering with perforated, plastic cover and frequently spraying of water. Similar process of maintaining humidity was practiced for hardening of banana (Jasrai and Wala 2000) and *Alpinia* (Rolf and

Table 1. Effect of 2.4-D, Kn, NAA separately or NAA + Kn, NAA + BAP and NAA + zeatin on callus differentiation from stock callus of *I. suffruticosum* after 45 days of culture.

2,4-D	NAA	Kn	BAP	zeatin	% of shoots*	No. of shoots/ callus*±SE
0.5 -2.0	-	-	-	-	Nil	Nil
2.0	-	0.5	-	-	20.0	08 ± 0.1
2.0	-	1.0	-	-	60.0	12 ± 0.2
2.0	-	2.0	-	-	60.0	14 ± 0.2
2.0	-	3.0	-	-	60.0	12 ± 0.2
-	-	0.5	-	-	40.0	02.0 ± 0.1
-	-	1.0	-	-	40.0	02.5 ± 0.1
-	-	2.0	-	-	45.0	08.2 ± 0.2
-	-	3.0	-	-	56.0	10.4 ± 0.2
-	-	4.0	-	-	95.0	15.2 ± 0.3
-	0.2	1.0	-	-	90.0	05.6 ± 0.2
-	0.2	2.0	-	-	80.0	06.0 ± 0.2
-	0.2	3.0	-	-	70.0	04.0 ± 0.1
-	0.2	4.0	-	-	45.0	03.0 ± 0.1
-	0.4	1.0	-	-	35.0	02.0 ± 0.1
-	0.4	2.0	-	-	90.0	03.0 ± 0.1
-	0.4	3.0	-	-	30.0	02.0 ± 0.1
-	1.0	2.0	-	-	25.0	03.0 ± 0.1
-	1.0	4.0	-	-	60.0	08.0 ± 0.1
-	1.0	8.0	-	-	Nil	Nil
-	-	-	0.25	-	10.0	00.2 ± 0.1
-	0.5	-	0.25	-	40.0	00.2 ± 0.1
-	1.0	-	0.25	-	8.0	00.2 ± 0.1
-	1.0	-	0.5	-	12.0	00.2 ± 0.1
-	1.0	-	1.0	-	10.0	00.2 ± 0.0
-	1.0	-	2.0	-	10.0	00.3 ± 0.1
-	1.0	-	2.5	-	8.5	00.5 ± 0.0
-	1.0	-	5.0	-	9.5	00.6 ± 0.1
-	2.0	-	5.0	-	Nil	Nil
-	5.0	-	1.0	-	7.5	05.9 ± 0.1
-	0.2	-	-	0.2	5.0	03.0 ± 0.1
-	0.2	-	-	0.4	4.0	03.0 ± 0.1
-	0.2	-	-	0.8	15.0	04.0 ± 0.2
-	0.2	-	-	1.0	12.5	03.0 ± 0.1
-	0.2	-	-	2.0	30.0	07.2 ± 0.2
-	0.4	-	-	2.0	10.0	04.0 ± 0.1
-	0.8	-	-	2.0	16.0	02.3 ± 0.1

*Data on average of ten replicates. The experiment was repeated for three times. Mean ± standard errors/deviations.

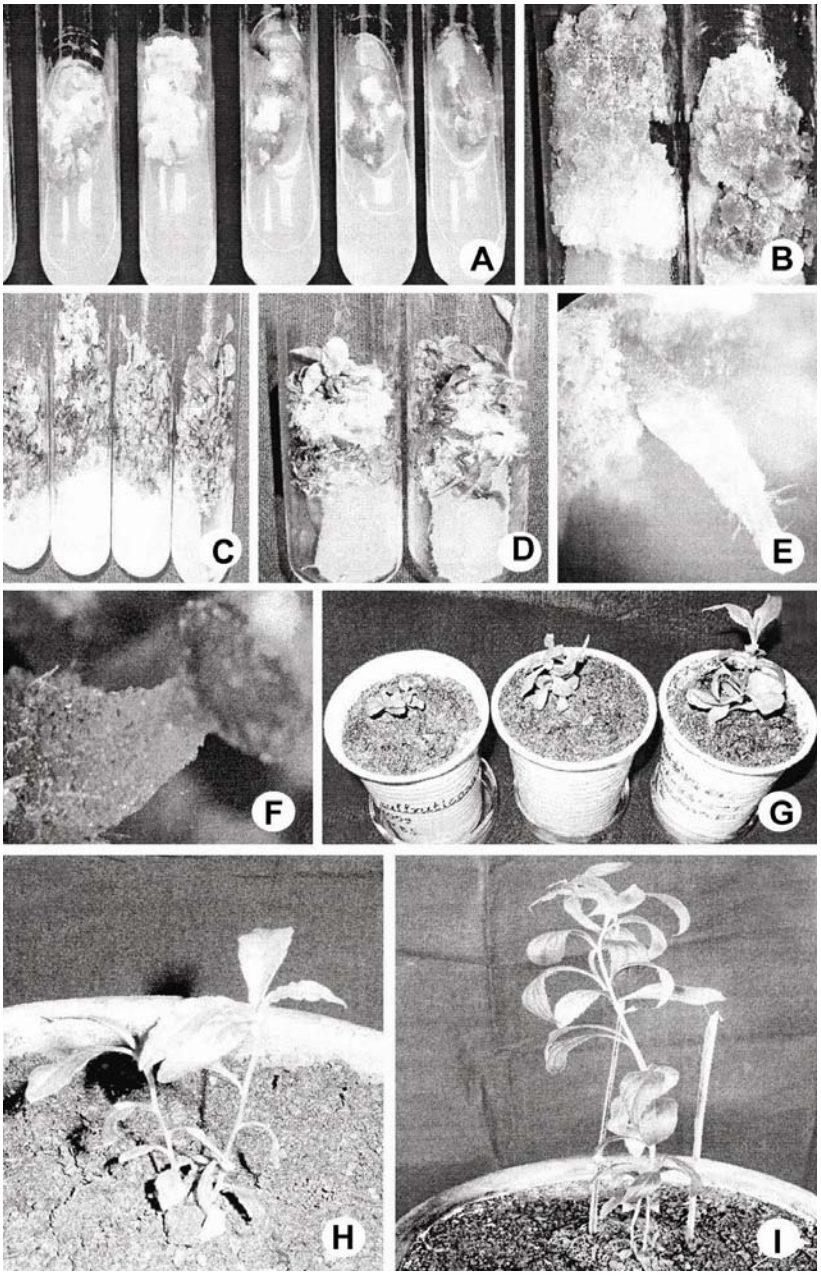

Fig. 1. A. Initiation of callus after eight to ten days. B. Formation of shoot buds and shoots after five to six weeks. C,D. Shoots are placed in rooting medium. E,F. Long roots with numerous root hairs. G. Rooted plants in plastic cups. H,I. Plants transferred to earthen pots for acclimation.

Ricardo 1995). The hardened plants were transferred to field after 35 days of good growth. Almost 70% of the regenerated plants survived and showed a

vigorous growth of shoots and roots with least morphological variations. However the height of the plantlets and size of the leaves were bigger than *in vivo* plants. Thus, a successful regeneration system culminating in transplantation to field has been accomplished. The tissue culture studies of *Ionidium suffruticosum* showed that, the MS supplemented with 2,4-D (0.5 mg/l) was optimal for callus induction whereas MS with Kn (4.0 mg/l) was best suited for both shoot and root induction in short duration.

Table 2. Effect of NAA, Kn and 2, 4-D separately and in combination on root induction in *in vitro* grown shoots after 45 days.

Growth regulators (mg/l)		% of root formation	No. of roots/ culture ± SE*	Length of roots (cm) ± SE*
NAA	Kn			
-	O.5	2.0	0.5 ± 0.0	0.5 ± 0.1
-	1.0	2.0	03.0 ± 0.1	0.8 ± 0.1
-	2.0	12.0	10.5 ± 0.1	0.8 ± 0.1
	3.0	18.0	12.5 ± 0.1	1.2 ± 0.1
-	4.0	24.0	18.2 ± 0.1	6.3 ± 0.2
0.5	-	-	Nil	Nil
1.0	-	10.0	2.0 ± 0.1	0.3 ± 0.1
2.0	-	-	Nil	Nil
1.0	0.2	12.0	28.0 ± 0.1	2.8 ± 0.2
2.0	0.2	62.0	68.0 ± 0.1	4.8 ± 0.2
2.0	0.4	30.0	8.0 ± 0.1	0.8 ± 0.2
2.0	0.8	09.0	2.0 ± 0.1	0.5 ± 0.1

*Data on average of ten replicates. The experiment was repeated for three times. Mean ± standard errors/ deviations.

In conclusion, we have developed an *in vitro* protocol for inducing rapid callus formation from leaf explant of *Ionidium suffruticosum* as well as regeneration of the plant from leaf callus via organogenesis. The leaf-based regeneration protocol is expected to useful for genetic improvement of this herb through recombinant DNA technology.

Acknowledgements

The authors are grateful to UGC, New Delhi for the financial assistance under CPE grant. The authors express their gratitude to the Chairman and members of K. L. E. Society, Belgaum, the Principal and the Head, Botany Department of P. C. Jabin Science College, Autonomous College, Hubli, Karnatak, India for the facilities provided and their continuous encouragement.

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