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Micropropagation of *Inula racemosa* Hook.f. A Valuable Medicinal Plant

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Abstract: A rapid *in vitro* regeneration protocol for induction of multiple shoots from nodal segments of *Inula racemosa* Hook.f. was developed. Leaf and nodal segments were inoculated on MS medium containing different concentrations of Benzylaminopurine (BAP) either alone or in combination with Naphthalene Acetic Acid (NAA) or Indole Butyric Acid (IBA) growth hormones. MS medium supplemented with BAP (0.25 mg L^{-1}) induced maximum number of shoots (20.7±0.8). The shoots were rooted on half strength of MS medium either alone or supplemented with (IBA) Indole butyric acid (1.0 mg L^{-1}). Direct rooting from leaves has also been developed from cultured plants. *In vitro* raised plantlets were acclimatized in green house and successfully transplanted to the field with a survival of 80%.

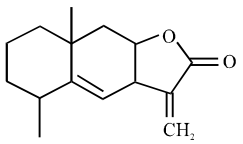
Key words: *In vitro*, micropropagation, medicinal plants, shoot multiplication

INTRODUCTION

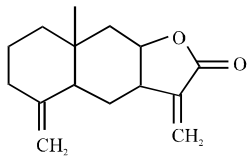
Medicinal plants constitute a very important bioresource in India because it has one of the richest plant based ethnomedical traditions in the world. The global market for medicinal plants and herbal medicines is estimated to be worth US $80 billion a year. International export trade in medicinal plants from India is 32600 tonns a year. The demand for medicinal plants has increased globally due to the resurgence of interest in herbal medicine standardized plant extract, Culinary herbs natural therapeutic essential oil and phytopharmaceuticals (Shawl and Qazi, 2004). Most of the demand is being met through collection of large quantities of these plant species and their parts from wild populations. The methods of extraction employed are almost crude and unscientific. As a consequence, the rates of exploitation may exceed those of local natural regeneration. The Indian Himalayan Region (IHR) is a rich reservoir of biological diversity in the world. High value medicinal plant species are threatened, their status ranging from low-risk, near threatened to critically endangered. There is thus an urgent need to develop and implement regeneration/conservation strategies for over exploited medicinal plant species. The common means of regeneration and propagation of medicinal plants include seed-based clonal and micropropagation methods. Seed based multiplication is the most effective, realistic and convenient means for most species.

Inula racemosa Hook.f. Poshkar (Asteraceae) is an important medicinal plant. Its roots are expectorant, seeds aphrodiac. The roots find use in ISM for cardiac asthama cough, pulmonary infections and skin diseases and as adultrant for *Saussurea costus* roots (Sarin *et al.*,1996). *Inula racemosa* is reported to have hypoglycaemic activity (Gholap and Kar, 2005). Roots are reported to contain sesquiterpene lactones, mainly alantolactones, isoalantolactone, alantolides, besides β-sitosterol, daucosterol (Tan *et al.*, 1998; Kalsi *et al.*, 1989). Alantolides displayed plant growth regulator activity (Kalsi *et al.*, 1989).

Isoalantolactone a major lactone showed anti-fungal properties against human pathogenic fungi (Tan *et al.*, 1998; Ketai *et al.*, 2000). The essential oil from roots has a strong aroma and is reported to mainly contain sesquiterpenes aplotaxene (Bokadia *et al.*, 1986).



Alantolactone



Isoalantolactone

Due to its various medicinal properties. It is being over exploited from the wild habitat. *In vitro* propagation of plants holds tremendous potential for the production of high quality plant based medicines. The present study reports a high frequency of multiple shoot regeneration in *Inula racemosa* from leaf and nodal explants obtained through seeds.

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MATERIALS AND METHODS

The seeds of *Inula racemosa* were collected from gene bank of RRL (CSIR) Srinagar during (sept-oct 2005). The seeds were dried for a fortnight after which they were stored in polythene bottles at room temperature. Shriveled and light seeds were removed before starting the studies. Seeds were surface-sterilized with 0.1% aqueous mercuric chloride for about 1-2 min. Thereafter they were washed thoroughly with tap water and kept submerged in distilled water for 24 h at 25±2°C. The seeds were transferred to Petri plates with one layer of filter paper made wet with distilled water and allowed to germinate in an incubator at 25±2°C under continuous illumination provided by fluorescent white light (PAR: 40 μmol m^{-2} sec^{-1}). Seed germination was recorded at periodic intervals, the radicle emergence (2-4 mm) serving as an index of germination (Fig. 1) (Sharma and Sharma, 2006). Germination Value (GV), which accounts for both germination rate and germination percentage was calculated (Fig. 2) according to Czabator's (1962) formula i.e., (Peak value×mean daily germination).

$$\text{Peak value} = \frac{\text{Maximum germination percentage}}{\text{Total No. of days to reach maximum}}$$

$$\text{Mean daily germination} = \frac{\text{Final germination percentage}}{\text{No. of days in the test}}$$

The apical buds were excised aseptically and inoculated on MS culture medium supplemented with BAP either alone or in combination with NAA or IBA adding 3% sucrose and gelled with 0.8% agar. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 0.1N HCl. The cultures were maintained at 25±2°C temperature

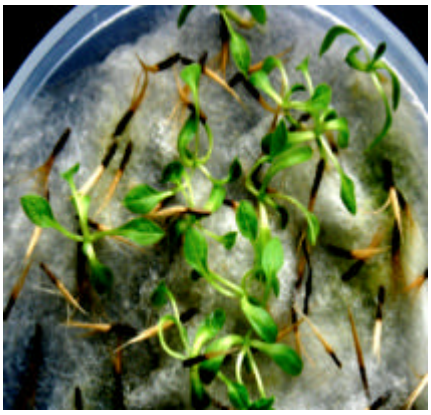

Fig. 1: Seed germination

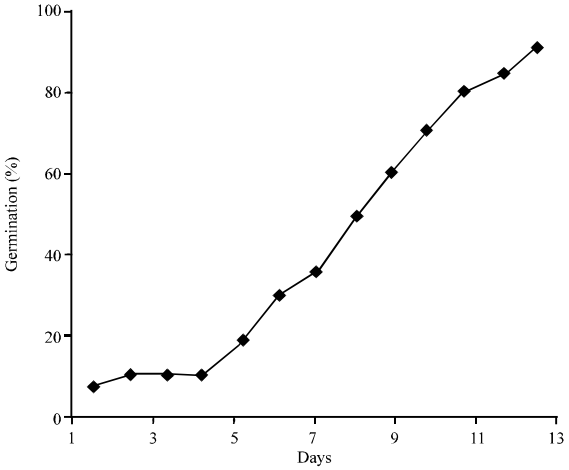

Fig. 2: Germination value (GV) of seeds of *Inula racemosa*

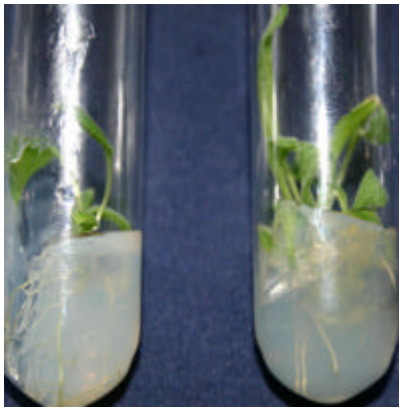

Fig. 3: Multiple shoot regeneration and root formation

with 16 h light and dark photoperiod (2500 lux) provided with fluorescent tubes and 60% relative humidity. For each treatment, 10 replicates were taken. The regenerates were allowed to grow under various treatments of plant growth regulators for 4-6 weeks to monitor growth, differentiation and other related morphogenetic responses. Average number of shoots (Fig. 3) was visually counted where as height of the shoots and root was measured. For callus induction, petiole explants (ca 2-3 cm in length) were obtained from plantlets growing *in vitro* on MS medium containing 0.50 and 1.0 mg L^{-1} BAP. The explants were placed on different levels of IBA, NAA and 2, 4-D (0.2-0.8 mg L^{-1}) in combination with 0.1-0.25 mg L^{-1} of BAP. Approximately 1g of callus was placed in each cultural vials containing different levels and combinations of cytokinins (BAP) and auxins (IBA, NAA) as detailed in the results (Table 1). Following regeneration of shoots complete plantlets were obtained on the growth and development medium (0.25 mg L^{-1} BAP

Table 1: Morphogenetic response of explants of *Inula racemosa* after 4 weeks on MS medium containing different concentrations and combinations of various auxins with BAP

		BAP						
	Concentrations							
Supplements	(mg L^{-1})	0	0.1	0.25	0.50	1.0	2.0	2.5
1	0	--	S	S	S	S	S	S
A	0.2	--	--	--	--	--	--	--
A	0.4	S	--	--	--	--	S	--
	0.6	S	S	S	--	--	--	S
	0.8	C	O	C	O	O	O	S
I	0.2	--	--	--	--	--	--	--
B	0.4	--	--	--	--	--	--	--
A	0.6	C	--	--	--	--	--	--
	0.8	C	C	--	--	--	--	--
N	0.2	C	C	C	S	S	--	--
A	0.4	C	C	C	C	--	--	--
A	0.6	C	C	S	S	S	--	--
	0.8	C	C	C	C	C	--	--
2	0.2	C	C	C	C	S	S	--
4	0.4	C	C	C	C	S	C	C
D	0.6	C	B	B	B		C	B
	0.8	C	B	B	B	B	C	B

Legend S --- Formation of shoot, B ---- Browning, O --- Formation of dark green nodules, C ---- Callusing, R --- Root formation, 4□ ----- Moderate response, ■ -----Extreme response

Fig. 4: Plantlet in jelly pot

Fig. 5: Field view

and 0.6 mg L^{-1} NAA). These were subsequently transferred to pots filled with a mixture of soil: sand: vermiculite (1:1:1). (Fig 4 and 5). Further the oil composition in the field grown plants was measured using a simple Clevenger apparatus. The oil percentage has been found to be 0.04% .

RESULTS

Seed germination studies: The chilled and sterilized seeds were kept for germination in petriplates for 2 weeks time. Maximum seed germination was observed (100%) after 13 days of inoculation under continuous illumination of 40 μM m^{-2} sec^{-1} at a temperature of 25±2°C. The cultures were kept as such for apical bud formation and shoot *in vitro* shoot regeneration. These results are improved than earlier findings in *Inula racemosa* (Sharma and Sharma, 2006).

***In vitro* culture initiation:** The nodal explants were placed on basal MS (Murashige and Skoog, 1962) medium supplemented with different concentrations and combinations of benzylaminopurine (BAP: 0.1, 0.25, 0.50, 1.0, 2.0 and 2.5 mg L^{-1}), Indole -3-acetic acid (IAA 0.4, 0.6 mg L^{-1}) for shoot proliferation and multiplication (Fig. 3). Routinely 25 mL of the molten medium was dispensed into culture tubes (25×150 mm), plugged with non-absorbent cotton sterilized at 121°C and 1.06 $kgcm^2$ pressure for 15min. The excised explants were inoculated in these culture vials and shoot proliferation was achieved after 4 weeks interval. The cultures were maintained at 25±2°C temperature with 16 h light and dark photoperiod (2500lux) and 60% relative humidity However, maximum regeneration frequency was obtained in basal MS media supplemented with 0.25 mg L^{-1} of BAP. For each treatment, 10 replicates were taken. The cultures were maintained by regular sub culturing at 4 weeks intervals on fresh medium with the same composition.

Induction of rooting and acclimatization: For root induction, the shoots were transferred on full and half strength of MS media supplemented with IAA and IBA (0.2, 0.4, 0.6 mg L^{-1}) and (0.2, 0.4, 0.6, 0.8 mg L^{-1}), respectively. MS half with IBA (1.0 mg L^{-1}), regenerated shoots exhibited maximum rooting from cut ends and an average of 3.6 roots per microshoot, 4.4 cm long after 20 days of inoculation. All the cultures were incubated at 25±2°C under 16 h photoperiod. Rooted micropropagules were thoroughly washed to remove the adhered gel and planted in 2.5 cm jelly pots containing a sterile mixture of sand: soil: vermiculite in the ratio of 1:1:1 and kept in green house (Fig. 4).

Table 2: Rooting response of microshoots on half or full strength MS medium with auxins

Auxins (mg L^{-1})	Response (%)	Average No. of roots±SD	Average root length (cm) ±SD
Control	0.0	0.0±0.0	0.00±0.0
MS+IAA (0.5)	40	1.5±0.5	1.3±0.2
MS+IAA (1.0)	60	2.2±0.4	1.6±0.1
MS+IAA (1.5)	80	2.4±0.4	1.8±0.2
1/2MS+IAA (0.5)	60	2.5±0.5	2.6±0.3
1/2MS+IAA (1.0)	80	2.6±0.4	4.4±0.8
1/2MS+IAA (1.5)	100	2.7±0.4	5.1±0.2
MS+IBA (0.5)	50	2.6±0.4	2.0±0.3
MS+IBA (1.0)	80	3.2±0.4	3.3±0.4
MS+IBA (1.5)	100	3.4±0.4	3.6±0.3
1/2MS+IBA (0.5)	90	3.2±0.4	2.8±0.2
1/2MS+IBA (1.0)	100	3.5±0.5	3.3±0.2
1/2MS+IBA (1.0)	100	3.6±0.4	4.4±0.4

Values are mean±SD Data based on 10 replicates per treatments

Observation of cultures and presentation of results: Ten cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots/ explant and percentage of rooting was statistically analyzed (Table 2).

DISCUSSION

With MS+0.6 mg L^{-1} NAA combination, 50% cultures differentiated in to shoots with fewer leaves. MS supplemented with 0.2 and 0.4 mg L^{-1} 2, 4-D separately induced the growth of rosette leaves in 4 weeks in 30% and 40% cultures, respectively. MS+0.6 mg L^{-1} IAA medium found to be the most effective with regard to apical buds forming little shoots in 70% cultures at end of 4 weeks. On MS+0.1 mg L^{-1} BAP the explants regenerated shoots bearing cluster of leaves. At the end of fourth week, well developed shoots were formed in about 80% of cultures. On MS+0.25 mg L^{-1} BAP shoot regeneration was maximum (Fig. 6).

On MS+0.5 mg L^{-1} BAP the apical bud grew to form rosette leaves in 85% cultures after which they do not show any appreciate growth. Addition of 1.0-2.0 mg L^{-1} BAP to MS basal decreased the rate of differentiation and also percentage of culture responding. Apical buds having cotyledonary leaves intact when grown on MS medium supplemented with various auxins-cytokinin combinations showed that capability of growing in to well differentiate plantlets. Although efficient procedure for seed germination and *in vitro* multiplication has been earlier attempted. but is of low frequency (Kaloo and Shah, 1997). The present study is an improved regeneration protocol as compared to the earlier findings with a regeneration frequency of 80%.

The established shoots were transferred on full and half strength of MS medium supplemented with IBA or IAA for the induction of rooting. Half strength of MS medium with IBA(1.0 mg L^{-1}) was found to be more suitable as compared to full strength of MS medium for rooting of shoots (Table 2) (Fig. 7). MS half strength with

Fig. 6: Elongated plantlet

Fig. 7: Shoot proliferation

Fig. 8: Roots from isolated leaf

IBA 1 mg L^{-1}, regenerated shoots exhibited rooting from cut ends and an average of 3.6 roots per microshoots with 4.4 cm long was observed after 20 days of inoculation. The responses of the explants on media containing different levels and combination of auxins and cytokinin are summarized in (Table 1). No callusing was observed in the absence of growth regulators. However, when a portion of the leaf lamina remained attached to the petiole explant, roots were produced from the cut end (Fig. 8). Similarly on media containing BAP alone at 0.5-1.0 mg L^{-1} concentration, no callusing was observed but roots were formed. On higher BAP levels root formation was inhibited (Mathur Ahuja, 1991).

Amongst the auxins, both IAA and IBA were found unsuitable for callus induction in both cases only slight callusing was observed at 0.8 mg L^{-1} level either alone or in combination with 0.2 to 0.5 mg L^{-1} of cytokinin .On low auxin levels and in combination with 0.2-2.0 mg L^{-1} cytokinin, rhizogenesis was the predominant response. However, as reported earlier (Becker and Schrall, 1980) callusing was observed on all levels of NAA ranging from moderate responses on 0.2 and 0.4 mg L^{-1} to an extensive response on 0.6 mg L^{-1} and 0.8 mg L^{-1}, in case of many other plants.

A slight degree of root formation took place on media containing low NAA: BAP ratio (viz 0.2 mg L^{-1} NAA with 2.0 mg L^{-1} BAP). On media with high 0.6-0.8 mg L^{-1} NAA and low BAP (0.2-0.5 mg L^{-1}) a fast growing, friable light green callus was obtained. However, on 0.8 mg L^{-1} level of 2, 4-D both with and without BAP, rapid browning of the callus took place within 25-30 days. Moderate callusing was also observed on media containing 0.2 mg L^{-1} of 2, 4-D either alone or in combination with 0.2-1.0 mg L^{-1} of BAP. In general, further increase in 2, 4-D levels led to browning and death of the explant within 20 days.

On the basis of above observations a callus stock (Fig. 9) was generated on 0.6 mg L^{-1} NAA with 0.25 mg L^{-1} BAP over two week duration with sub culturing every 4 weeks for experiments on regeneration from callus. Rooted plantlets grown *in vitro* were washed thoroughly to remove the adhering gel, transplanted to 2.5 cm earthen sterile pots containing garden soil: sand: vermiculite in the ratio of 1:1:1. About 96% of the rooted plantlets established in the greenhouse within 2-3 weeks of transfer. The plants grew well and attained 6-8 cm height within 4 weeks of transfer. The acclimatized plants were established in the field condition and grew normally without morphological variation and will be subjected to detailed phytochemical analysis in the forth coming studies using GC and GC/MS.

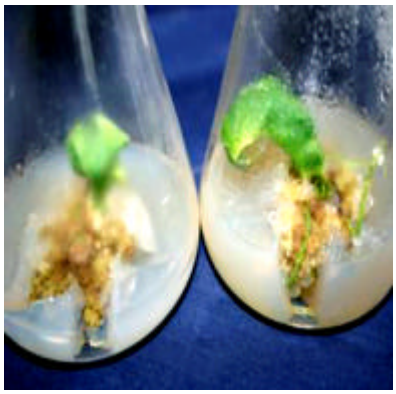

Fig. 9: Callus stock

Present study showed that the cytokinins are necessary for the regeneration of shoots. This observation is in conformity with a number of similar studies carried out on many other plants (Arora and Bhojwani, 1989; Sharma *et al.*, 1993).

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