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In vitro Propagation and Conservation of *Inula racemosa* Hook. F. an Endangered Medicinal Plant of Temperate Origin

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Abstract: *Inula racemosa* is an endangered medicinal plant. It is commonly known as Pushkarmool, Pushkar and Manu. The great sage Charaka has characterized it as *Hikka magrahana* (stops hiccups) and *Savasahara* (helpful in asthma). Also, he has cited it as the best medicament for pleurisy along with cough and asthma (http://enwikipedia.org/wiki/charaka-samhita). Due to the fragile nature of its habitat and exploitation due to its commercial medicinal properties, the species are facing the onslaught of indiscriminate over-exploitation. So far, this plant has not got the required attention from researchers, hence, except for a few efforts, not much work has been done for its cultivation and conservation. Plant tissue culture offers an attractive and quick method for its multiplication and further conservation. In the present investigation, effective procedures for micropropagation and *in vitro* conservation by vitrification were developed. In vitro propagation using aseptically grown seedlings and in vitro conservation via vitrification were standardized. The *in vitro* conserved material could be retrieved and multiplied normally on MS (Murashige and Skoog, 1962) medium fortified with 1.00 mg l-1BA (benzyl adenine) which has been recorded as the best performing medium for in vitro shoot multiplication. The conserved shoots showed normal in vitro propagation and after retrieval from vitrification, platelets were hardened and successfully established in the experimental fields under Nauni (Solan, HP) conditions at an elevation of around 1275 meters above mean sea level.

Keywords: Vitrification, *Inula racemosa,* Micropropagation.

1. Introduction

Inula racemosa Hook f. (Asteraceae) is widely used in traditional medicine in India and Tibet, as well as in drug industries for its antispasmodic, anti-asthmatic and digestive properties and as veterinary medicine also. This species grows in temperate regions of North-Western Himalayas and has been overexploited. Interest in the plant has been directed to domestication. Officially part of the plant is root. Current demand for the plant's root has motivated us to initiate micropropagation and *in vitro* germ-plasm conservation and multiplication. *Inula racemosa* is an endangered plant and hence there is a need for its cultivation and conservation (Anonymous, 1998). This work on micropropagation and conservation of *Inula racemosa* has been carried out in one of the research projects funded by the National Medicinal Plant Board, New Delhi.

2. Materials and Methods

2.1 *In vitro* propagation

Seeds were collected from Jammu and Kashmir and were surface sterilized using 0.2% solution of carbendazim for 10-15 minutes followed by treatment

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with a 0.1% solution of $HgCl_2$ (mercuric chloride) for 5-7 minutes. Sterilized seeds were washed with autoclaved distilled water for 5-7 times. Then placed in test tubes on MS basal medium with 0.8% Difco-bacto agar. Cultures were incubated at 25 ± 2^0C initially in the dark for 7-10 days. Later on, the seedlings were transferred at 16/8 hour photoperiod for further growth. 3-4 weeks old *in vitro* seedlings were divided into different segments and cultured in Erlenmeyer flasks with MS (Murashige and Skoog, 1962) basal medium supplemented with different concentrations of BA (Benzyl adenine) and Kn (Kinetin) in combination and single. The pH of the medium was adjusted to 5.8 and autoclaved at 121^0C for 15 minutes. Cultures were maintained in culture room at 25 ± 2^0C, 16/8 hours photoperiod, a photolux density of 50 µmole/ $s^{-1}m^{-2}$. Regenerated plants were further multiplied by sub-culturing *in vitro* shoot segments on the best responding medium which consisted of MS basal supplemented with 1.00 mg/l^{-1} (milligrams per litre) BA. *In vitro* regenerated shoots after a quick dip in 1000 ppm (parts per million) IBA (Indole butyric acid) were transferred to MS basal medium in order to regenerate roots.

2.2 *In vitro* conservation

In vitro shoot buds of *Inula racemosa* pre-cooled in refrigerator and those without a control was also kept which was not given vitrification treatment, however, it was cryopreserved.

Pre-cooling

↓

Kept in pre-culture medium V1 (MS salts + 0.5 M (Molar) sucrose

↓

***In vitro* shoot buds incubated overnight in dark at 4^0C**

↓

V1 solution replaced with a V2 solution (MS + 0.4 M sucrose+3M glycerol)

↓

***In vitro* shoots in V2 solution incubated at 4^0C for 20-30 minutes**

↓

V2 solution replaced with V3 (MS +40% glycerol+ 13% DMSO (Dimethylsulphoxide) + 15% ethylene glycol)

↓

Cryovials containing *in vitro* shoots were placed on ice for 60 minutes

↓

Cryovials containing *in vitro* shoot buds plunged quickly into the liquid nitrogen

↓

Cryovials were taken out and thawed immediately using a water bath maintained at 40^0C and agitated continuously and V3 solution was discarded

↓

Shoot buds were washed with a V4 solution (MS liquid + 1.2 M sucrose) to eradicate the effect of cryoprotectants and V4 solution was discarded

↓

Explants were placed on an autoclaved filter paper inside a petri dish containing 1 ml of liquid MS medium standardized earlier for micropropagation

↓

Kept in dark at 25 ± 2^0C

↓

Shoot buds transferred to fresh MS medium in flasks and initially kept in diffused light for a few days and thereafter they were shifted to light.

3. Results and Discussion

The seeds germinated after 7-10 days. Segments of young seedlings were placed in MS basal medium with different concentrations of BA and Kn (Kinetin) in combination and single. The present study showed that BA was necessary for the regeneration of shoots; which is in conformity with the results obtained for the same species (Kaloo and Shah, 1997) and *Saussurea obvallata* and *Eclipta alba* members of the same family (Dhar and Joshi, 2005; Dhaka and Kothari, 2005) hence proving that BA has a positive effect on shoot bud induction as well as multiplication especially for the members of the Asteraceae. The highest rate of shoot multiplication @ 7 shoots at the end of 6 weeks was obtained on 1 mg/l^{-1} BA. It was observed that BA at lower concentration was more effective, the same has been observed by Eellarova and Kimakova, 1999 in case of *Hypericum perforatum.* After bud sprouting, subsequent *in vitro* shoot multiplication was obtained on the same medium i.e. MS medium supplemented with 1.0 mg/l^{-1} BA. This medium produced vigorous *in vitro* shoots. Micropropagated shoots did not form roots in the same shoot multiplication medium as some plant species regenerate both roots and shoots simultaneously on the same medium. (Kaur *et al.*, 2000; Alagumanian *et al.*, 2004). Therefore, an experiment was conducted for root regeneration. *In vitro* formed shoots were given a quick dip in 1000 ppm of IBA before inoculating on to full strength MS basal medium with 3% sucrose and 0.8% agar. Diminished concentration of MS salts and sucrose did not induce rooting while in most of the plant species the reduction in these components does induce rooting (Ouma *et al.*, 2004; Joshi *et al.*, 2003; Bobrowski *et al.*, 1996; Kaur *et al.*, 2007). We observed that 100 percent plantlets developed multiple roots in four weeks.

Rooted plantlets were transferred to greenhouse in order to perform the acclimatization process. After ten days, the plants tolerated gradual uncovering and lowering of relative humidity. After fifteen days of transferring to pots, the plantlets were totally uncovered. At complete acclimatization, 95% plantlets survived and are being maintained in the experimental field of the department of biotechnology. Different stages of micropropagation of *Inula racemosa* are presented in plate 1.

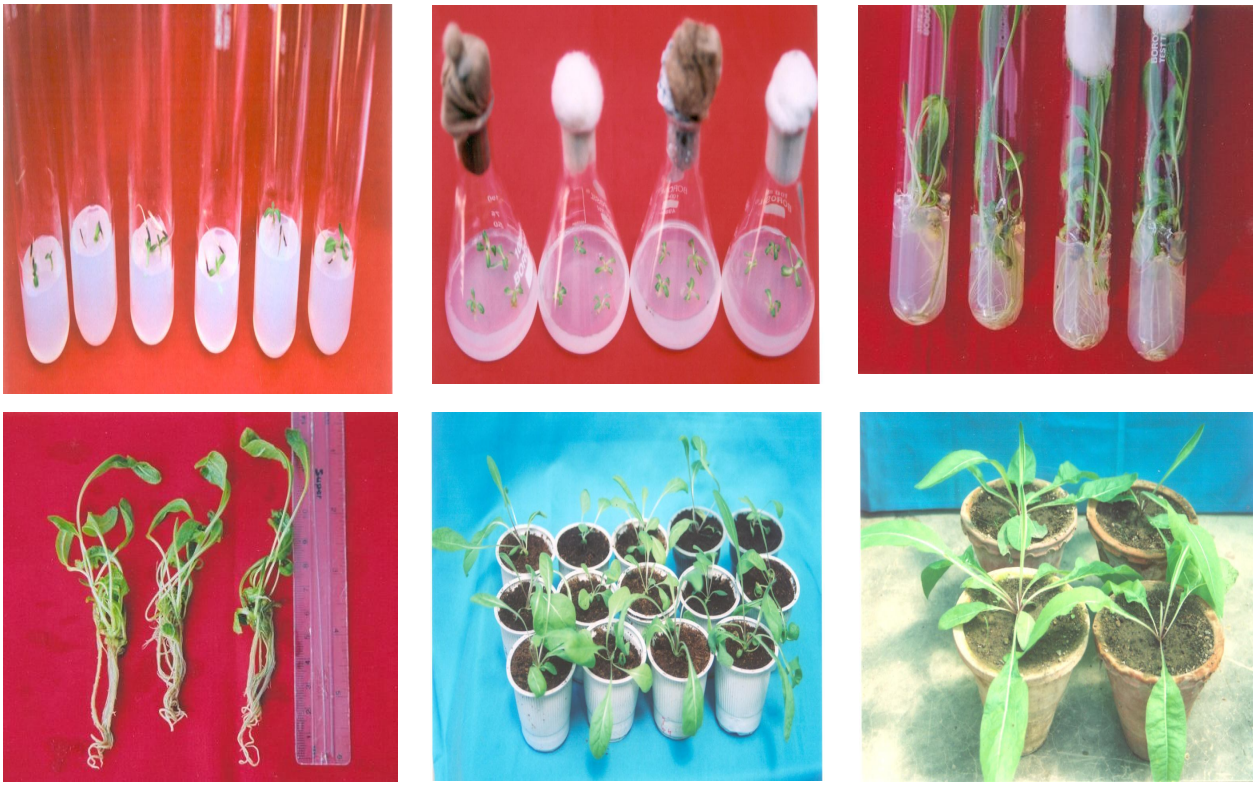

Plate 1. Different stage during *in vitro* propagation of *Inula racemosa* (a) germinating seedlings, (b) *in vitro* multiplying shoots (c) & (d) rooted plantlets, (e) & (f) hardened plants.

According to our understanding, this is the first report for *in vitro* conservation of *Inula racemosa* through vitrification and in this case shoot tips were used for the experiment. Shoot tips and other meristematic tissues are the commonly used explants for cryopreservation as there are lower chances for somaclonal variations as compared to calli or cell suspensions. The shoot tips were divided into two sets, each of twenty shoots. One of the two sets was given a precooling treatment while the other was used directly. Precooling, as well as thawing, was an important factor in determining survival of the shoot buds. The importance of the same has been reported as an imperative step for cryopreservation earlier (Niino *et al*., 1992; Hitmi *et al*., 2000). Conservation was done by treating the explants with a highly concentrated vitrification solution (mainly consisting of glycerol, DMSO and glacial acetic acid) and plunging them in liquid nitrogen for storage. This vitrification solution was first introduced for the cryopreservation of citrus (Sakai *et al.*, 1990). Shoot buds which were given a precooling treatment showed better survival rates of 64 percent and however without precooling, the retrieval rate was observed to be lower i.e. 20%. Control gave zero percent survival of shoot tips.

4. Conclusion

Inula racemosa Hook f. is listed as an 'endangered' medicinal herb of North Western Himalayas and hence efforts were made in the present investigations for its propagation as well as conservation to make the plant material available to the pharmaceutical industries. At sustainable basis, these studies will further help in future for mass propagation, multiplication and had laid foundation for *in vitro* conservation of the plant species that is presently endangered due to exploitation from its natural habitat.

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