

Conservation and Production of Ipecac (*Cephaelis ipecacuanha* Rich.) Plants from Long Term Shoot Cultures

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Abstract

In vitro germplasms of ipecac (*Cephaelis ipecacuanha* Rich.), an important medicinal plant, maintained through reduced growth conditions for more than 12-years, were used as source material for micropropagation. MS with different combinations of Kn (2 mg/l), BAP (2 mg/l), 2iP (2-3 mg/l), NAA (0.2 mg/l) and adenine (40-100 mg/l) as additive was used to induce fresh multiplication of shoots from the nodal meristems and direct shoot bud initiation on the internodal segments. Complete plant regeneration has been achieved from such long term cultures. Regenerated plants maintained their phenotypic and chromosomal stability. Eighty per cent hardened plants, survived in the field condition, are growing well and 25% of them produced flowers within one year. Long term preservation through reduced growth conditions and successful regeneration of morphologically stable plants with stable chromosome numbers ($2n = 22$) from such long term cultures of ipecae plants.

Introduction

Medicinal plant biotechnology offers a major role in production of natural products through plant tissue culture and bioengineering. Medicinal plants are important component of biodiversity. Out of 4,22,000 flowering plant species 35,000 - 70,000 are used for medicinal purpose world-wide. A recent survey revealed that 61% of 877 drugs introduced world-wide can be traced to or were inspired by natural products. Medicinal plant related trade in India is growing faster than the Indian domestic market. Recently a newfound-respect for the privileged structures inherent with natural products, which are safe, and without any side effects has escalated the use of medicinal plants enormously. Hence, there is a strong need for preservation of genetic diversity, cultivation and sustainable usage of important medicinal plant species for future use. With the advent of plant biotechnology, additional conservation strategies have now been

utilized for protecting valuable germplasms in the “*in vitro* botanical garden” indoors.

Long term preservation of genetic diversity *in vitro* is an important application of plant tissue culture. Different methods of *in vitro* germplasm preservation in a relatively little space for virtually unlimited periods are available (Bhojwani and Razdan 1996). Successful establishment of aseptic culture and genetic makeup of individual plant species are the important parameters to preserve the cultures for a long period of time and reuse the culture for production of morphological and chromosomally stable plant. *Cephaelis ipecacuanha*, a member of the family Rubiaceae is an important medicinal plant and the well-known drug ipecacuanha is obtained from the roots. Ipecacuanha is chemically a white crystalline bitter alkaloid named as emetine extracted from ipecacuanha root, and regarded as its peculiar emetic principle is popularly known in USA and Europe. Recently it has been enlisted in Japanese Pharmacopoea 14th edition (2001). It is used as an amoebicide in many different preparations and used as an emergency reagent for first aid treatments at hospitals. Ipecac plants are generally propagated through seed germination and vegetative cuttings. Inherent problem in seed viability after harvest poses a serious threat to commercial cultivation of this plant. Micropropagation from shoot tips and nodal explants excised from aseptically germinated seedlings in Ipecac has been reported by Jha and Jha (1989). Yoshimatsu and Shimomura (1991) and Yamuna et al. (1993) reported direct organogenesis and plant regeneration from internodal segments while Yoshimatsu and Shimomura (1994) described simple one step direct method of plant regeneration from cut end of root segments. However, there is no report of plant regeneration from long term cultures of ipecac. Considering the medicinal values of ipecac the present study was undertaken to see whether (i) reduced growth condition be an effective mode of germplasm preservation in ipecac, (ii) micropropagation from isolated nodal meristems and internodal segments is possible and (iii) the regenerants can maintain stability at the chromosomal level.

Material and Methods

Cultures were established for micropropagation of ipecac from nodal explants (Jha et al. 1989). Nodes of the plants with axillary meristem were used as initial explants. A protocol for direct organogenesis from internodal segments of the *in vitro* grown shoots was also reported earlier (Yamuna et al. 1993). Since then cultures of ipecac were maintained in half strength of MS with minimum concentration of Kn (0.25 mg/l) known as maintenance medium and was kept in diffused light at $22 \pm 2^\circ\text{C}$. In between subcultures were delayed up to 16 weeks.

The cultures were maintained over 12 years and mentioned as long term cultures (LTC).

Nodal meristems from the LTC stocks were placed on MS having different concentrations and combinations of BAP and Kn (2 mg/l each), NAA (0.5 mg/l) and adenine (40 - 100 mg/l). Internodal segments from the same LTC were also subjected to direct organogenesis with various concentrations of Kn (0.5 - 3.0 mg/l) and 2iP (0.5 - 3.0 mg/l).

Each culture contained full strength of MS formulations with 3% (w/v) sucrose and 0.7 % bacto-agar. The pH of culture media was adjusted to 5.6 before autoclaving for 15 min at 101°C at 1.05 kg/cm². All cultures were incubated at 22 ± 2°C, under a 16h day photoperiod with a light intensity of 45 µmol/m²/s (400 - 700 nm) photosynthetically active radiation (PAR) provided by 40w cool white fluorescent tubes/8 hr dark. All experiments were repeated twice. Statistical difference between mean tabulated values was estimated ($p \leq 0.05$) using DMRT with the Statistica Software v 5.0 (StatSoft 1995).

Young shoot tips were excised from randomly selected culture regenerants, in order to analyse the chromosome numbers. The shoot tips were pretreated with saturated solution of para dichloro benzene (PDB) for 4½ hr at 12 - 14°C and were kept in Carnoy's solution (acetic acid : chloroform : ethanol : : 1: 3 : 6:) for overnight at room temperature. Fixed shoot tips were hydrolysed with 1N HCl at 60°C for 10 min, stained in 2% aceto-orcein for 2 hr and squashed in 45% acetic acid to obtain well scattered metaphase plates on the glass slides. The slides were observed under high power, light microscope and chromosome numbers per cell were manually counted.

Results and Discussion

Ipecac germplasms in the form of *in vitro* grown shoot cultures were maintained in 250 ml borosil culture flasks containing 50 ml of half strength of MS with Kn 0.25 (mg/l). The maintenance medium did not affect the survival rate of shoots but substantial reduction of growth was noted during this prolonged period of preservation. Nodal meristems of LTC (Fig. 1) when placed on full strength MS with 3% sucrose and different concentrations and combinations of BAP/Kn, NAA and adenine (Table 1) induced multiple shoots within six - eight weeks of culture (Fig. 2) in all the sets mentioned in the Table 1. It is evident from our earlier published results (Jha and Jha 1989) that maximum shoot multiplication, 12.5 shoots/culture was achieved in MS medium with Kn (8 mg/l), NAA (0.5 mg/l) and adenine (200 mg/l). However in our present studies we have obtained maximum 10.5 shoots/culture. But the result has been obtained in a set having remarkably reduced amount of Kn, NAA and adenine (2, 0.2 and 40 mg/l, respectively, Fig. 3) which is desirable for cost effective multiplication.

Long-term habituation of culture may be the possible cause of lower requirement of growth regulators and additive. Such occurrences indicating habituation have been noted by various authors in different plants like tobacco, sugar beet and *Asparagus officinalis* (de Marsac and Jouanneau 1972, Kevars et al. 1982, Delbreil and Julien 1994). George et al. (2008) have supported the opinion that due to habituation, LTC may lose the requirement for auxin and/or cytokinin. The process points out to the fact that long term preservation without loosing the potential of micropropagation is possible in ipecac and it may reduce the production cost of micropropagated plants. Multiplication of shoots from long-term cultures is reported in *Phragmites australis*, *Juncus effuses*, *Betula pendula*, *Musa* sp. (Straub et al. 1988, Sarma and Rogers 2000, Ryyänen et al. 2005, Lakshmanan et al. 2007), but not in ipecac.

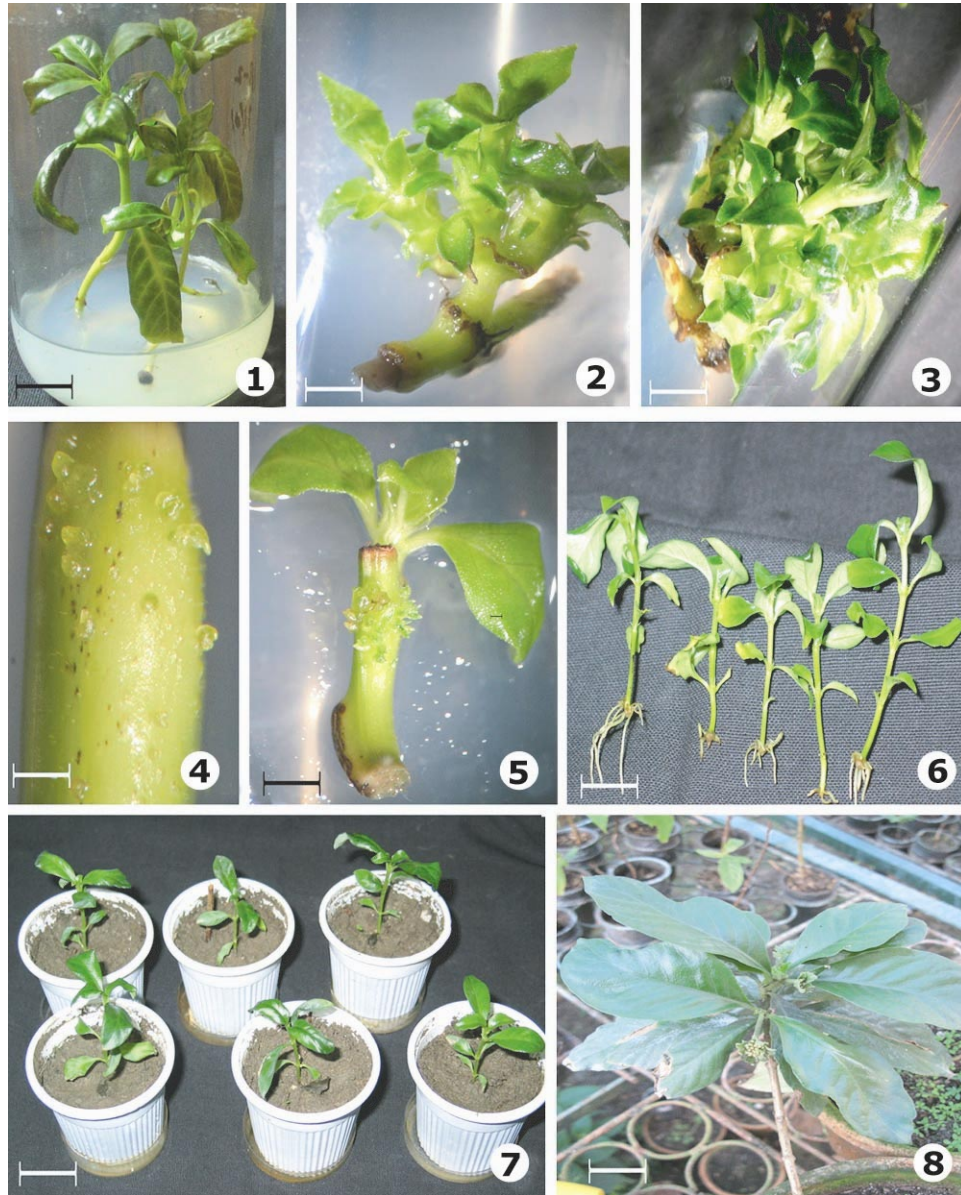
Table 1. Effect of various growth regulators and additives in shoot multiplication from nodal explants of *Cephalis ipecacuanha* (Set No. 1 to 4 describe multiplications prior to long term preservation; Set Nos. 5 to 8 describe the same from LTC).

Set. No.	Medium	BAP (mg/l)	Kn (mg/l)	NAA (mg/l)	Adenine (mg/l)	Mean no. of shoots
1	MS	2	-	0.5	-	0
2	"	-	8	-	-	9.5± 0.23 c
3	"	-	8	0.5	-	10.4± 0.14 b
4	"	-	8	0.5	200	12.5± 0.26 a
5	"	2	-	0.2	-	6± 0.17 e
6	"	-	2	0.2	-	8.16± 0.21 d
7	"	-	2	0.2	40	10.5± 0.19 b
8	"	-	2	0.2	100	8.31± 0.21 d

Each value represents the mean ± SE of three repeated experiments each with 12 explants. The mean followed by same letters within a column do not differ statistically according to DMRT at a 5 % probability level.

In another experiment we placed the internodal segments from LTC on full strength MS with or without growth regulators. Yamuna et al. (1993) reported direct organogenesis on both MS basal and MS with growth regulators. However, best result of 40 - 45 shoot buds/internodal segment was recorded on MS with 3.0 mg/l of 2iP within six - eight weeks of culture. On the other hand in our present studies direct organogenesis was obtained only on MS medium with 3.0 mg/l of 2iP (Table 2, Figs. 4, 5) within six - eight weeks. Maximum number of shoot buds recorded in our studies was 20/internodal segment. Induction of direct organogenesis on internodal segments from LTC is an important event not observed earlier in this species. It appears that some of the epidermal and hypodermal cells of internodal segments still retain the direct organogenic potentiality and in all the previous and present studies 2iP as PGR played a

consistent role for direct induction of shoot buds in Ipecac. In many occasions authors (Mokhtarzadeh and Constantin 1978, Nyochembeng and Garton 1998, Myers and Simon 1999) have highlighted the role of 2iP in direct and indirect regeneration of plants.



Figs. 1 - 8: 1. Shoots of Ipecac maintained for long term in culture, bar = 1.2 cm. 2, 3. Multiplied shoots of ipecac from nodal explants, bar = 0.5 cm. 4, 5. Showing direct shoot bud development on internodal segments of ipecac, bar = 0.5 cm. 6. Rooted shoots of ipecac prior to field transfer, bar = 1.5 cm. 7. Acclimatization and hardening of plants transferred to field conditions, bar = 6 cm. 8. 1 year old flowering ipecac plant, bar = 4 cm.

Induction of roots in regenerated shoots was obtained in 60% shoots (2 cm. long) when cultured on MS with 5.0 mg/l NAA for three - four weeks. Proliferation of roots take place when rooted shoots were transferred to liquid MS basal medium for another three - four weeks (Fig. 6). Rooted plants were hardened and transferred to potted soil containing vermicompost and sand in 1 : 1 ratio (Fig. 7) and 80 % of them survived in the field condition and we noted flowering in 25% of the plants within one year of field transfer. Flowering in tissue culture derived plants has not been reported earlier in ipecac (Fig. 8).

Table 2. Effect of various growth regulators and additives in direct shoot multiplication from internodal explants of *Cephalis ipecacuanha* maintained as LTC.

Set. No.	Medium	Plant growth regulators	Mean No. of shoots/node \pm SE ^a
1.	MS	Kn	0.5
2.	"	(mg/l)	1
3.	"		2
4.	"		3
5.	"	2iP	0.5
6.	"	(mg/l)	1
7.	"		2
8.	"		3

^aEach value represents the mean \pm SE of three repeated experiments each with 12 explants. The mean followed by same letters within a column do not differ statistically according to DMRT at a 5 % probability level.

Regeneration of plants from long-term cultures often may lead to epigenetic, genetical or cytological variations. These have been reported in several micropropagated plants such as 'Havana 425' tobacco plants (Meins and Binns 1978), *Populus tremuloides* (Rahman and Rajora 2001), *Actinidia deliciosa* (Palombi and Damiano 2002), Cavendish banana (Ramage et al. 2004) and in *Swertia chirata* (Chaudhuri et al. 2008). All these variations that arise during culture are dependent on genotype or cultural conditions. Genetic stability has been reported in long-term micropropagated shoots of banana (Lakshmanan et al. 2007).

Chromosomal stability in regenerated plants from long-term callus cultures of *Citrus acida* Roxb. has been reported by Chakravarty et al. (1999). Chromosomal analysis in our present studies from randomly selected shoot tips revealed maintenance of diploid chromosome numbers ($2n = 22$) in all the regenerants. It also confirms that no chromosomal variability was induced from the LTC explants among the regenerants. It indicates that reduced growth

conditions applied in our studies along with the inherent genotypic potentiality and stability of the species helped to maintain germplasms over such a long period of time in ipecac maintained morphological and chromosomal uniformity among the regenerants. Such mode of preservation can be utilized in other medicinal and economically important plants.

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