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Micropropagation of *Ilex khasiana*, a critically endangered and endemic holly of Northeast India

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Abstract

Background and aims *Ilex khasiana* is a rare and critically endangered holly endemic to the Khasi Hills of Meghalaya, India, and confined to a small number of pocket areas. In addition to conventional methods of propagation, endemic and threatened plants such as this could be more effectively multiplied and conserved using *in vitro* methods. Such techniques have the additional advantage of having a low impact on wild populations because they require a minimum of starting material. Our objective was to develop methodologies for the successful *in vitro* mass propagation of *I. khasiana*.

Methodology Seedlings were germinated *in vitro* under sterile conditions and nodal explants from these were transferred to Murashige and Skoog (MS) medium supplemented with 8.88 μM 6-benzyladenine and 4.64 μM kinetin.

Principal results This generated ~10 shoots per explant. In a second approach, callus was obtained from seedling-derived leaf discs cultured on MS medium supplemented with 2,4-dichlorophenoxyacetic acid and 6-benzyladenine. Approximately 12 adventitious shoots per callus were regenerated from 83.33 % of the calli after transfer to MS medium supplemented with 6.63 μM 6-benzyladenine. The most effective treatment for inducing root formation on the shoots was transfer of shoots to half-strength MS medium with 9.84 μM indole-3-butyric acid. Regenerated plantlets with well-developed shoots and roots were hardened and transferred to open soil with 70 % survival after 4 weeks.

Conclusions Both the methods described here are well suited for the mass multiplication of this critically endangered tree species.

Introduction

Application of tissue culture to plant conservation in India has been largely restricted to economically important species. However, the approach could usefully be extended to conserve all threatened plants so that vital biodiversity and the ecological networks it sustains can be preserved. *Ilex khasiana* (Aquifoliaceae) is a rare and critically endangered holly tree endemic to the Khasi Hills of Meghalaya, India, and confined to a few pockets. The species has been categorized as rare by Rao and Haridasan (1983) and is on the IUCN Red List of Threatened Species (2007). In nature, propagation by seed germination is unusual, the seeds failing to germinate because of incomplete and rudimentary embryos

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(Hu 1989). Also, any viable seeds have a prolonged period of dormancy (Upadhaya *et al.* 2009), making them problematic for use in conventional conservation work. As with many other woody species, vegetative propagation of this species is slow (Yadav *et al.* 1990). These conventional methods of propagation could be replaced to advantage by *in vitro* cultural methods capable of generating large numbers of propagules from a minimum of start-up plant material (Fay 1992, 1994; Krogstrup *et al.* 1992; Cuenca and Amo-Marco 2000). This approach would minimize damage to vulnerable remnant populations and compensate for the poor regeneration capacity of natural *I. khasiana* populations (Upadhaya *et al.* 2009).

The use of *in vitro* techniques in germplasm conservation is increasing and has been successfully applied to the conservation of several rare and endangered species, both for propagation and for long-term storage (Chandra *et al.* 2006; Sarasan *et al.* 2006). In the present investigation, we report the successful *in vitro* propagation of *I. khasiana* by means of shoot bud initiation and callus organogenesis.

Materials and methods

Plant material and culture initiation

Berries of *I. khasiana* were collected from mature trees during December 2008 and January 2009. Seeds were separated from the pulpy berries and thoroughly washed in running tap water for 10 min, immersed in 5 % (v/v) Teepol for 10 min and then rinsed three times with sterile distilled water. The seeds were sterilized in 70 % ethanol (v/v) for 1 min, then in 15 % (v/v) sodium hypochlorite solution for 15 min under aseptic conditions and washed four times with sterile distilled water, and finally placed on MS (Murashige and Skoog 1962) medium. The pH of the medium was adjusted to 5.8 ± 0.2 with 0.1 N NaOH or 0.1 N HCl before autoclaving at 105 kPa for 15 min at 121 °C. The cultures were incubated at 25 ± 2 °C under a 14-h photoperiod of cool-white fluorescent light (60.2 μmol m^{-2} s^{-1}) for germination and growth of seedlings to the two-leaf stage.

In vitro propagation from nodal segments

Cotyledonary nodal segments (~0.5 cm) from the seedlings were cultured in MS medium supplemented with the cytokinins 6-benzyladenine (BA) (2.22–11.11 μM) and kinetin (Kn) (2.32–11.60 μM) (Hi-Media, Mumbai, India) either singly or in combination. Explants that produced shoots were subcultured on to fresh media every 4 weeks. The number of shoots produced on each nodal explant was scored after 8 weeks of culture.

In vitro propagation from leaf discs

Callus induction and growth Leaf discs (2 × 4 mm) were excised from 2-month-old aseptic seedlings and placed on MS medium supplemented with different concentrations and combinations of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) (Hi-Media) and cytokinins (BA and Kn). Cultures were kept dark for the first 7 days and then incubated under a 14-h daily photoperiod of cool-white fluorescent light (60.2 μmol m^{-2} s^{-1}) to induce callus formation.

Adventitious shoot bud induction and multiplication To induce adventitious shoot bud induction and multiplication, calli were cultured in MS medium supplemented with various concentrations of BA and Kn. After 4 weeks, cultures were evaluated for numbers of shoots generated. Small shoots differentiating directly from callus were then detached and subcultured in fresh medium which was replaced every 4 weeks. The number of shoots produced on each explant was scored after 8 weeks of culture.

Root induction

In vitro-regenerated shoots (3–4 cm) were transferred to half-strength MS medium and one of several different concentrations of IBA (2.46–14.76 μM), IAA (2.85–17.13 μM) and NAA (2.68–16.48 μM). The number of roots per shoot was recorded after 4 weeks.

Acclimatization of plantlets

Successfully rooted plantlets were removed from the rooting medium, washed thoroughly to remove the adhering agar and transferred to plastic pots made of propylene (10 × 7 cm size) containing a mixture of black soil:vermiculite:sand (2:1:1 v/v); plants were acclimatized *ex vitro* in a greenhouse, covered with translucent plastic bags to ensure high humidity. The plantlets were irrigated with quarter-strength MS medium solution every 2 days for 2 weeks. The plantlets were then uncovered but kept under shade-netting for 2 weeks followed by transfer to natural light and earthenware pots (30 × 20 cm) containing garden soil for a further 4 weeks of hardening.

Statistical analysis

Ten replicates per treatment were taken and the experiment was repeated three times. Data were analysed by analysis of variance. Differences between means were compared using Tukey's test at the 5 % level of probability (software Origin 7.0).

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Table 1 Effect of different concentrations of BA and Kn applied separately or together on multiple shoot formation from nodal explants of *I. khasiana* cultured on MS medium.*

Plant growth regulators (μM)		Regeneration (%)	Number of shoots/explant ± SE	Total no. of shoots obtained (>3 cm)
BA	Kn			
2.22	–	33.33[e]	1.33 ± 0.09[f]	4
4.44	–	46.66[de]	2.23 ± 0.18[e]	18
8.88	–	63.33[c]	3.56 ± 0.19[d]	28
11.11	–	53.33[d]	2.76 ± 0.24[e]	22
–	2.32	33.33[e]	1.23 ± 0.10[f]	5
–	4.64	36.66[e]	1.20 ± 0.10[f]	5
–	9.29	53.33[d]	3.63 ± 0.25[d]	27
–	11.60	40.00[e]	1.53 ± 0.11[f]	6
4.44	2.32	66.66[bc]	6.70 ± 0.16[c]	54
8.88	4.64	86.66[a]	10.20 ± 0.22[a]	78
11.11	9.29	73.33[b]	7.36 ± 0.23[b]	60

Each treatment was replicated three times and each replicate consisted of 10 explants. Values represent means ± SE. Means having different superscript letters are significantly different from each other ($\alpha = 0.05$) according to Tukey's test.
*Data recorded after 8 weeks of culture.

Results and discussion

In vitro propagation from nodal segments

Nodal explants failed to respond to MS medium devoid of growth regulators. However, when cultured in medium supplemented with BA (2.22–11.11 μM) and/or Kn (2.32–11.60 μM), shoots were induced on the explants (Table 1). Media containing both BA (8.88 μM) and Kn (4.64 μM) in combination resulted in the maximum response and gave the largest number of shoots (Fig. 1A–C). In this treatment, 86.66 % of the nodal explants generated shoots with an average of 10.2 ± 0.22 shoots per explant. Higher concentrations of both BA (11.10 μM) and Kn (9.29 μM) were counterproductive. Lower concentrations, in combination, were also less effective. When BA or Kn were supplied separately, only one shoot emerged from each nodal explant. It is well documented that, in numerous species, cytokinins stimulate axillary shoot multiplication (Sansberro *et al.* 2000; Beena *et al.* 2003; Tandon *et al.* 2007). Our finding that a combination of both BA and Kn is more effective than when either is given separately reflects a similar report by Babu *et al.* (2003) where multiple shoots in camphor tree (*Cinnamomum camphora*) were initiated when BA and Kn were incorporated together in the medium. The need to optimize the concentrations of cytokinins is critical because both higher and lower concentrations of cytokinins are less effective in shoot formation. This has also been reported by Amin and Jaiswal (1993) and Bhatt and Dhar (2000) for other woody species.

In vitro propagation through callus organogenesis by leaf discs

Traditional approaches to plant regeneration from calli involve manipulating the relative ratio of auxin to cytokinin (Thomas and Maseena 2006) and also the type of growth regulator, their concentration and combination. Such manipulations influence the initiation and establishment of callus. In the present work, a combination of BA and 2,4-D supported callus induction (Table 2). Explants cultured in MS medium containing the auxins NAA and IBA singly or in combination with Kn or BA failed to respond. Callus initiation was observed on leaf explants within 4 weeks of culture in medium supplemented with 9.04 μM 2,4-D and 2.32 μM BA in combination (Fig. 1D). However, with the increase or decrease in 2,4-D concentration, development of callus decreased, highlighting the need to optimize the concentration used. Moreover, the yield of callus was low in media containing 2,4-D in combination with Kn. The initial 7 days of dark treatment to explants promoted callus formation. These results conform with previous findings with *Hyoscyamus muticus* (Basu and Chand

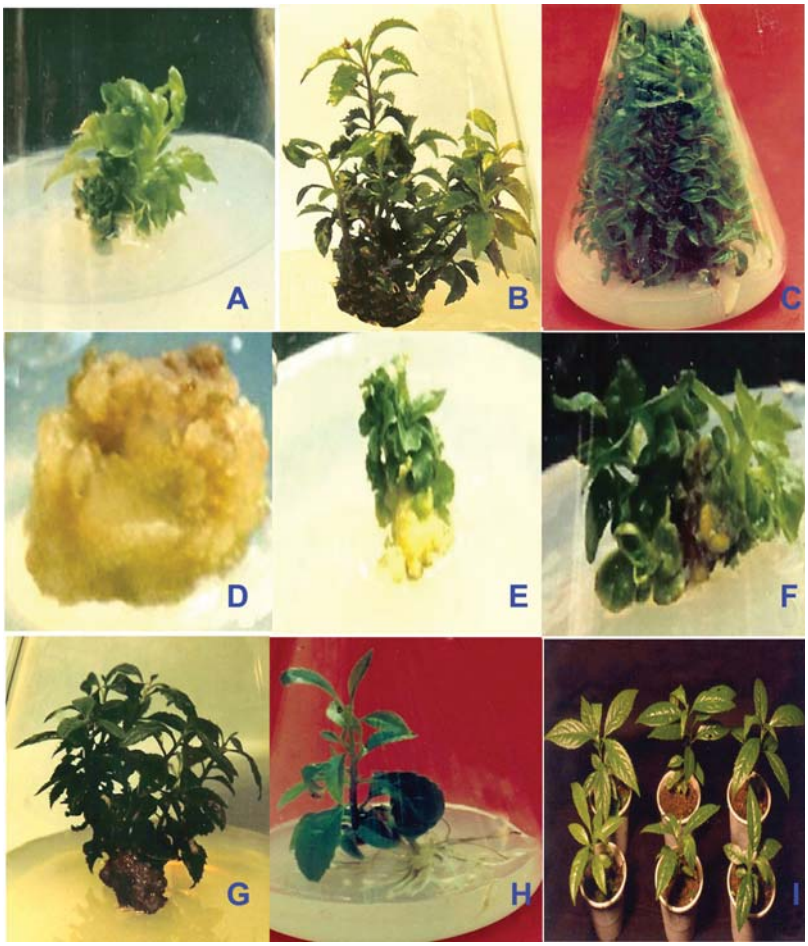

Fig. 1 ***In vitro* propagation of *I. khasiana*.** (A) Multiple shoot induction from nodal segment; (B, C) elongation in MS medium with 8.88 μM BA + 4.64 μM Kn; (D) callus initiation from leaf disc on MS medium supplemented with 9.04 μM 2,4-D + 2.20 μM BA; (E) shoot induction; (F, G) proliferation in MS medium with 6.63 μM BA; (H) *in vitro* rooted plantlets in MS medium with 9.84 μM IBA; (I) hardened plantlets in earthenware pots.

1996) and *Acacia mangium* (Xie and Hong 2001). A positive effect of a short inductive treatment of 2,4-D application has also been reported for apple (Yancheva *et al.* 2003).

With the aim of inducing shoot bud organogenesis and subsequent plant regeneration, the callus obtained in MS medium supplemented with 9.04 μM 2,4-D + 2.32 μM BA was used to regenerate whole plants by transferring to fresh MS medium supplemented with a range of cytokinin concentrations (Table 3). Maximum shoot bud proliferation (63.33 %) with an average of 12.16 ± 0.14 shoots per organogenic callus tissue (Fig. 1E–G) was achieved with 6.63 μM BA. As before, higher or lower concentrations were less effective. Benzyladenine was found to be essential for the regeneration of the calli. The superiority of BA for shoot induction may be due to the ability of plant tissue to metabolize natural hormones more readily than synthetic growth regulators. It is also possible that BA can induce production of natural hormones such as zeatin within the tissue (Sharma and Wakhlu 2003). The use of BA for stimulating shoot bud initiation in the induced calli has also been successfully accomplished in other species (Kang *et al.* 1994; Pretto and Santarém 2000; Gentile *et al.* 2002).

Root induction

The most challenging step in the successful regeneration of woody species is the induction of roots on newly induced shoots. For new root formation, shoots 3–4 cm long were excised from *in vitro*-formed shoot clumps and transferred to half-strength MS medium supplemented with IBA (2.46–14.76 μM), IAA (2.85–17.13 μM) or NAA (2.68–16.48 μM) (Table 4). Each of

Table 2 Effect of 2,4-D and BA or Kn on callus development from leaf discs of *I. khasiana* in MS medium.*

Plant growth regulators (μM)			% of callus initiation	Intensity of callus development	Nature of callus
2-4D	Kn	BA			
2.26	0.46	–	26.66	+	White and poor callus
4.52	2.32	–	43.33	+	Light brown and poor callus
6.78	4.64	–	50.00	++	White callus
9.04	2.32	–	56.66	++	Brown callus
11.30	4.64	–	36.66	+	Hard and poor callus
2.26	–	0.44	40.00	+	White callus
4.52	–	2.20	63.33	+	White callus
6.78	–	4.43	70.00	++	Shining and light brown callus
9.04	–	2.20	83.33	+++	Healthy callus
11.30	–	4.43	60.00	++	Hard with dark brown areas

Callus induction was initially done in dark period 7 days and then 14 h photoperiod at 25 ± 2 °C.
Each treatment was replicated three times and each replicate consisted of 10 explants.
*Data recorded after 4 weeks of culture.
+, poor callus; ++, medium; +++, profuse callus.

Table 3 Effect of various combinations of BA and Kn on morphogenetic responses of leaf discs from *in vitro*-germinated seeds of *I. khasiana* cultured on MS medium.*

Plant growth regulators (μM)		Mean no. of shoots buds ± SE	% of shoots
BA	Kn		
2.20	–	4.22 ± 0.15^{d}	43.33^{bc}
4.43	–	6.00 ± 0.14^{c}	50.00^{b}
6.63	–	12.16 ± 0.14^{a}	63.33^{a}
8.86	–	7.77 ± 0.22^{b}	56.66^{ab}
11.09		6.00 ± 0.18^{c}	40.00^{c}
–	2.32	1.44 ± 0.12^{f}	13.33^{e}
–	4.64	2.00 ± 0.14^{f}	20.00^{e}
–	6.96	4.16 ± 0.14^{d}	30.00^{d}
–	9.24	3.22 ± 0.10^{e}	40.00^{c}
–	11.61	2.05 ± 0.09^{f}	30.00^{d}

Each treatment was replicated three times and each replicate consisted of 10 explants. Values represent means ± SE. Means having different letters as superscripts are significantly different from each other ($\alpha = 0.05$) according to Tukey's test.
*Data recorded after 8 weeks of culture.

these auxins initiated rooting within 2 weeks. The rooting per cent, root quality and number of roots were, however, superior in IBA-containing medium (Fig. 1H). When 9.84 μM IBA was used, 93.33 % of excised shoots developed 5.56 ± 0.11 roots/shoot within 4 weeks of culture on half-strength MS. The promoting effect of IBA in rooting has been reported in several other plant species (Wawrosch *et al.* 2001; Joshi and Dhar 2003; Kumar *et al.* 2010).

Acclimatization of plantlets

Environmental conditions required for *ex vitro* growth of plants are different from those needed for *in vitro* cultivation (Hazarika 2003). In the acclimatization process, excessive water loss from plantlets is a major problem (Hazarika 2006). In our work, *in vitro*-regenerated plantlets were acclimatized *ex vitro* in a greenhouse. Fifty rooted plantlets were set out in this way (Fig. 1I) and irrigated with quarter-strength MS medium solution every 2 days for 2 weeks. The plantlets were then uncovered but kept under shade-netting for a further 2 weeks followed by transfer to natural light and earthenware pots (30 × 20 cm) containing garden soil. The plants were then grown on for 4 weeks, when 70 % of rooted plantlets were found to have survived and to have produced healthy new growth.

Conclusions and forward look

We report for the first time two protocols for the large-scale propagation of *I. khasiana*, a critically endangered holly of Northeast India. This is key to successful large-scale multiplication of *I. khasiana* since this species does not propagate readily under natural

Table 4 Effect of IBA, IAA or NAA on root production by *in vitro*-raised shoots of *I. khasiana* cultured on half-strength MS medium.*

Auxin (μM)			Rooting (%)	Number of roots/shoot	No. of regenerated plants obtained
IBA	IAA	NAA			
2.46	–	–	36.66^{de}	2.00 ± 0.19^{e}	11
4.92	–	–	63.33^{b}	4.96 ± 0.11^{b}	19
9.84	–	–	93.33^{a}	5.56 ± 0.11^{a}	28
14.76	–	–	56.66^{c}	4.63 ± 0.10^{b}	17
–	2.85	–	13.33^{g}	1.73 ± 0.10^{e}	4
–	5.71	–	20.00^{fg}	2.00 ± 0.20^{e}	6
–	11.42	–	36.66^{de}	3.63 ± 0.16^{c}	11
–	17.13	–	26.66^{f}	1.86 ± 0.08^{e}	8
–	–	2.68	23.33^{f}	1.36 ± 0.09^{e}	7
–	–	5.37	33.33^{ef}	1.50 ± 0.57^{d}	10
–	–	10.74	53.33^{c}	2.66 ± 0.13^{d}	16
–	–	16.48	43.33^{d}	2.88 ± 0.08^{d}	13

Each treatment was replicated three times and each replicate consisted of 10 explants. Values represent means ± SE. Means having different superscript letters are significantly different from each other ($\alpha = 0.05$) according to Tukey's test.
*Data recorded after 4 weeks of culture.

conditions. The two protocols readily produce plantlets from excised parts of germinated seeds and represent important tools for conservation of this species. Somaclonal variation induced by propagation via callus may also introduce a much needed source of novel variation. This may compensate for the lack of natural variation in the surviving populations of endangered species with genetic bottlenecks, as suggested by Fay (1992). Finally, callus cultures such as those we describe provide valuable material for long-term storage and preservation. This approach could also help conserve *I. khasiana* and other endangered plants of Northeast India and elsewhere.

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Contributions by the authors

All the authors contributed to a similar extent overall and have seen and agreed to the submitted manuscript.

Conflict of interest statement

None declared.

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