

In vitro propagation of some threatened plant species of India

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To prevent extinction of threatened species, *in vitro* regeneration protocols for the propagation of six threatened species were standardized. The regenerated micro-shoots were rooted in nutrient medium supplemented with low concentrations of auxin. The well-developed plantlets were successfully established in field conditions, thus improving the probability of self-sustenance of the introduced populations. The success story of these six threatened species reaffirms the role of *in vitro* propagation in conserving plants facing the threat of extinction.

Keywords: Conservation, *in vitro* regeneration, micro-shoots, threatened species.

Introduction

CONSERVATION of plant genetic resources can be achieved *in situ* as well as *ex situ*¹. Both cultivated and domesticated plant species are also maintained in their natural habitats as well as in field conditions^{2,3}. Due to habitat destruction and transformation of the natural environment, several species have been lost from the ecosystems. Therefore, *in situ* methods alone are insufficient for conserving the threatened species. Under these circumstances, *ex situ* conservation is a viable alternative for preventing extinction of threatened species. In some cases, it is the only viable strategy to conserve certain species. *In situ* and *ex situ* methods are complementary and not mutually exclusive. Selection of appropriate strategy should be based on a number of criteria including the status of the species and feasibility of applying the chosen methods⁴.

In vitro culture method is a powerful tool for propagation, conservation and management of commercially important and threatened plant species⁵⁻⁹. *In vitro* culture technique has been used for large-scale propagation of threatened species, thus improving the conservation status of the species. Further, *in vitro* techniques offer a safe way for international exchange of plant materials, enable

the establishment of extensive collections using minimum space, and allow supply of valuable material for recovery of wild threatened species populations¹⁰.

The present study was designed to demonstrate that the threatened species facing regeneration failure in nature and which are difficult to propagate through other cost-effective conventional vegetative and sexual propagation methods are best suited for *in vitro* propagation. We developed efficient protocols for *in vitro* plant regeneration of *Aconitum nagarum* Stapf, *Hypericum gaitii* Haines, *Podophyllum hexandrum* Royale, *Rhododendron macabeum* Watt ex Balf. f., *Rhododendron wattii* Cowan and *Vanda bicolor* Griff. using different explants and manipulation of plant growth regulators and culture conditions.

Materials and methods

Study species

Aconitum nagarum Stapf (Ranunculaceae): The genus *Aconitum* is represented by nearly 300 species in the world, of which India has 33 species. *A. nagarum* is a herb that grows at altitudinal range of 1600–3800 m amsl. The alkaloids produced from its rhizomes are used for cure of a wide range of ailments, and are used as arrow poison. The plant has antibacterial properties against *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli* and *Bacillus subtilis*³. Owing to its high medicinal value, *A. nagarum* is being exploited from its natural habitat and thus has become threatened.

Hypericum gaitii Haines (Hypericaceae): The genus *Hypericum* is represented by 494 species, of which 29 species occur in India¹¹. Among these 29 species, three, viz. *H. assamicum*, *H. gaitii* and *H. gracilipes* are endemic to India¹². *Hypericum* species can be used in the treatment of cancer and AIDS. It is popular as an antidepressant¹³. *H. gaitii* is a tall shrub, bushy with erect branches, and is a threatened species in the Eastern Ghats region of India owing to habitat loss and different anthropogenic activities. There is a preliminary report on *in vitro* axillary shoot multiplication of *H. gaitii*¹⁴.

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However, no report is available on direct plant regeneration from leaf and stem explants of *H. gaitii*.

Podophyllum hexandrum Royle (Podophyllaceae): *P. hexandrum* is a perennial threatened herbaceous plant species with a wide distribution range, i.e. 2000–4000 m amsl in the Indian Himalayan Region¹⁵. It is a source of anti-tumour drugs, viz. etoposide, etopophos and teniposide^{16,17}. The natural populations of *P. hexandrum* are low and steadily declining due to high demand of its rhizomes. *P. hexandrum* has a relatively long juvenile phase¹⁸. Propagation of the species is undertaken through vegetative means and seeds. Natural regeneration is poor due to erratic seed-setting, long seed dormancy (1–2 years) as an adaptation strategy to overcome harsh climatic conditions, and trampling by grazing animals. Hence, *in vitro* techniques can be an alternate and effective means of propagation¹⁹. Moreover, considering its medicinal importance, *in vitro* method offers stability in bioactive molecule production.

Rhododendron macabeum Watt ex Balf.f. and *R. wattii* Cowan (Ericaceae): *Rhododendron* is the largest woody plant genus in Ericaceae, represented by 1025 species in the world²⁰. In India, 135 species of rhododendrons have been recorded, among which 132 species are from North East (NE) India region (unpublished data). *R. wattii* and *R. macabeum* are endemic to Manipur and Nagaland, and the natural populations are scarce²¹. Anthropogenic activities like extraction for firewood and natural calamities such as forest fire during the dry season might have contributed to the rapid disappearance of these species. Considering the failure of both the species to regenerate through seeds, there is an urgent need to establish a suitable protocol for *in vitro* propagation^{22–24}.

Vanda bicolor Griff. (Orchidaceae): *V. bicolor* is a horticulturally important monopodial orchid reported only in NE India, Bhutan, Myanmar and Nepal²⁵. Due to habitat destruction, the species is now threatened. Considering its economic importance and poor natural regeneration, the present study was undertaken to develop an efficient protocol for mass propagation of the species for its commercial exploitation as well as conservation in the wild.

Plant material collection and culture condition

Aconitum nagarum: Matured fruits were collected during October and November in 2014 and 2015 from Khonoma village (Dzuko valley, altitude 2684 m amsl), Nagaland. Seeds were surface-sterilized with aqueous solution of HgCl₂ (0.2%, w/v) for 5 min, and subsequently rinsed 4–5 times with sterile pure water. Sterilized seeds were soaked in sterilized water. Shoot buds were collected from germinated seedlings grown during different seasons used as a source of explants.

Hypericum gaitii: *In vitro* shoot multiplication via shoot tip culture was established as reported by Swain *et al.*²⁵. Both young leaves (0.5 cm²) and internodal segments (0.25–0.5 cm) were excised from 8-week-old *in vitro* grown shoots, and were used as a source of explants for direct shoot bud regeneration without the intervening callus phase. The cultures were maintained in an incubation room with 16 h photoperiod and 3000 lux light intensity at 25° ± 2°C.

Podophyllum hexandrum: Matured fruits were collected during April and September in 2013 and 2014 from Martoli village region (3438 m amsl) of Pithoragarh district, Uttarakhand. Seeds were extracted, washed and treated with Bavistin (0.2%, w/v) for 10 min. They were dried under shade and stored at 4°C till use. The seeds were sterilized with HgCl₂ (0.04%) for 8 min and washed four times with sterilized distilled water. Subsequently, they were scarified for 3 min with H₂SO₄ (50%, v/v) before culture on nutrient medium.

Rhododendron macabeum and *Rhododendron wattii*: Matured seeds of both the species were collected during December 2015 from Dzukou valley (2575 m amsl), Nagaland. Seeds were washed with Tween-80 (1.0%, v/v) for ~5 min, and subsequently washed five times in sterile distilled water before culturing on agar gel Anderson medium (AM) enriched with sucrose (3%) for germination. The cotyledonary node and shoot tip explants obtained from *in vitro* derived seedlings were used for multiple shoot induction.

Vanda bicolor: Immatured seed pods of *V. bicolor* were collected during July 2014. Seed pods were surface cleansed with detergent (Labolene, India) and rinsed under running tap water. Surface-washed green pods were treated with HgCl₂ (0.2%) for 5 min followed by washing 4–5 times with sterile pure water. The pods were then dipped in ethanol (70%, v/v) and flamed before scooping out the seeds.

Initiation of culture

Cultures were initiated for all the species from different explant sources. For this, different nutrient media fortified differently were used. Tables 1 and 2 provide details of explant type(s), nutrient medium, supplements, plant growth regulators (PGRs) and culture condition(s). The multiple shoots/regenerated propagules developed on initiation medium were transferred to fresh medium either of same composition or of different combinations for multiplication.

Plant regeneration/multiple shoots induction, rooting, hardening and transplantation of regenerates

For shoot multiplication and regeneration, rooting and hardening of regenerates derived from different protocols were followed (Table 3).

Conservation of Threatened Plants of India

Table 1. Explants, nutrient media and supplements used for initiation of *in vitro* culture of different species

Species	Explants used for culture initiation	Basal medium and supplements	Culture conditions
<i>Aconitum nagarum</i>	Shoot buds from <i>in vitro</i> grown seedlings	MS medium + sucrose (3%) + BA (3–15 μ M) and NAA (1–2 μ M) singly or in combination	40 μ mol m ⁻² s ⁻¹ illumination with 12 h photoperiod at 25° ± 2°C
<i>Hypericum gaitii</i>	Leaf, internodal segments from <i>in vitro</i> grown plantlets	MS medium + sucrose (3%) + kinetin and BA (0.5–1.5 mg l ⁻¹) and NAA (0.25–0.50 mg l ⁻¹), either singly or in combination	55 μ mol m ⁻² s ⁻¹ light intensity with 16 h photoperiod 25° ± 2°C
<i>Podophyllum hexandrum</i>	Mature seeds	MS medium + sucrose (3%) + GA ₃ (0.1 μ M) and BA (1.0 μ M) in combination	42 μ mol m ⁻² s ⁻¹ illumination with 16 h photoperiod at 25° ± 2°C
<i>Rhododendron macabeaeanum</i>	Shoot apices and nodal segments from <i>in vitro</i> grown seedlings	AM, modified AM and WPM + sucrose (3%, w/v) + 2iP, BA and kinetin (1–8 mg l ⁻¹) separately	White fluorescent light with a 16 h photoperiod at 25° ± 2°C
<i>Rhododendron wattii</i>	Nodal segments from <i>in vitro</i> grown seedlings	AM, modified AM and WPM + sucrose (3%, w/v) + 2iP and BA (1–8 mg l ⁻¹) separately	
<i>Vanda bicolor</i>	Immature embryos	MS medium + sucrose (3%) + NAA and BA (3–9 μ M) either singly or in combination	40 μ mol m ⁻² s ⁻¹ illumination with 12 h photoperiod at 25° ± 2°C

MS: Murashige & Skoog; BA, Benzyl adenine; NAA, α -naphthaleneacetic acid; WPM, Woody plant medium; 2iP, N6(2-iso pentenyl) adenine; GA, Gibberellic acid; AM, Agar medium.

Table 2. Effects of nutrient media, plant growth regulators (PGRs) and adjuncts on *in vitro* morphogenetic response of explants of different species

Plant species	Optimum medium, PGRs and adjuncts	Morphogenetic pathway	% Response	No. of propagules developed per explant
<i>A. nagarum</i>	MS medium + sucrose (3%) + BA (6 μ M)	Shoot buds	95	16
<i>H. gaitii</i>	MS medium + sucrose (3%) + BA (1.0 mg l ⁻¹)	Direct shoot bud regeneration	85.6 (internodal segment), 73.8 (leaf)	86 (internodal segment), 56 (leaf explants)
<i>P. hexandrum</i>	MS medium + sucrose (3%) + GA ₃ (0.1 μ M) and BA (1.0 μ M) in combination	Seedlings	–	–
<i>R. macabeaeanum</i>	WPM + sucrose (3%) + 2iP (4 mg l ⁻¹)	Shoot buds	76	8
<i>R. wattii</i>	WPM + sucrose (3%) + 2iP (8 mg l ⁻¹)	Shoot buds	–	7
<i>V. bicolor</i>	MS medium + sucrose (3%) + NAA and BA (3 μ M in combination)	PLBs, plantlets	88	–

PLBs, Protocorm like bodies; other abbreviations are same as Table 1.

Aconitum nagarum: The micro-shoots developed in the initiation medium were transferred to the same medium for another 2–3 passages. In every subculture, the micro-shoots formed were separated and transferred to fresh regeneration medium for further proliferation. About 5–6 cm long micro-shoots with well-expanded leaves were selected for inducing roots. They were subjected to acclimatization and transplantation of the regenerates was carried out in the potting mix. The micro-shoots were maintained on MS medium containing NAA (0–5 μ M) and maintained at 16 h photoperiod at 25° ± 2°C. The well-rooted plantlets were taken out from the rooting medium and transferred to MS medium fortified with sucrose (3% w/v) devoid of PGRs and maintained in the laboratory for 3–4 weeks. The hardened plantlets were separated from the culture vials and washed with tap water. They were then transplanted on plastic pots containing a mixture of soil, decayed wood powder and coconut coir in the ratio 1 : 1 : 1. The pots were covered with holed transparent polybags and kept moist through

capillary water for 2 weeks. The acclimatized transplants were finally transferred to the greenhouse.

Hypericum gaitii: Leaf and internodal segments derived from *in vitro* raised shoots of *H. gaitii* were cultured on semi-solid basal MS medium supplemented with 3% (w/v) sucrose and different concentrations and combinations of BA (0.0–1.5 mg l⁻¹) or kinetin (0.0–1.5 mg l⁻¹), NAA (0.0–0.5 mg l⁻¹) for direct shoot-bud regeneration. The regenerated micro-shoots were maintained in a medium with similar composition at every 4 weeks interval. Fifteen replicates were employed for each treatment and the experiment was repeated four times. After 8 weeks, the regenerated micro-shoots (~2–3 cm length) were separated from the culture medium and transferred to half-strength MS semi-solid medium with 2% (w/v) sucrose and different concentrations of indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (0.0–1.0 mg l⁻¹) for induction of roots. Rooted plantlets were transferred to plastic cups containing sterile distilled water for

Table 3. Nutrient media, growth supplements and culture conditions for culture proliferation, rooting, hardening and transplantation of regenerates

Species	Optimum shoot proliferation medium and adjuncts	Rooting medium	Hardening	Potting mix for transplantation
<i>A. nagarum</i>	MS medium + sucrose (3%) + BA (6 μ M)	MS medium + sucrose (3%) + NAA (5 μ M) for 8 weeks	MS medium + sucrose (3%) for 4–6 weeks	Plastic pots containing soil : decayed wood powder : coconut coir (1 : 1 : 1 ratio, v/v). Transplants maintained under 75% shade for 4–6 weeks
<i>H. gaitii</i>	MS medium + sucrose (3%) + BA (1.0 mg l ⁻¹)	MS medium + sucrose (2%) + IBA (1 mg l ⁻¹) for 2 weeks	Rooted plantlets maintained in plastic glass containing sterile distilled water for 2 weeks	Polybags filled with garden soil, sand and cow dung at 2 : 1 : 1 ratio (v/v)
<i>P. hexandrum</i>	MS medium + sucrose (3%) + GA ₃ (0.1 μ M) and BA (1.0 μ M) in combination.	MS medium + sucrose (3%) + IAA (1.0 μ M)	In polybags containing soil and farmyard manure (3 : 1, w/w)	Polybags filled with soil and farmyard manure (3 : 1, w/w) for about 7–8 weeks
<i>R. macabeanum</i>	WPM + sucrose (3%) + 2iP (4 mg l ⁻¹)	WPM + AC (0.2%, w/v)	WPM + AC (0.2%, w/v)	Root trainer filled with coarse sand and soil at 1 : 1 ratio
<i>R. wattii</i>	WPM + sucrose (3%) + 2iP (8 mg l ⁻¹)	WPM + AC (0.2%, w/v)	Planted in root trainer filled with coarse sand and leaf moulds at 1 : 1 ratio and maintained in mist chamber	Polybags filled with coarse sand and leaf moulds in 1 : 1 ratio
<i>V. bicolor</i>	MS medium + sucrose (3%) + NAA and BA (3 μ M each) + 0.6% AC	As in proliferation medium. Simultaneous rooting on multiplication medium	Matrix of moss, charcoal pieces, brick pieces and decayed wood in the ratio 1 : 1 : 1 : 1 mixed with 1/10th of MS medium without sucrose and growth regulators for 5–6 weeks	Charcoal pieces, brick pieces and moss in 1 : 1 : 1 ratio. The transplants were maintained in polyhouse under 75% shade for 3–4 weeks

Incubation conditions for culture proliferation, rooting and hardening are the same as in the culture initiation shown in Table 1. AC, Activated charcoal; other abbreviations are same as Table 1.

primary acclimatization for 2 weeks in the growth chamber at 28°C with 70% relative humidity and 16-h photoperiod. Further, the plantlets were transferred to polybags containing a mixture of sterile sand : soil : well rotted cow-dung manure (1 : 1 : 1). They were kept in the greenhouse for secondary acclimatization before transferred to the field. Watering was done daily to maintain humidity and moisture.

Podophyllum hexandrum: Prominent cotyledonary tube with multiple leaves produced from germinating seeds was maintained on the germination medium for 2–3 weeks. Shoots were rooted by culturing on MS medium containing 1.0 μ M IAA. Rooted plants were kept for hardening in polybags containing soil and farmyard manure (3 : 1, w/w) for about 7–8 weeks.

Rhododendron macabeanum: About 2–3-month-old *in vitro* raised seedlings were used as explants, viz. apical shoots, nodal segments, and roots for multiple shoot induction. Different concentrations (1–8 mg l⁻¹) of N6 (iso pentyryl adenine), BA and kinetin (Kin) in woody plant medium (WPM) were investigated for multiple

shoot induction. Shoots were subcultured at 20 days intervals. Ten replicates were employed for each treatment and the experiment was repeated four times. About 1.5–2.0 cm long *in vitro*-raised shoots were transferred to liquid WPM medium using filter-paper bridge technique supplemented with different concentrations of IBA and α -naphthaleneacetic acid (NAA) (0.5–4.0 mg l⁻¹) for induction of roots.

Rhododendron wattii: Nodal segment explants (1.5–2.0 cm in length) from 2-month-old aseptic seedlings of *R. wattii* were cultured on different nutrient media (AM, modified AM and WPM) supplemented with different concentrations of BA and 2iP (1–12 mg l⁻¹) and different additives (100 mg l⁻¹ polyvinyl pyrrolidone (PVP), 100 mg l⁻¹ ascorbic acid and citric acid 10 mg l⁻¹) for multiple shoot induction. Cultures were sub-cultured at 3 week intervals. Individual shoots of ~1.5–2.0 cm length isolated from the shoot clump of *in vitro* culture were placed on AM, WPM containing activated charcoal (0.2%) and liquid medium using filter paper bridge technique supplemented with different concentrations of IBA and NAA (0.5–2.0 mg l⁻¹) for induction of roots. The *in*

in vitro grown plantlets were transferred to the greenhouse and planted in root trainer containing a mixture of coarse sand and leaf moulds (1 : 1) and the temperature of the greenhouse was maintained at 25°C.

Vanda bicolor: The PLBs and shoots developed from germinated seeds were transferred to MS medium supplemented with BA and NAA (0–9 µM, either singly or in combination) and activated charcoal (AC) (0.3–0.9%, w/v), and maintained for 2–3 passages at 4 weeks interval for plant regeneration and multiplication. Plantlets with well-developed roots were separated from the clumps and transferred for hardening²⁴. Plantlets were maintained under laboratory condition on a matrix of autoclaved moss, charcoal pieces, brick pieces and decayed wood in the ratio 1 : 1 : 1 : 1. MS nutrient medium (1/10th strength) without sucrose and growth regulators was applied for 5–6 weeks. The primary hardened plantlets were transferred to pots with charcoal pieces, brick pieces and moss in the ratio of 1 : 1 : 1. The transplants were maintained in the polyhouse at 75% shade for 3–4 weeks before transferring to the field.

Results and discussion

Cultures were initiated from different explants of various species as described above on different nutrient media



Figure 1. Different *in vitro* propagation stages of *Aconitum nagarum*. *a*, Excised shoot bud cultured on initiation medium showing signs of morphogenetic response by releasing the meristematic loci. *b*, Multiple shoots/micro-shoots developed in culture. *c*, Rooted plant. *d*, Regenerates transplanted in potting mix maintained in the polyhouse.

with manipulation of PGRs and additives. The explants exhibited differential responses (Table 2).

Aconitum nagarum

Culture was initiated from the shoot buds harvested during different seasons cultured on MS medium fortified with different PGRs. Among the different levels of PGRs used, BA alone was found to be beneficial for culture initiation against the combined treatment of BA and NAA (Table 2). Under the conditions provided in the present study, optimum morphogenetic response was registered on medium supplemented with (3%) sucrose and BA (6 µM). Within one week of culture, explants started responding by way of swelling and formation of loci (Figure 1 *a*). At lower concentration of BA, fewer shoot buds formed per explant cultured, while at higher concentration of BA delayed morphogenetic response was observed. Under optimum conditions, as many as 16 shoot buds were formed per explant in ~95% of culture (Figure 1 *b*). Different plants exhibited differential seasonal rhythm for growth and *in vitro* morphogenetic response. When BA-enriched medium was fortified with NAA, it supported lesser micro-shoot formation and there was callusing of the shoots at the basal part, exhibiting stunted growth. Earlier, Karuppusamy *et al.*²⁶ reported the synergistic effect of NAA and BA on nodal explants culture of *Hydrocotyle conferta*. Dhavala and Rathore²⁷ reported that cytokinin alone could not promote axillary bud-breaking in *Embelia ribes*, unless one of the auxins,

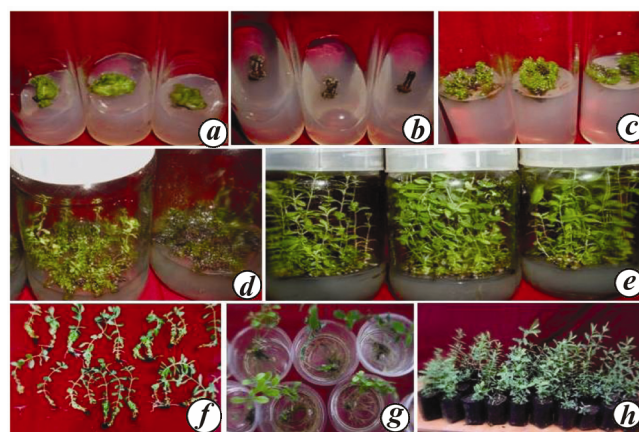


Figure 2. Direct plant regeneration of *Hypericum gaitti*. *a, b*, Proliferation of leaf (*a*) and intermodal explants (*b*) grown on MS medium supplemented with 1.0 mg l⁻¹ BA after 4 weeks of culture. *c, d*, Development of shoots from intermodal and leaf explants on MS medium supplemented with 1.0 mg l⁻¹ BA and 3% sucrose after 4 weeks of subculture. *e*, Elongation of shoots on MS liquid medium supplemented with 1.0 mg l⁻¹ BA and 3% sucrose after 4 weeks of third subculture. *f*, Induction of roots from micro-shoots on half-strength MS medium supplemented with 1.0 mg l⁻¹ IBA and 2% sucrose after three weeks of culture. *g*, *In vitro* raised plantlets acclimatized in distilled water and ready for transfer to greenhouse. *h*, *In vitro* raised plants grown in the green house after 2 months of transfer.

especially IAA was incorporated in the medium in conjunction with cytokinin. While in guava nodal segment culture, incorporation of GA₃ along with BA was the prerequisite for axillary bud-breaking²⁸. However, in *Adhata vatica*, axillary bud proliferation and multiple shoot initiation were optimum on MS medium containing BA alone²⁹.

Hypericum gaitii

Direct initiation of morphogenetic response was observed from leaf and internodal segments, which subsequently developed into green, globular structures on the cut surfaces as well as above the explants on MS basal medium supplemented with 0.5–1.5 mg l⁻¹ BA (Figure 2 a and b) followed by dark green shoot formation. The medium supplemented with kinetin alone or Kn + NAA or BA + NAA did not promote healthy morphogenesis. Healthy shoot-bud regeneration was observed on MS medium fortified with BA (1.0 mg l⁻¹) (Figure 2 c and d). High regeneration frequency (~85.6%) was achieved in the internodal explants compared to leaves (~73.8%) on MS medium containing 1.0 mg l⁻¹ BA (Table 2). Liu and Sanford³⁰ made similar observations in strawberry in a medium containing BA in combination with IBA. Induction of direct shoot-bud regeneration in the medium having either higher (>1.5 mg l⁻¹) or lower concentration (<0.5 mg l⁻¹) of BA resulted in lower frequency of shoot bud regeneration compared to the BA concentration of 0.5–1.5 mg l⁻¹. Similar results were reported in *Nyctanthes arbortristis*³¹ and *Plumbago zeylanica*³². There were differences among the treatments for both the percentage of cultures with shoot buds and the mean number of shoot buds per culture. The number of shoot buds/culture increased four-fold within 4 weeks of the third subculture,



Figure 3. Various conditions of germination of excised embryos on MS medium supplemented with 0.1 μM GA₃ + 1.0 μM BA. *a*, Plants in natural habitat bearing mature red berry; *b*, *c*, Expansion of excised embryo and multiple shoot formation in MS medium containing 0.1 μM GA₃ + 1.0 μM BA; *d*, Root induction in a micro-shoot on MS medium containing 1.0 μM IAA; *e*, Plants raised through excised embryos in polybags; *f*, Shoot proliferation and root induction in rhizome of *Podophyllum hexandrum*.

maintained for longer period without any loss in the morphogenetic potential (Figure 2 e). The regenerative potential in both the leaf and internodal explants was higher under 16 h photoperiod than continuous light. The synergistic effect of photoperiod and growth regulators on *in vitro* shoot bud differentiation as observed in the present study was also noted in *Lavandula latifolia*³³ and *Prunus* species^{30,34}.

Podophyllum hexandrum

Excised embryos germinated within one week of inoculation (Figure 3 b). A prominent cotyledonary tube with multiple leaves and distinct radicular portion was observed after 2–3 weeks (Figure 3 c). Under the given conditions, optimum response was registered on MS medium enriched with sucrose (3%), GA₃ (0.1 μM) and BA (1.0 μM) in combination. In the present study, 0.1 μM GA₃ (gibberellic acid) along with 1.0 μM BA advanced the time as well as improved the induction of somatic embryogenesis, and resulted in embryo formation.

Rhododendron macabeanum

Among the different PGRs studied, 2iP (4 mg l⁻¹) was a superior source of cytokinin compared to BA and kinetin, whereas many as six shoots developed per explant on AM. Among the different explants tested, nodal segments produced maximum number of multiple shoots than apical and root parts. Among different nutrient media tested, WPM supplemented with 2iP (4 mg l⁻¹) supported maximum number of multiple shoots formation (8), whereas mean shoot length of 2.1 cm was obtained (Table 2 and Figure 4 b).

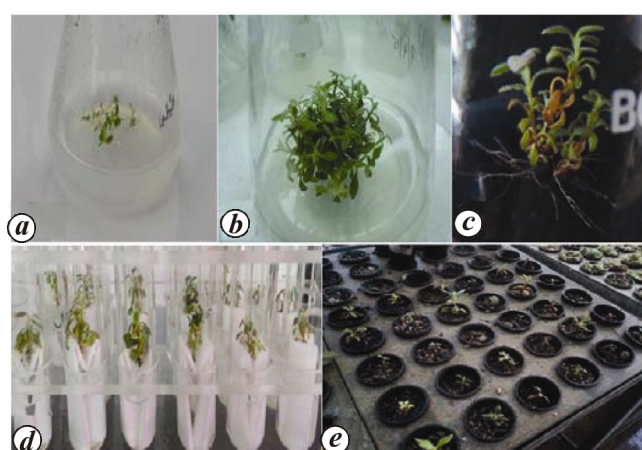


Figure 4. Different stages of *in vitro* propagation of *Rhododendron macabeanum* from nodal segments of *in vitro* grown seedlings. *a*, Seedling developed from the germinated seeds; *b*, Multiple shoot formation on initiation medium; *c*, Cultures under rooting in AC-rich medium; *d*, Rooting of micro-shoots on filter paper bridge; *e*, Regenerates in the potting mix.

Rhododendron wattii

Among the different media (AM, mAM and WPM) tested with different concentrations of cytokinins (2iP and BA), WPM with 8.0 mg l⁻¹ 2iP resulted in an average number of about 7 shoots per explant with a maximum shoot length of 2.3 cm after 13 weeks in culture (Table 2; Figure 5 b). However, with subsequent 3–4 subculture cycles (3 weeks interval), multiple shoots increased profusely (Figure 5 c).

Vanda bicolor

The first sign of seed germination was observed as changing of seed colour to yellowish-white followed by nodular swelling of seeds (Figure 6 a) after 25 days of culture, and subsequently converted into PLBs (Figure 6 b). Among the different concentrations of PGRs investigated for seed germination, a combined treatment of NAA and BA supported better germination over a single treatment of both. Under the given conditions, a combined treatment of BA and NAA (3 µM each) supported ~88% seed germination followed by PLBs formation within 48 days of culture initiation. The PLBs and germinating seeds were maintained for another two passages for further development. Under this condition, the PLBs grew into plantlets (Figure 6 c).

Multiple shoot induction/culture proliferation, rooting, hardening and transplantation of regenerates

The shoot buds/regenerated shoots developed on initiation medium were maintained on various nutrient media

fortified differently for culture proliferation and plant development (Table 3).

Aconitum nagarum

The micro-shoots were maintained for another two to three passages for culture differentiation and proliferation on initiation medium and with 3% (w/v) sucrose and 6 µM BA. The shoot buds were proliferated into young plantlets within 4–6 weeks on regeneration medium with fully expanded leaves (Figure 1 b). In many plant species, *in vitro* plant regeneration and culture proliferation were achieved in different growth media⁷. However, in the present study plant regeneration and culture proliferation were achieved in the initiation medium only. The effectiveness of cytokinin on plant regeneration and culture proliferation has been reported by earlier workers^{35–38}. The regenerated shoots were transferred to rooting medium having different concentrations of NAA for root induction. Among the different concentrations used, 5 µM NAA supported maximum root growth with 4–5 roots per plant within 8 weeks of culture (Table 3 and Figure 1 c). At lower concentration, fewer roots were formed with healthy shoot growth. The effect of auxin on root initiation in woody plant species has been reported earlier in *Quercus suber*³⁵, *Strobilanthes flaccidifolius*⁸ and *Cinnamomum tamala*⁹. The well-rooted plantlets were further transferred to MS medium without growth regulators and maintained for 4–6 weeks under laboratory condition. The hardened plantlets were removed from the culture vials and transplanted in plastic pots containing pot mixture of soil, decayed wood powder and coconut coir at 1 : 1 : 1 ratio (Figure 1 d). About 65% plantlets survived after two months of transfer. The hardened plantlets were transferred to the natural forest after 8 weeks.

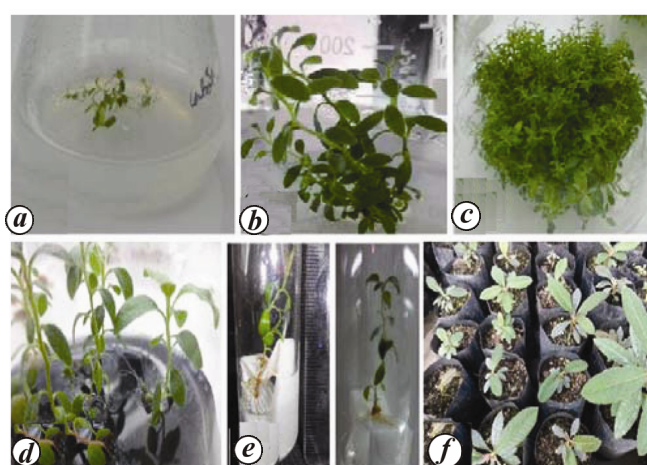


Figure 5. Different stages of *in vitro* propagation of *Rhododendron wattii* from nodal segments of *in vitro* grown seedlings. *a*, Seedling developed from the germinated seeds; *b*, Multiple shoot formation on initiation medium; *c*, Profuse proliferation of shoots; *d*, Cultures under rooting in AC-rich medium; *e*, Rooting of micro-shoots on filter paper bridge; *f*, Regenerates in the potting mix.

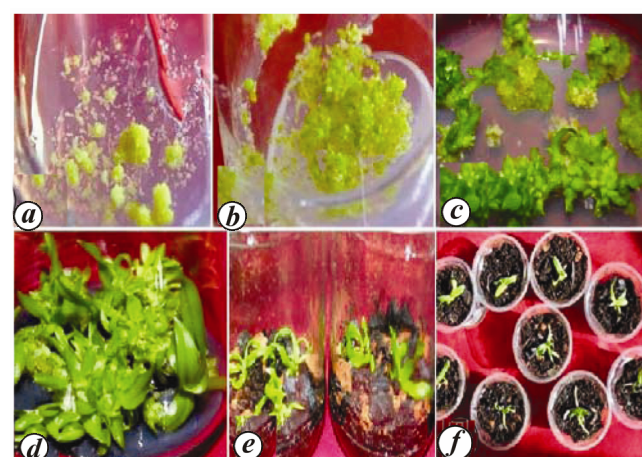


Figure 6. Different stages of *in vitro* propagation of *Vanda bicolor* from immature seeds. *a*, Immature seeds showing signs of germination; *b*, Germinated seeds converted to protocorm like bodies (PLBs); *c*, Plants developed from PLBs; *d*, Culture proliferation on AC-enriched medium; *e*, Plantlets under hardening condition; *f*, Potted plants.

Hypericum gaitii

Direct shoot-bud regeneration was achieved on MS medium fortified with 1.0 mg l^{-1} BA. The regenerated microshoots (~1–2 cm in length) were harvested and transferred to half-strength basal MS medium supplemented with various concentrations of IAA or IBA (0.0, 0.25, 0.5, 1.0 and 1.5 mg l^{-1}) with sucrose (2%) for induction of rooting. Among the different treatments, optimum rooting was achieved on medium supplemented with IBA (1 mg l^{-1}) after 3 weeks of transfer (Figure 2f). There was also an increase in shoot length after 4 weeks of culture. The rooting ability was reduced and led to root necrosis with increase in the concentration of IAA and IBA in the culture medium. Rooted plantlets grown *in vitro* were washed thoroughly in running tap water and transferred to 10 cm plastic glass containing sterile distilled water for primary acclimatization. The distilled water was changed at every two days interval. Within 1 week of transfer, some new root initials were obtained from micro-shoots (Figure 2g). Further, after 2 weeks of primary acclimatization, the rooted microshoots were transferred to polybags having soil mixture and kept in the greenhouse for secondary acclimatization. About 90% of the rooted plantlets were established in the greenhouse within 4 weeks of transfer. The plants attained 3–4 cm height within 12 weeks of transfer (Figure 2h). The acclimatized plants were established in the field.

Rhododendron macabeanum

Shoots developed on initiation medium were subcultured on WPM containing sucrose (3%) and AC (0.2%) for multiple shoots formation and culture proliferation. After 3–4 subcultures, multiple shoots showed profuse growth. The shoots were harvested at this stage for root induction. For rooting, two techniques were followed (Figure 4c and d). Liquid medium using paper bridge technique showed no signs of rooting for any of the concentrations of IBA and NAA (0.5, 1, 2 and 4 mg l^{-1}) (Figure 4d). Nutrient medium along with activated charcoal supported healthy rooting after two months of culture (Figure 4d). Well-rooted plantlets were hardened by transferring to root trainer containing a mixture of coarse sand and soil (1 : 1), and maintained in the greenhouse for acclimatization (Figure 4e).

Rhododendron wattii

Shoots proliferated while maintaining them on WPM containing sucrose (3%) and 2iP (8 mg l^{-1}). With 3–4 subculture cycles (3 weeks interval), multiple shoots increased profusely (Figure 5c). Multiple shoots were then separated from the shoot clumps and used for *in vitro* rooting. In AM and WPM supplemented with activated

charcoal (0.2%), 100% rooting was observed after 2 months in culture. However, WPM with 0.2% activated charcoal induced well-developed roots and broad green leaves (Figure 5d). Using the filter bridge technique, the highest percentage of *in vitro* rooting was obtained at IBA 0.5 mg l^{-1} (Figure 5e and Table 3). High concentration of auxin lowered the rooting percentage as well as root number, and induced callus formation from the roots. Plantlets with well-developed roots were washed with sterile distilled water and treated with systemic fungicide (Carbendazim, Dhanustin – 50% W.P., 0.1% w/v; 30 min). Then they were planted in root trainer containing a mixture of coarse sand and leaf moulds (1 : 1) (Figure 5f) and maintained in the greenhouse for acclimatization. After 2 months, plantlets were transferred to polybags containing coarse sand and leaf moulds (1 : 1). About 60% *in vitro* raised seedlings survived during hardening and acclimatization.

Vanda bicolor

The PLBs were transferred to MS medium supplemented with different PGRs and activated charcoal. Among the different combinations tested, a combined treatment of NAA and BA showed the best regeneration of plantlets with rooting. The optimum plant regeneration and culture proliferation was achieved on nutrient medium fortified with $3 \mu\text{M}$ NAA, $3 \mu\text{M}$ BA and 0.6% (w/v) activated charcoal. Under this culture medium, the plantlets became dark green in colour with healthy root systems (Figure 6d). Subsequently, the well-rooted plantlets were transferred to hardening medium consisting of a matrix of moss, charcoal pieces, brick pieces and decayed wood (Figure 6e). It was observed that during the hardening process, the roots adhered to the substrata. Similar observation was also reported by Deb and Imchen¹⁸ in other *Vanda* species. The hardened plants were transferred to potting mix and maintained in the greenhouse under 75% shade (Figure 6f). About 95% of the transplants survived after 3 months of transfer.

Conclusion

Efficient protocols have been established for six threatened species by manipulating the growth regulators and culture conditions. These protocols may facilitate conservation of the species and save them from extinction. The protocols can also be used for the production of clonal planting material at a commercial scale by the pharmaceutical industry. The success story of these six threatened species reaffirms the role of *in vitro* propagation in conserving the plants facing imminent threat of extinction.

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