Improving macropropagation and seed germination techniques for conservation of threatened species

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Populations of threatened plants are declining rapidly in natural habitats due to various anthropogenic activities. Reinforcement of the dwindling populations through reintroduction is a promising aspect for conservation of threatened plants. However, due to lack of standardized propagation methods of such plants, mass production of planting materials has become a challenge, thereby constraining the replenishment process. Identification of factors constraining the seed germination of threatened plants and addressing it effectively, are among the most cost-effective strategies for large-scale multiplication and subsequent conservation of the threatened species. Similarly, conventional low-cost vegetative propagation techniques such as grafting, air layering, and regenerating plantlets from root-suckers, apical meristems, and stem cuttings often prove more successful for multiplication than relatively costly micropropagation techniques. In this article, we present a few case studies on low-cost mass propagation techniques of threatened plant species of India through seed, stem/apical shoot cutting and air-layering, that helped in the restoration of the species.

Keywords: Conservation, seed germination, threatened plants, vegetative propagation.

Introduction

NATURAL populations of threatened plants are fast depleting because of exogenous factors such as habitat degradation, climate change, altered environmental conditions, environmental stress and biotic disturbances, as well as endogenous factors such as reproductive failure, genetic drift, and demographic stochasticity[1]. Reintroduction of nursery-raised seedlings in previously inhabited areas or in new suitable habitats helps in the reinforcement of depleted populations. This has been considered as a key conservation approach for preventing extinction of the species in the wild[2]. The broader aim of such reinforcement is to establish viable and self-sustaining populations having broad genetic base ensuring long-term survival of the species[3–5].

Large-scale production of planting materials for such reintroduction programnes is undertaken either through conventional propagation methods using both sexual (i.e. seeds) and vegetative means, or using micropropagation technique (i.e. tissue culture). Among these, sexual propagation through germination of seeds is the most desirable option, as it is able to bring in the much needed genetic variation within a species. It is important to mention that genetic variation can affect the long-term survival of a species, since it is a prerequisite for adaptation to environmental stresses such as climatic change, pollution, novel diseases, competitors and predators[6]. Loss of genetic variation is usually related to increased homozygosity and may lead to a rapid decline in reproductive fitness and population growth rates[7–9]. Nevertheless, reproductive bottlenecks, non-availability of seeds, poor propagule dispersal and germination necessitate vegetative propagation of the target species using different plant parts such as leaf, stem, root and other root-producing plant organs. Though techniques of propagation through seed germination and vegetative means have been standardized for a large number of horticultural and forestry plantation species, limited work in respect of threatened plant species of India has been undertaken. In this article, we demonstrate that if the propagation techniques are standardized through scientific improvement for large-scale production of planting materials using conventional low-cost methods such as seeds, stem/apical shoot cuttings and air layering, threatened species can be recovered successfully with relatively low cost compared to the use of expensive clonal micropropagation methods.

Materials and methods

Eight threatened species were selected for standardization of propagation techniques by improving the conventional macropropagation techniques through appropriate inputs.

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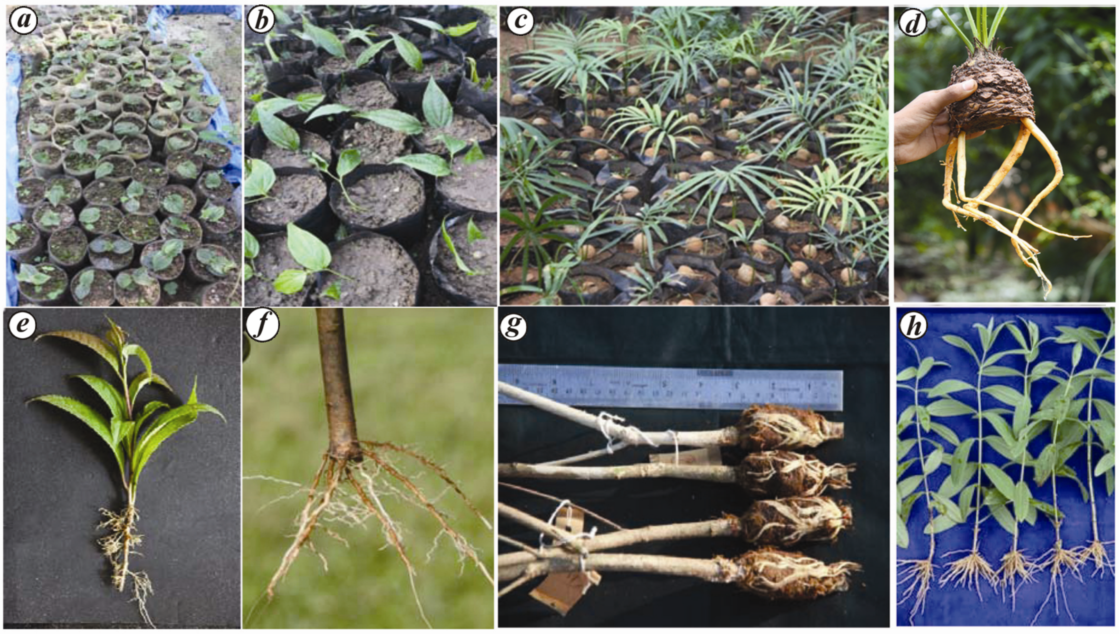

Figure 1. ***a***, *Piper haridsanii* seedlings generated through cuttings. ***b***, *Piper lonchites* seedlings generated through young shoot cuttings. ***c***, *Cycas sphaerica* seedlings generated from seeds. ***d***, Propagation of *Cycas sphaerica* through rooting of bulbils. ***e***, Root induction in young apical shoot cutting of *Ilex khasiana*. ***f***, Root induction in stem cuttings of *Lasiococca comberi*. ***g***, Root induction in branches of *Lasiococca comberi* through air-layering. ***h***, Rooting of cuttings in *Hypericum gaitii*.

Seed germination was improved in *Cycas sphaerica* Roxb. and *Ceropegia bulbosa* Roxb. Regeneration was standardized using stem/apical shoot cuttings in *Hypericum gaitii* Haines, and *Lasiococca comberi* Haines, *Piper haridasanii* Gajurel, Rethy & Y. Kumar, *Piper lonchites* Schult., *Ilex khasiana* Purk. and *Ilex venulosa* Hook.f. (Figure 1). Air layering method was adopted for *L. comberi* and rooting could be induced in bulbils of *Cycas sphaerica*. Table 1 provides a summary of the methodological improvements.

Results and discussion

Regeneration through seed germination

Cycas sphaerica (Cycadaceae): The distribution of *C. sphaerica* is restricted to Odisha and Andhra Pradesh in the Eastern Ghats of India, where wild populations of the species are rapidly declining. Matured fruits were collected from the trees and forest floor during January and February in 2013, 2014, 2015 and 2016, and kept under shade for 3–4 weeks for embryo maturation. The fruits were sown in a single layer in raised beds and were covered with thick layers of dry leaves and leaf moulds. These leaves attract termites that help in the process of decay and decomposition of the fibrous seed coats of *Cycas* seeds. The beds were then covered with a thin layer of soil and watered at weekly intervals. About 62% of the seeds started germinating after 90 days and emergence of first leaf was observed after 145 days.

A method of inducing roots from bulbils taken from matured trees of *C. sphaerica* was introduced to produce large-sized propagules. The cut ends of one-year-old bulbils were dipped in 1% solution of Bavistin in water and allowed to dry in shade for 24 h. They were planted in pots containing a mixture of coarse sand and leaf mould in equal proportion. Healthy and strong roots were produced after 53 days of planting in only 25% of the bulbils planted.

Lasiococca comberi (Euphorbiaceae): This is a threatened species with distribution in Odisha and Andhra Pradesh in India, and in Thailand. Seeds are oily in nature, frequently damaged by insects, and lose viability in 12–15 days of storage. Seeds from matured fruits were collected while the fruits were still attached to the plant and germination was achieved by immediately sowing the seeds in a nutrient-rich medium. The seeds need to be protected from ants and insects during the process of germination. About 46% of seeds germinated within 8–10 days.

Ceropegia bulbosa (Asclepiadaceae): This is a threatened, perennial, herbaceous, tuberous species distributed in India[10]. Due to over-exploitation for tubers, its population is depleting at an alarming rate. In general, species of *Ceropegia* are difficult to maintain under *ex situ* conditions[11]. Therefore, large-scale multiplication and reintroduction in its native habitats is the only way to rehabilitate this species. Generally, tubers are used for regeneration of the species. However, limited availability of the species in nature necessitates standardization of a

Table 1. Conventional macropropagation techniques and subsequent novel improvements in selected threatened species

Species	Conventional macropropagation technique	Novel improvements to the conventional macropropagation technique
Ceropegia bulbosa	Regeneration through tubers and seed germination	Seed germination improved seven times in sandy soil compared to gravel soil.
Cycas sphaerica	Propagation through bulbils	Healthy and strong root induction from bulbils after treatment with 1% solution of Bavistin in water.
Hypericum gaitii	Regeneration through stem cuttings	300 ppm IBA was the best in terms of root induction in apical stem cuttings.
Ilex khasiana	Seed germination	Regeneration through apical shoot cuttings was achieved without any chemical treatment.
Ilex venulosa	Seed germination	Regeneration through apical shoot cuttings was achieved without any chemical treatment.
Lasiococca comberi	Seed germination; regeneration through cuttings; air layering	Faster germination was achieved by sowing the seeds in a nutrient-rich media. 2000 ppm IBA was the best in terms of root induction in cuttings. 5000 ppm of IBA was the best treatment for induction of roots in 95.23% of branches.
Piper haridasanii	Regeneration through stem cuttings	One-node cutting had the best survival rate compared to two- and three-node cuttings.
Piper lonchites	Stem and shoot cuttings	Young shoot-tip cuttings performed better than cuttings with single and double nodes.

protocol for its mass multiplication using seeds. For successful multiplication through seeds, appropriate sowing medium is the most important factor.

We conducted an experiment to study the effect of soil texture on seed germination as well as seedling growth of *C. bulbosa*. The study was conducted in the Desert Botanical Garden of Central Arid Zone Research Institute, Jodhpur, during the rainy season of 2014. Two hundred seeds were collected from matured follicles of *C. bulbosa* plants from the Botanical Garden. Seed viability of *C. bulbosa* was tested using triphenyl tetrazolium chloride (TTC) staining method. In the germination experiment, seeds of *C. bulbosa* were sown in two potting media treatments in polybags: (i) sandy soil + FYM (3 : 1), and (ii) gravel soil + FYM (3 : 1). One hundred seeds were sown in each treatment in the first week of October 2014. Germination was recorded daily for 30 days.

TTC viability test revealed that 86.7% of seeds of *C. bulbosa* were viable, showing no inhibition in the germination of seeds. Seed germination started on the fourth day after sowing in both types of soil. Maximum seed germination was obtained in gravel soil on the 23rd day (85%) while in sandy soil, 65% germination was obtained on the 25th day. Thus, seed germination was 30% more in gravel soil than in sandy soil. Mean height of seedlings in sandy soil was maximum (3.7 cm). Mean number of leaves was also maximum (2.8) in sandy soil compared to seedlings in gravel soil. Number of buds was also more in sandy soil. The seedlings had 36%, 46% and 7.5% greater mean height, number of leaves and number of buds respectively, in sandy soil than gravel soil. Thus, sandy soil was the best sowing medium for the multiplication of *C. bulbosa*, and the seedlings exhibited better growth in sandy soil.

Regeneration through stem and apical shoot cuttings

Ilex khasiana and *Ilex venulosa* (Aquifoliaceae): *I. khasiana* has a restricted distribution range in the Khasi hills of Meghalaya, and has been categorized as critically endangered by IUCN. *I. venulosa* is distributed in Meghalaya and Arunachal Pradesh, and has been categorized as endangered by IUCN. Due to small population size and deteriorating habitat, both species are facing the risk of extinction[12]. Natural regeneration in both the species is limited due to poor seed viability and seedling establishment in nature[13]. Attempts to regenerate the species through stem cuttings failed under greenhouse condition. Hence, we used apical shoot cuttings to improve the conventional vegetative propagation. Shoot apical meristem is the region of meristematic cells that contains multipotent stem cells and produces primordia that develop into a plant. The study was carried out in the greenhouse of the Department of Botany, North-Eastern Hill University, Shillong during 2016. Young apical shoots measuring 8–12 cm in length were collected from juvenile plants of *I. venulosa* and *I. khasiana* in Sohrarim and Upper Shillong respectively. The young shoots were planted in beds prepared with sand and soil in 2 : 2 ratio. No plant growth regulator was administered. The cuttings produced roots within 25–30 days and developed into healthy plantlets in 80–90 days. This process was successful and was repeated several times to produce more than 1200 healthy plantlets of *I. khasiana* and about 400 plantlets of *I. venulosa*, which were then reintroduced in the field. Thus, the apical shoot cuttings from juvenile donor plants can be used for mass production of planting materials.

Hypericum gaitii (Hypericaceae): This is a threatened species with restricted distribution in Odisha, Jharkhand

and Andhra Pradesh. Trials were made at Regional Plant Resource Centre, Bhubaneswar to standardize propagation methods for production of planting materials for reintroduction. Since seed propagation proved unsuccessful, vegetative propagation method through rooting of cuttings in mist house conditions was standardized. Apical stems measuring 8–10 cm were treated with different concentrations and combinations of root promoting substances such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA). The treatment of 300 ppm IBA proved to be best in terms of number of cuttings producing roots (86.7%), mean length of roots (15.5 cm) and number of roots per cutting (29.7) in *H. gaitii* after 60 days of planting of stem cuttings. The vegetatively propagated plants were transferred to polybags for field planting.

Lasiococca comberi (Euphorbiaceae): Defoliated stem cuttings measuring 10–15 cm were collected from healthy trees of *L. comberi* from the forests and brought to the nursery in air-tight polybags to prevent desiccation. The basal ends of the explants were treated with various concentrations (1000–3000 ppm) of IBA, IAA and NAA for 15 min. Then the treated cuttings were planted in sand beds in mist house. The application of 2000 ppm of IBA was found to be the most suitable root-promoting substance producing roots in 73.3% of stem cuttings. The mean number of roots per cutting was 8.7, and the mean root length was 6.7 cm. The rooted cuttings were successfully transferred to polybags and hardened for field planting.

Piper haridasanii and *Piper lonchites* (Piperaceae): *P. haridasanii* and *P. lonchites* are two threatened *Piper* species found only in North East India. *P. haridasanii* was discovered as a new species from Arunachal Pradesh[14]. Since then, the species population has been located only in East Kameng[15]. *P. lonchites* is a native species of Malaysia. In India, it is found in Manipur, Meghalaya and Arunachal Pradesh[16,17]. These species are threatened because of restricted distribution and habitat destruction.

Piper species are successfully propagated through seeds, vegetative cuttings and tissue culture. However, the easiest and most successful method of propagation is through stems as the nodes of the stem have the ability to produce new shoots and roots. The erect species *P. lonchites* does not produce any runner shoots and regenerates through seeds. Due to unavailability of sufficient viable seeds, the species was multiplied through vegetative cuttings. The sprouting from cuttings was obtained without application of any growth hormones.

The study was carried out in the nursery of the Department of Forestry, North Eastern Regional Institute of Science and Technology, Arunachal Pradesh, during March 2016. Young and healthy stem cuttings of *P. haridasanii* and young shoots of *P. lonchites* were collected from small populations located in Itanagar. The collected samples were kept in cool and moist conditions till planting. As both species grow well in shade, the cuttings were raised in a shade house. Potting mixture and nursery beds were prepared following common nursery techniques. The nursery soil was prepared using sand, soil and cow dung in the ratio of 2 : 1 : 1. The planted cuttings were watered and monitored regularly. Observations on the survival of the cuttings were recorded from the time of first bud initiation till their establishment.

P. haridasanii: Three distinct types of cutting were made from the stem, i.e. cutting with one node, two nodes, and three nodes. The cuts were made just below the node at the base with a sharp knife. All cuttings were made 3–5 nodes below the shoot tip, as pre-experimentation from the young shoot tip did not show good propagation.

Observations on growth were made at 10 days interval. New buds began to emerge after one month in all the cuttings. Bud initiation in two- and three-node cuttings was comparatively slower, and took almost 40 days. However, it was observed that once the new buds emerged, the growth of the two-node cuttings was faster than those of the one- and three-node cuttings. Observation after three months revealed that the stem cuttings showed good survival percentage with a minimum of 70% for all the three types of cutting. However, the best performance was recorded for one-node cutting, where 92% survival rate was obtained.

P. lonchites: Stem and shoot cuttings were used for experimentation. For regeneration through stem cutting, matured to semi-matured stems were cut with one and two-nodes in the same manner as was done in case of *P. haridasanii*. To compare the performance of these cuttings, the young shoot tips were also planted. For regeneration through shoot cuttings, healthy plants were selected and shoot tips from all the small lateral branches were cut. Cuttings were made with 2–3 nodes depending on the length of the shoots. Each cutting was made with at least one fully developed leaf on the lower node.

New buds emerged in 40 days of planting the young shoot cuttings. New bud initiation of the one and two-nodes stem cuttings started later than that of the young shoot cuttings and was observed after 50 days. Leaf initiation of all the three cuttings started 60–70 days after its bud initiation, and it took almost 150 days to complete leaf initiation. However, leaf initiation was completed in 130 days in shoot-tip cutting. By the end of five months, the performance of the young shoot tip cutting was much better with survival percentage of 76 compared to 12% and 20% each of one-node and two-node cutting. Many of the stem cuttings started dying after 40 days, and only a few were able to survive. The growth rate and time taken for completion of leaf initiation were slower in *P. lonchites* than *P. haridasanii*.

Table 2. Number of seedlings of the selected species produced through vegetative and seed propagation techniques and planted in the wild

Species	Method of propagation					Number of seedlings produced	Number of seedlings planted in the wild
	Seed propagation	Apical shoot	Stem cutting	Air layering	Rooting of bulbils		
C. bulbosa	2150	–	–	–	–	2150	1750
C. sphaerica	500	–	–	–	100	600	250
H. gaitii	–	500	–	–	–	1500	–
I. khasiana	–	1200	–	–	–	1200	870
I. venulosa	–	400	–	–	–	400	300
L. comberi	3000	–	–	1500	–	4500	2242
P. haridasanii	–	–	5000	–	–	5000	500
P. lonchites	–	–	4000	–	–	4000	1450

Propagation by air-layering

Lasiococca comberi (Euphorbiaceae): The bark of one-year-old healthy stem was girdled in the forest during July 2015, and the exposed portion of the stem/bark was treated with different concentrations and combinations of IBA, IAA and NAA (1000, 2500, 5000, 7500 and 10000 ppm). The ringed surface was covered with moistened moss, wrapped with transparent polythene strip, and tied at both ends. The application of 5000 ppm of IBA was the best treatment for induction of roots in 95.2% of branches, with 14.5 roots per air-layered branch and mean root length of 12.5 cm. The rooted branches were cut from the lower end and transferred to polybags in nursery.

Conclusions

Vegetative and seed propagation techniques standardized for the eight selected threatened plants successfully produced several seedlings, which were subsequently planted in the natural habitats (Table 2). This demonstrates the necessity to improve the conventional propagation methods with appropriate scientific inputs for developing less expensive and simple techniques for recovering the threatened plants. The success of these eight threatened plants also indicates that the standard propagation protocols need to be worked out for other threatened plant species of India to take up species recovery and reintroduction programmes in different ecoregions.

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ACKNOWLEDGEMENTS. We thank the Department of Biotechnology, Government of India for providing financial support under the All-India Coordinated Project entitled 'Preventing extinction and improving conservation status of threatened plants of India through application of biotechnological tools' (Project No. BT/Env/BC/01/2010).

doi: 10.18520/cs/v114/i03/562-566