

***In Vitro* and Micropropagation for Conservation of Rare and Threatened medicinal Plant *Ceropegia* species - A Review**

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Abstract

India has a rich biological diversity due to its varied climatic, altitudinal variations and ecological habitats. There have been increasing rates of threats of depletion to these biological resources due to immense biotic and abiotic stresses. Indiscriminate collection of plants for their medicinal, ornamental, perfumery uses, etc. and habitat loss and degradation are potential causes of threats. Conventionally, are methods allow conservation to occur with ongoing natural evolutionary processes, *ex situ* conservation via *in vitro* propagation also acts as a viable alternative for increase and conservation of populations of existing bioresources in the wild and to meet the commercial requirements. A review highlighting various *in vitro* protocols developed for rare and threatened plant called *Ceropegia* species. India has been done to highlight the significance of *ex situ* conservation in cases where regeneration through conventional methods is difficult to undertake and species are left with low population in the wild.

Keywords: *Ceropegia*, *in vitro* propagation, micropropagation, conservation, threatened

Introduction

India is one of the twelve mega diversity countries of the world with a rich diversity of biotic resources (Bapat *et al.*, 2008). Out of 34 hotspots recognized, India has two major hotspots - the Eastern Himalayas and the Western Ghats. India harbours about 47 000 species of plants of which 17 000 are angiosperms (Bapat *et al.*, 2008). A total of 560 plant species of India have been included in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened species, out of which 247 species are in the threatened category. On a global basis, the IUCN has estimated that about 12.5% of the world's vascular plants, totaling about 34 000 species are under varying degrees of threat (Phartyal *et al.*, 2002). Human activity is the primary cause of risk for 83% of endangered plant species. Habitat destruction and loss are also a problem because they lead to the fragmentation of the remaining habitat resulting in further isolation of plant population (REC, 2008). From another side during the last 10 years an intense interest has emerged in "nutraceuticals" (or "functional foods") in which phytochemical constituents can have long term health promoting or medicinal qualities. Although the distinction between medicinal

plants and nutraceuticals can sometimes be vague, a primary characteristic of the latter is that nutraceuticals have a nutritional role in the diet and the benefits to health may arise from long term use as foods (Korver, 1998). In contrast, many medicinal plants possess specific medicinal benefits without serving a nutritional role in the human diet and may be used in response to specific health problems over short- or long-term intervals (Donald, 2000). There is indisputable interest towards traditional and alternative medicine world-wide (Matthys *et al.*, 2007) and at the same time an increasing application of herbs in medical practices, reported by World Health Organization (WHO, 2005). Nowadays the centuries-old tradition of medicinal plants application has turned into a highly profitable business on the world market. Numerous herbal products have been released like patented medical goods, food additives, herbal teas, extracts, essential oils, etc. (Lange, 2004).

Medicinal plants are precious part of the world flora. More than 80000 species out of the 2500 000 higher plants on Earth are reported to have at least some medicinal value and around 5000 species have specific therapeutic value. The contemporary phytotherapy and the modern allopathic medicine use raw materials from more

than 50 000 plant species (WHO, and WMS 2011). About two thirds of these fifty thousand plants utilized in the pharmacological industry are harvested from nature (Edwards, 2004). Small portions like 10%-20% of the plants used for remedies preparations are cultivated in fields or under controlled conditions (Vines, 2004). Ages old exploitation of the natural resources and the dramatically increased interest are a real thread for the biological diversity. Bad harvesting management and insufficient cultivation practices may lead to extinction of endangered species or to destruction of natural resources. Science has already recorded diminishing natural populations, lost in the genetic diversity, local extinction of many species and/or degeneration of their natural habitats (Peter *et al.*,2005). This alarming situation is raising the questions about special efforts which should be paid both to protection of the plant populations and to up-to-date knowledge concerning more reasonable and effective utilization of these plants (WHO, 2010).

Worldwide the constant expansion of herbs' trade, the insufficient cultivation fields, and the bad management of harvesting and overharvesting have led to exhaustion of the natural resources and reduction of the biodiversity. According to the data of the Food and Agricultural Organization (FAO) at the United Nations annually the flora bares irretrievable losses which destroy the natural resources and the ecological equilibrium (FAO, 2010). Four thousand to 10 000 medicinal species were endangered of disappearing at the beginning of this century (Edwards, 2004). To stop the violence against nature, efforts should be directed both to preservation of the plant populations and to elevating the level of knowledge for sustainable utilization of these plants in traditional, alternative, and allopathic medicine (WHO, 2010). This great issue is in the focus of science which offers different decisions to solve the global problem. Cultivation of the valuable species in experimental conditions is one of the approaches. The latter refers to application of classical methods for multiplication by cuttings, bulbs, and so forth, as well as by biotechnological methods of *in vitro* cultures and clonal propagation for production of enormous number of identical plants. The micropropagation is considered to have the greatest commercial and economic importance for the rapid propagation and *ex situ* conservation of rare, endemic, and endangered medicinal plants (Khan Mohamed *et al.*,2009; Tasheva and Kosturkova,2012).

Micropropagation, cell and callus cultures, metabolic engineering and genetic manipulations are especially appropriate for species which are difficult to propagate *in vivo* (Verpoorte, 1999).

Biotechnological methods seem appropriate ones with their potential for multiplication, selection and protection of medicinal plants. In this respect biotechnological approaches are convenient for use of cells, tissue, organs or entire organism which grow and develop in *in vitro* controlled conditions, and can be subjected to *in vitro* and genetic manipulations (Khan Mohamed *et al.*, 2009) to obtain desired substances (Rao Ramachandra and Ravinshankar, 2002). These methods are especially appropriate and reasonable to apply when the targeted species have high economical or trade value, or plant resources are limited concerning the availability of wild area or good healthy plants, or when the plants are difficult to grow (Misawa, 1994; Verpoorte *et al.*, 2002).

In vitro cultivation may be directed to development of different systems depending on the practical needs. At present production of a large number of identical plants by clonal micropropagation is the most prominent one. Complex and integrated approaches for cultivation of plant systems may be the basis for future development of new, effective, safe and high quality products. These scientific achievements might be used for the establishment of *ex situ* and *in vitro* collections, multiplication of desired species and to obtain raw material for the pharmaceutical and cosmetic industries (Julsing *et al.*, 2006). *In vitro* technologies offer some or most of the following advantages: easier extractions and purification of valuable substances from temporary sources; new products which may not be found in nature; absence of various environmental and seasonal effects, automation, better control of the biosynthetic pathways and flexibility in obtaining desired product; shorter production cycles and cheaper less costly products. Here should also be mentioned the potential of the sophisticated techniques of genetic engineering, which might be applied respecting the rules of contained use (Khan Mohamed *et al.*,2009; Verpoorte *et al.*, 2002). At present the methods of plant cell and tissue cultures have found many proper sites for application in the medicinal plants utilization. The achieved results and the confidence for further success drive the efforts for wider application of plant biotechnologies in more

spheres concerning medicinal plants (Tripathi and Tripathi, 2003).

Essence of *in vitro* culture

Plant cell methods and techniques were initially used in fundamental scientific investigations at the beginning of their development in the early 60-ties of the last century. Plant biotechnology is based on the totipotency of the plant cell (Butenko, 1999; Griga, 2001). This process of *de novo* reconstruction of an organism from a cell in differentiated stage is highly linked to the process of dedifferentiation when the cell is returning back to its early embryonic/meristematic stage. In this stage cells undergo division and may form non differentiated callus tissue or may dedifferentiate to form new tissue, organs and an entire organism. Morphogenesis *in vitro* is realized via two major pathways: (i) organogenesis when a group of cells is involved for *de novo* formation of organs and (ii) somatic embryogenesis when the new organism is initiated from a single cell.

Micropropagation

Micropropagation is a vegetative propagation of the plants *in vitro* conditions (in glass vessels under controlled conditions) leading to development of numerous plants from the excised tissue and reproducing the genetic potential of the initial donor plant. Usually tissues containing meristematic cells are used for induction of axillary or adventitious shoots but induction of somatic embryos can be achieved from differentiated cells as well. Micropropagation is used routinely for many species to obtain a large number of plants with high quality. It is widely applied to agricultural plants, vegetable and ornamental species, and Environmental Biotechnology - New Approaches and Prospective Applications in some less extent to plantation crops. One of the substantial advantages of micropropagation over traditional clonal propagation is the potential of combining rapid large-scale propagation of new genotypes, the use of small amounts of original germplasm (particularly at the early breeding and/or transformation stage, when only a few plants are available), and the generation of pathogen-free propagules (Altman, 1999). Compared to the other spheres of *in vitro* technologies clonal propagation has proved the greatest economical and market importance in industry including pharmaceutical industry which needs for raw

material from the medicinal plants is increasing constantly. It offers faster and alternative way for production of raw material and from another side overcoming the problems arising from the limited natural resources. At present, there is a long list of research groups worldwide investigating hundreds of medicinal species. Various success procedures and recipes for many of these species have been developed. However, there is not a universal protocol applicable to each species, ecotype, and explant tissue. From another side all these continuous tedious studies on the standardization of explant sources, media composition and physical state, environmental conditions and acclimatization of *in vitro* plants have accumulated information, continuously enriched, which is a good basis for elaboration of successful protocols for more species. Wider practical application of micropropagation depends on reduction of costs so that it can become comparative with seed production or traditional vegetative propagation methods (e.g., cuttings, tubers and bulbs, grafting), (Altman, 1999).

The genus *Ceropegia* L. is the largest genus of the tribe Ceropegieae with about 200 species distributed only in tropical and sub tropical regions of the Old World, ranging from the Spanish Canary Islands in the west, through Central, Southern, and Northern Africa, Madagascar, Arabia, India, South Asia to Northern Australia in the East (Good, 1952; Anonymous, 1992; Bruyns, 2003). The maximum diversity of *Ceropegia* occurs in South Africa followed by Kenya and Madagascar. Its species diversity eastwards diminishes in Arabia where only 10 species were recorded and only one species in Pakistan. The species of *Ceropegia* as a whole are under threat, owing to either destructive collection or habitat degradation. They are not only genetically depleted but also are scarcely available. Ansari, (1984) revised the Indian *Ceropegia* and reported 44 species, of which 28 are said to be endemic to India. Many species of the genus *Ceropegia* have now been added to the list of Indian endangered plants (BSI, 2002). These species are placed under the categories of rare, endangered, vulnerable, extinct, and threatened plants (Nayar and Sastry, 1987; Goyal and Bhadauria, 2006; Madhav Gadgil, 2004)

Economic importance of the genus *Ceropegia*

- The sweet-sour leaves are edible and are considered to be tonic and digestive (Kirtikar and Basu 1935)
- Used as an Antidote for snake bite Duraisamy Suresh and Paulsamy, 2010)
- The tubers are edible (Mabberly, 1987) and contain starch, sugars, gum, albuminoids, carbohydrates, fats, crude fibre, and the medicinally important alkaloid Ceropegin (Kirtikar and Basu, 1935; Nadkarni, 1976; Anonymous, 1980; Jain and Defillips, 1991).
- The root tubers also contain starch, sugars, gum, albuminoids, fats, crude fiber and valuable constituents in many traditional Indian Ayurvedic drug preparations that are active against ulcers, inflammation etc., (Adibatti *et al.*, 1991).
- The boiled or roasted tubers are edible and a rich source of carbohydrates (Nikam and Savanth, 2007).
- These species are of economic importance (Jagtap and Singh, 1999) due to their starchy edible tubers with medicinal value.
- The fresh tubers of these species are usually boiled before they are eaten to remove the bitterness. The active compound of tuberous roots is the alkaloid Ceropegin which is active against diarrhea and dysentery inflammation of gums and delirious fevers of parturition (Nadkarni, 1976).
- The tubers then again are a kind of energy source, among other things they are used to suppress fatigue. Furthermore the sticks with which the Toda stamp milk to butter (<http://www.biodiversitylibrary.org>).
- The alkaloid Ceropegin from the tubers of *C. bulbosa* was used in Bihar to cure cold, sneezing and eye diseases (Kirtikar and Basu, 1935).

Reasons for threatened status

Several species within this genus are rare and endangered. The major threats to these plants are habitat destruction and local peoples' use of the edible tubers as food. Due to the elaborate flower forms and ornamentation, several species have horticultural value (CITES, 2007). In most of the Indian *Ceropegia* species, the starchy tubers are prone to fungal infections and thus decay of tubers is a major problem in their cultivation and maintenance. They dislike organic manure and excess watering. Over exploitation of *Ceropegia* species for these tubers by humans and various animals, endemism, small and localized populations, and severe anthropogenic pressures

on the forest land have caused their decline in the wild influence of human activities, with this background, authors reviewed available literature on *in vitro* and micropropagation of *Ceropegia* species.

Conservation of *Ceropegia* through *in vitro* culture Micropropagation

Vishwanath and Patid, (1998) mainly focused on the *in vitro* techniques for conserving wild and endemic species of *Ceropegia* by mass multiplication for subsequent reintroduction in their natural habitat. Micropropagation involving a combination of axillary bud culture, shoot multiplication, somatic embryogenesis and *in vitro* tuber formation for *Ceropegia jainii*, a rare plant of the Indian sub continent, *C. bulbosa* var. *bulbosa* and *C. bulbosa* var. *lushii*, common species, was developed. Nodal explants from all species were cultured on 0.5 MS medium with 8.8 I.µM (2 mg/l⁻¹) N⁶-benzyl aminopurine (BA) to regenerate the axillary buds. These produced multiple shoots when transferred to multiplication medium consisting of 0.5 MS medium with 2.2 µM (0.5 mg/l⁻¹) BA, or microtubers when transferred to 0.5 MS medium with 22.2 µM (5 mg/l) -I BA and 23.2 µM (5 mg/l⁻¹) 0 kinetin. *In vitro* flowering occurred in *C. jainii* and not in the other two varieties when the plants were cultured on multiplication media with spermine at 0.25 µM (50 µM/ l⁻¹) as an additive. Shoot pieces produced callus on MS medium with 9.05 ktM (2 mg/l⁻¹) 2,4-dichlorophenoxy acetic acid. Regeneration of the calli by somatic embryogenesis was achieved when they were transferred to 0.5 MS medium with 2.2 µM (0.5 mg/l⁻¹) 0 BA. Rooting of the shoots was possible both by *in vitro* and *ex vitro* means.

Raghuramulu and Pullaiah (1999) developed a procedure for aseptic seed germination, callus induction and rapid micropropagation from mesocotyl and nodal segments of the aseptically grown seedlings in described. Explants were cultured on MS and B₅ media, supplemented with various hormones either alone or in combinations. For the inductions of the callus, 2,4-D (2 and 3mg/l) was found to be effective and NAA promoted profuse rooting. Mesocotyl and nodal explants were used to induce multiple shoots by culturing on the medium supplemented with various concentrations of BAP and KN (1-5mg/l) either alone or in combination. Combination of KN (0.3mg/l) and NAA (0.01mg/l) produced highest number shoots (3-4)

per explants, combination of BAP (5mg/l) KN (5mg/l) and NAA (0.5mg/l) produced healthy shoots with stout roots. Regenerated shoots were rooted on $\frac{1}{2}$ B₅ medium supplemented with 1.5 mg/l NAA and 0.25mg/l KN. The rooted explants were successfully transferred to the soil.

John Britto *et al.*, (2003) established a microtubers and *in vitro* flower in *Ceropegia bulbosa* Roxb. var. *bulbosa*. *C. bulbosa* were cultured on B₅ medium supplemented with different concentrations of BA and AdS each in combination with 0.05 mg/L NAA. Multiple shoot formation of up to 12 shoots was observed within one week in presence of BAP (3 mg/L) and NAA (0.05 mg/L). Shoots were multiplied by subculture on the same medium. Shoots of 3-4 cm length were rooted in medium supplemented with 2 mg/L IBA. The rooted plantlets were hardened and successfully established in pots at 70% success rate. *In vitro* flowering was observed at 0.5 mg/L BA+1 mg/L GA₃. Shoots transferred to the medium containing kinetin (0.05 mg/L) + IBA (2 mg/L) showed microtubers in 28 days while the shoots cultured in presence of 1 mg/L GA₃ +0.5 mg/L of BA showed 76% flowering leading to seed production and 65% of the seeds germinated in successive generations.

Beena and Martin, (2003) established an efficient somatic embryos from *Ceropegia candelabrum* Friable callus, developed from leaf and internodes explants grown on Murashige and Skoog (MS) medium supplemented with 4.52mM 2,4-dichlorophenoxyacetic acid (2,4-D), underwent somatic embryogenesis. Compared to solid media, suspension culture was superior and gave rise to a higher number of somatic embryos. Transfer of the friable callus developed on MS medium containing 4.52 mM 2,4-D to suspension cultures of half- or quarter-strength MS medium with lower levels of 2,4-D (0.23 or 0.45mM) induced the highest number of somatic embryos, which developed up to the torpedo stage. Somatic embryogenesis was asynchronous with the dominance of globular embryos. About 100 mg of callus induced more than 500 embryos. Upon transfer to quarter-strength MS agar medium without growth regulators, 50% of the somatic embryos underwent maturation and developed into plantlets.

Beena *et al.*, (2003) mainly focused on the rapid *in vitro* propagation of *Ceropegia candelabrum* L. (Asclepiadaceae) through axillary bud multiplication was established.

Murashige and Skoog (MS) medium with 8.87 μ M *N*⁶-benzyladenine (BA) and 2.46 μ M indole-3-butyric acid (IBA) was best suited for axillary bud proliferation inducing a mean of eight shoots /node. Excision and culture of the nodal segments from the *in vitro* shoots on fresh medium with same concentrations of BA and IBA facilitated development of more than 10 shoots / node. Subsequent cultures enhanced the rate of shoot proliferation. Shoots developed were rooted best on half strength MS with 0.49 μ M IBA. Starting from a single node explant, 250 rooted shoots were obtained within 120 days. Plantlets established in pots exhibited 90% survival. Plantlets successfully established in field exhibited morphological characters identical to mother plants.

Goyal and Bhadauria, (2006) developed highest frequency of shoots formation (85 %) from nodal using MS basal contains BAP (4.44 μ M/l) with GA (0.58 44 μ M/l) and NAA (2.85-11.42 μ M/l). Micro shoots were transferred on MS medium supplemented with different concentration of auxins IAA (2.85-11.42 μ M/l) and NAA (2.69-10.74 μ M/l) with BAP (3.55 μ M/l) for rooting. IAA (11.42 μ M/l) with BAP (3.55 μ M/l) showed highest rooting response.

Nair *et al.*, (2007) in their experiment *in vitro* flowering from *in vitro* regenerated shoots of *in vitro*-raised seedlings of rare and endemic *Ceropegia lawii*, *Ceropegia maccannii*, *Ceropegia oculata*, and *Ceropegia sahyadrica*, as well as the widely distributed *Ceropegia bulbosa* var. *bulbosa* and *Ceropegia hirsuta*. In their first experiments, the MS medium contained 87 mM sucrose and was supplemented with varying concentrations of BAP (4.4 to 26.6 μ M). For the second set of trials, varying concentrations of sucrose (87 to 233 mM) were tested in MS media containing a constant 4.4 μ M BAP. Sub-cultured apical as well as axillary buds flowered with similar frequencies after 30 d of incubation. For all six species, the highest percentage of flowering shoots was obtained with either 26.6 μ M BAP or 175mM sucrose. Although smaller in size, their *in vitro* flowers were morphologically comparable with *in vivo*-derived flowers. Variations among species were noted for the number of flower buds per shoot and the percentage of flower formation. Because all six species showed similar responses in both experiments, they have suggested that this protocol is applicable across the wide range of *Ceropegia* species.

Nikam and Savanth, (2007) focused on the callus cultures, micropropagation and domestication of *C. sahyadrica*. They had some proposals to improve this plant via genetic engineering in the field concerned with tubers just like potatoes etc. Pandit *et al.*, (2008). Developed microtuberization as a step towards conservation, we implied *in vitro* methods for the sustainable propagule production in *C. hirsuta*, *C. lawii*, *C. maccannii*, *C. oculata* and *C. sahyadrica*. Effects of explant, growth regulators, sucrose and photoperiod were studied. High frequency microtuber production was achieved with the seedling-apical buds, grown on MS medium containing 4-6 mg/l⁻¹ BAP, 3-8% (w/v) sucrose, under continuous illumination. Each microtuber, when subcultured proliferated to form a cluster of secondary microtubers. Every primary and secondary microtuber bore at least one shoot-bud and a root primordium. Each tuber (formed with any of the significantly effective treatments) weighed more than 500mg, enough to plant directly in non-sterilized soils. Microtubers could be produced and proliferated round the year. Proliferation could be solely attributed to *in vitro* procedures as these plants bear solitary tubers *in vivo*. Microtubers could be sprouted *in vitro* to prepare ready to pot plantlets. As, this novel method succeeded for all five species, though they belong to different eco-physiological backgrounds, we recommend its implementation in the conservation programs for a broader range of *Ceropegia* species, supported by other integrated strategies.

Nikam *et al.*, (2008) established a protocol for *in vitro* micropropagation of *C. hirsuta* through optimization of cytokinins and auxins and then transfer of plants to the field condition. The shoot regeneration was direct from nodal explants, which has been crucial for employing micropropagation techniques for plant regeneration and conservation as it ensures genetic stability.

Karuppusamy *et al.*, (2009) developed an *in vitro* techniques for conserving wild and endemic species of *Ceropegia intermedia* by axillary shoot multiplication. MS medium with 6.66 µM N⁶ benzyladenine (BA) was best for axillary bud proliferation inducing a mean of 5.51 shoots per node. Excision and culture of the nodal segments from the *in vitro* shoots on fresh medium with same concentration of BA facilitated development of more than 5 shoots per node. Subsequent subculture slightly enhanced up to third subcultures and decreased thereafter. Shoots

developed were rooted best on half strength MS with 5.37 µM NAA. Plantlets established in pots exhibited 75% survival.

Karuppusamy and Pullaiah (2009) reported that pollination in the species of *Ceropegia juncea* based on observation from *ex situ* grown plants. The study confirmed pollinators are small, mostly female Dipteran flies, ca 3mm long, which carry pollinaria on the proboscis. The most important factors for an insect to effect pollination appear to be the appropriate overall size, mouth parts and adjacent pads on which a pollinarium can attach. The complex and diverse floral morphology of the tubular corolla and their disposition as well as other commonly occurring features namely, vibratile corolla lobes and hairs, specialized hairs on corona, interior sculpturing, sliding zone and differential lighting within the flowers are important mechanisms for attracting insect pollinators. Stigmatic receptivity performs at anthesis and remains so far 24 hr. Flowers show pronounced protogyny. Manual pollinations showed that the species permits geitonogamous pollination. The percentage of fruit set in manually pollinated flowers is higher than that resulting from open pollination, confirming that pollination is a limitation for fruit set in the *ex situ*-grown population. Nevertheless, fruit and seed set are sufficiently high for *ex situ* conservation purposes.

Nikam and Savant, (2009) developed a protocol for the *in vitro* propagation and alkaloid accumulation in callus cultures of *Ceropegia juncea* Roxb. a source of "Soma" drug in Ayurvedic medicine. Multiple shoots and callus induction was optimized by studying the influence of auxins [IAA (Indole-3-acetic acid), NAA (2-Naphthalene acetic acid) and 2,4-D (2,4-Dichlorophenoxyacetic acid)] and cytokinins [BA (6-benzyladenine) and Kin (Kinetin)] alone and in combinations. The best response for multiple shoot induction was obtained in nodal explants on MS medium supplemented with 7.5 µM Kin (8.5 ± 3 shoots per explants). The shoots were rooted on half strength MS (Murashige and Skoog's) medium fortified with either IAA or NAA (0.5–2.0 µM). The plantlets were transferred directly to the field with 100 % success rate. Supplementation of MS medium with auxins and cytokinins enhanced the growth of callus but inhibited the shoot regeneration in nodal explants. Best callus induction and proliferation observed on MS + 1 µM 2,4-D+5 µM BA. However the maximum *ceropegin*

content (470 µg/g dry weight) was recorded in dried callus derived on MS+10 µM IAA+5 µM BA. Quantitative TLC (Thin layer chromatography) studies of the callus revealed a phytochemical profile similar to that of naturally grown plants. The calli were maintained by subculturing at 4 weeks interval on fresh parent medium over a period of 34 months. The optimized *in vitro* propagation and callus culture protocol offers the possibilities of using organ/callus culture technique for vegetative propagation and production of ceropegin alkaloid.

Murthy *et al.*, (2010) regenerated plant from direct somatic embryogenesis in *Ceropegia spiralis* from nodes, internodes and thin cell layer of the plant. Extensive callusing of TLCs (99.93±0.26%) was observed on the medium containing BAP (13.32µM) with 2,4-D (4.52 µM) On MS medium supplemented with Kinetin (4.56 µM) + casein (250mg/L) the highest number (8.40±1.14) of germinating somatic embryo was noticed. The ontogeny embryo was noticed from that plantlets were also observed.

Chandore *et al.*, (2010) reported *Ceropegia fantastica* Sedgw (Asclepiadaceae) is a critically endangered, endemic species in Western Ghats of India. The fruit and seed setting are very low and *in vitro* propagation is the only viable alternative for its sapling raising and restoring of this plants's population in the wild natural environment through reintroduction. Attempts have been made here for regeneration of this species through *in vitro* technique using nodal segments as explants and upto 13 multiple shoots were initiated on Murashige and Skoog's (MS) basal medium supplemented with 6- benzyl aminopurine (1.5mg⁻¹). Shoots were multiplied by routine periodic subcultures. The shoots of 3-4 cm length were isolated and rooted on MS basal medium (without Ca Cl₂) containing indole -3-butric acid (mg l⁻¹). The rooted plantlets were hardened plantlets in two successive years were transferred to their natural habitats of Western Ghats.

Kondamudi *et al.*, (2010) induced callus from node, internodes and thin cell layer explants an endangered medicinal plant *Ceropegia pusilla*. The best callus induction was found on the MS medium supplemented with 6-benzylaminopurine (BAP) 13.32 µM /l + 2, 4-dichlorophenoxy acetic acid (2,4-D) 0.45 µM /l from TCLs. After the initiation of the callus, it was immediately

transferred to MS medium supplemented with BAP along with other auxins like 2, 4-D, Indol-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), Naphthalene Acetic Acid (NAA). The regenerative calli were raised on the MS medium supplemented with 1.13 µM LG1 of 2, 4-D. Whereas, the organogenic calli was raised on the medium containing 22.7, 40.86, 45.4 µM /l of Thidiazuron (TDZ) induced 37.54±0.29, 37.12±0.18 and 34.32±0.17 shoots, respectively. On the media containing BAP 13.32 + IBA 0.49 to 1.23 µM LG1 micro shoots rooted best and 75% of the shoots were survived. The plantlets were established, acclimatized and thrived in green house conditions with 80%. The regeneration protocol developed in this study provides a basis for germplasm conservation and for further investigation of bio active constituents of this medicinal plant.

Murthy *et al.*, (2010) initiated the protocol to develop protocol for the rapid and large-scale *in vitro* propagation of *Ceropegia spiralis* through nodes, and *In vitro* flowering in Multiple shoots (14.37±0.12) induction was more successful using nodes as explants on MS medium supplemented with BAP 2.22µM induced multiple shoots after fourth subculture. *In vitro* flowering was observed on 0.5 MS medium with 3% sucrose supplemented with IAA 11.54 µM. The thin cell layers were obtained from the nodes and internodes of the plant and were cultured on the medium supplemented with BAP 13.32µM + NAA 0.537µM induced 17.34±0.55 shoots showing extensive growth. Later on the organogenesis was also induced on the medium containing BAP 13.32µM + 2, 4-D 1.130 µM. *In vitro* tuber formation by culturing both individual and multiple shoots on MS medium with 3% sucrose supplemented with BAP and different auxins at different concentrations, individually and in combination. Shoots developed were rooted best on 0.5 MS with NAA 10.74 µM. Optimum shoot and root multiplication was obtained within 8 weeks. *In vitro* plantlets were successfully weaned and transferred to soil with 90 % survival rate. Successfully, acclimatized plants were reintroduced into nature for their recovery.

Chavan *et al.*, (2011) reported a factors affecting *in vitro* propagation were evaluated for *Ceropegia attenuata* Hook., an endemic and endangered plant having ornamental potential but a limited reproductive capacity. Rapid shoot multiplication from nodal explants was

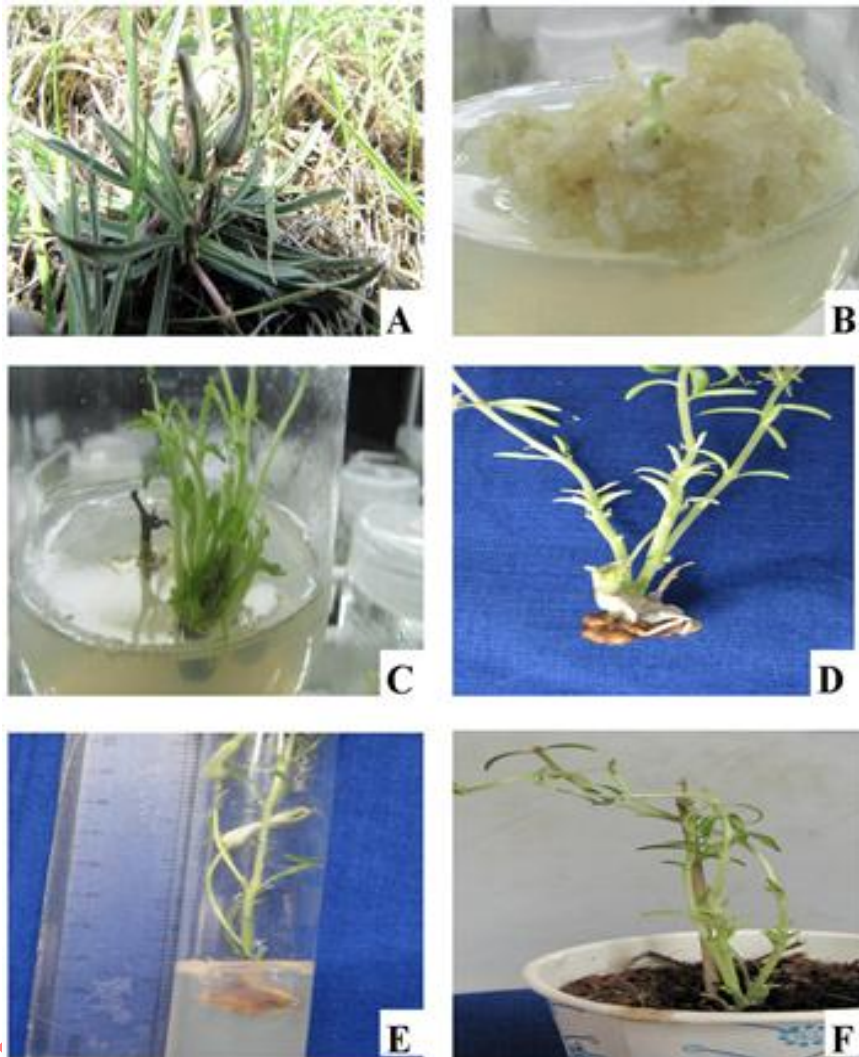
established using varying concentrations of cytokinins and auxins either alone or in combinations. The highest frequency of shoot induction was achieved when nodal explants were inoculated on Murashige and Skoog (MS) medium supplemented with 13.31 μM 6-benzylaminopurine with a mean of 12.9 ± 0.5 shoots per explant. High concentrations of TDZ (6.81–11.35 μM) and KN (6.78–11.61 μM) resulted in stunted and vitrified shoots. Factors implicated in the promotion of floral transition of the *C. attenuata* have been identified which are 4-amino-3, 5, 6-trichloropicolinic acid (picloram), 6-benzylaminopurine, sucrose and photoperiod. The highest frequency of flowering (100%) was obtained when axillary shoot explants were transferred to MS medium supplemented with picloram (4.14 μM) within 4 weeks of culture. Transfer of *in vitro* regenerated shoots to half strength MS medium with 2.46 μM indole-3-butyric acid (IBA) showed maximum root induction. The *in vitro* grown plantlets were successfully acclimatized in the glasshouse with 85% of survival and showed normal development. The developed protocol provided a simple, cost-effective approach for the conservation of endangered plant *C. attenuata* for replenishing its declining populations.

Krishnareddy *et al.*, (2011) developed the highest shoot multiplication rate of 20.65 ± 0.20 shoots/explant was achieved on Murashige and Skoog medium supplemented with BAP 8.87 μM + TDZ 4.54 μM . Excised shoots were rooted on half strength MS medium with IBA 4.90 μM + NAA 1.27 μM . 78% of the rooted shoots survived in the field. Combination of BAP 8.87 μM + TDZ 4.54 μM in *Ceropegia juncea* produced maximum number of 20.65 ± 0.20 shoots per explant and shoot length of 3.56 ± 0.03 cm with 76% response. For rooting shoots of 4 to 5 cm length were excised and transferred to half strength MS medium containing auxin for *in vitro* rooting. Half strength MS medium supplemented with auxins at different concentrations showed varied effect on *in vitro* rooting. Of the three auxins tested, IBA 4.90 μM was most effective for root induction. Combination of auxin were also tested. Thin delicate roots were noticed when the medium was supplemented with NAA and IAA. Half strength MS medium fortified with IBA 4.90 μM + NAA 1.27 μM was effective for better rooting in *Ceropegia juncea* and produced 8.23 ± 0.14 roots per explants and root length is 4.76 ± 0.14 cm. The highest mean length of longest roots 5.17 ± 0.11 cm with the root number of 4.62 ± 0.11

was recorded in IBA 4.90 μM + NAA 0.54 μM media composition.

Sri Rama Murthy and Kondamudi, (2011) established a thin cell layers of nodes and internodes of *Ceropegia spiralis* L. were cultured on MS supplemented with BAP 13.32 $\mu\text{M/l}$ + NAA 0.537 $\mu\text{M/l}$ induced 17.34 ± 0.55 shoots showing extensive growth. Later on the organogenesis was also induced on MS containing BAP 13.32 $\mu\text{M/l}$ + 2, 4-D 1.130 $\mu\text{M/l}$, whereas the medium with BAP 13.32 $\mu\text{M/l}$ + 2, 4-D 4.52 $\mu\text{M/l}$ has the highest callus producing ability in recalcitrants as well as in normal explants. Shoots developed were rooted best on 0.5 MS with NAA 10.74 $\mu\text{M/l}$. Optimum shoot and root multiplication was obtained within eight weeks. *In vitro* plantlets were successfully weaned and transferred to soil with about 90 per cent survival rate. So far, more than 650 weanlings have been produced successfully and reintroduced into nature for their recovery.

Muthukrishnan *et al.*, (2012) developed a reproducible protocol for *in vitro* propagation of *Ceropegia thwaitesii* Hook (Asclepiadaceae), an endemic plant species of Western Ghats, Tamil Nadu (India), was carried out. Auxiliary bud explants were cultured on Murashige and Skoog (MS) medium fortified with cytokinins (KIN), 6-benzyl adenine (BA), 2-isopentenyladenine (2-iP) and thidiazuron (TDZ) in various concentrations and in combination with auxins indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and naphthalene acetic acid (NAA). High frequency of shoot bud proliferation and multiplication was observed on KIN (13.94 μM) + IAA (28.54 μM). Rhizogenesis was observed on MS medium supplemented with IBA 2.46 μM and plantlets produced through micropropagation were hardened with the survival success of 73.33%. The efficient Micropropagation regeneration protocol developed would aid *ex situ* conservation of this endemic species. Krishnareddy and Pullaiah, (2012) they have develop *in vitro* techniques for conserving endemic species, *Ceropegia elegans* by axillary shoot multiplication. Murashige and Skoog (MS) medium with kinetin (Kn) 23.20 μM + indole-3-acetic acid (IAA) 5.71 μM was the best for axillary bud proliferation inducing a mean of 7.11 ± 0.07 shoots per node. Shoots developed were rooted best on half strength MS medium with 4.90 μM indole-3-butyric acid (IBA). Plantlets established in pots exhibited 72% survival rate.



A- Wild Plant with flower; B-Callus initiation from nodal explants cultured on MS +BA +NAA (0.5+0.2); C Shoot initiation from callus on MS +BA +NAA (0.5+0.1); D-Shoot along with tuber root formation on MS +BA +NAA (0.5+0.1); E- In vitro flowering on MS +BA +NAA (0.5+0.2); F- Acclimatized plantlet after five weeks.

Fig -1: *In vitro* propagation through nodal callus and multiple shoot proliferation of *Ceropegia pusilla*.

Sri Rama Murthy *et al.*, (2012) to form microtubers *in vitro* was studied, to conserve these endemic and endangered species. Murashige and Skoog's (MS) medium supplemented with various concentrations of cytokinins and auxins supported induction of two types of microtubers, that is, basal and aerial tubers. The nodal explants of *C. spiralis* best tuberized on MS medium supplemented with 6-benzylaminopurine (BAP) 13.32 μM + Naphthalene Acetic Acid (NAA) 1.34 to 2.68 μM . The aerial tubers were observed on Indole-3-Butyric Acid (IBA) 49.2 μM + 6% sucrose and

Indol-3-Acetic Acid (IAA) 11.54 μM + 3% sucrose augmented $\frac{1}{2}$ MS media. In another case, single roots were tuberized on $\frac{1}{2}$ MS medium supplemented with IAA 11.54 μM + 3% sucrose in *C. spiralis*. Whereas, in *C. pusilla*, the medium containing BAP 13.32 μM + 2, 4-D 0.11 μM + 3% sucrose played major role in the induction of basal tubers. Strength of the medium, plant growth regulators (PGRs) concentration and additives played an effective role in the formation of microtubers. If these valuable ornamental and medicinal plants were conserved, we may add another flower to the bouquet and a new drug as well.

Chavan *et al.*, (2013) The aim of this study was to optimize direct shoot organogenesis (DSO), indirect shoot organogenesis (ISO) and plant regeneration of threatened medicinal plant *Ceropegia santapau*, followed by analysis of genetic status and biochemical characterization of micropropagated plantlets. For optimization, cotyledonary nodes and cotyledons were used as source of explants in DSO and ISO respectively. The highest frequency of regeneration (88.0 %) for DSO with 8.1 ± 0.6 shoots per explant was obtained from cotyledonary nodes cultured on Murashige and Skoog's (MS) medium containing 2.0 mg L^{-1} 2iP. The best response for callus induction and proliferation was achieved with 1.5 mg L^{-1} PR (picloram) in which 97.5 % of cultures produced an average of $913 \pm 10.9 \text{ mg}$ (fresh weight) of callus. The highest frequency of shoot formation (92.5 %) with an average of 19.7 ± 0.3 shoots in ISO was obtained when calli were transferred to MS medium supplemented with 2.5 mg L^{-1} BAP and 0.4 mg L^{-1} IBA. Regenerated shoots were best rooted in half-strength MS medium with 2.0 mg L^{-1} NAA. Plantlets successfully acclimatized were morphologically indistinguishable from the source plant. Micropropagated plantlets subjected to random amplified polymorphic DNA and inter simple sequence repeats (ISSR) marker based profiling revealed uniform banding pattern in DSO-derived plantlets which was similar to mother plant. ISSR fingerprints of ISO-derived plants showed low variation. Method of regeneration, plant part and solvent system significantly affected the levels of total phenolics, flavonoids and antioxidant capacity. Assay of antioxidant activity of different tissues revealed that significantly higher antioxidant activity was observed in ISO-derived tissues than DSO-derived and mother tissues. RP-HPLC analysis of micropropagated plantlets showed the presence of three major phenolic compounds which were similar to those detected in mother plant. Rapid multiplication rate, genetic stability and biochemical parameter ensures the efficacy of the protocol developed for the propagation of this threatened medicinal plant.

Richa and Shekhawat (2013) developed a protocol for *in vitro* multiple shoot production was achieved in *Ceropegia bulbosa* var. *bulbosa*. Nodal segments obtained from mature plant were used as an explant and their morphogenetic potential was tested on, Murashige and Skoog's medium containing cytokinins alone or in combination with auxins. Application of

$8.88 \mu\text{M}$, 6-benzyladenine was most effective for shoot regeneration and resulted in an average of 9.7shoots/explant. Nodal segments obtained from *in vitro* proliferated shoots of *C. bulbosa*, were encapsulated in calcium alginate beads for large-scale clonal propagation, short-term conservation, germplasm exchange and distribution. Maximum percent response for conversion of encapsulated nodal segments into plantlets was 100%. Encapsulated nodal segments could be stored at low temperature (4°C) up to 60 days with a survival frequency of 50.7%. Morphologically identical plants generated *in vitro* were genetically identical when analyzed using random decamer primers (RAPD).

Mahendra Phulwaria *et al.*, (2013) developed an efficient protocol of shoot organogenesis and plant regeneration from seedling derived callus has been developed. Out of different explants *i.e.* epicotyl, hypocotyl, cotyledonary node and root, epicotyl was found to be the most suitable for establishment of cultures and further shoot regeneration. Callus was induced from epicotyl explants on MS medium containing 1.0 mg l^{-1} of 2,4-D. Proliferation of callus was observed on MS + 1.0 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} of BAP. The shoot regeneration was achieved when proliferated callus was transferred to MS medium supplemented with BAP either alone or in combination with IAA or NAA. Maximum number (25.5) of shoots were regenerated on MS medium containing 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. The shoots (100%) were rooted *ex vitro* when treated with IBA (100 mg l^{-1}) for 3 min. Rooted plantlets were hardened successfully and transferred to the field. The described method can be successfully used for the large-scale multiplication and conservation of germplasm of this important and threatened plant.

Kalimuthu *et al.*,(2013) developed an efficient protocol was described for the rapid *in vitro* multiplication of wild and endemic medicinal plant of *Ceropegia pusilla* by enhanced axillary bud proliferation from nodal explants. The physiological effects of growth regulators [6-Benzylaminopurine (BAP), Indole -3- Butric acid (IBA), Naphthalene Acetic Acid (NAA), with Murashige and Skoog (MS) medium were used. The highest number of shoots initiation (3.50 ± 0.34) and the maximum average shoot number from shoot subculture (15.40 ± 1.65) were recorded on MS medium supplemented

with BAP+NAA (2.5+0.5 mg/l). The microtubers formed in MS medium containing BAP (1.5mg/l) and NAA (0.5mg/l). Rooting was best achieved on MS Medium augmented with IBA (0.5mg/l) and the maximum average number of root (4.72 ± 0.66 cm) and maximum average number of root length (4.8 ± 0.49 cm) were recorded. The plantlets regenerated *in vitro* with well-developed shoot and roots were successfully established in pots containing mixture of decomposed coir waste, perlite and compost 1: 1:1 ratio and grown in a shade house with 81 ± 3.16 survival rate. Prabakaran *et al.*, (2013) study was undertaken to evaluate the most suitable concentration of plant growth regulators and suitable explants for callus induction and subsequent organogenesis of an endangered medicinal plant *Ceropegia pusilla*. The best performance of callus induction and morphogenesis was found on MS medium supplemented with 6- benzylaminopurine (BA) and naphthalene acetic acid (NAA), from node and internode the maximum account of callus initiation was recorded on MS medium supplemented with BA+NAA ($2.22\mu\text{M} + 5.37\mu\text{M}$) and the maximum % of callus induced on subculture is 5.47 ± 0.68 . Rooting was best achieved on MS medium augmented with IBA ($2.46\mu\text{M}$). The maximum number of roots (4.72 ± 0.66 cm) and root length (4.8 ± 0.49 cm) were recorded). Plantlets regenerated *in vitro* with well-developed shoot and roots were successfully acclimatized in pots containing a mixture of decomposed coir waste, perlite and compost 1: 1:1 ratio and grown in a shade house with 81 ± 3.16 percent survival rates.

Conclusions

The prime importance of *in vitro* propagation of rare, critically endangered, endangered and vulnerable plants would be to generate a large number of planting materials from a single explant without destroying the mother plant and subsequently their restoration in the natural habitat, thus conserving the biodiversity. The significance of an efficient *in vitro* protocol would be to obtain maximum number of plantlets in minimum period of time with proper rooting along with acclimatization in the field. The different regeneration systems which have been developed need to be field tested and the field data is collected so that the complete technology packages could be ready for commercialization and transfer to the user agencies (Anonymous, 2000).

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