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Research article

EFFECTIVE *EX SITU* CONSERVATION OF ENDANGERED SPECIES *BELOPERONE PLUMBAGINIFOLIA* NEES: A MEDICINAL PLANT

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ABSTRACT: *Beloperone plumbaginifolia* is a wild shrub, identified as an endangered medicinal species by the human exploitation for antidote properties. The establishment of *ex situ* conservation method, a simple and effective protocol was developed by us to conduct micropropagation abreast with genetic stability from regenerated clones by Random amplified polymorphic DNA marker. In *in vitro* tissue culture, high frequency bud break (45-65%) and multiple shoot (5±1 shoots/explants) was obtained from nodal explants inoculated in Murashige and Skoog medium (MS) fortified with benzylaminopurine alone (2.0 -2.5mg/l) in the 15th day. The kinetin hormone alone fortified, showed faster bud breaking (10th day) in all the concentration of (0.5-2.5 mg/l) and 4±1 shoots per explants was achieved on 1.5 mg/l. Auxin hormone of Indole-3 butyric acid along with Kinetin in ratio of 3.5:0.5 mg/l was found effective on root formation. Finally in acclimatization process, 73.6% of survival rate was found among the plants in the field after 3 months. Evaluated the genetic stability by RAPD random primer (OPK-15) was specified low variation occurred between mother plant and *in vitro* propagated clones. Jaccard's coefficient of similarity varied from 0.53 to 0.93 indicated high level of genetic similarities between mother plant and *in vitro* regenerated plants.

Keywords: Clones; e*x situ* conservation; endangered species; in vitro tissue culture; polymorphism

Abbreviations: BAP – N^6-benzylaminopurine; GA_3– Gibberellic acid; IBA – Indole-3 Butyric acid; KIN- Kinetin; MS- Murashige and Skoog medium; NAA– naphthalene acetic acid; RAPD – Random amplified polymorphic DNA

INTRODUCTION

The natural wealth of flora is increasingly threatened by the pressures of over-collecting, unsuitable agriculture and forestry practices, urbanization, pollution, habitat destruction, fragmentation and degradation, spread of invasive alien species and climatic change [21]. Today over 95% of the medicinal plants used by the industries in India are collected from the wild populations. This process leads to definite threat to the genetic stocks and to the diversity of medicinal plants. Concerning over these problems, finding and conserving an unfamiliar important medicinal plant throughout the world are significant to the scientific community. One of the unfamiliar threatened plants *Belopherone plumbagnifolia*, is a flowering plant belongs to the Class *Magnoliopsida*, Subclass *Asteridae*, Order *Lamiales* and Family *Acanthaceae*. It is a branched small shrub called Snake grass and it is commonly found in South America and South West Africa also some part of Southeast Asia and South India. In Brazil, it is grown as an ornamental plant [1]. It is a traditional herb used in Indian system of traditional medicine and folklore medicine. It is being widely used as an antidote for snake and scorpion bite by the tribal people [25]. Besides this, the extracts of the plants possess anticancer and antimicrobial activities and used for the treatment of Psoriasis [22]. The necessity of this medicinal plant increased dramatically in pharmaceutical sector due to the important medicinal value. But the Cultivation of medicinal plants is also difficult due to lack of standardized agronomic practices for unfamiliar species and unavailability of source planting materials. In order to meet the requirement and to overcome the low seed viability and low rate of germination in natural propagation, mass cultivation tissue culture protocol is needful. Reports says, recent years there has been an increased interest for *in vitro* culture techniques which is a viable tool for mass multiplication and germplasm preservation of rare, endemic and endangered medicinal plants [27].These technologies could be a cost effective means for the high volume production of elite planting materials throughout the year, without any seasonal constraints [3]. *In vitro* Micropropagation and re-introduction into the original or favorable habitats is the only strategy for the conservation of critically endangered, vegetatively propagated species. Screening the micropropagated plants, before re-introduction, using molecular markers is desirable to reduce the chances for inclusion of variable genotypes.

The only negative aspect considered on *in vitro* micropropagation was Phenotypic and genetic variations were reported to occur as consequences of the propagation process originating somoclonal variants [15]. The possibility of genetic alterations induced by the processes of tissue culture deserves special consideration when the objective is the multiplication of selected genotypes or germplasm conservation [4]. Molecular markers, which can detect modifications at the DNA level, are increasingly being used to access the fidelity of *in vitro* propagated plants. RAPD marker based fingerprinting is being used by several researchers to detect molecular alterations in *in vitro* regenerated plants, and different rates of variation were reported according to the species and the regeneration system adopted [7]. RAPD is an inexpensive and rapid method requires only small amount of genomic DNA and can produce high levels of polymorphism and may facilitate more effective diversity analysis in plants [28]. Development of efficient tissue culture protocol for the regeneration and organogenesis of this medicinal plant is a burden to researchers because there had been only one report available so far [25]. In the present study, we report the efficient in vitro regeneration of *B.plumbagnifolia* with high ratio of genetic stability compared with mother plant through analysis by RAPD marker.

MATERIALS AND METHODS

The plant tender twigs were collected from Herbal garden, Guindy campus, University of Madras, Tamil Nadu. Single nodal segments (1.5 cm long) were sectioned from defoliated twigs and taken to surface sterilization, washed with running tap water for 1 hr, treated with 70% ethanol for 30 sec, rinsed with sterile distilled water for four times, immersed in 0.1% $HgCl_2$ for 4-5 min, after the treatment of $HgCl_2$, nodes were thoroughly washed with sterile distilled water for 3-4 times. The plant tissue culture specific MS medium was prepared by standard procedure [17]. Medium adjusted to pH 5.8, followed by the addition of sucrose 30g/l and 0.75% (w/v) agar (Hi media). Borosil glass tubes (25 × 150 mm) each containing 15 ml of the culture medium, capped with plugs of nonabsorbent cotton were autoclaved at 120˚C for 15 min. All the surface sterilized explants were placed horizontally on MS medium by standard procedure of stock solutions with no growth regulators prepared. Nodal segments were inoculated in MS medium fortified with cytokinin growth regulator of BAP (0.5-2.5 mg/l) and Kinetin for multiple shoots proliferation (0.5-2.5 mg/l). Well developed shoots were transferred onto MS medium fortified with single concentration of GA_3 (0.5mg/l) for shoot elongation. For rhizogenesis, shoots used were excised from the elongated shoots transferred to fresh MS medium containing IBA (1-3.5 mg/l) and IAA (1-3.5 mg/l) along with kinetin (0.5mg/l). In each culture tube one explants was implanted. At least ten replications were maintained for each treatment and 20 explants were evaluated in each treatment. The cultures were maintained under 16 hr light provided with cool white fluorescent lamps at 25˚ ± 2˚ C. For hardening, the in vitro raised plantlets were removed from culture, washed thoroughly with tap water transferred to the greenhouse and planted in small polythene cups containing sterile soil and vermiculite (1:1) the cups were covered using plastic bags to maintain 85-90% humidity for two weeks. Subsequently the plants were transferred to the pots in garden, after four weeks they were planted in the field.

RAPD ANALYSIS

For the present study, fresh leaves of *B. plumbagnifolia* collected from the mother (an elite genotype from which the explants were collected) and its five randomly selected micropropagated progenies were used. Genomic DNA was extracted from collected leaves using standard procedure with necessary modifications [6]. The yield of the extracted DNA was quantified by Nanodrop 2000/2000c (Thermo Scientific Inc.,) and the purity of DNA was approximately 1.8 for both by calculating the ratio of absorbance at 260/280 nm. Polymerase chain reaction was carried out using RAPD decamer, OPK-15(5'-CTC CTG CCA A-3') specifically obtained from Operon technologies, Inc., USA have been screened and selected for further analysis based on their ability to detect distinct polymorphic amplified products across the five clones and control mother plant. The reactions were carried out in a DNA gradient thermocycler (L196GGD model Peltier thermocycler, Lark innovative technologies), each 20 µl reaction mixtures performed for 40 cycles [14]. The cycles performed were as follows; an initial denaturation at 94˚C for 5 min followed by denaturation at 94˚C for 1 min, annealing at 37˚C for 1min, 72˚C for 2 min and final extension step of 72˚C for 7 min. The PCR amplified products were subjected to electrophoresis in 2% agarose gels in 1× TAE buffer at 100 V for 2.5 hr. The gel product was stained with ethidium bromide and analyzed in UV transilluminator. Here the percentage of polymorphism was calculated as the proportion of amplification products which were polymorphism across all the genotypes to the total number of amplified products and Jaccard similarity index was calculated.

RESULTS AND DISCUSSION

Recent progress made in tissue culture technique has been successfully utilized on generating endangered and economically important plants. Morphogenic potential of stem nodal explants of *Beloperone plumbaginifolia* (Fig 1A) on MS medium supplemented with various concentrations of BAP and Kinetin individually is summarized in Table 1.

Table 1. Effect of Cytokinin growth regulators on shoot proliferation. Data (mean) were recorded after 15 & 45 days of inoculation.

Growth regulators (mg/l)	% of shoot formation	No. of shoots/explant	No. of leaves per shoots /culture	Av. Length of shoots(cm)
BAP 0.5	0%	0±0	0±0	Nil
1.0	0%	0±0	0±0	Nil
1.5	15%	1±0	2±0	1.5±0.2
2.0	65%	5±1	10±2	2.9±0.1
2.5	45%	2±1	4±0	1.8±0.3
Kinetin				
0.5	30%	2±0	4±1	1.5±0.2
1.0	45%	3±0	6±1	1.9±0.1
1.5	60%	4±1	8±0	3.0±0.2
2.0 2.5	40% 20%	2±1 1±1	6±0 2±0	2.3 ±0.2 1.3±0.2

Among the cytokinin concentrations tested, there was no sign of emergence shoot bud observed even after 15th day of inoculation on MS medium supplemented with BAP 0.5 mg/l and 1.0 mg/l. In the same day shoot bud breaking was recorded on 1.5 mg/l and 2.5 mg/l of BAP with 15% to 45% respectively. The maximum percentages of shoot initiation (65%) as well as 5±1 shoots per explants were recorded with BAP at 2.0 mg/l (Fig 1B) on the 13th day of inoculation [12, 24]. Two fold increased multiple shoots was found on the 45 days of culture bearing ten leaves in each shoot. In the other set of MS medium supplemented with kinetin promoted shoot bud initiation after 10th day of inoculation at all concentrations. Highest numbers of (4±1 shoots/culture) shoot buds were found in 1.5mg/l of KIN. After 45 days of culture, shoots reached maximum height of 3.0 cm bearing 8 leaves in same concentration. From the rest of KIN concentrations (0.5, 0.1, 2.0 and 2.5 mg/l) it was found that 20-45% of shoots per explants with two young leaves emerged in each shoot [18]. Of the percentage of shoot regeneration and number of shoots differed with both BAP and KIN hormone used. In contrast, the *B.plumbaginifolia* well responded with the KIN than BAP for shoot initiation and multiplication within 10 days. Similar shoot regeneration results were reported in *Graptophyllum pictum* [13]. The hormone GA_3 stimulates the elongation of shoots is well known as it has been found to promote cell division and elongation in the apical zone of shoots [8]. The shoots transferred to elongation medium fortified with 0.5mg/l of GA_3 attained good result. The shoots were obtained at the of height 5-7cm bearing 6-8 nodes per shoot in 2-3 weeks (Fig.1C).Similar outcome were reported in *Andrographis lineata Wall.ex.Nees* (AL) member of *Acanthaeceae* family [5] and *Andrographis echoides (L.) Nees* [9].

In general, auxin initiates and develops the root. Particularly, IBA and NAA were widely used for the development of in vitro root from the explants. The elongated shoots were separated individually and transferred to rooting medium. The rooting initiation was observed (Fig 1D) within 11 days of culture. Table 2 represents the effects of auxin (IBA) on the rooting at different concentrations with the presence of kinetin along with MS medium. Roots were developed from shoots of *Beloperone plumbaginifolia in* MS medium fortified with IBA 1.0 – 3.5 mg/l in combination with KIN 0.5mg/L in standard. No rooting response were observed in MS medium supplemented with NAA (1.0 – 3.5 mg/l) combination with KIN 0.5mg/L. Rooting was increased while increasing the concentration of IBA from 1mg/L to 3.5mg/L. Number of roots attained in all concentrations were 2- 10 per shoot. Maximum 90% (8±2) of rooting found at 3.5mg/L of IBA within the sixth week of culture was shown in (Fig 1D). The maximum obtained root length was 4.5 cm, was established by the action of IBA with KIN treatment. These findings are in agreement with those observed in other regenerated plant species of Phyla *nodfolia, Leptadenia reticulate* [2] and *Lins culinaris* Medik [19]. For gardening, *in vitro* developed 60 plantlets were transferred to the plastic cups containing a sterile soil and vermiculite (1:1) maintained inside the plant growth chamber and moistened with sterile water up to two weeks (Fig 1E). Over 50 (80%) plantlets survived by hardening on poly cups in two weeks. After which plantlets were transplanted to earthenware pots containing garden soil and kept under shade in a net house for next 2 weeks (Fig 1F). Plants were misted manually with sterilized water once a day during this period to avoid desiccation. However, survival of the plantlets were slightly decreased to 46 (73.6%) on transferring to outdoors under full sun to acclimate for a period 3months. Successful survival rate 71-72.3% was found among the acclimatized plants in fields (Fig 1G). This is the first successful attempt and simple tissue culture protocol could be utilized to conserve an endangered plant species as well.

Table 2. Effect of growth regulators on root generation per culture from in vitro grown shoot explants. Data (mean) were recorded after six weeks.

Growth regulators(mg/l)		% of root formation	No. of root per shoots/culture	Av. Length of root in cm
IBA	Kn			
1.0	0.5	10%	2±1	1.1±0.5
1.5	0.5	15%	3±1	1.3±0.2
2.0	0.5	20%	3±0	3.2±0.2
2.5	0.5	50%	4±0	3.0±0.2
3.0	0.5	75%	6±1	4.2±0.3
3.5	0.5	90%	8±2	4.5±0.1

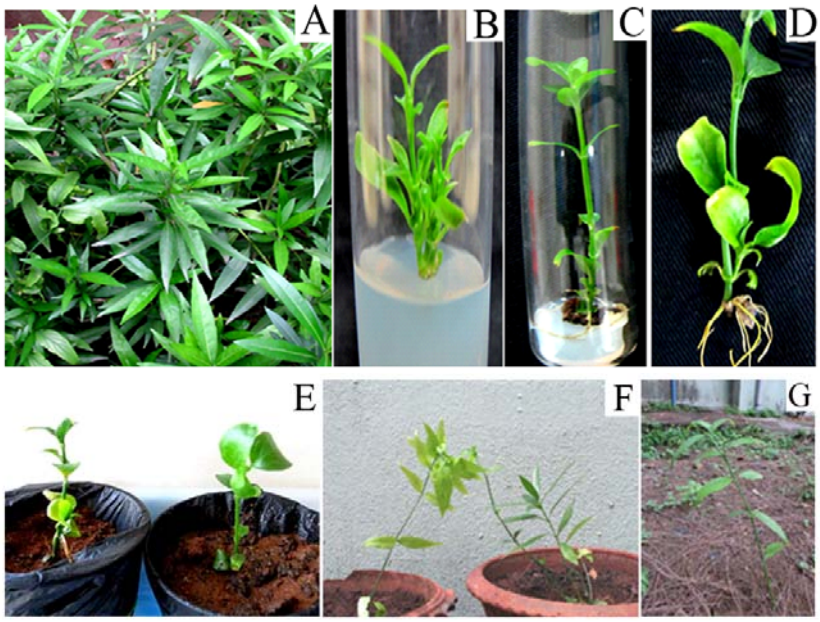

Figure 1 : In vitro propagation of Beloperone plumbaginifolia Nees., A. Mother Plant Culture; B. Initiation of shoot from nodal explants and Multible axillary shoots; C. Elongated shoot; D. Direct Rooting; E. Hardened plant in polycups; F. Plantlets in earthenwhere pots; G. Successful acclimazation.

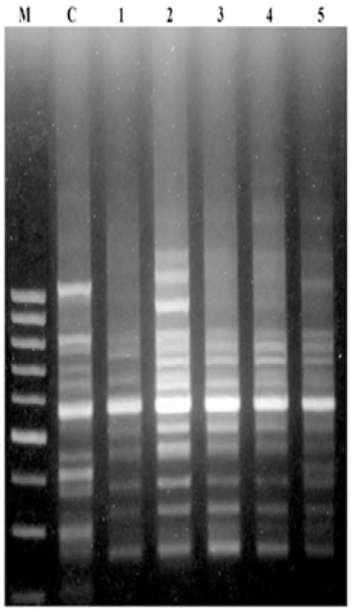

Figure 2 : M-Marker 100bp DNA ladder; C-Control (in vitro mother plant; 1-5: In vitro regenerated clones A-E respectively.

ASSESSMENT OF GENETIC STABILITY

Propagation from the vegetative cells under normal condition is expected to yield progenies genetically identical to the mother plant. However, invitro propagation through callus or direct organogenesis frequently results in variations [20]. In this regard, screening by RAPD fingerprinting had been useful in detecting variants at an early stage of plant development. Genetic stability was investigated among the *in vitro* cultured plant and mother plant using RAPD markers. The gel image (Fig 2) shows that the polymorphic banding patterns of mother plant and five *in vitro* regenerated plants from nodal explants using OPK-15 decamer primer. The size of amplified fragments obtained at the range of 100 bp to 1kb. By comparing the banding patterns of clones for a specific primer, polymorphic bands were identified and faint or unclear bands were not considered. Totally 11 bands were found from the mother plant and 10 – 13 bands were in micropropagated plants. Complete homology between the mother plant and any micropropagated plant was not observed. 9.0% and 18.18% of polymorphism frequency was found clone A, C, E and B, D respectively (Table 3). Jaccard's coefficient was performed and similarity matrix index was constructed between the mother and micropropagated plant [10]. Jaccard's similarity matrix index indicates that the scored for their presence '1' or absence '0' of bands for OPK-15 primer (Table 4). The index values were laid between the 1 and 0.53 and clone E (0.93**)** alone has attained high level similarity among the other plants. Clone A, B, C and D considered as molecular off types and therefore, not favored in a selection process aimed at preservation of genetic characteristics of an elite genotype except clone E micropropagated progenies. Thus micropropgated clones clearly shows genetic stability retained in the level of 91% to 82%. The mode of regeneration is known to play a major role on determining the degree of variation.

Table 3. Number of bands generated, polymorphic bands observed and the per cent polymorphism with OPK-15 primer.

S. No	Plant DNA source obtained	Primer	No. of bands generated	No. of polymorphic bands	(%) polymorphism
1	*In vivo* mother plant(control)	OPK-15	11	Nil	Nil
2	*In vitro* clone A	OPK-15	10	1	9.0
3	*In vitro* clone B	OPK-15	13	2	18.18
4	*In vitro* clone C	OPK-15	11	1	9.0
5	*In vitro* clone D	OPK-15	12	1	18.18
6	*In vitro* clone E	OPK-15	13	2	9.0

Table 4. Jaccard's similarity matrix of *Beloperone plumbagnifolia*., between *in vivo* mother plant and five *in vitro* clones.

	Mother Plant	Clone A	Clone B	Clone C	Clone D	Clone E
Mother Plant	1.00					
Clone A	0.53	1.00				
Clone B	0.67	0.73	1.00			
Clone C	0.67	0.87	0.87	1.00		
Clone D	0.67	0.87	0.87	1.00	1.00	
Clone E	0.73	0.80	0.80	0.93	0.93	1.00

CONCLUSION

The aim of work was to find high medium for micropropagation of *Beloperone plumbagnifolia* with native genetic resources. The creation of multiple shoots and roots were greatest than any other report [25] and also survival of the micropropagated plant by hardening was distinct to any other report [23, 26]. The micropropagation or regeneration is the most crucial aspect to retain genetic integrity with respect to mother plants. According to Pervious reports, tissue culture is a mutagenic technique causes cytogenetic, genetic and epigenetic variation collectively referred to as tissue culture- induced or somoclonal variation [11, 16]. In our study we have achieved the whole regeneration of endangered plant as well as the overall genetic stability of *B. plumbaginifolia* was retained with low genetic variation. Tissue culture has proved one of the crucial method in the *ex situ* conservation. Providing appropriate nutrient medium, growth regulators and environmental parameters helped in retaining the genetic viability of the plant. *B. plumbaginifolia* species under a severe degree of threat, tissue culture and RAPD marker analysis approaches may also be an appropriate method for DNA germplasm conservation significant in *ex situ* conservation strategy.

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