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Conservation Using *in vitro* Progenies of the Threatened Ginger-*Boesenbergia pulcherrima* (Wall.) Kuntze

N.P. Anish, M. Dan and M. Bejoy
Plant Genetic Resource Division, Tropical Botanic Garden and Research Institute,
Palode, Thiruvananthapuram, 695562 Kerala, India

Abstract: An *in vitro* method was developed for the rapid multiplication of *Boesenbergia pulcherrima* (Wall.) Kuntze, a threatened species of Zingiberaceae. Shoot tip explants developed from rhizome were transferred to different multiplication media containing various concentrations and combinations of BAP, KN and IAA. Though MS medium supplemented with single cytokinin simulated shoot development, showed poor multiplication. Shoot tip explants achieved better response in BAP than in KN. Addition of KN (0.5 mg L^{-1}) to BAP (1.0 mg L^{-1}) containing medium promoted the rate of multiplication and obtained average 5.8 shoots in 5 weeks. Since rooting was also observed along with caulogenesis, the plantlets were straight away transferred to greenhouse conditions. They were successfully established in the greenhouse condition with 85% survival. Hardened plantlets planted in the trial plot exhibited normal regeneration in the subsequent year and developed bulbils. In the following year, 65% of the tissue cultured plants exhibited regeneration. The vegetative and floral characters of the field grown plants were similar to their wild parents. Germination of bulbils produced from tissue cultured plants further confirm the suitability of *in vitro* plantlets for conservation purpose. The system suggests a feasible method for replenishing the wild population as the *in vitro* progenies accomplished their life cycle successfully in the field.

Key words: *Boesenbergia*, conservation, micropropagation, regeneration, Zingiberaceae

INTRODUCTION

The genus *Boesenbergia* (Zingiberaceae) comprises of about 60 species, distributed throughout the Indo-Malayan regions (Kress *et al*., 2002). In India, it is represented by five species (Jain and Prakash, 1995). *B. pulcherrima* (Wall.) Kuntze is a small delicate herb of about 15 cm, with much condensed, small rhizome and 4-6 elliptic-ovate leaves. Its distribution in India is in the Southern Western Ghats, reported only from two states, Karnataka and Kerala. The species has been identified as threatened (Nayar, 1997; Rao *et al*., 2003) and included in the IUCN Red List of Threatened Plants (Walter and Gillett, 1998). The family Zingiberaceae is well known for many economically important plants, but its wild relatives received only less attention and scientific studies on their propagation, biology and ecology are scanty.

In the wild, the populations of this threatened ginger are thin and fragmentary. Natural regeneration through seeds and bulbils seems to be insufficient to improve the populations of *B. pulcherrima*. In addition, human interactions, habitat specificity of preferring wet evergreen forests and lack of vegetative propagation techniques have compounded the problem and paved the way to figure this species under threatened category (Rao *et al*., 2003; Ramachandran, 1999). Thus, for such ill-fated taxa, alternative approach to facilitate conservation is required and *in vitro* techniques offer ways and means for resolving difficulties in the conservation of rare and threatened plants. In order to reduce the risk of extinction, there is a need to work out mass multiplication protocols and conservation approaches.

Propagation through tissue culture constitutes a powerful tool for *ex situ* conservation programmes especially for species with reduced populations or low seed production. This technique facilitates the rapid establishment of a large number of stock plants, putting minimum impact on endangered wild populations. Although there are a few reports on *in vitro* propagation of various gingers (Yasuda *et al*., 1987; Inden *et al*., 1988; Vincent *et al*., 1992; Anand and Hariharan, 1997; Prakash *et al*., 2004; Prathanturarug *et al*., 2005), there has been no report on tissue culture studies in the genus *Boesenbergia*.

In the present study, we developed an effective system for the rapid multiplication of *B. pulcherrima* and elucidated its use for the restoration of this endangered species.

Corresponding Author: M. Bejoy, Plant Genetic Resource Division, Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, 695 562 Kerala, India

MATERIALS AND METHODS

B. pulcherrima was collected from Thenmala forest, Kerala, India and the voucher specimens were deposited in the herbarium of Tropical Botanic Garden and Research Institute, Palode (TBGT No. 54601). Rhizomes along with a part of leaf sheath were washed thoroughly and treated with 2% (v/v) commercial bleach solution (Striliq, Combi Organic chemicals, New Delhi) and 0.2% (v/v) Labolene (Glaxo India, Bombay) for 30-40 min. The surface sterilisation was achieved in 15% (v/v) Striliq for 10-15 min and 0.1% $HgCl_2$ for 6 min followed by four rinses in sterile distilled water. Explants were than trimmed to 1-2 cm size prior to inoculation. MS medium (Murashige and Skoog, 1962) containing 30.0 g L^{-1} (w/v) sucrose, 7.0 g L^{-1} agar and adjusted to pH 5.7 served as the basal medium for the study. Various concentrations and combinations of BAP, KN and IAA were tested for their effectiveness on *in vitro* morphogenic responses (Table 1). Shoots obtained from initial cultures were isolated and used as the explant for all further experiments. All cultures were maintained at 24±2°C and a photoperiod of 16/8 h provided by cool white fluorescent tubes.

Deflasking and *in vivo* establishment: Plantlets showing well developed roots and shoots were washed carefully in running water to remove traces of agar. The shoot clumps were immersed in 3% commercial fungicide containing mancozeb 75% WP (Indofil M-45) for 5 min and planted in earthen pots containing river sand. They were maintained in a greenhouse under semi-shade (50%) and high humid (RH 75-85%) conditions for hardening. After 25 days of hardening individual plantlets were isolated and re-potted in small poly bags containing top soil, leaf mould and river sand (3:2:1) for further establishment. About 50 well established plants were transferred to an experimental plot in a demarked forest patch of TBGRI and observed the regeneration during the following year.

Statistical analysis: Each treatment contained a minimum of 6 replicates and the experiments were conducted in a completely random design. All data were taken after 5 weeks incubation, analyzed by ANOVA ($p \leq 0.05$) and the means were compared using Least Significant Difference (LSD) test.

RESULTS

Culture initiation and effect of plant growth regulators: The *B. pulcherrima* rhizome explants collected from the wild were heavily contaminated and standard surface sterilisation procedures could not eliminate infection substantially, that 35% of the explants were still contaminated. Further standardisation of the procedures

Table 1: Effect of plant growth regulators on *in vitro* multiplication of *B. pulcherrima* shoot tip explants

Treatments (mg L^{-1})*			Mean No. of	Average shoot length	Mean No. of
BAP	KN	IAA	shoots** ±SE	(mm)** ±SE	roots **±SE
0.5			3.0±0.3	29.7±4.8	11.2±1.5
1.0			3.5±0.6	21.2±2.5	9.8±1.9
2.0			2.8±0.3	32.4±1.7	7.2±1.7
	0.1		2.2±0.2	31.2±1.7	8.5±0.6
	0.5		2.3±0.2	45.1±3.7	9.7±0.9
	1.0		2.8±0.3	33.7±1.8	5.8±0.5
	2.0		2.0±0.5	35.0±3.9	5.8±0.7
	3.0		2.0±0.3	33.9±1.7	4.0±0.9
0.5	0.1		2.5±0.2	42.7±5.1	15.8±1.9
0.5	0.5		3.2±0.3	38.2±3.9	10.3±1.2
0.5	1.0		2.8±0.3	33.4±2.6	10.8±0.8
1.0	0.1		3.8±0.3	39.0±3.7	18.5±2.9
1.0	0.5		5.8±0.9	25.9±4.0	16.3±2.4
1.0	1.0		3.7±0.3	25.7±3.4	12.7±1.2
2.0	0.1		3.3±0.4	26.9±2.2	8.7±0.6
2.0	0.5		3.5±0.6	23.6±2.3	11.3±1.8
2.0	1.0		3.0±0.4	19.3±1.6	8.2±0.8
1.0		0.1	3.2±0.4	25.0±2.4	10.3±1.2
1.0		0.5	3.2±0.4	32.6±2.5	19.8±1.2
1.0		1.0	3.0±0.7	37.5±5.7	13.5±1.0
2.0		0.1	3.5±0.3	26.2±4.4	7.7±1.1
2.0		0.5	4.0±0.5	23.4±3.0	13.7±1.2
2.0		1.0	3.8±0.7	25.8±3.5	10.7±1.5
	LSD		4.2	28.6	4.0

*: Basal medium: MS+30.0 g L^{-1} sucrose + 7.0 g L^{-1} agar and pH 5.7; **: All data after 5 weeks of culture. LSD test-$p<0.05$

to three-step explant sterilisation followed in the study reduced the contamination rate significantly and 80% of the explants survived. The explants were initially treated with BAP or KN (0.5-3.0 mg L^{-1}) and 60% of the rhizome explants responded in BAP (1.0-2.0 mg L^{-1}) supplemented medium with the sprouting of 1-3 shoots in 6-7 weeks of incubation (data not presented). The shoots appeared to be developing directly from dormant buds present on the explants. Such shoots obtained from the primary explants were utilised for all further studies to avoid over collection of this threatened species from the wild.

Organogenic responses were observed in 5-10 days from *in vitro* shoot explants that were inoculated on MS basal medium supplemented with different concentrations and combinations of Plant Growth Regulators (PGRs). Early responses were visible with the differentiation of buds as small protuberance from the basal part. After 2-3 weeks of inoculation, explants on treatments showing higher response tended to have more buds. The number of shoot buds differentiated from shoot explants differed significantly when treated with specific PGR (Table 1). Though shoot multiplication was observed in single treatments of cytokinins, the rate of multiplication was poor. Among them, BAP (1.0 mg L^{-1}) offered better provision to develop an average of 3.5 shoots per explant. Beneficial effect of KN when treated along with BAP attributed to the improvement of multiplication in the present study. The rate of multiplication increased to 66% and an average of 5.8 shoots could be achieved when a combination of BAP (1.0 mg L^{-1}) and N (0.5 mg L^{-1}) was employed (Fig. 1). The application of BAP at 1.0 mg L^{-1} seems to be suitable for optimal multiplication, as concentrations above and below this level reduced shoot production. The buds initially developed were elongated into leafy shoots with simultaneous development of new buds from the base. Treatments to further the multiplication response with cytokinins supplemented with an auxin did not yield better performance. The presence of IAA (0.5 mg L^{-1}) along with BAP (2.0 mg L^{-1}) could support the production of only 4 shoots per explant, other combinations exhibited less stimulation in shoot multiplication. Buds differentiated at the base grew to an average size of 20-50 mm shoots with 1-3 leaves, which could be isolated for further multiplication. Growth of the *in vitro* shoots depended on the presence of PGR in the nutrient medium. Even though, the shoot elongation was invariably fair in all the treatments, maximum growth of 45 mm was observed in 0.5 mg L^{-1} KN and concentrations above this level adversely affected the growth response of newly formed shoots (Table 1).

Rhizogenesis was readily observed within 3 weeks in all treatments (Fig. 2). In 98% cultures, roots developed

Fig. 1: Induction of multiple shoots from the shoot tip explants cultured on MS + 30.0 g L^{-1} sucrose + 7.0 g L^{-1} agar + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} KN after 25 days

Fig. 2: Culture showing simultaneous root development after 40 days

simultaneously with caulogenesis. However, different PGR treatments affected the rhizogenic potential of *B. pulcherrima*. A combination of BAP and IAA produced most number of roots (19.8) followed by BAP and KN (Table 1). IAA at 0.5 mg L^{-1} seems to be optimum for rooting when treated with BAP. The cultures showed better rooting at lower concentrations of cytokinins, while their increase was inversely proportional to rooting responses (Table 1). Mean of roots recorded a reduction from 11.2 to 7.2 when BAP concentration was increased from 0.5 to 2 mg L^{-1}. Similar response was observed from cultures grown in KN.

Greenhouse transfer and field establishment: After 35-40 days in culture, the actively growing plantlets with profuse root system were transferred to greenhouse with 80-95% RH and recorded 85% survival in

Fig. 3: Germinating bulbils produced from tissue cultured plants grown in field conditions

2-3 weeks. Plantlets, after 4-5 weeks of establishment, were transferred to poly bags for further development. After 2-3 weeks these acclimatised plants grew well in poly-bags and showed uniform growth. They developed 2-3 leaves and bloomed in 60-80 days of *in vivo* growth. Some of the well established plants were transferred to a test plot in TBGRI campus, similar to their natural habitat, during the post monsoon (July) season in 2005. Though slow growth was observed initially, the plants picked up normal growth in 4-5 weeks. During field establishment 30% of the plantlets perished while the rest attained normal development and produced bulbils before entering into the natural dormant period. In the following year, 65% of the tissue cultured plants exhibited regeneration. Apart from normal regeneration of plants, small saplings from the bulbils were also noticed during the monsoon season from the study plot as well as from the pots maintained in the greenhouse (Fig. 3). The regenerants did not show any detectable variation in phenotypic characters to their wild parents.

DISCUSSION

The morphogenic capabilities of shoot cultures of the threatened ginger, *B. pulcherrima* were investigated for the first time. Shoot bud raised from rhizome explants behaved as the tissue of preference unveiling the importance of resident meristem and its possible application on *in vitro* multiplication. In the present study, the explants were found tolerant to the broad PGR regime tested indicating the high regenerative potential of the explant. However, the rate of bud induction depended on exogenous supply of PGR. Among the cytokinins, the efficacy of BAP on clonal multiplication of gingers has been reported by Balachandran *et al.* (1990), Hosoki and Sagawa (1977) and Chang and Criley (1993). *B. pulcherrima* also showed the superior effect of BAP over KN, when treated singly. Combination of the two cytokinins (1.0 mg L^{-1} BAP + 0.5 mg L^{-1} KN) was found more effective for the better shoot induction from the shoot explants. Efficiency of multiplication has been greatly improved by the combined use of phytohormones. This type of synergism and quantitative interaction of two or more growth regulators are of common occurrence in tissue culture (Minocha, 1987). The effectiveness of two cytokinins rather than singly or in combination with auxin were also reported in gingers like *Kaempferia galanga* and *Curcuma longa* (Vincent *et al.*, 1992; Shirgurkar *et al.*, 2001). When an auxin (IAA) was supplemented, higher level of BAP was found to be required for improving shoot differentiation. It appears that the excised tissue has sufficient endogenous auxins to balance with the exogenous cytokinin and the addition of auxins forced to increase the cytokinin requirement to attain critical auxin-cytokinin equilibrium. Whereas, IAA supplemented cytokinin medium has been found suitable in other members of Zingiberaceae such as *Curcuma aromatica* (Nayak, 2000) and *Alpinia calcarata* (Agretious *et al.*, 1996).

Though, best rooting was observed in a combination of BAP with IAA, single or combined use of cytokinins did not inhibit rhizogenesis in *B. pulcherrima*. Root initiation along with caulogenesis both in presence of cytokinins and auxins observed in the present study indicates the inherent root inducing tendency of explants of rhizome origin rather than the influence of hormones applied externally. Such response has been reported in other species also (Bejoy *et al.*, 2006), whereas 35% of shoot tip cultures of *Zingiber cassumanar* rooted readily and further rooting was achieved on basal medium conditioned with 10 g L^{-1} activated charcoal (Poonsapaya and Kraisintu, 1993).

Plantlets developed *in vitro* could be hardened in the greenhouse in one month suggesting the suitability of the *in vitro* protocol to restore the plantlets in heterotrophic condition. Perhaps, this may be due to the presence of small rhizome at the base of the plantlet, which helps to support the root system. Hardened plants of *B. pulcherrima* recorded 70% survival in the field, which is a good indication for the restoration of the species. *In vitro* techniques have been accepted as an alternative tool for multiplication and reintroduction of rare and endangered species (Fay, 1992). Successful reintroduction of micropropagated plants has been reported in a few species such as *Allium wallichii* (Wawrosch *et al.*, 2001), *Bletia urbana* (Rubluo *et al.*, 1989), *Paphiopedilum rothchildianum* (Grell *et al.*, 1988)

and *Decalepis arayalpathra* (Gangaprasad *et al*., 2005). This has demonstrated the human assisted support system for the effective restoration of rare and endangered species on a local scale (Bramwel, 1991). The production of bulbils before natural dormant phase from both greenhouse and field grown plantlets revealed the potential of tissue culture progenies for normal growth and development and germination of bulbils into saplings suggest the capability of *in vitro* regrenerants of *B. pulcherrima* for natural regeneration.

The present study suggests a feasible method for the restoration of threatened species through tissue culture mediated restocking. The micropropagated plants proved to be suitable for replenishing the wild population as they accomplish the life cycle successfully in the natural habitat. Therefore, the non-conventional cloning technique, without disturbing the wild germplasm, reported here suggests a suitable programme for the conservation of this threatened species.

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