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Rapid *in vitro* Clonal Propagation of *Mantisia spathulata* Schult, A Rare and Endemic Plant of Northeastern India for Recovery

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Abstract: *Mantisia spathulata* (Zingiberaceae) is a rare and endangered, endemic plant of North-East India. The plant has been rediscovered from Mizoram, India after two decades. Successful recovery of the plant has been achieved through an efficient true-to-type micropropagation protocol. Various concentrations of 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) were tried for *in vitro* plantlet production from rhizomatous shoot explants. Optimum shoot and root multiplication was obtained in 8 weeks using rhizomatous shoot explants cultured on Murashige and Skoog medium supplemented with 11.1 μM BAP and 2.7 μM NAA. The *in vitro* plantlets were successfully weaned and transferred to soil with about 90-93% survival rate. So far, more than 1000 plants have been produced successfully and reintroduced into nature for the recovery of this species.

Key words: Conservation, *ex situ*, *in vitro*, *Mantisia spathulata*, rhizome, Zingiberaceae

INTRODUCTION

The genus *Mantisia* (Zingiberaceae) comprising only four species viz. *M. spathulata* Schult, *M. radicalis* Dam and Dam, *M. wardii* Smith and *M. wengeri* Fischer is endemic to the Hilly areas of the Northeastern parts of India and Myanmar (Dam *et al*., 1997). Due to excessive deforestation, human settlements, burning of forest and shifting cultivation practices, *M. spathulata* has become very rare in the last several years. The rarity of this plant has reached to such a level that only countable few representatives are available. The plant is listed in the Red Data sheet of Indian plants as being rare and endangered species by Botanical Survey of India (www.envfor.nic.in/bsi/research.html) and is found only in the rocky hills of Lunglei, Mizoram, at 1200-1500 m above the sea level (Dam *et al*., 1997). Therefore, immediate steps are needed to propagate and conserve this plant species, which is possible through micropropagation from limited plant material available for experimentation. Recent study has also suggested that the Zingibers, *Globba* and *Mantisia* are phylogenetically related and for understanding this complex group of plants increased field collections of *Globba* and its relatives are critical as more new species will certainly be discovered (Williams *et al*., 2004). Some rare and endangered plants of Northeast India such as *Coptis teeta* (Tandon and Rathore, 1992), *Nepenthes khasiana* (Rathore *et al*., 1991) and *Lilium mackliniae* Sealy (Mao *et al*., 2002) have been conserved successfully through micropropagation in the last several years. A survey of literature shows that the micropropagation has been achieved in a number of plants belonging to the family Zingiberaceae (Hosoki and Sagawa, 1977; Borthakur *et al*., 1998; Shirin *et al*., 2000; Prathanturarug *et al*., 2004). Most of the Zingiberaceae plants are perennial and flower for a short period in their natural habitats during monsoon. The vegetative parts of rare Zingiberous plants are similar and therefore proper identification and collection can be done only during their blooming period. The floral shoot of *Mantisia spathulata* emerges before the onset of the vegetative shoots and results in the formation of floral buds. Flowers are highly ornamental with white-purple petals, yellow-lips and resemble a dancing girl that lasts for only 2-3 days producing immature reproductive capsules. The rhizome has a dormancy period and sprouts during the monsoon and therefore vegetative propagation of the plant is limited in natural conditions. Present report deals with the resurvey and collection of *Mantisia spathulata* Schult from the natural habitats after two decades and its recovery through *in vitro* clonal propagation.

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MATERIALS AND METHODS

Collection and initiation of aseptic cultures: A resurvey for finding the natural habitats of *M. spathulata* was conducted after a gap of two decades during the month of May at Lunglei, Mizoram, India. Two plants were collected from the natural habitat and maintained at the glass-house of North Eastern Hill University, Shillong, India. Newly sprouted rhizomatous shoots (20-30 mm) were thoroughly washed in running tap water for 60 min along with few drops of surface-disinfectant, Tween-20 (Merck, India). Finally, these were surface-sterilized with 0.2% cetramide (Hi-media, India) for 8 min followed by 0.1% mercuric chloride solution for 5-6 min. The explants were rinsed several times with sterile pure water to remove all the traces of mercuric chloride. Under aseptic conditions the leaf sheathes were removed from the shoots and an explant of size 5×10 mm was dissected by a transverse cut at the nodal point of rhizome. A single explant was inoculated in 20×150 mm test tube containing 15 mL of sterilized MS (Murashige and Skoog, 1962) initiation medium supplemented with BAP (2.2-22.2 μM) and NAA (0.5 μM) containing 0.8% (w/v) extra pure agar (Hi-media, India). MS medium with 3% (w/v) sucrose without growth regulator was used as a control. The pH of the medium was adjusted to 5.8, prior to autoclaving for 15 min at 1.06 kg cm^{-2} (121°C). The cultures were incubated at a temperature of 25±2°C under 12 h daily illuminations with white fluorescent light of 50 $\mu moles^{-2}$ sec^{-1} intensity. The *in vitro* shoots obtained in the induction medium were used for further multiplication.

Multiplication of *in vitro* shoot and root: The meristematic rhizomatous shoots (10-15 mm) obtained in the induction medium were cut into 2×5 mm sizes and inoculated in MS medium supplemented with a range of growth regulators, viz., BAP (0.0, 2.2, 11.1, 22.2, 44.4 μM) and NAA (0.0, 2.7, 13.4, 26.9, 53.7 μM) both singly and in combinations. Culture conditions and media used were similar to the induction medium. Observations were made on the shoot and root number per explant after 7-8 weeks of culture.

Statistical calculations: All experiments were repeated thrice with ten replicates per treatment. The data were analyzed using one-way analysis of variance (ANOVA) and comparison between the mean values of treatments were made by the Least Significant Difference (LSD) test. Multiplication ratio of the plantlets was calculated following the method of (Nadgauda *et al.*, 1978).

Weaning and transplantation: Eight weeks old plantlets were removed from the culture bottles, washed and transferred to plastic pots containing a mixture of soil, sand and humus in the ratio of 1: 0.5: 0.5. The plantlets were acclimatized inside the glass-house at 25±2°C at 80% RH initially for a week and then at 30±2°C under 60% RH for the next week. Established plants were then grown under natural conditions after two weeks. The weaned plantlets were then reintroduced in the natural habitat for recovery.

RESULTS AND DISCUSSION

Collection and establishment of aseptic cultures: After a gap of three years, plants of *Mantisia spathulata* flowered in the natural habitat (Fig. 1A and B) of Lunglawn at an altitude of 1183 m, longitude 92° 45' 30, Latitude 23° 52'30, Lunglei District, Mizoram (Fig. 1C). Thus, *Mantisia spathulata* could be successfully identified among other Zingiberous plants and collected from their natural habitats. A single flower remained for 2-3 days with the formation of reproductive capsules within a week that burst off before maturity. The dispersed immature seed does not germinate in the natural habitat due to infestation and fungal contamination. The plant was found growing in the rocky hills on weathered clayey soil containing slit that undergoes frequent landslides during monsoon. Further loss in the natural habitat would completely wipe out the remaining plants in the particular habitat. Thus, we found that loss of habitat due to landslides and lacks of seed germination in nature were the major factors for the plant becoming critically endangered. Contamination was a major problem in raising the aseptic cultures of *Mantisia spathulata*. Fungal contamination mostly predominated than the bacteria. However, a particular bacterial pathogen repeatedly limited the establishment of *in vitro* aseptic cultures. Using sterilization procedure described earlier, only 20% of the cultures produced contamination-free rooted shoots that were subcultured regularly. After three weeks, 2-3 axillary shoots of 5-10 mm sizes sprouted from the nodal portion of the inoculated rhizomatous shoot explants on MS initiation medium supplemented with 8.9 μM BAP and 0.5 μM NAA (Fig. 2A). No shoot multiplication was observed in the control.

Multiplication of *in vitro* shoots and roots: Of the various concentrations of BAP and NAA tested for the plantlet production, neither of the growth regulators used singly and in combinations at higher concentrations was effective in the production of normal healthy plantlets. Explants treated with higher concentrations of BAP produced few, thick pale green, stunted shoots without roots in 4 weeks. On the other hand, numerous thick and small roots were obtained at higher concentrations of

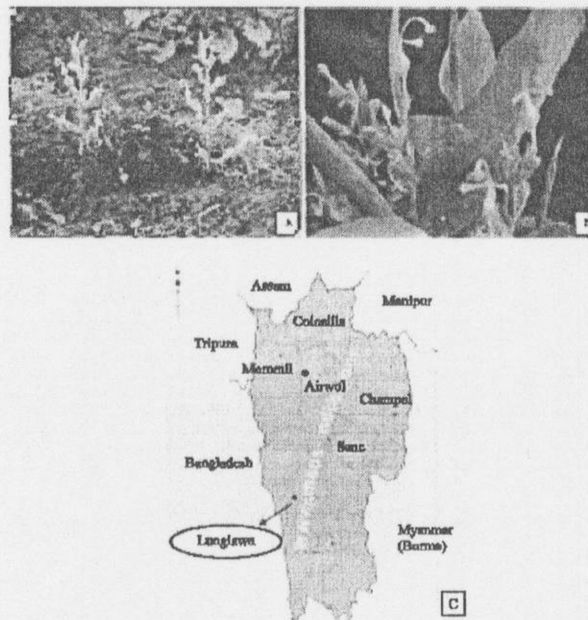

Fig. 1: (A) Flowering of *Mantisia spathulata* in the natural habitats after three years (B) A closed view of *Mantisia spathulata* flowers (C) Distribution of *Mantisia spathulata* in Mizoram

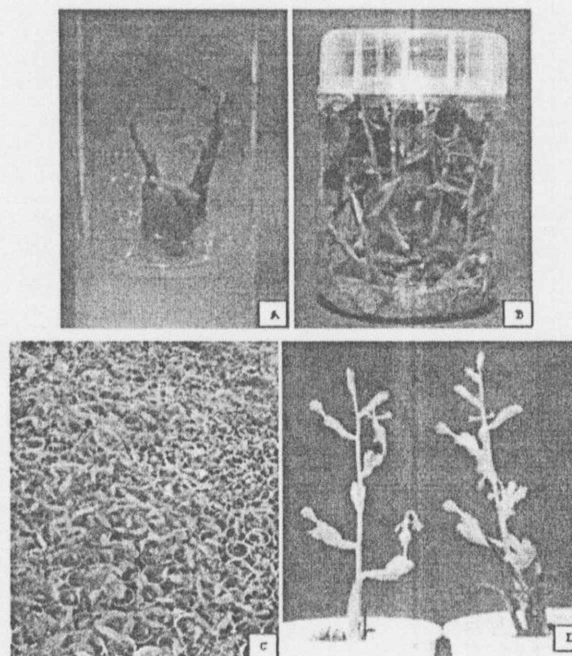

Fig. 2: Micropropagation of *Mantisia spathulata* (A) Shoot initiation on MS medium containing 8.9 μM BAP and 0.5 μM NAA after three weeks (B) Eight weeks old plantlets with multiple shoots and roots on MS medium containing 11.1 μM BAP and 2.7 μM NAA (C) Weaned plants after eight weeks of transfer (D) Flowering of morphologically invariant micropropagated plants under glass house condition after one year

Table 1: Effect of BAP and NAA on multiplication of *in vitro* plantlets of *M. spathulata*

Growth regulators (μM)		Mean No. of	Mean No. of
BAP	NAA	shoots/explant±SE	roots/explant±SE
0.0	0.0	0.4±0.21	1.2±0.33
2.2	2.7	2.4±0.66	6.0±1.16
11.1	2.7	5.2±0.95	8.6±0.82
22.2	2.7	4.2±1.03	6.4±0.66
44.4	2.7	1.4±0.53	4.6±0.72
LSD ($p<0.05$)		2.4	2.6
ANOVA		$F_{4,20}=5.62$*	$F_{4,20}=9.52$*

*Significant at $p<0.05$; ±SE (Standard error)

NAA which turned into callus after 10 weeks. Sprouting of shoots and roots occurred effectively in media containing a low concentration of NAA (2.7 μM) with all other concentrations of BAP (Table 1). Similar results were reported earlier for *Alipinia galanga* (Borthakur *et al.*, 1998) and *Kaempferia galanga* (Shirin *et al.*, 2000). Thus separate rooting medium as reported in other Zingiberous members like *Curcuma longa* Linn (Nadgauda *et al.*, 1978) and *Zingiber officinale* Rosc (Sharma and Singh, 1997) was not required. An optimum number of shoots (5.2±0.95) and roots (8.6±0.82) per explant was obtained in media containing 11.1 μM BAP and 2.7 μM of NAA. These are significantly higher ($p<0.05$) compared to those obtained at other concentrations and developed into normal healthy plantlets in 8 weeks. It was observed that 3 weeks old shoot buds (5-10 mm) before producing any roots if subcultured regularly at the same time interval maintains a high multiplication ratio. A single rhizomatous shoot produced 5.0 shoots within 3 weeks. Successive multiplication produced 9 shoots in the second subculture, 11 in the third and 8 in the fourth and fifth subculture, respectively. Thus, a single rhizomatous bud subcultured regularly at 3 weeks intervals can produce about 220 plantlets in 18 weeks which enables the rapid multiplication of *Mantisia spathulata*.

Weaning and transplantation: Plantlets of 100-150 mm size shoots were obtained within a short period of 8 weeks (Fig. 2B) without subculture; hence, expense for producing the plantlets was less. An initial weaning period of one week at 25±2°C with 80% RH followed by another week at 30±2°C at 60% RH in glass house was found to be significant for the higher survivability of the plantlets. About 90-93% of the plantlets were successfully weaned within 8 weeks (Fig. 2C). The weaned plantlets when transferred to bigger pots could grow as fast as the *ex situ* plants within 4 weeks. One year old weaned plants flowered in the glass house without any morphological variation (Fig. 2D). So far, more than one thousand morphologically invariant micropropagated plants have been developed and successfully reintroduced in their natural habitat. To the best of authors' knowledge, this is the first report, which describes the resurvey, collection and successful recovery of rare and endangered *M. spathulata* through our micropropagation protocol.

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