J. Orchid Soc. India, 32: 25-32, 2018 *ISSN 0971-5371*

RESTORATION OF *SMITHSONIA MACULATA* (DALZ.) SALDANHA, AN ENDEMIC AND VULNERABLE ORCHID OF WESTERN GHATS THROUGH *IN VITRO* PROPAGATION

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Abstract

Smithsonia maculata, an epiphytic and endemic orchid of Western Ghats region is vulnerable due to its poor fruit set and seedling establishment in the natural habitats, hence there is an urgent need for its micropropagation, and eco-restoration. Eight month old capsules showed 50% viable seeds and about 70% of these seeds germinated on Mitra *et al.* (1976, M) medium supplemented with organic additives. Coconut water (20%) as an additive supported vigorous growth of protocorms with deep green pigmentation and upon their sub-culture (90 days old) on M medium supplemented with coconut water (20%) or casein hydrolysate (0.05%), these protocorms developed into seedlings complete with roots and leaves in 2-3 months. Partially expanded leaves with meristematic base, separated from aseptically grown seedlings were also cultured on M medium fortified with 10 mgl^{-1} BAP and 1 mgl^{-1} IAA; this nutrient combination induced an average of 11.25 shoots/leaf in 6-9 months. Woody plant medium (Lloyed and McCown, 1980; WPM) enriched with 5% banana pulp induced early rooting of shoots in 2-3 months. The rooted plantlets and seedlings showed 90% and 63% establishment respectively in community pots and on tree trunks, in the garden site. Plants reinforced at Karamana river of Peppara Wildlife Sanctuary showed 48% survival after one year. The pilot trial on restoration through micropropagation is useful for further reintroduction and population enhancement for practical conservation of *Smithsonia maculata.*

Introduction

SMITHSONIA MACULATA is an epiphytic orchid species which grows on evergreen trees near river banks at an altitudinal range of about 650 m. It is endemic to Western Ghats region of Peninsular India. Its distribution is restricted to narrow pockets in the Western Ghats and was reported from Wayanad in Kerala and Hassan district of Karnataka (Manilal and Kumar, 2004). The genus *Smithsonia* comprises of 3 species and all are endemic to Western Ghats and *S. maculata* is the largest among them (Manilal and Kumar, 2004). Indiscriminate collection coupled with poor fruit set, seed germination and seedling establishment in the natural habitats limits the spread of the species in nature. Conservation Assessment and Management Plan Workshop (CAMP, 2001) placed the species under vulnerable category of orchids. Despite its small flowers (Figs. 1a, b), the species holds potentialities for horticultural exploitation through improvement programme. Thus, multiplication of species through biotechnological tools and planting it in the natural localities to enhance population size is necessary to reduce the prevailing threat. Asymbiotic seed germination of orchid seeds is an efficient *in vitro* propagation method for large-scale propagation of orchids for restoration as demonstrated in *Arundina graminifolia* (Sibin *et al.,* 2014), *Bletia urbana* (Rublo *et al.,* 1989), *Coelogyne cristata* (Kaur *et al.,* 2017), *C. nervosa* (Sibin and Gangaprasad, 2016), *Dendrobium* (Mohanty and Salam, 2017), *Dendrobium amoenum* (Pathak *et al*., 2016), *Dendrobium chrysanthum* (Anuprabha and Pathak, 2012), *Dendrobium aphyllum* and *Rhynchostylis retusa* (Bhattarcharjee and Hossain, 2015), *Gastrochilus calceolaris* (Pathak *et al.,* 2011), *Goodyera biflora* (Pathak *et al.,* 1992), *Ipsea malabarica* (Gangaprasad *et al.,* 1999), *Paphiopedilum rothschildianum* (Grell *et al.,* 1988), and *Paphiopedilum wardii* (Zeng *et al.,* 2012). Clonal propagation is also tried in other species such as *Arundina graminifolia* (Arora *et al*., 2014), *Dendrobium amoenum* (Arora *et al.,* 2016), *Epidendrum ilense* (Dodson, 1981), *Eulophia dabia* (Chauhan *et al.,* 2015), *Vanda coerulea* (Seeni and Latha, 2000) and *Vanda spathulata* (Decruse *et al*., 2003) for restoration purpose. In recent years, symbiotic germination is incorporated as a method for restoration of orchids as proved effective in *Aerides multiflora* (Bhatti *et al.,* 2017), *Dactylorhiza hatagirea* (Aggarwal *et al.,* 2010), *Dendrobium nobile* (Anuprabha *et al*., 2017), *Paphiopedilum spicerianum* (Borah *et al*., 2015), *Rhynchostylis gigantea* (Pathak *et al*., 2017) *Saccolabium papillosum* (Kaur and Pathak, 2015), and *Vanda coerulea* (Aggarwal *et al.,* 2012). In the present study, an attempt was made to propagate *S. maculata* through asymbiotic seed germination and further multiply it by regeneration using foliar meristem procured from aseptically grown seedlings. Seedlings obtained were reinforced into the natural locality, in the close proximity of naturally growing plants and these were monitored up to the flowering stage.

Received: March 4, 2018; Accepted: May 8, 2018

Materials and Methods

Stock Plants

One mother plant bearing 2 capsules (5 months old) collected from a natural population growing on *Garcenia gummi-gutta* at Karamana river basin near Bonaccord of Trivandrum District (8° 31' 26.90" N; 76° 56' 11.89" E), Kerala was brought to garden site and maintained in the field gene bank and subsequently, the capsules (3 cm long, 0.95 cm wide with 50% viable seeds) were harvested after 3 months.

Surface Sterilization

Immature seeds harvested from the capsules were used to raise seedlings. The capsules were washed thoroughly in running tap water using labolene, dipped in alcohol and flamed for 2-3 sec. The surface disinfected capsules were placed in sterile petriplates, split open and the seeds from each capsule were then transferred to 10 ml of sterile distilled water and subsequently, 2 ml seed suspension was transferred to liquid culture medium.

Culture Initiation

Seeds were transferred to liquid culture medium (Mitra *et al.,* 1976; M) supplemented with 20% (v/v) coconut water (CW) or 0.05% of peptone (P), or yeast extract (YE) or casein hydrolysate (CH) dispensed in 250 ml conical flasks. The cultures were maintained in a culture room at 25±2°C under an illumination of 30-50 $\mu M^{-2}s^{-1}$ and 12 h photoperiod. The cultures were swirled manually, once in a day to avoid clump formation. The protocorms developed after 90 days were transferred to agar-gelled M medium for their further growth and seedling development. The seedlings obtained after 3-4 months were transferred to Woody Plant Medium (Lloyed and McCown, 1980; WPM) supplemented with 5% (w/v) banana pulp with a view to enhance the seedling growth.

Multiplication

Continuous supply of *in vitro* plants was ensured through micropropagation using leaf segments procured from aseptically grown seedlings. Partially expanded leaves with meristematic base were vertically implanted with the base buried (3-5 mm) into agar-gelled M medium supplemented with BAP (5 and 10 mgl^{-1}) and IAA (0.2-5.0 mgl^{-1}) in various combinations.

In Vitro Root Initiation

Shoots raised in multiplication media were devoid of roots. After 5-6 months of initial inoculation, the shoots obtained were transferred to WPM containing 5% banana pulp for rooting.

Hardening and Field Establishment

Seedlings/plantlets complete with 3 to many leaves and 2 or more roots were deflasked and washed thoroughly in running tap water so as to remove any trace of nutrient medium. Washed plants were treated with 1% Indofil M-45 for 1h followed by washing in water and planted in 3 inches community pots with potting medium comprising charcoal and tile pieces (1:1). The potted plants were maintained in a rat proof net house and watered (sprinkling only) twice a day.

Reintroduction

Established seedlings after one year of transfer into community pots were wrapped in a wet paper and transported to the natural localities and tied directly onto trunks of trees which are natural hosts of *S. maculata* and a few associated species in the same habitat. The host trees include *Garcinia gummi-gutta*, *Kunstleria* spp., *Myristica malabarica,* and *Syzygium* spp.

Results

Presently, *Smithsonia maculata* seeds procured from 8 month old capsules successfully responded to *in vitro* asymbiotic germination. These observations are in line with earlier studies made in some orchid species including *Arundina graminifolia* (Sibin *et al.,* 2014), *Coelogyne cristata* (Kaur *et al.,* 2017), *Dendrobium amoenum* (Pathak *et al.*, 2016), *Dendrobium aphyllum* and *Rhynchostylis retusa* (Bhattarcharjee and Hossain, 2015), *Gastrochilus calceolaris* (Pathak *et al.,* 2011), *Goodyera biflora* (Pathak *et al.,* 1992), *Paphiopedilum rothschildianum* (Grell *et al.,* 1988), and *Paphiopedilum wardii* (Zeng *et al.,* 2012). Micropropagation using leaves from aseptically grown seedlings facilitates re-culture and thus ensures continuous supply of plants for restoration without disturbing natural populations for explants. Fifty per cent seeds were found to be viable and about 70% of these germinated on M medium supplemented with organic additives (20% coconut water, 0.05% either of peptone, casein hydrolysate and yeast extract); the seeds, however, did not show appreciable difference in germination percentage (Table 1). Coconut water (20%) supported vigorous growth of protocorms with deep green pigmentation as compared to other additives. Protocorms on CW supplemented medium attained an average diameter of 1.4 mm, while in presence of other additives, they attained 0.8-0.9 mm size only. In the basal M medium, protocorm growth was, however, very poor. Protocorms developed after

Table 1. Effect of organic additives in M medium on protocorm growth during seed germination in *S. maculata.*

Additives	Response of protocorms after 90 days	
	Pigmentation	Diameter (mm) (Mean ± SD, n=2)
0.05% CH	Pale green	0.910+0.290[b]
0.05% P	Pale green	0.82510.075[b]
0.05% YE	Pale green	0.910+0.020[b]
20% CW	Green	1 .400±0.090[a]
Nil	Pale green	0.370±0.120[c]

Means followed by the same letter do not differ significantly at 5% level based on Duncan's multiple range test.

90-days of culture in CW supplemented medium upon transfer to agar-gelled M medium supplemented with CW or CH developed into seedlings complete with roots and leaves in 2-3 months. Subsequent sub-culture of such seedlings in Woody Plant Medium (WPM) supplemented with 5% banana pulp supported vigorous growth and after 12 months, the seedlings were ready for transfer to community pots (Fig. 1c).

Continuous supply of *in vitro* plants was ensured through re-culture of seedling-derived leaves. Partially expanded leaves with meristematic base vertically implanted with the base buried (3-5 mm) into agar-gelled M medium supplemented with BAP and IAA showed initial swelling of the meristematic base in 15 days followed by the initiation of Protocorm-like bodies (PLBs) in 30-45 days (Fig. 1d). The protocorm like bodies differentiated leaves (Fig. 1e) in 3 months. Maximum number of shoots (10-11.25) /explant were obtained on the medium supplemented with 5 and 10 mgl^{-1} BAP + 1 mgl^{-1} IAA (Table 2). The culture transferred to fresh medium of the same composition facilitated enlargement of shoots with leaves and thus ready for transfer to rooting medium. Shoots raised in multiplication media even after 5-6 months of initial inoculation were devoid of roots and when transferred to WPM containing 5% banana pulp induced 2-3 healthy roots in 2-3 months period (Fig. 1g).

Table 2. Effect of BAP and IAA on M medium on shoot multiplication from leaf explants of *S. maculata.*

Growth regulators		Mean number of shoots ± SD; n= 2-3
BAP (mgl^{-1})	IAA (mgl^{-1})	
5.0	0.2	6.0±2.53[b]
5.0	0.3	6.0±2.55[b]
5.0	0.5	9.67±3.85[ab]
5.0	1.0	10.0±5.71[a]
5.0	2.0	8.33±2.86[ab]
5.0	3.0	5.0±2.16[c]
10.0	0.3	4.0±1.58[c]
10.0	0.5	5.0±0.82[c]
10.0	1.0	11.25±8.22[a]
10.0	3.0	6.5±1.5[b]
10.0	5.0	2.5±0.5[d]

Each treatment consisted of 3-5 replications. Observations recorded after 3 months of inoculation. Means followed by the same letter do not differ significantly at 5% level based on Duncan's multiple range test.

Table 3. Response of *S. maculata* seedlings reinforced at Karamana river basin of Peppara Wildlife Sanctuary and introduced at JNTBGRI campus.

S.No.	Host Plant	Number of seedlings tied	Survival after one Year	Survival after 16 years
Karamana river basin				
1	*Kunstlaria* sp.	25	5	0
2	*Myristica* sp.	64	35	20
3	Lianas	10	9	0
4	*Syzygium* sp.	26	9	0
5	*Xanthophyllum* sp.	11	2	2
6	*Garcenia* sp.	21	5	5
JNTBGRI campus				
7	*Terminalia paniculata*	30	19	0

Deflasked seedlings or plantlets possessing 3 or more leaves and 2 or more roots planted in community pots and maintained in a net house facilitated more than 90% establishment when observed after four months (Fig. 1f). The deflasked seedlings planted directly on trunks of trees growing in the garden site showed 63% establishment with new leaf and root growth when observed after 6 months (Fig. 2a) but none of them survived beyond 2 years. Seedlings reinforced at Karamana river basin of Peppara Wildlife Sanctuary showed 48% survival when observed after one year of planting (Table 3; Fig. 2b). After 16 years, the reinforced plants were located in 3 trees. The plants were at different growth stages. Out of 27 surviving plants, 4 plants were in flowering (Fig. 2c), 7 attained near flowering stage (Fig. 2d) and others were still at early stages (Fig. 2e).

Fig. 1. a-b. *Smithsonia maculata* during flowering and its inflorescence; c, Seedlings developed in WPM containing 5% banana pulp after one year; d-e, PLBs proliferated from the leaf base after 3 months and 6 months of culture (M + 10 mgl^{-1} BAP + 0.5 mgl^{-1} IAA); f, Seedlings established in a community pot; g, Development of roots in the shoots (WPM + 5% banana pulp).

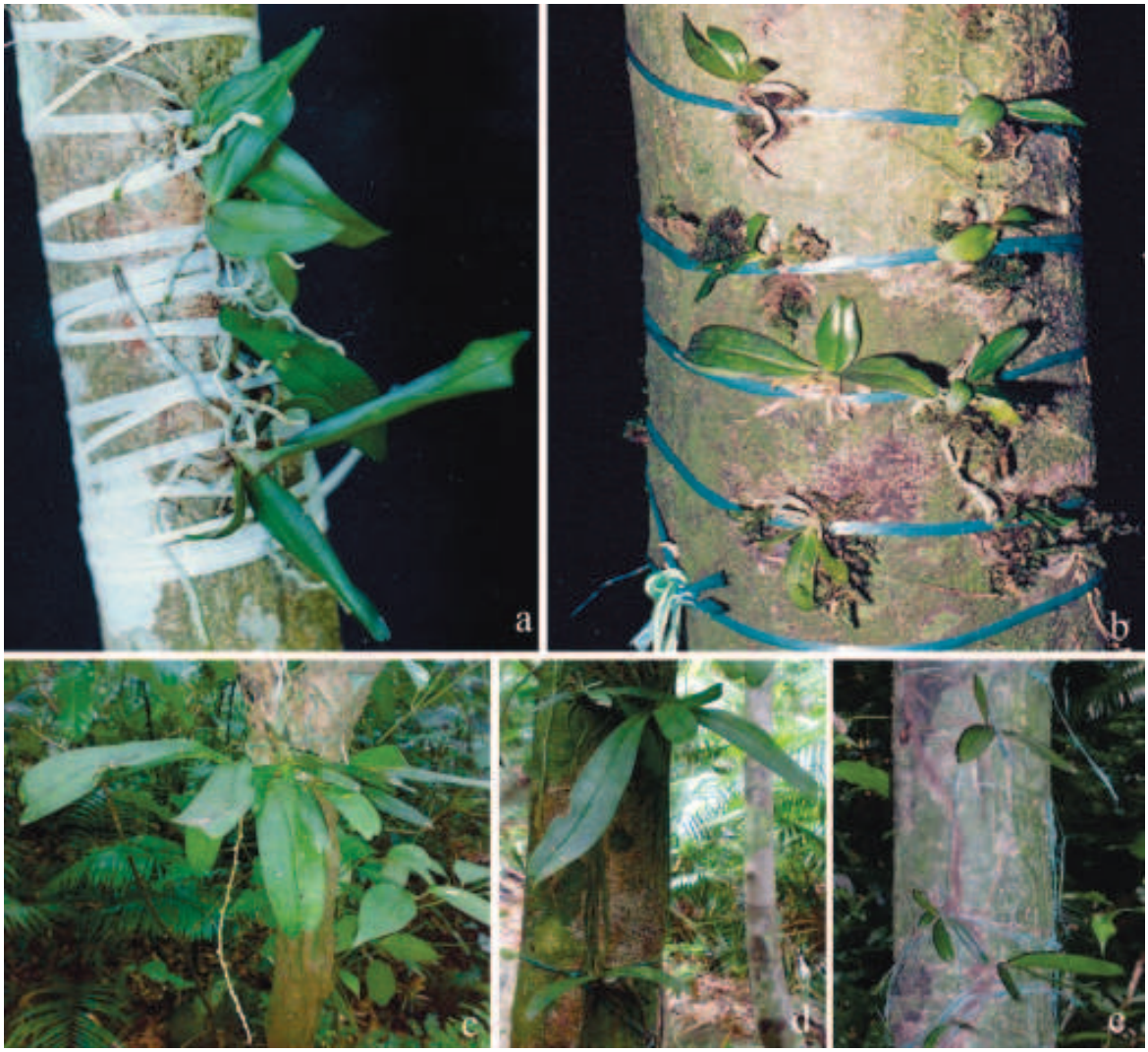

Fig. 2. a-e. Field transfer and reintroduction of *Smithsonia maculata* seedlings: a, Deflasked seedlings tied directly on the tree trunks in the orchid field conservatory showing good establishment after six months; b-e, Seedlings planted at Karamana river basin of Peppara Wildlife Sanctuary after one year (b) and 16 years (c-e) of reinforcement.

Discussion

Asymbiotic germination of seeds is the most common method practiced for the propagation of both epiphytic and terrestrial orchids even though symbiotic germination which has succeeded in some species has been recommended for utilization in restoration program (Aggarwal and Zettler, 2010; Bhatti *et al*., 2017; Stewart and Kane, 2006). Different media such as ¼ MS, ¼ MS, Knudson-C, and M are reported to support seed germination in *Ipsea malabarica, Paphiopedilum* spp., and *Eulophia cullenii* (Decruse *et al.,* 2013; Gangaprasad *et al.,* 1999; Long *et al.,* 2010; Pathak *et al.*, 2001; Zeng *et al.,* 2012). However, protocorm growth promoted by complex additives like peptone has been reported for orchid species like *Calopogon tuberosus* (Kauth *et al.,* 2006), *Epidendrum ibaguense* (Hossain, 2008), and *Spathoglottis plicata* (Curtis, 1947). Organic additives like coconut water, yeast extract and amino acid mixtures are known to enhance growth of protocorms of *Eulophia cullenii* (Decruse *et al.,* 2013). The present study revealed enhanced growth supported by CH, peptone and yeast but significantly higher growth by coconut water. Studies conducted in some terrestrial orchid seeds and protocorms revealed more efficient utilization of amino acids by young protocorms (Curtis, 1947; Malmgren, 1996; Spoerl, 1948). Greater preference for nitrogen from amino acids rather than from ammonium or nitrate salts has also been favored for some epiphytic orchids (Nadarajan *et al.,* 2011). As CH, peptone and yeast extract are mixtures of free amino acids in addition to vitamins and minerals, there is enhanced growth of protocorms, in their presence. The vigorous growth of protocorms in presence of coconut water may be because CW contains amino acids, vitamins, sugar, and plant growth regulators such

as cytokinin (Laurain *et al.,* 1993), as well as various inorganic ions such as phosphorus, magnesium, potassium, and sodium (Raghavan, 1977), which are beneficial for orchid seed germination and protocorm growth.

Multiplication of orchid species is also possible using different types of explants from axenic seedlings if supplemented with plant growth regulators. Protocorms were effective in *Vanda dearei* (Jawan *et al.,* 2010), pseudobulb segments in *Coelogyne stricta* (Basker and Narmatha Bai, 2006), *Dendrobium nobile* (Anuprabha *et al.*, 2017), segments of mini-rhizomes differentiated from protocorms in *Geodorum densiflorum* and *Eulophia cullenii* (Decruse *et al.,* 2013; Sheelavantmath *et al.,* 2000) and shoot tips in *Phaius tankervilliae* (Pant and Shrestha, 2011). Foliar meristem from axenic seedling also proved effective in a few species as *Aerides odorata* (Devi *et al*., 2013); *Cleisostoma racimeferum* (Temjensangba and Deb, 2005); *Doritaenopsis* hybrids (Park *et al.,* 2006), *Rhynchostylis gigantea* (Pathak *et al.*, 2017), and *Vanda coerulea* (Seeni and Latha, 2000). The present study proved that regeneration from basal meristem of leaf is very efficient to get high propagation rate as high as 11.25 shoots/explant. Hence, fast multiplication and continuous supply of plants can be assured using *in vitro* asymbiotic germination method and regeneration using leaf explants, in *S. maculata.*

Restoration efforts have been made in several orchids (Aggarwal *et al.,* 2010*,* 2012; Decruse *et al.*, 2003; Gangaprasad *et al.,* 1999; Grell *et al.,* 1988; Kaur *et al.,* 2017; Seeni and Latha, 2000). However, the present study demonstrated plant establishment and its growth and maintenance and to flowering stage. The difference in growth rate is possibly due to initial colonization of appropriate endomycorrhiza into the seedling roots as those seedlings planted very close to natural plants showed vigorous growth. As the reinforced seedlings were allowed to grow themselves in the natural localities without any external care, and it was observed that 42% were in good growing conditions after 16 years. Declining of natural population is probably due to poor fruit set rather than any other adverse conditions. The present results may be useful for reintroduction of many other orchid species for population enhancement and practical conservation.

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