Asymbiotic seed germination and *in vitro* seedling development of *Paphiopedilum villosum* (Lindl.) Stein, a valuable and vulnerable lady's slipper orchid from India

Orchids of *Paphiopedilum* genera, commonly known as lady's slipper orchids, are popular as potted plants. Nearly 131 species have been reported worldwide, among which 9 are found in the states of North East India. *Paphiopedilum villosum* (Lindl.) is one such species which is listed under vulnerable category in the latest version of the *IUCN Red Data Book*[1]. Its area of occupancy (AOO) is limited to only 56 sq. km in the world and its population trend has reduced significantly in recent decades. The species is protected under Schedule VI of the Wildlife Protection Act of India. Utilization of only cultivated specimens in the trade and long-term community-based conservation to protect the habitat and species has been recommended by IUCN. Development of a simple protocol for its multiplication is highly required to achieve the same. The seeds of this terrestrial species lack endosperm, cotyledon, root initials and do not germinate on their own *ex situ*[2]. *In vitro* propagation of terrestrial orchids is difficult due to their highly specific requirements for seed germination[3], and appropriate combination of auxin and cytokinins was found effective[4–6]. Several efforts have been made worldwide for *in vitro* seed culturing of *P. villosum*[7,8], *Paphiopedilum wardii*[9] and *Paphiopedilum spicerianum*[10]. Here we develop an easy and effective protocol for *in vitro* multiplication of this vulnerable category of orchids. We have used a combination of media and phytohormones in such a way that we can use a single medium which will enhance germination and bypass the process of callus formation and somatic embryogenesis and follow the path of seed germination–protocorm like bodies (PLB) formation–shoot induction/growth and root induction/growth efficiently in a sequential manner. Auxin concentration of 0.50 ppm has been standardized as optimum for all these processes[11]. Supplementation of different concentrations of 6-benzylaminopurine (BAP, @ 0.25, 0.50, 0.75 and 1.00 ppm) in combination with standard concentration of α-naphthaleneacetic acid (NAA; i.e. 0.50 ppm) was done to develop a common medium for the overall process of seed to plantlet development.

Seed setting was obtained by hand pollinating with mixed pollen (pollinia) collected from different accessions. Mature capsules were harvested at 180–200 days after pollination. Microscopic examination (Leica DM 2500) of seeds revealed that the average length and width at the widest part of seeds were 816.97 and 184.45 µm respectively (Figure 1 *c*). The capsules without any crack on their surfaces were utilized for seed culturing. Capsules were washed under running tap water and then soaked in 2.0% (w/v) suspension of carbendazim 50% wettable powder (trade name: Bavistin) for 30 min, followed by rinsing in sterile distilled water. These were further sterilized with 75% (v/v) ethanol for 30 sec and then dipped into 0.1% $HgCl_2$ for 10–15 min followed by thorough rinsing in sterile extra pure water. The capsules were then dipped in 90% ethanol and the surface incinerated using a α Bunsen burner for 4–5 sec. Finally, they were cut into two pieces by sterilized scalpel and the seeds extracted by sterilized spatula for inoculation on the medium. The extracted seeds were placed on the medium amended with sucrose, activated charcoal (AC) and plant growth hormones in 250 ml glass bottles (height 13 cm, circumference 24 cm), which were then sealed with a plastic cap and made airtight with parafilm. Three different full-strength basal culture media, namely MS (Murashige and Skoog medium)[12], G (Gomborg B5 medium)[13] and N (Nitsch medium)[14] were used. Sucrose (20 gl^{-1}), AC (2 gl^{-1}) and α-NAA (mol. wt. 186.21, @ 0.50 mgl^{-1}) were added with all the media. Additionally, BAP with mol. wt. of 225.26 in four doses (i.e. 0.25, 0.50, 0.75 and 1.00 mgl^{-1}) was added to G + NAA, N + NAA and MS + NAA. Thus a total of 12 treatment combinations were used for the experiment (Table 1). The pH of the media was adjusted to 5.8 by adding 1 N KOH or HCl. The media were autoclaved for 20 min at 121°C and slanted media were prepared. Solidification of media was done by adding 10 gl^{-1} agar.

At least 50 seeds were used per bottle, and the treatments were tested in triplicate. Seeds were placed under a 16/8 h (light/dark) photoperiod with light provided by 40-W cool-white fluorescent tube lights at temperature of 25 ± 2°C. No sub-culturing was carried out till PLBs were formed from the seeds. After the PLBs were formed inside the culture bottles, they were divided for sub-culturing in their respective amended medium and were kept undisturbed till the development of whole plants with full grown shoots and roots. No separate media was used for shoot and root induction. Data were recorded for various traits like time for initiation of germination (weeks), time for greening (weeks), time for PLB induction (weeks), time for shoot emergence (weeks), shoot length (cm), number of leaves/shoot, time for root initiation (weeks), number of roots/shoot and root length (cm). Data analysis was carried out by MS-Excel and SAS 9.2 software.

Earliest germination (6.67 ± 2.52 weeks) was recorded in Nitsch medium supplemented with 0.25 ppm BAP and

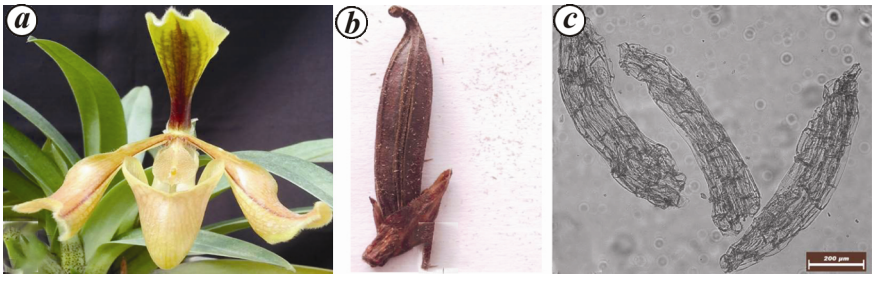

Figure 1. Flower (***a***) and mature capsule (***b***) with dispersed seeds. ***c***, Microscopic image (20X lens) of seeds of *Paphiopedilum villosum* (Lindl.) Stein.

Table 1. Effect of different media and plant growth regulators combinations on *in vitro* seed culture response in *Paphiopedilum villosum* (Lindl.) Stein

	Media traits								
Medium	Germination initiation time (weeks)	Greening time (weeks)	PLB induction time (weeks)	Shoot emergence time (weeks)	Shoot length (cm)	No. of leaves/shoot	Root initiation time (weeks)	No. of roots/ shoot	Root length (cm)
G + BAP (0.25) + NAA (0.50)	10.00 ± 2.00	12.00 ± 2.00	18.00 ± 2.00	24.00 ± 2.00	1.53 ± 0.31	3.00 ± 1.00	30.00 ± 2.00	2.33 ± 0.58	2.08 ± 0.16
G + BAP(0.50) + NAA (0.50)	8.00 ± 2.00	12.33 ± 1.53	19.00 ± 1.00	24.00 ± 2.00	1.48 ± 0.61	3.33 ± 1.53	32.00 ± 2.00	2.67 ± 1.53	1.65 ± 0.18
G + BAP (0.75) + NAA (0.50)	9.00 ± 2.00	12.33 ± 2.52	19.67 ± 1.53	25.67 ± 1.53	2.37 ± 0.85	3.00 ± 1.00	33.00 ± 2.65	1.67 ± 0.58	2.50 ± 0.56
G + BAP (1.00) + NAA (0.50)	9.00 ± 2.65	11.00 ± 2.00	18.33 ± 3.51	29.00 ± 2.00	2.15 ± 0.57	3.00 ± 1.00	36.00 ± 4.00	0.67 ± 0.58	1.83 ± 0.35
N + BAP (0.25) + NAA (0.50)	6.67 ± 2.52	10.33 ± 1.53	14.00 ± 2.00	20.00 ± 2.00	5.37 ± 1.32	5.67 ± 1.53	26.00 ± 2.00	4.33 ± 0.58	5.23 ± 1.00
N + BAP(0.50) + NAA (0.50)	8.67 ± 2.52	11.33 ± 2.52	20.67 ± 3.06	26.67 ± 3.06	2.01 ± 0.71	3.67 ± 1.53	32.33 ± 2.52	2.00 ± 1.00	3.03 ± 0.57
N + BAP (0.75) + NAA (0.50)	11.00 ± 3.00	14.67 ± 3.51	21.00 ± 3.00	27.00 ± 3.00	2.00 ± 0.17	3.33 ± 1.15	33.33 ± 3.06	1.67 ± 0.58	1.97 ± 0.57
N + BAP (1.00) + NAA (0.50)	9.00 ± 3.00	13.00 ± 3.00	20.00 ± 2.00	28.33 ± 2.52	1.61 ± 0.63	2.33 ± 0.58	35.67 ± 2.52	2.67 ± 0.58	1.43 ± 0.25
MS + BAP (0.25) + NAA (0.50)	9.67 ± 2.52	13.00 ± 2.00	22.00 ± 3.61	28.33 ± 3.51	2.53 ± 0.98	3.33 ± 1.53	34.00 ± 3.61	1.67 ± 1.15	1.83 ± 0.59
MS + BAP(0.50) + NAA (0.50)	11.67 ± 2.52	16.33 ± 3.06	22.00 ± 3.00	28.67 ± 2.08	2.70 ± 1.28	3.00 ± 1.00	35.33 ± 1.53	2.33 ± 0.58	2.63 ± 0.75
MS + BAP (0.75) + NAA (0.50)	8.33 ± 2.52	11.67 ± 2.08	18.67 ± 1.53	27.67 ± 3.79	2.05 ± 0.69	3.33 ± 1.53	33.33 ± 2.52	1.33 ± 0.58	2.70 ± 1.37
MS + BAP (1.00) + NAA (0.50)	9.33 ± 2.52	13.33 ± 4.16	21.00 ± 2.65	27.00 ± 2.65	2.90 ± 0.39	3.67 ± 1.53	32.33 ± 2.52	2.33 ± 0.58	1.87 ± 0.40
Variance among treatment means	1.75	2.70	4.86	6.80	1.08	0.64	7.46	0.82	1.03
P-Value in single factor ANOVA	0.60	0.34	0.05	0.01	0.00	0.35	0.01	0.00	0.00
LSD (0.05)	NA	NA	4.28	4.38	1.32	NA	4.48	1.34	1.11
LSD (0.01)	NA	NA	NA	5.93	1.78	NA	6.08	1.81	1.50

NA, Not applicable as null hypothesis is not rejected at that particular significance level; G, Gamborg; N, Nitsch; MS, Murashige and Skoog; NAA, α-Naphthaleneacetic acid; BAP, 6-Benzyl amino purine; LSD, Latin square design.

Figure 2. Micro-propagation through *in vitro* seed germination of *P. villosum* (Lindl.) Stein. ***a***, Inoculation of mature seed on Nistch medium supplemented with 0.50 mg/l NAA and 0.25 mg/l BAP; ***b***, Initiation of germination after inoculation; ***c***, Development of shoot primordia on the same medium; ***d***, Initiation of multiple shoots; ***e***, Multiplication and proliferation of shoots; ***f***, Complete plantlet with well-developed shoot and roots.

0.5 ppm NAA (hereafter known as M5), while it took 11.67 ± 2.52 weeks in case of MS medium containing 0.5 ppm BAP and 0.5 ppm NAA. PLBs formation was also recorded earliest (14.00 ± 2.00) in M5, which was significantly different (at 5% level) from all other treatments, except Gamborg medium + 0.25 ppm BAP + 0.50 ppm NAA. PLB induction was slowest in MS media irrespective of combination of hormones. The relative advantage of germination duration and PLB formation continued in further growth stages for a particular treatment. Shoot induction occurred after 20.00 ± 2.00 weeks, and root induction was observed at 26.00 ± 2.00 weeks, which were significantly earlier compared with other treatments. Highest shoot and root lengths besides number of leaves per shoot and number of roots per shoot were also observed in M5. Except for number of leaves per shoot, the other three parameters displayed significant differences among the treatments both at 5% and 1% level. The length of shoot varied from 1.48 cm to 5.37 cm, whereas the length of root varied from 1.43 cm to 5.23 cm among the treatments. The range for number for leaves/shoot in all treatments was narrow (3.00–3.67), except in case of Nistch medium containing 1.00 ppm BAP + 0.50 ppm NAA (2.33). The number of roots/shoot varied from 0.67 to 4.33 among the treatments. For the treatment containing Gamborg medium + 1.0 ppm BAP + 0.50 ppm NAA, many of the plantlets backed roots.

All the nine studied parameters were further subjected to multivariate analysis of variance (MANOVA), which tests for the difference in two or more vectors of means and uses the covariance between outcome variables in testing the statistical significance of the mean differences. The 12 different treatments were considered as class variables to compare their effect on the characters under study, besides comparing the effect of M5 versus the rest. Overall media effect was significant in MANOVA test criteria (P value = 0.01 in Wilks' lambda statistics). Contrast analysis clearly showed that M5 was significantly different from the other treatments (P value < 0.0001). The character combinations showing significant partial correlation coefficient between each other for the nine traits were IGW–NDGW (0.91, P value < 0.0001), IGW–PLBIW (0.42, P value = 0.04), NDGW–PLBIW (0.55, P value = 0.004), PLBIW–SEW (0.69, P value = 0.0001), PLBIW–RIW (0.64, P = 0.0006), RIW–SEW (0.89, P < 0.0001) and NLPS–RLC (0.47, P = 0.02).

Studies with related genus *Cypripedium* indicated that relatively low concentration of cytokinins is more effective for germination[3]. In the present study, increasing the concentration of BAP beyond 0.25 ppm delayed germination in all the three media. For induction of PLB or shoot buds, combinations of exogenous BAP and NAA have been used in many orchid species. Orchid explants have been reported to generate PLB in response to auxin and cytokinin[15]. In *Spathoglottis*, a lower ratio of BAP : NAA promoted PLB formation from seedling nodal explants, but a higher ratio induced shoot formation[16]. In the present study, M5 showed the earliest germination and PLB formation besides the best result for induction and growth of shoots and roots. In all the combinations of auxins and cytokinins, PLB developed into plantlets. However, lower concentration of auxins and cytokinins promoted healthy plantlet development and favourable root induction. With the initial growth advantage with lower BAP concentration, the M5 treatment could lead to earliest shoot emergence, root initiation, highest shoot length, number of leaves per shoot, number of roots per shoot and root length. Figure 2 shows the different growth stages.

Overall, the present study was able to identify a suitable single medium for developing plantlets of *P. villosum*, which provides a simple and cost-effective method for propagation of this rare and endangered orchid of high commercial value. In an effort to protect this species from being extinct, tissue culture-raised plantlets may be reintroduced in natural habitats after hardening.

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Received 16 January 2017; revised accepted 11 August 2017

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Name confusions in Indian cycads

Species and issues

Linnaeus[1] described the genus *Cycas* with the lone species *C. circinalis* L. from India, which was based on Rheede's, *Hortus malabaricus*[2], tab. 13-21. Roxburgh[3] included three species, viz. *C. circinalis* Willd., *C. revoluta* Thunb. and *C. sphaerica* Roxb. All three were reportedly introduced in the Botanical Garden, Howrah during 1798–99 from different countries (*C. circinalis*, Indonesia; *C. sphaerica,* Moluccas and *C. revoluta* cultivated in West Bengal gardens).

Dyer[4] recognized five species: *C. circinalis* L. from the Malabar coast and west Madras; *C. rumphii* Miq. from the Andaman Islands; *C. pectinata* Ham. from North East India (Assam, East Bengal and Sikkim), *C. beddomei* Dyer from east Madras (Cuddapah Hills), and *C. revoluta* cultivated in Indian gardens. Dyer equated *C. sphaerica* Roxb. under *C. circinalis* L. (since Rheede's figures and description of *Todda Panna* (=*C. circinalis* L.) matched well with those of *C. sphaerica*), and *C. circinalis* under *C. rumphii*, as he cited tt. 22 and 23 of Rumphius's of *Herbarium amboinense*. Sahni[5], after a century, attempted a revision of the Indian gymnosperms, but followed Dyer (l.c.) in synonymy. Hill[6] presented diagnostic features and an identification key of different species of Indian cycads. Further, he elaborated how Roxburgh's description of *C. circinalis* applies to *C. rumphii* and of *C. sphaerica* to *C. circinalis*.

Much had happened in Indian *Cycas* after Lindstrom and Hill[7] took up the revision of cycads of Southeast Asia that included its members from India as well. Their study was based on a couple of field trips in India (2000–2002), and scrutiny of Indian collections in major herbaria (A (Cambridge), B (Berlin), BM (London), BO (Bogor), E (Edinburgh), G (Geneva), K (Kew), L (Leiden), LAE (Papua New Guinea), NY (New York) and P (Paris)). They recognized eight species from India, including the two relatively recently described *C. annaikalensis* R. Singh & P. Radha (2006) (type: India, *Singh, Radha & Sharma (0491) 0144*, IPUH) and *C. indica* A. Lindstr. & K.D. Hill (2007) (Type: India, *Saldanha 15197*, E) and one new record, *C. nathorstii* J. Schust. (type: Sri Lanka, Thwaites 3689, K). *C. sphaerica*, which was synonymized under *C. circinalis* by Dyer (l.c.) was revived. Further, the collections established in the name of *C. rumphii* Miq., from the Andaman and Nicobar Islands were recognized by them as *C. zeylanica* (J.Schust.) A. Lindstr. & K.D. Hill. (type: Sri Lanka, *Thwaites 3862*, BM). The identity and distribution of various species are discussed below.

C. rumphii – C. zeylanica – C. edentata (Figure 1 *b* and *e*)

The shoreline habitat, glossy leaves, large seeds and spongy endotesta are the features shared by *C. rumphii* and *C. zeylanica*. However, *C. zeylanica* can be recognized by the distinct terminal spine in the microsporophyll and seeds without a crest[7]. In contrast, the microsporophyll in *C. rumphii* has rudimentary terminal spine and seeds with a crest. The mistaken identity of the Andaman and Nicobar Islands material (as *C. rumphii* and not as *C. zeylanica*) is possibly due to the proximity of these Islands to Indonesia where *C. rumphii* is more prevalent, than in Sri Lanka that has known distribution of *C. zeylanica*. There are no reports of the Burmese species, *C. edentata* de Laub. (type: Philippines, *Kondo & Edaño 38877*, L), from the Andaman Islands, and all the collections at PBL have been annotated as *C. rumphii*. It is difficult to establish the identity to these collections since only male/female specimens are present from different islands. The authors believe that these Islands might hold all three species, and not with distribution of *C. zeylanica* alone. A thorough exploration in the Andaman and Nicobar Islands might yield all three species since the Andaman Islands have a geographical affinity with Burma (*C. edentata*), and the Nicobar with Indonesia (*C. rumphii*) and Sri Lanka (*C. zeylanica*). This might even warrant a review on recent reported novelties, *C. andamanica* (type: India, *Prasad & M.V. Ramana 1288*, CAL) from North and Middle Andaman Islands[8] and *C. dharmrajii* from the Andaman Islands[9]. The former was reportedly allied to *C. edentata* and *C. zeylanica*, and the differences cited are more quantitative than qualitative. The conservation concerns are so forceful for both *C. edentata* (near threatened) and *C. zelyanica* (critically endangered or possibly extinct in Sri Lanka)[7], and may induce one to look for these species in newer possible localities