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Asymbiotic seed germination and *in vitro* propagation of *Brasiliorchis picta*

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Seed storage method for *in vitro* germination and propagation from leaves of *Brasiliorchis picta* was developed. Seeds were harvested and stored at -20 and -80°C for 1, 3, 6, and 12 months and were germinated on Knudson C (KC), Murashige and Skoog (MS), half-strength MS (½ MS macro- and micro-nutrients), and woody plant medium (WPM). Seeds stored at -20°C, the recommended temperature for seed banks, had a high germination rate (76.0%) when cultivated in WPM after 12 months of storage. WPM is the best medium for seed germination and seedling development for both harvested and stored seeds, regardless of storage time and storage temperature. Whole leaf and leaf transversal thin cell layers (tTCL) from 3-month-old *in vitro* grown protocorms were cultured in ½ MS supplemented with 6-benzyladenine (BA; 2.5, 5.0 and 10.0 µM) and thidiazuron (TDZ; 3.0, 6.0 and 9.0 µM) for 12 weeks. The highest frequency of regenerated protocorm-like bodies (PLBs) from explants (70.0%) occurred when whole leaves were cultured in medium containing 5.0 µM BA, whereas the best response for leaf TCL was with the basal section in medium containing 9 µM TDZ, in which PLBs developed in all regions of leaves. Plantlets were successfully acclimatized (with a survival rate of 97%) when vermiculite was used as a substrate.

Key words: Endangered species, conservation, germination rate, leaf explant, culture medium, micropropagation, Orchidaceae, thin cell layer.

INTRODUCTION

Brasiliorchis picta (Hook.) R. B. Singer, S. Koehler & Carnevali is an epiphytic species from the family Orchidaceae (Singer et al., 2007). It is native to Southern and Southeastern Brazil including the state of Mato Grosso (Barros et al., 2015) and reach Northeastern Argentina, in Misiones (Johnson, 2001). Its value is high on the ornamental flowers market, therefore, many plants of this species have been illegally collected from wild populations, putting this species at risk of becoming endangered. The flowers are showy, fragrant and long-lasting (10 days or more), combine cream or yellowish cream colorations with purple dots or maculation (Figure

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Figure 1. *Brasiliorchis picta* flower. Collection Smidt, EC, 988. Bar=1.0 cm.

1), and devoid of secretions or pluricellular trichomes that attract Meliponini bees *Trigona spinipes* (Singer and Koehler, 2004).

Storage of the orchid seeds gives us the opportunity to conserve orchids; however, there are very few studies on the longevity of orchid seeds (Suzuki et al., 2012). Many species produce enormous numbers of tiny seeds, which would allow the storage of large numbers in a reasonable small space. Orchid seeds are considered to display orthodox behavior, tolerate considerable desiccation, and storage longevity is thereby greatly increased when subjected to drying (to 5% moisture content) and freezing (-20°C), the conditions commonly utilized in orchid seed banks (Hong and Ellis, 1996; Seaton et al., 2013).

Orchid seeds do not contain endosperm and are very small, ranging in length from 0.05 to 6.0 mm (Arditti and Gani, 2000). They show quite a uniform pattern of germination and development starting with seed imbibition that leads to the rupture of the testa, release of the embryo and formation of a structure called the protocorm. Under *in situ* conditions, the germinating seed usually remains a protocorm until it is infected by a mycorrhizal fungus (Hosomi et al., 2011). Asymbiotic germination represents an ideal system for studying the growth and development of orchid seedlings (Kauth et al., 2008). It is an efficient method for large-scale propagation of orchids and is already used for many species (Zeng et al., 2012). The composition of culture medium is important for successful germination of orchid seeds and results vary significantly from one species to another (Suzuki et al., 2012). A wide variety of culture media has been successfully used for orchid seed germination, but Knudson C (Knudson, 1946) and Murashige and Skoog (1962) are probably the formulations most often used (Munoz and Jimenez, 2008).

Tissue culture methods have been extensively used, not only for rapid and large-scale propagation of orchids, but also for *ex situ* conservation (Murthy and Pyati, 2001). Among the various techniques of *in vitro* culture, thin cell layer technology has been successful in orchid mass multiplication for both commercial and conservation purposes (Teixeira da Silva and Dobránszki, 2013). Different explants, such as protocorms and juvenile leaves, have been successfully used for orchid micropropagation (Chen and Chang, 2002; Ferreira et al., 2015).

Successful micropropagation using leaf explants depends mostly on the age: young leaves respond better than the old ones (Chugh et al., 2009), as reported for *Coelogyne flaccida* (De and Sil, 2015). Other factors are the nutrient composition of the medium, the source of the leaf (*in vitro/in vivo*), the part of the leaf used, and the orientation of explants (Chugh et al., 2009). Whole leaves and transversal thin cell layers (tTCLs) have been successful at micropropagation of various orchid species, such as *Dorietanopsis* hybrid (Park et al., 2002), *Aerides crispum* (Sheelavanthmath et al., 2005), *Vanda testacea* (Kaur and Bhutani, 2009), *Phalaenopsis bellina* (Khoddamzadeh et al., 2011), *Aranda×Vanda coerulea* (Gantait and Sinniah, 2012), *Renanthera* (Wu et al., 2012) and *Epidendrum secundum* (Ferreira et al., 2015).

The aim of the present investigation was to study seed storage and *in vitro* germination of *B. picta* for use in conservation, as well as to establish a protocol for mass propagation of this orchid using leaves as explants.

MATERIALS AND METHODS

Plant, pollination, seed collection and storage

The plants of *B. picta* used in this study belong to the orchid collection of the Institute of Botany, São Paulo State, Brazil. Hand cross-pollination with flowers from different plants was performed and the resultant immature seed capsules, changing colouration from green to yellowish, were harvested after 3 months. The protocol of drying and storage followed that recommended by Seaton and Ramsay (2005) in their project: “Orchid Seed Storage for Sustainable Use” (OSSU). Capsules were initially washed under running tap water and a commercial detergent, and then immersed for 5 min in 70% ethanol, followed by three rinses in sterile, distilled water. The capsules were then split longitudinally with a scalpel and the seeds were placed on a Petri dish containing filter paper. Seeds were stored for seven days in a desiccator containing a saturated solution of calcium chloride ($CaCl_2$) at room temperature. The seeds were then transferred to tubes and placed into flasks containing silica gel. Finally, the seeds were stored in a freezer at -20 and -80°C for 1, 3, 6, and 12 months.

Seed sterilization, media and culture conditions

Seeds were surface-sterilized for 1 min with 70% ethanol, followed by immersion in 0.75% (v/v) sodium hypochlorite (NaOCl) plus 0.1% Tween 20® for 5 min and rinsed six times in autoclaved distilled water. Seeds were inoculated in Petri dishes (150 mm in

diameter and 20 mm in height) containing 40 ml of culture medium. Four basal media were tested in this study: Murashige and Skoog (MS) (Murashige and Skoog, 1962), half-strength MS (1/2 MS macro- and micro-nutrients), Knudson C (KC) (Knudson, 1946) and Woody Plant Medium (WPM) (Lloyd and McCown, 1980). The media were supplemented with 5.6 g L^{-1} agar Himedia®, 3% sucrose (w/v) and 0.1 g L^{-1} inositol. The pH was adjusted to 5.8 with 0.1 N NaOH or HCl before the addition of agar. The media was autoclaved for 20 min at 120°C. The cultures were maintained in a growth room with a temperature of 26 ± 2°C/18 ± 2°C (day/night) and under a 16-h photoperiod provided by white fluorescent tubes at an intensity of 40 µmol m^{-2} s^{-1}.

In vitro germination and seedling development

Seeds were inoculated under a stereomicroscope into Petri dishes containing culture medium. Four fields, each containing 100 seeds and three replicates per treatment in each Petri dish were marked. After 12 weeks, the seedling development was evaluated as one of five stages: I, rupture of the testa; II, protocorm with emerging rhizoids; III, protocorm with pointed shoot apex and rhizoids; IV, protocorm bearing one leaf; V, protocorm bearing two and more leaves and roots. The percentages of the different developmental stages were calculated by dividing the number of seeds/protocorms in each stage by the total number of seeds and protocorms (stages I to V) present in the samples. Seeds were considered germinated when the protocorm showed an initial shoot and emerging rhizoids. The germination percentage was determined after eight weeks.

Induction and regeneration of PLBs from leaves

Two leaves (1.0 cm in length) from 3-month old protocorms from *in vitro* germinated seeds were inoculated into Petri dishes with their abaxial face in contact with the culture medium ½ MS, supplemented with thidiazuron (TDZ; 0, 3.0, 6.0, and 9.0 µM) or 6-benzyladenine (BA; 0, 2.5, 5.0, and 10.0 µM). The experimental design was completely randomized with 3 replicates and 10 leaves per Petri dish. After 12 weeks, the percentage of explants forming protocorm-like bodies (PLBs) and the average number of regenerated PLBs per explant were determined.

Transversal thin cell layer (tTCL)

Leaves (1.0 cm in length) from 3-month old protocorms from *in vitro* germinated seeds were transversely sliced into six TCL sections of 1.0 mm thick (B1 and B2: basal; M1 and M2: middle section, and A1 and A2: apical section) using a sharp surgical blade. The leaf sections were cultured in Petri dishes containing 40 ml of ½ MS, supplemented with TDZ (0, 3.0, 6.0, and 9.0 µM) or BA (0, 2.5, 5.0, and 10.0 µM). The experimental design was completely randomized with three replicates and 10 sections of each region per Petri dish. After 12 weeks, the explants forming PLBs and the average numbers of regenerated PLBs per explant were determined. The values for the average number of PLBs were transformed by $\sqrt{x+0.5}$ and the percentages of explants forming PLBs were transformed by log x+0.5. Explants were transferred to ½ MS culture medium, without any growth regulator, for protocorm elongation and rooting.

Transplanting and acclimatization

Five-month old seedlings with two expanded leaves (5.0 cm long) from *in vitro* germination were transplanted to polystyrene trays containing: Plantmax®, vermiculite, coconut powder, a mixture of Plantmax®/vermiculite 1:1 (v/v), a mixture of Plantmax®/coconut powder 1:1 (v/v), a mixture of vermiculite/coconut powder 1:1 (v/v) and a mixture Plantmax®/vermiculite/coconut powder 1:1:1 (v/v). The seedlings were maintained in a greenhouse at room temperature and the survival percentage four months after transplanting was recorded. Seedlings were watered twice in a week. The experimental design was completely randomized, with six replicates and 10 plantlets per substrate.

Experimental design and statistical analysis

Experiments were performed using completely randomized designs. All data were statistically analyzed by analysis of variance (ANOVA). Means were compared by Tukey's test at $p < 0.05$ using Statistica 7.0 software.

RESULTS AND DISCUSSION

In vitro seed germination

Seed germination of *B. picta* starts with swelling (Figure 2A). Then it bursts its testa (Figure 2B) and develops into a round, green form a protocorm (Figure 2C) at approximately two weeks after they were placed on culture media, regardless of the kind of media. After four weeks, rhizoids could be observed emerging from the protocorm (Figure 2D) and an apex was apparent, followed by the development of the first leaf (Figure 2E) and the second leaf. Finally, the roots thereby giving rise to a seedling after 12 weeks (Figure 2F and G). With the exception of the KC medium, all protocorms developed to the last stages. The asymbiotic seed germination of *B. picta* followed the same developmental sequence as reported for the other orchid species.

Figure 3A presents stored (-20°C) and harvested seeds germinated on all the media tested, where the seed germination rate on WPM and MS media were higher than that for the other basal media, regardless of the storage period. After 12 months of storage, the germination percentage remained high in WPM and MS media (76.0%). However, lower germination percentages occurred with the KC medium, independent of the storage period, and there was a gradual reduction in seed viability after storage (Figure 3A). Germination of *B. picta* indicate a significant interaction of storage period and culture medium for both temperatures tested (-20°C: $F=2.41$; G.L=12; $P=0.006$; -80°C: $F= 4.60$; G.L=12; $P<0.001$).

There is an increase in germination rate for seeds stored at -20°C compared to seeds stored at -80°C, independent of storage period. Higher seed germination rate also occurred in WPM and MS media for seeds stored at -80°C. For all media tested, there was a reduction of seed germination after 3 months of storage (Figure 3B).

Figure 4 illustrates the protocorm development for seeds harvested and stored at -20°C for different periods 12 weeks after *in vitro* germination in different culture media. The best response of the initial development for

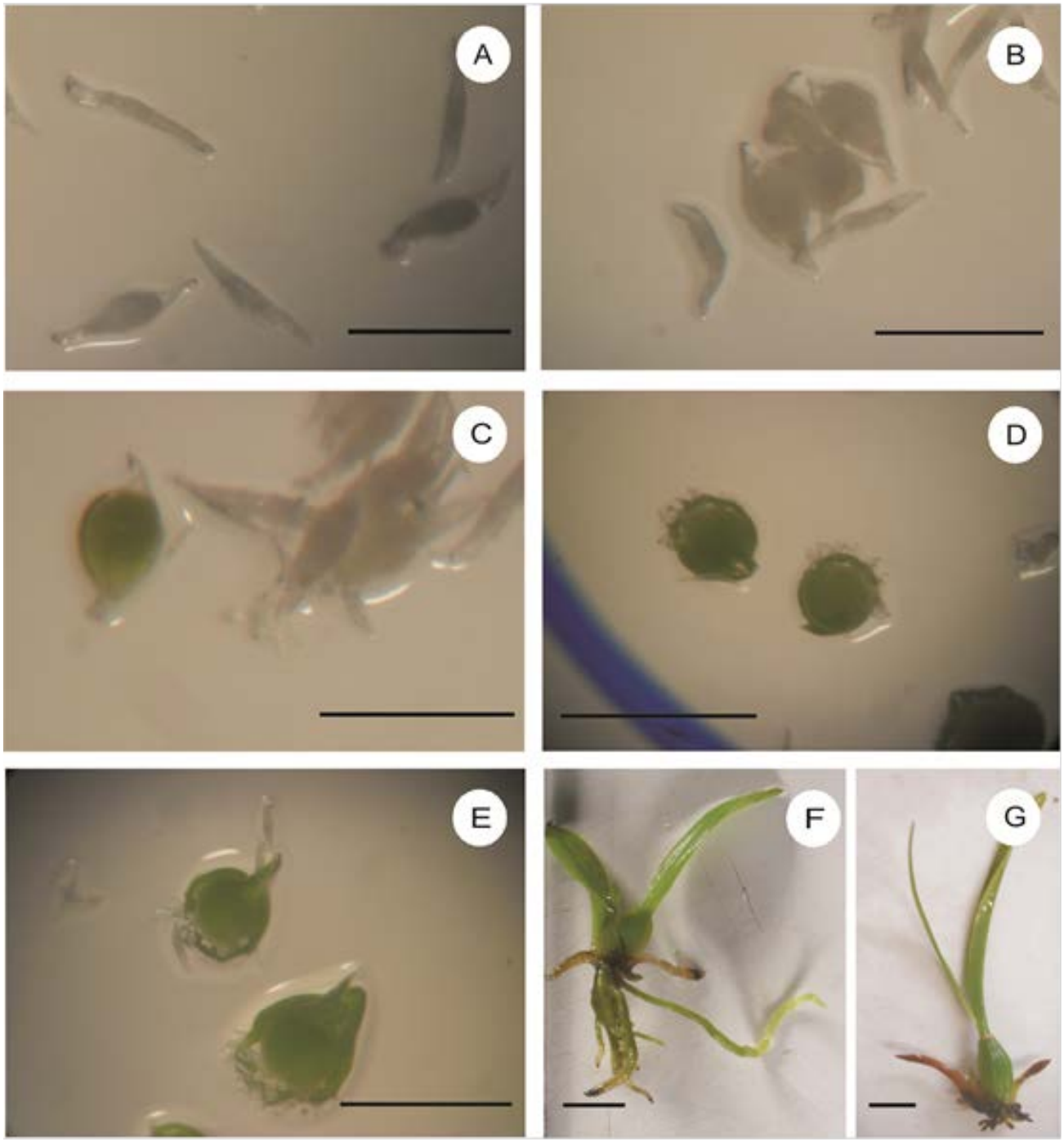


Figure 2. *Brasiliorchis picta*. Successive stages of asymbiotic seed germination and seedling development of cultured on Woody Plant Medium after storage at –20°C. A: swollen seed; B: embryo bursting from the testa after 4 weeks; C: protocorm stage; D: protocorm with emerging rhizoids after 7 weeks; E: protocorm with apex and rhizoids after 8 weeks; F, G: seedling with leaves and roots after 10 and 12 weeks, respectively. Scale bars: A-F: 1 mm, G: 10 mm.

freshly harvested and stored seeds was with WPM and MS media, where the former almost always had the highest germination rate in the initial and later stages. The KC medium showed the greatest delay in seedling development. An increase in the seed storage period caused a delay in germination and protocorm development stages. Figure 4 also indicates that the storage time increased seeds germinated better on WPM than those in the other media tested.

Seed banking is an efficient method for *B. picta*

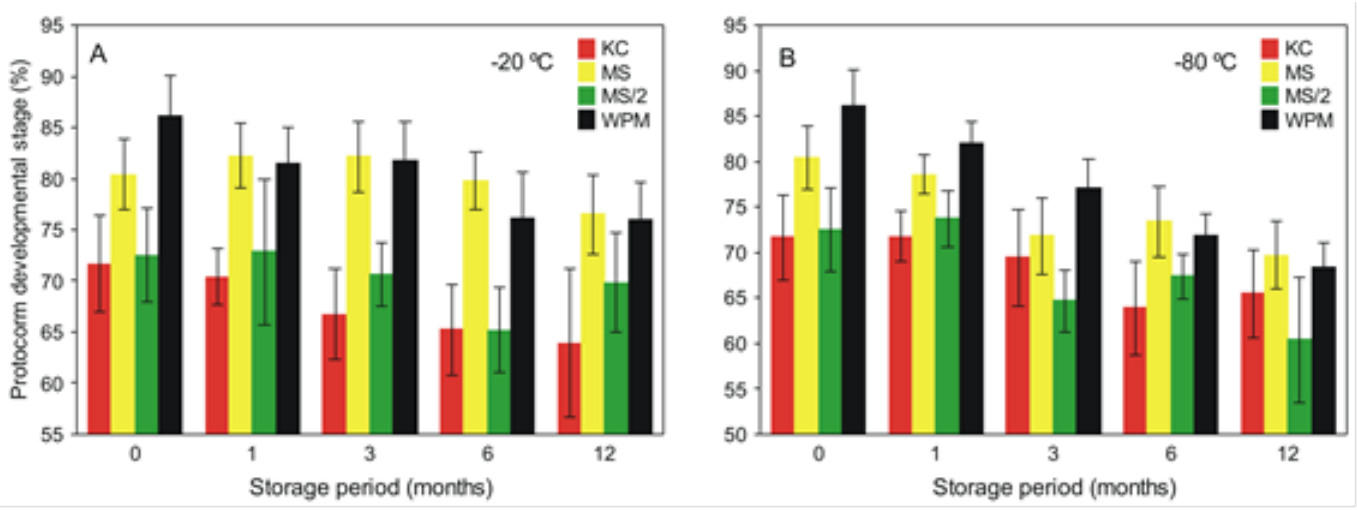


Figure 3. *Brasiliorchis picta.* Seed germination rate (%) harvested and stored at -20 and -80°C, A and B, respectively. Seeds were stored for 1, 3, 6 and 12 months and kept for 12 weeks in different culture media.

conservation considering that after 12 months of storage at -20°C, it was possible to germinate seeds with little loss of viability showing an orthodox behavior. The temperature of -20°C was recommended for orchid seed banks by Seaton et al. (2013). According to Machado Neto and Custodio (2005), seeds stored at -20°C and 5% moisture content can avoid attack of pathogens and activation of metabolic processes that would age seeds too fast, leading to rapid viability loss. The freshly harvested seeds had the highest germination rate, followed by those stored at –20°C, with a gradual reduction as storage period increased. Hay et al. (2010), who tested seed storage temperatures range -196 to +23°C also found that the ideal temperature to maintain the viability of several orchid species was -20°C including *Diuris laxiflora.*

Seed germination and seedling development of *B. picta* were affected by the type of medium, WPM with fresh harvested seeds and those stored at -20 and -80°C gave the best results: about 80, 76 and 69%, respectively. Plantlets cultured in WPM medium were more vigorous and showed no evidence of necrosis, as was also observed for plantlets developed with MS medium after eight weeks of culture. Suzuki et al. (2012) also reported that the germination and protocorm/seedling development are greatly influenced by different culture media and among different species the results vary significantly. They recommended the use of MS and KC medium for *Hoffmannseggella cinnabarina.* In the present study, seeds of *B. picta* cultured in MS medium initially showed good results, whereas the KC medium produced slow development of the protocorms and they were light-yellowish in color, like in research on *Cymbidium aloifolium* by Pradhan et al. (2013). Johnson and Kane (2007) also reported that protocorm development of *Vanda* hybrid seeds germinated on ½ MS was consistently more advanced than development with KC, and suggested that this may be linked to a low ammonium to nitrate ratio. MS is highly enriched with macro- and micro-elements, ½ MS and WPM media contain a low amount of macro- and micro-elements, but are enriched with different vitamins, whereas KC contains a low amount of macro- and micro- nutrients and lacks vitamins (Hossain et al., 2010). Thus, the nutrient regime for orchid culture is species specific and no single culture medium is universally applicable to all orchid species (Pradhan et al., 2013).

Induction and regeneration from whole leaves

After five weeks of culture, induction of PLBs was visible with white globular structures observed in the basal region of the leaves (Figure 5A). After eight weeks, greenish protuberances increased size and were observed on the surface of leaves. Approximately 30% of the explants produced 2.0 PLBs per responsive explant after 12 weeks of culturing on growth regulator-free WPM (Table 1). A similar response was observed for an orchid hybrid (*Aranda×V. coerulea)*, in which PLB formation was seen even with growth regulator-free medium, but the frequency was low with very few PLBs. In addition, the time taken for induction of PLBs was much longer as compared to the media with growth regulator. One logical explanation for the occurrence of PLBs in the control medium is endogenous cytokinin (Gantait and Sinniah, 2012).

Analysis of variance of the explants forming PLBs and the average number of PLBs per explant indicated that there were no statistical differences between treatments

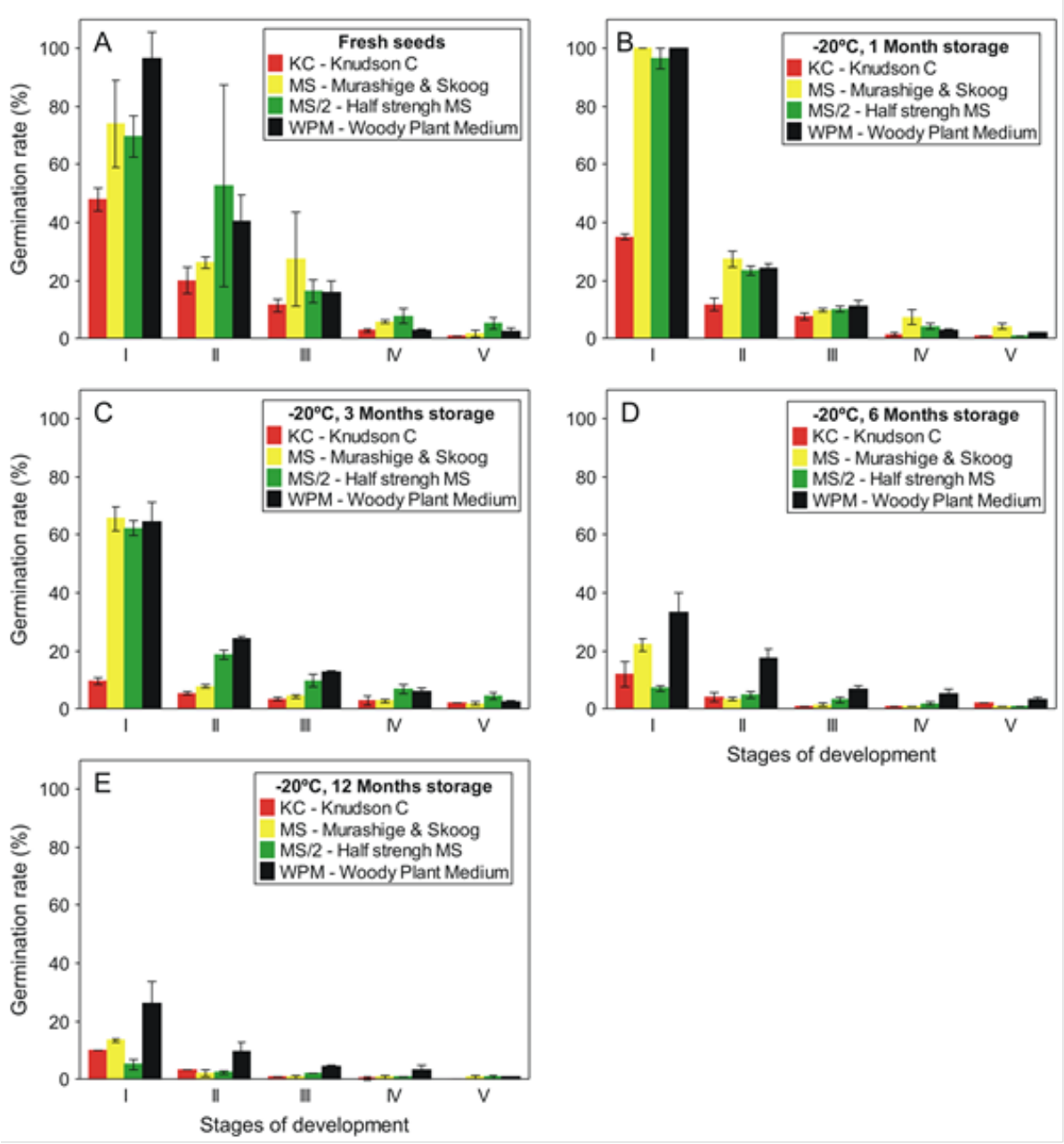


Figure 4. Effects of different culture media: KC, MS, ½ MS and WPM on development of *Brasiliorchis picta* from fresh seeds and seeds submitted to different storage periods at -20°C, after 12 weeks of cultivation. Stages of development: I- rupture of the testa; II- protocorm with emerging rhizoids; III- protocorm with pointed shoot apex and rhizoids; IV- protocorm bearing one leaf; V- protocorm bearing two or more leaves and roots.

(p>0.05). Cytokinin type and concentration did not influence regeneration of PLBs or the average number of shoots, but the explants cultured in half-strength MS medium containing 5.0 µM BA produced the highest percentage of new PLBs (70%) with 13.2 PLBs per explant (Table 1 and Figure 5B). Lesser results were obtained for leaf explants of *V. testacea,* for which the best regeneration of PLBs (48.7%) was obtained in Mitra medium containing a similar concentration of BA (4.4 µM) (Kaur and Bhutani, 2009), as used in this study. BA concentration also influenced the response of explants of *E. secundum*; leaves cultured in a medium supplemented with 1.0 to 5.0 µM BA regenerated more PLBs than the control (Ferreira et al., 2015). The addition of 5.0 µM of BA to WPM medium also induced high regeneration rate (70.0%), but the average number of PLBs per responsive explant was lower (2.0) (Ferreira et al., 2015). Similarly, for leaf explants of *Dendrobium* hybrids, BA was efficient

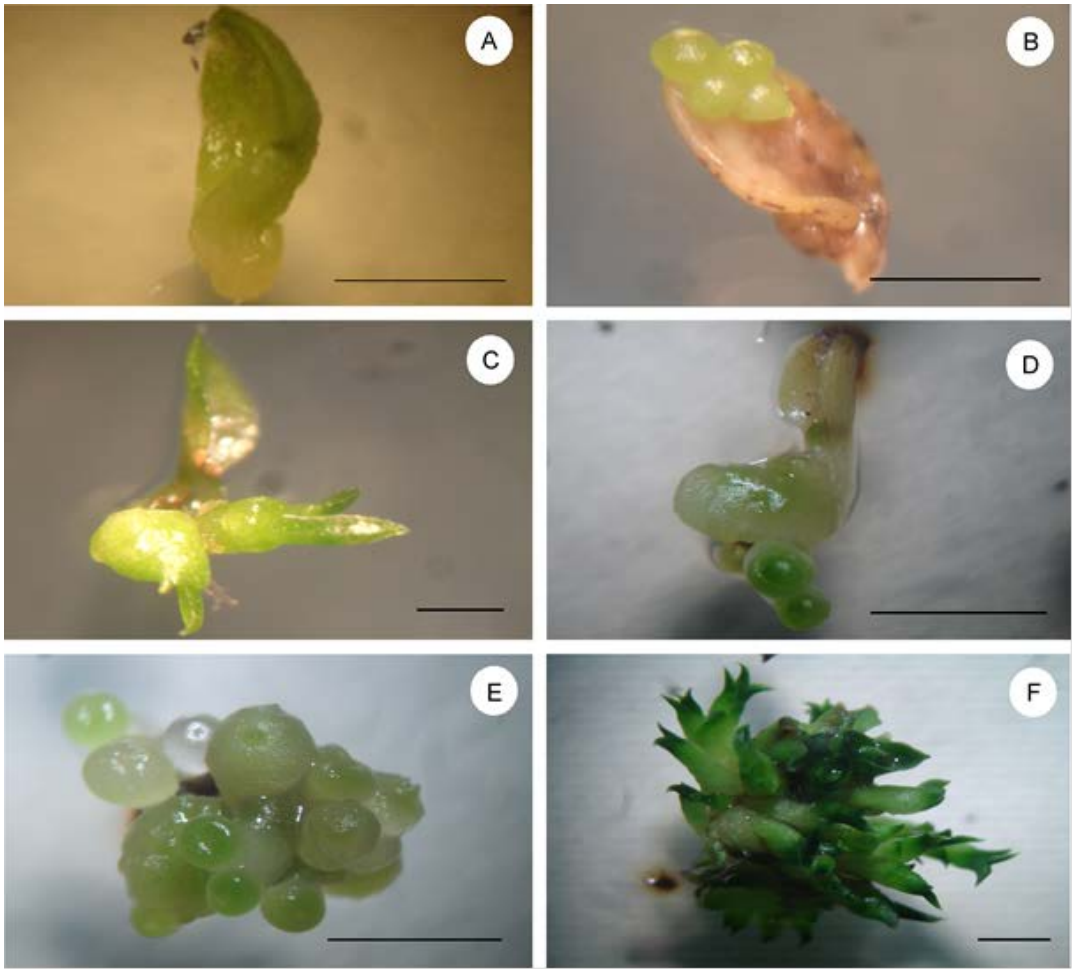


Figure 5. Plant regeneration and protocorm-like bodies (PLBs) developing from leaf explants of *Brasiliorchis picta* cultured on half-strength MS medium. A-B: Whole leaf with initiation of a PLB after five weeks and PLBs developing in medium supplemented with 5 µM BA after eight weeks of culture, respectively. C: PLBs cultured in medium with 6 µM of thidiazuron (TDZ) showing the first leaf primordial. D: PLBs developing from basal leaf section with medium supplemented with 3 µM. E-F: Formation of multiple PLBs along the basal leaf section in medium supplemented with 9 µM of TDZ after 8 and 10 weeks of culture, respectively (Scale bars = 1000 µm).

in MS medium, but the concentration used was ten times greater (44.4 µM) than that used in our study. This high concentration induced 60% shoot regeneration and 7.5 shoots per leaf explant (Martin and Madassery, 2006), which are lower than that for *B. picta* leaves. Since there were no significant differences in ratio of PLBs regeneration and average number of PLBs, it can be concluded that there is great variability in PLB formation. Therefore, cytokinin and/or culture time must be optimized and that future studies need to include more repetitions.

Transversal thin cell layer (tTCL) of leaves

Figure 5 presents the formation of PLBs after four weeks of culture, most frequently in the basal sections (B1) (Figure 5C to E) followed by B2 sections (Figure 5F), but the response of the middle region was poor (Table 2). PLBs were well differentiated on explants by the end of eight weeks and the first leaf primordia were observed after 12 weeks (Figure 5F). PLB formation was also observed on medium without growth regulator, but the frequency was low (26.7%) with very few PLBs (1.6) and only in the basal region (Figure 5). Among the cytokinins tested, the development of PLBs was more efficient in the presence of TDZ (Figure 5C to F), as compared to BA (Figure 5A and B). The best response was recorded for the medium containing 9 µM TDZ, in which PLBs developed in all regions of the leaves (Table 2, Figures 5E, 5F, and Figure 6). These responses demonstrate that the addition of cytokinin is necessary for organogenesis from leaf TCL and the medium supplemented with BA caused necrosis of PLBs, after 12 weeks (Table 2). The

Table 1. *Brasiliorchis picta*. Regeneration of protocorm–like bodies (PLBs) and the average number of PLBs per explant from leaves after 12 weeks of culture in Murashige and Skoog medium at half strength of salts (½ MS) supplemented with cytokinins.

Cytokinins (µM)	Regeneration of PLBs (%)	The average number of PLBs
Control	30.0 (±0.1)	2.0 (±0.5)
BA 2.5	40.0 (±0.3)	2.1 (±1.0)
BA 5.0	70.0 (±0.3)	13.2 (±6.6)
BA 10.0	60.0 (±0.2)	7.1 (±3.0)
TDZ 3.0	53.3 (±0.2)	8.2 (±4.6)
TDZ 6.0	53.3 (±0.3)	11.4 (±13.2)
TDZ 9.0	60.0 (±0.3)	9.0 (±2.0)
Mean	52.4	7.6

BA: 6-benzyladenine; TDZ: thidiazuron.

Table 2. *Brasiliorchis picta.* Effect of leaf tTCL regions (B1-B2 – basal region, M1-M2 – middle region and A1-A2 apical region) and cytokinins on protocorm-like-bodies (PLBs) regeneration after 12 weeks of culture in ½ MS medium.

Cytokinins (µM)	Regeneration of PLBs (%)						Mean
	B1	B2	M1	M2	A1	A2	
Control	26.7^{aA}	10.0^{bcA}	-	-	-	-	6.1
BA 2.5	40.0^{aA}	13.3^{cB}	-	-	-	-	8.9
BA 5.0	43.3^{aA}	23.3^{abcA}	-	-	-	3.3^{bcB}	11.7
BA 10.0	36.7^{aA}	30.0^{abA}	-	-	-	3.3^{bcB}	11.7
TDZ 3.0	43.3^{aA}	30.0^{abA}	-	-	-	15.0^{abB}	14.7
TDZ 6.0	46.7^{aA}	36.7^{aAB}	-	3.3^{aC}	-	16.7^{aB}	17.2
TDZ 9.0	46.7^{aA}	40.0^{aA}	3.3^{aB}	3.3^{aB}	3.3^{aB}	23.3^{aA}	20.0
Mean	40.5	26.2	0.5	0.9	0.5	8.8	-

Means within a column followed by the same lower case letter and means within a row followed by the same upper case letter do not differ significantly according to Tukey's test ($P \leq 0.05$). ½ MS Murashige and Skoog medium at half-strength of salts. BA: 6-benzyladenine; TDZ: thidiazuron.

results indicated a significant effect of the type of TCL and type of cytokinin on the formation of PLBs of *B. picta* (F=3.31; G.L=30; P<0.001).

The addition of cytokinin to culture medium was also necessary to produce PLBs from tTCLs of leaves of *B. picta*. While BA was more efficient at producing PLBs with whole leaves, TDZ provided better results for tTCL. TDZ was used alone for *P. bellina* and also formed PLBs directly from the surfaces of the leaf segments (Khoddamzadeh et al., 2011). The efficiency of TDZ for PLB induction was also reported for basal leaf sections of *Renanthera* (Wu et al., 2012). Foliar sections (1.0 mm thickness) of *Dorietanopsis* hybrid, cultivated in ½ MS and supplemented with 9 µM of TDZ, produced the highest ratio of PLB formation (72.3%) and the greatest number of PLBs per explant (18.0) (Park et al., 2002). In the present study, in the same conditions, the ratio of PLB regeneration was lower (46.7%), but the average number of PLBs was higher (23.1). If the number of PLBs formed in all the regions of the leaves in culture medium containing 9 µM of TDZ was added, 43.6 PLBs can be produced, indicating that the tTCL technique is promising for *B. picta.*

In the present study, young whole leaf and leaf segments of *B. picta* developed PLBs through direct organogenesis on media supplemented with cytokinins. The beginning of PLB formation occurred initially in the basal region for all explants after 4 weeks of culture. The middle leaf region was not responsive and the apical region produced a low frequency of PLB regeneration.

Like in others monocots, orchid leaf base is meristematic and produces plantlets when excised and cultured (Seeni and Latha, 1992). Similar response was also observed for *V. coerulea,* where the adventive meristematic cells spread all over the surface of the leaf base responded to favorable culture conditions initially by random mitotic divisions and then organization into PLBs (Seeni and Latha, 2000). Kaur and Buthani (2009) also observed that only basal sections of *V. testacea* responded to cultivation. Gantait and Sinniah (2012)

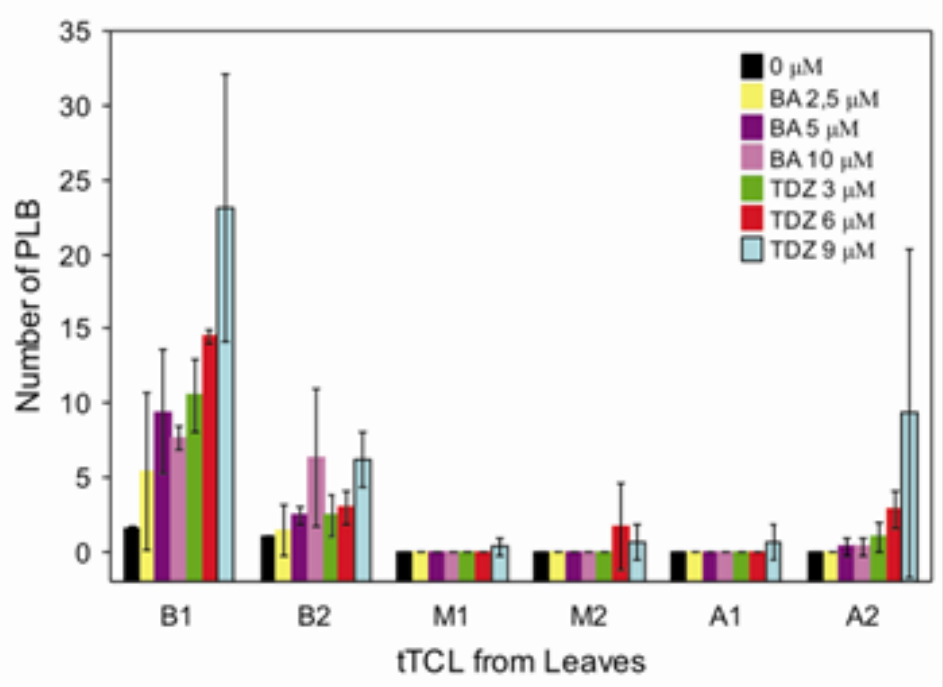


Figure 6. Effect of leaf tTCL region and cytokinin on the average number of protocorm-like-bodies (PLBs) of *Brasiliorchis picta,* after 12 weeks of culture in half-strength MS medium. BA: 6-benzyladenine; TDZ: thidiazuron; Leaf tTCL, B1-B2: basal region; M1-M2: middle region; A1-A2: apical region.

Table 3. *Brasiliorchis picta.* Survival of seedlings cultivated in a greenhouse in different substrates 12 weeks after transplanting.

Substrate	Survival rate (%)
Plantmax® (T1)	50^{e}
Vermiculite (T2)	97^{a}
Coconut powder (T3)	67^{cd}
T1+T2 (1:1)	60de
T1+T3 (1:1)	37^{f}
T2+T3 (1:1)	77^{bc}
T1+T2+T3 (1:1:1)	87^{ab}

Means within a column followed by the same lower case letter do not differ significantly according to Tukey's test ($P \leq 0.05$).

explained that the high potential of regenerating PLBs from leaf sections of the *Aranda*×*V. coerulea* hybrid can be attributed to the injury response of the cut surface of leaves activating quiescent cells and initiating cell multiplication.

Transplanting and acclimatization

Plantlets grew vigorously during 12 weeks of cultivation in a greenhouse and they showed higher survival rate after transplanting with vermiculite or a mixture of vermiculite, Plantmax® and coconut powder (1:1:1). The plantlets maintained in a substrate of only Plantmax® had higher mortality rate (Table 3).

Plantlets of *B. picta* were successfully acclimatized in a greenhouse using vermiculite as a substrate (97% survival rate after 12 weeks). They grew fast and formed new leaves. Faria et al. (2001) analyzed the growth of *Oncidium baueri* and *B. picta* plants in a greenhouse and showed that vermiculite is an excellent substitute substrate for xaxim (*Dicksonia sellowiana*), as observed in our study. However, they recommended a mixture of vermiculite and charcoal or carbonized rice husk substrates for *B. picta.* Similar response was obtained for plantlets of *Brasilidium forbesii* with 100% of survival rate in the greenhouse using vermiculite as a substrate (Gomes et al., 2015). The ideal substrate for ornamental plant cultivation should be available in great quantity, be easy to handle and cheap (Faria et al., 2001), as observed for vermiculite.

Based on the results obtained in our study, we recommend the use of WPM medium for seed germination and seedling development of *B. picta.* Seeds stored at -20^{o}C have a high germination rate, so it remains necessary to evaluate them for periods greater than 12 months. Whole leaves and leaf transversal thin cell layers from 3-month-old *in vitro* grown protocorms

can be used for PLBs regeneration when ½ MS is supplemented with 5 µM BA or 9 µM TDZ, respectively. Vermiculite can be used as substrate for transplanting and acclimatization of plantlets in a greenhouse.

Conclusion

An efficient method for seed conservation, for the *in vitro* germination and for the direct regeneration of a large number of plantlets from leaves of *B. picta* has been described. This report could be applied to mass-scale propagation as well as *ex situ* conservation for floriculture of this important, but threatened orchid species.

Conflict of interests

The authors have not declared any conflict of interests.

Abbreviations

BA, 6-Benzyladenine; **KC,** Knudson C; **MS,** Murashige and Skoog; **MS or MS/2,** half-strength MS macro- and micro-nutrients; **PLBs,** protocorm-like bodies; **TDZ,** thidiazuron; **tTCL,** transversal thin cell layer; **WPM,** woody plant medium.

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REFERENCES

Arditti J, Ghani A (2000). Numerical and physical properties of orchid seeds and their biological implications. New Phytol. 145:367-421.

Barros F de, Vinhos F, Rodrigues VT, Barberena FFVA, Fraga CN, Pessoa EM, Forster W, Menini Neto L, Furtado SG, Nardy C, Azevedo CO, Guimarães LRS (2015). Orchidaceae in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. Disponível em: <http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB64725>. Acesso em: 14 Set. 2015.

Chen JT, Chang WC (2002). Effects of tissue culture conditions and explant characteristics on direct somatic embryogenesis in *Oncidium* Gower Ramsay. Plant Cell Tissue Org. Cult. 69:41-44.

Chugh S, Guha I, Rao U. (2009). Micropropagation of orchids: A review on the potential of different explants. Sci. Hortic. 122:507-520.

De KK, Sil S (2015). Protocorm-like bodies and plant regeneration from foliar explants of *Coelogyne flaccida,* a hoticulturally and medicinally important and endangered orchid of eastern Himalaya. Lankesteriana 15(2):151-158.

Faria RTD, Rego LD, Bernardi A, Molinari H (2001). Performance of differents genotypes of Brazilian orchid cultivation in alternatives substrates. Braz. Arch. Biol. Technol. 44:337-342.

Ferreira DL, Smidt EC, Ribas LLF (2015). Efficient micropropagation of *Epidendrum secundum* Jacq. from leaves and protocorms. Afr. J. Biotechnol. 14:1122-1128.

Gantait S, Sinniah UR (2012). Rapid micropropagation of monopodial orchid hybrid (*Aranda* Wan Chark Kuan 'Blue'× *Vanda coerulea* Grifft. ex. Lindl.) through direct induction of protocorm-like bodies from leaf segments. Plant Growth Regul. 68:129-140.

Gomes LPG, Franceschi CRB, Ribas LLF (2015). Micropropagation of *Brasilidium forbesii* (Orchidaceae) through transverse and longitudinal thin cell layer culture. Acta Sci. Biol. Sci. 37(2):143-149.

Hay FR, Merritt DJ, Soanes JA, Dixon KW (2010). Comparative longevity of Australian orchid (Orchidaceae) seeds under experimental and low temperature storage conditions. Bot. J. Linn. Soc. 164:26-41.

Hong TD, Ellis, RH (1996). A protocol to determine seed storage behaviour. IPGRI Technical Bulletin No. 1. (J.M.M. Engels, JMM, Toll, J eds). International Plant Genetic Resources Institute, Rome, Italy. https://www.cbd.int/doc/case-studies/tttc/SeedStorage.pdf

Hosomi ST, Custódio CC, Seaton PT, Marks TR, Machado-Neto B (2011). Improved assessment of viability and germination of *Cattleya* (Orchidaceae) seeds following storage. *In Vitro* Cell. Dev. Biol. Plant. 48:127-136.

Hossain MM, Sharma M, Teixeira da Silva JÁ, Pathak P (2010). Seed germination and tissue culture of *Cymbidium giganteum* Wall. Ex Lindl. Sci. Hortic. 123:479-487.

Johnson AE (2001). Las Orquıdeas del Parque Nacional Iguazu´. Literature of Latin America (L.O.L.A.), Buenos Aires.

Johnson TR, Kane ME (2007). Asymbiotic germination of ornamental *Vanda*: *in vitro* germination and development of three hybrids. Plant Cell Tissue Org. Cult. 91:251-261.

Kaur S, Bhutani KK (2009). *In vitro* propagation of *Vanda testacea* (Lindl.) Reichb. f.–a rare orchid of high medicinal value. Plant Tissue Cult. Biotechnol. 19(1):1-7.

Kauth PJ, Dutra D, Johnson TR, Stewart SL, Kane ME, Vendrame WA (2008). Techniques and applications of *in vitro* orchid seed germination. In: Teixeira da Silva JA (Ed.). Floriculture, ornamental and plant biotechnology: advances and topics issues. Isleworth: Global Science Books, 2008. pp. 375-391.

Khoddamzadeh AA, Sinniah UR, Kadir MA, Kadzimin SB, Mahmood M, Sreeramanan S (2011). *In vitro* induction and proliferation of protocorm-like bodies (PLBs) from leaf segments of *Phalaenopsis bellina* (Rchb.f.) Christenson. Plant Growth Regul. 65:381-387.

Knudson L (1946). A new nutrient solution for the germination of orchid seed. Am. Orchid Soc. Bull. 14:214-217.

Lloyd G, MCCown B (1980). Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia,* by use of shoot-tip culture. Int. Plant Prop. Soc. 30:421-427.

Machado Neto NB, Custódio CC (2005). Orchid conservation through seed banking: ins and outs. Selbyana 26(1,2):229-235.

Martin KP, Madassery J (2006). Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. Sci. Hortic. 108:95-99.

Munoz M, Jimenez VM (2008). Capsule development, *in vitro* germination and plantlet acclimatization in *Phragmipedium humboldtii, P. longifolim* and *P. pearcei.* Lankesteriana 8:23-31.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.

Murthy HN, Pyati AN (2001). Micropropagation of *Aerides maculosum* Lindl. (Orchidaceae). *In Vitro* Cell. Dev. Biol. Plant 37:223-226.

Park SY, Yeung EC, Chakrabarty D, Paek KY (2002). An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of *Dorietaenopsis* hybrid using thin-section culture. Plant Cell Rep. 21:46-51.

Pradhan S, Regmi T, Parmar G, Pant B (2013). Effect of different media on *in vitro* seed germination and seedling development of *Cymbidium aloifolium* (L.) Sw. Nepal J. Sci.Technol. 14:51-56.

Seaton P, Kendon JP, Pritchard HW, Puspitaningtyas DM, Marks TR (2013). Orchid conservation: the next ten years. Lankesteriana 3:93-101.

Seaton P, Ramsay M (2005). Growing orchids from seeds. Kew: Royal Botanic Gardens.

Seeni S, Latha PG (1992). Foliar regeneration of the endareged Red

Vanda, *Renanthera imschootiana* Rolfe (Orchidaceae). Plant Cell Tissue Org. Cult. 29:167-172.
Seeni S, Latha PG (2000). *In vitro* multiplication and ecorehabilitation of the endangered Blue Vanda. Plant Cell Tissue Org. Cult. 61:1-8.
Singer RB, Koehler S, Carnevali G (2007). *Brasiliorchis*: a new genus for the *Maxillaria picta* Alliance (Orchidaceae, Maxillariinae). Novon 17(1):91-99.
Singer RB, Koehler S (2004). Pollinarium morphology and floral rewards in Brazilian Maxillariinae (Orchidaceae). Ann. Bot. 93:39-51.
Suzuki RM, Moreira VC, Pescador R, Melo Ferreira W (2012). Asymbiotic seed germination and *in vitro* seedling development of the threatened orchid *Hoffmannseggella cinnabarina*. *In Vitro* Cell. Dev. Biol. Plant 48:500-511.
Teixeira da Silva JÁ, Dobránszki J (2013). Plant thin cell layers: a 40-year celebration. J. Plant Growth Regul. 32:922-943.
Wu K, Zeng S, Teixeira da Silva JA, Chen Z, Zhang J, Yang Y, Duan J (2012). Efficient regeneration of *Renanthera* Tom Thumb 'Qilin'from leaf explants. Sci. Hortic. 135:194-201.
Zeng S, Wu K, Teixeira da Silva S, Zhang J, Chen Z, Xia N, Duan J. (2012). Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh, an endangered terrestrial orchid. Sci. Hortic. 138:198-209.