CAPSULE DEVELOPMENT, *IN VITRO* GERMINATION
AND PLANTLET ACCLIMATIZATION
IN *PHRAGMIPEDIUM HUMBOLDTII, P. LONGIFOLIUM* AND *P. PEARCEI*

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Abstract. Capsule development from pollination to full ripeness was evaluated in *Phragmipedium longifolium*, *P. pearcei* and *P. humboldtii*. Besides, seed viability, analyzed in each capsule by means of the tetrazolium chloride staining, was determined. Considering seed viability, germination rate was corrected and expressed as the rate of viable seeds that germinated in the presence and absence of light, on Knudson C and on half-strength Murashige and Skoog culture media. Capsule length remained constant during the evaluation period, while the diameter increased during the first 6-8 weeks and then stagnated. Capsule opening occurred 16 weeks after pollination in *P. longifolium*, after 9.8 weeks in *P. pearcei* and after 32 weeks in *P. humboldtii*. Seed viability averaged 44.7% in *P. longifolium*, 82.3% in *P. pearcei* and 34.3% in *P. humboldtii*. No significant effect of light conditions was evident in any of the species. However, a higher proportion of seeds of *P. longifolium* and *P. pearcei* germinated earlier on half-strength Murashige and Skoog medium than on Knudson C. Only 2.9% of the viable seeds of *P. humboldtii* germinated, while approximately 40% germination occurred in the other two species. Initial growth of the embryos was better in the dark on Knudson C medium, compared to the other treatments studied. Further growth of the seedlings took place under light conditions. Developed plants formed roots and were successfully acclimatized in the greenhouse.

Key words: capsule development, *in vitro* seed germination, pollination, *Phragmipedium*, terrestrial orchids, tetrazolium chloride, tropical orchids

Introduction

Slipper orchids belonging to the genus *Phragmipedium* (Subfamily Cypripedioideae Lindl.) are distributed in Meso and South America (Cox *et al.* 1998, Dressler 2003). They are seriously threatened because of alteration and destruction of their habitat and over collection from their natural environment (Arditti 1992, Salazar 1996).

Use of *in vitro* protocols has been foreseen as a successful approach for ex-situ conservation and reintroduction of endangered orchids (Stenberg and Kane 1998, Decruse *et al.* 2003, Sarasan *et al.* 2006). Plants regenerated from seeds have a broader genetic background than those developed by clonal propagation methods. Therefore, the former meet the goals of a reintroduction program better, in the sense of warranting sufficient genetic resources in the reintroduced population to undergo adaptive evolutionary change (Guerrant and Kaye 2007).

This strategy has been successfully employed for the reintroduction of the orchid species *Bletia urbana* (Rubluo *et al.* 1989), *Ipsea malabarica* (Gangaprasad *et al.* 1999) and *Spiranthes brevilabris* (Steward *et al.* 2003). An additional advantage of mass-propagating orchids for conservation purposes is that increasing availability of plants from preferred species with adequate phytosanitary standards and at affordable prices would reduce illegal collection from the wild populations (Ramsay and Dixon 2003, Salazar and Mata 2003).

Propagation of *Phragmipedium* through seeds does not seem to be extremely difficult, because commercial formulations for asymbiotic germination in this genus are available. However, the composition

of the culture media has remained elusive to the scientific community for commercial reasons. Most research on this and related genera is limited to studies on systematics and evolution (e.g., Cox *et al.* 1997, 1998). Investigations on phenology in this genus, describing characteristics of seed capsules and their development, are scarce (Arditti and Ghani 2000).

When proper conditions for germination are to be assessed, it is important to know the viability of the seeds under study. This allows distinguishing the proportion of seeds that do not germinate as a consequence of unfavorable germination conditions from those seeds that are not capable of germinating at all due to lack in viability. The most common method used to evaluate seed viability is the tetrazolium ($C_{19}H_{15}ClN_4$) stain. Dehydrogenases, which are active in living tissues, reduce the colorless tetrazolium chloride to a red compound, coloring viable seeds. The intensity of the tint could vary from pink to dark red (Singh 1981). This technique has been successfully used to test viability of orchid seeds (Lauzer *et al.* 1994, Vujanovic *et al.* 2000).

The aims of this study were to describe the development of *P. humboldtii, P. longifolium*, and *P. pearcei* capsules, to evaluate the seed viability and to establish a method for *in vitro* germination of mature seeds and for growing and acclimatizing plantlets of these three *Phragmipedium* species aiming at conservation.

Materials and methods

Pollination of flowers and capsule development. —
Manual pollination of flowers of *Phragmipedium humboldtii, P. longifolium*, and *P. pearcei*, growing at Lankester Botanical Garden, Universidad de Costa Rica, was conducted using pollen from a different plant. Capsule length, diameter and color were evaluated weekly, until full maturity. The time taken for each capsule to open was also recorded.

Seed viability. — The percentage of viable seeds was determined in each ripe capsule using the method of tetrazolium stain (Singh 1981). For that purpose, four subsamples of each of the two *P. humboldtii,* one *P. longifolium,* and four *P. pearcei* capsules were placed in tetrazolium chloride (1%, pH 6-7), in a water bath

(30°C) and in dark conditions for 24 h. Subsequently, they were transferred to Petri dishes and the percentage of viable (stained) seeds was determined with aid of a stereomicroscope (model 222279, Nikon). Seeds from the corresponding capsules were used in the germination studies.

In vitro *germination*. — Seeds from open capsules were sterilized in sodium hypochlorite (NaOCl, 0.6% w/v) and Tween 20 (1 drop/100 ml) for 10 min. They were subsequently screened through sterile Albet ® filter paper (quantitative quality), in the laminar hood. They were then rinsed three times with sterile distilled water, while adhered to the filter paper and, after decanting the water of the last rinse, distributed evenly, with a scalpel (blade No. 22) on 20 ml of semisolid culture medium contained in 90 mm-Petri dishes.

Knudson C (Knudson 1946) and half-strength Murashige and Skoog (1962) mineral salts, both supplemented with 1 mg l^{-1} thiamine, nicotinic acid and pyridoxine, together with 20 g l^{-1} sucrose, were compared for seed germination. pH was adjusted to 5.7 and media gelled with agar (0.8%). Media were autoclaved at 1.05 kg cm^{-2} for 25 min.

Additionally, two light regimes were evaluated during germination: dark conditions and photoperiod of 12 hours (10.9 μmol m^{-2} s^{-1}, Sylvania Supersaver Cool White, 32 W, F48%12/CW/SS). Cultures were grown at 25±1°C.

To determine the percentage of germination, three squares, of 1 cm^2 each, were drawn in each Petri dish. Areas of the Petri dishes in which individual seeds could be clearly observed using a stereomicroscope were selected to draw the squares. Total number of seeds in each section was counted at the first day of culture. Afterwards, the number of germinating seeds (those in which rupture of the testa occurred by the enlarging embryo) was assessed weekly. Three Petri dishes were evaluated for every combination of culture medium, light condition and species. Considering seed viability, germination rate was corrected and expressed as the rate of viable seeds that germinated in each treatment. For example, if 50% of the seeds of a capsule with a 50% germination rate, according to the tetrazolium test, germinated on culture medium, it was considered that 100% of the

viable seeds germinated. Besides, the percentage of dead protocorms (those that turned brown after germination) was calculated.

Protocorms growing in the dark were transferred to the same light conditions of the other treatment after six weeks of culture. Growing protocorms were subcultured every six weeks on the corresponding culture medium. Only actively growing and green protocorms were selected and individually subcultured. When the plantlets reached 1 cm in height, they were transferred into glass flasks (150 ml), eight plants per flask. Later on, when they were 2-3 cm tall, only five plants were cultured per flask.

Plants were acclimatized approximately 15 months after culture beginning, when they were 4-5 cm tall and had 2-3 roots around 1 cm in length. For that purpose, all remnants of the gelled culture medium were carefully removed from the roots with running water. One plant was potted per plastic container (3 x 3 x 4 cm), carefully covering the roots completely with the potting mix (peat moss). Plants were placed under mist irrigation (4 s watering every 15 min during the daylight hours). After six months in the greenhouse, the average number of roots and the size of the longest root were annotated.

Statistical analysis. — Capsule length and diameter, as well as the effect of culture medium and light conditions on germination for each species were analyzed with a multivariate test of significance (for repeated measures). Days needed for full maturity were compared between genotypes with an analysis of variance. Subsequently, the Post hoc Tukey's Honest-Significant-Difference-Test (HSD) for unequal N (Spjotvoll/Stoline) was used to determine significant differences in the parameters tested ($p<0.05$). All analyses were conducted using Statistica 6.1 (StatSoft Inc., Tulsa, Oklahoma, U.S.A.).

Results

Pollination of flowers and capsule development. — Blooming was observed in plants of *P. longifolium* and *P. pearcei* during the entire year, while *P. humboldtii* plants bloomed only in May and June. All manual-pollinated flowers (three of *P. humboldtii,* nine of *P. longifolium* and 16 of *P. pearcei*) developed capsules.
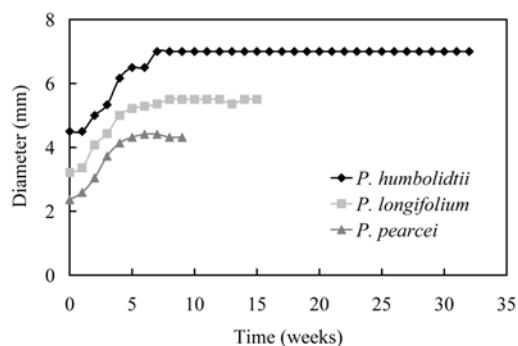

Figure 1. Diameter measured in capsules of *P. humboldtii, P. longifolium* and *P. pearcei* during development.

Capsule development, from pollination to opening, took longer in *P. humboldtii* (32 ± 0.6 weeks), than in *P. longifolium* and *P. pearcei* (16 ± 2.05 and 9.8 ± 0.6 weeks, respectively).

Significant differences ($p<0.001$) were measured in the capsule size of the three species (Fig 1). Largest capsules were those of *P. humboldtii*, with an average length of 182.7 ± 3.2 mm and a diameter of 7.0 ± 0.2 mm one week before opening. Capsules of *P. longifolium* were 60.02 ± 2.0 mm in length and 5.6 ± 0.1 mm in diameter, while those of *P. pearcei* were 42.5 ± 1.4 mm in length and 4.2 ± 0.1 mm in diameter at the same moment (Fig. 1). During development, length of the capsules did not vary in any of the species studied ($p>0.05$), while diameter increased during the first 6-8 weeks after pollination and then remained constant (Fig 1).

Capsules of *P. humboldtii* were light green and pubescent (Fig. 2A), while those of *P. longifolium* were dark purple without pubescence (Fig. 2B); capsules of *P. pearcei* were green-purple or totally purple, with short hairs (Fig. 2C). The capsules did not present notable changes in color or pubescence degree during maturation, except for those of *P. humboldtii,* which turned yellow 4-6 weeks before opening.

Seed viability. — A great difference in seed viability was observed between *P. pearcei* and the other species (Fig. 3). Average seed viability in the capsules of *P. humboldtii* was 34.3%, in *P. longifolium* 44.7% and in *P. pearcei* 82.3%. When the seed viability among different capsules of a single species was compared, values ranging from 20 to 80% were observed in *P. pearcei* and from 18 to 51% in *P. humboldtii*.
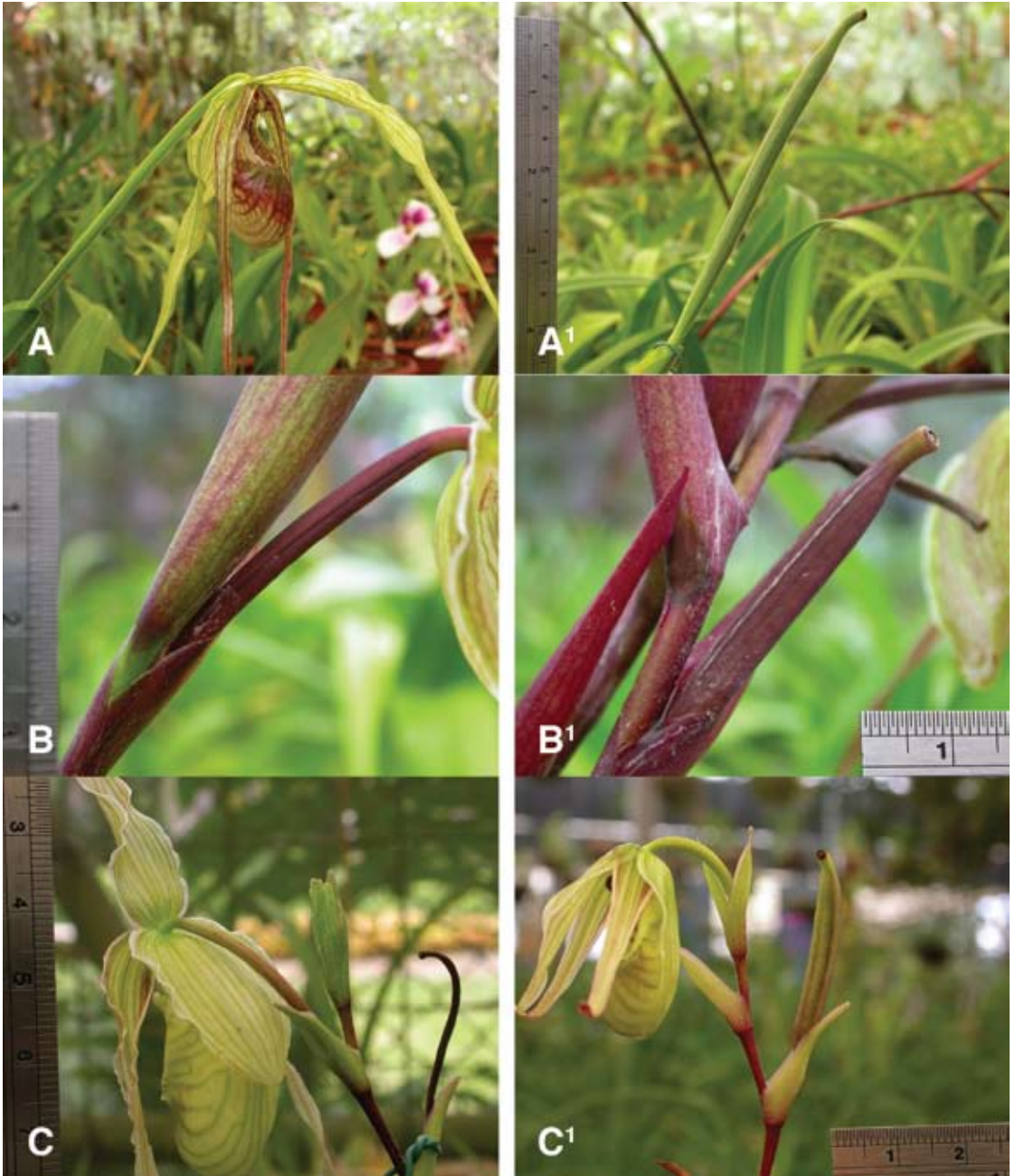
FIGURE 2. Development of capsules of *P. humboldtii* (A, A[1]), *P. longifolium* (B , B[1]) and *P. pearcei* (C , C[1]) at pollination day (left) and one week before capsule opening (right).

In vitro germination. — In order to consider only the viable seeds when calculating germination rate, percentage of seeds that germinated in the culture medium was corrected with the rate of viable seed measured for each capsule, as described in Materials and Methods. The corrected percentage of germination was lower in *P. humboldtii* (2.9%) than in *P. pearcei* (38.7%) and *P. longifolium* (41.3%), without significant differences between the last two (Fig. 3). Developing protocorms that subsequently died were observed only in *P. pearcei* (Fig. 3).
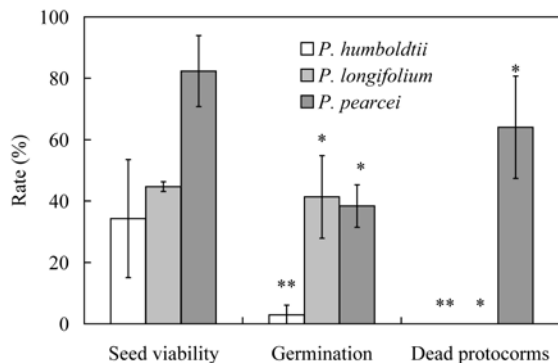
Figure 3. Seed viability, germination rate corrected with the percentage of seed viability of each capsule and death rate of germinated seeds in *P. humboldtii, P. longifolium* and *P. pearcei.* Data represent the mean of at least three replicates ± SD. *Four weeks after sowing. **Eight weeks after sowing.

Viable seeds of *P. longifolium* and *P. pearcei* started geminating during the first two weeks of culture. Afterwards, the germination rate decreased until, at week four, no additional germinating seeds were observed. On the other hand, germination in *P. humboldtii* occurred in a very low rate, with only a slight increase during the evaluated period of eight weeks (Fig. 4).

Culture medium had a significant effect on seed germination (Table 1), evident in *P. longifolium* and *P. pearcei* only during the first week by a higher germination rate on half-strength Murashige and Skoog medium. Thereafter, seed germination in these two species behaved very similarly (Fig. 4), without significant differences according to the Post-Hoc
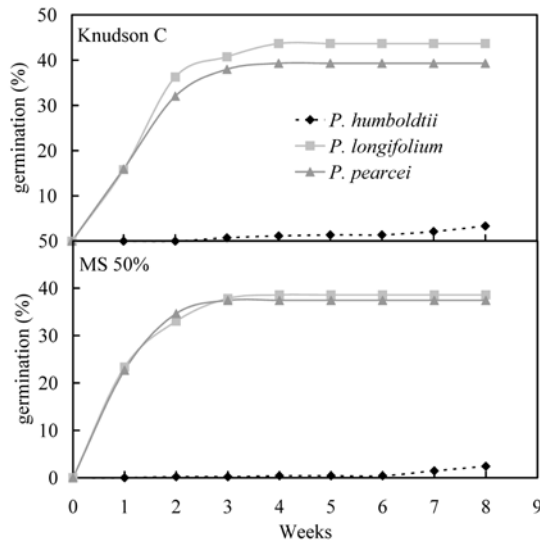

Figure 4. Germination rate in seeds of *P. humboldtii, P. longifolium* and *P. pearcei* along time on Knudson C and half-strength Murashige and Skoog media (MS 50%).

Tukey's Honest-Significant-Difference-Test (HSD) for unequal N (Spjotvoll/Stoline).

No effect of the light conditions tested could be observed on seed germination in any of the evaluated species (p>0.05) as presented in Table 1. However, among the protocorms cultured on the Knudson C medium, a higher growth rate was observed afterwards in those germinated under dark conditions. The protocorms germinated in the dark were placed under a photoperiod of 12 hours of light after six weeks of culture, and one week later they began to change their color from white to green. By that time,

Table 1. Multivariate test of significance of seed germination corrected with the viability rate for each capsule of the three *Phragmipedium* species studied in response to culture medium and light conditions.

| Source of variation	Test	Value	F	Effect df	Error df	p
Intercept	Wilks	0.031021	226.4653	4	29	0.000000
Species (A)	Wilks	0.038002	29.9406	8	58	0.000000
Culture medium (B)	Wilks	0.451880	8.7941	4	29	0.000089
Light conditions (C)	Wilks	0.757612	2.3195	4	29	0.080649
A×B	Wilks	0.469325	3.3328	8	58	0.003338
A×C	Wilks	0.421866	3.9122	8	58	0.000948
B×C	Wilks	0.691800	3.2299	4	29	0.026159
A×B×C	Wilks	0.454910	3.4992	8	58	0.002320
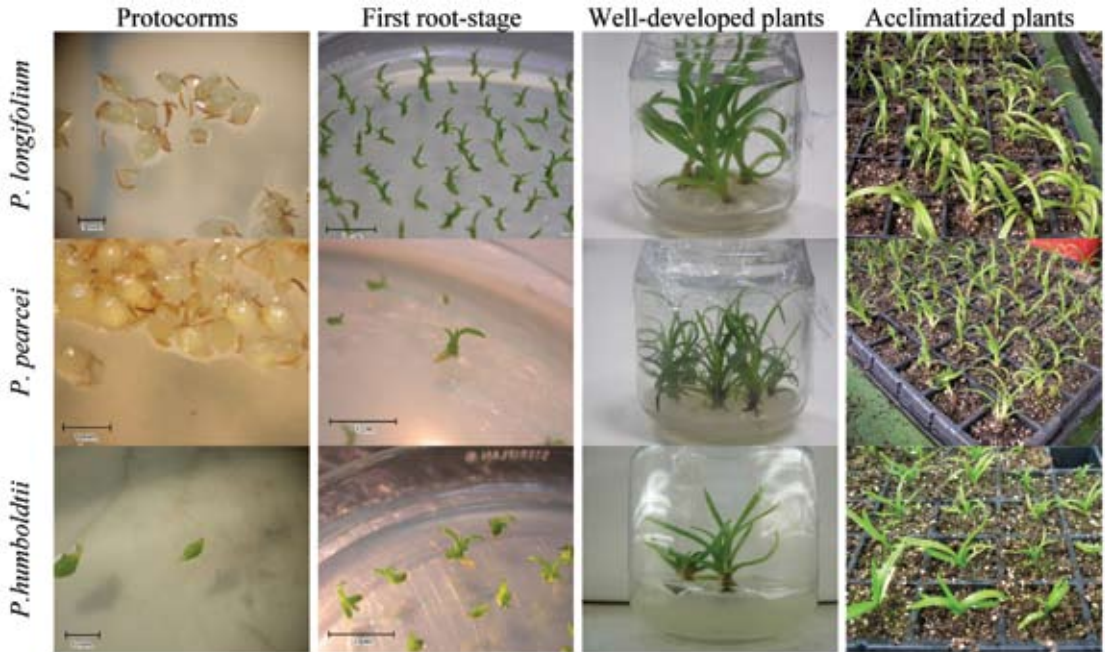
FIGURE 5. Stages during *in vitro* development of *P. pearcei, P. longifolium* and *P. humboldtii* cultured on MS 50%.

the protocorms of *P. pearcei* and *P. longifolium* began to develop the first leaf. On the other hand, the first leaf of *P. humboldtii* appeared in the eighth week of culture. The seedlings of *P. pearcei* and *P. longifolium* developed two or more leaves within the first three months, and began to develop roots in 3.5 months (Fig. 5). The plantlets of *P. humboldtii* developed their leaves in three months too, but began to develop roots only after five months in culture (Fig 5). Higher growth rate and better leaf development was observed with Knudson C (data not shown).

All plants were successfully acclimatized after ca. 15 months of culture *in vitro* (Fig. 5). After six months in the greenhouse, each plant of *P. pearcei* had 7.3±2.5 roots, with the longest one measuring 10.4±1.7 cm in length. At the same moment, the plants of *P. longifolium* had 3.6±0.9 roots, the longest one with 11.7±3.5 cm.

Discussion

The time it takes an orchid capsule to reach full-maturity varies according to genus and species. Times registered in this work for *P. pearcei, P. longifolium* and *P. humboldtii* (10, 16 and 32 weeks, respectively)

were considerably shorter than those measured in other species belonging to the Cypripedioideae subfamily. For example, *Paphiopedilum callosum* capsules required between 47 and 48 weeks to open (Arditti 1992). Moreover, it is not unusual to observe differences in this respect between species belonging to the same genus. For example, while capsules of *Laelia gouldiana* needed 17 weeks to open, those of *L. purpurata* required 31 weeks. Similarly, in *Dendrobium kingianum* the time until full ripeness was 20 weeks, while in *D. nobile* it was 41 weeks (Arditti 1992).

Capsule development in the three *Phragmipedium* species studied characterized by an initial growing period of around eight weeks, followed by a maturation period in which the fruit did not increase in size until opening (Fig. 1). This growing pattern is commonly observed in many fruits. It is characterized by a sigmoid arrangement, in which cell division and elongation initially occur, followed by growth halt and finally the fruit reaches maturity (Agustí 2000). Those phases could vary in duration, depending on the species or genotype, as it was observed in the three *Phragmipedium* species studied here.

Knowing the time the capsule of a particular orchid species requires until full maturity is very useful to determine proper harvesting time for *in vitro* germination. Ripe capsules usually have larger amounts of viable seeds than unripe ones. Moreover, collecting seeds after capsule opening usually reduces success during *in vitro* establishment due to contamination problems and damage caused by the sterilization process, and should be avoided when possible.

Seed viability measured in *Phragmipedium* (Fig. 3) is similar to that observed in other terrestrial orchids. For example, the seed viability of *Cypripedium acaule* varied from 20% to 40% in the capsules studied by Lauzer *et al.* (1994), and the viability of *Calopogon tuberosus* was 35% in the study conducted by Kauth *et al.* (2006). High values, as measured in *P. pearcei* in this study, have been observed in mature capsules of *Ophrys* (Kitsaki *et al.* 2004). Given that seed viability can vary significantly among capsules within the same species (up to 60%, as observed in *P. pearcei*), it is important to evaluate viability in each capsule. This is necessary to assess the real effect of particular conditions on seed germination based on the actual amount of viable seeds per capsule.

The seeds of *P. pearcei* and *P. longifolium* germinated faster (2-3 weeks) (Fig. 4) than those of several terrestrial orchids. Henrich *et al.* (1981) showed that 7-12 weeks were necessary for the beginning of germination in 17 terrestrial orchid species. In the study of Shiau *et al.* (2002), the seeds of *Anoectochilus formosanus* germinated eight weeks after sowing. On the other hand, there are other terrestrial orchids that germinate faster, as in the case of *C. tuberosus*, whose seeds germinated one week after sowing and reached the maximum germination in 4-6 weeks (Kauth *et al.* 2006).

The percentage of germination in terrestrial orchids varies among species. Henrich *et al.* (1981) found 100% germination in *Orchis fucssi* and *Epipactis gigantea*, 75% in *Goodyera oblongifolia* and *Spiranthes romanzoffiana*, 50% in *Platanthera stricta* and *Orchis macula*, and 25% in other seven species, *Platanthera dilatata*, *Liparis laeselii* and *Cypripedium reginae* among them. These authors also measured very low germination rates (1%) in *Cypripedium calceolus*, *C. candidum*, *Plantanthera hiperboria* and *P. flava*. However, they did not evaluate the viability of the seeds used; therefore the real percentage of germination of viable seeds could not be determined.

The seeds of *P. humboldtii* had very low germination rate (2.9%) in spite of their relatively high viability (34.3%). However, the large quantity of seeds in each capsule (data not shown) and the low rate of dead protocorms (Fig. 3) allowed the regeneration of enough plants using the protocol developed in this work for conservation and reintroduction of this species.

It has been considered that both germination and embryo staining (e.g., with tetrazolium chloride) are comparable viability tests. However, although the latter test has been successfully employed with epiphytic tropical orchids and several European and North American terrestrial orchids, there are some reports on inconsistencies in several species, probably attributed to variation in the permeability of the seed coat (reviewed by Vujanovic *et al.* 2000). In one of these cases, Lauzer *et al.* (1994) did not find any correlation between the percentage of germination and the percentage of seeds stained with tetrazolium chloride in *Cypripedium acaule*. They attributed these differences to a prolonged pretreatment with NaOCl (40 min with 0.6% w/v NaOCl, as compared to 10 min with the same concentration in our study), a compound that can promote dormancy release in terrestrial orchid seeds (St-Arnaud *et al.* 1992).

Numerous orchid species grow better in the dark, mainly during the first phases of development. Activated carbon has also been used to darken the culture medium and, in this way, improve germination (Arditti and Ernst 1993). In some species, the role of light during germination is not clear; for example, some authors recommend germination of *C. tuberosus* in the dark and others in light conditions (reviewed by Kauth *et al.* 2006). The light or dark treatments did not have any effect over germination in the *Phragmipedium* species studied. However, larger protocorms in the three species were obtained in dark conditions. The protocorms that germinated in the dark were white because they do not produce

chlorophyll; when they were placed in the light, the chlorophyll synthesis began, and their color changed to green, as observed in other orchids (Arditti & Ernst 1993).

A considerable number of culture media has been successfully employed and even devised specifically for orchid seed germination, some of them being only slight modifications of others (Arditti 1967). Knudson C (Knudson 1946) and Murashige and Skoog (1962) mineral salts are probably the most commonly used formulations for asymbiotic germination of orchid seeds. Since the latter one contains high amounts of nitrogen, especially in form of ammonium, dilutions are frequently employed, such as in this investigation. For many orchids, the effect of media on germination and development appears to be genotype dependent. When Knudson C and half-strength Murashige and Skoog media were compared, some genotypes developed better in the former and others in the latter (Johnson and Kane 2007). There are also examples of equal response in both media (Roy and Banerjee 2002). This behavior could be related to the differences in the ammonioum:nitrate ratio of both media. Contrary to the results obtained with *Phragmipedium* in this work, half-strength Murashige and Skoog promoted better development of the developing seedlings of hybrid *Vanda*, as compared to Knudson C (Johnson and Kane 2007).

Although, to the best of our knowledge, acclimatization of *in vitro*-generated *Phragmipedium* orchids has not been reported in the scientific literature, this task should not be very difficult, since it seems to be routinely conducted by commercial growers. Very high survival rates during acclimatization, as reported in the present work, indicate that plants generated *in vitro* were in adequate conditions and could adapt with few problems to the reduced relative humidity in the greenhouse and become autotrophic. This seems to be the case in the present work, since 100% success was obtained in this step.

In this work an efficient protocol for seed germination and plantlet growth of three *Phragmipedium* species was described. The combination of Knudson C medium and culture in the dark during the first six weeks seems to be appropriate to reach a good germination percentage

and initial protocorm development. Following the method described in this work it was possible to grow plants of the three *Phragmipedium* species studied and to achieve their acclimatization in the greenhouse. A great quantity of plants could be produced by *in vitro* germination, maintaining larger genetic variability than with clonal propagation. This is a useful tool for the conservation of these species.

LITERATURE CITED

Agustí, M. 2000. Crecimiento y maduración del fruto. *In*: Azcón-Bieto, J. & M. Talón (eds.). Fundamentos de Fisiología Vegetal. Mc Graw-Hill, Madrid, Spain, 419-433.

Arditti, J. 1967. Factors affecting the germination of orchid seeds. Bot. Rev. 33: 1-97.

Arditti, J. 1992. Fundamentals of orchid biology. John Wiley & Sons, U.S.A. 691 p.

Arditti, J. & R. Ernst. 1993. Micropropagation of orchids. John Wiley & Sons, New York, U.S.A. 682 p.

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

Cox, A.V., A.M. Pridgeon, V.A. Albert & M.W. Chase. 1997. Phylogenetics of the slipper orchids (Cypripedioideae, Orchidaceae): nuclear rDNA ITS sequences. Plant Syst. Evol. 208: 197-223.

Cox, A.V., A.M. Pridgeon & M.W. Chase. 1998. The utility of DNA sequence data in orchid systematics: an example from the slipper orchids (Cypripedioideae). *In*: C.E.B. Pereira (ed.). Proceedings of 15th World Orchid Conference. Naturalia, France, 265-273.

Decruse, S.W., A. Gangaprasad, S. Seeni & V.S. Menon. 2003. Micropropagation and ecorestoration of *Vanda spathulata*, an exquisite orchid. Plant Cell Tiss. Organ Cult. 72: 199-202.

Dressler, R.L. 2003. Orchidaceae. *In:* Hammel, B.E., Grayum, M.H., Herrera, C. & Zamora, N. (eds.). Manual de plantas de Costa Rica, vol III: Monocotiledóneas (Orchidaceae-Zingiberaceae). Missouri Botanical Garden. Saint Louis, Missouri, U.S.A., 1-595.

Gangaprasad, A.N., W.S. Decruse, S. Seeni & S. Menon. 1999. Micropropagation and restoration of the endangered Malabar daffodil orchid *Ipsea malabarica.* Lindleyana 14: 38-46.

Guerrant, E.O. & T.N. Kaye. 2007. Reintroduction of rare and endangered plants: common factors, questions and approaches. Austr. J. Bot. 55: 362-370.

Henrich, J.F., D.P. Stimart & P.D. Ascher. 1981. Terrestrial orchid seed germination *in vitro* on a defined medium. J. Amer. Soc. Hort. Sci. 106: 193-196.

Johnson, T.R. & M.E. Kane. 2007. Asymbiotic germination of ornamental *Vanda*: in vitro germination and development of three hybrids. Plant Cell Tiss. Organ Cult. 91: 251-261.

Kauth, P.J., W.A. Vendrame & M. Kane. 2006. *In vitro* seed culture and seedling development of *Calopogon tuberosus.* Plant Cell Tiss. Organ Cult. 85: 91-101.

Kitsaki, C.K., S. Zygouraki, M. Ziobora & S. Kintzios. 2004. *In vitro* germination, protocorm formation and plantlet development of mature versus immature seeds from several *Ophrys* species (Orchidaceae). Plant Cell Rep. 23: 284-290.

Knudson, L. 1946. A new nutrient solution for the germination of orchid seed. Amer. Orchid Soc. Bull. 15: 214-217.

Lauzer, D., M. St-Arnaud & D. Barabé. 1994. Tetrazolium staining and *in vitro* germination of mature seeds of *Cypripedium acaule* (Orchidaceae). Lindleyana 9: 197-204.

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

Ramsay, M.M. & K.W. Dixon. 2003. Propagation science, recovery and translocation of terrestrial orchids. *In:* Dixon, K.W., S.P. Kell, R.L. Barrett & P.C. Cribb (eds.). Orchid conservation. Natural History Publications, Malaysia, 259-288.

Roy J., & N. Banerjee. 2002. Optimization of *in vitro* seed germination, protocorm growth and seedling proliferation of *Vanda tessellate* (Roxb.) Hook Ex G Don. Phytomorphology 52: 167-178.

Rubluo, A., V. Chávez & A. Martínez. 1989. *In vitro* seed germination and re-introduction of *Bletia urbana* (Orchidaceae) in its natural habitat. Lindleyana 4: 68-73.

Salazar, G.A. 1996. Conservation threats. *In:* Hágsater, E & V. Dumont (eds.). Orchids - Status survey and conservation action plan. IUCN. Gland, Switzerland and Cambridge, U.K., 6-10.

Salazar, V.M. & M. Mata. 2003. Micropropagación y conservación de orquídeas mexicanas en el Jardín Botánico Clavijero. Lankesteriana 7: 151-153.

Sarasan, V., R. Cripps, M.M. Ramsay, C. Atherton, M. McMichen, G. Prendergast & J.K. Rowntree. 2006. Conservation *in vitro* of threatened plants – progress in the past decade. *In Vitro* Cell. Devel. Biol. – Plant 42: 206-214.

Shiau, Y.J., A.P. Sagare, U.C. Chen, S.R. Yang & H.S. Tsay. 2002. Conservation of *Anoectochilus formosanus* Hayata by artificial cross-pollination and *in vitro* culture of seeds. Bot. Bull. Acad. Sin. 43: 123-130.

Singh, F. 1981. Differential staining of orchid seeds for viability testing. Amer. Orchid Soc. Bull. 50: 416-418.

St-Arnaud, M., D. Lauzer & D. Barabé. 1992. *In vitro* germination and early growth of seedlings of *Cypripedium acaule* (Orchidaceae). Lindleyana 7: 22-27.

Stenberg, M.L. & M.E. Kane. 1998. *In vitro* seed germination and greenhouse cultivation of *Encyclia boothiana* var. *erythronioides,* an endangered Florida orchid. Lindleyana 13: 101-112.

Steward, S.L., L.W. Zettler & J. Minso. 2003. Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. Selbyana 24: 64-70.

Vujanovic, V., M. St-Arnaud, D. Barabé & G. Thibeault. 2000. Viability testing of orchid seed and the promotion of colouration and germination. Ann. Bot. 86: 79-86.