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In vitro asymbiotic germination, protocorm development, and plantlet acclimatization of *Aplectrum hyemale* (Muhl. ex Willd.) Torr. (Orchidaceae)

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Lauzer, D., S. Renaut, M. St-Arnaud and D. Barabé (Institut de recherche en biologie végétale, Jardin botanique de Montréal, 4101 rue, Sherbrooke Est, Montréal, Québec, Canada H1X 2B2). *In vitro* asymbiotic germination, protocorm development, and plantlet acclimatization of *Aplectrum hyemale* (Muhl. ex Willd.) Torr. (Orchidaceae). J. Torrey Bot. Soc. 134: 344–348. 2007.—A method to significantly improve the germination and development of plantlets of *Aplectrum hyemale* was developed. Seeds of this terrestrial orchid were collected in a natural stand, surface disinfected and grown *in vitro* on a gellified growth medium in absence of symbiotic fungi. Seeds were sowed whole or after excision of the seed coat. Embryos that were removed from the seed coat showed a significant increase in germination and survival percentages compared to whole seeds. Embryos grew to form a ramified protocorm, irrespective of seed treatment, followed by plantlet formation. Plantlets were successfully acclimatized and transferred to soil conditions. Asymbiotic *in vitro* culture is therefore shown as a potential tool to produce viable plantlets for use in natural site restoration.

Key words: *Aplectrum hyemale*, orchid, rare species, seed coat, seed dormancy.

Tropical orchids can generally be easily germinated without a symbiotic mycorrhizal association under *in vitro* conditions. Terrestrial orchids, on the other hand, are generally much more recalcitrant to germinate and may also have a complicated dormancy pattern (Johansen and Rasmussen 1992). As such, they are one of the most difficult group of higher plants to grow from seeds. Orchid species from temperate climates have a strong seed coat, impermeable to water and nutrients, which is thought to be a significant factor in the often extended period of seed dormancy in natural habitats (Van Waes and Debergh 1986, Rasmussen 1995, Arditti and Ghani 2000). Since Knudson (1922) developed an asymbiotic germination technique, a growing number of orchid species have been produced *in vitro*. However, germination of temperate terrestrial orchids remains limited to a few genera (Oliva and Arditti 1984), notably species from *Cypripedium* (Leroux et al. 1995), *Spiranthes* (Zelmer and Currah 1997), *Platanthera* (Zettler and McInnis 1994), and *Ophrys* (Kitsaki et al. 2004). In fact, these species seem to have species-specific requirements making the development of a single technique for germination more difficult. Numerous procedures have been tested to overcome the factors thought to prevent germination (e.g., asymbiotic/symbiotic germination, use of mature/immature seeds, light/dark treatments, sonication, sterilization and scarification treatments) (Arditti and Ghani 2000, Ramussen 1995), enlightening our limited knowledge of the mechanisms implicated in the release of dormancy and the germination of terrestrial orchids.

Aplectrum hyemale (Muhl. ex Willd.) Torr., a temperate terrestrial orchid from northeastern North America, is considered rare in most locations of its distribution area (Brumback and Mehrhoff 1996, Richburg 2004, USDA 2006). Only two threatened locations of this species are still known in Quebec province (Eastern Canada) from the several ones where herbarium data indicates the plant originally grew (Lavoie 1994). It is the only representative of its genus and is being threatened by a multitude of events, most importantly by human encroachment on old growth deciduous forests, its natural habitat (Richburg 2004). The seeds of this species have little reserve and depend on a mycorrhizal association to develop in nature (Oliva and Arditti 1984). To our knowledge, there are no studies that have looked at the potential of *in vitro* culture to produce *Aplectrum hyemale* seedlings suitable for eventual reintroduction to its natural habitat, and *in vitro* asymbiotic culture

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technique from seeds has not yet been developed (Richburg 2004).

Studies by Lauzer et al. (1994) and Miyoshi and Mii (1988) have reported that breakdown of the seed coat can promote germination in terrestrial orchids. Moreover, the orchid seed coat is known to be highly resistant to water (Arditii and Ghani 2000) while water imbibition is vital to trigger germination (Fenner 1985, Yoder et al. 2000). The aim of this paper is therefore to assess the effect of manual seed coat removal on seed germination of *Aplectrum hyemale*.

Materials and Methods. ORIGIN AND VIABILITY OF SEEDS. Mature (ripe) fruits of *Aplectrum hyemale* were collected during fall (September) in a mature maple forest located at St-Armand, Quebec, Canada (45°2′N, 73°2′W). Seeds were removed from the fruits, air dried and stored in small bottles with blue silica gel for 2 months at 4°C. For surface disinfection, seeds were placed in small bags made of a Nitex nylon membrane (100 μm mesh, Sefar Canada Inc.), dipped and agitated in a 0.6% sodium hypochlorite solution containing 0.1% Tween 20 for 20 min at 200 rpm, and rinsed three times with sterile water. Viability of the seeds was estimated by staining in 1% 2, 3, 5-triphenyl-tetrazolium chloride (TTC) solution (pH 6.5) for 4 days at 32 ± 1°C (Lauzer et al. 1994). Orange to red embryos were considered as viable.

EXCISION OF EMBRYOS AND GERMINATION. After surface disinfection, a group of seeds was used for excision of the seed coat. The embryos were extracted from the seed coat under sterile conditions using surgical dissecting needles under a binocular microscope. Whole seeds and isolated embryos were then sowed in 100 mm diameter Petri dishes filled with 20 ml of germination medium containing MS salts (Murashige and Skoog 1962), thiamine HCl (5 mg l^{-1}), nicotinic acid (10 mg l^{-1}), calcium pantothenate (5 mg l^{-1}), kinetin (1 mg l^{-1}), naphtaleneacetic acid (0.1 mg l^{-1}), potato extract (1.2 g l^{-1}, Difco), dextrose (19 g l^{-1}) and Bacto-agar (7 g l^{-1}, Difco) in distilled water (St-Arnaud et al. 1992). Five trials were performed. Each trial included 5 replicates of 80 to 150 whole seeds each and 5 replicates of 10 to 15 embryos each. Cultures were incubated for 6 months in the dark at 25°C. At one-month intervals, all seeds or embryos were examined under a binocular microscope and germination was noted. Embryos that had doubled in size were considered as having begun germinating. Among the germinated seeds or embryos, protocorms that survived and developed into a plantlet were also assessed after 6 months of culture. Individuals were ranked in four different classes; stage 1: embryo doubled in size, stage 2: protocorm without differentiation, stage 3: first scale, and stage 4: first leaf. Individuals in stage 1, 2 or 3 were considered in the germination stage, while individuals in stage 4 were considered as viable seedlings. Seedlings were then transferred to GA-7 vessels (Magenta Corp. Chicago, IL) containing a growth maintenance media (Phytamax Orchid Maintenance Medium, Sigma-Aldrich P6668) without plant regulators, and incubated at 25°C under a 16 hour photoperiod in low light conditions provided by Cool White fluorescent lamps (Sylvania) at 35 μmol m^{-2} s^{-1}. Plants were subsequently transferred every three months to fresh growth maintenance medium. When transferred, old degenerating corms and leaves were removed and roots were trimmed to about 1 cm in length.

ACCLIMATIZATION OF PLANTLETS. Rooted seedlings (3 replicates of 10 seedlings) were transferred to soil (1:1 Pro-Mix and vermiculite) and acclimatized in greenhouse. The relative humidity was maintained at 100% for the first week, and was then gradually lowered to 40% over one week. The plantlets were grown under greenhouses conditions for 4 months at 23 ± 2°C, and then transferred into a cold room (4°C) for 6 months before being taken back to the greenhouse.

Results. EFFECT OF SEED COAT EXCISION ON GERMINATION. Surface disinfection had no effect on seed viability, as estimated with TTC staining (Table 1). Disinfected *Aplectrum hyemale* seeds showed a mean embryo viability of 65.9%. Excision of the seed coat significantly increased germination (Table 2). Undissected seeds germinated slowly and reached 2.1% after six months, while excised embryos germinated faster and reached 39.9%. Most germinated seeds or embryos did not reach the stage of fully formed protocorms (Fig. 1C), only grew to stage 1 or 2 (Fig. 1B), became orange-brown and died. Nevertheless, after a six month period, a small proportion of

Table 1. Viability of seeds of *Aplectrum hyemale* after 20 min surface disinfection in 0.6% sodium hypochlorite.

Treatment[1]	Total number	Percentage of viable seeds[2] (TTC Staining)
Disinfected	842	65.9
Control	311	63.0

[1] Seeds were surface disinfected for 20 min in a 0.6% sodium hypochlorite solution, or soaked in sterile water as control treatment.

[2] Values are not significantly different at the $P = 0.05$ level (χ^2 test, df = 1, $F = 19.3$).

germinated seeds or embryos survived and produced a protocorm that grew successfully to give rise to plantlets (Fig. 1D). Notably, this percentage was significantly higher for the dissected embryos (6.46%) than for the intact seeds (0.1%).

DEVELOPMENT FROM SEED TO PLANTLET. Ungerminated *Aplectrum hyemale* seeds showed globular embryos enclosed in the seed coat (Fig. 1A). During germination of intact seeds, embryos first grew to form a white round protocorm breaking the seed coat (Fig. 1B). This protocorm elongated and ramified to form a multiple branched structure (Fig. 1C). Protocorm development that successfully led to plantlet formation was similar whether the seeds were sown intact or the embryos had been excised. Green leaves differentiated on developing protocorms (Fig. 1D). Most of the time, the leaf first grew inside the culture medium before emerging in the culture vessel. As the leaf developed, a corm formed at its base (Fig. 1E). Once the corm had reached its maximum size (about 15 mm), the leaf turned brown and withered. One shoot, seldom two, developed on the corm, producing another leaf on a short rhizome on which roots were produced. This process went on continuously, on a 2 to 3 month basis under *in vitro* conditions. Eventually, several corms linked by short sections of rhizomes could be observed in the culture vessel. Old corms degenerated as new ones were produced (Fig. 1F). The ramified corms produced can be subdivided and transferred to fresh medium. This allowed for *in vitro* propagation of the plants. All the plantlets transferred to soil survived the acclimatization process and the 6 month cold treatment (Fig. 1G).

Discussion. This is the first report of successful germination of *Aplectrum hyemale* seeds *in vitro*, and of seedling development and acclimatization *ex vitro*. The results clearly show that seed coat limits germination of seeds under asymbiotic *in vitro* conditions.

Arguably, observing the seeds for a period of time longer than 6 months might have yielded slightly higher germination percentage.

Table 2. Effect of excision of the seed coat on the percentage of germination and plantlet development over a six months growth period.

		Percentage of seeds developed to growth stage[2]				
Treatment[1]	Incubation (months)	1	2	3	4	Total[3]
Whole seeds						
	1	0.8	0.0	0.0	0.0	0.8
	2	1.1	0.4	0.0	0.0	1.5
	3	1.2	0.4	0.0	0.0	1.5
	4	1.1	0.9	0.0	0.0	2.0
	5	1.0	1.0	0.1	0.0	2.1
	6	1.0	1.0	0.0	0.1	2.1
Isolated embryos						
	1	33.1	0.4	0.0	0.0	33.5
	2	23.2	13.3	1.1	0.0	37.6
	3	23.2	13.3	1.1	0.0	37.6
	4	13.7	20.5	4.9	0.0	39.2
	5	11.8	21.3	6.8	0.0	39.9
	6	11.8	21.3	0.4	6.5	39.9

[1] Total number of seeds inoculated was 2248 for undissected seeds and 263 for isolated embryos. Values were estimated from 5 independent trials, and each trial included 3–5 replicate Petri dishes with 50–200 seeds or 10–15 dissected embryos per dish.

[2] Growth stage: 1 = embryo doubled in size (germinated), 2 = protocorm without differentiation, 3 = first scale, and 4 = first leaf (viable plantlet).

[3] Total percentage of seeds or embryos developed over the six months growth period.

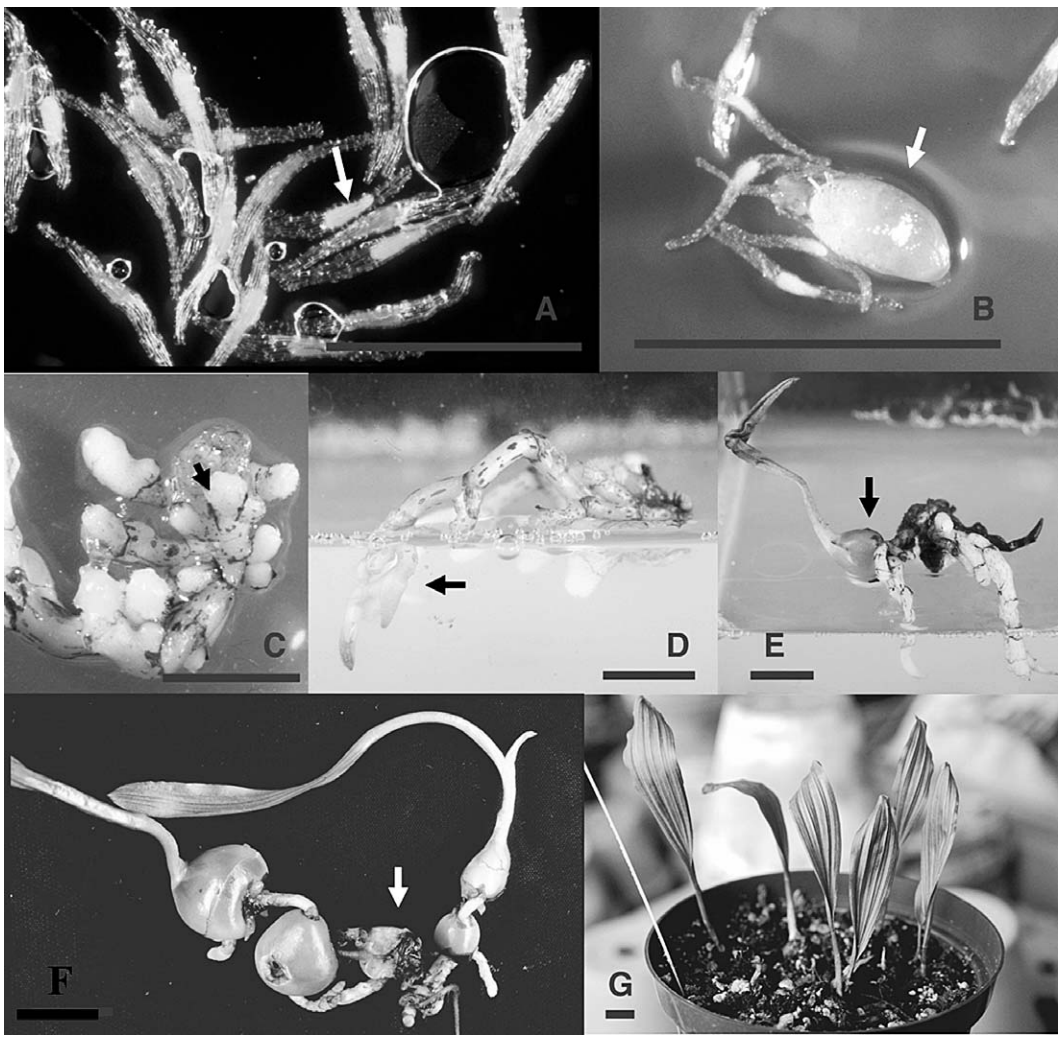

FIG. 1. *Aplectrum hyemale* growing asymbiotically *in vitro* at various phases of its development: A. Ungerminated seeds with the globular embryos enclosed in the seed coat; B. Germinating seeds showing embryos forming a white round protocorm breaking the seed coat (stage 2); C. Germinating seeds at a later stage showing protocorms elongated and ramified to form a multiple branched structure (stage 3); D. Plantlet formation from successful protocorms showing green leaves differentiated (stage 4); E. A corm formed at the base of the leaf; F. Plantlet with leaves showing old degenerating corms; G. *A. hyemale* plantlets acclimatized and transferred to soil conditions. Scale bar = 1 cm.

However, most seeds that were still in the stage 1 or 2 after a 6 month period did not survive and eventually died (data not shown), such that only seeds in the stage 3 could have potentially produced more viable plantlets. This percentage is small (0% for the undissected seeds and 0.4% for the dissected embryos) and would not have significantly altered the results.

The germination and plantlet formation rates obtained with the culture of excised embryos is lower than the proportion of seed viability estimated by TTC staining. This discrepancy between the proportion of viable seeds and those that germinate might reflect special requirements for nutrients that were non-optimal in the germination conditions used in the present study. It has also previously been pointed out that the TTC staining assay, based on the activity of dehydrogenases, could produce an over-estimation of seed viability (Lauzer et al. 1994, Vujanovic et al. 2000).

Our results suggest that seed coat imposed dormancy and that the embryo is not dormant

and can germinate when removed from the seed coat. The inhibition of germination probably reflect an ecological adaptation of the species which produces seeds in the fall but which should only germinate the following spring. By preventing water imbibition of the seed (Arditii and Ghani 2000), the seed coat must therefore play a key role in preventing early germination. It assures that seeds will only germinate once their seed coat has been damaged by the combined effects of frost and the activity of soil microorganisms, i.e., after a variable period of time that should lead to next spring (see Rasmussen 1995).

In vitro culture sheds new light on germination and early development of *Aplectrum hyemale*. While it is not possible to directly extrapolate *in vitro* results to *in vivo* conditions, the results show that this species can propagate asexually under certain conditions, and therefore its propagation may occur both asexually and sexually in nature. These aspects of the biology of the species had not been documented in the literature since they are very difficult to study in natural conditions due to the small size of the seeds and the rarity of the species. Despite the fact that the proportion of *in vitro Aplectrum hyemale* germinated seeds or embryos were relatively low, the present study shows that several plantlets can be produced vegetatively from a single germinating seed or embryo. Asymbiotic *in vitro* germination followed by vegetative multiplication can therefore be considered as a way to produce plantlets for restoration projects.

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