EX SITU GERMINATION AS A METHOD FOR SEED VIABILITY ASSESSMENT IN A PEATLAND ORCHID, PLATANTHERA BLEPHARIGLOTTIS

MARC-ANDRÉ LEMAY1,2,4, LAURENT DE VRIENDT2,4, STÉPHANIE PELLERIN3,4, AND MONIQUE POULIN2,4,5

2Université Laval, Pavillon Paul-Comtois, Faculté des sciences de l’agriculture et de l’alimentation, Département de phytologie, 2425 rue de l’Agriculture, Québec, QC, Canada, G1V 0A6; 3Institut de recherche en biologie végétale, Jardin botanique de Montréal, Université de Montréal, 4101 Sherbrooke Est, Montréal, QC, Canada, H1X 2B2; and 4Québec Centre for Biodiversity Science, McGill University, 191205 Dr. Penfield Avenue, Montréal, QC, Canada, H3A 1B1

1Manuscript received 10 October 2014; revision accepted 6 February 2015.

The authors thank D. Auclair, M. Paquet, L. Rochefort, J. L’Espérance-Hamel, M. Chénier and M. Labbé for technical support provided throughout the experiment, H. Crépeau for statistical advice, K. Grislis for stylistic revision of the manuscript, L. Zettler for providing some of the literature, and all the landowners who kindly granted permission to access the study sites. J. Freudenstein and two anonymous reviewers provided useful comments on a previous version of the manuscript. This study was supported by the NSERC: an Undergraduate Student Research Award to M.A.L., a Postgraduate Scholarship to L.D.V. and a Discovery grant to S.P. (RGPIN-2014-05367) and M.P. (RGPIN-2014-05663).

Seed germination or viability is routinely assessed in ecology, for example, to study ecotypic differentiation (Kauth and Kane, 2009) or plant reproductive success in response to habitat fragmentation (Menges, 1991), reproductive isolation (De hert et al., 2009) or plant reproductive success in response to habitat fragmentation (Rasmussen and Whigham, 1993; Rasmussen, 1995; Lauzer et al., 2007). Yet these impediments to viability assessment studies should be overcome, since the conservation status of many of the approximately 25 000 orchid species is of concern (Swarts and Dixon, 2009).

Several protocols have been developed to accommodate orchids’ stringent germination requirements. Seeds can be germinated in vitro on artificial media (e.g., Van Waes and Debergh, 1986b; Kauth et al., 2006) that provide the nutrients necessary for germination and development supplied in nature by mycorrhizal fungi. In vitro protocols can be either asymbiotic, in which seeds are germinated without fungal inoculum, or symbiotic, in which the culture medium is inoculated with mycorrhizal strains compatible with the studied species (Dowling and Jusaitis, 2012). In vitro asymbiotic protocols have been implemented successfully (Kauth et al., 2008, 2011; Kauth and Kane, 2009) to study ecotypic differentiation in Calopogon tuberosus nonviable seeds. Developing such protocols has been notoriously difficult in the case of orchids, owing to their small seed size and impermeable seed coat, physiological and physical dormancy patterns, and reliance on mycorrhiza for germination and subsequent development (Rasmussen and Whigham, 1993; Rasmussen, 1995; Lauzer et al., 2007). Yet these impediments to viability assessment studies should be overcome, since the conservation status of many of the approximately 25 000 orchid species is of concern (Swarts and Dixon, 2009).

Several protocols have been developed to accommodate orchids’ stringent germination requirements. Seeds can be germinated in vitro on artificial media (e.g., Van Waes and Debergh, 1986b; Kauth et al., 2006) that provide the nutrients necessary for germination and development supplied in nature by mycorrhizal fungi. In vitro protocols can be either asymbiotic, in which seeds are germinated without fungal inoculum, or symbiotic, in which the culture medium is inoculated with mycorrhizal strains compatible with the studied species (Dowling and Jusaitis, 2012). In vitro asymbiotic protocols have been implemented successfully (Kauth et al., 2008, 2011; Kauth and Kane, 2009) to study ecotypic differentiation in Calopogon tuberosus nonviable seeds. Developing such protocols has been notoriously difficult in the case of orchids, owing to their small seed size and impermeable seed coat, physiological and physical dormancy patterns, and reliance on mycorrhiza for germination and subsequent development (Rasmussen and Whigham, 1993; Rasmussen, 1995; Lauzer et al., 2007). Yet these impediments to viability assessment studies should be overcome, since the conservation status of many of the approximately 25 000 orchid species is of concern (Swarts and Dixon, 2009).

Several protocols have been developed to accommodate orchids’ stringent germination requirements. Seeds can be germinated in vitro on artificial media (e.g., Van Waes and Debergh, 1986b; Kauth et al., 2006) that provide the nutrients necessary for germination and development supplied in nature by mycorrhizal fungi. In vitro protocols can be either asymbiotic, in which seeds are germinated without fungal inoculum, or symbiotic, in which the culture medium is inoculated with mycorrhizal strains compatible with the studied species (Dowling and Jusaitis, 2012). In vitro asymbiotic protocols have been implemented successfully (Kauth et al., 2008, 2011; Kauth and Kane, 2009) to study ecotypic differentiation in Calopogon tuberosus nonviable seeds. Developing such protocols has been notoriously difficult in the case of orchids, owing to their small seed size and impermeable seed coat, physiological and physical dormancy patterns, and reliance on mycorrhiza for germination and subsequent development (Rasmussen and Whigham, 1993; Rasmussen, 1995; Lauzer et al., 2007). Yet these impediments to viability assessment studies should be overcome, since the conservation status of many of the approximately 25 000 orchid species is of concern (Swarts and Dixon, 2009).

Several protocols have been developed to accommodate orchids’ stringent germination requirements. Seeds can be germinated in vitro on artificial media (e.g., Van Waes and Debergh, 1986b; Kauth et al., 2006) that provide the nutrients necessary for germination and development supplied in nature by mycorrhizal fungi. In vitro protocols can be either asymbiotic, in which seeds are germinated without fungal inoculum, or symbiotic, in which the culture medium is inoculated with mycorrhizal strains compatible with the studied species (Dowling and Jusaitis, 2012). In vitro asymbiotic protocols have been implemented successfully (Kauth et al., 2008, 2011; Kauth and Kane, 2009) to study ecotypic differentiation in Calopogon tuberosus nonviable seeds. Developing such protocols has been notoriously difficult in the case of orchids, owing to their small seed size and impermeable seed coat, physiological and physical dormancy patterns, and reliance on mycorrhiza for germination and subsequent development (Rasmussen and Whigham, 1993; Rasmussen, 1995; Lauzer et al., 2007). Yet these impediments to viability assessment studies should be overcome, since the conservation status of many of the approximately 25 000 orchid species is of concern (Swarts and Dixon, 2009).
var. tuberosus, as well as to grow Dactylorhiza sambucina seeds to seedling stage to assess inbreeding depression in this species (Juillet et al., 2007). On the other hand, symbiotic protocols have been used successfully to study the effects of cold stratification on germination of Platranthera leucophaea (Bowles et al., 2002). However, both of these in vitro protocols require axenic conditions to avoid contamination by fungi and bacteria and can thus be difficult to implement without specialized equipment. Moreover, the development of in vitro protocols requires the selection of an appropriate medium for the germination of the species under study (see e.g., Kauth et al., 2006), which implies that experimental protocols for species whose germination has not been previously studied must incorporate an additional step. Symbiotic germination methods are generally more efficient in promoting germination than axenic protocols (Dowling and Jusaitis, 2012), but remain more difficult to implement due to the time and specialized knowledge required to isolate mycorrhizal fungal strains (e.g., Zettler and Hofer, 1998).

Protocols that germinate seeds on their natural substrates have also been developed. For instance, Rasmussen and Whigham (1993) devised an in situ orchid germination protocol by which seeds are sown in small packets, buried in the field, and recovered later to score germination. This protocol has been widely used by ecologists ever since, notably to study orchid seed banks (Whigham et al., 2006), availability of germination microsites (Jacquemyn et al., 2007), and effects of inbreeding depression on germination (Sletvold et al., 2012). However, time between seed sowing and retrieval can last several months to several years (e.g., Rasmussen and Whigham, 1993; Whigham et al., 2006), a clear tradeoff compared with the few weeks needed for germination assays conducted in the laboratory with an in vitro protocol.

To avoid germinating orchid seeds, ecologists have used other measures of plant reproductive success such as seed mass (Wallace, 2003) or capsule set (Newman et al., 2013). Some have also tested for seed viability using specialized stains (Wallace, 2003; Faast et al., 2011), most commonly triphenyltetrazolium chloride (TTC; Van Waes and Debergh, 1986a) or fluorescein diacetate (Pritchard, 1985). Yet, seed mass or seed number may not always accurately reflect the seeds’ potential to germinate (Wallace, 2003), and the viability rates provided by staining methods can be more or less accurate depending on the species (Dowling and Jusaitis, 2012) or even the ecotype (Kauth et al., 2011) under consideration. Therefore, viability testing seeds should preferably be conducted concurrently with germination tests, as either method may underestimate actual seed germinability. Clearly, adapting in situ germination protocols on natural substrates to ex situ protocols, which are more easily and rapidly implemented, would greatly facilitate ecological studies involving orchid germination potential. In this context, Brundrett et al. (2003) successfully germinated ex situ seeds from four different Australian orchid genera on substrate sampled from the study species’ natural habitat, in this case, sandy soil from urban Banksia woodlands. Germination can be scored after only a few weeks using this protocol. Although first developed to study mycorrhizal fungi distribution and inoculum potential, we hypothesized that this protocol may also be used as a method for seed germination assessment per se. In this study, we therefore tested whether an ex situ protocol based on a natural substrate could be used in ecological studies to rapidly assess the viability of orchid seeds by observing their germination. We carried out ex situ germination of Platranthera blephariglottis (Willd.) Lindley var. blephariglottis (referred to hereafter as P. blephariglottis; Orchidaceae) on Sphagnum substrate collected in bogs where the orchid grows naturally. We then compared the viability estimates obtained through germination with those obtained by viability staining using TTC. Since we observed great variability in seed testa color among individual plants and testa properties are known to influence viability staining results (Van Waes and Debergh, 1986a; Dowling and Jusaitis, 2012), we also tested whether intraspecific variation of this variable in P. blephariglottis had an effect on viability rates. We hypothesized that seed testa color would have a significant effect on viability rates estimated by the TTC method, but not on those estimated by the ex situ germination assay.

MATERIALS AND METHODS

Study species—Platranthera blephariglottis (Orchidaceae) is a terrestrial orchid native to eastern North America, from Newfoundland to Florida (Sheviak, 2002). In the province of Quebec, its presence is limited to ombrotrophic peatlands (bogs), where it usually grows in open habitats, frequently near Larix laricina (Du Roi) Koch and/or Picea mariana (Mill.) BSP thickets (FloraQuebeca, 2009). A recent study (Laroche et al., 2012) suggests that its presence and abundance are indicators of bog integrity, and consequently assessing seed viability in this species in the context of ecological studies is of particular interest. High in vitro germination rates (90% after 3 mo) of P. blephariglottis have been previously reported (Anderson, 1990), but our own attempts at in vitro germination were inconclusive (unpublished data) and the need for simpler seed viability assessment protocols remains for the reasons outlined earlier. Apparently, no other studies have been carried out on the germination of this species.

Capsule and sphagnum moss collection—Twenty populations of P. blephariglottis were selected for this study, each located in a distinct bog in southern Quebec. In this region, peatlands form an archipelago on lowlands dominated by deciduous and mixed forests, and agriculture. Average annual temperature (1981–2010) at the nearest meteorological station (Drummondville) is 6.4°C, and average annual precipitation is 1113.3 mm, about 22% of which falls as snow (Environment Canada, 2014). Platranthera blephariglottis flowers in July throughout the study area, and after dehiscence, seed dispersal takes place from the end of August throughout the fall and even into the winter. The maximum distance between populations was approximately 125 km.

Ten individuals per population were randomly selected for capsule collection. The selection was made by sampling the first individual found in 10 directions generated at random from a common point located in the center of the population. Four capsules were collected per individual between 29 and 31 August 2013, for a total of 800 capsules representing 200 individual plants. Capsules were mature but not yet dehiscent at the time of collection. Individuals bearing less than four intact capsules were rare, and were systematically excluded from selection. Capsules were kept in a desiccator with the desiccant Drierite (Drierite, Xenia, Ohio, USA) for 2 wk at room temperature. Seeds originating from the same individual were then pooled in 1.5 mL microtubes and kept in a refrigerator at 4°C for 3 mo prior to preparation of the germination plates and 6 mo before viability testing. Average seed testa color was determined visually for each individual on a four-level scale (1: dark brown testa, 2: light brown testa, 3: yellowish testa, 4: transparent testa) to assess the effect of seed testa color on viability staining and germination. Seed testa color was considered an indirect measure of suberin content of the testa, as darker seed coats usually contain more suberin (Van Waes and Debergh, 1986a). Heterogeneity in seed testa color was evident, but individuals were unevenly distributed between color classes, with 14 having dark brown, 39 light brown, 61 yellowish, and 86 transparent testae.

Sphagnum moss material was collected concurrently with the capsules in each of the 20 bogs. Approximately 2 L of Sphagnum mosses were collected from each site, from a depth between 5 and 15 cm from the surface and at a distance of less than 30 cm from one plant of P. blephariglottis, since the density of germination-promoting mycorrhizal fungi is likely to be higher in the vicinity of their host (Diez, 2007; Jacquemyn et al., 2007). Sphagnum material from all populations was then thoroughly mixed together so as to thread the mosses and distribute mycorrhizal fungi uniformly. The resulting mixture was kept at 4°C in plastic bags for 3 mo prior to use.
Viability staining using triphenyltetrazolium chloride (TTC)—Viability was tested using the standard TTC staining method according to a protocol adapted from Van Waes and Debergh (1986a). Preliminary tests were conducted to determine the optimal pretreatment soaking time in a sodium hypochlorite (NaClO) solution. Sodium or calcium hypochlorite pretreatments are routinely used in TTC staining protocols in orchids to weaken the impermeable testa and allow the stain to reach the embryo cells, but the pretreatment duration must be determined carefully to avoid killing the embryos through overexposure to hypochlorite (Dowling and Jusaitis, 2012). For that purpose, seeds from 20 randomly chosen individuals were pooled and then divided into 25 microtubes (1.5 mL). Seeds were treated with a 1% NaClO (m/v) + 0.1% Tween 20 (V/V) solution for a duration of either 0 (no NaClO pretreatment), 15, 30, 45, or 60 min, with five replicates of approximately 80 seeds for each pretreatment duration. Seeds from all five treatments were then thoroughly rinsed and soaked in distilled water for 24 h at room temperature in darkness. Following preliminary tests, seed viability was determined for approximately 100 seeds from each of the 200 individual plants sampled following the protocol described above, using a NaClO pretreatment time of 15 min, since this treatment yielded the best results (see Results).

Ex situ germination—For the ex situ germination assay, the 200 individuals were randomly distributed among 40 germination plates. Germination plates were prepared according to a protocol adapted from Brundrett et al. (2003). First, polystyrene petri dishes (100 mm diameter × 15 mm height) were filled to a height of about 1 cm with Sphagnum moss. A 90-mm-diameter nylon filter with a pore size of 100 µm (NY1H09000, EMD Millipore, Billerica, Massachusetts, USA) was then placed on top of the Sphagnum moss. Squares of 7 × 7 mm cut from nitrocellulose filters with a pore size of 0.45 µm (HAWP04700, EMD Millipore) were subsequently moistened with distilled water and placed on the nylon filter in five clusters of three squares each (Fig. 1). Approximately 100 seeds from a single individual were deposited on each cluster of filter squares, so that seeds from five different individuals were sown per plate.

Following sowing, plates were sealed using a single layer of parafilm to prevent drying, then wrapped in aluminum foil to avoid inhibiting germination, which can occur in case of exposure to light (Rasmussen, 1995). Petri dishes were kept in a refrigerator at 4°C for 11 wk to break potential seed dormancy and then incubated in growth chambers in darkness at 20°C for 9 wk. A cold treatment was applied, since this has been shown to promote germination of several temperate terrestrial orchids (Øien et al., 2008; Kauth et al., 2011). Following incubation, germinated seeds were scored under a dissecting microscope. All seeds that had either swollen noticeably and/or produced rhizoids were considered germinated, based on Zettler and McInnis (1994), who considered the production of rhizoids to be the first stage of germination in a related species, P. integrilabia. Kauth et al. (2006) also considered imbibition and swelling of the seeds to be the first evidence of germination in Calopogon tuberosus. Seeds were scored according to a binary scheme (germination occurred/did not occur) both to facilitate comparison with the TTC staining viability estimates, and because the vast majority of germinated seeds were found at the same germination stage, i.e., embryos noticeably swollen and presence of several, often long, rhizoids (Fig. 2).

Statistical analyses—A binomial model with hypochlorite pretreatment duration as fixed factor was used with the GLIMMIX procedure of SAS software (SAS Institute, 2011) to determine which pretreatment duration yielded the highest viability rates. A mixed binomial model was similarly used with the SAS GLIMMIX procedure to study the effect of seed testa color and method (ex situ germination and TTC staining) on the estimated viability rate of the

![Fig. 1](image1.png)

Fig. 1. Example of an ex situ germination plate used in this study. Each plate comprised five clusters of three filter squares, with approximately 100 seeds from one plant of *Platanthera blephariglottis* distributed on the three squares of each cluster. The 200 individuals were randomly distributed among 40 such plates. Scale bar = 2 cm.

![Fig. 2](image2.png)

Fig. 2. Seeds of *Platanthera blephariglottis* assessed for germination. (A) Germinated seeds, with swollen embryos and rhizoids. Scale bar = 600 µm. (B) Ungerminated seeds, without swollen embryo or rhizoids, except for the seed indicated by an arrow which was considered germinated. Scale bar = 400 µm.
seeds. Seed testa color and method were considered as fixed factors and population and individual plants as random factors to account for variation that might be caused by seed origin. The mixed binomial model used took into account the heterogeneity in the number of individuals per seed testa color class. All pairwise comparisons were made using the protected Fisher least significant difference (LSD) test.

RESULTS

Viability staining—Hypochlorite pretreatment duration had a significant effect on the percentage of seeds shown to be viable upon staining ($F_{3,20} = 58.83, P < 0.0001$), with pretreatment durations of 15 min and 30 min yielding the highest viability rates (Table 1). Although results obtained with 15 and 30 min pretreatment times did not differ significantly from each other, we chose to conduct larger-scale viability staining tests using a 15-min pretreatment duration to prevent any possibility of harmful effects on seed viability from extended exposure to hypochlorite that might not have been detected owing to our small sample size. Overall, 27.9% of the 20543 seeds assessed afterward for TTC viability staining were scored as viable, with individual plant viability rates ranging from 0 to 89.1%.

Ex situ germination—Of the 19050 seeds assessed for ex situ germination, a total of 66.1% germinated, with individual plant germination rates ranging from 7.6 to 98.4%. Most germinated seeds had swollen embryos, rhizoids, and an intact testa, but no seed developed into a protocorm. There was no visible evidence of mycorrhizal infection of the seeds, but since no thorough search for mycorrhizal fungi was carried out, signs of mycorrhizal infection might have been noted at more advanced stages of germination had the seeds been allowed to continue maturing. There were no obvious signs of predation of seeds by soil-dwelling organisms, even though some were occasionally observed in the plates.

Effects of seed testa color on TTC staining and ex situ germination—Differences in germination and viability rates varied according to seed testa color (Table 2). However, in a given seed testa color class, germinability estimates obtained using ex situ germination were consistently higher (1.4 to 2.5 times) than those obtained with viability staining, indicating that TTC staining underestimated seed germinability (Fig. 3). The significant interaction between the method used for viability assessment and seed testa color stemmed from the fact that seed testa color had no effect on ex situ germination rates, whereas TTC viability estimates declined with increasing paleness of the seed testa (Fig. 3).

**Table 1.** Effects of hypochlorite (1% NaClO + 0.1% Tween 20) pretreatment duration on seed viability of *Platanthera blephariglottis* estimated using triphenyltetrazolium chloride staining.

<table>
<thead>
<tr>
<th>Pretreatment duration (min)</th>
<th>Viability rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.7 ± 1.0*</td>
</tr>
<tr>
<td>15</td>
<td>37.4 ± 2.0*</td>
</tr>
<tr>
<td>30</td>
<td>31.7 ± 3.2*</td>
</tr>
<tr>
<td>45</td>
<td>16.9 ± 3.4*</td>
</tr>
<tr>
<td>60</td>
<td>14.1 ± 2.9*</td>
</tr>
</tbody>
</table>

Notes: Reported viability rates are the mean ± SE from five replicates of an average of 80 seeds. Viability rates with different superscript letters differ significantly at $\alpha = 0.05$.

**Table 2.** Effects of method (ex situ germination or triphenyltetrazolium chloride viability staining) and seed testa color on viability rate of *Platanthera blephariglottis* assessed using a binomial mixed model.

<table>
<thead>
<tr>
<th>Variable</th>
<th>df</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td>3, 177</td>
<td>6.61</td>
<td>0.0003</td>
</tr>
<tr>
<td>Color</td>
<td>1, 196</td>
<td>1928.17</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Method × color</td>
<td>3, 196</td>
<td>83.09</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Notes: A total of 200 individuals each with an average of 100 seeds was evaluated for each method ($n_{total} = 39593$).

DISCUSSION

The relatively high germination rate obtained in our assay suggests that the ex situ protocol tested here could be successfully used to assess orchid seed viability in ecology studies. Although the possibility that our protocol failed to trigger germination of every viable seed cannot be ruled out, it still appears to provide more accurate estimates than the triphenyltetrazolium chloride (TTC) staining assay, which obviously underestimated viability rates.

Perhaps a more problematic aspect of the TTC analysis is the fact that it clearly showed a bias with respect to seed testa color. Seeds with a paler testa were indeed more likely to be inaccurately scored as nonviable, although this trait has no relevance to seed germination as evidenced by similar germination rates regardless of seed testa color. The observed bias is probably due to the fact that the hypochlorite pretreatment killed several of the embryos of paler seeds, while making testae of the darker seeds permeable without affecting their embryos. The embryos...
of darker seeds would indeed have been protected by the higher suberin content, which reduced the exposure time of the embryo itself to hypochlorite. The fact that the suberin content of orchid testae influences hypochlorite treatment outcome has been noted previously (Harvais, 1980; Van Waes and Debergh, 1986a), and viability staining protocols are well known to be frequently inaccurate (Dowling and Jusaitis, 2012). However, to our knowledge, this is the first time that intraspecific variation in seed testa color of ecotypically undifferentiated individuals is shown to have an impact on viability staining results (such variations have, however, been previously observed by Kauth et al., 2011 for different ecotypes of Calopogon tuberosus). Our results therefore underline the systematic bias that this variation may introduce in seed viability assessment when hypochlorite pretreatments are involved. Adjusting hypochlorite pretreatment protocols may not resolve this situation, as it can be expected that shorter pretreatment durations would result in fewer viable dark seeds staining red. As shown by the results of our preliminary tests, reducing the soaking time in hypochlorite would indeed lead to an important reduction in viability staining rates. Calcium hypochlorite, which performs better than sodium hypochlorite for some orchid species (Rasmussen, 1995), might possibly represent a solution to this dilemma. Similarly, the use of fluorescein diacetate instead of TTC for staining could improve results, as it enters cells more freely and is often more accurate (Dowling and Jusaitis, 2012).

Several techniques have been used previously in ecological studies to assess seed germination or viability of orchids, but the ex situ germination protocol tested here compares favorably to several of them for certain purposes. Although in vitro germination may be better suited to propagation by enabling seeds to develop into plantlets, the ex situ protocol described here is a simple alternative to time-consuming protocol development and axenic condition requirements when obtaining large protocorms is not requisite. Moreover, the use of natural substrate, as opposed to in vitro artificial media, enables adequate nutrient concentrations and may provide the seeds with mycorrhizal fungi that assist in germination, although we did not evaluate this in our study. These natural conditions would probably be more likely to trigger germination of every viable seed (a prerequisite of viability assessment protocols) if field conditions are reproduced, as was the case here when seeds were subjected to a cold treatment. Also, and more importantly, since most in vitro (whether symbiotic or asymptotic) seed sterilization protocols rely on hypochlorite treatments, such methods can be expected to introduce a bias similar to that observed in our experiment for TTC staining.

Ex situ germination assays could also be used as a complement to well established in situ germination studies. Ex situ germination enables common garden studies in experimental designs that require germination of seeds from multiple sites. The effects of different conditions on germination can also be tested with ex situ germination, since the more common in situ germination is contingent upon the natural conditions of the study site. Moreover, the extended period of time usually required to obtain in situ germination results is highly constraining when the purpose of the study is not to investigate potential germination at a particular study site, but rather to assess viability of the seeds themselves. Ex situ germination also enables time-lapse observation of germination, which is much more difficult to do using in situ protocols. The ex situ protocol presented here is therefore not intended to replace the more widely used in situ protocol, but could rather be used as an alternative to simple seed viability assessment in orchids.

Until now, the ex situ protocol investigated here has been almost exclusively used as a seed baiting technique to gain insight into the distribution and ecology of orchid mycorrhizal fungi. Our results underline that this protocol can be used to assess orchid seed viability per se in ecological studies, with substrate collected from the study site(s) replacing the more commonly used agar-based media of in vitro protocols. This ex situ protocol was previously used with dry to mesic substrates (e.g., Brundrett et al., 2003 and De Long et al., 2013), but never with substrates from wetlands, although it may be especially appropriate for peatland or other wetland species because it enables incubation in continuous darkness throughout the germination assay. Specifically, the use of parafilm prevents dessication, making further wetting of the substrate unnecessary, and avoiding possible inhibition due to light exposure during such a procedure. Moreover, Aewsakul et al. (2013) found that commercial peat moss promoted orchid mycorrhizal fungi growth and seed germination more effectively than soil and coir dust, which may similarly be true for natural Sphagnum moss as used in our experiment. Although we did not observe evidence of mycorrhizal infection, previous studies using ex situ germination have successfully isolated mycorrhizal fungi from seeds germinated using this protocol (Brundrett et al., 2003).

Under the protocol presented here, no seeds reached protocorm stage, which might cast doubt on the extent to which the results obtained represent a measure of seed quality. Although seed quality could certainly be evaluated more accurately by observation of more advanced stages of germination, early stages nonetheless indicate the presence of living tissue and metabolic processes in the seed. Compared with staining methods, this protocol thus not only identifies a higher percentage of seeds as viable, but also detects living tissue in a much more reliable way than viability staining, which only demonstrates the presence of enzymes able to alter TTC.

In conclusion, we found that ex situ germination can be used successfully to assess seed viability of P. blephariglottis. Our results also highlight that viability staining using the standard TTC method may introduce a bias toward scoring paler seeds as nonviable, a condition which is not necessarily indicative of incapacity to germinate. Studies focusing on orchids should therefore avoid conducting viability staining tests without concurrently carrying out germination assays. The ex situ protocol described here provides a simple, presumably unbiased alternative to viability staining for viability assessment in ecological studies. It is possible that many other orchid species could be germinated ex situ on substrate collected from their natural habitat. Furthermore, this protocol could be used to gain insight into the germination ecology of the several endangered North American orchid species that inhabit peatlands or other types of wetlands and consequently lead to the development of appropriate conservation measures. For instance, we used this method to investigate the effect of ecological isolation of populations on seed viability of P. blephariglottis (L. De Vriendt et al., unpublished manuscript). Several other questions related to orchid conservation can undoubtedly be answered using this efficient, easy to implement protocol.

LITERATURE CITED


Afonso, A., S. Castro, J. Loureiro, L. Mota, J. Cerca de Oliveira, and R. Torres. 2014. The effects of achene type and germination


FlorQuébec. 2009. Plantes rares du Québec méridional. Les publications du Québec, Quebec City, Quebec, Canada.


