A Preliminary Investigation on the Effect of Seed Physiological Stage, Concentration and Duration of Exposure to Calcium Hypochlorite on In Vitro Germinability and Seedling Development of *Phalaenopsis amabilis* Orchids

A.M. Mweetwa¹, G.E. Welbaum¹ and D. Tay²

¹ Department of Horticulture, Saunders Hall, Virginia Tech, Blacksburg, Virginia 24061, USA
² USDA-ARS, Ornamental Plant Germplasm Center, 670 Tharp Street, The Ohio State University, Columbus, Ohio 43210, USA

Keywords: germplasm preservation, seed sterilization, seed vigor, seed storage

Abstract

For commercial propagation, orchid seeds are germinated aseptically in vitro. Successful establishment is dependent on harvesting seeds that are sufficiently mature to develop into protocorms and sanitation procedures that minimize contamination in culture. However, there are no standardized procedures for sanitizing orchid seeds and there is insufficient information about the optimum state of orchid seed development for best germination. *Phalaenopsis amabilis* fruits 90, 105, and 120 days after pollination (DAP), surface treated with 15% calcium hypochlorite (CH) for 30 min, were compared with seeds treated with 0, 5, 10, or 15% CH for 5, 10, or 15 min. Ninety six percent of untreated seeds from 90 DAP fruit, surface sanitized with 15% CH, produced protocorms within 40 days after sowing (DAS). Seed sanitization with 5% CH and exposure durations greater than 5 min significantly reduced protocorm percentages. Seeds treated with 10 or 15% CH showed inhibited protocorm development at all exposure times. Protocorms developed root hairs and shoot primodia by 50 DAS and an average of one leaf and root by 85 DAS after treatment with either 0 or 5% CH. Only 27 and 22% of seeds developed into protocorms but not roots or shoots following treatment with 10 or 15% CH, respectively. *P. amabilis* flowers were hand pollinated and fruits harvested 90, 105, and 120 DAP while still green for seed developmental analysis. Cell number per seed was estimated by counting nuclei stained with 4'-6-diamidino-2-phenylindole using confocal microscopy. Germination percentage and cell number per embryo increased from 14 to 61% and 41 to 66%, respectively, during fruit development from 90 to 120 DAP. Harvesting fruits 120 DAP while still green and using fruit sanitization before plating seeds produced the highest percentage of protocorms. Seed sanitation with greater than 5% CH dramatically reduced protocorm and seedling development.

INTRODUCTION

The development of orchid ovules follows a pattern different from most angiosperms. At pollination, there are no developed ovules in most orchid species. According to Nadeau et al. (1996), hormonal signaling associated with pollination leads to ovule development in *Phalaenopsis*. *Phalaenopsis* species commit to ovule development between 28 and 48 d after pollination, and ovule development is nearly complete 77 d after pollination when fertilization generally occurs (Nadeau et al., 1996). Other studies have verified that fertilization occurs 70-120 days after pollination (DAP) and embryo development begins within about 4 weeks (Arditti, 1992; Rasmussen, 1995). The number of cells in a mature orchid embryo differs among species ranging from 8 to as many as 734 cells in *Bletilla straita* (Arditti, 1992).

Immature orchid embryos germinate more readily in vitro. Immature *Cypripedium* seeds with 9-12 cells, living testa cells, and embryos about 66% the size of mature embryos germinated better than fully mature ones (Rasmussen, 1995). The ability of
immature seeds to germinate well has been attributed to the protein mobilization that occurs during rehydration in vitro and an undeveloped embryonic envelope that may repel water (Rasmussen, 1995). Mature seeds are considered by some to have greater potential for propagation (Miyoshi and Mii, 1998). Because there is little information on when to harvest Phalaenopsis seed pods for best seed quality, a developmental study was initiated to see how seed maturity affected germinability and storability.

Sowing orchid seeds in vitro avoids the difficulty of establishing seedlings with their fungal symbionts. The use of tissue culture has enabled commercial mass production of orchids. Seeds must be sanitized for aseptic culture. Sodium and calcium hypochlorite solutions are extensively used to stimulate seed germination and reduce contamination in culture. Calcium hypochlorite is very effective at lower concentrations and short exposures (Blischak, 2005). Stimulatory effects of calcium hypochlorite have been reported in some orchid species as well. Ervin and Wetzel (2002) observed a 50% reduction in mean time to germination of Juncus effusus, while Vujanovic et al. (2000) also observed increased germinability of seeds of Cypripedium species after treatment with calcium hypochlorite. Miyoshi and Mii (1998) found that germination of Cypripedium macranthos was stimulated after treatment with sodium hypochlorite (1% available chlorine) for 15 to 30 min and calcium hypochlorite (3.2% available chlorine) for 5 to 7h. These studies suggest seed genotype, concentration of sanitizing agent and duration of exposure are important factors affecting seed germinability and the prevention of contamination. This study was carried out to evaluate how well calcium hypochlorite could control microbial contamination in cultured seeds of Phalaenopsis amabilis from different stages of development and whether calcium hypochlorite had any detrimental side effects on seed germination or seedling development.

MATERIALS AND METHODS

Plant Material

Budding mericlonal P. amabilis plants were grown in a shaded greenhouse with maximum and minimum temperatures of 30 and 19°C, respectively, in Blacksburg, VA. Pollinations were synchronized so that harvesting of multiple pods could occur on the same date. Flowers were hand-pollinated by removing the anther cap and pollinia with forceps and then placing the pollinia on the stigma.

Harvest Dates

Seed capsules were harvested at 90, 105, and 120 DAP to provide a broad range of developmental changes (Vujanovic et al., 2000). Stems were uniformly cut with a knife approximately 1 cm below the pod attachment. Pods were placed in self-sealing bags for transport and short-term storage to minimize desiccation. At each harvest, pod weight, diameter, and color were recorded.

Seed Germination and Viability Testing

Seeds were collected from pods, placed in microcentrifuge tubes, mixed with 1 ml of 15% w/v calcium hypochlorite, vortexed for 7 min and incubated for an additional 8 min. Seeds were spread on Petri dishes containing Phytamax Orchid Maintenance Medium (Sigma-Aldrich) supplemented with filter-sterilized coconut water. The Petri dishes were incubated at 20°C and protocorm and seedling development were recorded for 120 days. Seeds that developed into protocorms were counted as germinated.

Seed viability was also assessed using tetrazolium triphenyl chloride (TZ) staining. Seeds were pre-soaked on moist filter in 60 mm Petri dishes for 6 h at 25°C then 15 ml of 1% w/v 2,3,5-triphenyl-2H-tetrazolium chloride adjusted to pH 6.5 with NaOH was added and the mixture incubated in the dark at 30°C for 24h (ISTA, 1985). Seeds were viewed under a microscope and pink to red stained seeds were considered viable. Data were collected from three grids randomly placed on each Petri plate. The percentage of stained seeds within each grid was determined and averaged for each plate.
Determination of Cell Number per Embryo

Seeds at different stages were stained for 10 min at 4°C in an aqueous 0.1 mg ml⁻¹ solution of 4’ 6 diamidino-2-phenylenidole (DAPI), a stain specific for nuclei, and visualized with a confocal microscope (Zeiss LSM 510 Laser). Nuclei were counted to determine embryo cell number at each stage of developmental.

Capsule and Seed Sterilization

Pods were surface treated with 15% calcium hypochlorite for 30 min then opened with a sterile scalpel under a laminar flow hood. Seeds were teased from the pod with the scalpel and plated directly on Phytamax Medium or sterilized further with 5, 10, or 15% calcium hypochlorite for 5, 10, or 15 min before plating. Control seeds were harvested from surface sterilized capsules and plated without treatment. Germination data were then collected as described above.

Statistical Analysis

Each treatment was replicated three times in a Completely Randomized Design (CRD). Data were analyzed using the SAS statistical software and means separated by LSD₀.₀₅. Graphs and linear regressions were performed using Microsoft Excel software.

RESULTS

Pods from all three stages of development were green at harvest (Table 1) and did not correspond with the green, fading, and brown pod color changes anticipated (Vujanovic et al., 2000). Mean pod diameter increased from 90 to 105 but not between 105 and 120 DAP (Table 1). Mean pod weight or length did not change between 90 and 120 DAP (Table 1). Seeds were stained with DAPI to count nuclei and estimate the number of embryo cells during development (Fig. 1). At 105 and 120 DAP, the stain was less effective and required much longer incubation times compared to 90 DAP. This suggested that the permeability of the testa or embryo envelope might have decreased during development. To test this hypothesis, seeds were briefly exposed to different solvents in an attempt to increase the permeability of DAPI. Treating seeds with 80% ethanol for 24h increased DAPI staining. In an attempt to identify an impermeable layer in the orchid testa, seeds were stained with Sudan Black, a lipid specific stain. Positive staining with Sudan Black suggested a lipophilic layer does develop in the testa between 90 and 120 d and this may be responsible for the reduced DAPI staining observed (data not shown). The number of cells estimated by counting positively stained DAPI nuclei increased between 90 and 120 DAP and was the best indicator of orchid embryo development during this period (Fig. 1, Table 1).

At 90 DAP, the percentage of embryos positively stained with TZ was greater than the percent germination (Fig. 2). Both the percent germinability and viability increased between 90 and 105 DAP and the difference between percent viability and germinability decreased as well (Fig. 2). There was no significant change in either germination or viability percentage between 105 and 120 DAP (Fig. 2). Overall DAP was positively correlated with both viability and germinability (Fig. 2).

Capsule decontamination alone resulted in 96% germination, e.g. protocorm development eventually leading to orchid seedlings, without significant contamination in culture. Brief exposure to 5% calcium hypochlorite did not reduce germinability but there was a gradual decline as exposure was increased to 15 min (Figs. 3 and 4). Brief exposures to 10 or 15% calcium hypochlorite reduced protocorm development and changing the duration of exposure caused only minor changes (Figs. 3 and 4). Protocorm development was tracked for 85 d after treatment to see how calcium hypochlorite exposure affected embryo development. Exposure to low concentrations (5%) calcium hypochlorite slowed seedling development compared to untreated seeds (cf. Fig. 4A and B). Seeds exposed to 15% calcium hypochlorite showed even greater inhibition of protocorm and subsequent root and shoot development (Fig. 4C).
DISCUSSION

The highest percent germination and viability were achieved later in development 105 to 120 DAP (Fig. 2). Immature *C. formosanum* seeds germinated better than more mature ones (Lee et al., 2005). Nagashima (1994) also found that seeds germinated best at the completion of embryogenesis. Pods harvested 90, 105, and 120 DAP were all green. We anticipated that these stages would correspond to the green, fading, and brown stages (Vujanovic et al., 2000). Seed pods in this study developed during the winter under short daylengths and cool greenhouse temperatures that may have delayed pod maturity compared to other studies. The orchid pods sampled in this study did not represent the broad range of developmental stages desired, based on pod characteristics. Our results also suggest that pod development may vary dramatically with environment and genotype, making it difficult to compare results based solely on DAP. Because of this variation, pod color may be a more satisfactory means of tracking development of orchid seeds (Vujanovic et al., 2000).

More mature seeds showed greater tetrazolium staining as well as in vitro germinability (Fig. 2). At 90 DAP, less than half of the seeds appeared to be viable and less than 15% produced protocorms, indicating these embryos were quite immature and still developing. The discrepancy between TZ testing and germinability in immature seeds may be an indication that some seeds were dormant or viable but incompetent to germinate because further maturation was required. Even in 120 DAP seeds, only 61% produced protocorms and only slightly more stained positive with TZ, indicating that development was incomplete or a high percentage of seeds failed to develop and were dead or possibly dormant. Hirano et al. (2005) suggested that the accumulation of inhibitory substances or increased impermeability of the embryo during maturation might account for low germination in more mature seeds. *C. formosanum* seeds harvested at 135 DAP showed poor germinability due to the development of a cuticle in the caraspace (Lee et al., 2005). The presence of this hydrophobic cuticle may help the seeds survive in harsh environments (Lee et al., 2005) and to protect the embryo from desiccation (Yeung et al., 1996). The development of an embryo barrier was suggested in our study when embryo penetration by DAPI was limited in 105 and 120 DAP seeds. The high correlation between TZ staining and germinability suggests that TZ was able to penetrate the embryo more effectively than DAPI. Water did not seem to be excluded from 105 and 120 DAP seeds when viewed under a microscope. The orchid embryo envelope has been described as being a multicellular layer of dead collapsed cells rich in pectin (Prutsch et al., 2000). Prutsch et al. (2000) previously suggested that the orchid embryo envelope was semi-permeable. In this regard, there seem to be similarities between some seeds from the family Cucurbitaceae that develop an apoplastic semipermeability in an envelope surrounding the seed later in development (Yim and Bradford, 1998). In cucurbit seeds, this envelope has likely evolved to protect embryos from the harsh chemical environment of decaying fruit and soil (Welbaum, 1993). Additional characterization of the orchid impermeable barrier is needed to determine its characteristics and how they change during development and affect propagation.

Since orchid embryos are very small and translucent, we were able to count nuclei to track embryo development (Fig. 1). Cell number, estimated by DAPI staining of nuclei, increased with DAP and was one of the few differences noted in this study (Table 1). The number of cells in the mature embryos of different orchid species is known to range from as few as 8 to as many as 734 (Arditti, 1992). In future studies, cell number can be easily counted and used to study orchid embryo development using DAPI stain and confocal microscopy.

With the exception of 5% calcium hypochlorite treatments for 5 min, all seed treatments with hypochlorite adversely affected protocorm development (Fig. 3). Our results do not agree with other findings that calcium hypochlorite stimulated germination of orchid seeds. Vujanovic et al. (2000) observed an increase in the germinability of *Cypripedium* sp. after treatment. In *Juncus effusus*, Ervin and Wetzel (2000) found that
seeds germinated faster if treated with calcium hypochlorite. It is possible that the effect of the calcium hypochlorite depends on the species or seed maturity. In immature seeds that are highly permeable, embryos may be easily damaged. In mature seeds or species with thick testae, hypochlorite may improve germination by scarification. According to Hicks (2004) Phalaenopsis viability drops sharply with extended exposure times or high concentrations of hypochlorite and the observed negative effect is a function of pH instead of actual chlorine concentration.

In this study, best P. amabilis seed quality was obtained at 105 and 120 DAP (Fig. 2). Seed contamination was not a significant problem in this study and seed disinfectant treatments were not needed for successful propagation. Culturing P. amabilis seeds under sterile conditions from surface sterilized green pods produced little contamination and satisfactory seed vigor. Directly treating seeds with calcium hypochlorite at the concentrations and exposures used here runs the risk of damaging sensitive embryos. Calcium hypochlorite may be most effective on seeds harvested from senescing pods with more surface contamination or resilient testae.

ACKNOWLEDGEMENTS
We thank J. Harper and the Mid-American Orchid Congress for their financial support, R. Veilleux and his lab for assistance with tissue culture, J. McDowell and C. DeCourcy for assistance with confocal microscopy. We also thank J. Burr for helping maintain plants and F.C. Chen and R. Griesbach for providing orchid seeds.

Literature Cited

Tables

Table 1. Pod weight, length, diameter, color, and nuclei number 90, 105, and 120 DAP.

<table>
<thead>
<tr>
<th>DAP days</th>
<th>Mean pod weight (grams)</th>
<th>Mean pod length (cm)</th>
<th>Mean pod diameter (cm)</th>
<th>Pod color</th>
<th>No. of nuclei stained with DAPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>3.2 a</td>
<td>3.5 a</td>
<td>2.0 b</td>
<td>Green</td>
<td>41 c</td>
</tr>
<tr>
<td>105</td>
<td>3.3 a</td>
<td>4.0 a</td>
<td>3.9 a</td>
<td>Green</td>
<td>58 b</td>
</tr>
<tr>
<td>120</td>
<td>4.1 a</td>
<td>4.5 a</td>
<td>4.0 a</td>
<td>Green</td>
<td>66 a</td>
</tr>
</tbody>
</table>

*Means represent the average of at least five pods with separation by LSD₀.₀⁵.

Figures

Fig. 1. Visualization of cell number during orchid seed pod development. Nuclei stained blue with 0.1 mg ml⁻¹ (in water) 4'-6-Diamidino-2-phenylindole (DAPI) could be counted using confocal microscopy. Embryo cell number increased with pod maturity.
Fig. 2. Seed germinability and viability testing using in vitro and TZ staining. TZ staining estimated higher viability than germination tests showed. Both TZ staining and in vitro germinability increased with maturity, suggesting that mature seeds lack dormancy and are higher quality seeds.

Fig. 3. Protocorm development following pod (capsule) and seed decontamination with 5, 10, or 15% calcium hypochlorite for 5, 10, or 15 mins. Seeds were obtained from a pod surface sterilized with 15% calcium hypochlorite for 30 min before seed removal. Pod sterilization and 5% calcium hypochlorite were least damaging to seeds and produced the highest percentage of protocorm development. The protocorm development for untreated control seeds receiving only pod decontamination was 96%.
Fig. 4. Phalaenopsis seedlings 50 and 85 days after sowing. Pod sterilization only (A), seed decontamination with 5% calcium hypochlorite for 10 min (B), or seed decontamination with 15% calcium hypochlorite for 10 min (C). Protocorms developed root hairs and shoot primordia by 50 DAS and an average of one leaf and root by 85 DAS after treatment with either 0 or 5% CH. Only 27 and 22% of seeds developed protocorms but not roots or shoots following treatment with 10 and 15% CH, respectively.