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In vitro seed culture and seedling development of *Calopogon tuberosus*

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Abstract

A major obstacle to native orchid production is difficulty in seed germination. Culture media and light effects on seed germination of *Calopogon tuberosus* var. *tuberosus*, a native orchid with horticultural potential, were studied. Culture media included Knudson C, Malmgren modified terrestrial orchid, and PhytoTechnology orchid seed sowing. Effects of 8 weeks continual darkness, 8 weeks 16-h photoperiod, 2 weeks dark followed by 6 weeks 16-h photoperiod, 4 weeks dark followed by 4 weeks 16-h photoperiod, and 6 weeks dark followed by 2 weeks 16-h photoperiod were examined. Percent seed germination was highest on Knudson C after 8 weeks culture; however, seedling development was enhanced on PhytoTechnology seed sowing medium during 8 weeks culture under a 16-h photoperiod. This suggests that while KC and darkness promoted seed germination, P723 and light enhanced further seedling development. Seedlings of *C. tuberosus* readily acclimated to greenhouse conditions.

Abbreviations: KC – Knudson C; MM – Malmgren modified terrestrial orchid medium; NAD – nicotinamide adenine dinucleotide; NADP – nicotinamide adenine dinucleotide phosphate; P723 – Phyto Technology orchid seed sowing medium

Introduction

Evident by recent increases in wholesale prices, orchids are now the second most popular potted floriculture crop with 2004 wholesale prices estimated at $128 million (USDA, 2005). Orchids have recently become popular houseplants. In particular, *Phalaenopsis* and its hybrids comprise 75–90% of all orchid sales in the United States (Griesbach, 2002; Nash, 2003).

While sales of popular tropical orchid hybrids are quickly increasing in the United States, production and sales of native terrestrial orchids of the United States have been slowly increasing. Several factors contribute to the lack of full commercialization of native orchids. First, production is centralized within hobby growers and small, specialized nurseries. Second, these nurseries offer only a limited selection of showy taxa. Third, the small market for native terrestrial orchids is influenced by a lack of knowledge from the consumer, lack of interest by industry, and difficulties in seed propagation.

Seed germination studies are often unreliable or unpractical (Arditti et al., 1985). Stoutamire (1974) observed that many native orchids flower after 2–8 years from seed culture with most requiring 4 or 5 years after seed germination. The optimization of seed culture for native orchids could provide an efficient method to produce larger quantities of faster flowering, vigorous plants.

Calopogon tuberosus var. *tuberosus* (L.) Britton, Sterns and Poggenberg, the common grasspink, is a widespread cold-hardy terrestrial orchid of eastern North America. The species is characterized by grass-like leaves, pink flowers, and corms. Plants

commonly inhabit open roadsides and bogs, and grow in full-sun (Luer, 1972). Advances in seed culture and breeding of *C. tuberosus* var. *tuberosus* (will be referred to as *C. tuberosus)* have led to increased yet limited production. Although seed culture of *C. tuberosus* has been studied, many of the published studies are contradictory in regards to culture media and photoperiod, and often do not include seedling development or acclimatization data (Henrich et al., 1981; Meyers and Ascher, 1982; Arditti et al., 1985; Whitlow, 1996). This study was completed to develop an efficient seed culture protocol, as well as investigate the role of photoperiod and culture media on protocorm and seedling development.

Materials and methods

Seed source and sterilization

Mature seed capsules of *Calopogon tuberosus* were collected from Goethe State Forest, Levy County, FL in July and August 2004. Seeds were removed from dehisced capsules, pooled, and stored in scintillation vials at 21–23 °C in a desiccator over anhydrous calcium sulfate to absorb excess moisture. Seeds were surface sterilized for 2 min in sterile scintillation vials containing 8 ml of an aqueous solution consisting of 0.33% sodium hypochlorite, 5% ethanol, and 90% sterile distilled deionized water. Following surface sterilization, seeds were rinsed three times for 2 min each in sterile distilled deionized water. Solutions were drawn from the vial with a disposable 1000 μl sterile pipette tip that was replaced after each use. Seeds were then suspended in sterile deionized distilled water, and a sterile inoculating loop was used for inoculating culture vessels. The average number of seeds per individual inoculation was approximately 93 ± 27.

Media preparation

Three basal media (Table 1) commercially prepared and modified by PhytoTechnology Laboratories, L.L.C (Shawnee Mission, KS) were assessed for germination efficiency: Malmgren modified terrestrial orchid medium (MM) (Malmgren, 1996), modified Knudson C (KC) (Knudson, 1946), and PhytoTechnology orchid seed sowing medium (cat. #P723). Malmgren medium without pineapple powder was obtained from PhytoTechnology Laboratories. Malmgren and KC were further modified to be consistent with P723 by the addition of 2% sucrose and 0.8% TC® agar (PhytoTechnology, Shawnee Mission, KS) as well as 0.1% charcoal to KC. Media were adjusted to pH 5.7 with 0.1 N KOH before the addition of agar and dispensed into 1000 ml flasks prior to autoclaving for 40 min 117,700 Pa and 121 °C.

Sterile media were dispensed as 50 ml aliquots into square 100×15 mm petri dishes (Falcon "Integrid" Petri Dishes, Becton Dickinson, Woburn, MA). The bottom of each petri dish was divided into 36, 13×13 mm cells. Of the 36 cells, the interior 16 were used for seed inoculation to avoid media desiccation at the edges of the petri dish. Five of the 16 interior cells were randomly selected for seed inoculation. Ten replicate petri dishes were inoculated per light and media treatment. Petri dishes were sealed with a single layer of Nescofilm (Karlan Research Products, Santa Rosa, CA).

Table 1. Comparative mineral salt content of P723, MM, and KC as prepared by PhytoTechnology Laboratories

	P723	MM	KC
Macronutrients (mM)			
Ammonium	5.15	–	10.03
Calcium	0.75	0.24	2.12
Chlorine	0.75	–	3.35
Magnesium	0.62	0.81	1.01
Nitrate	9.85	–	8.37
Potassium	5.01	0.55	5.19
Phosphate	0.31	0.55	1.84
Sulfate	0.08	0.10	4.91
Micronutrients (μM)			
Boron	26.68	–	–
Cobalt	0.03	–	–
Copper	0.03	–	–
Iron	50.10	99.99	89.90
Iodine	1.25	–	–
Manganese	25.00	9.12	33.60
Molybdenum	0.26	–	–
Zinc	9.22	–	–
Total N (mM)	15.00	–	18.40
$NH_4^+ : NO_3^-$	0.52	–	1.12
Total (mM)	22.64	2.36	36.93

Light treatments

The effects of five light treatments were evaluated:

- 8 weeks 16-h photoperiod;
- 8 weeks continual darkness;
- 2 weeks dark followed by 6 weeks 16-h photoperiod;
- weeks dark followed by 4 weeks 16-h photoperiod;
- 6 weeks dark followed by 2 weeks 16-h photoperiod.

Light was provided by General Electric F96T12 fluorescent lights at an average of 92 μmol m^{-2} s^{-1} measured at culture level and 25 ± 2 °C. Cultures in continual darkness were maintained at 24 ± 0.6 °C. Cultures maintained under the 8-week 16-h photoperiod were evaluated weekly for 8 weeks to determine percent germination. After incubation in 2, 4, and 6 weeks dark, cultures were placed under previously described light conditions and evaluated weekly until week 8. Seeds cultured in the 8-week dark period were evaluated once at week 8. Seedling development was evaluated using the six stages of seedling development (Table 2) modified from Stenberg and Kane (1998).

In vitro *seedling development*

After 16 weeks culture, seedlings were transferred to PhytoTech Culture Boxes (PhytoTechnology, L.L.C., Shawnee Mission, KS) (95 mm×95 mm×110 mm) containing 100 ml of corresponding medium. Seedlings from each initial 8-week treatment were maintained separately. Fifteen seedlings were transferred to each vessel. After 8 weeks culture in PhytoTech Culture Boxes and 24 weeks total culture, 50 seedlings from each initial treatment combination (photoperiod×medium) were randomly selected for evaluation of leaf length and number, root length and number, and fresh and dry weight. Seedlings were placed in a drying oven for 48 h at approximately 60 °C to measure dry weight.

Table 2. Six stages of orchid seed development. Adapted from Stenberg and Kane (1998) and Mariat (1952)

Stage	Description
1	Imbibed seed, swollen and greening still covered or partially covered by testa
2	Enlarged seed without testa
3	Protocorm with pointed shoot apex and rhizoids
4	Protocorm with developing leaves and rhizoids
5	Seedling with one or more leaves and one or less developing roots
6	Seedling with evident roots and two or more leaves

Acclimatization

Potting media effects on seedling *ex vitro* acclimatization were studied. Four potting media were examined: Chilean sphagnum moss (Better Gro Orchid Moss, Sun Bulb Company, Arcadia, FL), vermiculite, Fafard mix 2 (Conrad Fafard, Inc., Agawam, MA) and a 1:1 (v/v) peat moss/sand mixture. Fafard mix 2, a commercially available mix, consists of 55% Canadian peat, perlite, and vermiculite. Potting media were randomly assigned to nine plugs (5.08 cm diameter×6.35 cm tall) in a 38-cell plug tray. Eight replicate plug trays were used. Only seedlings cultured on P723 in the 16-h photoperiod were utilized since they were the most developmentally advanced and provided a large number of seedlings. One randomly selected 6 month-old seedling was placed in each plug cell. Seedlings were acclimatized from March though May 2005.

Plug trays were covered with clear vinyl humidity domes to prevent desiccation and placed under 50% shade cloth in the greenhouse with an average light level of 795 μmol m^{-2} s^{-1} measured at noon. After 2 weeks, domes were partially removed and completely removed one week later. Seedlings were watered weekly at 3–4 day intervals. After one-month acclimatization, seedlings were fertilized weekly with 150 ppm N–P–K balanced liquid fertilizer (Peter's 20–20–20, The Scott's Company, Marysville, OH). Average temperatures ranged from 21.6 ± 2.0 °C to 28.8 ± 3.0 °C, and humidity levels ranged from 51 to 97%. After 12 weeks, seedlings were examined for growth and development including leaf length and number, root length and number, fresh and dry weight, corm diameter, and survivorship. The longest leaf and root were measured, and corm diameters were measured at the widest portion of the corm.

Statistical analysis

Germination rates were calculated by dividing the number of seeds in stages 1–6 by the total number

of seeds in the subsample. The percentage of protocorms and seedlings in a developmental stage was calculated by dividing the number of seeds in that stage by the total number of germinated seeds. Germination, development, and acclimatization data were analyzed using general linear model procedures, least square means, and LSD at $\alpha = 0.05$ in SAS v 8.02. Germination counts were arcsine transformed to normalize variation.

Results

Seed germination

Seeds became swollen quickly after inoculation onto culture media, and germination commenced within the first 2 weeks of culture. The overall visual contamination rate of cultures was 13%. A tetrazolium test (Lakon, 1949) of *C. tuberosus* seeds from the Goethe State Forest population yielded 35% viable embryos (S. Stewart, unpublished data).

Within both the 8-week dark and 8-week 16-h photoperiod treatments, seed germination was highest on KC (Figure 1). Seed germination rates decreased on KC to MM to P723, respectively in the 8-weeks continual darkness; however, this trend was not seen in the 8-week 16-h photoperiod since seed germination between MM and P723 was non-significant. Between the 8-week dark treatment and 8-week 16-h photoperiod the lowest seed germination was observed on P723 under continual darkness. Seed germination on all media was significantly lower in the dark pretreatments than the 8-week continual dark and 8-week 16-h photoperiod (Figure 1).

Seed germination under the 8-week 16-h photoperiod commenced by week 1 regardless of

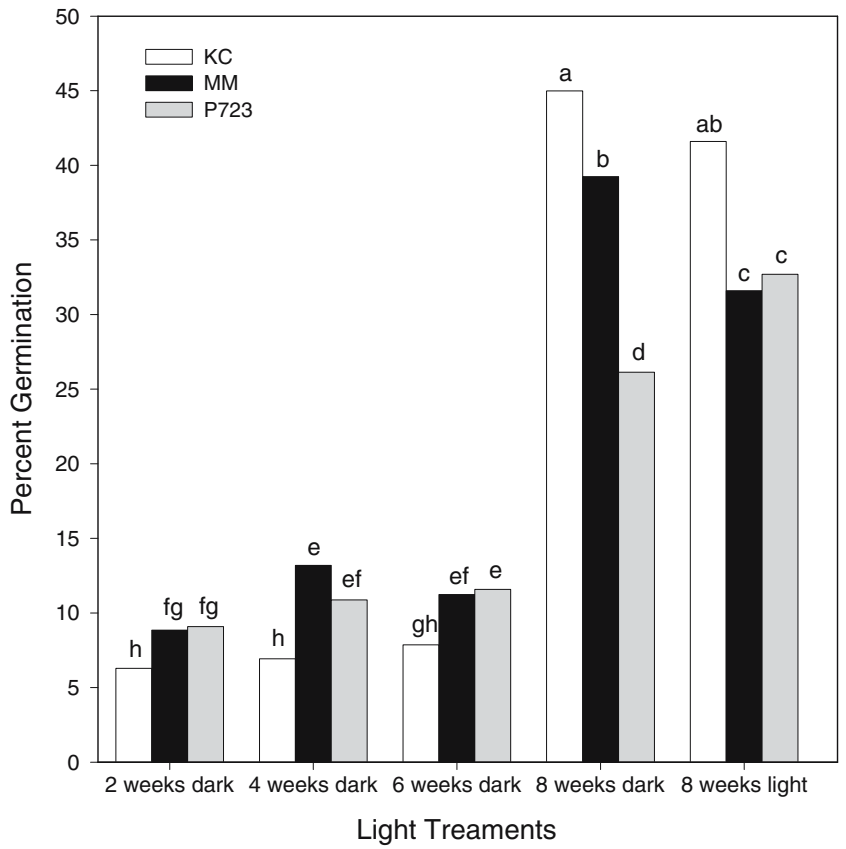

Figure 1. Effect of culture media and light treatment on percent seed germination after 8 weeks culture. Histobars with the same letter are not significantly different across light treatments ($\alpha = 0.05$). KC – Knudson C; MM – Malmgren modified terrestrial orchid medium; P723 – orchid seed sowing medium.

media (Figure 2). Throughout the initial 8-week culture period in the 16-h photoperiod, maximum germination occurred at 4, 5, and 6 weeks culture on KC, P723, and MM, respectively. Seed germination on KC increased from week 1 to 4, germination on P723 increased quickly from week 1 to 2, and germination on MM increased steadily from week 1 (Figure 2). After peak germination, percent germination either remained the same or decreased slightly due to embryo and protocorm death.

Although seed germination was highest on KC, subsequent seedling development was not enhanced on this medium, but rather on P723. Seedlings developed to stage 4, 5, and 6 after week 2, 4, and 7 on KC, respectively (Figure 3a). On MM seedlings developed to stages 4, 5, and 6 after week 2, 5, and 7, respectively (Figure 3b). Seedlings on P723 developed to stages 4, 5, and 6 after weeks 2 and 5 (Figure 3c). At week 8 total seed germination on P723 in the 8-week 16-h photoperiod was 33%, and nearly 85% of the protocorms developed to at least Stages 5 and 6. Of the 41% germination on KC in the 8-week 16-h photoperiod, approximately 52% developed to Stages 5 and 6. Protocorms developed to Stages 5 and 6 slowly on MM in the 8-week 16-h photoperiod with about 30% exhibiting advanced development. Seeds incubated in complete darkness did not develop to Stage 6 on any media, but a small percentage of seedlings did develop to Stage 5 within 8 weeks.

Protocorm and seedling development

Seedlings on P723 were highly developed compared to those on KC and MM after 12 weeks *in vitro* culture (Figure 4). Seedlings cultured on KC in continual darkness were etiolated, while those cultured on KC in the 16-h photoperiod were chlorophyllous (Figure 4a, b). Embryos germinated on MM in the 8-week dark period developed to Stages 5–6 while embryos cultured in 8-week 16-h photoperiod were less developed (Figure 4c, d). Protocorms cultured in the 8-week dark period on MM were etiolated with numerous rhizoids, a characteristic of Stages 3 and 4. The most advanced seedlings developed on P723 in the 8-week 16-h photoperiod (Figure 4e). The majority of embryos germinated on P723 developed to

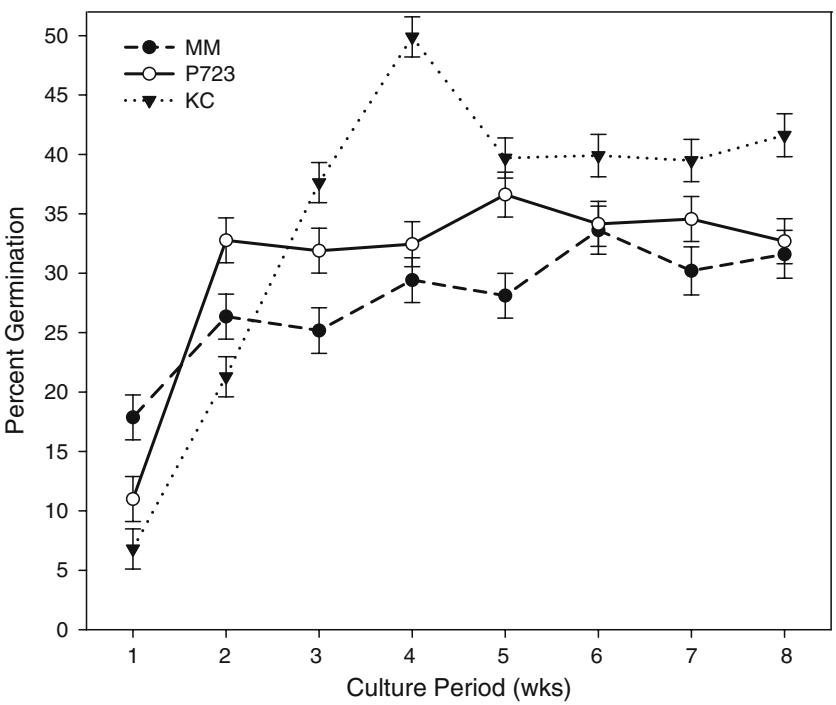

Figure 2. Eight week timecourse of *Calopogon tuberosus* seed germination in a 16-h photoperiod. Error bars represent ± S.E. KC – Knudson C; MM – Malmgren modified terrestrial orchid medium; P723 – orchid seed sowing medium.

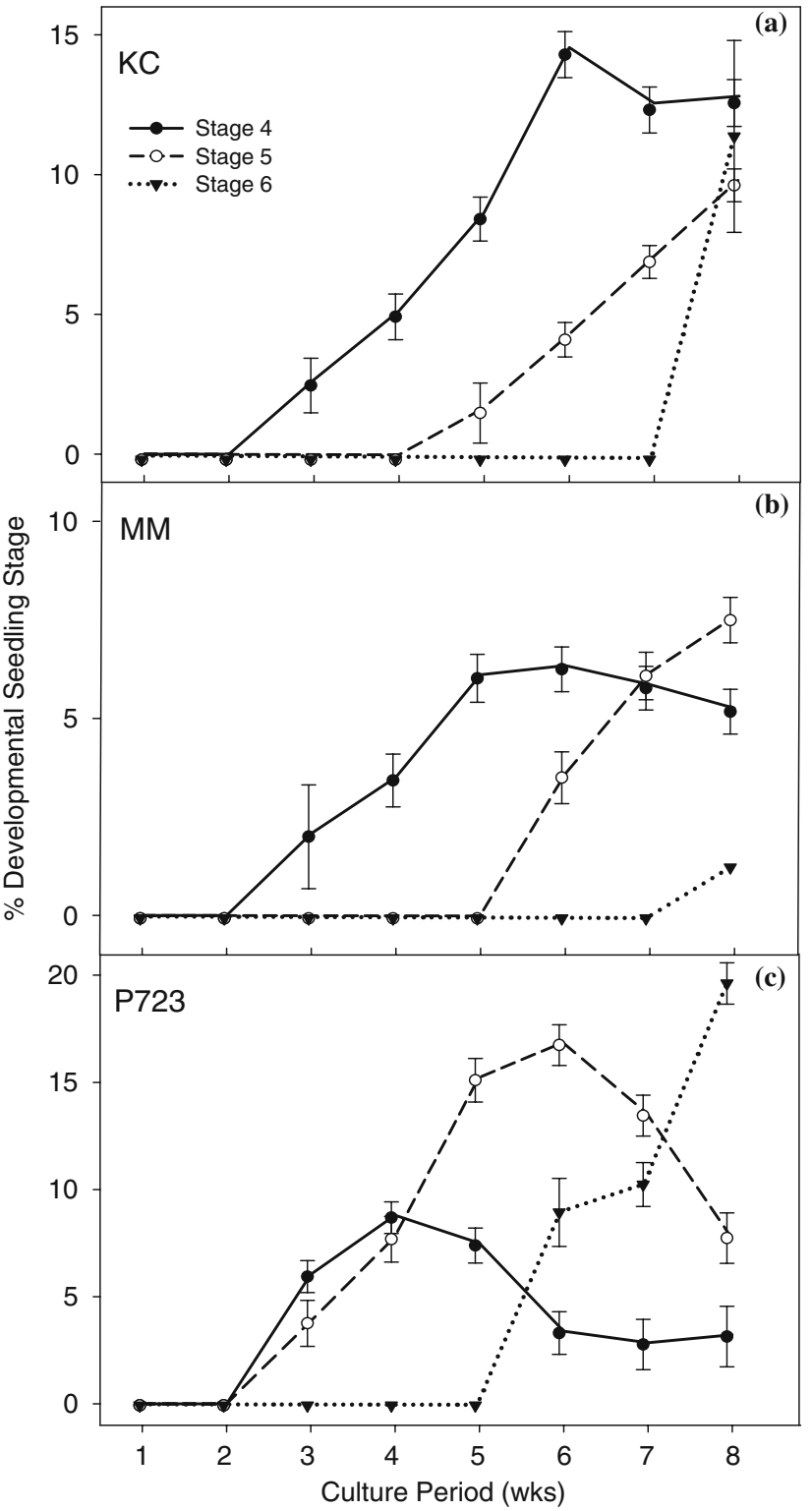

Figure 3. Effect of culture media on weekly protocorm and seedling development of *Calopogon tuberosus*. Seeds were cultured in a 16-h photoperiod for 8 weeks. Each point on the graphs represent the mean response of 10 replicate plates each with five subsamples. (*a*) Seeds cultured on KC. (*b*) Seeds cultured on MM. (*c*) Seeds cultured on P723. Error bars represent ± S.E. KC – Knudson C; MM – Malmgren modified terrestrial orchid medium; P723 – orchid seed sowing medium.

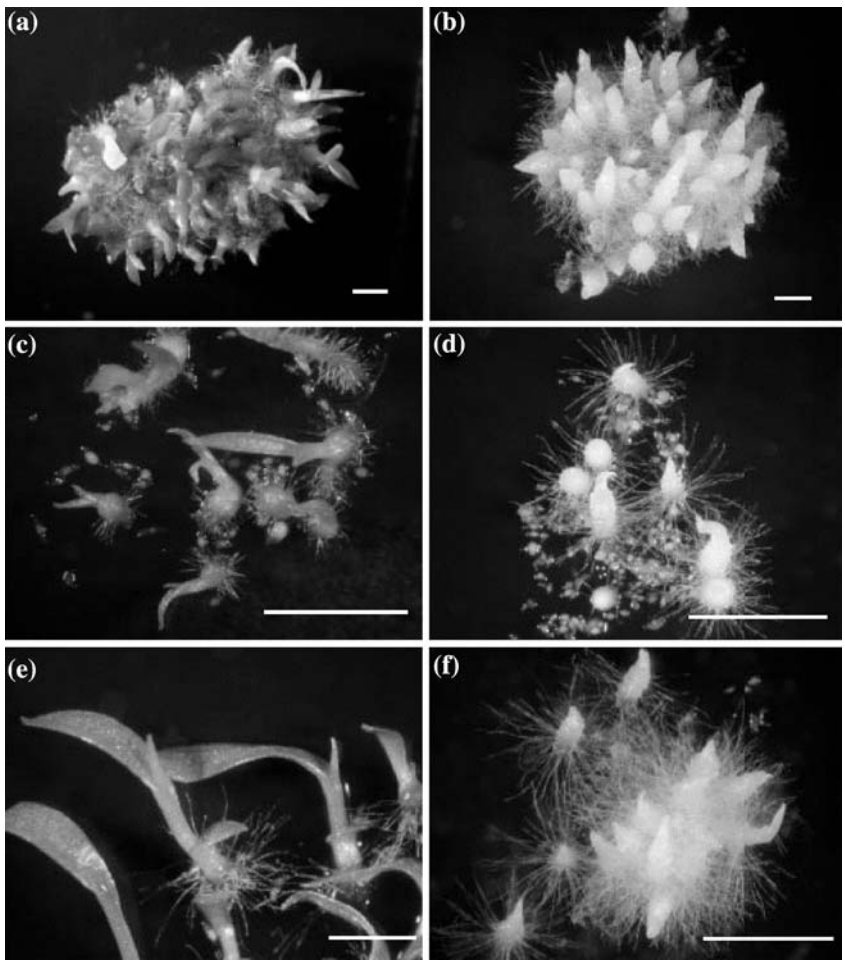

Figure 4. Comparative effects of media and light on protocorm and seedling development of *Calopogon tuberosus* after 8 weeks culture. (*a*) Seeds cultured on KC; 8-week 16-h photoperiod. (*b*) Seeds cultured on KC; 8-weeks continual darkness. (*c*) Seeds cultured on MM; 8-week 16-h photoperiod. (*d*) Seeds cultured on MM; 8-weeks continual darkness. (*e*) Seeds cultured on P723; 8-week 16-h photoperiod. (*f*) Seeds cultured on P723; 8-weeks continual darkness. KC – Knudson C; MM – Malmgren modified terrestrial orchid medium; P723 – orchid seed sowing medium. Scale bars = 5 mm.

Stage 6. Embryos cultured on P723 in the 8-week dark period were etiolated with numerous rhizoids.

After 24 weeks culture *in vitro*, corm development was evident on seedlings cultured on P723 regardless of initial light treatment (Figure 5a). Seedlings cultured on P723 developed longer leaves and roots than those cultured on other treatments (Table 3). The lowest leaf production occurred on KC in the initial 8-week dark period (Table 3). Seedlings cultured on P723 produced the highest root number. Seedlings cultured on P723 in the initial 8-week 16-h photoperiod had significantly greater fresh weight compared to seedlings in all other treatments (Table 3). Conversely, dry weight was higher for seedlings cultured on P723 in the initial 8-week continual dark period than those cultured in all other treatments (Table 3).

Acclimatization

Seedlings exhibited vigorous growth after 7 weeks cultivation (Figure 5b). Original shoots on corms showed signs of browning after 4 weeks and completely died back after 7 weeks. Among all treatments 53% of seedlings regenerated new growth within the 12-week acclimatization. Seedling survival was high in all potting media. Survivorship on sphagnum, vermiculite, peat/

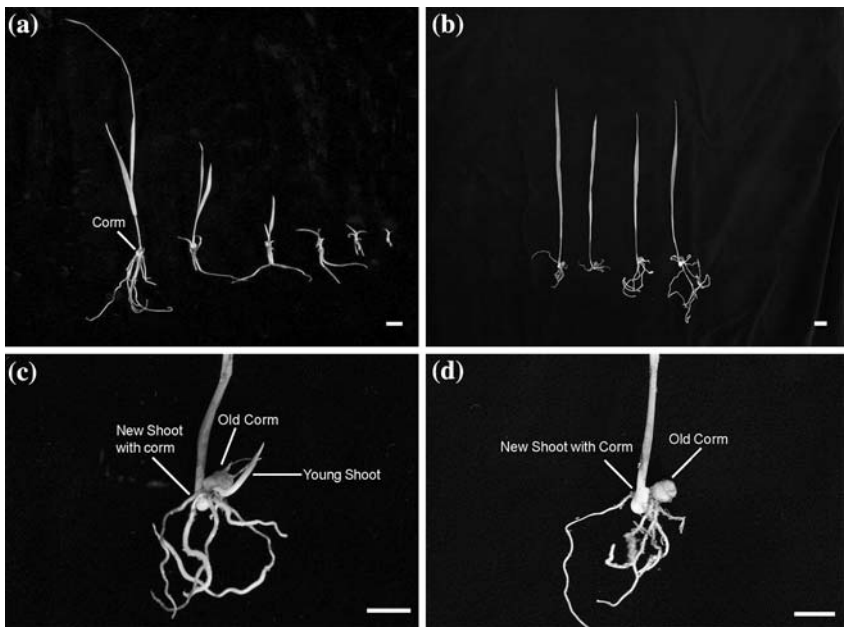

Figure 5. Seedling development of *Calopogon tuberosus*. (*a*) *In vitro* seedling development after 24 weeks culture. From left to right seedling cultured on: P723 and initially incubated in a 16-h photoperiod; P723 and initially incubated in complete darkness; KC and initially incubated in a 16-h photoperiod; KC and initially incubated in complete darkness; MM and initially incubated in a 16-h photoperiod; MM and initially incubated in complete darkness. (*b*) Seedling development after 12 weeks greenhouse acclimatization. From left to right seedling cultured in: 1:1 peat/sand; vermiculite; Fafard mix; sphagnum moss. (*c*) 12 week greenhouse cultured seedling with original corm and 2 new shoots. (*d*) 12 week greenhouse cultured seedling with original corm and new shoot. Scale bars = 1 cm.

sand, and Fafard mix was 89, 90, 90, and 88%, respectively. Seedling leaf production and root lengths were similar among potting media (Table 3). Root number was significantly different between seedlings cultured in sphagnum and peat/sand (Table 3). Leaf lengths, corm diameter, fresh

Table 3. Seedling development after 24 weeks *in vitro* culture and 12 weeks *ex vitro* greenhouse acclimatization

	Leaf #	Leaf length (mm)	Root #	Root length (mm)	Fresh Wt (mg)	Dry Wt (mg)	Corm (mm)
In vitro							
KC-light	3.12b	7.82c	1.40b	5.53b	7.38c	0.75c	–
KC-dark	1.96d	5.42c	1.31b	5.52b	5.44c	0.52c	–
MM-light	3.18ab	10.4c	1.17b	12.1b	12.8c	2.38b	–
MM-dark	2.60c	6.34c	1.40b	12.3b	11.1c	1.42c	–
P723-light	3.53a	63.6a	2.52a	38.7a	110.3a	2.37b	–
P723-dark	3.18ab	38.3b	2.67a	35.7a	75.1b	7.04a	–
Ex vitro[a]							
Sphagnum	1.27a	88.2a	2.80b	48.7a	136.9a	28.5a	5.88a
Vermiculite	1.16a	62.9b	3.22ab	35.8a	95.6b	17.1b	5.25b
Peat/sand	1.26a	85.6a	4.05a	38.9a	141.9a	27.4a	6.03a
Fafard mix	1.22a	82.4a	3.87ab	41.2a	130.1a	25.2a	5.77ab

[a]Seedlings evaluated *ex vitro* were produced on P723 medium.
Peat/sand-1:1 (v/v); Corm-diameter length; KC – Knudson C; MM – Malmgren modified terrestrial orchid medium; P723 – PhytoTechnology orchid seed sowing medium; light-24 weeks 16-h photoperiod; dark-8 weeks continual darkness followed by 16 weeks 16-h photoperiod. Numbers with same letter are not significantly different at $\alpha = 0.05$.

weight, and dry weight were significantly less on seedlings cultured in vermiculite compared to all other potting media (Table 3). New shoot regeneration commenced from original corms after 8 weeks under greenhouse conditions, and new corms and shoots formed on several seedlings (Figure 5c, d).

Discussion

Successful *in vitro* seed germination has been previously reported for *C. tuberosus*. Arditti et al. (1985) reported near 100% germination on a modified Curtis medium in 2 months and 5–10 cm long seedlings in 4 months. Henrich et al. (1981) reported 25% germination in 29 days on Norstog medium under continual darkness and 25 °C. Anderson (1990) reported 90% germination in a liquid Knudson medium. Stoutamire (1974) reported rapid seed germination in 2 to 3 weeks in sterile distilled water under fluorescent lights. In our study, the highest germination rates in 8 weeks occurred on KC under complete darkness (45%) or a 16-h photoperiod (41%) at 23–25 °C, but seedling development was enhanced on P723 in the 16-h photoperiod.

Many of the previous seed germination studies were published in conference proceedings (Anderson, 1990; Whitlow, 1996) or as brief protocols with other orchid species (Henrich et al., 1981; Arditti et al., 1985). These studies do not include complete data from germination through acclimatization. We examined the effects of dark and light incubation combined on seed germination and subsequent seedling development complete with data. We also included seedling development and acclimatization data, and recorded observations on corm development.

A major difference between the three culture media is the form and concentration of nitrogen (Table 1). While KC and P723 contain inorganic nitrogen sources (ammonium and nitrate), MM, as prepared by PhytoTechnology Laboratories, contains the amino acid glycine. Malmgren (1992, 1996) and Van Waes and Debergh (1986) found that germination rates of several cold-hardy terrestrial orchids were higher on media containing amino acids. Malmgren suggested that an organic nitrogen source (amino acids) might be more readily available than an inorganic nitrogen source due to the simplified form of nitrogen. Glycine was reported to inhibit germination compared to ammonium nitrate (Spoerl and Curtis, 1948; Raghavan, 1964). Seed germination with other amino acids, such as arginine, proline, and glutamine, was similar to that of ammonium nitrate; however, response to various amino acids differs among species, and further investigation should be employed.

Nitrogen in the form of ammonium was found to be more effective than nitrate or nitrite for the germination of orchid seeds (Curtis, 1947; Raghavan and Torrey; 1964). Both Knudson C and P723 contain ammonium and nitrate. Curtis and Spoerl (1948) found that a high ratio of ammonium to nitrate (4.2:1) was beneficial for germination of *Cattleya* seeds. The ammonium to nitrate ratio in P723 is 0.5:1, while the ratio in KC is 1.12:1. Ammonium content was highest in KC, contributing to the high overall germination rates of *C. tuberosus*. Raghavan and Torrey (1964) found that nitrate reductase activity was evident after 60 days culture, and therefore nitrate uptake was unavailable until then. P723 contained slightly more nitrate than KC, promoting the excelled growth of seedlings cultured on P723.

The inclusion of several organic nutrients to P723 improved the effectiveness of this medium for germination and development. P723 contains 0.2% peptone, an additional source of nitrogen, but the exact amount of nitrogen contributed by peptone is uncertain. Curtis (1947) found that the addition of 0.05% peptone to culture media significantly increased seed germination of *Paphiopedilum* and *Vanda* species. Seed germination of *Platanthera clavellata* (*Habenaria clavellata*), a native orchid of the United States, was reduced in the presence of peptone (Curtis, 1947). Peptone was also responsible for increased uniformity of seedling development. Niacin has also been found to be beneficial for orchid seed germination (Arditti, 1967). Niacin and its derivatives are essential for metabolism as components of NAD^+ and $NADP^+$. P723 contains nicotinic acid, a derivative of niacin.

The roles of light and dark in seed germination have not been sufficiently determined for *C. tuberosus*. While Whitlow (1996) recommended light incubation, others recommended dark incubation (Henrich et al., 1981; Myers and Ascher, 1982). Anderson (1990) found that

germination was higher in complete darkness than light, but seedlings were larger in light and corm formation was initiated. However, the data in these studies were not complete. Results of our study have shown that light incubation is more beneficial for subsequent seedling development than continual darkness.

While *C. tuberosus* seeds germinate in the presence of light, seed germination of cold-hardy terrestrial orchids is often inhibited by light incubation (Arditti et al., 1981; Van Waes and Debergh, 1986). Many cold-hardy terrestrial orchids inhabit shaded forest floors where seeds are not exposed to large quantities of red light, the most effective portion of the spectrum for seed germination (Rasmussen and Rasmussen, 1991). Canopy under-stories are poor in red light but rich in far-red light, which inhibits seedling development (Anjah et al., 2003). Rasmussen and Rasmussen (1991) reported that red light followed by continual darkness stimulated seed germination of *Dactylorhiza majalis*, an European orchid, compared to continual darkness. Seeds that are stimulated by red light to germinate develop more readily in open vegetation (Rasmussen and Rasmussen, 1991). Stoutamire (1964, 1974) found that species of wet, open or partially shaded locations, such as *C. tuberosus*, become photosynthetic quickly in sterile distilled water under fluorescent light. Since seed germination of *C. tuberosus* was similar between light and dark incubation, seeds can have the ability to germinate on, near, or below the surface of soil.

The negative effects of a dark pretreatment before light exposure on symbiotic and asymbiotic seed germination have been reported in other cold-hardy orchid species (Rasmussen et al., 1990; Zettler and McInnis, 1994). Under natural conditions seeds of terrestrial orchids germinate in soil, and are first exposed to light upon capsule dehiscence. Rasmussen and Rasmussen (1991) suggested that northern terrestrial orchid seeds could remain above ground due to the hydrophobic testa, thus being exposed to light. Although seeds were stored in light before inoculation in our study, light exposure may only stimulate nutrient mobilization but not lead to germination (Rasmussen and Rasmussen, 1991).

Calopogon tuberosus perennates via corm formation and a new corm develops each growth cycle (Luer, 1972). A new shoot subsequently develops from the apical meristem of each corm. Since *C. tuberosus* is perennial, the growth cycle occurs once per year with new shoots actively growing between April and July throughout the range. In culture small seedlings began corm production *in vitro* by 4 months. During the first 8 weeks, seedling bases became swollen. Corm formation *in vitro* occurred regardless of seedling size, medium type, and light treatment, suggesting that initial corm formation may be developmentally, not environmentally, controlled.

Acclimatization for *C. tuberosus* has been studied, but no data was reported. Whitlow (1996) acclimatized *C. tuberosus* seedlings after 15 months and several cold storage treatments *in vitro*. Alternatively, Myers and Ascher (1982) acclimatized seedlings under greenhouse conditions after one or more leaves fully expanded. Anderson (1990) recommended acclimatization after 2 months in culture. In our study, original shoots died soon after greenhouse culture, but corms persisted throughout the acclimation period. Interestingly, new shoots formed within several weeks of original shoot senescence. Since *C. tuberosus* has a perennial growth habit with one new growth cycle starting in subsequent years, production of new shoots within several weeks was surprising, suggesting non-dormancy of acclimatized the corms. However, the corm dormancy requirement of plants from more northern latitudes may differ significantly.

In conclusion, this study demonstrated the benefits of KC for seed germination, as well as light and P723 for seedling development of *C. tuberosus*. In a commercial setting a seed culture protocol that produces a higher quantity, as well as larger seedlings in a short period of time is valuable. Flowering has been reported from 2 to 3 years (Riley, 1983) as well as 41 months (Stoutamire, 1964) from seed germination for *C. tuberosus*. Culturing seeds first on KC and subsequent transfer to P723 can enhance seed germination and seedling development. The regenerative capacity of *C. tuberosus* to form corms *in vitro* provides an opportunity for propagule development of a commercially viable production system, which facilitates storage. Preliminary results show that *in vitro* derived corms exhibit the capacity to generate new corms and shoots when divided and cultured on a medium with an auxin or cytokinin (P. Kauth, personal observation). Since *C. tuberosus* is widespread, requirements for seed culture

and *in vitro* corm development may differ between populations and genotypes, and should be further investigated. Although asymbiotic seed germination of *C. tuberosus* provides efficient means to produce large and numerous seedlings for commercial purposes, symbiotic seed germination should be explored for conservation and ecological purposes.

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