Plant Cell Tiss Organ Cult (2009) 96:235–243
DOI 10.1007/s11240-008-9480-z

ORIGINAL PAPER

Asymbiotic seed germination and in vitro seedling development of *Cyrtopodium punctatum*: a propagation protocol for an endangered Florida native orchid

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Received: 12 June 2008 / Accepted: 17 November 2008 / Published online: 30 November 2008

Abstract *Cyrtopodium punctatum* Lindley is an endangered epiphytic orchid restricted in the United States to southern Florida. Due to its ornamental value, the species was extensively collected from the wild during the past 100 years. Today, only a few plants remain in protected areas. As part of a conservation plan for the species, procedures for asymbiotic seed germination were developed. Five asymbiotic orchid seed germination media (*Phyto*Technology Orchid Seed Sowing Medium, Knudson C, Malmgren Modified Terrestrial Orchid Medium, Vacin & Went Modified Orchid Medium, and ½-strengh Murashige & Skoog) were examined for their effectiveness in promoting seed germination and protocorm development under a 16/8 h L/D photoperiod and dark (0/24 h L/D). The influence of photoperiod on growth and development was also examined. Seeds were germinated under a 16/8 h, 12/12 h, 8/16 h L/D photoperiod, at 25 ± 3°C and allowed to develop in vitro for 10 weeks. After 10 weeks, developing seedlings were transferred to Sigma Phytatrays and returned to their assigned photoperiod treatments for continued seedling development for an additional 15 weeks. Highest germination occurred in 0/24 h L/D on *Phyto*Technology Orchid Seed Sowing Medium and seedlings displayed more advanced development when cultured under 16/8 h L/D photoperiod after 15 weeks in Phytatrays. Thirty-five week old seedlings potted in coconut husk growing medium exhibited 90% survival following 5 weeks acclimatization to greenhouse conditions. This asymbiotic seed germination protocol for *C. punctatum* will facilitate future reintroduction projects involving this endangered species.

Keywords Orchid · Seed germination · Native · Conservation · Cigar orchid

Abbreviations

½MS	½-strength Murashige and Skoog
dd	Distilled deionized
FPNWR	Florida Panther National Wildlife Refuge
KC	Knudson C
L/D	Light/dark
MM	Malmgren modified terrestrial orchid medium
P723	*Phyto*Technology orchid seed sowing medium
VW	Vacin & went modified orchid medium

Introduction

The genus *Cyrtopodium* is comprised of 42 species of Neotropical origin that can be found from southern Florida to Argentina (Batista and Bianchetti 2004; Romero-González 1999; Romero-González and Fernández-Concha 1999). *C. polyphyllum* and *C. punctatum* are the only two species found in the United States; however, *C. polyphyllum* has been naturalized (Brown 2005). *Cyrtopodium punctatum* is found in southern Florida, as well as Cuba,

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Hispaniola, Puerto Rico, and the northwestern Caribbean coast of South America (Romero-González and Fernández-Concha 1999). Very few species in the genus are epiphytic with *C. punctatum* being one of them.

Cyrtopodium punctatum, also known as the cigar orchid, is listed as endangered in the state of Florida (Coile and Garland 2003). The species was over collected during the past century and today only a few plants still exist in remote protected areas. Early accounts in the literature refer to *C. punctatum* as abundant throughout southern Florida, especially in cypress swamps of the Big Cypress Basin (Ames 1904; Luer 1972). The remaining plants in Florida are found in small populations in protected areas such as Everglades National Park, Big Cypress National Preserve, and the Florida Panther National Wildlife Refuge. Over the past 10 years, careful observations of plant populations in the Florida Panther Wildlife Refuge indicated limited seed production in the remaining plants. Pollination biology observations of *C. punctatum* revealed that limited seed production is a consequence of reduced pollination (Dutra 2008). Consequently, the long-term sustainability of remaining populations is in question.

Asymbiotic seed propagation techniques have been applied to the conservation of endangered and threatened orchid taxa and may be useful in the re-introduction of *C. punctatum*. Stenberg and Kane (1998) developed an effective protocol for the asymbiotic production of the epiphytic orchid *Prosthechea boothiana* var. *erythronioides* (syn. = *Encyclia boothiana*), an epiphytic orchid. Stewart and Kane (2006) developed an asymbiotic germination protocol for *Habenaria macroceratitis*, a rare terrestrial orchid. Other authors have successfully developed methods for asymbiotic seed propagation for the purpose of plant conservation (Dutra et al. 2008; Light and MacConaill 2003; Shimada et al. 2001; Thompson et al. 2001).

The goal of this research was to develop effective protocols for the asymbiotic seed germination of *C. punctatum*. Culture variables evaluated included the effects of mineral nutrient formulation (media type) and photoperiod on germination and subsequent seedling development.

Materials and methods

Seed Source and sterilization procedure

Seeds were obtained from a naturally pollinated capsule collected at the Florida Panther National Wildlife Refuge Unit 51 (Collier Co., Florida) on February 23, 2007. The capsule was dried over silica desiccant for 70 days at 25 ± 3°C after which dehisced seeds were collected and transferred to a 20 ml scintillation vial and placed in cold storage at −10°C (May 4, 2007). Seeds were surface sterilized for 3 min in a sterile scintillation vial containing a solution consisting of 5 ml ethanol (100%), 5 ml 6.0% sodium hypochlorite, and 90 ml sterile distilled water, followed by three repetitive 30 s rinses in sterile distilled water. Sterile glass pipettes were used to remove the sterilization solution and rinse water. Seeds were then left suspended in the final water rinse and immediately sown onto the germination media.

Asymbiotic media, light and dark effects on seed germination (Experiment 1)

Five agar-solidified asymbiotic orchid seed germination media (Table 1) were examined for their effectiveness in promoting germination and subsequent protocorm development of *C. punctatum* seeds under dark versus light culture conditions. With the exception of P723 medium, all media were purchased from *Phyto*Technology Laboratories, LLC (Shawnee Mission, KS). *Phyto*Technology Orchid Seed Sowing Medium (P723) was prepared using concentrated stock solutions according to the *Phyto*Technology Laboratories formulation. The five media screened were: KC (#K400; Knudson 1946), P723, MM (#M551; Malmgren 1996), VW (#V895; Vacin and Went 1949) and ½MS (#M5524; Murashige and Skoog 1962). All media were modified to contain final concentrations of 0.8% TC® agar, 2.0% sucrose, and 0.1% activated charcoal. All media were adjusted to pH 5.8 prior to autoclaving at 117.7 kPa for 40 min at 121°C. Autoclaved medium (ca. 50 ml medium/plate) were dispersed into square 100 × 15 mm Petri plates (Falcon "Integrid" Petri Plates, Becton Dickinson, Woburn, MA). The bottom of each plate was divided into 36, 13 × 13 mm cells. Only the 16 interior cells were used for inoculation to avoid uneven medium drying. Five of the 16 interior cells were selected randomly for inoculation using a computerized random number generator. Surface sterilized seeds were inoculated onto the surface of sterile germination medium using a sterile bacterial inoculating loop. Plates were sealed with one layer of NescoFilm (Karlan Research Products, Santa Rosa, CA) and incubated under dark (0/24 h L/D) or light (16/8 h L/D; provided by cool-white florescent at 60 μmol m^{-2} s^{-1}) photoperiod at 25 ± 3°C. Approximately 77 seeds were sown into each plate (average seeds/plate = 76.5; average seeds per cell = 15.3). Eight replicate plates were used for each germination medium per photoperiod treatment. Seed germination and protocorm development stage percentages were recorded every other week for 10 weeks. At the end of Week 8, plates from dark incubation (0/24 h L/D) were transferred to a 16/8 h L/D photoperiod. Seedling development was scored on a scale of 1–5 (Table 2; modified from Stewart et al. 2003).

Table 1 Nutrient composition of germination media used for the asymbiotic seed germination of *Cyrtopodium punctatum*

	KC	P723	MM	VW	½-MS
Macronutrients (mM)					
Nitrogen–Ammonium	13.82	5.15		7.57	10.31
Calcium	2.12	0.75	0.73	1.93	1.50
Chlorine	3.35	1.50			3.1
Magnesium	1.01	0.62	0.81	1.01	0.75
Nitrogen–Nitrate	10.49	9.85		5.19	19.70
Potassium	5.19	5.62	0.55	7.03	10.89
Phosphate	1.84	0.31	1.03	3.77	0.63
Sulfate	8.69	0.71	0.92	8.71	0.86
Sodium		0.10	0.20	0.20	0.10
Micronutrients (μM)					
Boron		26.7			50
Cobalt		0.026			0.053
Copper		0.025			0.5
Iron	90	50	100	100	50
Iodine		1.25			2.50
Manganese	30	25	10	30	50
Molybdenum		0.26			0.52
Zinc		9.22			14.95
Organics (mg/l)					
D-Biotin			0.05		
Casein hydrolysate			400		
Folic acid			0.5		
L-Glutamine					
Glycine			2.0		
myo-Inositol		100	100		
Nicotinic acid		1.0			
Peptone		2,000			
Pyridoxine · HCl		1.0			
Thiamine · HCl		10			
Total mineral salt concentration (mM)	46.72	24.72	4.35	35.54	48.01
Total inorganic N (mM)	24.31	15.00	0	12.76	30.01
NH_4:NO_3	1.32	0.52	0	1.46	0.52

KC Knudson C, *MM* Malmgren Modified Terrestrial Orchid Medium, *P723* *Phyto*Technology Orchid Seed Sowing Media, *½MS* Half-strength Murashige & Skoog, *VW* Vacin & Went Orchid Medium, (modified from Dutra et al. 2008)

Table 2 Seedling developmental stages of *Cyrtopodium punctatum* (modified from Stewart et al. 2003)

Stage	Description
1	Intact testa
2	Embryo enlarged, testa ruptured (=germination)
3	Appearance of protomeristem
4	Emergence of two-first leaf primordia
5	Elongation of shoot and further development

Influence of photoperiod on germination and seedling development (Experiment 2)

Based on the differential effects of the asymbiotic media on germination and growth (Experiment 1), P723 medium was selected to further examine photoperiodic effects on germination and seedling growth. Following inoculation of surface sterilized seed onto the germination medium, the plates were sealed with Nescofilm and incubated under a 16/8 h, 12/12 h, or 8/16 h L/D photoperiod at 25 ± 3°C, following the same methods outlined for Experiment 1. Approximately 111 seeds were sown into each Falcon "Integrid" Petri Plate (average seeds/plate = 111.2 average seeds per cell = 22.3). Eight replicate plates were used for each germination medium per photoperiod treatment. Seed germination data and protocorm development stages were measured starting at 2 weeks and continuing every other week for a total of 10 weeks. Germination and seedling development were scored on a scale of 1–5 (Table 2).

To further evaluate seedling development, seedlings (Stages 4–5) cultured in the photoperiod treatments were transferred after 10 weeks from the Integrid Petri Plates to Sigma-Aldrich (St. Louis, MO) Phytatrays containing 100 ml P723 medium. Nine seedlings were transferred into each Phytatray, with 10 Phytatrays per photoperiod treatment (90 seedlings per photoperiod; 30 Phytatrays total). Phytatrays were sealed with one layer of NescoFilm and the seedlings were then returned to their respective photoperiodic treatments. Seedlings developed for an additional 15 weeks (10 weeks in germination +15 weeks in seedling development = 25 total weeks). After the total 25 week culture period, fresh and dry weight, leaf length and number, root length and number, and shoot number were recorded.

Greenhouse acclimatization

After 35 weeks culture, *C. punctatum* seedlings, previously cultured on P723 medium, were rinsed to remove residual medium, potted in coconut husk in 38-cell plug trays before being transferred to greenhouse conditions. Coconut husk was used since *C. punctatum* is often found growing on tree bark and stumps. Plug trays were covered with clear vinyl humidity domes to prevent desiccation during early acclimatization and placed under shade (239 µmol m^{-2} s^{-1}) in the greenhouse. After 1 week, the plastic domes were lifted slightly to lower the relative humidity in each plug tray, and completely removed 1 week later. After 4 weeks, seedlings were grown under increased light (1,025 µmol m^{-2} s^{-1}). Seedlings were watered once daily and fertilized weekly with 150 mg/l Peter's 20–20–20 liquid fertilizer (The Scott's Company, Marysville, OH).

Statistical analysis

Seed germination and development data from the asymbiotic seed germination experiment (Experiment 1) were analyzed using ANOVA (general linear model procedures and least square means procedures). The percentage of seedlings in each stage was obtained by dividing the number of seeds in each germination and development stage by the total number of viable seeds in the subsample. Percent germination data were arcsine transformed to normalize variation.

Germination and seedling development data from the photoperiod experiment (Experiment 2) were analyzed using ANOVA (general linear model procedures and Waller–Duncan procedures at $\alpha = 0.05$). SAS v 9.1.3 (SAS, 2003) was used for all data analysis.

Results

Asymbiotic media, light and dark effects on seed germination (Experiment 1)

Regardless of media, highest germination was achieved under complete darkness (0/24 h L/D, Fig. 1). Germination, as evidenced by testa rupture (Fig. 3a), was first observed at Week 2 under the dark treatment (Fig. 2). Protocorms produced in both photoperiod treatments were achlorophyllous, but became chlorophylous in light (16/8 h L/D) when they developed to Stage 3. Since cultures maintained under dark (0/24 h L/D) were only exposed to light at Week 8, the protocorms that had reached Stages 3–4 in dark were achlorophyllous with numerous rhizoids but became chlorophyllous soon after exposure to light. Immediately after testa rupture (Stage 2), a protocorm formed (Stage 3) with the appearance of a protomeristem. Stage 4 protocorms possessed two leaf primordia (Fig. 3b) and by Stage 5, shoot elongation commenced (Fig. 3c). At Stage 5 roots were also evident on some seedlings (Fig. 3d).

Seeds cultured on P723 and VW media had the highest percent germination among all media in light (Fig. 1; 27.3 and 26.1%, respectively) when compared to ½-MS (12.9%), KC (10.0%), and MM (12.5%). Advanced seedling development (Stages 4 and 5) was observed in light (16/8 h L/D) only on P723, ½-MS, and VW (Stage 4 only; Fig. 4). In darkness, seeds cultured on all media exhibited both high germination percentages (Fig. 1) and advanced (Stage 4) seedling development (Fig. 4). However, Stage 5 protocorms only developed on P723, ½-MS, and VW in

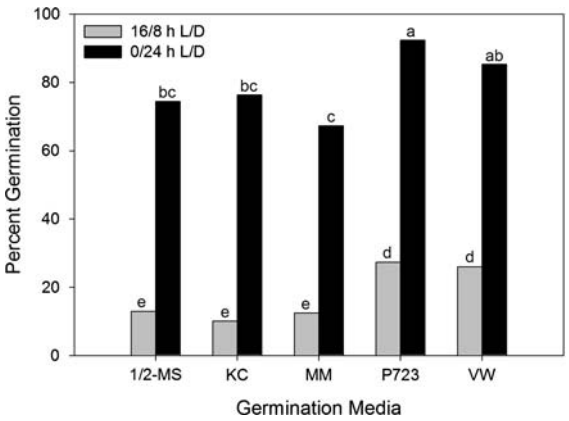

Fig. 1 Comparative effects of culture media and photoperiod on germination of *C. punctatum* seeds after 10 weeks asymbiotic culture (Experiment 1). Seedlings cultured under 0/24 h L/D where transferred to a 16/8 h L/D photoperiod after 8 weeks. Histobars with the same letter are not significantly different ($\alpha = 0.05$). *KC* Knudson C, *MM* Malmgren Modified Terrestrial Orchid Medium, *P723* *Phyto*Technology Orchid Seed Sowing Medium, *½MS* Half-strength Murashige & Skoog, *VW* Vacin & Went Orchid Medium

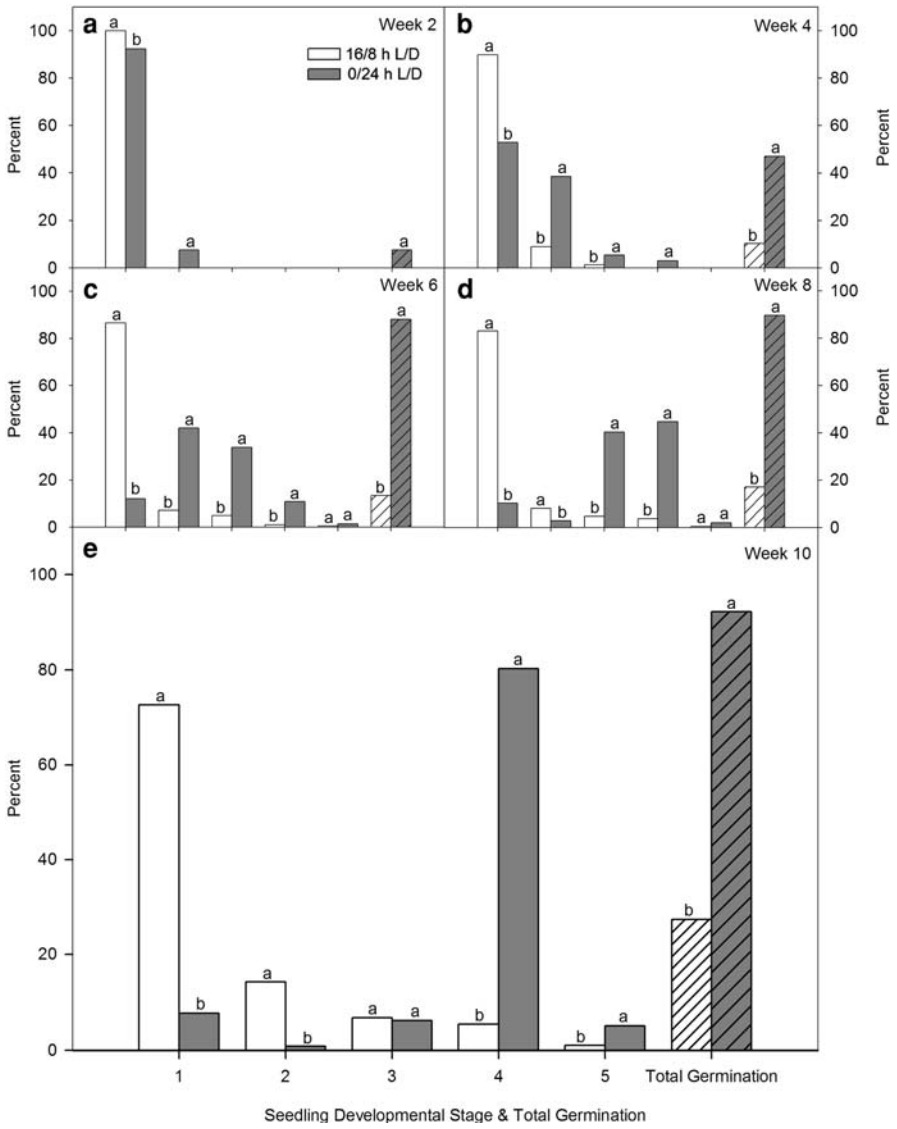

Fig. 2 Progression of in vitro seedling development stages of *C. punctatum* seeds cultured on P723 medium under a 0/24 h and a 16/8 h L/D photoperiod over 10 weeks culture (Experiment 1). Seedlings cultured under a 0/24 h L/D photoperiod where transferred to a 16/8 h L/D photoperiod after 8 weeks. Histobars within each developmental stage with the same letter are not significantly different ($\alpha = 0.05$)

darkness. Of the media screened, the highest percentages of advanced seedling development stages (Stages 4 and 5) were observed by Week 10 on P723 medium regardless of the photoperiod (Fig. 4). However, on P723 medium the percentage of advanced development seedling development (Stage 4) was significantly higher in complete darkness (Fig. 2).

Influence of photoperiod on germination and seedling development (Experiment 2)

A significant main effect of photoperiod on germination was observed as soon as 2 weeks in seed germinated under the three photoperiods for 10 weeks. Seed germination (Stage 2) under the 8/16 h L/D photoperiod was statistically higher after 2 weeks culture than under either the 12/12 h or 16/8 h L/D photoperiod (Table 3). Total germination under the 8/16 h L/D photoperiod was also significantly higher than in either intermediate or long day photoperiods throughout the 10 week culture period. Significantly higher numbers of seedlings in advanced developmental stages (Stages 4 and 5) were also observed under an 8/16 h L/D photoperiod, regardless of culture duration. Stage 4 protocorms (Fig. 3b) were first observed by Week 4 at significantly higher percentages than in the 12/12 h L/D and 16/8 h L/D photoperiods (Table 3). Stage 5 protocorms (Fig. 3c,d) were first observed at Week 10 and also occur in significantly higher percentages under the 8/16 h L/D than under the other photoperiods. Seedling growth and development was then assessed after an additional 15 weeks culture following transfer into Phytatrays culture vessels (Fig. 3f). Seedlings cultured under a 16/8 h L/D photoperiod displayed significantly greater root production, fresh and dry root biomass, and total fresh and dry weights measurements

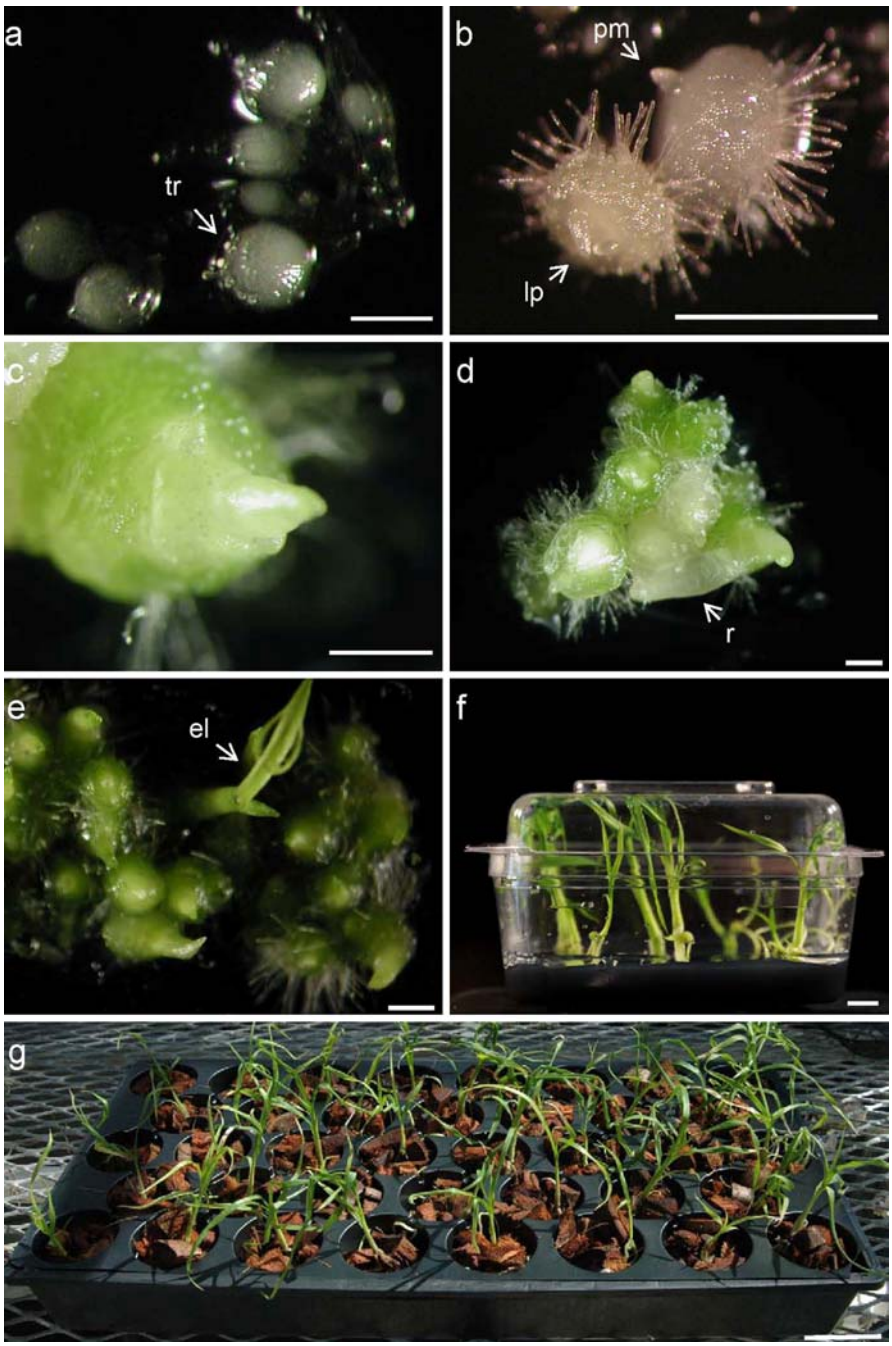

Fig. 3 Protocorm and progression of seedling development of *C. punctatum* cultured on P723 medium. **a** Stage 1 seed (intact testa) and Stage 2 protocorms (tr = testa ruptured). **b** Stage 3 protocorm (pm = protomeristem) and stage 4 protocorm (lp = leaf primordia). **c** Stage 5 protocorm (elongation of shoot). **d** Stage 5 protocorms (r = root). **e** Seedling with expanded leaves (el). **f** Seedlings at 25 weeks culture. Scale bars = 0.5 cm (**a**–**f**). **g** Seedlings after 1 week acclimatization in the greenhouse. Scale bar = 5 cm

than seedlings cultured under 8/16 h or 12/12 h L/D photoperiods (Table 4).

Greenhouse acclimatization

After 2 weeks acclimatization under greenhouse conditions, the leaves of all seedlings quickly abscised when the humidity domes were completely removed. However, the original basal shoots remained alive. By Week 3, new shoots began forming at the base of the old shoots and were emergent by Week 4. Seedlings exhibited 90% survival after 5 weeks greenhouse acclimatization.

Discussion

Low seed production in *C. punctatum* natural populations threatens the long-term sustainability of these populations. This study indicates that *C. punctatum* seedlings can be produced in vitro using asymbiotic seed germination

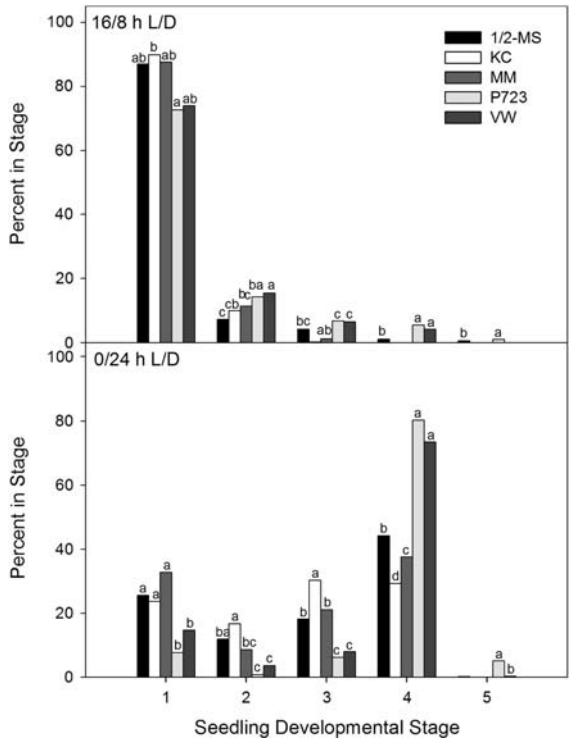

Fig. 4 Comparative effects of culture media and photoperiod on in vitro seedling development of *C. punctatum* after 10 weeks asymbiotic culture (Experiment 1). Seedlings cultured under a 0/24 h L/D where transferred to a16/8 h L/D photoperiod after 8 weeks. Histobars with the same letter within each seedling developmental stage are not significantly different ($\alpha = 0.05$). *KC* Knudson C, *MM* Malmgren Modified Terrestrial Orchid Medium, *P723 Phyto*Technology Orchid Seed Sowing Medium, *½MS* half-strength Murashige & Skoog, *VW* Vacin & Went Orchid Medium

techniques. The use of manual pollination to promote seed capsule formation combined with this asymbiotic seed culture protocol and the subsequent re-introduction of seedlings provides a means to increase *C. punctatum* populations and perhaps increase the genetic diversity currently existing in these very limited populations (Dutra 2008).

Both germination rate and seedling development were affected by asymbiotic culture media and photoperiod. Although seeds germinated on all culture media screened, only P723 medium supported the highest germination percentages and advanced seedling development (Stages 4 and 5). Possibly the presence of peptone in the culture media may have promoted the growth and advanced development of *C. punctatum* seedlings since P723 medium is the only medium used that contained peptone. A positive effect of peptone on seedling development was documented by Kauth et al. (2006) in *Calopogon tuberosus*, a terrestrial species. While KC supported highest seed germination for *C. tuberosus*, seedlings grown on P723 medium displayed enhanced seedling development.

The promotive effect of darkness on seed germination was remarkable. Epiphytic orchid species are thought to generally germinate in either light or dark (Arditti 1967; Arditti and Ernst 1984). However, species specific light and dark requirements for germination are often not examined. Our results showed that highest seed germination was achieved in dark for this epiphytic species with moderate gains in germination also being observed under short day (8/16 h L/D) but not intermediate (12/12 h L/D) or long day (16/8 h L/D) photoperiods. While more research is warranted, these similar responses suggest that photoperiod might play a role in seed germination in situ. Interestingly, inhibition of seed germination following light exposure has been demonstrated in many temperate terrestrial orchid species (Arditti et al. 1981; Ernst 1982; Van Waes and Debergh 1986; Yamazaki and Miyoshi 2006). The genus *Cyrtopodium* is mostly comprised of terrestrial species, however; a few species in the genus can be found growing epiphytically. *C. punctatum* is only found growing as an epiphyte on the trunks of trees or on stumps of logged

Table 3 Photoperiodic effects on total in vitro seed germination and the seedling developmental stage of *Cyrtopodium punctatum* during 10 weeks culture in Falcon "Integrid" petri plates on P723 medium (Experiment 2)

Culture duration	8/16 h L/D						12/12 h L/D						16/8 h L/D					
	Seedling developmental stage (%)[a]						Seedling developmental stage (%)						Seedling developmental stage (%)					
	1	2	3	4	5	TG[c]	1	2	3	4	5	TG	1	2	3	4	5	TG
Week 2	93.8b[b]	6.2a	0.0a	0.0	0.0	6.2a	97.4a	2.6b	0.0a	0.0	0.0	2.6b	97.2a	2.4b	0.14a	0.0	0.0	2.5b
Week 4	76.3c	17.8a	2.8a	3.1a	0.0	23.7a	86.2a	11.9b	1.4b	0.5b	0.0	13.3b	81.5b	17.1a	1.2b	0.2b	0.0	18.5c
Week 6	70.5b	15.9a	5.8a	7.8a	0.0	29.5a	78.1a	16.0a	3.4b	2.5b	0.0	21.9b	75.8a	18.8a	2.1b	2.1b	0.0	23.0b
Week 8	63.8b	10.0a	14.5a	11.6a	0.0	36.1a	72.9a	10.6a	11.3a	5.1b	0.0	27.0b	71.1a	11.1a	13.3a	4.5b	0.0	28.9b
Week 10	62.6b	4.3b	16.5a	14.9a	1.7a	37.4a	71.0a	7.9ab	15.1a	5.8b	0.2b	29.0b	69.9a	6.8a	17.7a	5.6b	0.0b	30.1b

[a] Numbers 1–5 = seedling developmental stage (see Table 2)

[b] Percentage values with the same letter within the same developmental stage and week are not significantly different at $\alpha = 0.05$

[c] TG = total germination (Stages 2–5)

Table 4 Photoperiodic effects on seedling development of *Cyrtopodium punctatum* after 25 weeks culture on P723 medium

Photoperiod	Shoot #	Leaf #	Shoot length (mm)	Leaf width (mm)	Root #	Root length (mm)	Total fresh wt (mg)	Fresh shoot wt (mg)	Fresh root wt (mg)	Total dry wt (mg)	Dry shoot wt (mg)	Dry root wt (mg)
8/16 h L/D	1.06b[a]	4.51a	61.99a	2.26a	3.47b	67.19b	21.25b	7.29a	13.96b	2.16b	0.73a	1.39b
12/12 h L/D	1.23a	4.49a	59.31a	2.25a	3.63b	78.86a	22.31b	7.26a	15.06b	2.42b	0.72a	1.52b
16/8 h L/D	1.28a	4.33a	69.86a	2.15a	4.45a	87.07a	28.98a	9.26a	19.71a	3.31a	0.91a	1.97a

Seeds were germinated in Falcon "Integrid" petri plates and then seedlings were transferred after 10 weeks to Sigma-Aldrich Phytatray culture vessels and cultured for an additional 15 weeks (Experiment 2)

[a] Each value represents the mean response of 90 seedlings per treatment. Values with the same letter are not significantly different at $\alpha = 0.05$

cypress trees where seeds are likely to germinate in bark crevices.

Photoperiod appears to have a significant influence on later seedling growth and development in *C. punctatum*. Growth and development of 10 week old seedlings transferred to Phytatrays and cultured for an additional 15 weeks, was significantly enhanced in a long day (16/8 h L/D) photoperiod compared to culture under either short day (8/16 h L/D) or intermediate (12/12 h L/D) photoperiods. Root number, root fresh and dry weights, seedling fresh and dry weights were significantly greater under a 16/8 h L/D photoperiod. In situ, *C. punctatum* forms a root basket that may function for detritus accumulation and water absorption (Dressler 1981). During the wet summer months in Florida under longer natural photoperiods, *C. punctatum* plants produce roots. Enhanced shoot and root growth under 16/8 h L/D in vitro may reflect a similar plant developmental response to environmental conditions occurring in situ.

Acclimatized seedlings showed a high survival rate after 5 weeks acclimatization to greenhouse conditions, however, great care should be taken to control insect pests such as scales after acclimatization. *C. punctatum* seedlings proved highly susceptible to hard scale infestation and monthly pesticide applications were needed.

A reliable asymbiotic seed culture method for the plant conservation of *C. punctatum* has been described. We recommend germinating seeds on P723 in the dark for 8 weeks followed by seedling development under a 16/8 h L/D photoperiod. Seedlings successfully acclimatized to greenhouse conditions can be used for reintroduction and conservation purposes. This should aid in increasing the long term sustainability of remaining *C. punctatum* populations.

Acknowledgements The authors thank the US Fish and Wildlife Service's Florida Panther National Wildlife Refuge and the American Orchid Society for providing financial support for this project. We also thank Phil Kauth, Timothy Johnson, Nancy Philman, Scott Stewart and two anonymous reviewers for their valuable input. Brand names are provided for reference only; the authors do not solely endorse these particular products.

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