Asymbiotic and Symbiotic Seed Culture of Polystachya Concreta (Jacq.) Garay & H.R. Sweet From Ecuador

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Abstract

Protocols for seed germination and seedling development were developed for the orchid, *Polystachya concreta*. Mature seeds were collected from native populations in northwest Andes Mountains of Ecuador. Putative mycorrhizal fungi were isolated from roots of orchid seedlings and juvenile plants *in situ*. Seeds and pure fungal cultures were transported to the laboratory located at the University of Florida, Gainesville, Florida, USA.

Four asymbiotic orchid seed germination media: Knudson-C (KD-C), Vacin and Went Modified Orchid Medium (VW), ½ X Strength Murashige & Skoog (½ X MS), and *Phyto*Technology Orchid Seed Sowing Medium (P723) were examined for their effectiveness in promoting seed germination and protocorm development of *P. concreta* in both 0/24 h and 12/12 h Light/Dark photoperiod at 23 ºC.

Germination occurred regardless of medium and photoperiod treatments. Germinated *P. concreta* seeds attained the seedling developmental stage on all media, except KN-C. Protocorm like bodies were observed when seeds were germinated in complete darkness for the initial 21 days of the experiment.

Effects of putative mycorrhizal fungi isolated from native populations on seed germination and seedling development were also examined. Both germination and seedling development were promoted by *Tulasnella calospora* (UAMH 9824) isolated from *Spiranthes brevilabris* in Florida, USA*. Ceratobasidium* strains isolated from different orchid species from Ecuador did not promote seed gemination and seedling development. *Tulasnella* strains from Ecuador only enhanced seed germination on *P. concreta*. Results of this study will aid in the development of efficient seed germination protocols for the conservation of native orchids from Ecuador.

Introduction

The Orchidaceae is perhaps the largest family of flowering plants with ca. 27,000 species worldwide (Dressler 2005, Chase et al. 2015, The Plant List 2020). Orchids are distributed worldwide, with The Tropics being the region with the greatest orchid diversity (Arditti 1967; Dressler 1993). Specifically, the northern Andes of South America, has been cataloged as a biodiversity hotspot (Myers 2000). An example of this species richness area is exemplified in Ecuador, the smallest country located in Andean South America where more than 4,200 orchid species has been described (Dodson 2003; Endara 2011).

The global diversity and distribution of orchids is the result of complex relationships with pollinators, mycorrhizal fungi (Davis et al. 2015; Swarts and Dixon 2009; Waterman and Bidartondo 2008) and for epiphytic orchids with host trees (Fay et al. 2015; Rasmussen and Rasmussen 2018). Orchid seeds do not contain endosperm or cotyledons. Therefore, they depend largely on nutrients from the mycorrhizal association (Rasmussen 1995). The presence of compatible fungal endophytes is a critical requirement for orchid seed germination, seedling development and establishment *in situ* (Rasmussen et al. 2015). Adult plants may develop different mycobiont associations or may not longer require any association as their photosynthetic capacity increases (Bidartondo and Reed 2008; Rasmussen et al. 2015).

Different patterns of specificity towards mycorrhizal fungi throughout the orchid life cycle have been described mostly for terrestrial orchid species. Some orchids seem to be associated with a high proportion of fungi available on-site during germination, whereas a smaller subset of these mycobionts were able to promote seedling development (Bidartondo and Read 2008; Bonnardeaux et al. 2007). Degree of mycobiont specificity varies among orchids species. High levels of specificity have been reported for a widespread orchid *Pheladenia deformis* (Davis et al. 2015). Some epiphytic adult orchids have also shown high mycorrhizal specificity while others are mycorrhizal generalist with the capacity to grow in different habitats (Otero et al. 2002; Otero et al. 2004; Silva et al. 2020).

One of the widely distributed orchid species is *P. concreta*, a member of the pantropically distributed genus *Polystachya*. Its distribution extends throughout the neotropics, tropical African forests, Indian Ocean islands, Southern India, Sri Lanka and Southeast Asia (Russell 2010). Their altitudinal range extends from sea level up to about 2,400 m. *Polystachya concreta* is an epiphytic orchid that grows on various types of trees without preference for a particular phorophyte (Kolanowska et al. 2020). Plants of *P. concreta* are variable in vegetative characters. They can grow as robust or small herbs with long inflorescences containing numerous small flowers with yellow or yellowish green colors (Fig. 1) (Kolanowska et al. 2020; Russell 2010).

Limited information is available about mycorrhizal endophytes associated with *P. concreta*. *Epulorhiza epiphytica* has been isolated from roots of *P. concreta* in the Atlantic rain forest in Brazil (Pereira et al. 2003), and *T. calospora* from the roots of *P. concreta* adult plants from the state of Minas Gerais, Brazil (Nogueira et al. 2014). Considering, the broad range of habitats where it is possible to find *P. concreta* (Kolanowska et al. 2020; Russell 2010), high levels of specificity for their mycorrhizal associates are less likely to occur for this orchid species (Kolanowska et al. 2020). Currently, *P. concreta* is not considered as a vulnerable or endangered species. However, studies of ecological niche modeling have estimated a significant loss of habitats for this species in the Neotropics due to future climate change (Kolanowska et al. 2020). Habitat loss, overcollection of orchids for their commercial use, lack of control of extremely rare and vulnerable orchids, and an inefficient national system of protected areas also represent major threats for orchid conservation in Ecuador (Cuoco and Cronan 2009; Leon-Yanez et al. 2010). Therefore, *in situ* orchid conservation efforts alone may not provide viable solutions and as such, may need to be complemented with *ex situ* conservation strategies (Fay 2018; Seaton et al. 2010).

Application of *in vitro* seed culture techniques could be a viable approach to facilitate the conservation of many native Ecuadorian orchid species. Many rare and endangered orchids species have already successfully been germinated and propagated using *in vitro* seed culture protocols (Dutra et al. 2008; Hughes and Kane 2018; Hoang et al. 2017; Kendon et al. 2020). Mycorrhizal associations can be established under sterile and controlled environmental conditions using orchid *in vitro* seed germination combined with co-culture with its mycobiont (Zettler et al. 2017; Zettler 1997). Although, successful symbiotic germination *in vitro* does not guarantee that the same association occurs *in situ* (Rasmussen et al. 2015). Symbiotic seed culture can provide insight into some aspects of the mycorrhizal association

such as the physiological compatibility, patterns of *in vitro* mycobiont specificity, and dependency during germination and seedling development (Bonnardeaux et al. 2007).

Scientific research on *in vitro* seed germination and subsequent protocorm and seedling development is very limited for Ecuadorian Orchids (Gonzales and Cueva 2014). Levels of specificity and dependency of orchid species for certain fungal species and other critical factors that affect seed germination and seedling development need to be experimentally evaluated to successfully use this approach for conservation of native orchid species from Ecuador (Rasmussen et al. 2015; Fay 2018).

Therefore, the objectives of the present study were to: 1) evaluate the effects of photoperiod and medium composition on *in vitro* seed germination and seedling development on *P. concreta*; 2) document the morphological development from seed to the mature seedling stage; 3) screen putative mycorrhizal fungi isolated from native Ecuadorean orchids roots by testing their effectiveness on promoting symbiotic seed germination and seedling development; and 4) determine the level of *in vitro* specificity and dependency of this native orchid species for their mycorrhizal fungi. The overall goal was to develop an effective protocol to reproduce orchids species with the potential to be reintroduced with the aim of achieving their long-term conservation.

Materials And Methods

Seed Collection

Mature seed capsules of *P. concreta* (Jacq.) Garay & H.R. Sweet were collected from a native population in the Northern tropical Andes of Ecuador on 23 August 2018. Plants with closed mature capsules were located along the roadside to Chical (0º48'36.5"N 78º12'55.30"W, 1370 m.a.s.l.), Carchi Province, Ecuador. Upon return to the lab, capsules were place in a sealed container containing $CaSO_4$ desiccant at 22±2 ºC. After 24 hours, seeds were removed from the capsules and stored in glass vials over $CaSO_4$ for shipping to the laboratory located at the University of Florida, Gainesville, Florida, USA. Seeds were stored at -10 ºC in the dark at 0% relative humidity until used in the experiments. Two vials of seeds were maintained at 22±2 ºC in the dark for 16 weeks before transferred to storage conditions.

Seed Viability Testing

Seed viability tests using triphenyl tetrazolium chloride (TTC) were performed in seeds before being transfer to storage cold conditions and after 7 weeks in storage. Approximately 5 mg of seed contained in 1.5 ml microtube was preconditioned in a 10% sucrose solution for 24 hours at 22±2 ºC. Solutions were removed and seeds were washed twice with deionized distilled (dd) water. A 1% TTC solution was added and the microtubes were incubated in the dark at 36 ºC for 24 hours. The TTC solution was removed and replaced with dd water before samples were stored at 4 ºC until their evaluation of seed viability using a light microscopy. Seeds with rose-red stained embryos were considered viable, while those with white embryos were recorded as non-viable (Hosomi et al. 2011; Hosomi et al. 2012).

Seed Sterilant Screening

To optimized seed sterilization procedures, sodium hypochlorite (NaOCl) and calcium hypochlorite [$Ca(OCl)_2$] solutions were used to test their individual effectiveness as orchid seed surface sterilants. Seeds surface sterilization was evaluated using a 0.3% or 0.5% sodium hypochlorite/ethanol solution or 2% calcium hypochlorite in a sterile 1.5 ml sterile microtube for 1, 3 and 5 minutes. The 0.3 % and 0.5% sodium hypochlorite solutions were prepared by combining 250 or 416 µl 6.0% sodium hypochlorite (Clorox bleach), 250 µl 100% ethanol and 4,500 or 4,334 ml sterile dd water. The calcium hypochlorite solution was prepared by dissolving 307 mg $Ca(OCl)_2$ (65% available chlorine) in 10 ml sterile dd water. This gave a final solution providing 2% available chlorine. The solution was filtered through sterile white filter paper (Whatman ®, Qualitative 1, Cat# 1001-125) immediately before use. After sterilization, solutions were removed with a micropipette by placing the sterile pipette tip into the bottom of the microtube. Seeds were then rinsed three times for 1 min. each with sterile dd water.

After sterilization, three 60 µl aliquots of the sterile seed solution were pipetted and each was placed on a separated section into a Petri dish (100x15mm) containing 30 ml ½ X Strength Murashige & Skoog (½ MS; *Phyto*Technology Laboratories, Shawnee Mission, KS, USA Cat# M524, Murashige and Skoog 1962). Seeds then were distributed uniformly using a sterile inoculating loop. Four Petri dishes per treatment were sealed with one layer of sealing film (*Phyto*Technology Laboratories, Shawnee Mission, KS, USA Cat# A003) and incubated at 23 ºC under a 12h-Light /12h-Dark photoperiod for 3 weeks. Light was provided by cool-white florescent tubes (General Electric F20T12/CW) at 35 µM m^{-2} s^{-1} (PAR) in a Percival I-35LL growth chamber (Percival Scientific, Boone, IA).

Asymbiotic Seed Germination

Four asymbiotic orchid seed germination media: 1) Knudson-C (KND-C; *Phyto*Technology Laboratories, Shawnee Mission, KS, USA Cat# K400, Knudson 1946); 2) Vacin and Went Modified Orchid Medium (VW; PhytoTechnology Laboratories, Shawnee Mission, KS, Cat# V891, Vacin and Went 1949); 3) ½ X Strength Murashige & Skoog (½ MS; *Phyto*Technology Laboratories, Shawnee Mission, KS, Cat# M524, Murashige and Skoog 1962); and 4) *Phyto*Technology Orchid Seed Sowing Medium (Cat# P723) were examined for their effectiveness in facilitating seed germination and seedling development of *P. concreta*. To standardize the concentrations of 2% sucrose, 0.8% agar and 0.1% activated charcoal, all basal media were modified as follows: 20 g/L sucrose was added to 1/2 MS, 8 g/L TC agar (*Phyto*Technology Laboratories, Shawnee Mission, KS, USA Cat# A175) to KND-C and ½ MX, 1 g/L TC agar to VW, and 1 g/L activated charcoal to KND-C, ½ MS, and VW. KND-C and ½ MS was adjusted to pH 5.8. All media were sterilized by autoclaving at 117.7 kPa and 121 ºC for 40 min.

Based on preliminary sterilant screening, seed sterilization was performed using a filtered solution of 2% calcium hypochlorite for 1 min. followed by three 1-min. rinses with sterile dd water. After sterilization, three 60 µl aliquots containing between 60-80 sterile seeds each, were pipetted and distributed uniformly using a sterile inoculating into Petri dishes (100x15mm) containing 30 ml of each of the four asymbiotic

seed germination media. Petri dishes were sealed with one layer of sealing film and incubated under both 0/24 h and 12/12 h Light/Dark photoperiod at 23 ºC. The complete darkness treatment was achieved by wrapping stacked Petri dishes in heavy duty aluminum foil.

Plates were first inspected for seed germination after 1-week culture. At 3 weeks Petri dishes from the 0/24 h photoperiod were unwrapped from the aluminum foil and placed under a 12/12 h Light/Dark photoperiod for the rest of the experiment. Seed germination and seedling developmental stages were assessed on a scale of 0-6 (Table 1). Germination and seedling growth and development were recorded every 2 weeks after the first week using a stereomicroscope.

Seedling Development Experiment

Survival and further development of *P. concreta* seedlings were evaluated using three media: 1) P723; 2) PhytoTechnology Orchid Maintenance/Replace Medium (Cat# P748); and 3) PhytoTechnology Orchid Multiplication Medium (Cat# P793). After 16 weeks on P723, 12 Stage 6 seedlings were transferred into each of four Phytatray™ II (Sigma-Aldrich, Cat# P-5929) vessels containing 125 ml of each medium. Vessels were sealed with one layer of sealing film and incubated under a 12h-Light /12h-Dark photoperiod at 23 ºC. Percent survival and seedling length were recorded after 25 weeks.

Symbiotic Seed Germination

Isolation of putative mycobionts

Isolation of root endophytes from root cortical tissue was performed at IDgen, a commercial molecular diagnostic laboratory, located in Quito, Ecuador. Standard methods described by Zettler et al. (2003) were applied. Briefly, roots from young orchids or seedlings, when available, were collected in the field and transported to the laboratory. Roots were rinsed with sterile distilled water, then surface sterilized with a 0.4% sodium hypochlorite/ethanol solution, followed by three 1-minute rinses with sterile distilled water. This solution was prepared by combining 8 ml 5% sodium hypochlorite (Clorox bleach), 5 ml 98% ethanol and 87.0 ml sterile distilled water.

Roots were cut into 1 cm long segments starting at the tip. Each segment was placed into a separate sterile Petri dish containing 200 µl sterile distilled water. Root segments were macerated using sterile scalpel and forceps to separate and break open the cortical cells containing fungal pelotons. Warm Fungal Isolation Medium (FIM) (Clements et al. 1986) containing 10ml/L streptomycin sulfate antibiotic, was slowly added to the dish. To uniformly distribute the macerated tissue, dishes were gently swirled before solidification of the medium. Using a dissection microscope, plates were observed after 24 hours for active hyphal growth emerging from the pelotons. Individual pelotons with hyphal tips were subcultured using a sterile scalpel into ½ Potato Dextrose Agar (PDA, PhytoTechnology Laboratories, Shawnee Mission, KS, USA Cat# P772) and incubated for 24-72 hours at 22 ± 2 ºC. Hyphal tips or pelotons were then transferred to fresh ½ PDA solidified on a 45º angle in glass culture tubes. Glass tubes were sealed with a layer of sealing film and incubated at 22 ± 2 ºC for at least 48 hours and then

stored at 4 ºC until their transportation to the laboratory located at the University of Florida, Gainesville, Florida, USA.

Fungal strains with morphological features that have been previously assigned to the form-genus *Rhizoctonia* (Currah et al. 1997) were subcultured on ½ PDA for further identification to the genus level using Sanger sequencing of the ribosomal DNA internal transcribed spacer (ITS) sequences (Otero et al. 2002). Subcultures of putative mycorrhizal fungi were transferred to fresh ½ PDA plates every 4 months and maintained at 10 ºC in the dark.

Symbiotic seed culture

Fungal strains, isolated from different native orchid species from Ecuador and Florida, USA, were used to examine their ability to enhance seed germination and seedling development of *P. concreta.* Symbiotic treatments consisted of one fungal culture provided by Dr. Lawrence W. Zettler (Illinois College, Jacksonville, IL): *Tulasnella calospora* strain (University of Alberta Microfungus Herbarium UAMH 9824) isolated from a primary lateral root of *Spiranthes brevilabris* in Levy County, Florida, USA in 1999 (Stewart et al 2003), four fungal strains isolated from roots of plants collected from native orchids species from Ecuador: two *Tulasnella* sp. strains isolated one from *Lepanthes acarina* (EC16-LPA2T [UAMH12450]) and the other from *Campylocentrum* sp. (EC17-CM4T [UAMH 12451]), and two *Ceratobasidium* strains isolated from *Ericina pumilla* (EC18-ER2C) and *Macroclinium* sp. (EC18-MC4C [UAMH12454]). Few pelotons were observed in cortical tissue of roots of *P. concreta* collected from juvenile or adult plants. Unfortunately, no putative mycorrhizal fungi could be isolated therein.

Based on the asymbiotic media screen responses, P723 was included as the asymbiotic treatment control. A second control treatment consisted of the symbiotic medium with seeds but without fungal inoculation (Fig. 9d). Oatmeal Agar (OMA; 2.5 g/L oats flour, 7 g/L agar (*Phyto*Technology Laboratories, Shawnee Mission, KS, USA Cat# A175)) was used as the symbiotic seed germination medium. A triangle-shaped cellophane membrane (BIO-RAD, Cat# 1650922) with one black filter paper strip (Thomas Scientific, USA Cat# 4740C10) on each side were placed on the surface of the symbiotic medium after it solidified (Fig. 9d). Medium was sterilized by autoclaving at 117.7 kPa at 121 ºC for 40 min. Approximately 30 ml of cooled but molten sterile OMA was dispensed in each (100x15 mm) plastic Petri dish. Seeds were removed from cold storage and maintained at 22±2 ºC for at least 4 hours before sterilization. Seed sterilization was performed using the same procedure described for the asymbiotic seed germination experiment. After sterilization, 60 µl aliquots containing 60-80 seeds were placed on the surface of each 1 cm X 4 cm black sterile filter paper.

Fungal inoculation was performed by cutting a 1X1X0.5 cm3 ½ PDA block containing active growing fungal mycelia harvested from the edges of a week-old culture in the case of *Tulasnella* and 2 day-cultures of *Ceratobasidium* (Fig. 10). The PDA block was placed into each Petri dish on the center of the membrane (Fig. 9e). Petri dishes were sealed with one layer of sealing film and incubated under in both 0/24 h and 12/12 h Light/Dark photoperiods at 23 ºC. Plates were examined for evidence of germination after 5 days. Germination and seedling development stages (Table 1) were recorded every 5 days until

day 20, and then weekly, thereafter until 9 weeks. Roots from seedlings produced on the symbiotic medium were finely sliced and stained using an aqueous 0.5% Toluidine Blue Staining Solution to evaluate the presence of fungal pelotons in the cortical tissue.

Experimental Design and Statistical Analysis

Treatment effects were evaluated using a completely randomized experimental design. Each factor or combination of factors consisted of 7 replicates with 3 sub-replicates. The experimental unit consisted of a Petri dish containing 30 ml culture medium with three areas or three filter paper strips with ca. the same number of seeds on the surface. These areas were considered as sub-replicates. All experiments were repeated once. Numbers of seeds in each developmental stage (Table 1) were recorded at each data collection time point. Percentages of individuals in each stage were calculated for each treatment by dividing the number of seeds in each stage by the total number of viable seeds in each sub replicate. Percent data were arcsine transformed to normalize variation prior to analysis. Data were analyzed using general linear model procedures with a multifactor Analysis of Variance (ANOVA). Mean separation was assessed using Tukey's Methods at $\alpha = 0.05$ significant level. Statistical Analysis were completed with JMP Pro15 (SAS Institute Inc.).

The time-to-event data, in this case time from sowing to germination (Stage 2), were analyzed using the survival analysis package in R studio 3.2.5. Survival analysis is a statistical method that deals with time to events. It has many important applications in clinical research and epidemiology as well as other disciplines. It is believed that time-to-event analysis provides the most appropriated method to analyze germination, because the analysis is based on the distribution of germination times of individuals seeds rather than on cumulative germination (McNair et al. 2012). A Kaplan-Meier estimator was used to estimate the survival functions with 95% confidence intervals. Log-rank or Stratified log-rank test were performed to evaluate differences between survival curves at the $\alpha = 0.05$ significant level and multiple pair wise comparisons were performed using log-rank test with a Bonferroni correction to adjust *p*-values at $\alpha=0.05$ significance level.

Results

Seed Viability Testing

Percentage viability of *P. concreta* seeds before being transferred to storage conditions was 92.2%. Low temperature storage conditions did not affect seed viability as 94.8% of seeds were viable after being subjected to cold storage conditions for 7 weeks. Seeds that were stored at 22±2 ºC in the dark for 16 weeks after field collection also maintained viability. Base on TTC testing, a 93.1% seed viability was observed.

Seed Sterilant Screening

No visual bacterial or fungal contamination was observed with any of the seed sterilization treatments. Twenty percent of culture plates in the control treatment (no surface sterilization) were discarded due to visual contamination. Germination percentage was not negatively affected when 2% $Ca(OCl)_2$ was used as the sterilant from 1 up to 3 min.. Sterilization times longer than 1 min. in 0.3% NaOCl significantly reduced germination percentage. Significantly lower germination percentages were observed in seeds sterilized with 0.5% NaOCl for 1 min. compared to the other treatments. Longer sterilization periods resulted in the complete absence of germination (Fig. 2).

Asymbiotic Seed Germination and Seedling Development

The morphological development from seed to mature seedling was documented for the first time for *P. concreta* (Fig. 3 a-h). Rapid germination (Fig. 3 a-b) was observed, with greater than 75 % germination occurring within a week in all treatments. Seeds that were incubated in complete darkness 24h-Dark/0h-Light (88% at 7 days) germinated relatively faster than seeds incubated under 12h-Dark/12h-Light (81% at 7 days). After 21 days, when all treatments were exposed to a 12-h photoperiod, a significant difference in germination percentages between photoperiod treatments was not observed. Seed germination percentages approached a plateau after 21 days in all treatments (Fig. 4).

Significant differences in germination percentage over time between the two photoperiods were also observed within each asymbiotic seed germination medium type (Stratified long-rank test p=3e-7) (Fig. 5). Significantly faster germination was observed when seeds were sown either in VW or ½ MS media under 24h-Dark/ 0 h-Light compared to 12h-Dark/12h-Light. Whereas higher germination percentages were observed in seeds incubated under 12h-Dark/12h-Light than 24h-Dark/ 0h-Light photoperiod in P723 across all times. Cumulative germination percentages after 35 days were not significantly different between the two photoperiods when seeds were sown in ½ MS. However, seeds sown in VW attained significantly higher germination percentage under 24h-Dark/ 0h-Light photoperiod for 21 days compare to seed under a constant 12-h photoperiod. The effect of photoperiod on seed germination was not significant in KND-C medium according to the log-rank test p-value= 0.13.

All (100%) of the germinated seeds transitioned to globular-shaped protocorms with rhizoids (Stage 3, Table 1; Fig. 3c) in all treatments by 21 days culture (Fig. 6). Non-green protocorms with more rhizoids were observed in plates incubated under complete darkness. Protocorms with leaf primordia and rhizoids (Stage 4, Table 1; Fig. 3d) were also observed at 21 days, with significantly higher percentages observed under 24h-Dark/0h-light than in 12h light/12h-Dark on P723, ½x MS and VW (Fig. 6). By 35 days, Stage 3 and 4 protocorms in the initial 21-day period of complete darkness became chlorophyllous in all medium tested expert in KND-C. Only white protocorm with lead primordia were observed in KND-C.

At 35 days, seedlings with the first elongated leaf developing from the center of an elongated leaf primordia (Stage 5, Table 1; Fig. 3f) were observed on P723 with significantly higher percentages compared to other media in both photoperiods. Higher percentages of these Stage 5 seedlings were observed when seeds were germinated under complete darkness for 21 days only on P723. Whereas on

VW and ½ MS, the higher percentages of Stage 5 seedlings were observed under 12h-Light/12h-Dark photoperiod compared with plates maintained under the 21-day initial darkness period. The significant reductions in seedling production on VW and ½ MS media was due to protocorms like bodies production (Fig. 3e) instead of the formation of the second leaf and further development.

Stage 6 (elongation of the second leaf, Fig. 3g) development was observed on all media at 49 days in both photoperiods except in KND-C. At the end of the experiment (77 days), P723 supported the highest percentage of Stage 6 seedling development (90%) when seeds were initially incubated for 21 days under complete darkness (Fig. 6). Whereas on VW and ½ MS, significantly higher percentages of Stage 6 seedlings were observed in plates incubated under a constant 12-h photoperiod compared to plates under complete darkness for 21 days at the beginning of the experiment (Fig. 6).

The highest (100%) seedling survival was observed on P748. Survival was significantly reduced when seedlings were cultured either on P723 or P793. Significantly longer seedlings were observed on P748 compare with P723 and P793 (Table 2). Protocorm-like bodies (Fig. 3i) at the base of seedlings growing on P793 were observed, resulting in a significant reduction in seedling length compared to the other treatments.

Symbiotic Seed Germination

Seed germination and seedling development were both promoted in *P. concreta* when seeds were co-cultured with *Tulasnella* fungal strains isolated from different orchid species. Significantly higher rates of germination in seeds incubated under 12h-Dark/12h-Light compared to 24h-Dark/0h-Light and significant differences in gemination rates among the symbiotic treatments were observed (Fig. 7). No germination was observed when seeds were co-cultured with either of the two *Ceratobasium* fungal strains (CM4C, ER2C) under any of the photoperiods tested (Fig. 7). At most 25% of seeds germinated on the symbiotic control treatment. However, they exhibited the lowest germination rates compared with P723 or the other symbiotic treatments.

Significant improvement in germination rates was observed when seeds were co-cultured with *Tulasnella* LPA2T or CM4T compared with the control (Fig. 7). Significant higher germination rates were observed when seeds were cocultured with the *Tulasnella* strain US266 compared with *Tulasnella* strains collected from orchid species from Ecuador: LPA2T or CM4T. The highest germination rates were observed in seeds sown on P723. Only after 5 days culture 71% of seeds germinated under 24h-Dark/0h-Light photoperiod and 48% under 12h-Dark/12h-Light (Fig. 7).

Globular protocorms (Stage 3) were observed at 10 days cultures only in seeds germinated on P723 or inoculated with US266. At 35 days, globular green protocorms were observed in plates inoculated with LPA2T. Development of leaf primordia (Stage 4) was observed at 28 days only on protocorms germinated on P723 or co-cultured with US266 (Fig. 8). Leaf primordia elongation and formation of the first leaf (Stage 5) was observed at 35 days again only on P723 or in the presence of US266. Seedlings with two leaves (Stage 6) were observed after 49 days culture on both P723 and US266 (Fig. 8). Uniform

production (Fig. 9f) with significantly higher percentage of Stage 6 seedlings were observed on P723 compared with US266 (Fig. 9), however larger and longer seedling were produced with US266 (Fig. 9c). Stained fungal pelotons were observed in roots of seedlings cocultured with US266 (Fig. 9b).

Discussion

Our results showed that asymbiotic germination techniques can be used to obtain high germination percentages and produce seedlings (40mm) of *P. concreta* from seeds collected from a native population in Ecuador. Early development of seedlings (two first leaves and short roots) was possible using symbiotic seed culture with mycorrhizal fungi of the *Tulasnella* genus isolated from different orchid species. Seedlings were not acclimatized to ex vitro conditions due to restrictions outlined in the import permit (USDA/APHIS P37-16-0556) that prohibited the ex-vitro culture of these non-native orchid species in the United States.

Polystachya concreta has a uniquely wide distribution, it is the only species that can be found on all continents in a variety of habitats (Kolanowska et al. 2020). In Ecuador, it is possible to find it growing in all three continental regions: Coastal, Andean, and Amazonian (Tropicos.org. Missouri Botanical Garden). Small populations or individual adult plants were observed in most of the collection sites in the east side of the northern tropical Andes during three field collections in Ecuador from 2016 to 2018. However, plants with mature capsules were only observed during August to September 2018, which are considered dry season months (INAMHI 2015). This indicated that relative these dry months may be the ideal season to collect mature seeds of this orchid species.

Viability and germination potential in orchid seeds may be accurately predicted using Triphenyl tetrazolium chloride (TTC) staining (Hosomi et al. 2010, Hosomi et al. 2012). Percent viability of *P. concreta* (92%) did not differ from the actual maximum germination percentage (96%), indicating for this orchid species, TTC staining can be used as reliable test to estimate germination capability (Fig. 9). However, for other orchid species, researchers have reported lower viability percentages than actual germination rates using TTC staining (Johnson and Kane 2011; Rafter et al. 2016). High viability percentages have been also reported for *P. concreta* seeds from Argentina using TTC staining (Lallana et al. 2019).

Seed surface sterilants and exposure time should be screen for newly collected seeds before testing other factors that affect for affect germination and seedling development (Swarts and Dixon 2017). Sodium hypochlorite (NaOCl) and calcium hypochlorite [$Ca(OCl)_2$] have been commonly with orchid seeds (Swarts and Dixon 2017). Chemical sterilants also served as a scarification treatment to remove suberin from the seed surface, which results in improvement of water imbibition thus increasing germination percentages (Kauth et al. 2008; Rasmussen 1995). However, strong hypochlorite concentrations and extended sterilization times can negatively affect germination by damaging the orchid embryo. In our study, surface sterilization of *P. concreta* seeds was achieved in all combination treatments of sterilant concentration and exposure time. However, germination percentage was significantly reduced compared

with the non-sterilization control treatment, when 2% $Ca(OCl)_2$ for 5 minutes, 0.3% NaOCl for more than 1 min. or 0.5% NaOCl were used as sterilant treatments. Significant reductions in seed germination percentages were also observed in several Brazilian epiphytic orchids when seeds were sterilized with 0.4% or 0.8% NaOCl for more than 5 min. (Alvarez-Pardo et al. 2006). High germination percentages observed in seeds that were not sterilized before sowing indicated that *P. concreta* seeds did not need a scarification pretreatment before germination.

Responses to light are highly variable among orchid species. Light has been reported as an inhibitory factor for germination especially for terrestrial orchid species as well as for some epiphytic species (Rasmussen et al. 2015; Dutra et al. 2009; Zi et al. 2014). In our study, significant differences in cumulative germination percentage at the end of the experiment were not observed, thus supporting the general assumption of that epiphytic orchid seeds germinated either in the light and in the dark (Arditti 1967). However, a delayed effect of light on seed germination was observed, seeds cultured under complete darkness reached maximum germination percentages faster than seeds germinated under a12h-light photoperiod. A similar response to light has been reported in *Bletia purpurea* where culture under a 12h- photoperiod resulted in significantly lower germination than cultured in darkness for 3 weeks. However, nearly 100% germination occurred in all treatments by week 6 (Johnson and Kane 2011).

Effects of light on *in vitro* seed germination also varies with the composition of the culture medium. Significant higher percent germination of *Bletia purpurea* seed was observed under light compared to dark in the absence on sucrose, but when media were supplemented with 10 or 50 mmol sucrose, differences were not significant between illumination treatments (Johnson and Kane 2011). Our results showed that the effect of light on *in vitro* germination of *P. concreta* seeds varied between culture medium type. Light enhanced germination rate was observed when seeds were sown on P723 compared to that observed on ½ MS or VW. Probably due to the differences in composition of mineral and organic nutrients between media tested.

Knudson-C medium (Knudson 1946) has been widely used to germinate and propagate orchids (Rasmussen 1995). However, our results indicated that while germination of *P. concreta* is supported on KND-C, subsequent seedling development was not. A low percentage of etiolated protocorms produced on KND-C contained underdeveloped leaf primordia and few rhizoids. It has been also reported that Knudson medium was not suitable for optimal seedling development in other epiphytic orchids (Avila-Diaz et al. 2009). The limited development of *Calopogum tuberosus* seedlings on KND-C was attributed to the presence of high nitrate concentration (Kauth et al. 2006). Orchid protocorms or germinating seeds are not able to synthesize nitrates due to absence of nitrate reductase activity in these early growth and development stages (Raghavan and Torres 1964).

Although faster and higher cumulative germination was observed when seeds were sown under complete darkness for initially 21 days on ½ MS and VW, seedling development was negatively affected. Formation of protocorm like-bodies (PLB) instead of Stage 5 and 6 seedlings was observed when seeds were

incubated under complete darkness. Induction and proliferation of PLB is one of the preferred methods for commercial clonal propagation of orchids (Cardoso et al. 2020). However, clonal propagation by PLB production is not recommended for conservation purposes, as it results in low genetic diversity of the out planted plant material which will not ensure the long-term persistence of restored populations (Johnson et al. 2010; Kettenring et al. 2014). On the other hand, seedling production on P723 was significantly improved when seeds were exposed to 21 days of complete darkness at the beginning of the culture period. No PLBs were observed on P723.

The orchid medium P723 differs from the other media tested by the presence of organic compounds such as peptone (2000 mg/l), an organic nitrogen source that can supply auxin-like compounds and several amino acids (Kauth et al. 2008). High germination percentages and uniform early seedling development observed in *P. concreta* may be due to presence of peptone in P723. High germination percentage and uniform early seedling development in *Calopogon tuberosus* and *Cyrtopodium punctatum* have been also attributed to the presence of peptone (Dutra et al. 2009; Kauth et al. 2006). However, advanced seedling development was not supported on P723. Maximum survival and larger seedlings were observed only on P748, which contains double the amount of mineral salts than P723. This indicates the importance of mineral nutrition during advance seedling development in *P. concreta*. The significant reduction in seedling length observed on P793 can be explained by the induction of PLB at the base of the seedlings and formation of multiple shoots. PLB formation is likely due to the presence of 6-Benzylaminopurine (BA) in the medium. Cytokinins have been supplemented to culture medium to induce formation of PLBs in numerous orchid species (Ng and Saleh 2011).

Compatible mycorrhizal fungi are critical for germination and seedling development of orchid species *in situ* (Rasmussen et al. 2015). Information regarding host specificity and the roles of mycobionts through the orchid life cycle is limited for epiphytic species in the neotropics. To our knowledge, this is only the second report that fungal strains isolated from orchid species from Ecuador have been screened for their potential to promote orchid seed germination and seedling development (Durán-López et al. 2019). In our study, fungal isolates belonging to the genera *Tulasnella* and *Ceratobasidium*, isolated from different orchid species located in different habitats and geographical locations than *P. concreta,* were used to assess *in vitro* specificity and dependency for the mycobiont during germination and early seedling development of this native orchid species from Ecuador.

Ceratobasidium fungal strains completely inhibited germination of *P. concreta* seeds. In contrast, other *Ceratobasidium* strains enhanced germination and seedling development in other epiphytic orchid species (Hoang et al. 2017; Otero et al. 2004). However, those mycobionts were isolated from the same orchid species. *Polystachya concreta* showed a preference for *Tulasnella* strains as mycobionts, but they may not require a specific species from this genus during germination as all *Tulasnella* strains screened promoted significantly higher percent germination compared with the symbiotic control (OMA with no fungal inoculation). However, only US66 (*T. calospora* - UAMH 9824) was able to facilitate further seedling development. US266 has been applied to inducing seedling formation in germinated *P. concreta* seeds from south Florida (L. Zettler, personal communication). The mycorrhizal association between *P.*

concreta and US266 was confirmed by the presence of pelotons in the root cortical tissue of *in vitro* germinated seedlings roots. Conceivably this is likely to occur *in situ* as only *T. calospora* and other *Tulasnella* sp. have been isolated from *P. concreta* so far (Pereira et al. 2002; Nogueira et al. 2014). Thus, indicating the preference of this orchid for *Tulasnella* species as mycobionts and the possible specificity for only a subset of them during early seedling development. A similar specificity pattern has been also described for some terrestrial orchid species, where only a subset of fungi that promoted germination were able to support early seedling development (Bidartondo and Read 2008; Bonnardeaux et al. 2007).

US266 has been nicknamed as the “Super Fungus” due to its ability to stimulate germination in a numerous orchid species from different geographic locations (Zettler et al. 2007; Zettler and Dvorak 2021). An association with this broadly distributed non-specific orchid symbiont during early development stages may help to explain the pantropical geographical distribution of this orchid species (Kolanowska et al. 2020). The ability of US266 to promote faster germination and seedling development is probably due to its faster growth rate compared with other the *Tulasnella* strains (Fig. 10). Faster growth can result in faster and higher colonization of fungus in the orchid tissue, thus providing faster and more nutrients and carbohydrates to support germination and seedling development.

Delayed germination in the presence of CM4T and LPA2T compared with US266 can be explained by their differences in fungal hyphae growth rates. After 15 days, LPA2T hyphae reached the seeds on the filter paper whereas CM4T reached seeds only after 35 days in culture, which corresponded with the time when seeds started to germinate with each isolate, regardless of the photoperiod. The easy visualization of the hyphae growing on OMA was made possible by adding the cellophane membrane (Fig. 9d). The black filter paper was also useful to determine when hyphae reach the seeds and the black background also facilitated distinguishing the different seedling stages.

A reliable asymbiotic seed germination and seedling development protocol for *P. concreta* from Ecuador has been optimized. We recommend seed germination on P723 with a short initial culture period of complete darkness to improve subsequent seedling development. For conservation purposes, it is important to test putative mycorrhizal fungi isolated from the same or sympatric species (Steward and Kane 2007). Thus, increasing the availability of compatible symbionts or addressing questions of specificity which directly will impact the success of conservation efforts (Swarts and Dixon 2009). The eventual release of seedlings with their fungal mycobiont from the same geographic location will be less risky from an ecological standpoint (Zettler et al. 2007; Zettler et al. 2017). Future research should be also focused on acclimatization of seedlings obtained from asymbiotic versus symbiotic protocols. Understanding the critical role of the mycobionts on seedling development and establishment will be useful to ensure the long-term sustainability of the restore populations (Zettler et al. 2003) in the original location or in a safe site when assisted migration is used as a conservation strategy. (Fay et al. 2018; Seaton et al. 2010; Swarts and Dixon 2009).

Declarations

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Competing Interests

All authors state that there are no financial or non-financial interests that are directly or indirectly related to this work submitted for publication.

Author Contributions

All authors significantly contributed to various aspects of the study. Plant field locations and identification of the plants from which seed capsules and root samples were collected were determined by Luis E. Baquero. Lawrence Zettler provided training in the isolation, culture and identification of mycorrhizal fungi and symbiotic seed culture techniques. Experimental designs, interpretation of results and discussion were provided by Paulina H. Quijia-Lamina, Michael E. Kane and Lawrence W. Zettler. The first draft of the manuscript was written by Paulina Quijia-Lamina and all authors commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Tables

Table 1

Seed germination and seedling developmental stages of *Polystachya concreta*. Adapted from Stewart and Zettler (2002)

Stage	Description
0	No germination, viable embryo, testa intact
1	Swollen embryo, filling the seed coat
2	Continued embryo enlargement, rupture of testa (GERMINATION)
3	Embryo is released from the seed coat, Appearance of globular protocorm, production of rhizoid(s)
4	Emergence of leaf primordia
5	Elongation of leaf primordia and appearance of the first leaf (SEEDLING STAGE)
6	Elongation of the second leaf and further development

Table 2

Effects of medium type on survival and growth of 16-weeks old *Polystachya concreta* seedlings germinated on P723 at 23 °C under 12h-Dark/12h-Light photoperiod after 25 weeks culture. Treatments with the same letter are not significantly different (α=0.05).

Medium type	Survival (%)	Seedling length (mm)
P748	100.0 [a]	40.5 [a]
P723	52.1 [b]	16.1 [b]
P793	31.3 [b]	14.8 [b]

Figures

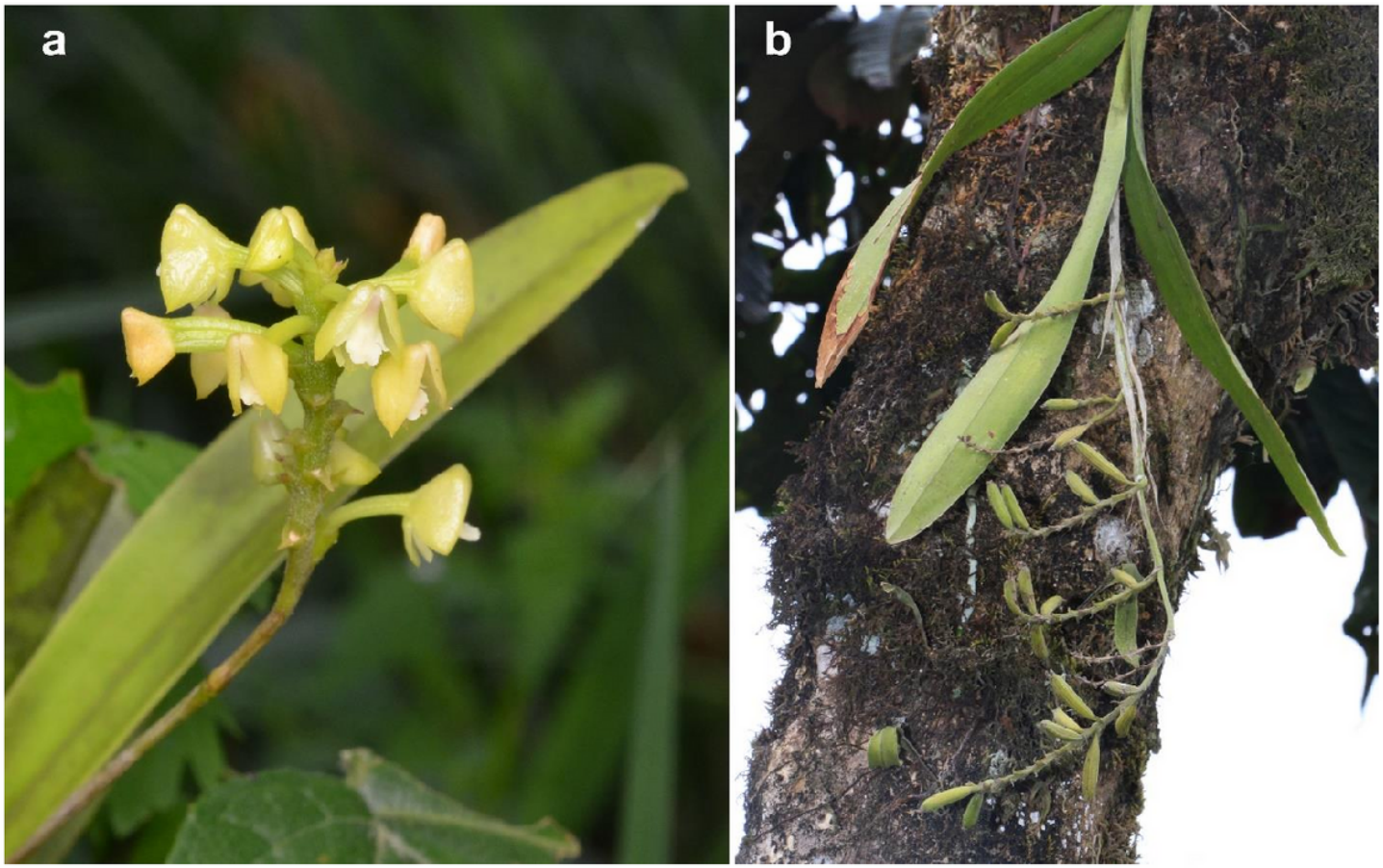

Figure 1

Polystachya concreta in flower (a) and bearing mature seed capsules growing in the Northern Andes Mountains of Ecuador (b). Photo (a) by Luis E. Baquero with permission.

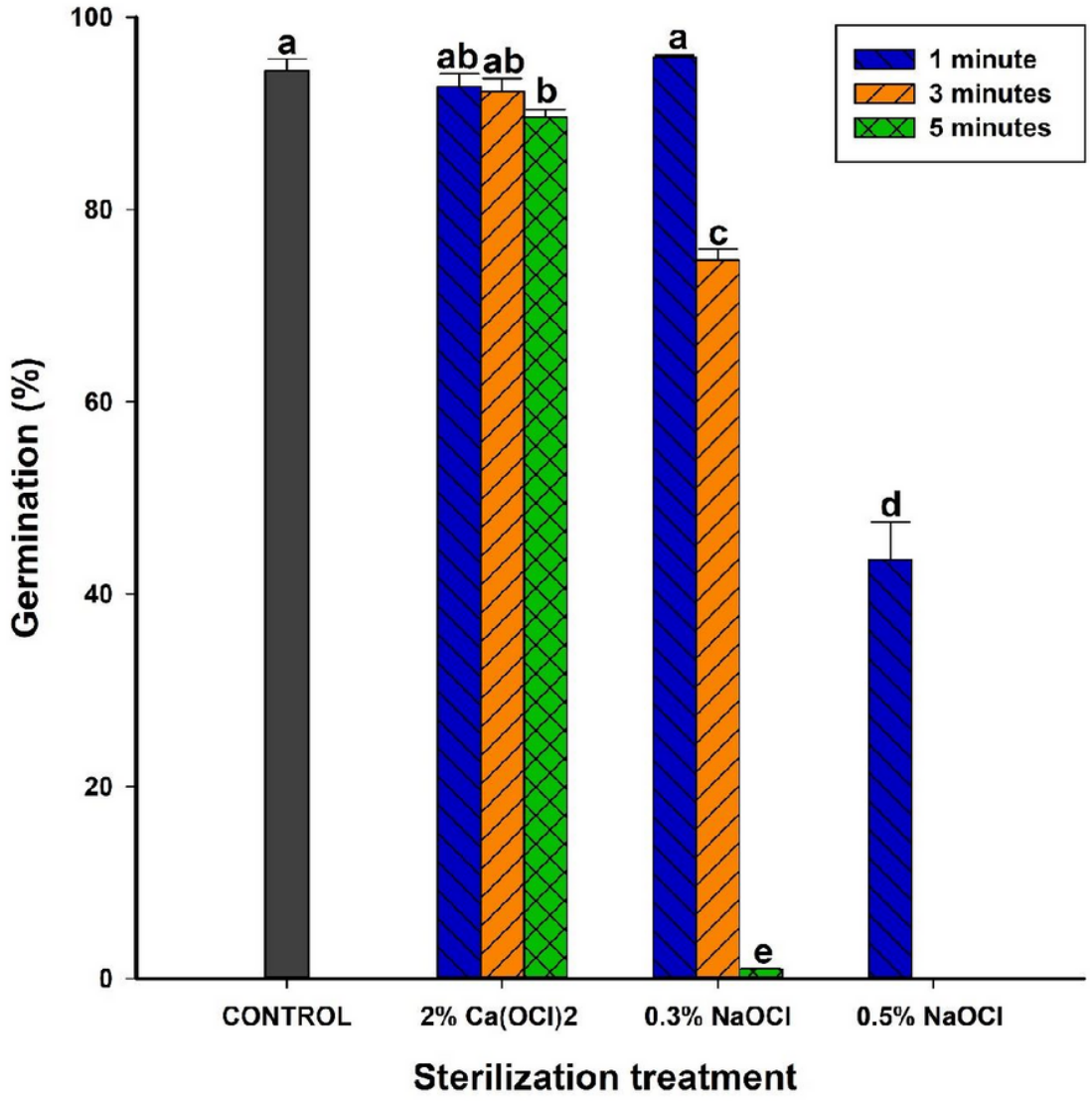

Figure 2

Effects of seed surface sterilization treatments on germination of *Polystachya concreta* seeds incubated under 12h-Light/12h-Dark at 23°C for 2 weeks. Histobars with the same letter are not significantly different (α=0.05).

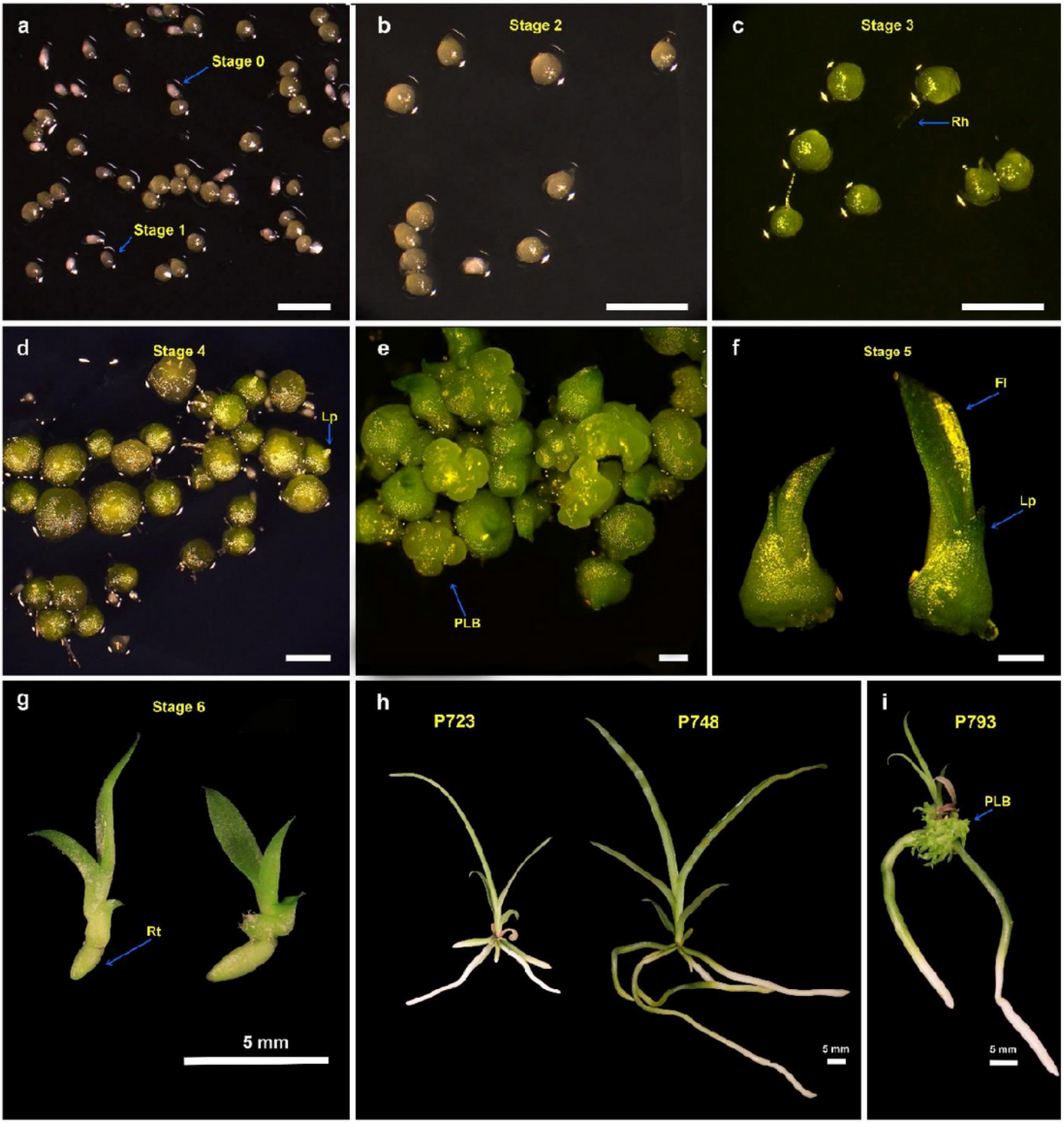

Figure 3

Asymbiotic seed germination and early seedling development of *Polystachya concreta* on P723. (a) Stage 0 and imbibed seeds (Stage 1). (b) Germinated seeds after 7 days. (c) Globular protocorms with rhizoids (Rh). (d) Protocorms with leaf primordia (Lp). (e) Protocorm-like bodies on VW from seeds germinated in the dark (f) Seedling with elongated leaf primordia (Lp) and formation of first leaf (Fl). (g)

Seedlings with two leaves and roots (Rt) after 16 weeks. (h-i) Seedling development on three medium types. Scale bars = 500 µm (a-f).

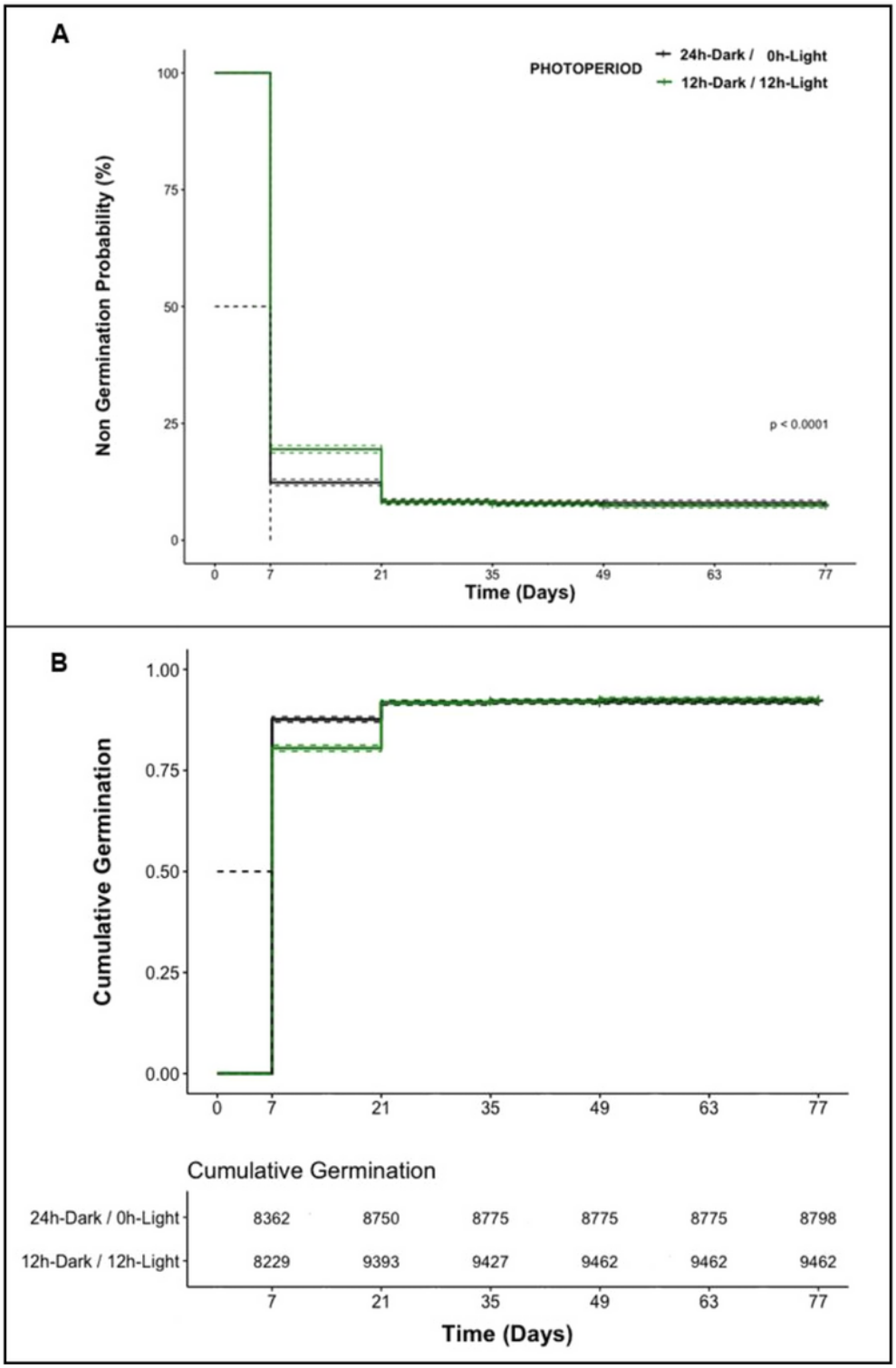

Figure 4

Kaplan Meier estimator of non-germination (survival) (A) and cumulative germination (B) functions for *Polystachya concreta* seeds incubated under different photoperiods at 23 °C for 77 days with 95% confidence intervals. Dotted lines indicating median overall germination times.

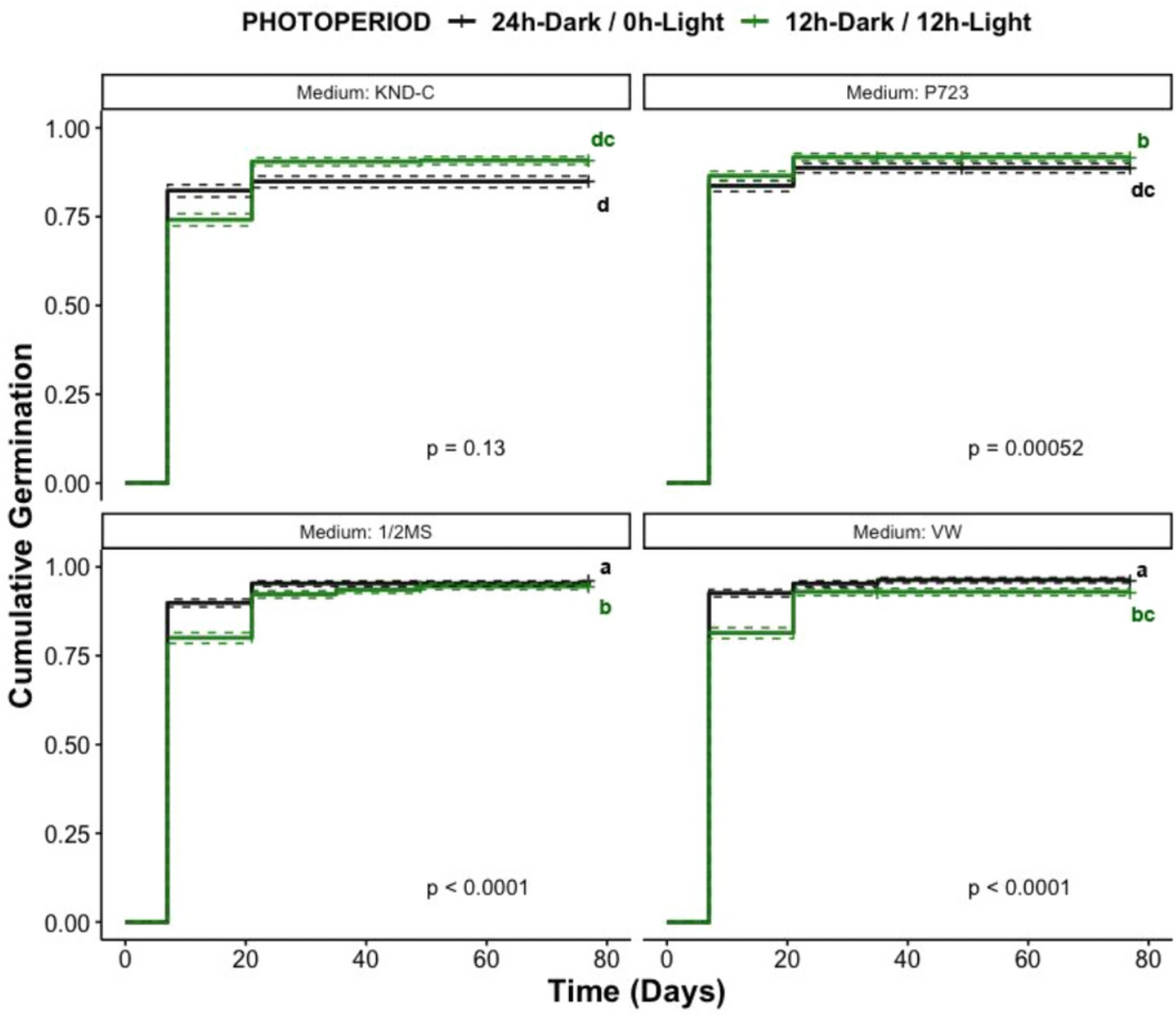

Figure 5

Cumulative proportion of germinated *Polystachya concreta* seeds sown on different asymbiotic medium types and incubated under different photoperiods. Curves with same letters are not significantly different according to a multiple pair wise comparison log-rank test with a Bonferroni correction to adjust p-values at α=0.05 significance level.

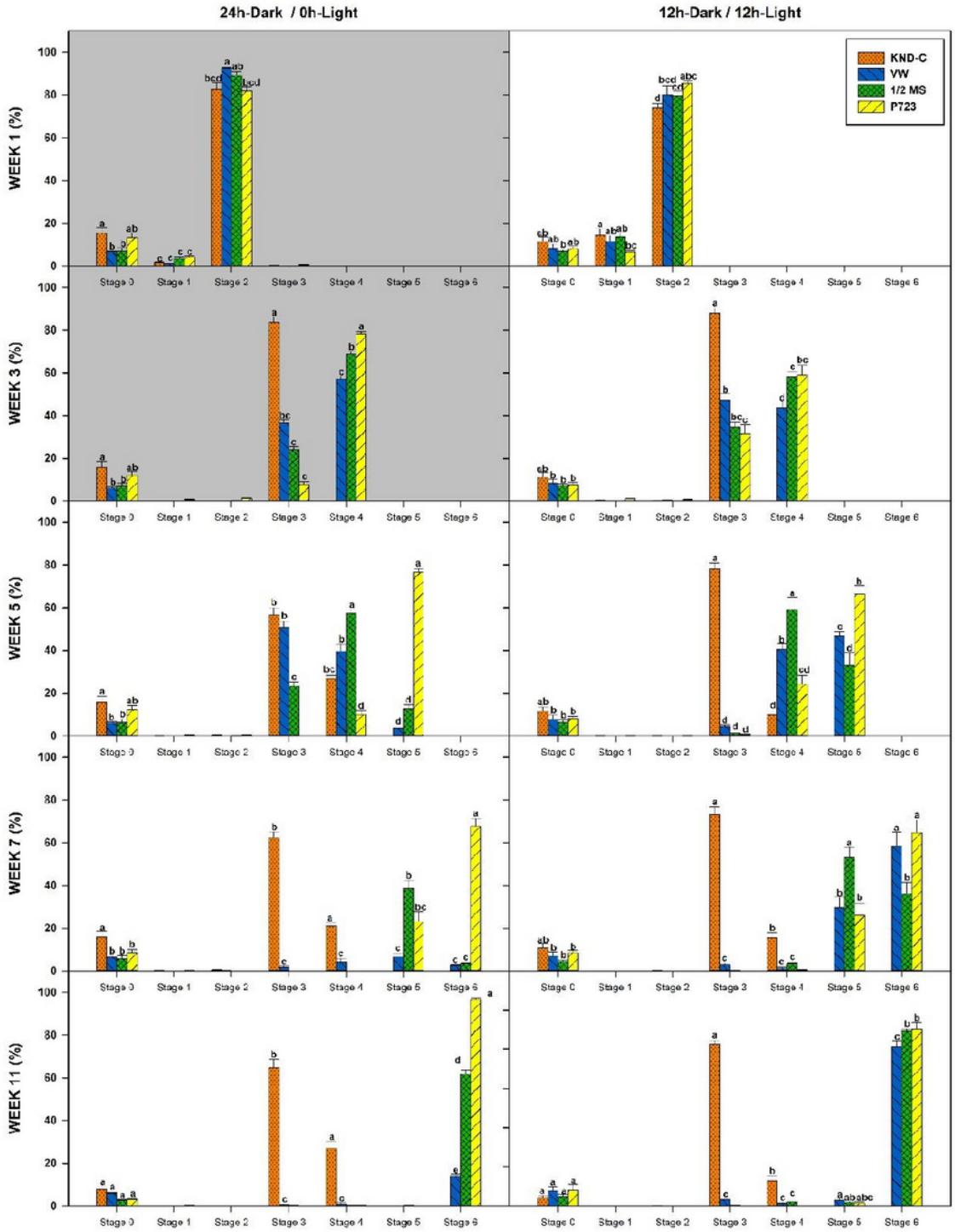

Figure 6

Comparative effects of photoperiod and asymbiotic medium type on seed germination and early seedling development of *Polystachya concreta* incubated at 23 °C for 11 weeks. Histobars with the same letter are not significantly different (α=0.05).

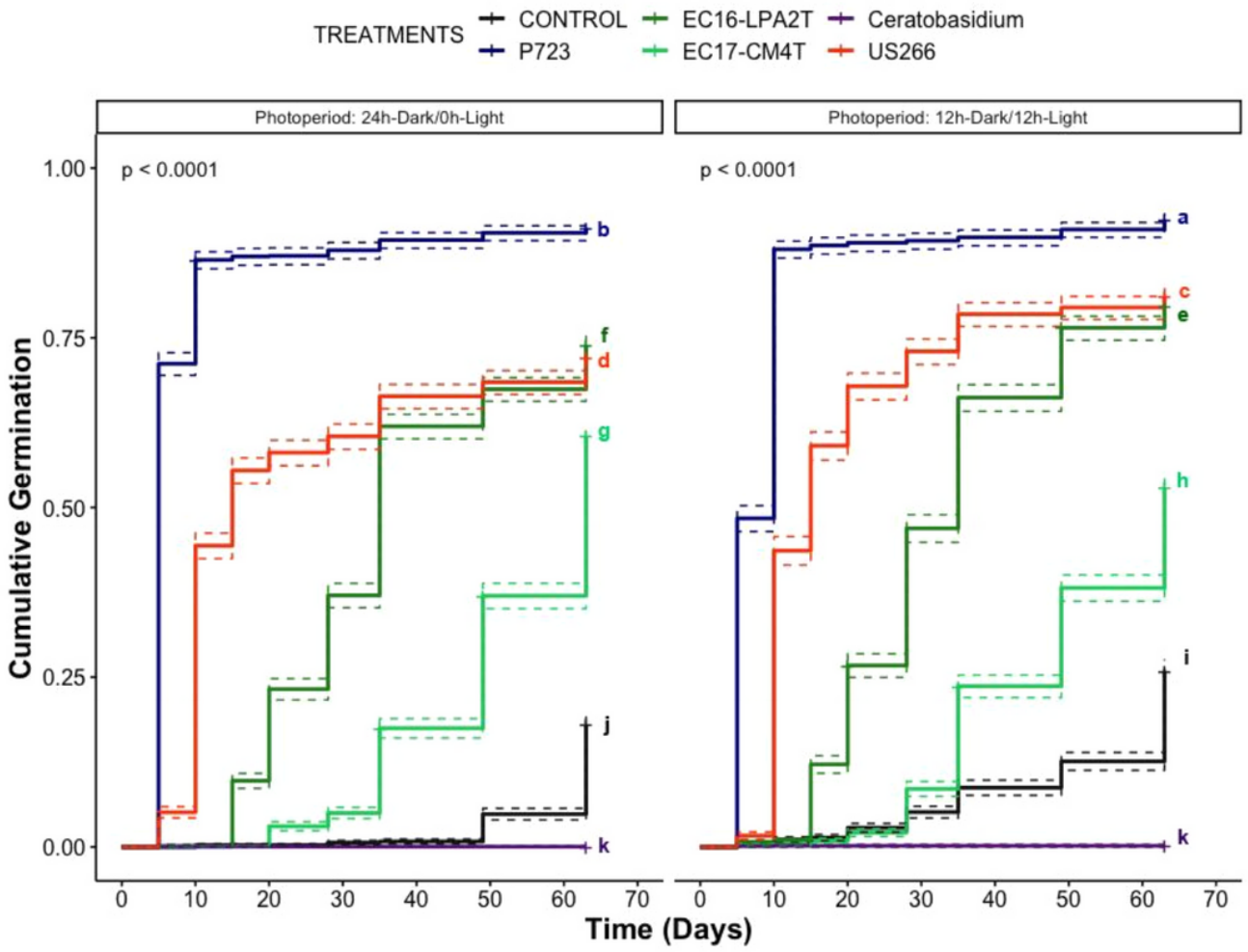

Figure 7

Cumulative proportion of germinated *Polystachya concreta* seeds co-cultured with different fungal strains under two photoperiods. Curves with same letters are not significantly different according to a multiple pair wise comparison log-rank test with a Bonferroni correction to adjust p-values at α=0.05 significance.

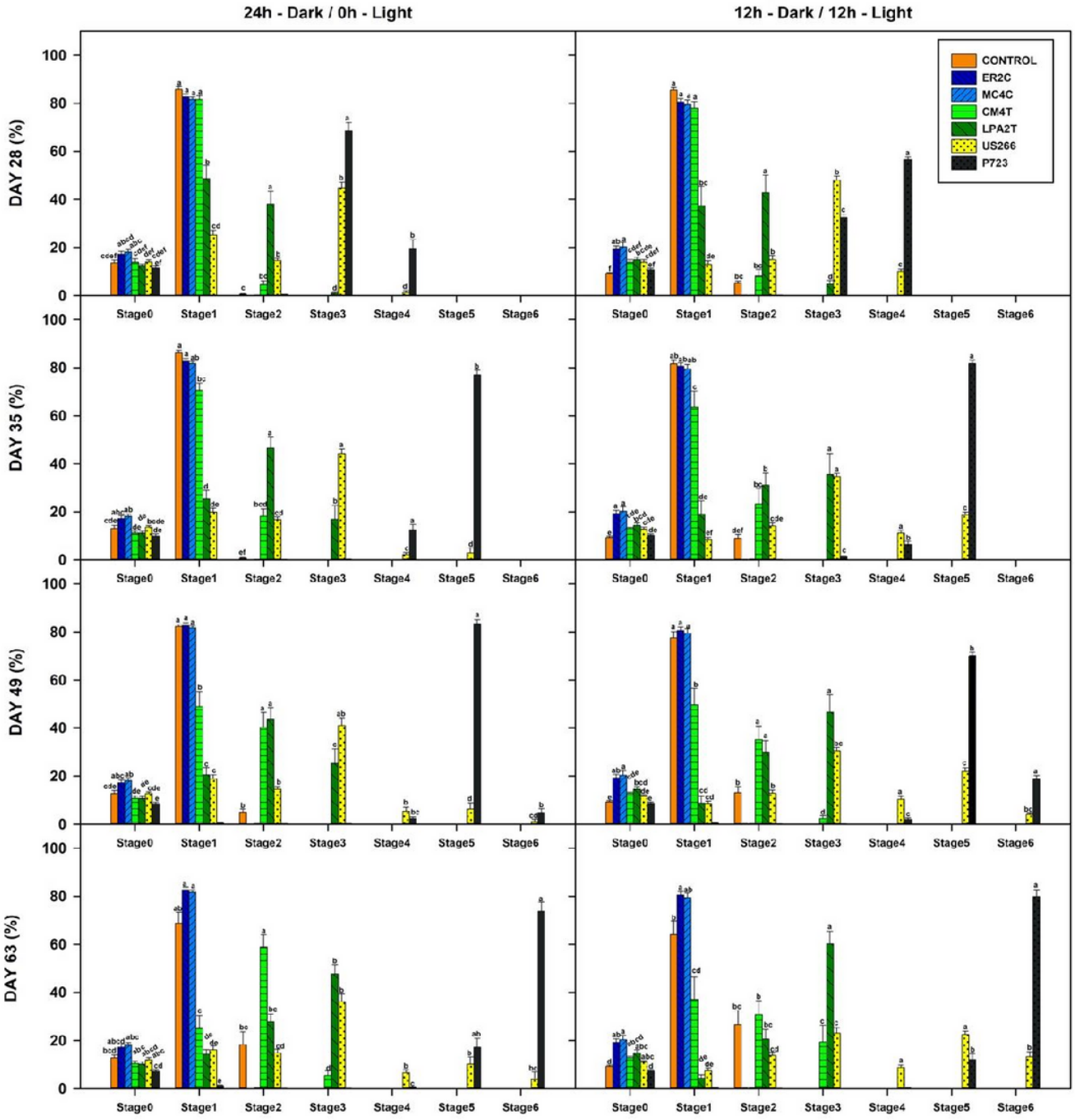

Figure 8

Comparative effects of photoperiod and symbiotic treatments on seed germination and seedlings development of *Polystachya concreta* incubated at 23 °C for 63 days. Histobars with the same letter are not significantly different (α=0.05).

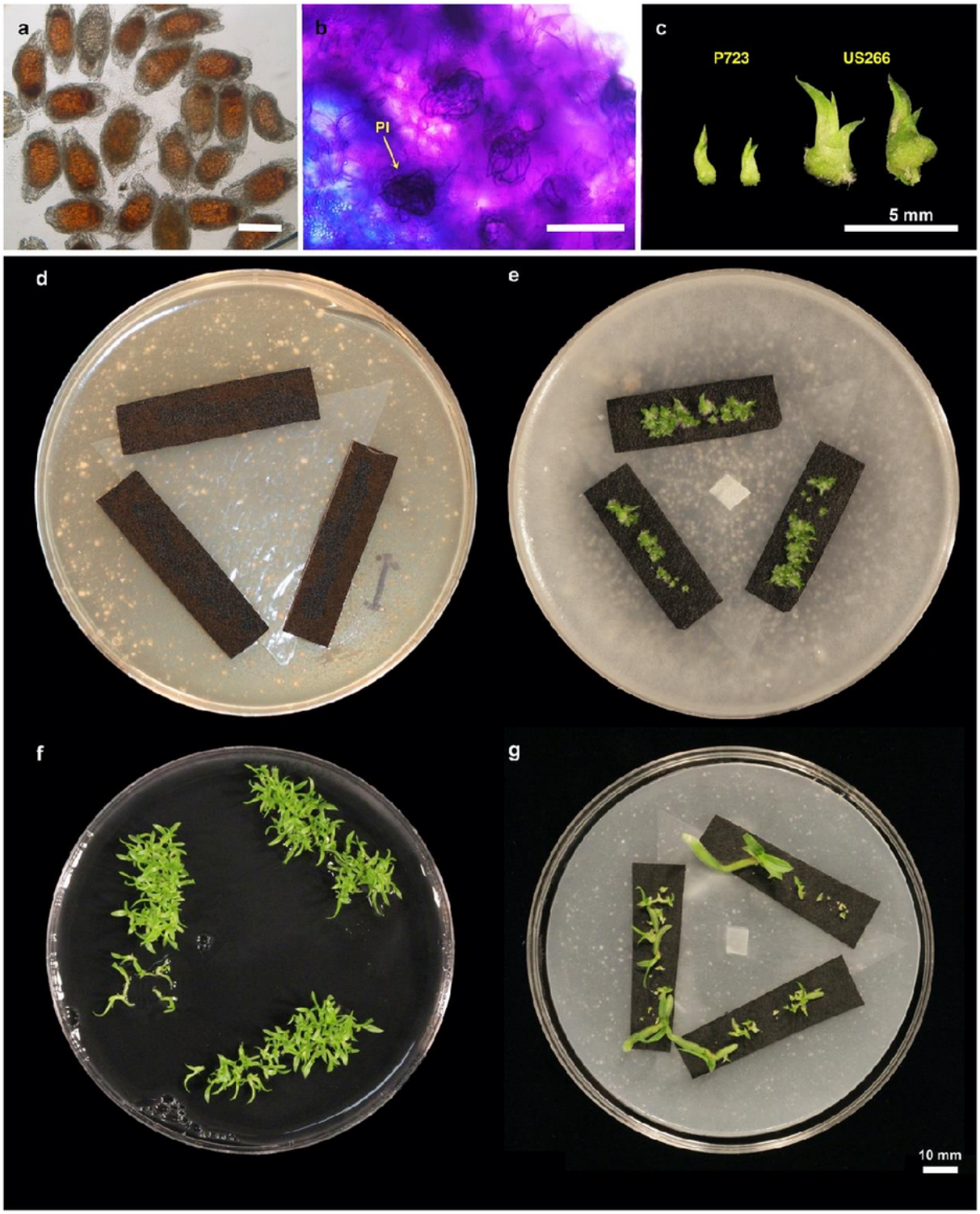

Figure 9

Seeds and symbiotic seed culture of *Polystachya concreta* (a) Seeds of *P. concreta* preconditioned in 10% sucrose followed by a TTC viability test: viable seeds (red embryos) and non-viable seeds (white embryos). (b) Peloton (Pl) formation in root cortical tissue of *P. concreta* seedling co-cultures with US266. (c) Seedlings growing on P723 or US266. (d) Control asymbiotic plate with a cellophane membrane and black filter papers strips over OMA solidified agar. (e) Seedling co-cultured with US266 after 9 weeks. (f)

Uniform seedling production of *P. concreta* sown on P723. (g) Seedling co-cultured with US266 after 22 weeks. Scale bars = 100 µm (a-b).

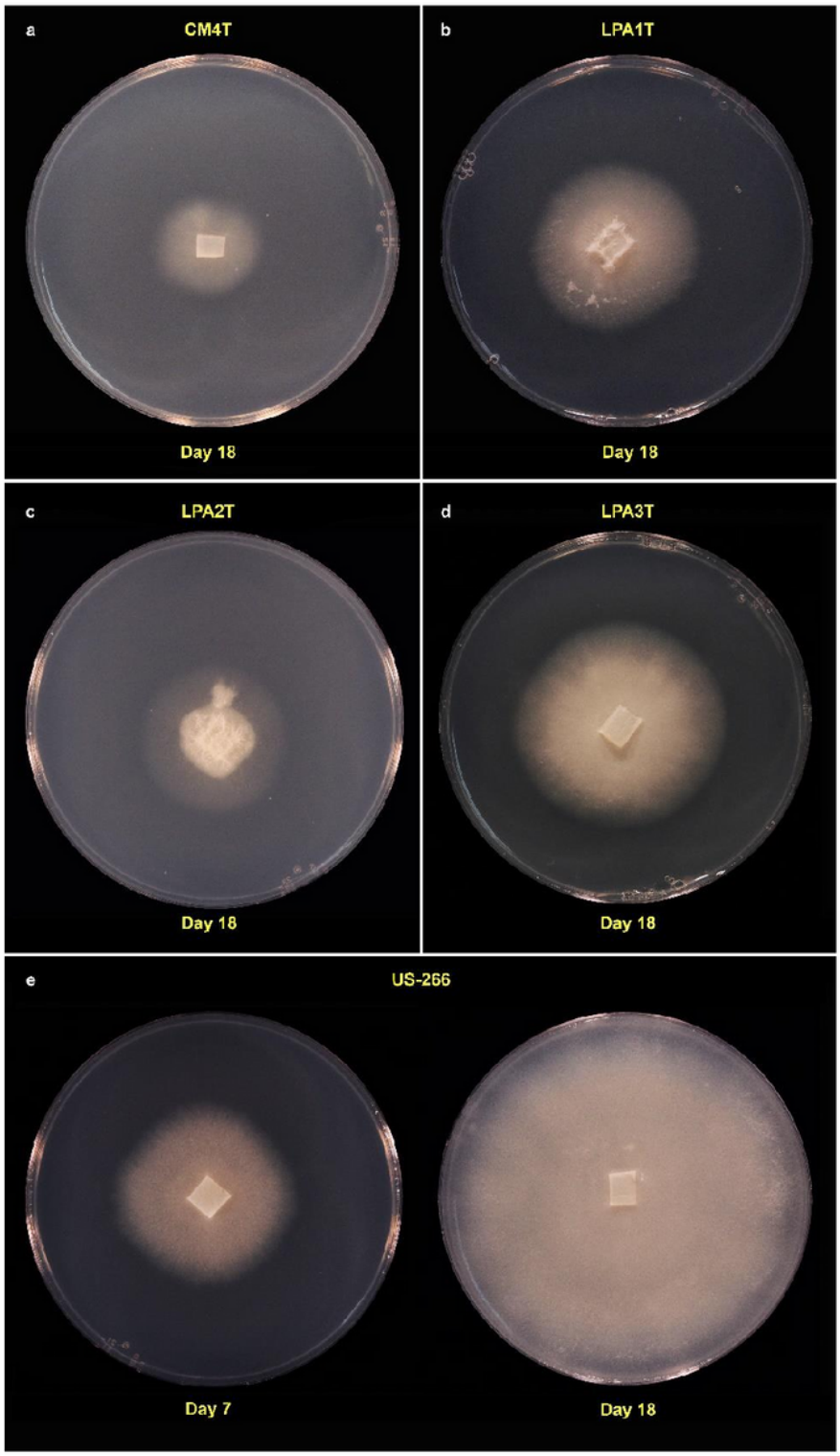

Figure 10

Growth of different *Tulasnella* strains after 7 and 18 days. (a-d) Isolated from native orchid species from Ecuador and (e) *Tulasnella calospora* strain (University of Alberta Microfungus Herbarium UAMH 9824) isolated from a primary lateral root of *Spiranthes brevilabris* in Levy County, Florida.