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Original research article

Asymbiotic seed germination and *in vitro* seedling development of *Paphiopedilum spicerianum*: An orchid with an extremely small population in China

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ABSTRACT

Paphiopedilum spicerianum is listed as one of the country's Wild Plants with Extremely Small Populations (PSESP). Procedures were developed for asymbiotic seed germination and seedling development aimed at producing seedlings for reintroduction. The highest germination was achieved in RECW with a 24 h dark cycle after pretreatment with 1% NaOCl for 40 min after 30 days from germination. However, these protocorms remained white and did not develop further. Although germination was lower under the same conditions in MSCW, it resulted in healthier and greener protocorms. Of four suitable media tested to promote seedling formation, Hyponex No 1 medium with 1.0 mg l^{-1} α-naphthalene acetic acid, 0.5 g l^{-1} activated charcoal and 10% banana homogenate was the most effective. Advanced seedling development was seen in all six tested media during a 4 month growing period, with the highest leaf growth rate seen in the same media used for seedling formation, supplemented with 1.0 mg l^{-1} 6-benzyladenine added to promote leaf growth. Fluorescein diacetate (FDA) tests on seeds showed that higher salt concentrations in the medium and longer duration of exposure to NaOCl reduce germination because of damaging effects on the testa and the embryo cells.

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1. Introduction

The conservation of rare and endangered species using *in vitro* methods of plant propagation are largely similar to other species but require overcoming the challenges due to limited amount of available plant material (Fay, 1992; Sarasan et al., 2006). When developing *in vitro* methods of propagation for plants of conservation importance, seeds are better suited compared to methods using tissue culture because they allow us to maintain a wider genetic base. Here, we report on fast and reliable tests that can be used to assess the suitability of growth media and tissue culture, while developing germination and seedling development protocols, for the critically endangered orchid species, *Paphiopedilum spicerianum* (H.G. Reichenbach)

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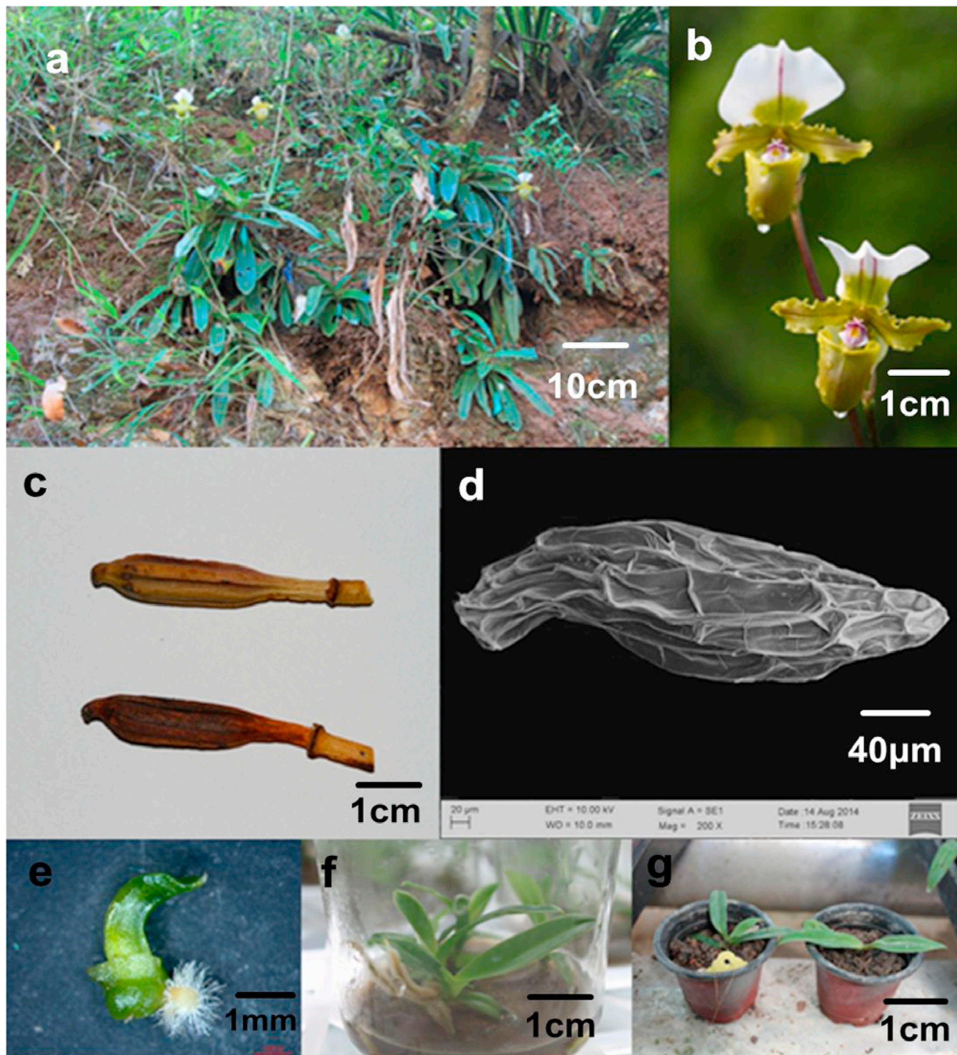

Fig. 1. *P. spicerianum* plants in the wild with flower, capsule, seed and plants developed from asymbiotic seed germination ready for reintroduction. a. Plant of *P. spicerianum* on a soil covered cliff in the wild. b. Flower of *P. spicerianum* from plants maintained at XTBG nursery. c. Mature capsules of *P. spicerianum* at 356 DAP. d. Scanning electron micrograph of *P. spicerianum* seed. e and f. The seedlings after protocorms were transplanted into 3.0 g l^{-1} Hyponex No 1 with 1.0 mg l^{-1} NAA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate medium for 1 month and 3 months, respectively. g. Seedlings transplanted into soil containing pots ready for re-introduction into the wild.

Pfitzer. Such tests, if requiring few seeds, allows conservation practitioners to save the limited seeds for the development of plantlets that can be assigned for re-introduction in the wild (Stewart, 2008; Swarts and Dixon, 2009; Ren et al., 2012).

Paphiopedilum spicerianum is found from Northeast India to Southwest Yunnan in China and Myanmar and has been heavily harvested for horticultural value in recent years (Liu et al., 2009). In China, only a single population of about 10 mature individuals was found in 2006 in the Pu'er Prefecture of Yunnan Province (Ye and Luo, 2006). These were sub-terrestrial plants that grow on steep river banks (Fig. 1(a)) and their habitat is seriously threatened due to the surrounding monoculture of coffee plantations. As a member of the family Orchidaceae, *P. spicerianum* is protected under the appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; CITES, 2013) and in China, among 38 other orchid species, it falls under the "Conservation Program for Wild Plants with Extremely Small Populations" (PSESP; (State Forestry Administration of China, 2012)). Reintroduction is favored as a conservation strategy for PSESP plants, which are defined as species with low population numbers due to recent and serious human disturbance and exclude naturally rare species (State Forestry Administration of China, 2012; Ren et al., 2012; Ma et al., 2013).

Due to their specialized biology, it is difficult to germinate orchid seeds and obtain enough viable plantlets for reintroduction in the wild. Although the discovery in 1922 by Knudson made it possible for orchid seeds to be germinated asymbiotically and despite the success of using asymbiotically produced plants in conservation of many endangered and threatened orchid taxa (Sgarbi et al., 2009; Mohanty et al., 2012), asymbiotic *in vitro* germination methods have to be

developed species-by-species since the conditions of tissue culture at each step are largely species-specific (Arditti, 1967; Zeng et al., 2013). *P. spicerianum* belongs to the subfamily Cypripedioideae, which is known to be very difficult to germinate (Arditti, 1967; Kauth et al., 2008; Zeng et al., 2013).

Asymbiotic germination success depends on seed conditions such as seed capsule maturity and origin, as well as physical germination conditions and the constituents in the growth media (Arditti, 1967; Kauth et al., 2008; Zeng et al., 2013). In the genus *Paphiopedilum*, the effect of maturity of the seed capsule (measured as the number of days after pollination [DAP]) on germination success varies by species (Lee, 2007; Long et al., 2010). Cross pollination in *Paphiopedilum* has been shown to produce seeds that have higher germination success, compared to seeds obtained through self-pollination (Stimart and Ascher, 1981). Further, growth media conditions such as composition, carbon source, plant growth regulators, organic nutrient additives (Chen et al., 2004a,b; Long et al., 2010), pretreatment duration (Lee, 2007), and light availability (Stimart and Ascher, 1981) can also significantly influence successful germination of *Paphiopedilum* species. It is time and resource consuming to develop asymbiotic germination protocols for endangered species and the ability to test the best conditions is severely limited by seed availability.

At the Xishuangbanna Tropical Botanical Garden (XTBG), Yunnan, China, as part of an integrated conservation plan focusing on the prevention of the extinction of rare and endangered orchids, we developed an asymbiotic *in vitro* seed germination protocol for *P. spicerianum*. We tested which medium is best for each stage of seed germination by manipulating the media composition, duration of pretreatment with 1% NaOCl, and light availability. Based on previous studies on other *Paphiopedilum* species, and our own preliminary tests, we hypothesized that the highest germination would be in Murashige and Skoog medium (MS; Murashige and Skoog, 1962) supplemented with coconut water (Chen et al., 2002, 2004a,b; Zeng et al., 2012), under dark conditions (Pierik et al., 1988), and when pretreated with 1% NaOCl for 60 min (Lee, 2007). We then tested four media in fresh subculture for seedling formation and six media for advanced seedling development. While the protocol was being developed we used scanning electron microscopic techniques and Fluorescein Diacetate (FDA) staining methods to determine the effect of the tested conditions on the seed coat and embryo integrity. This study is the first to report on comprehensive empirical tests for developing an asymbiotic seed germination protocol for *P. spicerianum*, combined with staining and imaging techniques that can be used more broadly in other rare and endangered species.

2. Materials and methods

2.1. P. spicerianum plant material and seed collection

P. spicerianum (Fig. 1(a)) is confined to shaded and moist places in soil-covered cliffs or on rocks in forests at elevations between 900 and 1400 m (Liu et al., 2009). Flowering takes place from September to November in the wild and mature, nearly dehisced fruits can be obtained at 11 months to one year following pollination (Liu et al., 2009). In September 2012, out of 38 plants that were found in Pu'er, southern Yunnan, 18 plants were collected for *in situ* conservation at XTBG. In November 2012, assisted outcross pollination was performed between three different flowering individuals maintained at XTBG (Fig. 1(b)) and in October 2013, two nearly dehisced mature capsules (Fig. 1(c)) were collected when the capsule color became brown (356 DAP). Then, seeds were harvested under sterile conditions and germination experiments were conducted directly on the collected seeds. A subset of seeds was tested for viability using the FDA test, as follows. All seeds were soaked in 1% NaOCl for 15 min and rinsed twice in de-ionized water and stored in water for 16 h in 4 replicates in syringes fitted with nylon cloth with 45 μm diameter holes. Seeds from each replicate were placed on a microscope slide and physically mixed with a pipette in a 1:1 ratio with 0.5% (w/v) FDA dissolved in acetone. The reaction was allowed to progress for 15 min to allow for the accumulation of fluorescein in living cells (Clarke et al., 2001). Seeds were then examined with an UV-fluorescence microscope (LSM710 ImagerZ2, Zeiss, Germany) for assessing the condition of the testa and embryo cell viability (Copeland and McDonald, 2001). The fresh seeds were also used directly without any pretreatment to obtain micrographs (Fig. 1(d)) using a scanning electron microscope (EVO LS10, Zeiss, Germany). Further experiments on the harvested seeds were conducted in tissue culture rooms under sterile conditions at $25 \pm 2.0\,^{\circ}$C and at 45% relative humidity (RH).

2.2. Preliminary assessment of suitable medium composition for germination (stage 1)

For our experiments we modified two media; agar was reduced from 10 to 6 g l^{-1} and sucrose from 30 to 20 g l^{-1} in MS medium (hereafter referred to as modified MS) and agar was reduced from 16 to 6 g l^{-1} and fructose was replaced with sucrose in Robert Ernst medium (hereafter referred to as modified RE; cf. Arditti, 1982). To assess the effect of reduced salt and addition of coconut water (CW) as a supplement, the harvested seeds were tested in six compositions: (1) 1/2 strength modified MS; (2) 1/2 strength modified MS supplemented with 10% CW; (3) 1/4 strength modified MS; (4) 1/4 strength modified MS supplemented with 10% CW; (5) modified RE with 1.0 g l^{-1} activated charcoal; (6) modified RE with 1.0 g l^{-1} activated charcoal and 10% CW. In all media pH was adjusted to 5.8 using 1 mol l^{-1} NaOH or 1 mol l^{-1} HCl. Each treatment was replicated 15 times in 200 ml sterile clear glass jars with 30 ml of media solution. Approximately 100 seeds, each sterilized for 5 min in 1% NaOCl, were placed in each jar under sterile conditions. Seed placement was done by suspending seeds in 0.1% autoclaved Agar solution using a sterile syringe and pipetting out 1 ml for each replicate. After seed placement, the exact

number of seeds in each jar was counted. Seeds were then allowed to germinate in tissue culture rooms under a 12 h dark and 12 h light cycle. We used cool white fluorescent lights with approximately 200 μ mol m^{-2} s^{-1}, for the photoperiod cycle condition. In orchids germination and seedling development are divided into six stages including stage 0 the ungerminated seed and were scored as described by Arditti (1967). Germination (stage 1), defined as imbibed seeds still covered by testa and with a white and green color visual to the eye, was assessed three months after seed sowing (Arditti, 1967).

2.3. Secondary assessment of suitable light and pretreatment conditions for germination, protocorm formation and protocorm development (stages 1–3)

Following the preliminary assessment (see results section), two media, 1/4 strength modified MS supplemented with 10% CW and modified RE with 1.0 g l^{-1} activated charcoal and 10% CW, were selected for assessing the effect of light (12 h dark and 12 h light cycle compared to 24 h dark cycle) and pretreatment duration (1% [w/v] NaOCl for 0, 40, 60 or 80 min) on germination, protocorm formation and protocorm development. A subset of the pretreated seeds, in four replicates in each pretreatment, was assessed for seed viability using the FDA tests as described above and compared with non-treated seeds. All treatments were visualized using a UV-fluorescence microscope (LSM710, ImagerZ2, Zeiss, Germany). Another subset was transferred to growth media using the same method as above, and each treatment with 15 replicates (15, 200 ml sterile glass jars each containing approximately 100 seeds) was germinated in the same tissue culture rooms. After seed placement in the jars the exact number of seeds was counted under a microscope. The 24 h dark cycle was obtained by placing the jars in black cloth covered boxes. Seed germination, protocorm formation and development were assessed 40, 70 and 90 days after sowing. We defined protocorm formation (stage 2) as continued embryo enlargement and testa rupture, and protocorm development (stage 3) as appearance of a promeristem (Arditti, 1967).

2.4. Assessment of suitable conditions for seedling formation (stage 4)

After 90 days, developed green protocorms obtained from the above experiment were used to test for seedling formation in four media: (1) 1/2 strength modified MS with 0.2 mg l^{-1} indol-3-butylic acid (IBA) and 2.0 g l^{-1} activated charcoal; (2) 3.0 g l^{-1} Hyponex No 1 (Hyponex Corporation, USA) with 1.0 mg l^{-1} NAA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate; (3) Modified MS with 1.0 mg l^{-1} NAA, 0.2 mg l^{-1} IBA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate; (4) 2.0 g l^{-1} Hyponex No 1 with 2.0 g l^{-1} peptone, 1.0 mg l^{-1} NAA, 1.0 g l^{-1} activated charcoal and 10% banana homogenate. Protocorms were transferred into 200 ml sterile glass jars under sterile conditions and each treatment contained 15 jars with each jar containing 3 protocorms. The protocorms were allowed to grow in 12 h light and 12 h dark photoperiod cycles. Seedling formation (stage 4), defined as emergence of first leaf (Arditti, 1967), was assessed after two months of growth and seedling survival.

2.5. Assessment of suitable conditions for advanced seedling development (stage 5)

The seedlings developed were further assessed for advanced seedling growth under six media conditions: (1) Modified MS with 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} 6-BA and 2.0 g l^{-1} activated charcoal; (2) 3.0 g l^{-1} Hyponex No 1, 0.5 mg l^{-1} NAA and 1.0 g l^{-1} activated charcoal; (3) 3.0 g l^{-1} Hyponex No 1, 2.0 mg l^{-1} 6-BA, 0.5 mg l^{-1} NAA and 1.0 g l^{-1} activated charcoal; (4) Modified MS with 0.2 mg l^{-1} IBA, 0.2 mg l^{-1} 6-BA and 2.0 g l^{-1} activated charcoal; (5) 3.0 g l^{-1} Hyponex No 1, 3.0 g l^{-1} peptone, 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} 6-BA and 1 g l^{-1} activated charcoal; and (6) 3.0 g l^{-1} Hyponex No 1, 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} 6-BA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate. Three seedlings each were transferred into 200 ml sterile glass jars under sterile conditions and each treatment contained 8 jars. The effect of these media on advanced seedling development (stage 5) was assessed using leaf length and leaf width growth rates. We did not take any destructive biomass assessments because all seedlings were assigned for re-introduction trials in the wild (Fig. 1(g)).

2.6. Assessment of the impact of high salt concentrations on seed structure and cellular integrity

At the end of experimentation, we conducted a further test to explore why the seeds were able to germinate significantly higher in low concentration media. Seeds were pretreated with 1% NaOCl for 40 min and then rinsed twice with sterile deionized water and allowed to be soaked in one of three treatments; 1/4 strength MS medium solution; full strength MS medium solution and RE medium. After 16 h of soaking in each medium in four replicates the seeds were then subjected to the FDA test as described above and visualized using UV-fluorescence microscope (LSM710 ImagerZ2, Zeiss, Germany).

3. Statistical analysis

We did not conduct statistical analysis on the preliminary test of suitable medium composition for germination (stage 1) because germination and viable protocorm formation were observed only in two media and both media were selected for further assays.

Table 1
The effect of length of pretreatment (washing with 1% NaOCl for 0, 40, 60, and 80 min), media (Murashige and Skoog, 1962 supplemented with 10% coconut water [MSCW], Robert Ernst (cf. Arditti, 1982) supplemented with 10% coconut water [RECW]), and light (12 h light and 12 h dark cycle vs 24 h dark cycle) on the asymbiotic seed germination of *Paphiopedilum spicerianum* (the values presented are Chi-squared statistics with associated significance). Seed germination was assessed at 40, 70 and 90 days after germination.

	Days after sowing (day)		
	40	70	90
Factor			
Pretreatment ($df = 3$)	578.62***	1212.13***	1219.90***
Media ($df = 1$)	284.57***	522.00***	522.08***
Light ($df = 1$)	91.88***	135.08***	137.60***
Pretreatment × Media ($df = 3$)	170.08***	292.44***	304.03***
Pretreatment × Light ($df = 3$)	37.04***	51.65***	49.14***
Media × Light ($df = 1$)	17.57**	37.80***	36.35***
Pretreatment × Media × Light ($df = 3$)	0.12 ns	1.29 ns	1.10 ns

ns not significant.
** $P < 0.01$.
*** $P < 0.0001$.

For the secondary assessment of suitable conditions for germination, protocorm formation and protocorm development (stages 1–3), we counted the number of seeds that successfully reached or passed the protocorm development stage for each treatment as successful events (coded as 1). Those seeds that did not reach or pass this stage were assessed as failures (coded as 0). We tested whether medium, light condition, pretreatment duration and the interaction between these three factors, influenced the probability of success of reaching or passing the protocorm formation stage by using generalized linear models (Sileshi, 2012) with a binomial distribution function in R project epicalc package (version 3.1.1 R Development Core Team, 2011). We conducted multiple comparisons to test within-treatment differences after adjusting for alpha at 0.05 (Bretz et al., 2010) using the Tukey test in the mvtnorm and multcomp packages of R.

Since seedling formation (stage 4) was only successful in one of the four tested media, we did not conduct a statistical analysis for the assessment of suitable conditions for seedling formation. For the assessment of suitable conditions for advanced seedling development, we calculated the relative leaf length growth rate (RLLGR) as: $RLLGR = (LL_n/LL_0)/t$, where LL_0 is the leaf length of the seedlings at the final measurement and LL_n is the leaf length of the seedlings at the end of the experiment, and t is the elapsed time. The relative leaf width growth rate was calculated as (RLWGR): $RLWGR = (LW_n/LW_0)/t$, where LW_0 is the leaf width of the seedlings at the final measurement and LW_n is the leaf width of the seedlings at the end of the experiment, and t is the elapsed time. For analysis using *RLLGR* and *RLWGR* as response variables, we fitted general linear mixed models appropriate for unbalanced data (some treatments were contaminated by fungi and were discarded) using the lme4 package in R, specifying the media as the fixed effect and replication of growth jars as a random effect. Within the context of the fitted model, single degree of freedom contrasts were calculated using Tukey adjustments (alpha = 0.05) to compare between media, using mvtnorm and multcomp packages of R.

4. Results

4.1. *P. spicerianum* plant material and seed collection

The viability tests on the fresh seeds showed that on average 51.5 ± 6.3% seeds contained viable embryos assessed through the green fluorescence of the living cells in the testa and embryo.

4.2. Preliminary assessment of suitable medium composition for germination (stage 1)

In the preliminary experiments using six media we were able to germinate adequate seeds from 1/4 strength modified MS supplemented with 10% CW (79 ± 8% germination of viable seeds) and modified RE with 1.0 g l^{-1} activated charcoal and 10% CW (57 ± 8% germination of viable seeds). All other RE media gave seeds that germinated but subsequently died and all other MS media had lower seed germination compared to the 1/4 treatment supplemented with 10% CW.

4.3. Secondary assessment of suitable light and pretreatment conditions for germination, protocorm formation and protocorm development (stages 1–3)

When we further assessed germination in the above two media, we found that at 40 days after germination, all three factors – pretreatment, medium and light – were significant in affecting the probability of germination (Table 1). Pretreatment had the most significant effect on the probability of germination followed by medium and light (all P <

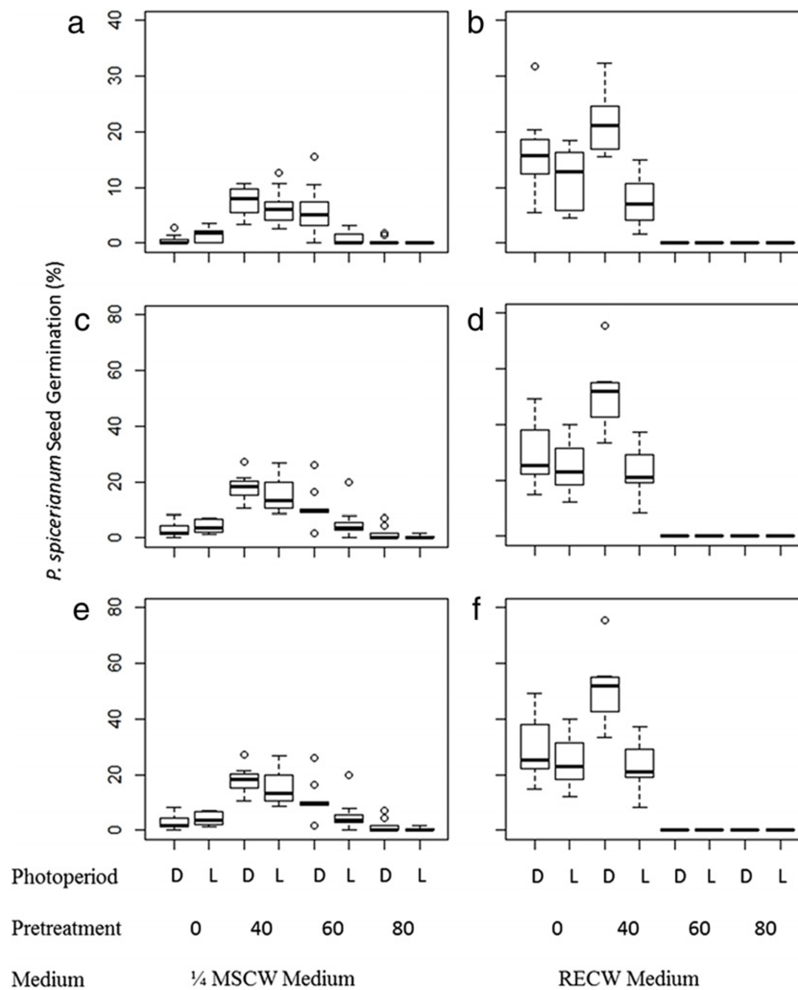

Fig. 2. Percentage germination of *P. spicerianum* seeds assessed 40, 70 and 90 days after experiment began. Photoperiod D = seeds germinated in 24 h dark cycle and photoperiod L = seeds germinated in 12 h dark and 12 h light cycle. 1/4 MSCW = 1/4 strength modified Murashige and Skoog medium supplemented with 10% coconut water and RECW = modified Robert Ernst medium supplemented with 10% coconut water. Panels a and b show germination percentages after 40 days from experiment start time, panels c and d show germination percentages after 70 days, and panels e and f show germination percentages after 90 days. The thick black lines in each box plot indicate the 50th percentile values of each species, the lower and upper sides of the box represent the 25th and 75 percentile, and the error bars the 10th and 90th percentiles. The circles above or below the 10th and 90th percentile lines represent data points that are more than 3 times the standard error for the observation (outliers).

0.0001; Table 1; Fig. 2). There were strong interaction effects between all factors (pretreatment duration and medium $P < 0.0001$; pretreatment duration and light $P < 0.0001$; medium and light $P < 0.01$; Table 1). Although highest germination percentage was observed in RECW medium after pretreatment for 40 min with 1% NaOCl and kept in dark (21.65 ± 1.88 % germination; Fig. 2(b)), all protocorms remained white and subsequently browned and died (Fig. 2(a) and see photo for visual comparison in Fig. 3). In MSCW, the same conditions gave the highest germination percentage (7.50 ± 0.75% germination; Fig. 2(a)) for that medium, but germinated seeds further developed into healthy green protocorms (Fig. 3), unlike in the RECW medium (Fig. 2(b)). At 40 days, when light versus dark conditions were compared, seed germination probability was significantly higher in the dark condition for 60 min pretreated seeds in MSCW media and for 40 min pretreated seeds for RECW media (Table 2). When pretreated for 60 min with 1%NaOCl, none of the seeds germinated in RECW medium either in the dark or in the 12 h photoperiod. Seeds pretreated for 80 min with 1% NaOCl did not germinate in either medium.

When germination was assessed at 70 days, seeds pretreated with NaOCl showed no difference between dark and light treatments, but germination percentage was very low (0.97 ± 0.42% germination for both dark and light treated seeds; Fig. 2(c), (d)). In contrast germination was higher in the dark treatment with 40 min pretreatment with 1% NaOCl (16.37 ± 4.94% in MSCW and 50.25 ± 17.76% in RECW). When seed germination was assessed at 90 days, the results were similar to those at 70 days (Tables 1 and 2; Fig. 2(e), (f)). In the dark with 40 min pretreatment with 1% NaOCl, MSCW medium had 18.05 ± 5.44% germination compared to 51.02 ± 18.04% in RECW.

Fig. 3. Comparison of seed germination in two different media. a. Green shoot formation from protocorm in 70 days old seed germinated in 1/4 strength modified Murashige and Skoog medium supplanted with 10% coconut water. b. 70 days old browned and dead protocorm grown in Robert Ernst medium supplemented with 10% coconut water.

Table 2
The effect of light versus dark conditions on the probability of germination for *Paphiopedilum spicerianum* seeds germinated in Murashige and Skoog (1962) media supplemented with 10% coconut water (MSCW) and Robert Ernst (cf. Arditti, 1982) supplemented with 10% coconut water (RECW) media (estimation ± SE) for different pretreatment times (washing with 1% NaOCl for 0 min, 40 min, 60 min, 80 min). Seed germination was assessed at 40, 70 and 90 days after germination. Multiple comparisons are given for each treatment in comparison to the light condition (negative values indicate that the probability of protocorm formation in the dark condition is higher compared to the probability under light photoperiod). We provide the estimates and their standard errors, and the associated significance of the multiple comparison using adjusted *P* values and Tukey comparison method.

	Days after sowing (day)		
	40	70	90
MSCW medium			
Time in pretreatment (min)			
0	0.87 ± 0.67 ns	0.39 ± 0.38 ns	0.37 ± 0.38 ns
40	−0.21 ± 0.20 ns	−0.15 ± 0.14 ns	−0.21 ± 0.14 ns
60	−1.78 ± 0.36***	−0.91 ± 0.21**	−0.80 ± 0.20**
80	ng	−1.32 ± 0.78 ns	−1.32 ± 0.78 ns
RECW medium			
Time in pretreatment (min)			
0	−0.35 ± 0.15 ns	−0.22 ± 0.12 ns	−0.23 ± 0.12 ns
40	−1.19 ± 0.17***	−1.25 ± 0.12***	−1.27 ± 0.12***
60	ng	ng	ng
80	ng	ng	ng

ns not significant.
ng no germination.
** $P < 0.01$.
*** $P < 0.0001$.

The FDA test gave further insights into why seeds pretreated for 60 min or 80 min with 1% NaOCl failed to germinate while the 40 min seeds had better germination success. In the UV micrographs obtained, when compared with non-treated seeds (Fig. 4(a)), the seeds pretreated for 40 min with 1% NaOCl had dissolved testa cells at the long end of the seeds, but the testa cells covering the embryo remained intact and embryo cells remained alive (Fig. 4(b)). In the seeds that were pretreated for 60 min with NaOCl, we observed that the testa was ruptured and close to 50% of the embryos were assessed as non-viable. In the 80 min pretreated seeds, the embryo protruded out of the testa covering, and many of the embryo cells were dead and almost all embryos were assessed as non-viable.

4.4. Assessment of suitable conditions for seedling formation (stage 4)

Of the four growth media tested, seedling formation was observed only in 3.0 g l^{-1} Hyponex No 1 with 1.0 mg l^{-1} NAA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate (Fig. 1(e), (f) shows seedling growth after one and three months, respectively). In all other treatments the 90 day old protocorms became brown and died.

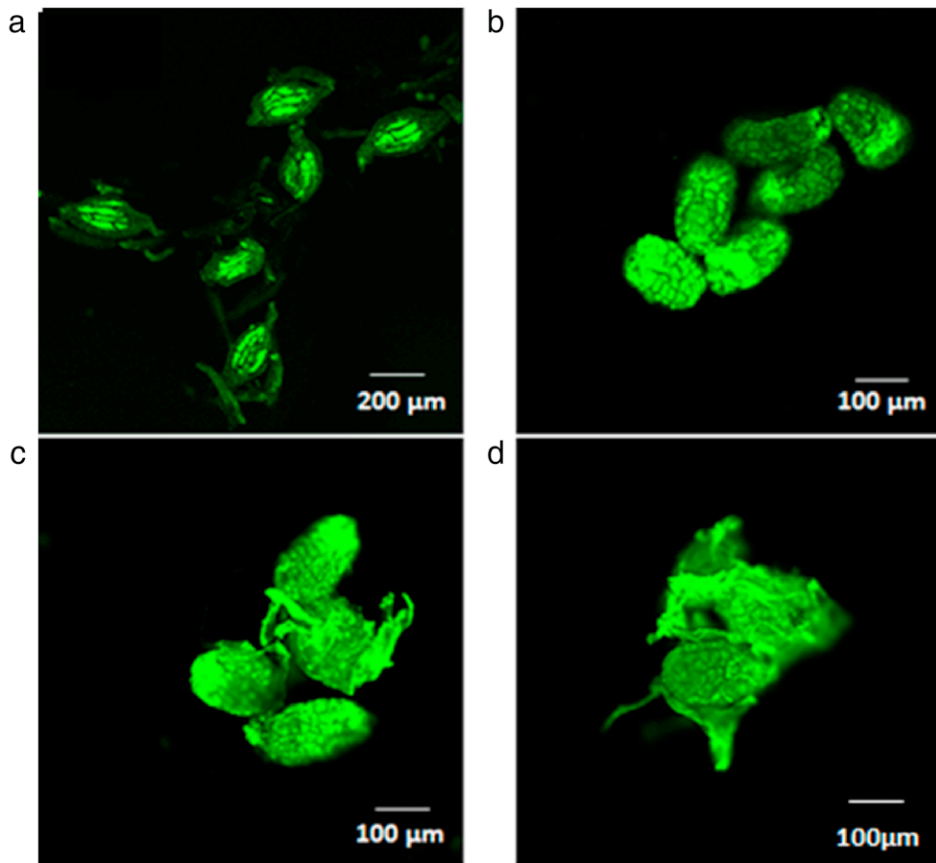

Fig. 4. Comparison of seeds in different pretreatment durations with 1% NaOCl after Fluorescent Diacetate staining. a. Non-treated seeds, b. Seeds pretreated for 40 min, c. Seeds pretreated for 60 min and d. Seeds pretreated for 80 min.

4.5. Assessment of suitable conditions for advanced seedling development (stage 5)

Advanced seedling development was significantly affected by the composition of the medium, when measured through seedling leaf length growth rate ($F_{5,36} = 3.652$, $P < 0.01$). Leaf length growth rate was highest and leaves remained green in color in 3.0 g l^{-1} Hyponex No 1, 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} 6-BA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate medium (Fig. 5). However, this medium was not significantly different compared to 3.0 g l^{-1} Hyponex No 1, 3.0 g l^{-1} peptone, 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} 6-BA and 1 g l^{-1} activated charcoal medium or compared to modified MS with 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} 6-BA and 2.0 g l^{-1} activated charcoal medium. All other media were significantly different and gave lower leaf length growth rates compared to 3.0 g l^{-1} Hyponex No 1, 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} 6-BA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate medium (all $P < 0.05$). The first three media tested produced yellow leaves but plantlets remained alive even at 6 months after growth in these media. Relative leaf width growth rate was not significantly affected by the growth medium. In all media, we obtained on average three to four shoots for cluster bud proliferation per germinated seed.

4.6. Assessment of the impact of high salt concentrations on seed structure and cellular integrity

The FDA test showed that seeds treated for 16 h in 1/4 strength MS medium had well imbibed seeds (Fig. 6(a)), while seeds placed in full strength medium showed signs of shrinkage (Fig. 6(b)). In the seeds treated with RE, we observed that, close to 50% of the embryo cells were dead (Fig. 6(c)).

5. Discussion

The choice of medium, conditions of growth and pretreatment duration significantly affected the germination of *P. spicerianum* seeds. As has been reported for other *Paphiopedilum* species (Pierik et al., 1988; Chen et al., 2004a,b; Ding et al., 2004; Long et al., 2010; Zeng et al., 2012), the preliminary tests for germination confirmed that *P. spicerianum* germinated best in low salt media. The ideal medium for the seed germination of *Paphiopedilum* species is known to be species-specific.

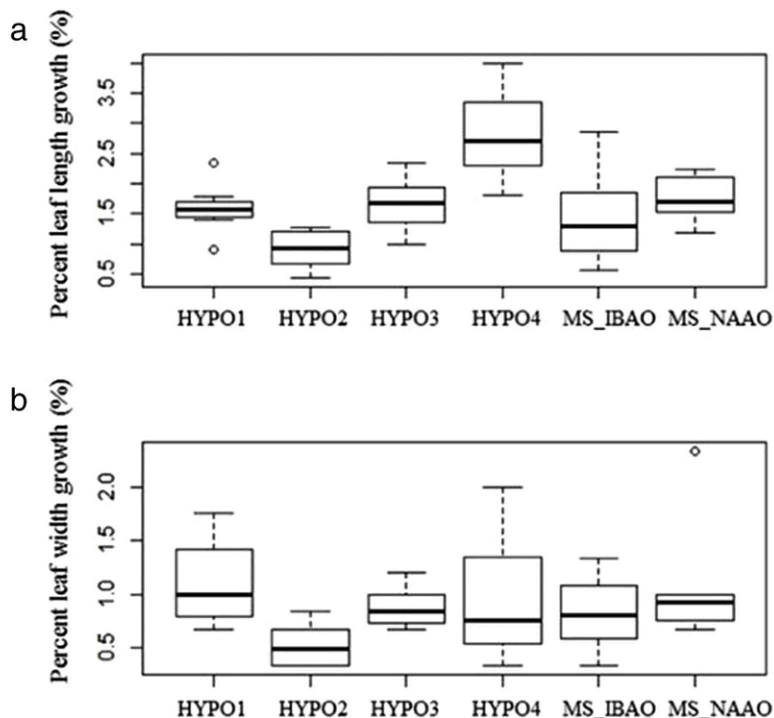

Fig. 5. Advanced seedling development measured as leaf length and width growth of the longest leaf in each seedling grown in different media. Media are: 3.0 g l^{-1} Hyponex No 1, 0.5 mg l^{-1} NAA and 1.0 g l^{-1} activated charcoal (HYPO1); 3.0 g l^{-1} Hyponex No 1, 2.0 mg l^{-1} 6-BA, 0.5 mg l^{-1} NAA and 1.0 g l^{-1} activated charcoal (HYPO2); 3.0 g l^{-1} Hyponex No 1, 3.0 g l^{-1} peptone, 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} 6-BA and 1 g l^{-1} activated charcoal (HYPO3); 3.0 g l^{-1} Hyponex No 1, 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} 6-BA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate (HYPO4); Modified MS with 0.2 mg l^{-1} IBA, 0.2 mg l^{-1} 6-BA and 2.0 g l^{-1} activated charcoal (MS-IBAO) and Modified MS with 0.2 mg l^{-1} NAA, 2.0 mg l^{-1} 6-BA and 2.0 g l^{-1} activated charcoal (MS-NAAO). a. Leaf length growth as a percentage of the original measurement and b. Leaf width growth as a percentage of the original measurement. The thick black line in each plot indicate the 50th percentile values of each species, the lower and upper sides of the box represent the 25th and 75 percentiles, and the error bars the 10th and 90th percentiles. The circles above or below the 10th and 90th percentile lines represent data points that are more than 3 times the standard error for the observation (outliers).

The constituents of the medium can range from modified Thomale GD (*Paphiopedilum* hybrids; Flamee, 1978 cf. Zeng et al., 2012), Knudson C (KC; *P. delenaii*; Nhut et al., 2005), 1/4 MS (*P. ciliolare*; Pierik et al., 1988), to 1/2 MS (*P. wardii*; Zeng et al., 2012). Furthermore, even for the same species, *P. micranthum*, there have been reports of different media giving best conditions for germination (RE medium; Chen et al., 2004a,b; 1/5 MS; Ding et al., 2004).

In our further testing for suitable media for germination, we found that viable protocorms of *P. spicerianum* were formed under low salt concentrations in 1/4 MS medium, but the number of seeds that germinated in 1/4 MS was lower than in RE medium. This is similar to what Pierik et al. (1988) found when comparing KC and 1/4 MS; KC gave a higher number of germinated seeds but all seeds died without further development. KC in composition is very similar to RE medium and our seeds remained white when grown in RE medium, did not develop chlorophyll and later became brown and died. The main difference between MS and both KC and RE is that it has many more micronutrients needed for plant growth.

When we assessed the seed structure and embryo cell viability using the FDA test, we found that the seeds imbibed in 1/4 strength MS medium retained their integrity, while in full strength MS medium seeds showed shrinkage (Fig. 6). This result may indicate that the full strength MS medium solute concentration is too high for *P. spicerianum,* leading to solute leakage from the seed. Although we did not see the seed shrinkage in RE treated seeds, we observed localized cellular death in the embryo in these seeds, visible as black spaces within the embryo, indicating that the constituents of the RE media may be toxic to some of the embryo cells. It is not clear from this investigation why or which elements in the RE media may cause cell death and this should be further investigated. However, because seeds in RE medium imbibed, became swollen and changed color to a whitish hue, but did not further develop green cells, we can assume that the toxicity may affect chlorophyll formation following cell expansion.

As has been known with other *Paphiopedilum* species, low salt concentration was not the only factor that affected seed germination and further protocorm development. Photoperiod and the duration of pretreatment significantly affected the probability of germination. We found that, except in the untreated seeds and seeds pretreated for 40 min and germinated in MSCW media, for all other treatments in which seeds germinated, a 24 h dark photoperiod increased the probability of germination compared to the 12 h light and 12 h dark photoperiod. Pierik et al. (1988) reported that there was a negative correlation between germination and the increase in the intensity of irradiance for *P. ciliolare* seeds. They found that the dark treatment for 24 h cycle at 30 days from germination resulted in the highest germination for *P. ciliolare*. Zeng et al.

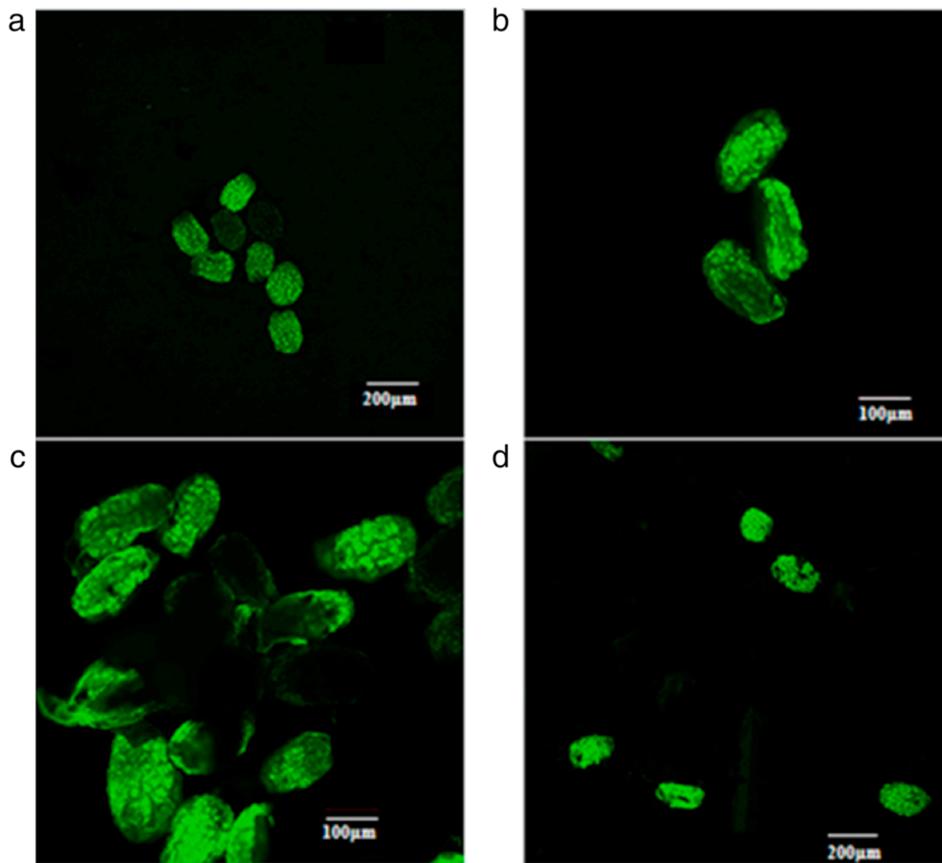

Fig. 6. Seed structure and cell assessment after soaking in different media and staining with fluorescent diacetate. a. Seeds soaked in 1/4 strength MS medium solution, b. Seeds soaked in full strength MS medium solution and c and d. Seeds soaked in RE medium solution. All seeds were pretreated with 1% NaOCl for 40 min before soaking in each medium solution.

(2012) reported that the duration of the dark pretreatment significantly affected the germination percentage; 45 days of dark pretreated seeds had the highest germination percentage. Other research has reported that the media can modulate the influence of light on germination. Stimart and Ascher (1981) reported that modified Thomale GD medium in darkness strongly promoted seed germination as well as protocorm survival of *Paphiopedilum* hybrids, while germination was best in Burgeff EG-1 when seeds were given light, though these seeds later died. The study by Zeng et al. (2012) reported that continuous dark treatment of seeds did not produce seedlings, suggesting that light is an important ecological cue in seedling development, although it is not clear why the dark treatment promotes seed germination of terrestrial orchids. It is widely accepted that epiphytic orchids require light for seed germination while terrestrial species require dark conditions. However, this is a complex phenomenon and our results and others show that the requirement for light for germination in *Paphiopedilum* genus is species-specific as well as germination stage-specific.

Asymbiotic *in vitro* germination of ripe orchid seeds can fail due to mechanical or physiological mechanisms that maintain seed dormancy. Seed dormancy can be broken by certain temperature regimes, lengthy imbibition, chemical softening of the testa with $Ca(OCl)_2$ or NaOCl, or by mechanical damage (Zeng et al., 2013). Studies on orchid seed germination have shown that immature seeds germinate better than mature seeds because during maturity integuments may become increasingly more impermeable to water (Ramsay and Stewart, 1998; Kauth et al., 2008). However, this can be species-specific; in *Cypripedium debile*, a species in the same subfamily as *Paphiopedilum*, mature seeds germinate better compared to immature seeds because of sparse cuticular deposition of testa cells that makes the testa less hydrophobic (Hsu and Lee, 2012). In the genus *Paphiopedilum*, the effect of maturity of the seed capsule, measured as number of days after pollination (DAP), is known to vary by species. In *P. godefroyae* optimum DAP ranged from 90 to 120 (Lee, 2007), whereas in *P. villosum* var. *Densissimum* DAP ranged from 170 to 190 (Long et al., 2010). In our study, we were not able to test the effect of seed maturity on germination success because we had only two seed capsules to obtain seeds that came from an extremely small population of *P. spicerianum*, and all experiments were conducted on 356 DAP seeds. It is important to note that in an earlier investigation by Lee (2007), *P. spicerianum* seeds obtained from greenhouse maintained plants gave 40%–50% germination when 120–180 DAP seeds were used. The best germination in this study was obtained at 90 days from germination and averaged $18.05 \pm 5.44\%$. Therefore, less mature seeds may be better for increasing germination percentage.

Stimulatory effects of NaOCl or $Ca(ClO)_2$ on *Paphiopedilum* seeds are also species-specific and the optimum duration for pretreating seeds can range from 10 to 75 min (Liao and Chen, 2006 cf. Lee, 2007; Zeng et al., 2012). Lee (2007) showed that *Paphiopedilum* seeds pretreated between 40 to 80 min generally improved the germination percentage, while Zeng et al. (2012) reported that in *P. wardii* 1% NaOCl for 40 min or 0.5% NaOCl for 60 min significantly increased germination. In both investigations, stronger NaOCl concentrations and longer durations inhibited germination. We too found that 40 min pretreatment with 1% NaOCl was the best for obtaining germination and viable protocorm development on seeds extracted from 356 DAP capsules of *P. spicerianum*. Our UV micrographs obtained on FDA tests of seeds pretreated with NaOCl confirmed that seeds pretreated for longer durations were more likely to lose testa integrity and result in embryo cell death (Fig. 3). This explains why germination was lower or absent in seeds pretreated for longer durations than 40 min. Thus, our study also supports the hypothesis that the living testa cells may be important in providing nutrition and activating embryo cells for protocorm development (Zeng et al., 2013).

Enhancement of media with plant growth regulators, carbohydrates that provide an energy source, organic amendments that stimulate growth, and beneficial vitamins, are all thought to be advantageous during formation and heterotrophic growth of orchid seedlings (Arditti, 1967, 1982; Kauth et al., 2008). Similar to what we found in *P. spicerianum*, Zeng et al. (2012) found in *P. wardii* that Hyponex medium supplemented with banana homogenate was the most suitable for seedling formation. Banana homogenate may promote seedling formation because of added nutrients and adequate amounts of sugars that are supplied to the medium. While Zeng et al. (2012) also had peptone as a plant growth regulator, in our study the same medium with added peptone did not result in seedling formation. Similar to *P. wardii*, however, *P. spicerianum* responded well to the addition of NAA to promote root development, and activated charcoal was important for absorbing harmful chemicals produced during seedling formation. In contrast, Long et al. (2010) found that in four *Paphiopedilum* species the addition of NAA and BA resulted in green leaf formation, but further subculturing of plantlets was not successful. The Long et al. study also demonstrated that there was no consistency in shoot multiplication when treatment concentrations of NAA and BA were varied (Long et al., 2010). In our study, the same MS basal media that worked well for seed germination was not successful in seedling formation. In summary, our results and previous studies on *Paphiopedilum* species indicate that the best medium for seedling formation has to be determined individually for each study species.

When we assessed advanced seedling development we found that the same medium that supported seedling formation, containing 3.0 g l^{-1} Hyponex No 1, 1.0 mg l^{-1} NAA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate medium, was the best for advanced seedling development, but with the addition of 1.0 mg l^{-1} 6-BA. BA is known to promote seedling leaf formation in other *Paphiopedilum* species but in varying concentrations (Huang et al., 2001). In our study, three media tested for seedling leaf length growth rate faired equally. In the other three media, leaf color was yellow but plants remained alive even at the end of 4 months. In our study we obtained an average of three to four shoots per germinated seed. However, in most cases *Paphiopedilum* produces fewer shoots per node (Chen et al., 2004a,b; Hong et al., 2008).

The major limitation of our study is seed availability for experimentation. This is an unavoidable constraint when working with PSESPs. In our approach to developing the seed germination, seedling formation and seedling development, we used a variety of media that contain different components and different concentrations based on our experiences with species from the same genera, communication with experts in the field and the published literature. It would have been ideal to develop our protocols in a more logical and step by step approach. Further limitations include an inability to know how well the seedlings produced survive in the field; ongoing experiments will test this (see Fig. 1. g; seedlings transplanted into soil containing pots ready for re-introduction into the wild). Zeng et al. (2012) were able to successfully re-introduce another asymbiotically produced *Paphiopedilum* species, giving an indication that this method may be successful in developing seedlings for re-introduction. We also did not test the effect of capsule maturity on seed germination (Lee, 2007). Lastly, our experiments were conducted on seeds obtained from a single population and comparison of asymbiotic protocol with other populations found in northern India and Myanmar (Liu et al., 2009) are strongly recommended, since different populations may have differences in germination and growth requirements due to local adaptations. Despite these limitations, we provide here a first roadmap for asymbiotic seed germination of one of the most vulnerable terrestrial orchid species in the world that has high horticultural potential. Species with small population sizes are prone to stochastic demographic fluctuations that lead to extinction (Purvis et al., 2000; Melbourne and Hastings, 2008). Thus, it is critical and time sensitive to develop integrated conservation approaches for *P. spicerianum*. Asymbiotic germination is a convenient method for large scale production of plants that can be used for re-introduction, as well as commercial propagation, thereby reducing the collection pressure on wild populations.

6. Conclusion

Based on our study, we recommend the following for enhancing seed germination and obtaining viable protocorm development for this species: pretreatment of seeds should be done with 1% NaOCl for 40 min and seeds should be germinated in a 24 h dark photoperiod for approximately 30 days in 1/4 strength MS media modified by reducing agar to 6 g l^{-1} and sucrose to 20 g l^{-1} and supplementing with 10% coconut water. For seedling formation, we recommend growing 90 day old protocorms in 3.0 g l^{-1} Hyponex No 1 with 1.0 mg l^{-1} NAA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate for six months before transferring to advanced plant growth media. For advanced plant growth, we recommend the same media used for seedling formation, supplemented with 1.0 mg l^{-1} 6-BA. We conclude that the FDA

test can effectively be used to screen a small number of seeds for pretreatment and growth media suitability. Such screening is critical for the germination of rare and endangered species that have limited seeds for testing and protocol development.

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