

Influence of Photoperiod and Culture Medium on the Speed of Asymbiotic Seed Germination and Seedling Development in *Spathoglottis plicata*

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Abstract. *Spathoglottis plicata* is an attractive, easy-to-grow, and floriferous terrestrial orchid that has become the most important horticulturally cultivated *Spathoglottis* species in Southeast Asia. The present research was conducted to study the asymbiotic seed germination and seedling development of this orchid under the influence of various photoperiod and medium treatments. Seeds from 28-day-old capsule were sown on five culture media, including half-strength Murashige and Skoog medium (1/2 MS), Orchid Seed Sowing Medium (OSSM), BM-1 Terrestrial Orchid Medium (BM-1), Vacin and Went Modified Orchid Medium (VW), and Knudson C Orchid Medium (KC), and incubated under 0/24-hour or 16/8-hour light/dark photoperiod. Seed germination occurred in all photoperiods and media tested but at different paces. Seeds subjected to total darkness germinated more rapidly in the first 3 weeks than those subjected to light. However, seed germination under light overtook or even exceeded seed germination under dark starting on the fourth week of culture. Seedlings grown on the OSSM and VW media showed the fastest development as they reached the advanced stage (Stage 6) within 11 weeks of culture. Seedlings on the BM-1 medium were the slowest to evolve, as they required more than 16 weeks' time to complete all the developmental stages. Light-incubated advanced stage seedlings were subcultured on the same medium until leaves and roots were well developed and acclimatized in the greenhouse with 100% survival.

With the increased popularity of orchids, a need exists for the introduction of new commercially valuable species to uphold consumer's attention. In recent years, commercial production and cultivation of native orchid species have increased considerably. The genus *Spathoglottis* includes about 50 species distributed in Southeast Asia and the Pacific region. All members of the genus *Spathoglottis* are terrestrial and most grow at low moderate altitudes (Beltrame, 2006). Many species have attractive purple, pink, white, and yellow medium- to large-sized flowers, which make them worthy subjects for cultivation. The most widely cultivated species of the genus is *Spathoglottis plicata*, commonly known as the garden orchid or ground orchid. Due to its hardiness and fast growth, it often is used in hybridizing with other mountain *Spathoglottis* that are difficult to grown in lowland to produce more interesting varieties. In Taiwan, native *S. plicata* is found in forest margins and grassy lowlands or slopes (Chen and Bell, 2009) or cultivated in gardens. Unlike most of the orchids, which require cooler environment to thrive, a mature *S. plicata* plant can tolerate the high summer temperatures and

continues to bloom throughout the year in the central and southern part of the country. It has become a valuable ornamental plant in the market with great future potential.

S. plicata is conventionally propagated by the separation of the pseudobulbs, which is time consuming with low proliferation and survival rates. The propagation by seed in the nature is hampered by the limited nutrients present in minute and dust-like seeds. With the advancement of tissue-culture technology, asymbiotic seed germination is now commonly used to ensure its propagation. Although there are reports on the asymbiotic seed germination of *S. plicata* using different medium formulations (Aswathi et al., 2017; Hossain and Dey, 2013; Minea et al., 2004; Thakur and Dongarwar, 2012), none of them examine in great depth the influence of culture media and incubation conditions on the speed of seed germination and seedling development. For commercial and amateur growers, as well as future breeders of *S. plicata*, a tissue-culture protocol that ensures rapid, uniform, and high rates of seed germination and seedling development is crucial. Hence, the present study was undertaken to examine the effect of different photoperiod and medium treatments on the speed of seed germination and seedling development in *S. plicata*.

Plant material. *S. plicata* plants were maintained in a greenhouse under natural light conditions, 70% relative humidity and 27/22 °C day/night temperature. When the flowers were fully opened, they were hand-pollinated by transferring pollinia onto the stigma of the same flower. Seed capsules were harvested 28 d after pollination.

Asymbiotic seed germination. The capsules were surface-sterilized using 70% ethanol for 1 min followed by 1% NaOCl (with one drop of Tween-20) for 20 min under constant agitation. The capsules were rinsed three times with sterile distilled water to remove the chemical sterilant. The sterilized capsules were dissected longitudinally into two halves and seeds were collected from the capsules and sown on the surface of the culture medium (≈100–200 seeds per petri dish). Five media were tested for seed germination and development, including the OSSM (P723; PhytoTechnology Laboratories, Lenexa, KS), BM-1 (B141; PhytoTechnology Laboratories), VW (V895; PhytoTechnology Laboratories), KC (K400; PhytoTechnology Laboratories), and 1/2 MS medium (Murashige and Skoog, 1962) (Table 1). The media were sterilized by autoclaving at 1.05 kg·cm⁻² and 121 °C for 15 min before being dispensed in 9-cm petri dishes with 25 mL per dish. The petri dishes were sealed with double layers of parafilm before being incubated at 25 °C under 0/24-h or 16/8 h-light/dark photoperiods provided by cool fluorescent lights (Phillips F20T12/CW; Philips North American Corporation, Andover, MA) at ≈40 ± 10 μmol·m⁻²·s⁻¹. The influence of photoperiod and culture medium on seed germination was assessed over a period of 4 weeks. A germinated seed refers to a seed that has initiated germination (i.e., embryo swollen, Stage 1). The germination percentage was evaluated by dividing the number of germinated seeds by total number of seeds sown in each petri dish. Five replicate petri dishes were used for each medium treatment and the average was obtained from the five replicates.

Seedling development. Germinated seeds that proceeded through subsequent development stages are collectively termed seedlings in this study. Seedling development was scored on a scale of 0 to 6 (i.e., Stage 0: testa intact; Stage 1: embryo swollen; Stage 2: testa ruptured; Stage 3: appearance of protomeristem; Stage 4: emergence of first leaf; Stage 5: elongation of first leaf and its further development; Stage 6: emergence of second leaf) (Table 2). The influence of culture medium on the speed of seedling progression through six developmental stages under light conditions was evaluated with the aid of a dissecting microscope (Olympus SZ6045; Olympus, Tokyo, Japan) by determining the percentage of seedlings at each stage over a period of 16 weeks. The percentage of seedlings at a given development stage was evaluated by dividing the number of

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Table 1. Composition of culture media used for asymbiotic seed germination of *Spathoglottis plicata*.

Component (mg/L)	Medium					
	1/2 MS	KC	VW	BM-1	OSSM	
Macronutrients						
NH ₄ NO ₃	825	500				412.5
(NH ₄) ₂ SO ₄		500	500			
CaCl ₂ ·H ₂ O	166.1					83
Ca(NO ₃) ₂		347.2				
Ca ₃ (PO ₄) ₂			200			
MgSO ₄ ·7H ₂ O	90.35	122.13	122.1	100		75.18
KCl		250				
KNO ₃	950		525			475
KH ₂ PO ₄	85	250	250	300		42.5
Chelated iron						
Na ₂ EDTA·2H ₂ O	18.63		37.26	37.25		18.65
FeSO ₄ ·7H ₂ O	13.9	25	27.8	27.85		13.93
Micronutrients						
H ₃ BO ₃	3.1			10		1.65
CoCl ₂ ·6H ₂ O	0.0125			0.025		0.0063
CuSO ₄ ·5H ₂ O	0.0125			0.025		0.0063
MnSO ₄ ·H ₂ O	8.45	5.682	5.088	25		4.23
MoO ₃ ·2H ₂ O	0.125			0.25		0.0625
KI	0.415					0.2075
ZnSO ₄ ·7H ₂ O	4.3			10		2.65
Organics						
Activated charcoal						1,000
Casein, enzymatic hydrolysate				500		
Peptone from meat						2,000
D-biotin				0.05		
Folic acid				0.5		
Glycine (free base)	1			2		
<i>myo</i> -Inositol				100		100
MES (free acid)						500
L-Glutamine	50			100		100
Nicotinic acid (free acid)	0.25			5		1
Pyridoxine·HCl	0.25			0.5		1
Thiamine·HCl	0.05		0.4	0.5		10
Sucrose	15,000	20,000	20,000	20,000		20,000
Agar	8,000	7,000	7,000	5,000		8,000
pH	5.7	4.5	5.8	5.5		5.8

1/2 MS = half-strength Murashige and Skoog medium; KC = Knudson C Orchid Medium; VW = Vacin and Went Modified Orchid Medium; BM-1 = BM-1 Terrestrial Orchid Medium; OSSM = Orchid Seed Sowing Medium.

Table 2. Stages of seedling development in *Spathoglottis plicata*.

Stage	Description
0	Testa intact
1	Embryo swollen
2	Testa ruptured
3	Appearance of protomeristem
4	Emergence of first leaf
5	Elongation of first leaf and its further development
6	Emergence of second leaf

prevent desiccation during the early stage of acclimatization and the polythene sheet was gradually open every week until it was completely removed after 4 weeks. Irrigation started at the fifth week with a frequency of twice a week using tap water.

Data analysis. Complete randomized design was used for all the experiments. Data were analyzed using Waller–Duncan at $\alpha = 0.05$ by SAS, version 9.00 (SAS Institute, 2002).

Results

Overview of seed germination, seedling development, and seedling acclimatization. An overview from seed germination to seedling acclimatization is presented in Fig. 1. The flowers (Fig. 1A) were self-pollinated and started to senesce 3 d after pollination. The seed capsules were cylindrical, green, and matured fast on this species (Fig. 1B). Green-pod sowing was started 28 d after pollination. Seeds incubated under 16/0-h light/dark photoperiod became chlorophyllous and developed to Stage 1 (Fig. 1C) within 1 week of culture. Once the embryos reached Stage 1, they quickly progressed to the protocorm stage (Stage 2) with a loosened testa. Thereafter, the protocorms entered

Stage 3, which was characterized by completely detached testa, well-defined shoot apices (i.e., protomeristem), and/or outgrown rhizoids (Fig. 1D). Following the formation of the protomeristem, the protocorms reached Stage 4, where the leaf primordium was initiated. At the early seedling stage (Stage 5), the elongation of the first leaf was observed (Fig. 1E). Stage 6 was distinguished by having two expanded leaves (Fig. 1F). Seeds cultured under 0/24-h light/dark photoperiod also were capable of evolving through characteristic development stages, but seedlings reaching Stage 6 were achlorophyllous and etiolated (data not shown) and so were of inferior quality. Light-incubated seedlings were subcultured on the same medium until the acclimatization stage without the use of any plant growth regulator (Fig. 1G). All the seedlings survived acclimatization in the greenhouse using the peatmoss as the potting substrate (Fig. 1H).

Influence of photoperiod and culture medium on seed germination. The percentage of germinated seeds following different photoperiod and medium treatments over a 4-week period is shown in Fig. 2. After 1 week of culture, seeds initiated germination on most of photoperiod and medium treatments except in the 1/2 MS-light and VW-light

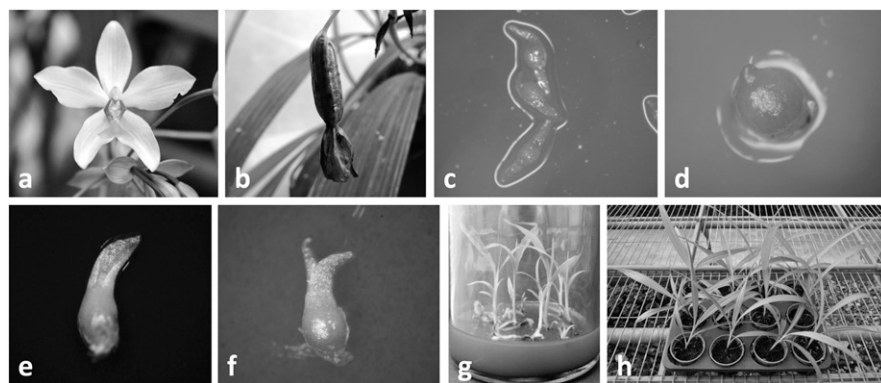


Fig. 1. Overview of asymbiotic seed germination, seedling development, and seedling acclimatization in *Spathoglottis plicata*. (A) *S. plicata* flower. (B) Immature seed capsule. (C) Stage 1 seedlings with swollen embryos. (D) Stage 3 seedling with distinguishable protomeristem. (E) Stage 5 seedling with elongated first leaf. (F) Stage 6 seedling with two expanded leaves. (G) Seedlings before acclimatization. (H) Seedlings after acclimatization.

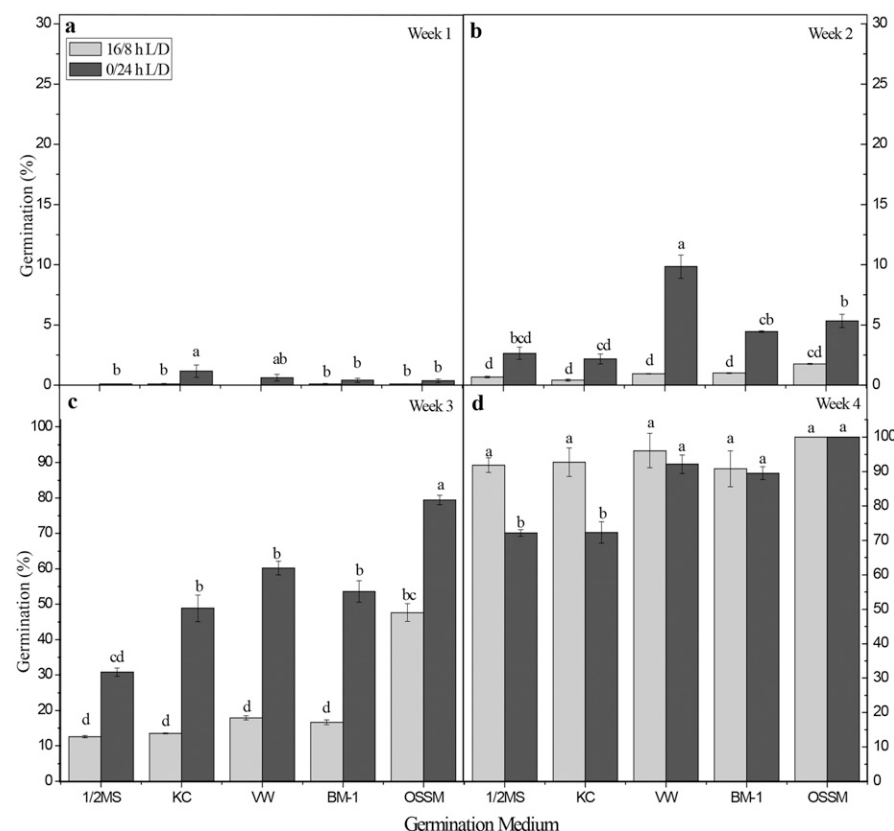


Fig. 2. Percentages of germinated seeds of *Spathoglottis plicata* under the influence of different photoperiod and medium treatments over a 4-week culture period. Means (\pm SE) with the same letter are not significantly different ($\alpha = 0.05$).

treatments. After 2 weeks of culture, the percentage of germinated seeds incubated in the dark was greater compared with the light except for the 1/2 MS and KC media, where no significant difference was found between light and dark treatments. The greatest percentage of germinated seeds was recorded with the VW medium (9.83%). After 3 weeks of culture, all the media showed comparatively greater seed germination rates in the dark than in the light except for

the 1/2 MS medium, where no difference was found between light and dark. Among the dark treatments, the OSSM medium recorded the greatest rate of germinated seeds (79.45%). After 4 weeks of culture, the percentage of germinated seeds incubated in the light was as high as or even superior to the percentage of germinated seeds incubated in the dark in all the media tested. Except for the KC and 1/2 MS dark treatments, which recorded only 72.17 and

72.32% of germinated seeds, respectively, the remaining treatments showed more than 90% of germinated seeds, with the OSSM medium recording 100% of germinated seeds.

Influence of culture medium on seedling development. The percentage of seedlings present at different development stages on different media is presented in Fig. 3. In week 1, only few seeds in the KC (0.09%), BM-1 (0.10%), and OSSM (0.08%) media progressed to Stage 1 of development. In week 2, some seeds in the 1/2 MS (0.66%) and VW (0.95%) media also showed Stage 1 of development. In week 3, seedlings in the OSSM medium showed the most rapid development, as some were found at Stages 2 (9.91%), 3 (12.92%), and 4 (2.77%) of development. A few seedlings in the 1/2 MS (0.37%), KC (0.36%), and VW (1.16%) media also evolved into Stage 3 of development, but at significantly lower rates than the OSSM medium. At week 4, all the Stage 0 seeds and Stage 1 seedlings in the OSSM medium evolved into Stages 3 and 4 of development. The greatest percentage of seedlings at Stage 4 was observed with the OSSM medium (85.37%), followed by VW (41.79%), KC (19.82%), and 1/2 MS (14.71%) media. None of the seedlings on the BM-1 medium showed Stage 4 of development. In week 5, 49.40% and 0.61% of seedlings on the OSSM medium were in Stages 5 and 6 of development, respectively. A few seedlings on the VW medium (0.25%) also advanced to Stage 5, but the majority of the seedlings remained at Stages 3 and 4. In week 6, the percentages of seedlings on the OSSM medium at Stage 5 and Stage 6 increased to 60.98% and 31.55%, respectively. In week 7, no more seedlings were found at developmental stages 1 and 2 in all the media tested. Seedlings on the BM-1 medium showed the slowest development, with 43.5% of seedlings still remaining at Stage 3. Seedlings started to show Stages 5 and 6 of development on the 1/2 MS, KC, and VW media. However, the greatest percentage of seedlings (72.61%) at Stage 6 was still recorded with the OSSM medium. In week 8, 35% of seedlings on the BM-1 medium were still found at Stage 3 of development, whereas those on the other media had all moved to more advanced stages. The OSSM medium contained the greatest percentage of Stage 6 seedlings (84.40%), and this was followed by VW (66.05%), KC (31.27%), and 1/2 MS (15.34%) media. In week 9, the percentages of seedlings at Stage 6 of development were comparable between the OSSM (89.33%) and VW (80.51%) media. In week 10, a few seedlings (0.90%) on the BM-1 medium first entered Stage 5 of development. The greatest percentages of seedlings at Stage 6 were still recorded on the OSSM (97.67%) and VW (97.82%) media, respectively. In week 11, all the seedlings on these two media reached Stage 6 of development. In week 12, there was first appearance of seedlings at Stage 6 on

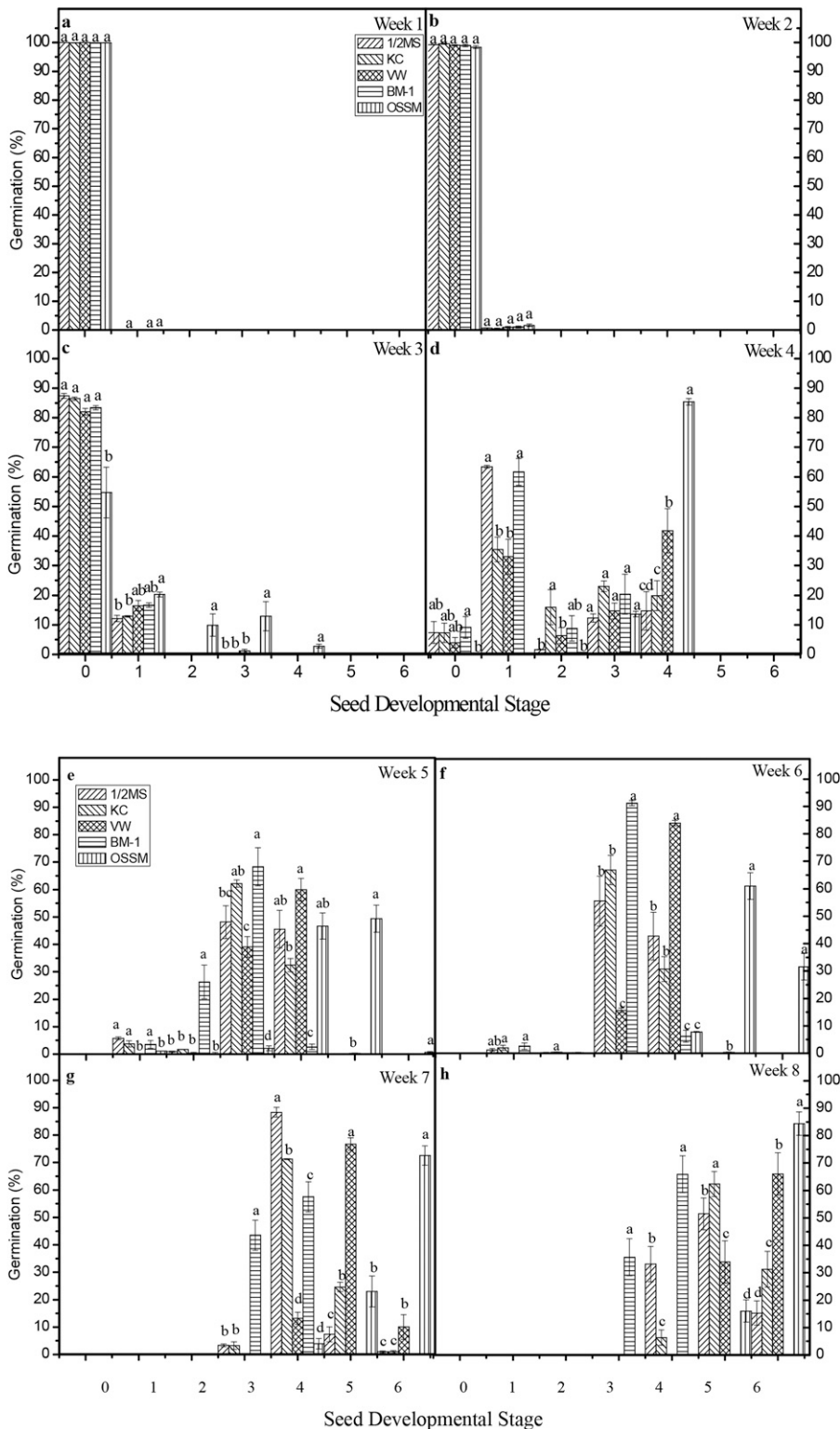


Fig. 3. Percentages of *Spathoglottis plicata* seedlings at different development stages on different media under light condition over a 16-week culture period. Means (\pm SE) within each development stage with the same letter are not significantly different ($\alpha = 0.05$).

the BM-1 medium. All the seedlings from the 1/2 MS and KC media progressed to Stage 6 in week 13. Seedlings on the BM-1 medium continued their development in weeks 14 and 15, and eventually, 98.93% of them reached Stage 6 of development in week 16.

Discussion

The influence of light and darkness on seed germination often is overlooked and not well understood for terrestrial orchid species and has not been examined before for *S. plicata*. Complete darkness often is consid-

ered to promote germination of terrestrial orchids. Seed germination in many temperate terrestrial orchids often is inhibited by light incubation (Arditti et al., 1981; Ernst, 1982; Van Waes and Debergh, 1986). Many terrestrial orchids grow in more shaded environments than their epiphytic counterparts, and light may not reach the habitat floor as readily. Upon dehiscence, seeds of terrestrial orchids may not germinate until they are buried. *S. plicata* may have improved germination in complete darkness, possibly due to its terrestrial background. The negative impact of light in terrestrial orchid seed germination also was recorded by Arditti et al. (1981), where light decreased germination of *Calypso bulbosa* and *Epicactis gigantea* seeds. Results in this study have further demonstrated the importance of light in seedling development of *S. plicata*. Comparative results were found with *Calopogon tuberosus*, where seeds at an advanced stage (Stage 6) were only observed under light incubation (Kauth et al., 2006). Similarly, although light was not a limiting factor for early stages of seed germination, it was essential for subsequent seedling development in *Dendrobium aequum* (Parthibhan et al., 2012). The authors observed that the 0/24-h light/dark photoperiod supported seeds up to the protocorm stage with poor seedling growth; seedling development occurred only when longer photoperiods were used. Nevertheless, results in our study indicate that *S. plicata* seeds incubated in the dark were capable of reaching the advanced seedling stage, as opposed to the findings of Kauth et al. (2006) and Parthibhan et al. (2012), where seedling growth was arrested in the absence of light. This may suggest the lower light requirement for seedling development of *S. plicata* compared with the other two orchid species.

Successful in vitro seed germination has been reported for *S. plicata* using various medium formulations. Hossain and Dey (2013) found that the maximum seed germination percentage was observed with the Phytamax medium (95%) followed by the OSSM medium (90%) after 5 weeks of culture. Thakur and Dongarwar (2012) obtained 87.3% germination and complete seedling development (reaching Stage 6) within 11 weeks when the seeds were cultured on 1/2 MS medium. Kheawwongjun and Thammasiri (2008) obtained near 100% germination using the modified semisolid VW medium. Minea et al. (2004) reported greater than 65% seed germination after 3 to 4 weeks on semisolid or liquid Knudson C medium. Aswathi et al. (2017) reported germination rates of 95%, 90%, and 25% using B5, KC, and MS media, respectively. In the present study, where we compared the efficiency of seed germination and seedling development between five different culture media, it was observed that all the *S. plicata* seeds were capable of germinating and reaching the Stage 6 of development no matter which medium was used if they were given sufficient time. However, the speed of

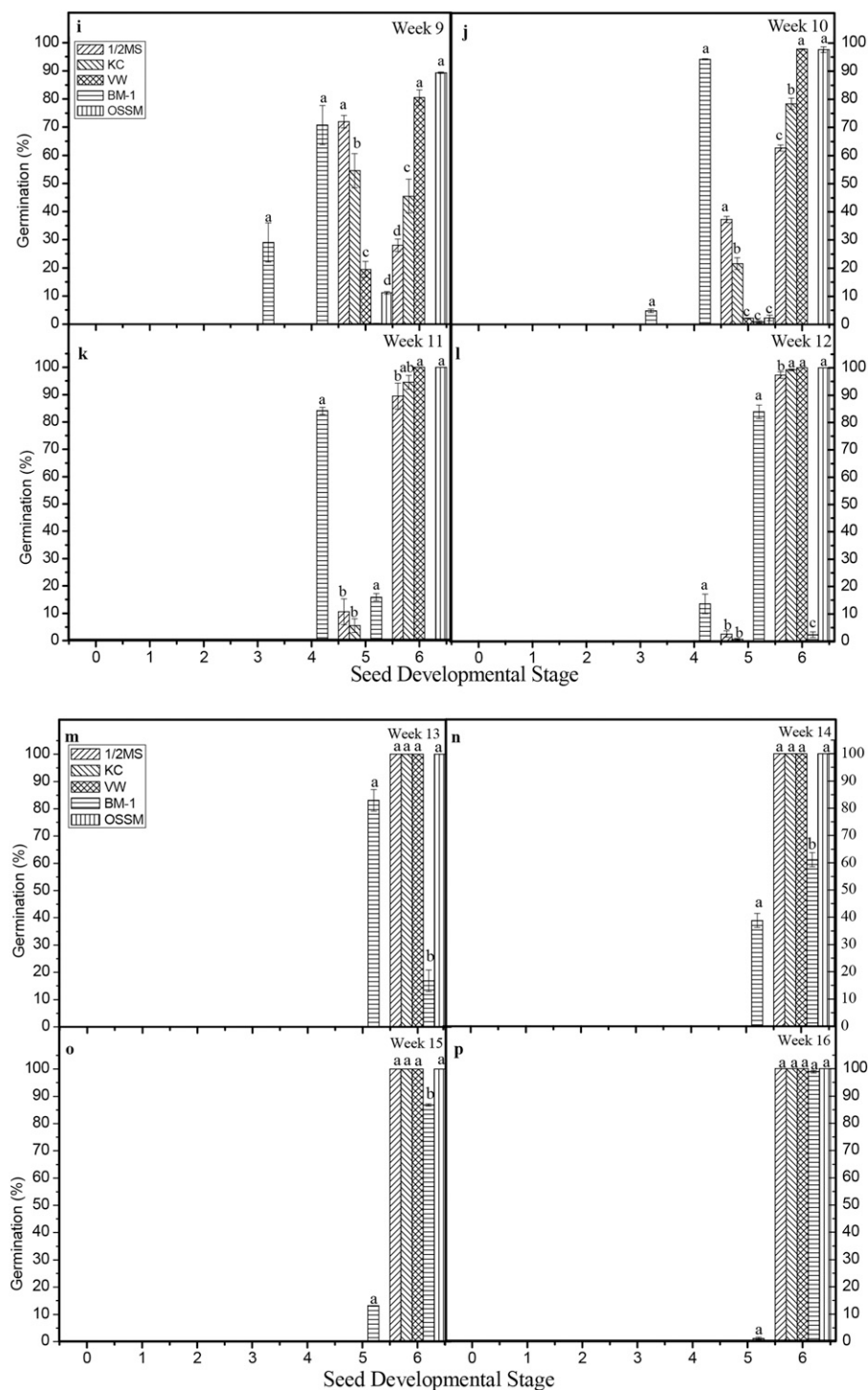


Fig. 3. (Continued)

seedling development depended largely on the medium used. Seedlings on the OSSM and VW media evolved the fastest as they reached Stage 6 of development in 11 weeks' time, followed by the seedlings on the KC and 1/2 MS media, which required 13 weeks; seedlings on the BM-1 medium evolved the slowest and required more than 16 weeks' time to complete seedling development.

The medium composition is one of the most important factors governing the success of micropropagation. Among the medium components, nitrogen often has been regarded as one of the key ingredients. The

source of nitrogen in the culture medium has been shown to affect the germination of different orchid species such as *Bletia purpurea*, *Calopogon tuberosus*, *Platanthera ciliaris*, and *Habenaria macroceratitis* (Anderson, 1996; Dutra et al., 2008; Kauth et al., 2006; Stewart and Kane, 2006). Anderson (1996) suggested that since the symbiotic fungi naturally provide the orchid seeds with organic nitrogen, an organic nutrient supplement would benefit germination and development of *Platanthera ciliaris* seeds in vitro. Stewart and Kane (2006) also showed that an organic form of nitrogen could advance the in

vitro growth and development of *Habenaria macroceratitis* seeds. In this study, the slowest seed germination and seedling development was observed with the BM-1 medium. The BM-1 medium contained only organic nitrogen source in the form of amino acids, namely glycine and glutamine. Although Malmgren (1996) suggested that an organic nitrogen source provided in the form of an amino acid might be more readily available due to simplified forms of nitrogen, glycine, the simplest amino acid, has been found to decrease germination of *Cattleya* seeds from 53% to 41% (Raghavan, 1964). Curtis and Spoerl (1948) also demonstrated that glycine significantly reduced germination of *Cattleya* seeds. Raghavan (1964) found that only certain amino acids were as effective as ammonium in germinating *Cattleya* seeds. Seed germination in the presence of other amino acids (i.e., arginine, proline, glutamine, etc.) was comparable with germination with ammonium nitrate. On the opposite end, the OSSM medium, which showed the fastest seedling development in this study, contained both organic and inorganic nitrogen. Besides amino acids, the medium also contained peptone. The beneficial effect of peptone on asymbiotic seed germination of orchids also has been reported previously. Curtis (1947) reported that the seed germination percentages of *Paphiopedilum insigne* and *Paphiopedilum hirsutissimum* were 30% greater with 2 g/L peptone than without peptone. In a more recent study, Hossain and Dey (2013) found that the maximum germination of mature seeds of *S. plicata* (i.e., 95% and 90%) was recorded in Phytamax and OSSM media, respectively, which both contained 2 g/L peptone. It also should be noted that in this study, the fact that the VW medium showed comparable performance with the OSSM medium (seedlings reaching Stage 6 of development in 11 weeks' time) may suggest that the presence of inorganic nitrogen alone in the medium was sufficient for seed germination and seedling development in *S. plicata*. The other two media used in this study, namely 1/2 MS and KC, contained also inorganic nitrogen as the nitrogen source, but in both the nitrate and ammonium forms. Since they allowed slower seedling development than the VW medium, this may imply that although ammonium was beneficial for *S. plicata* seed germination and seedling development, the presence of nitrate together with ammonium in the medium was unfavorable. Our finding is in agreement with Curtis (1947) and Raghavan and Torrey (1964), where the seeds of a *Cattleya* hybrid germinated more efficiently on media containing an ammonium source rather than a nitrate or nitrite source. Several researchers reported that inorganic nitrogen in the form of nitrate may limit germination, possibly due to low nitrate reductase activity during seed germination and early protocorm development (Malmgren, 1996; Raghavan and Torrey, 1964; Van Waes and Debergh, 1986).

Phosphate availability in the culture medium also may affect seed germination in *S.*

plicata. The VW medium contained a greater phosphate concentration (3.77 mM) than all the other media tested (i.e., 1.84 mM in KC, 0.31 mM in OSSM, 0.63 mM in 1/2 MS, and 2.20 mM in BM-1). The VW medium supported a high number (100%) of advanced-stage seedlings (Stage 6) within 11 weeks of culture, and this may be due to a greater phosphate concentration available to developing seeds of *S. plicata*. Dutra et al. (2008) observed the same pattern with the VW medium, which supported a high number (35.5%) of advanced-stage seedlings (Stage 6) of *Bletia purpurea*, whereas the KC, 1/2 MS, and BM-1 media only supported high numbers of Stages 4 and 5 seedlings. Dijk and Eck (1995) studied the effects of phosphate concentration on seedling growth of *Dactyloctenium aegyptium*, and their results showed that those cultured in the presence of high concentrations of phosphate (10 mM) had a greater fresh weight than the control treatment (1 mM).

Molybdenum is an important element that should be included in any plant culture medium (Gamborg et al., 1976). Although required in small quantities, molybdenum is essential for nitrogen assimilation, biosynthesis of abscisic acid, purine catabolism, and sulfur reduction (Mendel and Hänsch, 2002). Breddy (1953) found that the addition of molybdenum in combination with other micronutrients to the culture media increased both seed germination and seedling growth of orchids. In contrast, greater ammonium nitrate concentrations significantly decreased the concentration of molybdenum in leaf tissue of eggplant (Villora et al., 2002). The reduced molybdenum concentration was responsible for a decrease in the capacity of the plant to reduce nitrate to ammonium and ammonium to amino acids (Kauth et al., 2006). The OSSM medium in this study may have enhanced germination, growth, and development of *S. plicata* seeds due to the presence of molybdenum in the medium. Kauth et al. (2006) also found that *Calopogon tuberosus* seeds germinated better and developed faster to Stage 6 seedlings on the OSSM medium.

Niacin has been found to be beneficial for orchid seed germination (Arditti, 1967). The OSSM medium contained nicotinic acid, a derivative of niacin, which is essential for metabolism of components NAD⁺ and NADP⁺ (Kauth, 2005). Consequently, metabolism may be more efficient in seeds of *S. plicata* cultured on the OSSM medium than on other media assessed. Effectiveness of

niacin also was reported by Kauth et al. (2006), where increased growth and vigor were observed in seeds of *Calopogon tuberosus*.

In conclusion, the photoperiod and culture medium were two important factors governing the speed of seed germination and seedling development in *S. plicata*. The fastest and complete seed germination and seedling development was obtained with the OSSM and VW media under 16/8-h light/dark photoperiod incubation.

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