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Scientific article

Asymbiotic seed germination and in vitro development of orchid *Papilionanthe* Miss Joaquim

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Abstract

Orchids with their sheer variety of species are amazing, a major sharer in global floriculture trade. *Papilionanthe* Miss Joaquim, well known hybrid orchid, also recognized as official National flower of Singapore is a resilient, sun loving orchid, which blossoms throughout the year. Though a seed pod derived from a single flower contains millions of dusty seeds, due to lack of endosperm, seeds cannot germinate without the help of symbiont fungus. Commercial production of seedlings thus remains challenging. Therefore, in this study, we report a novel asymbiotic seed germination protocol standardized for 'Papilionanthe Miss Joaquim' (a hybrid of *Papilionanthe hookeriana* x *Papilionanthe teres*) which could be adapted for mass cultivation in a commercial setup. Seed of Papilionanthe Miss Joaquim were treated with 0.1% H_2O_2 and 0.1% KNO_3 to increase the germination rates. Seed pods were also subjected to various regimes of surface sterilization methods to reduce contaminations. Employing Carbendazim (1% w v^{-1}), Tricyclazole (1% w v^{-1}), Sodium hypochlorite (0.5% v v^{-1}) and Ethanol (80% v v^{-1}) in surface sterilization process, resulted in highest percentage of aseptic cultures. Seeds were inoculated on to Murashige and Skoog (MS) medium supplemented with BAP (6-Benzylaminopurine), NAA (1-Naphthaleneacetic acid) and 2,4-Dichlorophenoxyacetic acid (2,4-D) combination of 3 mg L^{-1} BAP (6-Benzylaminopurine) + 0.5 mg L^{-1} NAA (1-Naphthaleneacetic acid) found to be most effective to induce germination (84.67 ± 3.2%). Subsequently, germinated seedlings were subjected to different levels of BAP and NAA levels to achieve the highest number of plantlets. thus, multiplied plantlets were later subcultured onto MS medium containing 0.5 mg L^{-1} BAP and 3 mg L^{-1} NAA to induce rooting. Consequently, developed plantlets were acclimatized on a substratum containing coconut husk and charcoal pieces.
Keywords: *Papilionanthe hookeriana x Papilionanthe teres,* plant growth regulators, seed treatment, *Vanda* Miss Joaquim.

Resumo

Germinação assimbiótica de sementes e desenvolvimento in vitro da orquídea *Papilionanthe* Miss Joaquim

As orquídeas, com sua grande variedade de espécies, são de grande importância ao comércio global de flores. *Papilionanthe* Miss Joaquim, conhecida como orquídea híbrida, também reconhecida como flor oficial nacional de Cingapura é uma orquídea resiliente, amante do sol, que floresce durante todo o ano. Embora uma vagem derivada de uma única flor contenha milhões de sementes semelhantes a um pó muito fino, devido à ausência de endosperma, as sementes não podem germinar sem a ajuda de um fungo simbionte. A produção comercial de mudas, portanto, continua sendo um desafio. Portanto, neste estudo, relata-se um novo protocolo de germinação de sementes assimbióticas padronizado para *Papilionanthe* Miss Joaquim' (um híbrido de *Papilionanthe hookeriana* x *Papilionanthe teres*) que pode ser adaptado para cultivo em massa em uma escala comercial. Sementes de *Papilionanthe* Miss Joaquim foram tratadas com 0,1% H_2O_2 e 0,1% KNO_3 para aumentar as taxas de germinação. As vagens de sementes também foram submetidas a vários métodos de esterilização de superfície para reduzir contaminações. Empregando Carbendazim (1% P/V), Triciclazol (1% p v^{-1}), Hipoclorito de Sódio (0,5% v v^{-1}) e Etanol (80% v v^{-1}) no processo de esterilização de superfície, resultou em maior porcentagem de culturas assépticas. As sementes foram inoculadas em meio Murashige e Skoog (MS) suplementado com 3 mg L^{-1} de BAP (6-Benzilaminopurina), + 0,5 mg L^{-1} de NAA (ácido 1-Naftalenoacético) e combinação de ácido 2,4-Diclorofenoxiacético (2,4-D) de 3 mg L^{-1} de BAP (6-Benzilaminopurina) + 0,5 mg L^{-1} de NAA (ácido 1-naftalenoacético) mostrou-se mais eficaz para induzir a germinação (84,67 ± 3,2%). Posteriormente, as plântulas germinadas foram submetidas a diferentes níveis de BAP e NAA para atingir o maior número de plântulas. Essas plântulas foram multiplicadas e posteriormente subcultivadas em meio MS contendo 0,5 mg L^{-1} de BAP e 3 mg L^{-1} de NAA para induzir o enraizamento. As mudas obtidas foram aclimatizadas em substrato contendo casca de coco e pedaços de carvão.
Palavras-chave: *Papilionanthe hookeriana* x *Papilionanthe* teres, reguladores de crescimento vegetal, tratamento de sementes, *Vanda* Miss Joaquim.

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Introduction

Orchids are members of the Orchidaceae family, one of the largest in plant kingdom (Simpson, 2010). Their vibrant flowers with alluring hues are mesmerizing, charming and have an added merit of longevity. Orchid floriculture is a rapidly growing global business, making up to 8% of the global $21 billion floriculture trade (Hegde, 2020). Slow growth, poor seed germination rates make the propagation of orchid varieties challenging. Unlike most seeds, orchids seeds do not have an endosperm (Zhang et al., 2017). Seeds germinate symbiotically with fungi (Mala et al., 2017). The fungi supply sugars, inorganic and organic nutrients required for the initiation of seed germination (Yeh et al., 2019). In the wild, millions of seeds perish due to the lack of mycorrhizal fungal associations. Hence, symbiotic seed germination remains a challenging prospect of this trade (Chen et al., 2015).

Asymbiotic seed germination is commercially promising for the mass cultivation of orchids. Large-scale commercial cultivation of orchids is possible through *in-vitro* asymbiotic seed germination using a plant growth regulators supplemented artificial medium (Stewart and Kane, 2006) such as Murashige and Skoog (MS) media. In plant micropropagation, commonly cytokinins such as zeatin, kinetin, 6-benzylaminopurine (BAP), thidiazuron (TDZ) etc are used to induce multiple shoots. Auxins such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-Naphthaleneacetic acid (NAA), etc, are used to induce rooting. These plant growth regulators bring about various signal transduction mechanisms via different types of post translational modifications. For e.g., cytokinins bind to histidine binding receptor of endoplasmic reticulum, thus triggering autophosphorylation of the receptor, which in turn phosphorylates transcription factors to regulate the expression of genes such as type A and type B RRs (response regulators) (Leuendorf and Schmulling, 2021) To standardize micropropagation technique for a plant species, it is necessary to examine auxins and cytokinins in various combinations and concentrations. Micropropagation via seeds has an added advantage over vegetative parts, due to availability of seeds in large numbers. During hybridization experiments, i.e., cross pollination of different varieties of orchids to obtain new cultivar, such hybrid seeds can be effectively germinated to observe the variations in the next generation of plants. Whereas hybrids seeds cannot be germinated otherwise in a high numbers in natural conditions (Utami and Hariyanto, 2019; Peak et al., 2011).

Papilionanthe Miss Joaquim is a hybrid orchid derived from the crossing of *Papilionanthe hookeriana* (pollen parent) *x Papilionanthe teres* var. *andersonii* (female parent) (Khew and Chia, 2011). *Papilionanthe* Miss Joaquim grows better in high humidity and sunlight. The plant grows in dense clumps which require support. The plant blooms when its top grows about 50cm above the support. The flowers are violet rose in color with an orange centre. It has two petals, three sepals, and a single lip. The inflorescence has 10-12 flowers.

Materials and Methods

Plant material selection and molecular identification

The seed pods were collected from a local orchid nursery. Plant sample is identified using molecular identification through partial gene sequencing of the large subunit of the ribulose-bisphosphate carboxylase gene (rbcL) and Maturase K (matK) genes using Sanger sequencing. Accession numbers ('LC648642' for rbcL and 'LC656313'for matK) were obtained from the DNA Data Bank of Japan (DDBJ). Flowers (Figure 1A) of *Papilionanthe* Miss Joaquim orchid plant (Figure 1B) were subjected to artificial self-pollination. Unopened seed pods of different seed maturity levels were selected for asymbiotic seed germination.

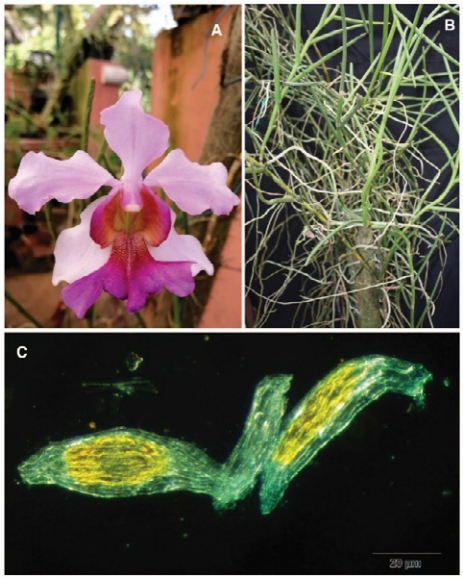

Figure 1. A) Flower of *Papilionanthe Miss Joaquim.*; B) *Papilionanthe* Miss Joaquim plant on a vertical support; C) Seeds of *Papilionanthe* Miss Joaquim under the microscope.

Surface sterilization and seed treatment scheme on seed germination

Unopened seed pods of different maturity levels were selected for the surface sterilization. Seed pods were soaked in deionized water for 10min and treated with fungicide solution (1% w v^{-1} carbendazim and 1% w v^{-1} tricyclazole in water) for 15 min and rinsed. Pods were later treated with 0.5% sodium hypochlorite solution and rinsed, followed by treatment with 70% ethanol for 1 min. Seeds pods were quickly flamed for 10 seconds. The sterile pods were slit opened longitudinally. The exposed seeds were scraped using a sterile blade and forceps and collected in a centrifuge tube. Seeds were treated with 0.1% hydrogen peroxide (H_2O_2) and 0.1% potassium nitrate (KNO_3) for one minute, washed and centrifuged at 500 RPM to remove excess water. The pellet was resuspended in autoclaved liquid MS medium. And proceeded for the inoculation of seeds onto MS medium containing different combinations of growth regulators. Figure 1C presents the microscopic image of the orchid seed

Asymbiotic seed germination

Approximately 0.5 g of seeds suspended in 2 mL MS medium were inoculated onto agar supported solid MS medium supplemented with indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), 6-benzylaminopurine (BAP), 2,4-Dichlorophenoxyacetic acid (2,4-D), 4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid (Picloram), Thidiazuron (TDZ) and Kinetin (Kin) at varying concentrations (0.0, 0.5, 1.0 and 3.0 mg L^{-1}). The cultures were incubated at 25-28 °C for 60 days under a light regime of light: dark (16:8) provided by cool white fluorescent tubes (~270 µmol photons m^{-2} s^{-1}).

Seedling development

Germinated seeds at the protocorm stage (60 days after the inoculation) and seedlings (2 mm length) were subcultured onto MS medium containing different combinations of BAP and NAA growth regulators to study the effects on maturation and root development. Emphasis was placed on faster growth and root development. Effect of supplementation of 2,4-D at 0.5 mg L^{-1} and 1 mg L^{-1} were tested to investigate its callogenesis effects during seed germination process. The effect of NAA and BAP from 0.0, 0.5, 1.0 and 3 mg L^{-1} on rooting was analysed. Mature *in-vitro* grown plantlets with 5 cm shoot and 6-8 cm root were selected for acclimatization. Coconut husk, brick pieces and charcoal pieces were used as substratum. Acclimatization was carried out at 25-27°C, 75-90% relative humidity. Watering was done on alternative days.

Liquid culture

To study the scope of liquid culturing system during the course of seed germination and acclimatization process, an attempt was made to grow seeds and *in-vitro* germinated seedlings in liquid culture. The seeds and seedlings were cultured using Stuart flasks containing 50 mL liquid MS media supplemented with 3mg L^{-1} BAP + 0.5 mg L^{-1} NAA. Flasks were slowly rotated using a vertical rotatory board rotated via a belt-connected pulley and a geared motor as described in (Steward et al., 1958). The cultures were incubated at 25-28 °C for 60 days with under a light regime of light: dark (16:8) provided by cool white fluorescent tubes (~270 µmol photons m^{-2} s^{-1}).

Statistical analysis

All experiments were carried out in triplicates. Factorial experiments are embedded in completely randomized experimental designs. Results were represented as mean ± standard error. Significance was measured using one-way ANOVA and Tukey's test for post-hoc analysis with probability level set to $p < 0.05$. Jamovi, a R-based open-source statistical tool (Jamovi Version 1.6**)** was used to carryout statistical analysis.

Results and Discussion

Effect of surface sterilization and pre-treatment of explants

Results of various surface sterilization regimes to control the contamination are presented in Figure 2. Bacterial and fungal contaminants, if any, may outgrow and compete with the orchid seeds resulting in eventual seed death. Hence, surface sterilization is crucial in eliminating contamination.

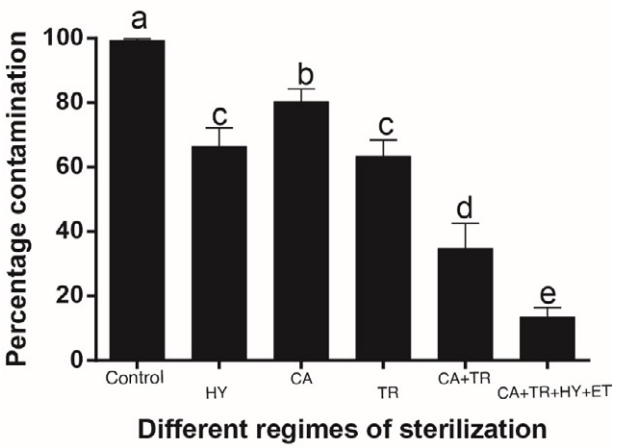

Figure 2. Effect of various surface sterilization regimes on contamination control. A combination of carbendazim, tricyclazole, sodium hypochlorite and ethanol were found to be the best surface sterilization regime to control the contamination. HY: Sodium hypochlorite (0.5% v v^{-1}), CA: Carbendazim (1% w v^{-1}), TR: Tricyclazole (1% w v^{-1}), ET: Ethanol (80% v v^{-1}) in sterile distilled water.

Controlling infection without affecting seed viability is vital to obtaining high rates of seed germination. Carbendazim at 1% w v^{-1} resulted in 80.4% ± 3.8% contamination, whereas tricyclazole at 1% w v^{-1} lead to 63.4% ± 4.90% contamination. However, a mixture of fungicides yielded a significantly better reduction of contamination (34.8% ± 7.68%). Bacterial contamination was controlled by sodium hypochlorite treatment. Ethanol treatment and subsequent flaming of seed pods significantly reduced contamination resulting in only 13.4% ± 2.90% contamination compared to control (99.4% ± 0.40% contamination).

During surface sterilization, sodium hypochlorite may penetrate the seed pod causing direct contact with the seeds. The alkalinity and cytotoxic effects of the solution causes the seeds to turn dark resulting in germination failure (Mercado and Bayona, 2020). It is noted that, while slicing the seed pod, the blade introduces surface contaminants into the inner tissues of the seed pods. Slitting the seed pods with forceps instead of slicing them with a blade proved better at reducing contamination.

Results of different seed treatment scheme are presented in figure 3. Seeds without treatment resulted in a significantly low germination rate compared to the treated set. Soaking seeds in a solution of 0.1% hydrogen peroxide and 0.1% potassium nitrate resulted in a significant enhancement of germination rate (83.0% ± 1.30%) compared to seeds inoculated without any treatment (33.8% ± 1.50%).

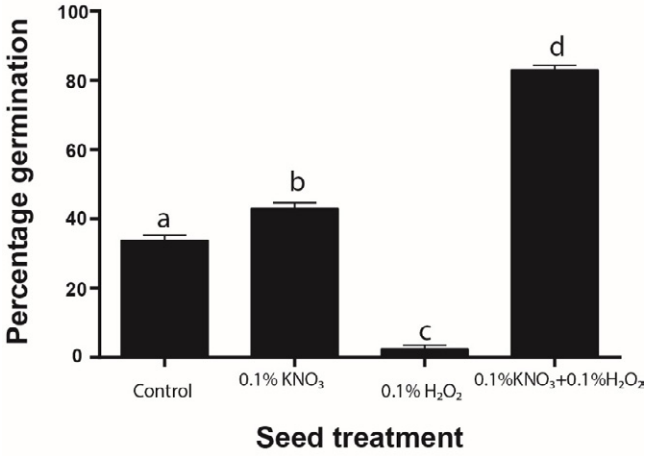

Figure 3. Effects of seed treatment regimens on contamination control. (0.1% w v^{-1}) KNO_3 and (0.1% v v^{-1}) H_2O_2 were used to enhance the seed germination rate.

The usage of hydrogen peroxide alone resulted in a low germination rate (2.5% ± 1.10%). Unlike fast-growing plants, orchids require extended incubation of up to 18 months. Subculturing is a tedious process due to the number of seedlings. Hence establishing clean culture conditions is imperative. Surface sterilizing agent such as sodium hypochlorite is pH basic. It damages the cell wall and causes discolouration and death of cells during the seed treatment step.

Usage of 0.5% hydrogen peroxide acts as a protecting agent which reverts seeds to the normal colour with the added benefit of protection against contamination. As described in the following equation, sodium hypochlorite will react with hydrogen peroxide to produce gaseous oxygen and sodium chloride. Hence pH is shifted back to the normal range (Djimeli et al., 2014; Trautmann et al., 2021) $NaOCl + H_2O_2 à O_2↑ + NaCl + H_2O$.

The maturation of the seed pods significantly affected the germination response as presented in Figure 4.

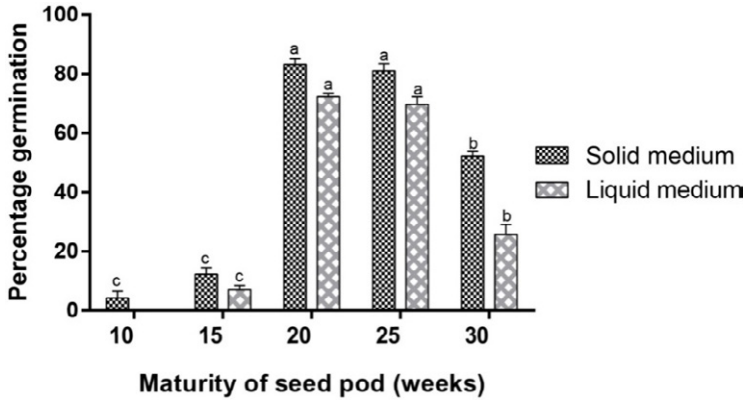

Figure 4. Effect of seed pod maturation and type of the medium on the germination rate.

Seeds obtained from the 20-week old seedpod post artificial pollination yielded the highest germination rate in agar-supported medium (83.3% ± 1.70%) and liquid medium (72.3% ± 1.20%). Immature seedpods displayed reduced germination. Older seed pods exhibited gradually declined germination rates. Ripe, yellow pods could not be processed since seed pods split open during the surface sterilization process. Seeds responded faster in the liquid medium compared to agar supported medium; however, the germination rate was significantly higher (83.3 % ± 1.70%) in agar supported medium compared to the liquid medium. Protocorms in liquid culture failed to develop and produce cotyledons.

Asymbiotic seed germination and effect of growth regulators on seeds

The seeds successfully germinated on MS medium supplemented with 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} 2,4-D (Figure 5A).

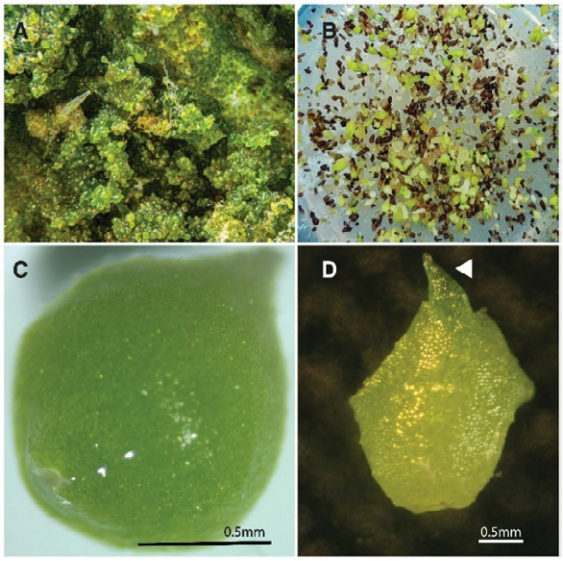

Figure 5. A) Seeds of *Papilionanthe* Miss Joaquim responding to germination medium (3 mg L^{-1} with 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA on protocorm bodies. Seeds that turned yellow and dark did not produce shoots. C) Microscopic image of single seed responding to germination medium (MS medium containing 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA). Embryo is ovoidal, swollen and multicellular. This stage is also known as protocorm. D) Appearance of cotyledons from protocorm 9 weeks after the inoculation on to maturation medium (MS medium containing 3 mg L^{-1} BAP + 1 mg L^{-1} NAA).

A germination rate of 90.67% ± 2.30% was achieved in 35 days, which was significantly higher than the other combinations of the growth regulators. Lower rates of germination were observed when supplemented with 3 mg L^{-1} BAP alone. Germination response was noted when seeds turned from white to a bulged green colour to indicate the formation of protocorms (Figure 5C). Effect of different combinations of BAP, NAA and 2,4-D on seed germination and maturation is presented in Table 1.

Table 1. Effect of different growth regulators incorporated singly and in combinations in Murashige and Skoog (MS) medium on germination and early seedling development and rooting stages. The values represent means ± SD from three replicates and numbers followed by the same letter in the same column are not significantly different ($p < 0.05$) from each other

Plant growth regulators in MS medium (mg L^{-1})			Seed germination (%)	Protocorms developed intocotyledons (%)	Number of Multiple shoots formed	Number of Roots formed
BAP	NAA	2,4D				
0	0	0	12.67 ± 1.8^{efg}	13.67 ± 0.9^{fg}	01.67 ± 0.7^{cd}	01.67 ± 0.3^{efgh}
0	0.5	0	00.33 ± 0.3^{i}	11.33 ± 0.3^{fgh}	01.00 ± 0.6^{cd}	03.33 ± 0.3^{cdf}
0	1	0	00.33 ± 0.3^{hi}	07.33 ± 0.9^{ghi}	00.33 ± 0.3^{d}	03.67 ± 0.3^{cd}
0	3	0	00.00 ± 0.0^{i}	00.33 ± 0.3^{i}	00.00 ± 0.0^{d}	04.67 ± 0.3^{bc}
0.5	0	0	20.33 ± 0.9^{e}	26.00 ± 1.2^{e}	01.33 ± 0.7^{cd}	00.67 ± 0.3^{gh}
0.5	0.5	0	19.00 ± 0.6^{e}	71.33 ± 3.2^{ab}	01.33 ± 0.3^{cd}	01.33 ± 0.3^{fhg}
0.5	1	0	15.33 ± 0.7^{ef}	44.00 ± 2.1^{c}	02.00 ± 0.0^{cd}	05.67 ± 0.3^{b}
0.5	3	0	09.33 ± 1.5^{fgh}	11.67 ± 1.8^{fgh}	01.00 ± 0.0^{cd}	08.33 ± 0.3^{a}
1	0	0	04.00 ± 0.6^{hi}	30.33 ± 2.9^{de}	01.67 ± 0.3^{cd}	00.00 ± 0.0^{h}
1	0.5	0	31.67 ± 1.2^{d}	63.67 ± 2.3^{b}	03.33 ± 0.3^{bc}	01.00 ± 0.6^{fgh}
1	1	0	37.00 ± 1.2^{cd}	38.67 ± 2.2^{cd}	01.00 ± 0.6^{cd}	01.67 ± 0.3^{efgh}
1	3	0	06.33 ± 1.8^{ghi}	00.33 ± 0.3^{i}	00.33 ± 0.3^{d}	02.67 ± 0.3^{def}
3	0	0	02.33 ± 0.3^{hi}	46.67 ± 2.3^{c}	05.33 ± 0.7^{ab}	00.00 ± 0.0^{h}
3	0.5	0	84.67 ± 3.2^{a}	79.33 ± 4.8^{a}	05.33 ± 0.3^{ab}	01.33 ± 0.3^{fgh}
3	1	0	42.00 ± 4.0^{c}	82.00 ± 1.5^{a}	02.00 ± 0.6^{cd}	02.00 ± 0.6^{defg}
3	3	0	16.00 ± 0.6^{ef}	21.33 ± 1.9^{ef}	00.67 ± 0.3^{d}	01.33 ± 0.3^{fgh}
3	0.5	0.5	90.67 ± 2.3^{a}	09.33 ± 1.5^{ghi}	06.33 ± 0.9^{a}	01.67 ± 0.3^{efgh}
3	0.5	1	62.33 ± 1.5^{b}	01.33 ± 0.3^{hi}	01.00 ± 0.6^{cd}	00.00 ± 0.0^{h}

BAP in combination with IBA or kinetin failed to induce germination. Seeds inoculated onto individual weedicide growth regulators like 2,4-D, TDZ and Picloram did not germinate. However, 0.5 mg L^{-1} 2,4-D + 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA combination induced 90.67% ± 2.30 germination, whereas 1.0 mg L^{-1} 2,40-D + 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA combination induced 62.33% ± 1.50% germination. However, in both cases, protocorms showed discolouration and failed to give rise to shoots (Figure 5B). Higher 2,4-D (>3 mg L^{-1}) concentrations resulted in seed browning. Seeds inoculated onto 3 mg L^{-1} 2,4-D + 3 mg L^{-1} BAP resulted in profuse callogenesis which did not mature into plantlets. Hence, MS medium with 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA was selected to induce germination for further experiments. After 2-3 months, the seed protrusions developed cotyledons (Figure 5D). Weedicide growth regulators like 2,4-D induce non-morphogenic callus in the orchid seed germination process at elevated concentrations (Budisantoso et al., 2017). The observance of callogenesis in our study supports this. Low concentrations of these growth regulators are known to induce cell division in general and may break the dormancy of the orchid seeds to induce germination (Cardoso et al., 2020; Silpa and Thomas, 2021).

Effect of plant growth regulators on germinated seedlings

Maturation medium containing 3 mg L^{-1} BAP + 1 mg L^{-1} NAA combination was found to be effective at inducing cotyledon formation. 82.0% ± 1.5% of the protocorms germinated to give rise to cotyledons. Germinated seedlings in different levels of BAP and NAA combinations (Table 1) induced multiple shoots. Plantlets inoculated onto combinations of IBA, TDZ, Kinetin and picloram failed to grow. Plantlets upon exposure to these growth regulators turned yellow and eventually perished. Germinated seedlings did not grow in a liquid medium. The tip of the leaflets and protocorms turned yellow. The highest number of shoots (6.3 ± 0.9) were observed in 0.5 mg L^{-1} 2,4-D + 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA (Figure 6A) from shoot initials.

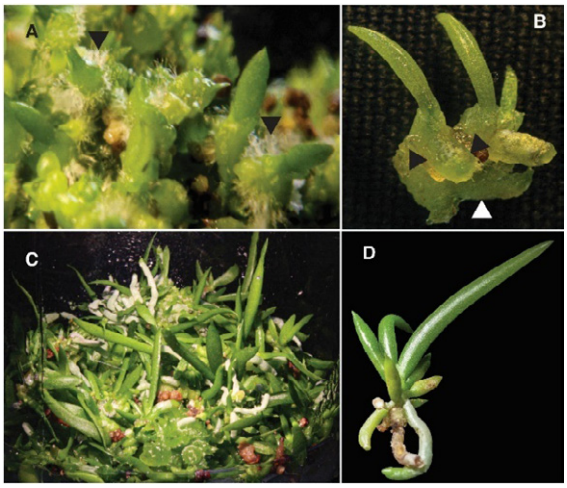

Figure 6. A) Germinated protocorm bodies cultured onto multiplication medium (MS media supplemented with 0.5 mg L^{-1} 2,4-D + 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA). Leaflets and hairy rhizoids are indicated by a black arrow mark. B) Formation of root initials from the separated shootlets. Rooting medium contains MS medium supplemented with 0.5 mg L^{-1} BAP + 3 mg L^{-1} NAA. White arrow indicates callus-like structures which have the potential of forming multiple shoots; C) Root formation on rooting medium 0.5 mg L^{-1} BAP + 3 mg L^{-1} NAA. Rooting was prevalent in plants that were not in direct contact with the solid medium. D) Mature plantlet of *Papilionanthe* Miss Joaquim ready to be acclimatized.

An increase of 2,4-D concentration to 1 mg L^{-1} resulted in a significant reduction in the number of shoots (1.0 ± 0.9) and eventually callusing of the seedlings. Multiple shoot initials were obtained from 0.5 mg L^{-1} 2,4-D + 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA medium. These shootlets were separated and subsequently used for rooting. Highest number of roots (08.33 ± 0.3) were formed in MS media supplemented 0.5 mg L^{-1} BAP + 3 mg L^{-1} NAA (Figure 6B-C). The rooting stage takes about five months. Supplementation of 0.5 mg L^{-1} GA_3 with 0.5 mg L^{-1} BAP + 3 mg L^{-1} NAA enhanced leaf elongation. However, prolonged exposure (> 1 month) inhibited the overall growth and inhibited rooting. Thus, GA_3 was excluded from the media. Mature plantlets with developed shoots and roots (Figure 6D) were ready for the acclimatization process.

Due to slow growth, media depletion was a considerable problem. Subculturing plantlets resulted in a risk of contamination and slow root formation. During the multiplication stage, monthly supplementation of 5 mL of Liquid MS medium to the agar medium containing 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA sustained leaf formation. At the rooting stage, roots developed rapidly when not in direct contact with the agar medium (Figure 6C). This indicates that reduction of water content in the media trigger root formation. Growing plantlets only in liquid media resulting in discolouration and death of the plantlets (Figure 7A).

Subculturing of germinated seedlings on multiplication medium (0.5 mg L^{-1} 2,4-D + 3 mg L^{-1} BAP+ 0.5 mg L^{-1} NAA) results in callus like root initials (Figure 7B) which gave rise to multiple leaflets (Figure 7C). Spraying of 1 mg L^{-1} GA_3 in sterile water onto mature plantlets in the rooting medium resulted in the yellowing of the plants (figure 7D).

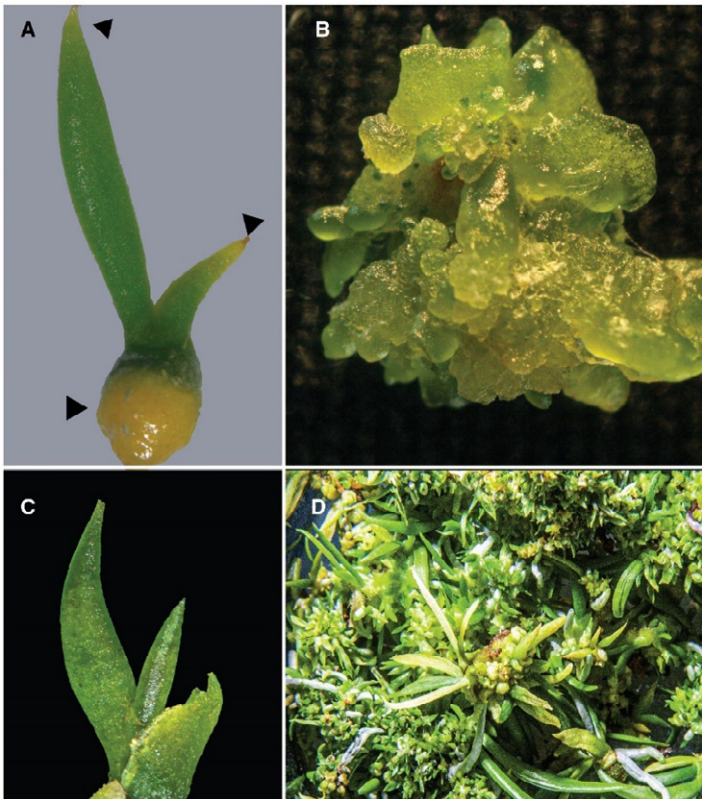

Figure 7. A) Effect of liquid medium on germinated seedlings: The tip of the leaflets and protocorm turned yellow as indicated by a black arrow. Plantlets eventually turned dark and dead. B) Effect of multiplication medium (0.5 mg L^{-1} 2,4-D + 3 mg L^{-1} BAP + 0.5 mg L^{-1} NAA) on germinated protocorm bodies: Callus-like structure with shoot initials was formed, which later develops into leaflets. C) Leaflets formed on multiplication medium. D) Effect of 1 mg L^{-1} GA_3 on plantlets on rooting medium. Plant growth was stunted and leaves turned yellow

Plantlet acclimatization

Plantlet survival was successful on acclimatization with coconut husk and charcoal pieces. Spraying with 1 mg L^{-1} BAP and 0.5 mg L^{-1} gibberellic acid in water once a month increased the length of the shoots. Increased exposure to concentrated GA_3 resulted in the yellowing of leaf tips. Orchids are slow-growing plants. Acclimatisation is one of the most challenging steps in the overall seed germination exercise (Shah et al., 2019; Vettorazzi et al., 2019). Fungal contamination is a major impediment in open-air culturing (Kunakhonnuruk et al., 2018). Usage of controlled chambers, maintaining a sterile environment increase the economics of production. Contaminants aside, the substratum selection also plays a key role in the survival of the seedlings. Due to high moisture, organic substances such as plant husk, tend to exudate phenolics and other metabolites. Considering that high organic and inorganic nutrients support the growth of the microflora and contribute to contamination (Kang et al., 2020), nutrient management and contamination control have to be achieved hand in hand waterlogging in the hardening substratum should be avoided as the presence of water negatively affects the rooting process and increases the probability of contamination. This investigation reports a successful protocol of asymbiotic seed germination of the ornamental orchid, *Papilionanthe* Miss Joaquim as depicted in Figure 8.

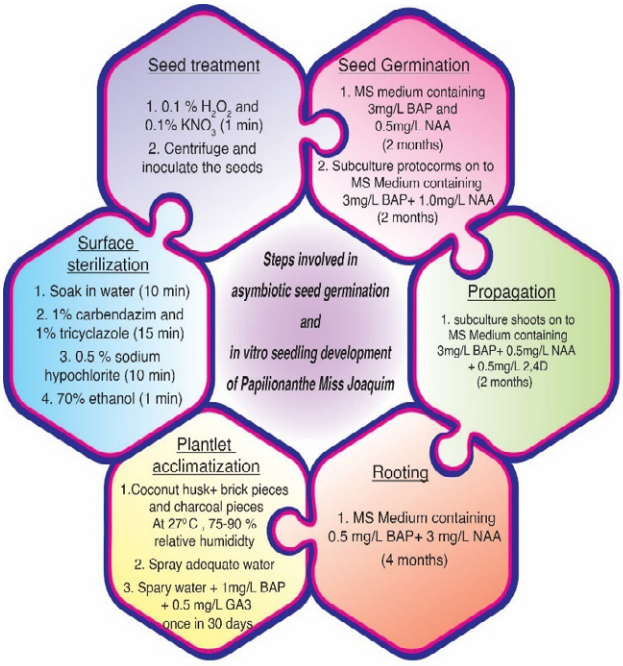

Figure 8. Schematic representation of steps involved in asymbiotic seed germination and in vitro seedling development of *Papilionanthe* Miss Joaquim. Various steps with key points and approximate duration of each phase are represented in the respective stage.

Conclusions

A surface sterilization regime with carbendazim, tricyclazole, sodium hypochlorite, and ethanol is effective in controlling contamination. Seeds treated with 0.1% H_2O_2 and 0.1% KNO_3 yielded a high percentage of germination rates. MS medium containing 3 mg L^{-1} BAP and 0.5 mg L^{-1} NAA was found to be optimal for inducing seed germination. 0.5mg L^{-1} BAP and 1 mg L^{-1} NAA were found to be efficient at cotyledon induction. Protocorms matured well in MS medium containing 0.5 mg L^{-1} BAP + 1 mg L^{-1} NAA (maturation medium). Highest numbers of multiple shoots were obtained in MS media supplemented with 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP + 1 mg L^{-1} NAA (multiplication medium). The highest number of roots were formed in the 0.5 mg L^{-1} BAP + 3 mg L^{-1} NAA combination. Plantlets with roots were acclimatized on the substrate containing coconut husk and charcoal pieces. This protocol can be successfully adapted for large-scale commercial mass multiplication of the *Papilionanthe* Miss Joaquim orchid.

Ethics Statement

No human subjects, lab animals, red-listed, endangered or invading species were used in the experiments; hence ethics committee approval was not required for this study.

Author Contribution

SSP: performed experiments, contributed to the manuscript, statistical analysis, designed the experiment. **SI:** performed experiments, contributed to the manuscript, scientific observation, designed the experiment. **RT:** Performed early stages of experiments. **QL:** supplying the explants, logistical assistance. **SK:** logistical assistance, experimental design. **LD'S:** funding acquisition, experimental design. **SKN:** experimental design, funding acquisition.

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