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Asymbiotic Seed Germination and *in vitro* Seedling Development of *Paphiopedilum liemianum* Fowlie, an Endangered Terrestrial Orchid in Northern Sumatra, Indonesia

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ABSTRACT

Paphiopedilum liemianum Fowlie, is a terrestrial orchid species and endemic in Northern Sumatra, Indonesia. However, due to a deep dormancy that the seed display at dispersion and the difficulty to obtain uniform plant in a short time period, micropropagation may be a feasible alternative. Micropropagation by *in vitro* seed germination techniques have been applied to the conservation of endangered orchid. Four months old seeds of *P. liemianum* germinated on five different basal media. All media were supplemented with 2.5 μM α -naphthalleneacetic acid (NAA) and cultures were incubated in the dark for 4 weeks followed by protocorm development at condition a 16/8 h L/D photoperiod. Germination percentage was 78.8% in Vacin and Went (VW) medium were significantly higher than other basal media. To evaluated the effect of organic nutrient additives on seed germination and protocorm development, the seed were cultured on VW medium amended with different of organic nutrient. Additives, especially 10% Coconut Water (CW) to VW medium improved the protocorm development well, with 33.3% the protocorm development to stage 5 (seedling). The seedlings were cultured on VW medium supplemented with different concentrations (0.0, 1.0, 2.0, 3.0 and 4.0 μM) of thidiazuron (TDZ). Healthy plantlets with developed leaves and roots were planted in pots with sphagnum moss and grown under *ex vitro* condition and the result was 76% survival rate after 4 weeks.

Key words: Coconut water, *in vitro* seed germination, seedling development, thidiazuron, vacin and went medium

INTRODUCTION

Paphiopedilum is a genus of the flowering plant family Orchidaceae, well known as Slipper Orchids, is characterized by resemblance of the pouch-shaped lip to a lady's slipper (Lin *et al.*, 2000; Chen *et al.*, 2004). This genus is terrestrial orchids with about 70 species that are native to South and South East Asia and a distribution that extends from the ASEAN region up to Papua New Guinea (Teob, 1989). Their flowers are variety of shapes, size, colors and the most popular commercially available orchids among the Orchidaceae family and a highly valued ornamental plant (Cribb, 1998; Ng and Saleh, 2011; Hong *et al.*, 2008).

Although, *Paphiopedilum* produce abundant seeds, this orchid is relatively rare in the wild due to the absence of endosperm in their seed (Arditti and Ernst, 1993; Pierik *et al.*, 1988; Long *et al.*, 2010). In nature, the *Paphiopedilum* are endangered population because of destruction and very

restricted habitat and all species were included to the list of endangered and threatened species in Appendix I of the convention on international trade in endangered species of wild fauna and flora (CITES, 2011).

Paphiopedilum liemianum Fowlie, is a terrestrial orchid species which is endemic in Northern Sumatra, Indonesia. It was found growing among rocks or at the root of the tree at the elevation of 500-1200 m. This species is characterized by pouch shaped-lip, rounded at the ends, pink and fade towards the edge, leathery and ribbon leaves with a prominent middle rib, leaf color dark green at upper surface and green with purple grape spotting at lower surface with trichomes at the edge leaves. Inflorescence emerges from the tip of the stem, supporting approximately to 8 flowers. The flowers stay fresh for up to 3 weeks. They are marketed as a pot plant with high value because of its unique and exotic flowers. This species is unique because of spiral corolla. For the micropropagation and conservation of this endangered species, information concerning its developed an effective protocol for the asymbiotic production of the terrestrial orchid *P. liemianum* is thus of great importance to study. Knowledge of the factors that affect asymbiotic seed germination required in the design of experiments for *in vitro* propagation studies. Micropropagation by *in vitro* seed germination techniques have been applied to the conservation of endangered and threatened orchid and may be useful in the re-introduction of *P. liemianum*.

There are many reports of the micropropagation of some *Paphiopedilum* species and their hybrids by the *in vitro* seed germination (Pierik *et al.*, 1988; Long *et al.*, 2010; Ding *et al.*, 2004; Lee, 2007; Tay *et al.*, 1988; Stimart and Ascher, 1981; Nagashima, 1982; Zeng *et al.*, 2012, 2013). However, there is still lack of information about the asymbiotic seed germination on *P. liemianum*.

In this study, the effects of germination media and organic nutrient additives on asymbiotic mature seed germination of *P. liemianum* were evaluated. The effects of growth regulator on *in vitro* seedlings development were also investigated. The aim of this study was to formulate the simple protocol for propagation of threatened orchid species by *in vitro* in large scale.

MATERIALS AND METHODS

Seed source and sterilization procedure: The mature capsules that are 4 months old after hand pollination were collected from DD Orchid Nursery, Junrejo Village, East Java, Indonesia. The capsules were soaked in commercial detergent liquid (Sunlight, Unilever, Indonesia) for 4 min for eliminated the dust, followed was washed 3 times with sterile-distilled water and drained. The capsules surfaces were disinfected using 70% ethanol and then flamed for 3 times in a laminar air flow cabinet. The capsules were sliced transversally and longitudinally into four parts with a sterile surgical blade in a sterile petri dish. The mature seeds from the capsules were removed and pooled into the petri dish with the help of a sterilized spatula.

Basal media effect on asymbiotic seed germination: To identify the suitable medium for seed germination and protocorm development, the seed were sowing onto five basal media: (1) Vacin and Went (Vacin and Went, 1949), (2) Quarter-strength Murashige and Skoog: 1/4 Murashige and Skoog macro-and micro-nutrient (Murashige and Skoog, 1962), (3) Half-strength MS (1/2 MS macro-and micro-nutrient), (4) Knudson C orchid medium (Knudson, 1946) and (5) Robert and Erast (Arditti, 1982). All media were supplemented with 2.5 μ M α -naphthalleneacetic acid (NAA, from Merck-Schuchardt), 3% sucrose and solidified with 0.2% gellan gum (Phytigel; Sigma Chemical Co., St. Louis, MO) and adjusted to a pH of 5.6 with NaOH and HCl prior to autoclaving

Table 1: Seed germination and protocorm development stages of *Paphiopedilum liemianum* fowlie modified from Yamazaki and Miyoshi (2006) and Miyoshi and Mii (1995)

Stages	Description
0	Ungerminated: No growth of embryo occurs
1	Enlarged embryo: Covered by testa
2	Testa ruptured: Embryo emerges from the testa (germination)
3	Embryo is discharged from the testa: Appearance of the shoot apex and or rhizoids (protocorm)
4	Emergence of first leaf
5	Presence of two or more leaves, roots primordial present (seedling)

at 120°C and 105 kg cm⁻² for 15 min. For each treatment, approximately 200 seeds were cultured in a 150 mL culture flask containing 25 mL of medium which were covered with aluminium foil. All experiments consisted of three independent replicates with 5 cultures flasks per replication. All the cultures were incubated in the dark for 4 weeks followed by protocorm development under a 16/8 h L/D photoperiod at 23±2°C. Culture were observed at 12 weeks after inoculation to determinate the effect of basal medium on seed germination and protocorm development using a Tension stereomicroscope, Nikon SMZ-1, Japan. The process of seed germination to protocorm development was classified into the following six stages according to the developmental stages of embryos (Table 1), which were modified from Yamazaki and Miyoshi (2006) and Miyoshi and Mii (1995). Germination was considered to have occurred only if testa ruptured and if embryo emerges from the testa (Stage 2, Fig. 1d). The percentage of seed at the different developmental stage was counted by dividing the number of seed in each stage by the total number of seed×100 (including viable and non viable seed).

Organic nutrient additives effect on asymbiotic seed germination: To evaluate the effect of organic nutrient additives on seed germination and protocorm development, the seed were cultured on VW medium which is the most suitable medium concluding from the first experiment result. The seeds were placed on VW medium amended with 10% Coconut Water (CW), 100 g L⁻¹ tomato mash, 100 g L⁻¹ potato mash, 2 g L⁻¹ peptone and without organic nutrient additives as control treatment. The source of these additives were fresh tomato *Lycopersicum esculentum* Mill, potato *Solanum tuberosum* L, that were skinned and homogenized in a blender. All the cultures were incubated in the dark for 4 weeks followed by protocorm development under a 16/8 h L/D photoperiod. All the cultures were maintained for 12 weeks at 23±2°C. The percentage of seed at the different developmental stage was counted by dividing the number of seed in each stage by the total number of seed×100 (including viable and non viable seed).

Evaluation of the effects of growth regulator on seedling development: After 12 weeks of culture, seedling derived from seeds of mature capsules were sub cultured to fresh media. Seedlings, 4-6 mm in length were grown on VW medium supplemented with Plant Growth Regulators (PGRs) with 1, 2, 3 and 4 µM thidiazuron (TDZ) and without PGRs as control treatment. Each treatment consisted of three independent replicates with five culture flasks per replicate containing four to five seedlings in each flask. After 16 weeks culture, weight and length of plantlet, number of leaves and roots, length and maximum width of leaf, length and maximum diameter of root were recorded. Following *P. liemianum* plantlets with 2-3 leaves bearing 2-3 roots (approximately 3-4 cm in height) were taken out from the culture flask and washed with running water to remove any



Fig. 1(a-l): Asymbiotic seed germination and seedlings development of *Paphiopedilum liemianum* Fowlie, (a) Flowering plant of *Paphiopedilum liemianum* fowlie, (b) Stage 0, seed, no growth of embryo occurs, (c) Stage 1, enlarged embryo, (d) Stage 2, testa ruptured (= germinated), (e) Stage 3, embryo is discharged from the testa (= protocorm), (f). Stage 4, emergence of first leaf, (g) Stage 5, present of two or more leaves, roots primordial present (= seedling), (h) Asymbiotic seed germination and protocorm development on VW medium supplemented with 2.5 μM NAA, (i) Seedlings *in vitro* on VW medium supplemented with 2.5 μM NAA and 2 g L⁻¹ peptone, (j) Seedlings growth on VW medium supplemented with 2.5 μM NAA and 10% coconut water (CW: v/v), (k) Developed plantlet with 3-4 leaves and roots after 16 weeks of culture on VW supplemented with 4 μM thidiazuron and (l) Transplanted plantlets after 4 weeks acclimatize in the greenhouse. em: Embryo, fl: First leaf, rh: Rhizoids, rp: Root primordial, sa: Shoot apex, t: Testa. Scale bars: (a) 5 cm, (b) 177 μm , (c) 230 μm , (d) 240 μm , (e) 870 μm , (f) 2.5 mm, (g) 3.5 mm, (h) 1.5 cm, (i) 1.6 cm, (j) 1.6 cm, (k) 1.25 cm and (l) 3.5 cm

residual agar. The plantlets were then placed into 10 cm diameter plastic pots containing shagnum moss. All the pots were then kept to the greenhouse (temperature $26\pm 2^{\circ}\text{C}$, light intensity 3700 lux, 14 h photoperiod and sprayed with water twice a day) for acclimatization.

Experimental design and statistical analysis: This experiment was arranged in a Completely Randomized Design (CRD). The data of all the experiments was analyzed using one-way analysis of variance (ANOVA) with SPSS 14. Subsequent mean separation was followed by the Duncan's Multiple Range Test (DMRT) with level of significance at $p = 0.05$ (Duncan, 1955).

RESULTS

Asymbiotic seeds germination and protocorm development: Dry seeds of *P. liemianum* were used as explants of this study that have brownish yellow are $816\ \mu\text{m}$ long, $142\ \mu\text{m}$ wide and the embryos consist only undifferentiated cells surrounded by a testa (Fig. 1b). The morphological development stage of *P. liemianum* from seed to seedling was documented (Fig. 1b-g). The seed germination stages starts about 3 weeks after inoculation with an enlarged embryo (Fig. 1c). Six weeks after inoculation, the embryos become swollen, the testa begins ruptured and embryo so white (Fig. 1d). Following embryos is discharged from the testa, shoot apex become able to be seen at one side of protocorm and appear rhizoids at the other (Fig. 1e). When protocorm reaches a size of about 2.5 mm, the protocorms fastly turn green, followed by the first leaf forms respectively (Fig. 1f). Generally, the roots primordial presence when the second leafs present (Fig. 1g).

Basal medium effect on asymbiotic seed germination and development of *Paphiopedilum liemianum* fowlie: The effect of five different basal media on asymbiotic seed germination of *P. liemianum* after 12 weeks in culture is shown in Table 2. The results show that type of basal media significantly affected on asymbiotic seed germination and development of *P. liemianum*. The highest percentage seed germination was obtained on VW medium (78.8%). The seed germination rate in the RE medium (63.9%) was lower than in VW medium, but higher than in half-strength MS (60.6%), quarter-strength MS (58.2%) and KC (55.6%). VW medium was also found suitable for protocorms development, 28.6% protocorms developed to stage 5 seedlings, were significantly higher than other basal media.

Organic nutrient additives effect on asymbiotic seed germination: The results of Table 3 show that seeds, cultured on VW medium supplemented with $2\ \text{g L}^{-1}$ peptone had highest percent

Table 2: Basal medium effect containing $2.5\ \mu\text{M}$ NAA on asymbiotic seed germination and development of *Paphiopedilum liemianum* fowlie for 12 weeks culture

Basal medium	Seedling developmental stage (%)						Total germination (stage 2-5)
	Stage 0	Stage 1	Stage 2 (germinated)	Stage 3 (protocorm)	Stage 4	Stage 5 seedling	
KC	36.8±0.9 ^e	7.6±0.4 ^a	6.3±0.9 ^a	10.2±0.5 ^a	14.4±0.6 ^b	24.7±1.5 ^c	55.6±1.1 ^a
RE	24.0±0.7 ^b	12.1±0.7 ^b	15.6±1.0 ^c	13.5±0.9 ^d	15.0±0.7 ^c	19.8±1.8 ^a	63.9±0.8 ^d
VW	13.8±0.6 ^a	7.4±0.6 ^a	11.9±1.0 ^c	16.5±0.7 ^e	21.8±0.6 ^d	28.6±1.4 ^d	78.8±0.8 ^c
1/2 MS	24.9±0.7 ^c	14.5±0.7 ^d	13.8±0.6 ^d	12.6±0.5 ^c	14.3±0.5 ^{ab}	19.9±1.3 ^a	60.6±0.7 ^c
1/4 MS	29.1±0.7 ^d	12.7±0.6 ^c	9.2±0.6 ^b	11.7±0.7 ^b	13.8±0.5 ^a	23.5±1.0 ^b	58.2±0.7 ^b

Values followed by same the letters within a column are not significantly different at $p\leq 0.05$ according to DMRT. Each mean is based on microscopic observation

Table 3: Organic nutrient additives effect on asymbiotic seed germination and development of *Paphiopedilum liemianum* fowlie on VW medium containing 2.5 μ M NAA for 12 weeks culture

Organic nutrient	Seedling development stage (%)						Total germination (stage 2-5)
	Stage 0	Stage 1	Stage 2 (germinated)	Stage 3 (protocorm)	Stage 4	Stage 5 (seedling)	
Control	39.8±0.6 ^d	10.5±0.8 ^c	9.4±0.5 ^b	8.5±0.5 ^c	8.6±0.6 ^a	23.2±1.1 ^a	49.7±1.0 ^a
Tomato 100 g L ⁻¹	37.9±1.0 ^c	8.8±0.5 ^b	13.0±0.7 ^c	6.4±0.4 ^a	9.1±0.6 ^{ab}	24.8±1.1 ^b	53.3±0.8 ^b
Potato 100 g L ⁻¹	39.4±0.7 ^d	10.9±0.6 ^c	8.4±0.7 ^a	7.5±0.5 ^b	9.3±0.8 ^b	24.5±1.1 ^b	49.7±1.0 ^a
Peptone 2 g L ⁻¹	20.9±1.2 ^a	6.3±0.5 ^a	16.9±0.8 ^d	14.1±0.6 ^c	14.6±0.5 ^d	27.2±0.8 ^c	72.8±1.2 ^d
10% CW	24.9±0.7 ^b	8.4±1.0 ^b	8.4±0.9 ^a	11.0±1.0 ^d	14.0±1.1 ^c	33.3±1.3 ^d	66.7±1.1 ^c

Values followed by same the letters within a column are not significantly different at $p \leq 0.05$ according to DMRT. Each mean is based on microscopic observation

Table 4: Effects of growth regulator on seedlings development of *Paphiopedilum liemianum* fowlie on VW medium for 16 weeks culture

TDZ (μ M)	Plantlet		Leaf (mm)			Root (mm)		
	Weight (g)	Length (mm)	No.	Length	Maximum width	No.	Length	Maximum diameter
0	16.8±0.2 ^a	31.5±1.1 ^a	2.3±0.4 ^a	25.6±1.9 ^a	7.3±0.6 ^a	2.0±0.5 ^a	7.7±0.9 ^a	1.1±0.2 ^a
1	17.9±0.2 ^b	34.3±0.8 ^c	2.6±0.4 ^b	27.5±1.0 ^b	8.9±0.8 ^c	2.2±0.4 ^{ab}	9.5±0.6 ^b	1.2±0.3 ^a
2	38.9±0.1 ^d	32.3±0.7 ^b	3.5±0.5 ^c	29.3±0.7 ^c	8.1±1.0 ^b	3.3±0.4 ^c	9.6±0.4 ^b	1.6±0.3 ^b
3	23.9±0.1 ^c	43.4±1.0 ^d	2.4±0.5 ^{ab}	30.3±0.7 ^d	9.5±0.5 ^d	2.4±0.4 ^b	11.6±1.4 ^c	1.8±0.2 ^c
4	64.6±0.1 ^e	47.9±0.8 ^e	4.6±0.5 ^d	30.2±0.9 ^d	12.5±0.6 ^e	4.1±0.5 ^d	11.5±0.7 ^c	2.3±0.4 ^d

Values followed by same the letters within a column are not significantly different at $p \leq 0.05$ according to DMRT

germination among all treatment (72.8%, respectively) when compared VW containing 10% CW (66.7%), tomato 100 g L⁻¹ (53.3%), whereas both VW medium containing potato 100 g L⁻¹ and without any organic amendments (control) showed seed germination percentage are same (49.7%).

Effects of growth regulator on seedling development: Table 4 shows the effects of growth regulator (PGRs) TDZ on seedling development. Seedling measuring about 4-6 mm in length were cultured on VW medium supplemented with various concentration of TDZ (0-4 μ M). The presence of PGRs in the VW medium had significantly resulted in a better response than without PGRs. In the treatment without PGRs showed a low leaf number and root number. Here also slow leaf and root growth were observed. The maximum response was obtained on VW medium supplemented with 4 μ M TDZ with an average weight 64.6 g per plantlet, an average length 47.9 mm per plantlet, an average maximum width 12.5 mm per leaf and an average maximum diameter 2.3 mm per root. Among the four TDZ concentration tested, the 4 μ M TDZ also proved to be more effective in increasing the leaf and root formation. In 3 μ M TDZ-containing medium also showed more growth induction with an average length 30.3 mm per leaf and an average length 11.6 mm per root, however there is no significant difference with 4 μ M TDZ.

DISCUSSION

We found the germination occurred on all media testing, but germination percentage in VW medium were significantly higher than other basal media and only VW medium supported high number of advanced stage 5 seedlings. The stimulate effect of the VW medium on asymbiotic seed germination percentage and protocorm development, which possible be due to the VW medium

contained phosphate at a relatively higher concentration (4.74 mM) compared to RE (2.21 mM), KC (1.84 mM), half-strength MS (0.62 mM) and quarter-strength MS (0.32 mM). This result is similar to Dutra *et al.* (2008) working with *Bletia purpurea* terrestrial orchid cultured on VW medium more optimal for aymbiotic seed germination than on KC, ½ MS, MM, BM-1, P723. Phosphate availability in the culture media also had significantly effect to growth and development of *Bletia purpurea* seedlings. Teixeira Da Silva *et al.* (2005) reported the source and concentration of phosphate plays a decisive role in the success of protocorm-like body and callus induction and development in hybrid *Cymbidium*. In different, Zeng *et al.* (2012) reported a higher percentage of germination for *Paphiopedilum wardii* on ½ MS than ¼ MS, MS, KC, VW, RE, Thomale GD, Hyponex. Tay *et al.* (1988) reported that seeds of *Paphiopedilum sukhakulii* cultured on Norstog medium to be optimal for *in vitro* germination than on Burgeff EG-1 medium. Johnson *et al.* (2007) reported P723 medium to be most suitable for germination of *Eulopia alta* terrestrial orchid than on ½ MS, KC, MM, VW, while Stewart and Kane (2006) working with *Habenaria macroceratitis* terrestrial orchid obtained on LM and KC medium, significantly higher than on ML, MS, VW, MM media. In addition, same medium the ideal for seed germination of the differ *Paphiopedilum* species; for example: *Paphiopedilum ciliolare*, *Paphiopedilum delenatii*, *Paphiopedilum villosum* var. *densissimum* have been shown that on KC medium to be most suitable for germination of these species (Pierik *et al.*, 1988; Nhut *et al.*, 2005; Long *et al.*, 2010).

Effect of organic nutrient additives on seed germination and protocorm development had already tested in a large number orchids such as *Epidendrum ibaguense* Kunth (Hossain, 2008); *Ophrys spuneri* (Kitsaki *et al.*, 2004); *Rhychostylis retusa* Blume (Thomas and Michael, 2007); *Cymbidium findlaysonianum* Lindl (Towaro *et al.*, 2008); *Cleisostoma racemiferum* Lindl (Temjensangba and Deb, 2006); *Grammatophyllum scriptum* (Abbas *et al.*, 2011); *Grammatophyllum speciosum* (Khampa *et al.*, 2010); *Acampe papillosa* (Piri *et al.*, 2013). We therefore, investigated the effects of organic nutrient on seed germination and protocorm development in *P. liemianum*. Organic nutrient additives in culture media as CW, tryptone, peptone, banana homogenates, potato homogenates, apple mash, lactalbumin hydrolysate, chayote mash for seed germination and protocorm development in some terrestrial orchids has been (Pierik *et al.*, 1988; Long *et al.*, 2010; Zeng *et al.*, 2012), however showed different results.

As shown in Table 3, we found the germination occurred on all organic nutrient additives evaluated. Although seed germination percentage was maximum on VW medium supplemented with 2 g L⁻¹ peptone and 14.1% protocorm remained in stage 3, however only 27.2% protocorm development to stage 5, but rather on 10% CW (33.3%). Our results indicated that for aymbiotic seed germination and subsequent protocorm development, VW supplemented with 10% CW was found to be most suitable medium for *P. liemianum*. This facilitating effect of CW may be because CW contain carbohydrate, vitamins, amino acid, organic acid, organic ion and enzyme which are usually important to plant cell development (Nitsch, 1951). Shantz and Steward (1952) and Shantz and Steward (1955) showed CW at concentration of 10-15%, it enhanced seed germination of many orchid species *in vitro*. This result of the study supported by Zeng *et al.* (2012) reported seed germination and protocorm development percentages of *Paphiopedilum wardii* Sumerh to be ca 65% highest with 10% CW than potato and banana homogenate, peptone and tryptone. Coconut water induced seed germination has been reported by Long *et al.* (2010) with *Paphiopedilum villosum* var. *dentissimum*. Of the five organic nutrient used, 10% CW was found to be most potential in which highest frequency of seed germination ca 10%, germination using

potato and apple homogenates were <3%, while those with lactalbumin hydrolysate and chayote mash were 0%. In contrast, Pierik *et al.* (1988) reported seed germination percentages of *Paphiopedilum ciliolare* to be ca 80% higher with tryptone than banana homogenates.

In this study, the maximum seedling growth of *P. liemianum* were obtained when they were cultured onto VW medium containing the highest level of TDZ (4 µM). The highest number of leaf and root was also observed when seedling grown on this medium. Thomas and Michael (2007) reported that addition of TDZ showed maximum response on seedling growth and development of *Rhynchostylis retusa*. This result is also partially agreed with Shiau *et al.* (2005) where they found cytokinins including TDZ, promoted the seedling growth of *Haemaria discolor*. In contrast, Vogel and Macedo (2011) observed that the addition of high concentration of TDZ abnormal shoot formation in *Cyrtopodium glutuniferum*. This indicated that effects of cytokinin supplementation to the culture medium on the seedling development of orchids varies with the species.

Healthy plantlets with developed leaves and roots were planted in pots sphagnum moss and grown under greenhouse condition. These plantlets adapted well in the *ex vitro* condition and the result was recorded 76% survival rate after 4 weeks (Fig. 11).

CONCLUSION

The conclusion of this study, asymbiotic seed germination techniques can be used for producing large numbers of *P. liemianum* seedlings and the plantlets can be successfully *ex vitro* acclimatized.

AUTHOR'S CONTRIBUTIONS

The work presented here was carried out in collaboration between all authors. ESWU and HP designed the experiment, TS and SH hand pollination, collected the data and analyzed the data, ESWU carried out the laboratory experiments and wrote the manuscript, HP revised the manuscript. All the authors agreed on the contents of the paper and post no conflicting interest.

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