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Study on the Production of the Tissue Culture Plantlets of Two Endangered Orchid Species, *Paphiopedilum wardii* Summerh and *Paphiopedilum bellatum* Pfitzer, Using in-vitro Seed Tissue Culture Technique

Nwe Nwe Win - Research Assistant-2
Ei Ei Zin - Research Assistant-2
Khin Pa Pa Shwe - Assistant Research Officer
Aung Zaw Moe - Research Officer

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Contents

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Abstract

Ng and Saleh (2011) stated that the propagation of *Paphiopedilum* using the conventional method of dividing the mother plant has been reported to be ineffective. This is because only a small number of new plants can be obtained from a single mother plant. As market demand for these species continues to increase, it has become necessary to develop techniques that enable large-scale production. Among these, tissue culture is one of the most effective methods for achieving a high rate of vegetative propagation. At the induction stage of *P. belluum*, both 1/4 MS1 and 1/4 MS2 media were found to have a combined effect on germination. However, for *Paphiopedilum belluum* and *P. wardii*, only the 1/4 MS2 medium showed a significant impact on the maximum size, minimum size, and number of protocorms, as well as on the germination of *P. wardii*. For the multiplication stage of *P. bellatum* and *P. wardii*, it was confirmed that only the T2 medium had a significant effect among all the parameters tested. Therefore, this study confirms that the type of stock solution, the proportion of stock solutions, the type of hormones used, and the hormone concentration ratios all have significant effects. This study can contribute to the development of in situ and ex situ conservation strategies for the sustainable development and conservation of the endangered *Paphiopedilum wardii* and *P. bellatum.*

Keywords: *Paphiopedilum, in vitro, seed germination, induction, multiplication*

အစေ့တစ်သျှူး မွေးမြူခြင်း နည်းစနစ်ကိုအသုံးပြု၍ မျိုးသုဉ်းရန် အန္တရာယ်ရှိသော *Paphiopedilum wardii* နှင့် *Paphiopedilum bellatum* သစ်ခွမျိုးစိတ် ၂ မျိုး၏ တစ်သျှူးပျိုးပင်များ ပွားများခြင်းအား စမ်းသပ်လေ့လာခြင်း

နွယ်နွယ်ဝင်း - သုတေသနလက်ထောက်-၂၊ အိအိဇင်- သုတေသနလက်ထောက်-၂၊
ခင်ပပရွှေ - လက်ထောက်သုတေသနအရာရှိ၊ ဦးအောင်ဇော်မိုး - သုတေသနအရာရှိ

စာတမ်းအကျဉ်း

Paphiopedilum သစ်ခွမျိုးစိတ်များသည် မိခင်အပင်တစ်ပင်မှ အပင်အသစ် အနည်းငယ် သာဖွံ့ဖြိုးသည့်အတွက် ရိုးရာနည်းလမ်းဖြစ်သော ပင်ပွားခွဲ၍မျိုးပွားခြင်းနည်းသည် ထိရောက်မှုမရှိကြောင်းဖော်ပြထားပါသည်။ စျေးကွက်တွင် ဝယ်လိုအားမြင့်မားလာသဖြင့် ဤမျိုးစိတ်များကို အများအပြား ထုတ်လုပ်နိုင်မည့် မျိုးပွားနည်းစနစ် လိုအပ်လျက်ရှိပါသည်။ မျိုးပွားနည်းများအနက် တစ်သျှူးမျိုးပွားခြင်းနည်းလမ်းသည် ပင်ပိုင်းမျိုးပွားနှုန်း (vegetative propagation rate) ကို အမြင့်ဆုံး ရရှိစေနိုင်သော နည်းလမ်းတစ်ခု ဖြစ်ပါသည်။ *P. bellatum* ၏ စတင်မွေးမြူခြင်း အဆင့်တွင် ¼ MS1 နှင့် ¼ MS2 ပေါင်းစပ်ပါဝင်သည့် မျိုးပွားအာဟာရ (Media) နှစ်မျိုးစလုံးသည် အညှောက်သစ်များ ပေါက်ရောက်မှုအပေါ် သက်ရောက်မှု ရှိကြောင်းတွေ့ရှိရပါသည်။ သို့သော် *Paphiopedilum bellatum* နှင့် *P. wardii* မျိုးစိတ်များတွင် protocorms ၏ အကြီးဆုံး အရွယ်အစား၊ အငယ်ဆုံးအရွယ်အစား နှင့် အရေအတွက် ဖွံ့ဖြိုးမှုကို ဖြစ်ထွန်းစေပြီး၊ *P. wardii* ၏ အညှောက်သစ်ပေါက်ရောက်မှုအပေါ်တွင် ¼ MS2 medium တစ်မျိုးတည်းသာ အရေးပါသက်ရောက်မှုရှိကြောင်း တွေ့ရှိရပါသည်။ *P. bellatum* နှင့် *P. wardii* မျိုးစိတ်များ၏ ပွားများ မွေးမြူခြင်းအဆင့် အတွက် စမ်းသပ်လေ့လာသော parameter အားလုံးတွင် T2 medium တစ်မျိုး တည်းသာသက်ရောက်မှုရှိကြောင်း အတည်ပြုနိုင်ခဲ့ပါသည်။ ဤလေ့လာမှုမှ အခံဖျော်ရည် အမျိုးအစားနှင့် အချိုးအစား၊ အသုံးပြုသည့် အာဟာရမျိုးပွားစာအမျိုးအစားနှင့် အချိုးအစားကွဲပြားမှုတို့ သည် အညှောက်သစ် ဖြစ်ထွန်းမှုအပေါ် သက်ရောက်မှုရှိကြောင်း အတည်ပြုနိုင်ပါသည်။ ဤလေ့လာမှုသည် မျိုးသုဉ်းပျောက်ကွယ်ရန် အန္တရာယ်ရှိနေသည့် *P. wardii* နှင့် *P. bellatum* မျိုးစိတ်များ၏ မျိုးရိုးဗီဇထိန်းသိမ်းကာကွယ်ရေးနှင့် ပြန့်ပွားခြင်းလုပ်ငန်းများတွင် အသုံးချနိုင်မည် ဖြစ်ပါသည်။

List of abbreviations

AC Activated charcoal
BA 6-Benzyladenine
CW Coconut water
DAP Days After pollination
FDA Flavin adenine dinucleotide
HCL Hydrochloric Acid
KC Knudson (KC; 1946)
KOH Potassium Hydroxide
NAA a-Naphthalene acetic acid
LSM Laser Scanning Confocal Microscope
NaOH Sodium Hydroxide.
NaOCL Sodium hypochlorit

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1. Introduction

The orchid family is among the most diverse in the plant kingdom, comprising approximately 25,000 to 35,000 species worldwide. Within this family, the genus Paphiopedilum includes about 100 species, most of which are terrestrial orchids, with a few being epiphytic (Sein Hla Bo, 2014). Notably, these orchids are characterized as pseudobulb sympodial orchids. In Myanmar, 14 species of Paphiopedilum have been recorded (Ye Lwin Aung et al., 2020). However, these populations face increasing threats, as deforestation, high market demand, and illegal harvesting are destroying native orchid ecosystems and placing several species at risk of extinction. Among the Myanmar orchids, the entire *Paphiopedilum* genus, which is endemic to the region, is listed in CITES Appendix I. A single orchid fruit can contain up to 72,000 seeds. However, the fruit size of orchids in the *Paphiopedilum* genus is much smaller than that of other orchid groups. Orchid seeds are also called "hollow seeds" because they lack the endosperm that allows them to germinate. Orchid seeds have low natural germination rates. They only reproduce with the help of fungi (Mycorrhiza) in the environment, which provide the nutrients needed for the plant to grow. A particular species of orchid also requires a specific type of fungus to help it grow from seed. This is why orchids are not found in a specific area where they grow and spread. Without the presence of fungi would not be able to spread. There is no possibility of propagation. It is essential to preserve and permanently preserve natural orchid species by using the mature green fruit of the orchid species (Asymbiotic or Sterile culture Method). Additionally, the entire *Paphiopedilum* genus is included in CITES Appendix (1).

Therefore, it is essential to preserve natural orchids in the long term and maintain them continuously by using the tissue culture method, which involves the (Asymbiotic or Sterile culture Method). This research was conducted because the Asymbiotic or Sterile culture method is essential for the sustainable development of natural orchids. I have proposed revisions to remove repetitive statements, clarify the explanation of orchid-fungus relationships, and improve the overall flow. The update also emphasizes the importance of the Asymbiotic (Sterile Culture) Method for sustainable orchid conservation.

2. Objectives

1. To know the best proportion effect of stock solution type and the ratio of stock solution *Paphiopedilum wardii* and *Paphiopedilum bellatum* in vitro seed technique.
2. To know the best type of hormone and their ration of hormone in vitro *Paphiopedilum wardii* and *Paphiopedilum bellatu*m of inducton & Multiplication stage in vitro seed technique.

3. Literature Review

3.1 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 $moll^{-1}$ NaOH or 1 $moll^{-1}$ 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 $molm^{-2}$ 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).

3.2 *Paphiopedilum. 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 ± 2.0 °C and at 45% relative humidity (RH).

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

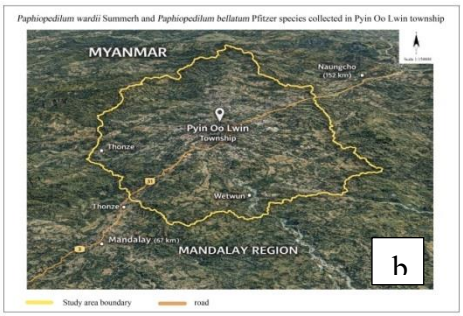
Following the preliminary assessment (see results section), two media, 1/4 strength modified MS supplemented with 10% CW and modified RE with 1.0 g l−1 activated charcoal and 10% CW, were selected for assessing the effect of light (12 h dark and 12 h light cycle compared to 24 h dark cycle) and pretreatment 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.

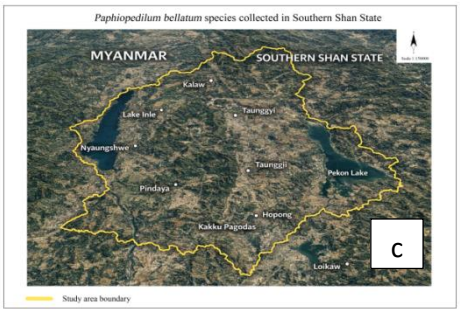
All treatments were visualized using a UV-fluorescence microscope (LSM710, ImagerZ 2, Zeiss, Germany). Another subset was transferred to growth media using the same method as above, and each treatment with 15 replicates (15, 200 ml sterile glass jars each containing approximately 100 seeds) was germinated 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).

4. Materials and Method

4.1. Location of the collected mature fruits/capsules for tested two orchid species

Hakaborazi National Park located in Kachin State, Myanmar encompasses various elevations, with zones including the sub-tropical zone (around 2,450 to 2,750 meters), a temperate zone above that, transitioning to semi-deciduous and evergreen forests, and finally an alpine zone with bare rock and snow above 3,350 meters figure (a). Pyin Oo Lwin is located at approximately 22.035° N latitude and 96.457° E longitude, with an elevation of around 3,570 feet (or 1,088 meters) figure (b). Southern Shan State is an administrative division and is located in Shan, Myanmar figure (c). The estimated terrain elevation above sea level is 824 meters figure (c). Seventeen mature fruits of *Paphiopedilum wardii* were collected from Khakaborazi National Park in Kachin State figure (a). Twenty-seven mature fruits of *Paphiopedilum wardii* were collected in Southern Shan State, and eight mature fruits of *P. wardii* were collected from PyinOo Lwin figure (a). Twenty-five mature fruits *of Paphiopedilum bellatum* were collected in Southern Shan State and Pyin Oo Lwin Township figure (b).





4.2. Sterilization of the mature Fruits or Capsules for two species

The mature Fruits or Capsules were first scrubbed with a soft brush in running tap water to remove any debris using a dishwashing detergent solution. Next, the samples were dipped and sterilized with 2.3% Kasugamycin $1ml^{-1}$ and Hominy $0.1gl^{-1}$ for 10 minutes, thoroughly rinsed with wash double-distilled water three times to ensure cleanliness. After that, they were dipped and sterilized with Clorox solution $10ml^{-1}$ containing 1-2 drops of 'Teepol' as wetting agent for 10 minutes, and then rinsed thoroughly with sterile double-distilled water three times. They were dipped and sterilized with $0.1gl^{-1}$ mercuric chloride for 5 minutes and then rinsed thoroughly with sterile double-distilled water three times. The sterilized fruits were air-dried for five minutes in a laminar flow chamber within an induction room that had been irradiated with UV light. Subsequently, in a laminar flow clean room, the fruits were immersed in 95% ethanol and flamed with a torch for 20 seconds.

4.3. Basal culture media and general culture conditions for induction or germination (stage 1)

The basal culture medium consisted of quarter-strength Murashige and Skoog (MS; Murashige and Skoog, 1962) macro- and micro-elements, supplemented with 100 mg/L myo-inositol, 1 mg/L niacin, 1 mg/L pyridoxine, 1 mg/L thiamine-HCl, 20 g/L sucrose, and 6 g/L agar (plant tissue culture grade). The pH of the medium was adjusted to 5.8 using 1 N KOH or HCl. The medium was then autoclaved at 121 °C and 1.06 kg/cm² pressure for 15–20 minutes before use. The sterilized fruits were air-dried for five minutes in a laminar flow chamber within an induction room that had been irradiated with UV light. Subsequently, in a laminar flow cleanroom, the fruits were immersed in 95% ethanol and flamed with a torch for 20 seconds. Capsules were dissected longitudinally using a sterile surgical blade, and both immature and mature seeds were sterilized within the capsules.

1. (1/4 MS1) Quarter-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with agar (6 g/L), sucrose (20 g/L).
2. (1/4 MS2) Quarter-strength MS medium (Murashige and Skoog, 1962) supplemented with agar (6g/L), sucrose (20 g/L), NAA (0.1 mg/L), and coconut water (CW, 10%).
3. (KC) Knudson C (KC) medium (Knudson, 1964) was supplemented with agar (6–8 g/L), sucrose (20 g/L), activated charcoal (1 g/L), and coconut water (CW, 10%).
4. (HY1) Hyponex 7–6–19 medium was supplemented with agar (6 g/L), sucrose (20–30 g/L), NAA (0.1 g/L), and coconut water (CW, 10%).

All four media types were prepared in 25 × 150 mm glass culture bottles, each containing 40 ml of medium. The pH of all media was adjusted to 5.8 before autoclaving. Culture bottles were incubated at 21 ± 2 °C under an 8/16-hour day/night photoperiod with a light intensity of 150 $\mu molm^{-2}$ s^{-1}. Humidity level was between 40-50%. For each treatment, either 0.015±0.01g of seeds were used per vessel, and all treatments were performed in triplicate. The study was carried out in a completely randomized design with three replications. The parameters of number of protocroms, maximum and minimum (protocroms size) and germination were assessed accordingly.

4.4. **Multiplication of protocorm for plantlets development**

For each nutrient treatment, three protocorms (ranging from 0.05 to 0.5 cm in size) derived from the induction culture stage of the two native species *Paphiopedilum wardii* and Paphiopedilum bellatum were placed in each culture vessel, and all treatments were conducted in triplicate. For the stage, six different media were used as treatments and designated as T1, T2, T3, T4, T5, and T6, respectively. Of the six media, three were Murashige and Skoog (MS) media, each supplemented with different proportions of chemical components. Out of the six media, three were Murashige and Skoog (MS) medium, Knudson C (KC; Knudson, 1964)

medium, and two were Hyponex N06.5–6–19 media, each supplemented with different proportions of chemical components.

Six different media were used as treatment and these were

Treatments	stock	Agar	Coconut (Milk)	NAA (mg l^{-1})	6BAP	Peptone	Banana	AC(gl^{-1})	Sucrose
T1	1/4MS	8 gl^{-1}	10%	0.001	-	1gl^{-1}	100gl^{-1}	1	10 gl^{-1}
T2	1/4Ms	6-10 gl^{-}	20%	0.5	1mgl^{-1}	1gl^{-1}	-	1	10gl^{-1}
T3	Hyponex	8gl^{-1}	10%	0.05	-	1gl^{-1}	150gl^{-1}		20 gl^{-1}
T4	Hyponex	6-10gl^{-1}	-	1.0	1mgl^{-1}		10%	0.5	30-20%
T5	KC	6 gl^{-1}	100 ml l^{-1}	0.1	4mgl^{-1}	-	-		20gl^{-1}
T6	1/2MS	6 gl^{-1}	150ml l^{-1}	0.1	-	-	150gl^{-1}	-	30gl^{-1}

All six media types were prepared in 25 × 150 mm glass culture bottles, each containing 40 ml of medium. The pH of all media was adjusted to 5.8 before autoclaving. Culture bottles were incubated at 21 ± 2 °C under an 8/16-hour day/night photoperiod with a light intensity of 150 $\mu molm^{-2}$ s^{-1}. Humidity level was between 40-50%. The study was carried out in a completely randomized design with three replications. The parameters of number of plantlets, maximum and minimum (plantlet), maximum and minimum (root size) were assessed accordingly.

4.5. Data analysis

Data was analyzed by using statistical product and service solutions (SPSS) version 26. All the variables for every treatment were compared by using Kruskal-Walls one way ANOVA (Non-Parametric Method). Pairwise comparison was applied to compare among treatments. The types of treatments are four for the induction stage in in vitro seed culture of one species. Each treatment, either 0.015± 0.01 g of seeds were used per vessel of total numbers are 120), for the induction stage in in vitro seed culture of one species. The types of treatments are six for the multiplication stage in in vitro seed culture of one species. Each nutrient treatment, three protocorms (ranging from 0.05 to 0.5 cm in size) derived from the induction culture stage, were used per vessel of a total number of 180 for the multiplication stage of one species.

5. Result and Discussion

5.1 Effect of different medium on *Paphiopedilum bellatum* in *vitro* seed culture for the induction stage of germination

A Kruskal–Wallis test was conducted to examine the effects of treatments in the induction or germination of *Paphiopedilum bellatum*, based on different proportions of stock solution types, hormone types, and basal media ratios. A significant result was found, H (3) =12.551, p<.05, $^{W}H=0.32$ / $X^2(3)$ =12.551, p.006, indicating that the treatments or media groups differed from each other. Basal media 1/4MS1 (Mean Rank =69), media 1/4MS2 (Mean Rank =69), media HY1 (Mean Rank =53), and media KC (Mean Rank =51). So, there was a significant difference between them. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the six pairs of groups. There was no difference in evidence between some pairs among groups. There are differences in evidence among groups in some pairs. Table 1 shows that there was a statistically significant difference (adjusted significance = 0.049) between the KC–

1/4MS1 and KC–1/4MS2 media pairs. It was observed that these two pairs of groups had the same adjusted significance. Figures 1 and 2 present the pairwise comparisons of treatments (KC–1/4MS1 and KC-1/4MS2), showing differences between the pairs of groups. Therefore, for the germination of *P. bellatum* using in vitro seed culture, the pairwise treatment comparisons KC–1/4MS1 and KC–1/4MS2 media demonstrated differences among the groups. Accordingly, Figures 1 and 2 present the differences between the average rank of treatment and the mean rank of treatments.

Table 1: Pairwise Comparisons of Treatments For *paphiopedilum bellatum* of Germination by in vitro seed culture.

Treamet (media)-Treatment (media)	Test Statistis	Std,Error	Std. Test Statistic	Sig.	Adj.Sig[a]
KC-HY1	2.000	6.810	.294	.769	1.000
KC-1/4MS1	18.000	6.810	2.643	**.008**	**.049**
KC-1/4MS2	18.000	6.810	2.643	**.008**	**.049**
HY1-1/4MS1	16.000	6.810	2.350	**.019**	.113
HY1-1/4MS2	16.000	6.810	2.350	**.019**	.113
1/4MS1-1/4MS2	.000	6.810	.000	1.000	1.000

Each row tests the null hypothesis that the treatment 1 and treatment 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Shown in Table 1, the significance level for pairs of groups the treatments or media KC–HY1 and 1/4MS1–1/4MS2 is greater than 0.05; therefore, no significant difference was observed between these treatments. We used A Kruskal-Wallis test to assess the significance of differences across the treatments. Additionally, Dunn's pairwise analysis was performed to adjust significance levels. As presented in Table 1, based on the adjusted significance (Adj. Sig.) values, the treatments KC–HY1 and 1/4MS1–1/4MS2 were found to have adjusted significance levels less than 0.05. Although the significance levels for the treatments HY1–1/4MS1 and HY1–1/4MS2 are greater than 0.05, indicating no significant difference, their adjusted significance (Adj. Sig.) values were less than 0.05. Furthermore, as shown in Table 1, the pairs of groups KC–1/4MS1 and KC–1/4MS2 have both adjusted significance and significance levels less than 0.05, indicating significant differences between these pairs of groups. Therefore, among the four media tested, 1/4MS1 and 1/4MS2 are suitable for the induction stage of germination in *Paphiopedilum bellatum* in vitro seed culture. However, about the number of protocorms and the maximum and minimum sizes of protocorms at the induction stage, further consideration is required.

(1/4 MS2) Quarter-strength MS medium contains coconut water and NAA, whereas Basal (1/4 MS1) Quarter-strength MS medium does not contain coconut water or NAA. However, as shown in Figures 1 and 2, quarter-strength MS2 (1/4 MS2) and quarter-strength MS (1/4 MS1) media exhibited similar results during the germination induction stage.

Therefore, for the germination at induction stage, Basal (1/4 MS1) Quarter-strength MS medium is considered more suitable. At 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) (Chen et al., 2015). 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 (Chen et al., 2015).

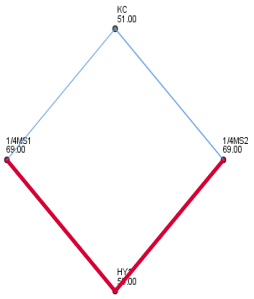


Figure1: Pairwise Comparisons of Treatments For *p.bellatum* of germination

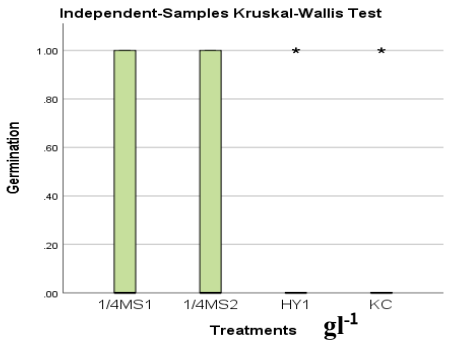


Figure2: Effect of four teaments for *p. Bellatum* of germination by in *vitro* seed culture

As shown in Figure 2, the KC and HY1 media or treatments exhibit outliers. The first quartile (Q1) and third quartile (Q3) are shown for the 1/4MS1 and 1/4MS2 media; however, they are not displayed for the KC and HY1 media. Additionally, among the four media or treatments, the second quartile or median, as well as the maximum and minimum values, are not displayed.

5.2. Effect of different media on the maximum protocorm size of *Paphiopedilum bellatum* during the induction stage in in vitro seed culture.

A Kruskal–Wallis test was conducted to examine the effects of treatments on the maximum protocorm size of *Paphiopedilum bellatum*, based on different proportions of stock solution types, hormone types, and basal medium ratios.For size of maximum the size of the maximum protocorm of *Paphiopedilum bellatum*, a significant result was H (3) =11.551, p<.05.^{W}H= 0.1/ X^2(3) =11.551, p.009, ^{W}H=0.1, indicating that the treatments or media are different from each other. Basal media 1/4MS1 (Mean Rank =18.38), media 1/4MS2 (Mean Rank =19.04), media HY1 (Mean Rank =6.00), and media KC (Mean Rank =7.67). So, there was significant difference between them. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the six pairs of groups. There was no difference in evidence in some pairs among groups. Additionally, there was a difference in evidence among groups in some pairs. Table 2 shows a statistically significant difference (adjusted p-value = 0.037) between the groups in the KC–1/4MS2 media pairs. Figures1 and 2 present pairwise comparisons of the KC–1/4MS2 treatments, I illustrating differences among the group pairs. Therefore, for *Paphiopedilum bellatum*, regarding the maximum protocorm size in in vitro seed culture, the pairwise treatment comparisons of KC–1/4MS2 media demonstrated differences among the groups. Accordingly, Figures 3 and 4 show the differences between the average rank of each treatment and the overall mean rank.

Table 2 : Pairwise comparisons of treatments for *Paphiopedilum bellatum of the* maximum protocorm size during the induction stage of in vitro seed culture.

Treamet (media)-Treatment (media)	Test Statistics	Std Error	Std. Test Statistic	Sig.	Adj.Sig[a]
HY1-KC	-1.667	6.304	-.264	.791	1.000
HY1-1/4MS1	12.375	4.766	2.597	**.009**	.056
HY1-1/4MS2	13.042	4.766	2.737	**.006**	**.037**
KC-1/4MS1	10.708	5.328	2.010	**.044**	.267
KC-1/4MS2	11.375	5.328	2.135	**.033**	.197
1/4MS1-1/4MS2	-.667	3.370	-.198	.843	1.000
Each row tests the null hypothesis that the treatment 1 and treatment 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05. a. Significance values have been adjusted by the Bonferroni correction for multiple tests.					

Table 2 shows that the significance levels for the treatment pairs HY1–KC and 1/4MS1–1/4MS2 are greater than 0.05, indicating no significant differences according to the Kruskal–Wallis test. Among the six media pairs, HY1–1/4MS1, HY1–1/4MS2, KC–1/4MS1, and KC–1/4MS2 have p-values less than 0.05, showing significant differences. Dunn's pairwise analysis was used to adjust the significance levels. Based on the adjusted significance (Adj. Sig.) values in Table 2, only the HY1–1/4MS2 pair has an adjusted significance less than 0.05.Therefore, HY1–1/4MS2 was difference in evidence between some pairs among groups.

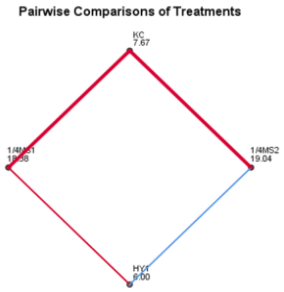


Figure3: Pairwise Comparisons of Treatments for *P. bellatum* of the maximum protocorm size

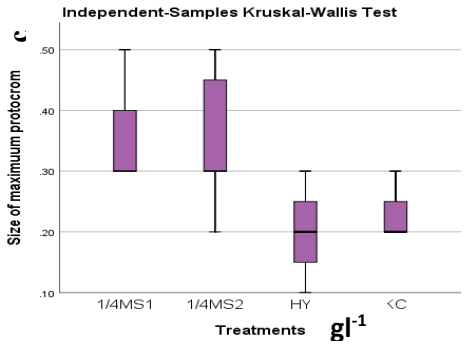


Figure 4: Effect of four treatments for *P. bellatum* of the maximum protocrom size

As shown in Figure 4, among the four treatments or media, only the HY1 medium exhibits a distinct median. It also reveal that the third quartile (Q3) and maximum values of the HY1 and KC media are identical. In addition, the first quartiles of the 1/4MS1 and 1/4MS2 media are the same, and the third quartile (Q3) of the 1/4 MS2 medium is the highest among the four treatments or media. Therefore, among the four media tested, 1/4MS2 media is suitable for the induction stage of maximum protocrom size of *Paphiopedilum bellatum* in vitro seed culture.

5.3. Effect of different media on the minimum protocorm size of *Paphiopedilum bellatum* during the induction stage in in vitro seed culture.

A Kruskal–Wallis test was conducted to examine the effects of treatments on the minimum protocorm size of *Paphiopedilum bellatum*, based on different proportions of stock solution types, hormone types, and basal medium ratios. For size of minimum protocorm of

Paphiopedilum bellatum, a significant result was H (3) =12.229, p<.o5.^{W}H= 0.32/ X^2(3) =12.229, p.007, ^{W}H=0.32, indicating
. Dunn's pairwise tests were carried out for the six pairs of groups. There was no difference in evidence in some pairs among groups. Additionally, there was a difference in evidence among groups in HY1-1/4MS2 pair. Table3.shows a statistically significant difference (adjusted p-value = 0.024) between the groups in the HY1-1/4MS2media pairs. Figures 5 and 6 present pairwise comparisons of the HY1-1/4MS2treatments, illustrating differences among the group pairs. Therefore, for *Paphiopedilum bellatum*, regarding the minimum protocorm size in in vitro seed culture, the pairwise treatment comparisons of HY1-1/4MS2media demonstrated differences among the groups. Accordingly, Figures 5 and 6 show the differences between the average rank of each treatment and the overall mean rank.

Table 3 : Pairwise comparisons of treatments for the minimum protocorm size of Paphiopedilum bellatum during the induction stage of in vitro seed culture.

Treamet (media)-Treatment (media)	**Test Statistics**	**Std,Error**	**Std. Test Statistic**	**Sig.**	**Adj.Sig[a]**
HY1-1/4MS1	8.708	5.053	1.724	.085	.509
KC-1/4MS1	8.708	5.649	1.542	.123	.739
HY1-1/4MS2	14.542	5.053	2.878	**.004**	.024
KC-1/4MS2	14.542	5.649	2.574	**.010**	.060
HY1-KC	.000	6.684	.000	1.000	1.000
1/4MS1-1/4MS2	-5.833	3.573	-1.633	.103	.615
Each row tests the null hypothesis that the treatment 1 and treatment 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05. a. Significance values have been adjusted by the Bonferroni correction for multiple tests.					

Table: 3 shows that the significance levels for the treatment pairs HY1–KC, 1/4MS1–1/4MS2, HY1-1/4MS1 and KC-1/4MS1are greater than 0.05, indicating no significant differences according to the Kruskal–Wallis test. Among the six media pairs, HY1-1/4MS2 and KC-1/4MS2 have p-values less than 0.05, showing significant differences. Dunn's pairwise analysis was used to adjust the significance levels. Based on the adjusted significance (Adj. Sig.) values in Table 4, only the HY1–1/4MS2 pair has an adjusted significance less than 0.05. Therefore, among the four media tested, 1/4MS2 is the most suitable for achieving the minimum protocorm size in *Paphiopedilum bellatum* in vitro seed culture during the induction stage of germination. Therefore, among the four media tested, 1/4MS2 media is suitable for the induction stage of minimum protocrom size of *P. bellatum* in vitro seed culture.

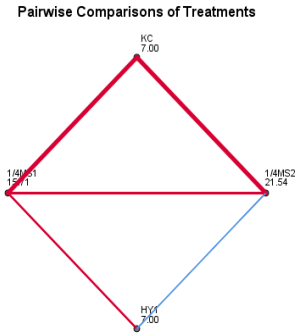


Figure5: Pairwise Comparisons of Treatments for *P. bellatum* of the minimum protocorm size

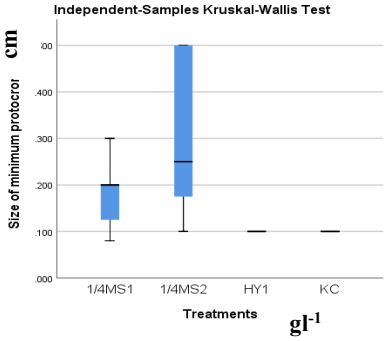


Figure 6: Effect of four treatments for *P. bellatum* of the minimum protocrom size

As shown in Figure 6, among the four treatments or media, only the 1/4MS1 medium exhibits both maximum and minimum values. In addition, among the four treatments or media, only the 1/4MS2 medium shows a median. For the four treatments or media, the 1/4MS2 medium also displays the third quartile (Q3) and the median.

5.4. Effect of different media on the number of protocorms of *Paphiopedilum bellatum* during the induction stage in in vitro seed culture.

A Kruskal–Wallis test was conducted to examine the effects of treatments on the number of protocorms size of *Paphiopedilum bellatum*, based on different proportions of stock solution types, hormone types, and basal medium ratios. For number of protocorm of *Paphiopedilum bellatum*, a significant result was H (3) = 16.997, p<.05.^{W}H= 0.38/ X^2(3) =16.997, p.001, ^{w}H= 0.38, indicating that the treatments or media are different from each other. Basal media 1/4MS1 (Mean Rank =17.04), media 1/4MS2 (Mean Rank =21.83), media HY1 (Mean Rank =5.00), and media KC (Mean Rank =3.17). So, there was significant difference between them. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the six pairs of groups. There was no difference in evidence in some pairs among groups. Additionally, there was a difference in evidence among groups in some pairs. Table 4 shows statistically significant differences (adjusted p-values = 0.009 and 0.008) between the KC–1/4MS2 and HY1–1/4MS2 media pairs in Dunn's pairwise comparisons. As shown in Figures 7 and 8, the four treatments differ from one another. The mean rank analysis of the treatments, as shown in Figures 7 and 8, revealed that among the four media, 1/4MS2 is the most suitable for producing the highest number of protocorms of *Paphiopedilum bellatum* during the induction stage in in vitro seed culture. KC is not the most suitable medium among the treatments for producing the highest number of protocorms of *Paphiopedilum bellatum* during the induction stage of in vitro seed culture.

Table 4: Pairwise comparisons of treatments for number of protocorms of P.bellatum during the induction stage of in vitro seed culture

Treamet (media)-Treatment (media)	Test Statistis	Std,Error	Std. Test Statistic	Sig.	Adj.Sig[a]
KC-HY1	1.833	6.930	.265	.791	1.000
KC-1/4MS1	13.875	5.857	2.369	**.018**	.107
KC-1/4MS2	18.667	5.857	3.187	**.001**	**.009**
HY1-1/4MS1	12.042	5.239	2.299	**.022**	.129
HY1-1/4MS2	16.833	5.239	3.213	**.001**	**.008**
1/4MS1-1/4MS2	-4.792	3.704	-1.294	.196	1.000

Each row tests the null hypothesis that the treatment 1 and treatment 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 4 shows that the significance levels for the treatment pairs KC-HY1 and 1/4MS1-1/4MS2are greater than 0.05, indicating no significant differences according to the Kruskal–Wallis test. Among the six media pairs, KC-1/4MS1, KC-1/4MS2, HY1-1/4MS1and HY1-1/4MS2have p-values less than 0.05, showing significant differences. Dunn's pairwise analysis was used to adjust the significance levels. Based on the adjusted significance (Adj. Sig.) values in Table 4, KC-1/4MS2 and HY1-1/4MS2 pair has an adjusted significance less than 0.05. Therefore, among the four media tested, 1/4MS2 is the most suitable for achieving the number of protocorm size in *Paphiopedilum bellatum* in vitro seed culture during the induction stage of germination. Therefore, among the four media tested, 1/4MS2 media is suitable for the induction stage of number of protocrom size of *P. bellatum* in vitro seed culture.

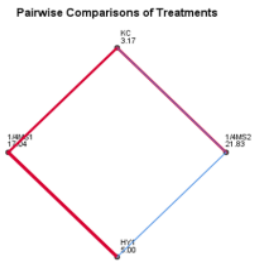


Figure7: Pairwise Comparisons of Treatments for the number of protocorms of *P. bellatum*

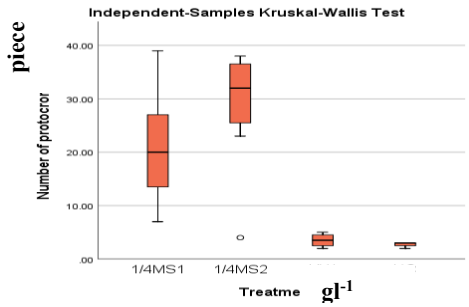


Figure 9: Effect of four treatments for the number of protocorms of *P. bellatum*

Table 5 : This section presents the significance levels (e.g., p-values) for all evaluated parameters, including germination and minimum, maximum and minimum protocroms size, and the number of protocroms of *Paphiopedilum bellatum* during the induction stage, based on the results of statistical analyses such as ANOVA and the Kruskal–Wallis test.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Size of maximuum protocrom is the same across categories of Treatments.	Independent-Samples Kruskal-Wallis Test	.009	Reject the null hypothesis.
2	The distribution of Size of minimum protocroms is the same across categories of Treatments.	Independent-Samples Kruskal-Wallis Test	.007	Reject the null hypothesis.

3	The distribution of Number of protocroms is the same across categories of Treatments.	Independent-Samples Kruskal-Wallis Test	.001	Reject the null hypothesis.
4	The distribution of Germination is the same across categories of Treatments.	Independent-Samples Kruskal-Wallis Test	.006	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .050.

5.5. Effect of different media *on Paphiopedilum wardii* in vitro seed culture for the induction stage of germination

A Kruskal–Wallis test was conducted to examine the effects of treatments on the induction stage of germination of *Paphiopedilum wardii*, based on different proportions of stock solution types, hormone types, and basal medium ratios. For number of protocorm of *Paphiopedilum wardii*, a significant result was H (3) = 29.88, p<.o5.^{W}H= 0.49/ X^2(3) =29.88, p.000, ^{W}H=0.49, indicating that the treatments or media are different from each other. Basal media 1/4MS1 (Mean Rank =71), media 1/4MS2 (Mean Rank =77), media HY1 (Mean Rank =47), and media KC (Mean Rank =47). So, there was significant difference between them. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the six pairs of groups. There was a difference in evidence in pairs of groups. Table 5 shows that Dunn's pairwise tests were conducted for the six pairs of groups. Most group pairs exhibited differences, while a few pairs showed no difference. However, about the number of protocorms and the maximum and minimum sizes of protocorms at the induction stage, further consideration is required.

Table 6 : Pairwise Comparisons of Treatments for the germination of *Paphiopedilum wardii* by in vitro seed culture.

Treatment (media)-Treatment (media)	Test Statistis	Std,Error	Std. Test Statistic	Sig.	Adj.Sig[a]
HY1-1/4MS1	24.000	7.071	3.394	**.001**	**.004**
KC-1/4MS1	24.000	7.071	3.394	**.001**	**.004**
HY1-1/4MS2	30.000	7.071	4.243	**.000**	**.000**
KC-1/4MS2	30.000	7.071	4.243	**.000**	**.000**
HY1-KC	.000	7.071	.000	1.000	1.000
1/4MS1-1/4MS2	-6.000	7.071	-.849	.396	1.000

Each row tests the null hypothesis that the treatment 1 and treatment 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 6, the significance levels of HY1–KC and 1/4MS1–1/4MS2 are greater than ($P < 0.05$); therefore, no significant difference was observed between these pairs. In addition, according to the adjusted significance (Adj. Sig.), the treatment or media group pairs HY1–1/4MS1, KC–1/4MS1, HY1–1/4MS2, and KC–1/4MS2 were found to have values less than ($p < 0.05$). Thus, the significance levels of these treatment or media pairs are below ($P < 0.05$), indicating that these pairs of treatment or media groups show significant differences. Therefore, among the four treatments or media used for the germination of *Paphiopedilum wardii* by in vitro seed culture, 1/4MS2 and 1/4MS1 were found to be more suitable in figure (10). However, medium 1/4 MS2 shows a slight advantage over medium 1/4 MS1 in figure (9). Knudson C medium) were evaluated for their effectiveness in achieving maximum seed germination and early seedling development (Kaur, 2016). The effect of seed maturity of *Paphiopedilum* on in vitro germination showed that on HY1 medium, seeds collected at 180 and 190 days after pollination achieved 80% germination; seeds collected at 190 days after pollination showed 75% germination; while seeds collected at 200, 210, 220, and 190 days

after pollination showed 65% germination (Zeng et al., 2015). Pierik et al. (1988) reported highest seed germination of P. ciliolare on KC and 1/4 MS medium and much lower levelson MS medium; KC resulted in plant death(Zeng et al., 2015).

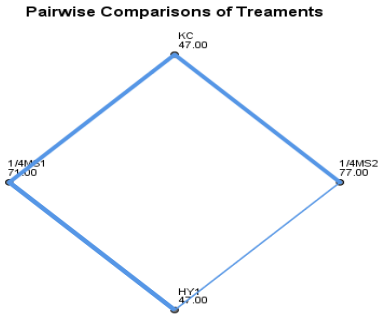


Figure (9) Pairwise Comparisons of Treatments For *p. wardii* of Germination

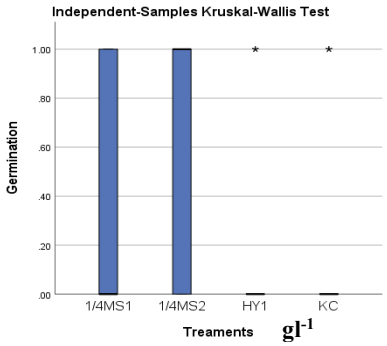


Figure (10) Effect of four teaments for *p.wardii* of Germination by in *vitro* seed culture

5.6. Effect of different media on the maximum protocorm size of *Paphiopedilum wardii* during the induction stage in vitro seed culture

A Kruskal–Wallis test was conducted to examine the effects of treatments on the maximum protocorm size of *Paphiopedilum wardii* during the induction stage, based on different proportions of stock solution types, hormone types, and basal medium ratios. For number of protocorm of Paphiopedilum wardii, a significant result was H (3) = 10.534, p<.015.^{W}H= 0.296/ X^2(3) =10.534, p.015, ^{W}H=0.296, indicating that the treatments or media are different from each other. Basal media 1/4MS1 (Mean Rank =15.82), media 1/4MS2 (Mean Rank =22.5), media HY1 (Mean Rank =6.5), and media KC (Mean Rank =6.5). So, there was a significant difference between the mean ranks of some treatments. Additionally, it was found that there was no significant difference, and the mean ranks of some treatments were similar. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the six pairs of groups. There was not difference in evidence in pairs of groups.

Table 7 : Pairwise comparisons of treatments for *Paphiopedilum wardii* of the maximum protocorm size during the induction stage of in vitro seed culture.

Treamet (media)-Treatment (media)	Test Statistis	Std,Error	Std. Test Statistic	Sig.	Adj.Sig[a]
HY1-1/4MS1	9.321	7.140	1.306	.192	1.000
KC-1/4MS1	9.321	7.140	1.306	.192	1.000
HY1-1/4MS2	16.000	7.060	2.266	**.023**	.141
KC-1/4MS2	16.000	7.060	2.266	**.023**	.141
HY1-KC	.000	9.445	.000	1.000	1.000
1/4MS1-1/4MS2	-6.679	3.409	-1.959	**.050**	.300
Each row tests the null hypothesis that the treatment 1 and treatment 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05. a. Significance values have been adjusted by the Bonferroni correction for multiple tests.					

As shown in Table 7, the significance levels of HY1–1/4MS1, HY1–KC, and KC–1/4MS1 are greater than ($P < 0.05$); therefore, no significant differences were observed among

the groups for these treatments or media pairs. The treatment or media pairs HY1–1/4MS2 and KC–1/4MS2 have significance levels equal to (P < 0.05); therefore, these treatment or media pairs showed significant differences among the groups. No adjusted significance (Adj. Sig.) was found for any of the six pairs of groups. Therefore, there was no difference in evidence among the pairs of groups.

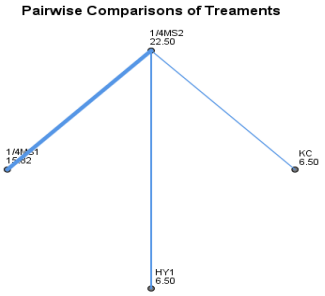


Figure 11: Pairwise Comparisons of Treatments for *P.wardii* of the maximum protocorm size

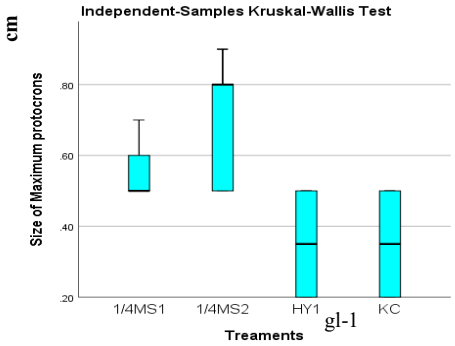


Figure 12: Effect of four treatments for *P. wardii* of the maximum protocrom size

In Figure 11, for each treatment or media, the mean rank of each medium shown in Figure 11, 1/4MS2 was found to be the most suitable among the four treatments or media for achieving the maximum protocorm size of *Paphiopedilum wardii*. Treatments or media of mean rank 1/4MS2 are found to be more suitable among the four treatments or media for the maximum protocorm size of *Paphiopedilum wardii*. During further testing of suitable media for germination, we found that viable protocorms of *P. spicerianum* were formed on ¼ MS2 medium (Chen,Goodale,Fan,Gao, 2015). This result is similar to the findings obtained when comparing KC and ¼ MS media (Chen, Goodale, Fan, & Gao, 2015). Although the number of seeds that germinated was higher on the KC medium, all of the seeds died without further development (Chen, Goodale, Fan, Gao, 2015).

In Figure 12, present the boxplots of the four reatments or media including the third quartile (Q3), first quartile (Q1), median, error bars, and maximum values1/4MS2 was found to be more suitable among the four treatments or media for the effect of different media on the minimum protocorm size of *Paphiopedilum wardii* during the induction stage in in vitro seed culture.

5.7. Effect of different media on the minimum protocorm size of *Paphiopedilum wardii* during the induction stage of in vitro seed culture

A Kruskal–Wallis test was conducted to examine the effects of treatments on the minimum protocorm size of *Paphiopedilum wardii* during the induction stage, based on different proportions of stock solution types, hormone types, and basal medium ratios. For size of minimum the size of the minimum protocorm of Paphiopedilum bellatum, a significant result was H (3) =17.784, p<.o5.^{W}H= 0.38/ ^{X}2(3)=17.784, p.000, ^{W}H=0.38, indicating that the treatments or media are different from each other. Basal media 1/4MS1 (Mean Rank =14.75), media 1/4MS2 (Mean Rank =24.21), media HY1 (Mean Rank =4.25), and media KC (Mean Rank =1.75). So, there was significant difference between them. A Kruskal-Wallis test provided very strong evidence of a difference (p < 0.001) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the six pairs of groups. There was a difference in evidence in pairs of groups.

Table 8 : Pairwise comparisons of treatments for the minimum protocorm size of *Paphiopedilum wardii* during the induction stage of in vitro seed culture.

Treamet (media)-Treatment (media)	Test Statistis	Std,Error	Std. Test Statistic	Sig.	Adj.Sig[a]
KC-HY1	2.500	9.802	.255	.799	1.000
KC-1/4MS1	13.000	7.410	1.754	.079	.476
KC-1/4MS2	22.456	7.328	3.064	**.002**	**.013**
HY1-1/4MS1	10.500	7.410	1.417	.156	.939
HY1-1/4MS2	19.956	7.328	2.723	**.006**	**.039**
1/4MS1-1/4MS2	-9.456	3.538	-2.673	**.008**	**.045**

Each row tests the null hypothesis that the treatment1 and treatment2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 8, the significance levels of KC–HY1, KC–1/4MS1, and HY1–1/4MS1are greater than ($P < 0.05$); therefore, no significant differences were observed among the groups for these treatments or media pairs. It was also found that these treatments or media pairs were not significant at the adjusted significance (Adj. Sig.) level across all six group comparisons.

In contrast, the significance levels of KC–1/4MS2, HY1–1/4MS2, and 1/4MS1–1/4MS2 are less than ($P < 0.05$), and all six group comparisons were significant at the adjusted significance (Adj. Sig.) level. Therefore, these three treatment or media pairs showed significant differences among the six group comparisons.

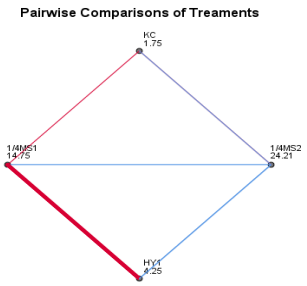


Figure 13: Each node shows the average rank of treatments for the minimum protocorm size of *P. wardii* during the induction stage.

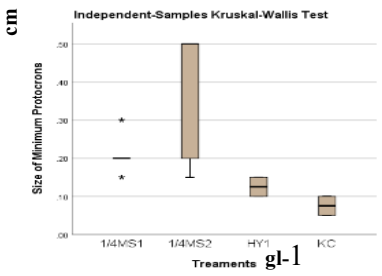


Figure 14: Effect of four treatments for *P. wardii* of the minimum protocrom size

As shown in Figure 13, by examining the mean ranks of each treatment or medium, 1/4MS2 was found to be the most suitable among the four treatments or media for the minimum protocorm size of *Paphiopedilum wardii*. Figure 14 presents the boxplots for the four treatments or media, showing the third quartile (Q3), first quartile (Q1), median, error bars, outliers, and minimum values. Based on examination of these results, the 1/4MS2 treatment or medium is the most appropriate among the four media for the effect of different media on the minimum protocorm size of *Paphiopedilum wardii* during the induction stage in in vitro seed culture.

5.8. Effect of different media on the number of protocorms of *Paphiopedilum wardii* during the induction stage of in vitro seed culture.

A Kruskal–Wallis test was conducted to examine the effects of treatments on the number of protocorm size of *Paphiopedilum wardii* during the induction stage, based on different proportions of stock solution types, hormone types, and basal medium ratios. For

number of protocorm of *Paphiopedilum waidii*, a significant result was H (3) = 11.492, p<.05.^{W}H= 0.31/ X^2(3) =11.492, p.000, ^{W}H =0.31, indicating that the treatments or media are different from each other. Basal media 1/4MS1 (Mean Rank =16.71), media 1/4MS2 (Mean Rank =22, 44), media HY1 (Mean Rank =4.25), and media KC (Mean Rank =3.00). So, there was significant difference between them. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the six pairs of groups. There was a difference in evidence in pairs of groups.

Table9: Pairwise comparisons of treatments for number of protocorms of Paphiopedilum during the induction stage of in vitro seed culture.

Treamet (media)-Treatment (media)	Test Statistis	Std,Error	Std. Test Statistic	Sig.	Adj.Sig[a]
KC-HY1	1.000	10.199	.098	.922	1.000
KC-1/4MS1	13.929	7.710	1.807	.071	.425
KC-1/4MS2	21.353	7.624	2.801	**.005**	**.031**
HY1-1/4MS1	12.929	7.710	1.677	.094	.561
HY1-1/4MS2	20.353	7.624	2.670	**.008**	**.046**
1/4MS1-1/4MS2	-7.424	3.681	-2.017	**.044**	.262

Each row tests the null hypothesis that the treatment 1 and treatment 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 9, the significance levels of the pairs KC–HY1, KC–1/4MS1, and HY1–1/4MS1 are greater than the significance level ($P < 0.05$); therefore, no significant differences were observed between these pairs of treatments or media among the groups. In addition to these pairs, the 1/4MS1–1/4MS2 treatment or medium was also not significant at Adj. Sig. Across all six pairs of groups. In contrast, the significance levels of KC–1/4MS2, HY1–1/4MS2, and 1/4MS1–1/4MS2 are less than ($P < 0.05$). Therefore, there was evidence of differences between these pairs of groups. The KC–1/4MS2 and HY1–1/4MS2 treatments or media were significant at the adjusted significance level (Adj. Sig[a]). among the six treatment or media pairs. Hence, these two pairs of treatments or media showed significant differences among the six pairs of groups.

Table 9 : This section presents the significance levels (e.g., p-values) for all evaluated parameters, including germination and minimum, maximum and minimum protocroms size, and the number of protocroms of Paphiopedilum bellatum during the induction stage, based on the results of statistical analyses such as ANOVA and the Kruskal–Wallis test.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Size of Maximum protocrons is the same across categories of Treaments.	Independent-Samples Kruskal-Wallis Test	.015	Reject the null hypothesis.
2	The distribution of Size of Minimum Protocrons is the same across categories of Treaments.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
3	The distribution of Number of protocroms is the same across categories of Treaments.	Independent-Samples Kruskal-Wallis Test	.009	Reject the null hypothesis.
4	The distribution of Germination is the same across categories of Treaments.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .050.

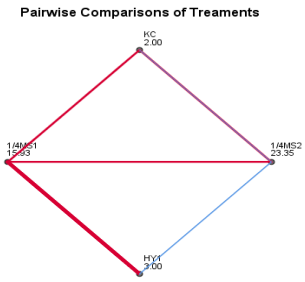


Figure 15: Each node shows the average rank of treatments for the number protocorm of *P. wardii* during the induction stage

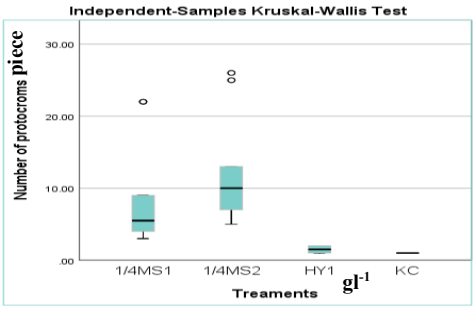


Figure 16: Effect of four treatments for the number protocorm of *P. wardii* during the induction stage

As shown in Figure 13, by examining the mean ranks of each treatment or medium, 1/4MS2 was found to be the most suitable among the four treatments or media for the number of protocorms of *P. wardii* during the induction stage. Figure 14 presents the boxplots for the four treatments or media, showing the third quartile (Q3), first quartile (Q1), median, error bars, outliers, and minimum values. Based on examination of these results, the 1/4MS2 treatment or medium was the most appropriate among the four media for the effect of different media on the number of protocorms of *Paphiopedilum wardii* during the induction stage in in vitro seed culture. Effect of basal media 1/4MS and 1/2M S were 78% Seed germination frequency (%) or description on propagation in vitro of *Paphiopedilum* (Songjun; et al., 2015). Pierik et al. (1988) reported highest seed germination of P. ciliolare on KC and 1/4 MS medium and much lower levels on MS medium; KC resulted in plant death. Although the KC and HY media contain coconut water and NAA, the low germination rate may be due to the small proportion of well-developed (viable) seeds. In addition, the fact that the number of protocorms and the maximum and minimum sizes of protocorms were lower than those observed on 1/4 MS2 suggests that the KC (Knudson, 1964) medium and Hyponex N06.5–6–19 medium may contain lower levels of macronutrients and micronutrients than the Murashige and Skoog (MS) medium.

5.9. Effects of different media on the maximum plantlet height of *Paphiopedilum bellatum* during the multiplication stage of in vitro seed culture.

A Kruskal–Wallis test was conducted to examine the effects of treatments on the maximum plantlet height of *Paphiopedilum bellatum* during the multiplication stage, based on different proportions of stock solution types, hormone types, and basal medium ratios. For the maximum plantlet height of *Paphiopedilum bellatum* during the multiplication stage, a significant result was H (5) = 12.371, p<.05.^{W}H= 0.26/ X^2(3) =12.371, p.03, ^{W}H=0.26, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank =5.5), media or treatment T2 (Mean Rank =19.8), media or treatment T3 (Mean Rank =9.71), media or treatment T4 (Mean Rank =9.5), media or treatment T5 (Mean Rank= 9.25) and media or treatment T6 (Mean Rank=82.93). So, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There was no difference in evidence in all pairs of groups.

Table 11: Pairwise comparisons of treatments for the maximum plantlet height of *Paphiopedilum bellatum* during the multiplication stage

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T1-T5	-3.750	7.572	-.495	.620	1.000
T1-T4	-4.000	6.913	-.579	.563	1.000
T1-T3	-4.214	6.071	-.694	.488	1.000
T1-T6	-8.000	7.572	-1.056	.291	1.000
T1-T2	-14.300	5.865	-2.438	**.015**	.222
T5-T4	.250	6.913	.036	.971	1.000
T5-T3	.464	6.071	.076	.939	1.000
T5--T6	-4.250	7.572	-.561	.575	1.000
T5-T2	10.550	5.865	1.799	.072	1.000
T4-T3	.214	5.225	.041	.967	1.000
T4-T6	-4.000	6.913	-.579	.563	1.000
T4-T2	10.300	4.985	2.066	**.039**	.582
T3-T6	-3.786	6.071	-.624	.533	1.000
T3-T2	10.086	3.732	2.703	**.007**	.103
T6-T2	6.300	5.865	1.074	.283	1.000

Each row tests the null hypothesis that the media 1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 10, among the 15 pairs of treatments or media, only three pairs, T1 to T2, T4 to T2, and T3 to T2, are found to have significance levels less than 0.05. However, no pairs of treatments or media were significant at the adjusted significance level (Adj. Sig.) among the 15 pairs of groups. Therefore, there was no evidence of differences between the pairs of groups.

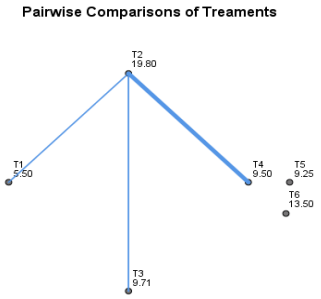


Figure 17: Each node shows the average rank of treatments for the plantlet of maximum height *of P. bellatum* during the multiplication

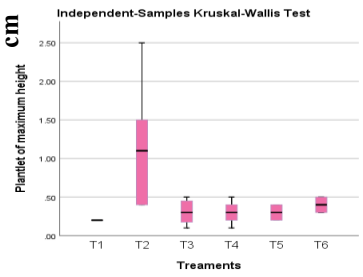


Figure 18: Effect of four treatments for plantlet of maximum height of *P. bellatum* during the multiplication stage

As shown in Figure 17, by examining the mean ranks of each treatment or medium, the T2 medium was found to be the most suitable among the six media. For the maximum plantlet height of *Paphiopedilum bellatum* during the multiplication stage in vitro seed culture, the T6 and T1 treatments or media are identified as the second most suitable and the least suitable, respectively, among the six treatments or media.

Figure 18 presents the boxplots for the six treatments or media, showing the third quartile (Q3), first quartile (Q1), median, error bars, maximum, and minimum values. Based on examination of these results, the T2 treatment or medium was the most appropriate among the six media for the maximum plantlet height of *Paphiopedilum bellatum* during the

multiplication stage in in vitro seed culture. The T6 and T1 treatments or media were the second most suitable and the least suitable, respectively, among the six treatments or media. T6 medium is the regular medium for the multiplication stage in the tissue culture lab of Forest Research Institute FRI lab (tissue lab). T4 medium is the Leaf length growth rate, and the highest leaves remained green in color (Chen,Goodale,Fan,Gao, 2015).

5.10. Effects of different media on the minimum plantlet height of *Paphiopedilum bellatum* during the multiplication stage of in vitro seed culture

A Kruskal–Wallis test was conducted to examine the effects of treatments on the minimum plantlet height of *Paphiopedilum bellatum* during the multiplication stage, based on different proportions of stock solution types, hormone types, and basal medium ratios. For the minimum plantlet height of *Paphiopedilum bellatum* during the multiplication stage, a significant result was H (5) = 12.371, p<.05.^{W}H= 0.30/ X^2(3) =16.557, p.005, ^{W}H=0.30, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank =4.75), media or treatment T2 (Mean Rank =20.55), media or treatment T3 (Mean Rank =10.55), media or treatment T4 (Mean Rank =6.5), media or treatment T5 (Mean Rank= 13) and media or treatment T6 (Mean Rank=8.5) so, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There was not difference in evidence in all pairs of groups.

Table 11: Pairwise comparisons of treatments for the minimum plantlet height of *Paphiopedilum bellatum* during the multiplication stage

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T1-T5	-3.750	7.572	-.495	.620	1.000
T1-T4	-4.000	6.913	-.579	.563	1.000
T1-T3	-4.214	6.071	-.694	.488	1.000
T1-T6	-8.000	7.572	-1.056	.291	1.000
T1-T2	-14.300	5.865	-2.438	**.015**	.222
T5-T4	.250	6.913	.036	.971	1.000
T5-T3	.464	6.071	.076	.939	1.000
T5-T6	-4.250	7.572	-.561	.575	1.000
T5-T2	10.550	5.865	1.799	.072	1.000
T4-T3	.214	5.225	.041	.967	1.000
T4-T6	-4.000	6.913	-.579	.563	1.000
T4-T2	10.300	4.985	2.066	**.039**	.582
T3-T6	-3.786	6.071	-.624	.533	1.000
T3-T2	10.086	3.732	2.703	**.007**	.103
T6-T2	6.300	5.865	1.074	.283	1.000

Each row tests the null hypothesis that the media 1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 11, among the 15 pairs of treatments or media, only four pairs, T1–T2, T4–T2, T6–T2, and T3–T2—were found to have significance levels less than $p< 0.05$. However, no treatment or media pairs were significant at the adjusted significance level (Adj. Sig.) among the 15 pairs of groups. Therefore, there was no evidence of differences between the pairs of groups.

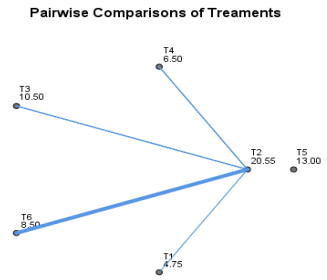


Figure 19: Each node shows the average rank of treatments for the plantlet of minimum height of *P. bellatum* during multiplication stage

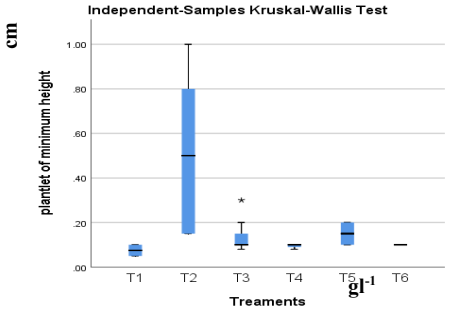


Figure 20: Effect of four treatments for plantlet of minimum height of *P. bellatum* during the multiplication stage

By examining the mean ranks of each treatment or medium shown in Figure 19, it was found that the T2 medium is the most suitable among the six media. For the minimum plantlet height of *Paphiopedilum bellatum* during the multiplication stage of *in vitro* seed culture, the T5 and T1 treatments or media were identified as the second most suitable and the least suitable, respectively, among the six treatments or media. As shown in Figure 20, the box plots for the six treatments or media present the third quartile (Q3), first quartile (Q1), median, error bars, interquartile range (IQR), outliers, and maximum and minimum values. Based on examination of these parameters, the T2 treatment or medium is the most suitable among the six treatments or media for the minimum plantlet height of *Paphiopedilum bellatum* during the multiplication stage of *in vitro* seed culture. The T6 and T1 treatments or media were determined to be the second most suitable and the least suitable, respectively, among the six treatments or media. T4 medium is Leaf length growth rate, and the highest leaves remained green in color (Chen,Goodale,Fan,Gao, 2015).

5.11. Effects of different media on the number of plantlets of *Paphiopedilum bellatum* during the multiplication stage of in vitro seed culture.

Kruskal–Wallis test was conducted to examine the effects of treatments on the number of plantlets height of *Paphiopedilum bellatum* during the multiplication stage, based on different proportions of stock solution types, hormone types, and basal media ratios. For the number plantlet of *Paphiopedilum bellatum* during the multiplication stage, a significant result was H (5) = 12.440, p<.05.^{W}H= 0.26/ ^{X}2(5) =12.440, p.029, ^{W}H=0.26, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank =4.50), media or treatment T2 (Mean Rank =19.55), media or treatment T3 (Mean Rank =11.79), media or treatment T4 (Mean Rank =8.67), media or treatment T5 (Mean Rank= 8.25) and media or treatment T6 (Mean Rank=10.75) so, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There was no difference in evidence in all pairs of groups.

Table 12: Pairwise comparisons of treatments for the number of plantlets of *P. bellatum* during the multiplication stage

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T1-T5	-3.750	7.442	-.504	.614	1.000
T1-T4	-4.167	6.793	-.613	.540	1.000
T1-T6	-6.250	7.442	-.840	.401	1.000
T1-T3	-7.286	5.967	-1.221	.222	1.000
T1-T2	-15.050	5.764	-2.611	**.009**	.135

T5-T4	.417	6.793	.061	.951	1.000
T5-T6	-2.500	7.442	-.336	.737	1.000
T5-T3	3.536	5.967	.593	.553	1.000
T5-T2	11.300	5.764	1.960	**.050**	.749
T4-T6	-2.083	6.793	-.307	.759	1.000
T4-T3	3.119	5.135	.607	.544	1.000
T4-T2	10.883	4.899	2.222	***.026***	.395
T6-T3	1.036	5.967	.174	.862	1.000
T6-T2	8.800	5.764	1.527	.127	1.000
T3-T2	7.764	3.667	2.117	**.034**	.514

Each row tests the null hypothesis that the media1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05
a Significance values have been adjusted by the Bonferroni correction for multiple tests.

Among the 15 treatment or media pairwise comparisons (T1–T2, T4–T2, T6–T2, and T3–T2), only four pairs were found to have significance levels less than P< 0.05. However, among the 15 treatment or media pairs, no significance was observed at the adjusted significance level (Adj. Sig.). Therefore, there was no statistically significant difference in evidence between the pairs of groups for the number of plantlets of *P. bellatum* during the multiplication stage.

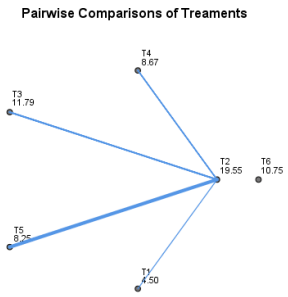


Figure 21: Each node shows the average rank of treatments for the number plantlets of *P. bellatum* during multiplication stage

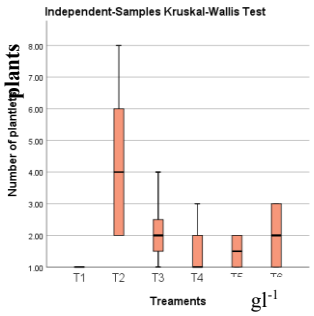


Figure 22: Effect of four treatments for the number of plantlets of *P. bellatum* during the multiplication stage

As shown in Figure 21, by examining the mean ranks of each treatment or medium, it was found that the T2 medium is the most suitable among the six media. For the number of plantlets of *Paphiopedilum bellatum* during the multiplication stage, the T3 and T1 treatments or media were identified as the second most suitable and the least suitable, respectively, among the six treatments or media.Among the six treatments or media, the box plots present the third quartile (Q3), first quartile (Q1), median, error bars, interquartile range (IQR), outliers, and maximum and minimum values. By examining these parameters, the T2 treatment or medium was found to be the most suitable among the six treatments or media for the number of plantlets of *Paphiopedilum bellatum* during the multiplication stage. The T3 and T6 treatments or media were identified as the second most suitable, while the T1 treatment or medium was the least suitable among the six treatments or media. T4 medium is Leaf length growth rate, and the highest leaves remained green in color (Chen,Goodale,Fan,Gao, 2015).

5.12 Effects of different media on the maximum root size of *Paphiopedilum bellatum* during the multiplication stage in in vitro seed culture

Kruskal–Wallis test was conducted to examine the effects of treatments on the maximum root size of *P. bellatum* during the multiplication stage, based on different

proportions of stock solution types, hormone types, and basal media ratios. For the maximum root size of *P. bellatum* during the multiplication stage, a significant result was H (5) = 12.620, p<.05.^{W}H= 0.26/ X^2(5) =12.620, p.027, ^{W}H=0.26, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank =4.75), media or treatment T2 (Mean Rank =19.50), media or treatment T3 (Mean Rank =10.86), media or treatment T4 (Mean Rank =14), media or treatment T5 (Mean Rank= 7.5) and media or treatment T6 (Mean Rank=6.75) so, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There not was difference in evidence in pairs of groups.

Table 14: Pairwise comparisons of treatments for the maximum root size of *P. bellatum* during the multiplication stage

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T1-T6	-2.000	7.584	-.264	.792	1.000
T1-T5	-2.750	7.584	-.363	.717	1.000
T1-T3	-6.107	6.081	-1.004	.315	1.000
T1-T4	-9.250	6.923	-1.336	.182	1.000
T1-T2	-14.750	5.875	-2.511	**.012**	.181
T6-T5	.750	7.584	.099	.921	1.000
T6-T3	4.107	6.081	.675	.499	1.000
T6-T4	7.250	6.923	1.047	.295	1.000
T6-T2	12.750	5.875	2.170	**.030**	.450
T5-T3	3.357	6.081	.552	.581	1.000
T5-T4	6.500	6.923	.939	.348	1.000
T5-T2	12.000	5.875	2.043	**.041**	.616
T3-T4	-3.143	5.234	-.601	.548	1.000
T3-T2	8.643	3.738	2.312	**.021**	.311
T4-T2	5.500	4.993	1.102	.271	1.000

Each row tests the null hypothesis that the media 1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05
a Significance values have been adjusted by the Bonferroni correction for multiple tests.

Among the 15 treatment or media pairwise comparisons (T1–T4, T6–T4, T5–T2, and T3–T2), only four pairs were found to have significance levels less than P< 0.05. However, among the 15 treatment or media pairs, no significance was observed at the adjusted significance level (Adj. Sig.). Therefore, there was no statistically significant difference in evidence between the pairs of groups for the maximum root size of *P. bellatum* during the multiplication stage.

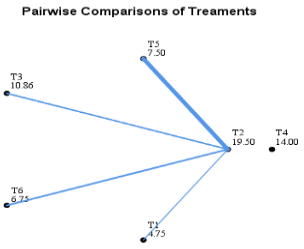


Figure 23: Each node shows the average rank of treatments for the maximum root size of *P. bellatum* during multiplication stage

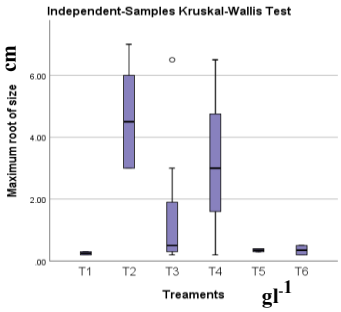


Figure 24: Effect of six treatments for the maximum root of *P. bellatum* during the multiplication stage

As shown in Figure 23, by examining the mean ranks of each treatment or medium, it is found that the T2 medium is the most suitable among the six media. For the maximum root size of *Paphiopedilum bellatum* during the multiplication stage, the T4 and T1 treatments or media were identified as the second most suitable and the least suitable, respectively, among the six treatments or media.As shown in Figure 24, the box plots for the six treatments or media present the third quartile (Q3), first quartile (Q1), median, error bars, interquartile range (IQR), outliers, and maximum and minimum values. Based on examination of these parameters, the T2 treatment or medium is the most suitable among the six treatments or media for the maximum root size of *Paphiopedilum bellatum* during the multiplication stage. The T4 and T1 treatments or media were identified as the second most suitable and the least suitable, respectively, among the six treatments or media. T4 medium is Leaf length growth rate, and the highest leaves remained green in color (Chen,Goodale,Fan,Gao, 2015).

5.13. Effects of different media on the minimum root size of *P. bellatum* during the multiplication stage of in vitro seed culture

Kruskal–Wallis test was conducted to examine the effects of treatments on the minimum root size of *P. bellatum* during the multiplication stag, based on different proportions of stock solution types, hormone types, and basal media ratios. For the minimum root size of *P. bellatum* during the multiplication stage, a significant result was H (5) = 12.194, p<.05.^{W}H= 0.26/ X^2(5) =12.194, p.032, ^{W}H=0.26, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank =4.5), media or treatment T2 (Mean Rank =18.50), media or treatment T3 (Mean Rank =10.14), media or treatment T4 (Mean Rank =18.17), media or treatment T5 (Mean Rank= 7.25) and media or treatment T6 (Mean Rank=8.50). So, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There was no difference in evidence in all pairs of groups.

Table14: Pairwise comparisons of treatments for the minimum root size of *P. bellatum* during the multiplication stage.

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T1-T5	-2.750	7.491	-.367	.714	1.000
T1-T6	-4.000	7.491	-.534	.593	1.000
T1-T3	-5.643	6.006	-.939	.347	1.000
T1-T4	-13.667	6.839	-1.998	**.046**	.685
T1-T2	-14.000	5.803	-2.413	**.016**	.238
T5-T6	-1.250	7.491	-.167	.867	1.000
T5-T3	2.893	6.006	.482	.630	1.000
T5-T4	10.917	6.839	1.596	.110	1.000
T5-T2	11.250	5.803	1.939	.053	.788
T6-T3	1.643	6.006	.274	.784	1.000
T6-T4	9.667	6.839	1.414	.157	1.000
T6-T2	10.000	5.803	1.723	.085	1.000
T3-T4	-8.024	5.170	-1.552	.121	1.000
T3-T2	8.357	3.692	2.264	**.024**	.354
T4-T2	.333	4.931	.068	.946	1.000

Each row tests the null hypothesis that the media1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05

a Significance values have been adjusted by the Bonferroni correction for multiple tests.

Among the fifty treatments or media using pairwise comparisons (T1–T4, T1–T2, and T3–T2), only three pairs were found to have significance levels less than P< 0.05. However, among the 15 treatment or media pairs, no significance was observed at the adjusted significance level (Adj. Sig.). Therefore, there was no statistically significant difference in evidence between the pairs of groups for the minimum root size of *P. bellatum* during the multiplication stage.

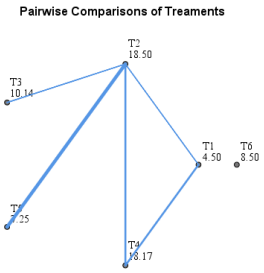


Figure 25: Each node treatment shows the average rank of treatments for the minimum root size of *P. bellatum* during the multiplication stage

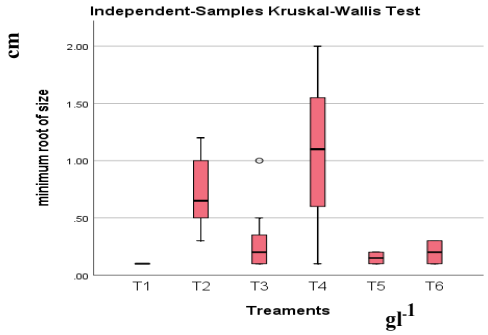


Figure 26: Effect of six treatments for the minimum of *P. bellatum* during the multiplication stage

As shown in Figure 25, by examining the mean ranks of each treatment or medium, the T2 medium was found to be the most suitable among the six media for the maximum root size of *Paphiopedilum bellatum* during the multiplication stage. For the maximum root size of *Paphiopedilum bellatum* during the multiplication stage, the T4 and T1 treatments or media were identified as the second most suitable and the least suitable, respectively, among the six treatments or media.

As shown in Figure 26, the box plots for the six treatments or media present the third quartile (Q3), first quartile (Q1), median, error bars, interquartile range (IQR), outliers, and maximum and minimum values. The analysis explores two aspects of root size characteristics by using distinct statistical measures to establish suitability. When considering only the third quartile (Q3) and the interquartile range (IQR), the emphasis was on identifying treatments with more consistent and less variable data, highlighting the T4 treatment or medium as the most suitable, as it indicates a more clustered root size distribution. However, when the error bars and the median are also taken into account, it was determined that the T2 treatment or medium is the most suitable among the six treatments or media for the minimum root size of *P.bellatum* during the multiplication stage. This dual approach ensures a comprehensive assessment, addressing both data variability and central tendency.

Table 15: This section presents the significance levels (e.g., p-values) for all evaluated parameters, including maximum and minimum plantlet height, maximum and minimum root size, and the number of plantlets of *Paphiopedilum bellatum* during the multiplication stage, based on the results of statistical analyses such as ANOVA and the Kruskal–Wallis test.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Plantlet of maximum height is the same across categories of Treaments.	Independent-Samples Kruskal-Wallis Test	.030	Reject the null hypothesis.
2	The distribution of plantlet of minimum height is the same across categories of Treaments.	Independent-Samples Kruskal-Wallis Test	.005	Reject the null hypothesis.

3	The distribution of Number of plantlets is the same across categories of Treaments.	Independent-Samples Kruskal-Wallis Test	.029	Reject the null hypothesis.
4	The distribution of Maximum root of size is the same across categories of Treaments.	Independent-Samples Kruskal-Wallis Test	.027	Reject the null hypothesis.
5	The distribution of minimum root of size is the same across categories of Treaments.	Independent-Samples Kruskal-Wallis Test	.032	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .050.

5.14. Effects of different media on the maximum plantlet height of *P. wardii* during the multiplication stage of in vitro seed culture.

Kruskal–Wallis test was conducted to examine the effects of treatments on the maximum plantlet height of *P. wardii* during the multiplication stage, based on different proportions of stock solution types, hormone types, and basal media ratios. For the maximum plantlet height of Paphiopedilum wardii during the multiplication stage, a significant result was H (5) = 73.288, p<.05.^{W}H= 0.64/ X^2(5) =73.288, p.000, ^{W}H=0.64, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank =94.78), media or treatment T2 (Mean Rank =132.62), media or treatment T3 (Mean Rank =65.86), media or treatment T4 (Mean Rank =86.40), media or treatment T5 (Mean Rank= 33.32) and media or treatment T6 (Mean Rank=62.72) so, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There was no difference in evidence between some pairs among groups. There are differences in evidence among groups in some pairs.

Table16: Pairwise comparisons of treatments for the maximum plantlet height of *P. wardii* during the multiplication stage.

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T5-T6	-29.124	12.674	-2.298	.022	.323
T5-T3	32.280	13.556	2.381	**.017**	.259
T5-T4	52.817	12.578	4.199	**.000**	**.000**
T5-T1	61.194	12.885	4.749	**.000**	**.000**
T5-T2	99.033	12.578	7.874	**.000**	**.000**
T6-T3	3.157	12.985	.243	.808	1.000
T6-T4	23.693	11.960	1.981	**.048**	.714
T6-T1	32.071	12.283	2.611	**.009**	.135
T6-T2	69.910	11.960	5.845	**.000**	**.000**
T3-T4	-20.536	12.892	-1.593	.111	1.000
T3-T1	28.914	13.191	2.192	**.028**	.426
T3-T2	66.753	12.892	5.178	**.000**	**.000**
T4-T1	8.378	12.184	.688	.492	1.000
T4-T2	46.217	11.859	3.897	**.000**	**.001**
T1-T2	-37.839	12.184	-3.106	**.002**	**.028**

Each row tests the null hypothesis that the media1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05 a Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 16, among the 15 treatment or media pairwise comparisons, seven pairs T5–T4, T4–T2, T5–T1, T6–T2, T3–T2, T4–T2, and T1–T2 are found to have significance levels and adjusted significance (Adj. Sig[a].) with p-values less than $P < 0.05$. Because the adjusted values were also below the threshold, these seven pairs showed a difference in evidence between the pairs of groups. Among the remaining eight pairs, four pairs were found to have significance levels with p-values less than P<0.05; however, the remaining eight pairs were determined to show no difference in evidence between the pairs of groups.

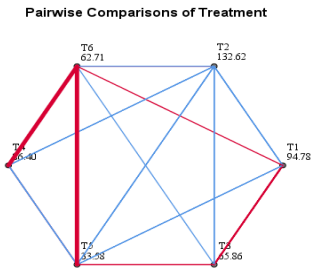


Figure 27: Each node treatment shows the average rank of treatments for the maximum root size of *P. wardii* during the multiplication stage

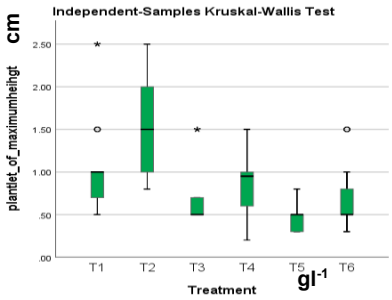


Figure 28: Effect of six treatments for the maximum plantlet height of *P. wardii* during the multiplication stage

As shown in Figure 27, by examining the mean ranks of each treatment or medium, T2 medium is found to be the most suitable among the six media for achieving the maximum plantlet height of *Paphiopedilum wardii* during the multiplication stage. For the same parameter, T4 and T1 treatments or media were identified as the second and third most suitable, respectively, among the six treatments or media. In contrast, T5 treatment or medium was observed to be the least suitable among the six media for the maximum root size of *Paphiopedilum bellatum* during the multiplication stage.

As illustrated in Figure 28, the boxplots for the six treatments or media present the third quartile (Q3), first quartile (Q1), median, error bars, interquartile range (IQR), mild (potential) outliers, extreme (far-out) outliers, and maximum and minimum values. The T2 treatment or medium is the highest among the six treatments or media for the maximum plantlet height of *P. wardii* during the multiplication stage. At first glance, T4 treatment or medium appears to be the second best among the six treatments or media. However, upon closer examination of the third quartile (Q3) and the median, which overlap in T1 treatment or medium, it can be confirmed that T1 treatment or medium is actually the second most suitable among the six treatments or media for achieving the maximum plantlet height of *P. wardii* during the multiplication stage.

5.15. Effects of different media on the minimum plantlet height of *P. wardii* during the multiplication stage of in vitro seed culture.

Kruskal–Wallis test was conducted to examine the effects of treatments on the minimum plantlet height of *Paphiopedilum wardii* during the multiplication stage, based on different proportions of stock solution types, hormone types, and basal media ratios. For the number plantlet height of *Paphiopedilum wardii* during the multiplication stage, a significant result was H (5) = 54.026, p<.05.^{W}H= 0.55/ X^2(5)=54.026, p.000, ^{W}H=0.55, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank =88), media or treatment T2 (Mean Rank =128), media or treatment T3 (Mean Rank =78), media or treatment T4 (Mean Rank =78), media or treatment T5 (Mean Rank= 65) and media or treatment T6 (Mean Rank=34) so, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There was no difference in evidence between some pairs among groups. There are differences in evidence among groups in some pairs.

Table17: Pairwise comparisons of treatments for the minimum plantlet height of *P. wardii* during the multiplication stage.

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T6-T5	18.798	12.369	1.520	.129	1.000
T6-T3	31.887	12.673	2.516	**.012**	.178
T6-T4	31.969	11.672	2.739	**.006**	.092
T6-T1	41.569	11.987	3.468	**.001**	**.008**
T6-T2	81.569	11.672	6.988	**.000**	**.000**
T5-T3	13.089	13.230	.989	.322	1.000
T5-T4	13.171	12.275	1.073	.283	1.000
T5-T1	22.771	12.574	1.811	.070	1.000
T5-T2	62.771	12.275	5.114	**.000**	**.000**
T3-T4	-.082	12.581	-.007	.995	1.000
T3-T1	9.682	12.873	.752	.452	1.000
T3-T2	49.682	12.581	3.949	**.000**	**.001**
T4-T1	9.600	11.890	.807	.419	1.000
T4-T2	49.600	11.573	4.286	**.000**	**.000**
T1-T2	-40.000	11.890	-3.364	**.001**	**.012**

Each row tests the null hypothesis that the media1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05 a Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 17, the significance levels and adjusted significance values (Adj. Sig.) for the six pairs of treatments or media T6–T1, T6–T2, T5–T2, T3–T2, T4–T2, and T1–T2 are found to be less than $p < 0.05$ among the fifty treatment or media pairs. Therefore, these six pairs show evidence of significant differences between groups.

In contrast, for the two treatment or media pairs T6–T3 and T6–T4, the significance levels are less than $p < 0.05$, but the adjusted significance values (Adj. Sig.) are greater than 0.05. Consequently, these two pairs, together with the remaining seven pairs, did not show significant differences between groups.

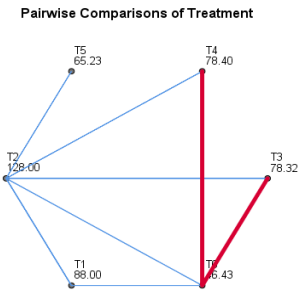


Figure 29: Each node treatment shows the average rank of treatments for the minimum root size of *P. wardii* during the multiplication stage

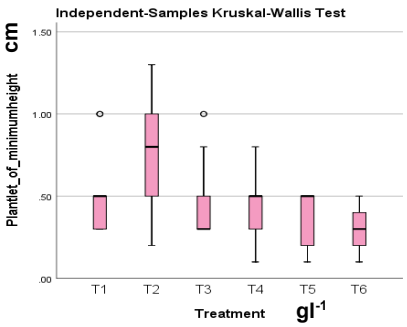


Figure 30: Effect of six treatments for the minimum root of *P. bellatum* during the multiplication stage

As shown in Figure 29, examination of the mean ranks of each treatment or medium indicates that T2 medium is the most suitable among the six media for achieving the minimum plantlet height of *Paphiopedilum wardii* during the multiplication stage. For the maximum plantlet height of *P. wardii* during the multiplication stage, T1 treatment or medium is identified as the second most suitable among the six treatments or media, while T4 and T3 treatments or media ranked third among the six treatments or media. In contrast, T6 treatment or medium was found to be the least suitable among the six media for the minimum plantlet height of *P. wardii* during the multiplication stage. T6 medium is used regularly during the

multiplication stage of orchids in the Biotechnology Laboratory of the Forest Research Institute (FRI), Forest Department.

As illustrated in Figure 30, the boxplots for the six treatments or media display the third quartile (Q3), first quartile (Q1), median, error bars, interquartile range (IQR), and maximum and minimum values. Among the six treatments or media, the T2 treatment or medium is the best for the maximum plantlet height of *P. wardii* during the multiplication stage. At first glance, T1, T4, and T3 treatments or media appear to be the second best among the six treatments or media. However, T1 treatment or medium shows a more compact distribution of values, without the wide maximum and minimum error bars observed in T4 and T3. Therefore, T1 treatment or medium can be identified as the second best among the six treatments or media. The addition of a higher amount of peptone (1.0 and 2.0 g/L) inhibited multiple shoot formation (Songjun; et al., 2015). T1, T2 and T3 are supplemented with peptone (1.0 g/L) for multiplication stage in in vitro seed culture of *Paphiopedilum wardii* and *P.* bellatum.

5.16. Effects of different media on the number of plantlets of *P. wardii* during the multiplication stage of in vitro seed culture

Kruskal–Wallis test was conducted to examine the effects of treatments on the number of plantlets height of *Paphiopedilum wardii* during the multiplication stage, based on different proportions of stock solution types, hormone types, and basal media ratios. For the number plantlet of *Paphiopedilum wardii* during the multiplication stage, a significant result was H (5)= 45.633, p<.05.^{W}H= 0.50/ ^{X}2(5)=45.633, p.000, ^{W}H=0.50, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank =98.67), media or treatment T2 (Mean Rank =117.07), media or treatment T3 (Mean Rank =56.07), media or treatment T4 (Mean Rank =86.55), media or treatment T5 (Mean Rank= 42.71) and media or treatment T6 (Mean Rank=74.60) so, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There was no difference in evidence between some pairs among groups. There are differences in evidence among groups in some pairs.

Table18: Pairwise comparisons of treatments for the number of plantlets height of *P. wardii* during the multiplication stage.

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T5-T3	13.746	13.676	1.005	.315	1.000
T5-T6	-31.895	12.786	-2.495	**.013**	.189
T5-T4	43.842	12.689	3.455	**.001**	**.008**
T5-T1	55.958	12.999	4.305	**.000**	**.000**
T5-T2	74.358	12.689	5.860	**.000**	**.000**
T3-T6	-18.149	13.100	-1.385	.166	1.000
T3-T4	-30.095	13.006	-2.314	**.021**	.310
T3-T1	42.212	13.308	3.172	**.002**	**.023**
T3-T2	60.612	13.006	4.660	**.000**	**.000**
T6-T4	11.947	12.066	.990	.322	1.000
T6-T1	24.063	12.391	1.942	.052	.782
T6-T2	42.463	12.066	3.519	**.000**	**.006**
T4-T1	12.117	12.291	.986	.324	1.000
T4-T2	30.517	11.963	2.551	.011	.161
T1-T2	-18.400	12.291	-1.497	.134	1.000

Each row tests the null hypothesis that the media1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05 a Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 18, among the fifteen pairs of treatments or media, eight pairs T5–T6, T5–T4, T5–T1, T5–T2, T3–T4, T3–T1, T3–T2, and T6-T2 are found to have significance levels lower than $p < 0.05$. Among these eight pairs, the significance levels and adjusted significance values (Adj. Sig.[a]) of six treatment or media pairs—T5–T4, T5–T1, T5–T2, T3–T1, T3–T2, and T6–T2 were less than $p < 0.05$ among the fifteen treatment or media pairs. Therefore, these six treatment or media pairs showed significant differences among the fifteen treatment or media pairs. In contrast, the adjusted significance values (Adj. Sig.[a]) of the nine treatment or media pairs T5–T3, T5–T6, T3–T6, T3–T4, T6–T4, T6–T1, T4–T1, T4–T2, and T1–T2 are greater than $p < 0.05$. Therefore, these nine treatment or media pairs showed no significant differences among the fifteen treatment or media pairs.

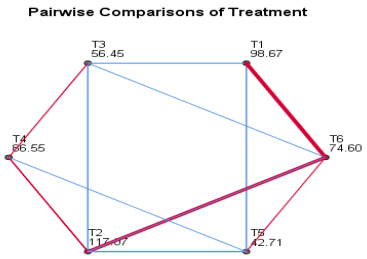


Figure 31: Each node treatment shows the average rank of treatments for the number of plantlets size of *P. wardii* during the multiplication stage

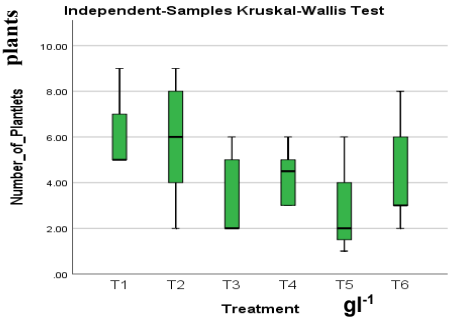


Figure 32: Effect of six treatments for the number of plantlets of *P. bellatum* during the multiplication stage

As shown in Figure 31, based on examination of the mean ranks of each treatment or medium, the T2 medium is the most suitable among the six media for the number of plantlets (height) of *Paphiopedilum wardii* during the multiplication stage. The T1 treatment or medium was identified as the second most suitable among the six treatments or media; the T4 treatment or medium was found to be the third most ideal among the six treatments or media. In contrast, the T5 treatment or medium was found to be the least suitable among the six treatment media for the number of plantlets (height) of *P. wardii* during the multiplication stage.

As shown in Figure 32, the box plots of the six treatments or media illustrate the third quartile (Q3), first quartile (Q1), median, error bars, interquartile range (IQR), and maximum and minimum values. The T2 treatment or medium is the best among the six treatments or media for the number of plantlets (height) of *Paphiopedilum wardii* during the multiplication stage. The T1 treatment or medium ranked second best among the six treatments or media, whereas the T5 treatment or medium was the poorest among the six treatments or media for the number of plantlets (height) of *Paphiopedilum wardii* during the multiplication stage. The highest number of shoots formed on medium supplemented with 2.0 g/L tryptone–peptone with an average of 2.8 shoots forming per single shoot explant (Poniewozik et al., 2020).

5.17. Effects of different media on the maximum root size of *P. wardii* during the multiplication stage of in vitro seed culture

Kruskal–Wallis test was conducted to examine the effects of treatments on the maximum root size of *P. wardii* during the multiplication stage, based on different proportions of stock solution types, hormone types, and basal media ratios. For the maximum root size of *P. wadii* during the multiplication stage, a significant result was $H(5) = 88.189$, $p<.05$.$^{W}H= 0.69/ X^2(5) =88.189$, p.000, $^{W}H=0.69$, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank = 102.28, media or treatment T2 (Mean

Rank =134.93), media or treatment T3 (Mean Rank =69.36), media or treatment T4 (Mean Rank =87.1), media or treatment T5 (Mean Rank= 27.9) and media or treatment T6 (Mean Rank=54.66) so, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There was no difference in evidence between some pairs among groups. There are differences in evidence among groups in some pairs.

Table19: Pairwise comparisons of treatments for the maximum root of *P. wardii* during the multiplication stage.

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T5-T6	-26.759	12.854	-2.082	.037	.560
T5-T3	41.468	13.749	3.016	**.003**	**.038**
T5-T4	59.204	12.757	4.641	**.000**	**.000**
T5-T1	74.382	13.068	5.692	**.000**	**.000**
T5-T2	107.038	12.757	8.391	**.000**	**.000**
T6-T3	14.708	13.170	1.117	.264	1.000
T6-T4	32.445	12.130	2.675	**.007**	.112
T6-T1	47.623	12.457	3.823	**.000**	**.002**
T6-T2	80.278	12.130	6.618	**.000**	**.000**
T3-T4	-17.736	13.075	-1.357	.175	1.000
T3-T1	32.914	13.379	2.460	**.014**	.208
T3-T2	65.570	13.075	5.015	**.000**	**.000**
T4-T1	15.178	12.357	1.228	.219	1.000
T4-T2	47.833	12.027	3.977	**.000**	**.001**
T1-T2	-32.656	12.357	-2.643	**.008**	.123

Each row tests the null hypothesis that the media1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05 a Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 19, among the fifteen pairs of treatments or media, eleven pairs (T5–T3**,** T5–T4, T5–T1, T5–T2, T6–T1, T6–T2, T6–T4, T3–T1, T3–T2, T4–T2, and T1–T2) are found to have significance levels less than $p < 0.05$. The significance was determined using Dunn's pairwise test, followed by Kruskal–Wallis tests to confirm pairwise differences.

Among these, eight pairs**,** namely T5–T3**,** T5–T4, T5–T1, T5–T2, T6–T1, T6–T2, T3–T2, and T4–T2, show both significance levels and adjusted significance values (Adj. Sig.) less than $p < 0.05$ among the fifteen treatments or media pairs. The Bonferroni correction was applied to adjust the significance values, ensuring the robustness of our analysis.

Therefore**,** these six treatment or media groups exhibited significant differences among the fifteen pairs, whereas the remaining eight pairs showed no significant differences among the treatment or media pairs.

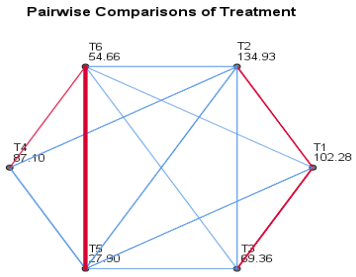


Figure 33: Each node treatment shows the average rank of treatments for the maximum root size of *P. wardii* during the multiplication stage

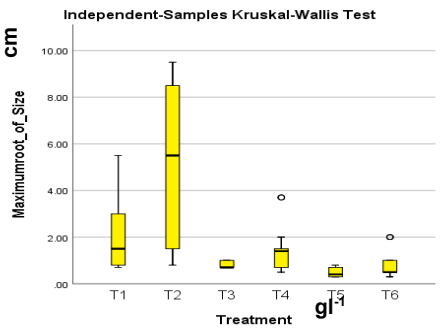


Figure 34: Effect of six treatments for the maximum root size of *P. wardii* during the multiplication stage

As shown in Figure 33, based on the examination of the mean rank of each treatment or medium, T2 medium was found to be the most suitable among the six media for achieving the maximum root number of *Paphiopedilum wardii* during the multiplication stage (figure 37). T1 treatment or medium was identified as the second most ideal among the six media. In contrast, T5 treatment or medium was found to be the least effective among the six media (Figure 33).

In the box plot shown in Figure 34, the third quartile (Q3), first quartile (Q1), median, error bars, interquartile range (IQR), mild (potential) outliers, extreme (far-out) outliers, and maximum and minimum values are presented. T2 treatment or medium is found to be the most suitable among the six media for achieving the maximum root number of *Paphiopedilum wardii* during the multiplication stage (Figure 34). T1 treatment or medium was identified as the second most **ideal** among the six media, whereas T5 treatment or medium is found to be the least effective among the six media (Figure 34). The most roots were obtained on the media supplemented with KIN 1 mg•dm–3 (3.84). Similar number was noted in presence of BA 0.5 and 1 mg•dm–3 (3.06 and 2.68 respectively) (Poniewozik et al., 2020). The addition of a higher amount of peptone (1.0 and 2.0 g/L) inhibited multiple shoot formation (Zeng et al., 2015).

5.18 Effects of different media on the minimum root size of *P. wardii* during the multiplication stage in in vitro seed culture

Kruskal–Wallis test was conducted to examine the effects of treatments on the minimum root size of *P. wardii* during the multiplication stage, based on different proportions of stock solution types, hormone types, and basal media ratios. For the minimum root size of *P. wadii* during the multiplication stage, a significant result was H (5) = 60.644, p<.05.^{W}H= 0.58/ X^2(5) =60.644, p.000, ^{W}H=0.58, indicating that the treatments or media are different from each other. Media or treatment T1 (Mean Rank = 92.33, media or treatment T2 (Mean Rank =129.75), media or treatment T3 (Mean Rank =70.64), media or treatment T4 (Mean Rank =80.40), media or treatment T5 (Mean Rank= 37.19) and media or treatment T6 (Mean Rank=67.55) so, there was a significant difference between the mean ranks of all treatments. A Kruskal-Wallis test provided very strong evidence of a difference ($p < 0.001$) between the mean ranks of at least one pair of groups. Dunn's pairwise tests were carried out for the fifty-five pairs of groups. There was no difference in evidence between some pairs among groups. There are differences in evidence among groups in some pairs.

Table20: Pairwise comparisons of treatments for the minimum root of *P. wardii* during the multiplication stage.

media 1-media2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T5-T6	-30.364	12.698	-2.391	.017	.252
T5-T3	33.449	13.582	2.463	**.014**	.207
T5-T4	43.213	12.602	3.429	**.001**	**.009**
T5-T1	55.146	12.909	4.272	**.000**	**.000**
T5-T2	92.563	12.602	7.345	**.000**	**.000**
T6-T3	3.085	13.010	.237	.813	1.000
T6-T4	12.848	11.983	1.072	.284	1.000
T6-T1	24.782	12.306	2.014	**.044**	.661
T6-T2	62.198	11.983	5.190	**.000**	**.000**
T3-T4	-9.764	12.916	-.756	.450	1.000
T3-T1	21.697	13.216	1.642	.101	1.000
T3-T2	59.114	12.916	4.577	**.000**	**.000**
T4-T1	11.933	12.207	.978	.328	1.000
T4-T2	49.350	11.881	4.154	**.000**	**.000**
T1-T2	-37.417	12.207	-3.065	**.002**	**.033**

Each row tests the null hypothesis that the media1 and media 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05 a Significance values have been adjusted by the Bonferroni correction for multiple tests.

As shown in Table 20, for the minimum root size of *Paphiopedilum wardii* during the multiplication stage, nine treatment or media pairsT5–T3, T5–T4, T5–T1, T5–T2, T6–T1, T6–T2, T3–T2, T4–T2, and T1–T2were found to have significance levels less than $p < 0.05$ among the fifteen treatment or media pairs.Among these, seven pairsT5–T4, T5–T1, T5–T2, T6–T2, T3–T2, T4–T2, and T1–T2—showed both significance levels and adjusted significance values (Adj. Sig.) less than $p < 0.05$ among the fifteen treatment or media pairs. The significance was determined using Dunn's pairwise test, followed by Kruskal–Wallis tests to confirm pairwise differences.Therefore, these seven treatment or media pairs exhibited significant differences among the fifteen pairs, whereas the remaining eight treatment or media pairs showed no significant differences among the treatment or media pairs.

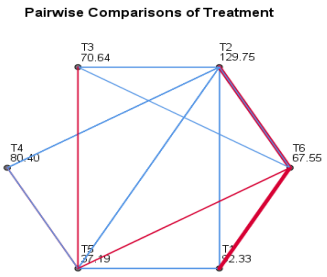


Figure 35: Each node treatment shows the average rank of treatments for the minimum root size of *P. wardii* during the multiplication stage

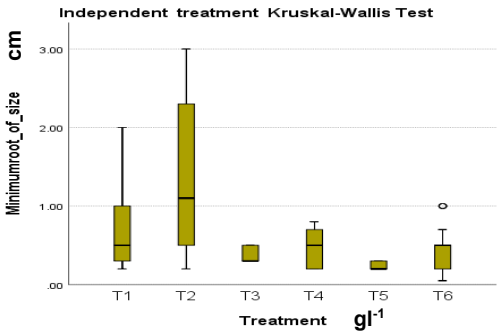


Figure 36: Effect of six treatments for the minimum root size of *P. bellatum* during the multiplication stage

As shown in Figure 35, based on the examination of the mean rank of each treatment or medium, T2 medium is found to be the most suitable among the six media for achieving the minimum root of *Paphiopedilum wardii* during the multiplication stage (Figure 35). T1 treatment or medium was identified as the second best among the six media, whereas T5 treatment or medium was found to be the least effective among the six media for the minimum root size of *P. bellatum* during the multiplication stage figure 37.

In the box plot shown in Figure 36, the third quartile (Q3), first quartile (Q1), median, error bars, interquartile range (IQR), mild (potential) outliers, extreme (far-out) outliers, and maximum and minimum values are presented. For the minimum root of *P. wardii* during the multiplication stage, T2 treatment or medium was found to be the best among the six media, T1 was the second best, T4 treatment or medium was the third best, and T5 treatment or medium was the least effective among the six media for the minimum root size of *P. bellatum* during the multiplication stage figure 37. The highest multiplication rate was obtained on the media supplemented with BA 0.5 mg•dm–3 (2.92) in comparison to KIN 2.5 or 5 mg•dm–3 (1.38 and 1.0 respectively) (Poniewozik et al., 2020). Similar number was noted in presence of BA 0.5 and 1 mg•dm–3 (3.06 and 2.68 respectively) (Poniewozik et al., 2020). The addition of a higher amount of peptone (1.0 and 2.0 g/L) inhibited multiple shoot formation (Zeng et al., 2015).

Table 21: This section presents the significance levels (e.g., p-values) for all evaluated parameters including maximum and minimum plantlet height, maximum and minimum root size, and the number of plantlets of *Paphiopedilum wardii* during the multiplication stage based on the results of statistical analyses such as ANOVA and the Kruskal–Wallis test.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of maximum plantlet of height is the same across categories of Treatment.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
2	The distribution of minimum Plantlet of height is the same across categories of Treatment.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
3	The distribution of Maximum root of size is the same across categories of Treatment.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
4	The distribution of Minimum root of size is the same across categories of Treatment.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
5	The distribution of Number of Plantlets is the same across categories of Treatment.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .050

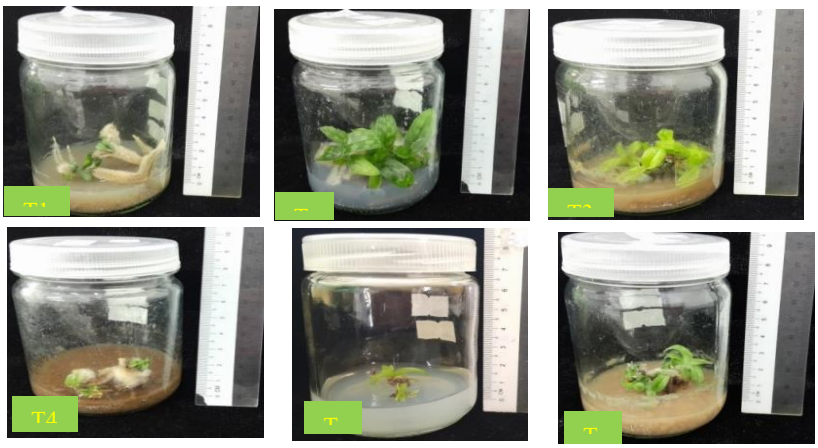


Figure 37: Condition of *P. wardii* plantlets in culture bottles on six different media after 10 months of the multiplication stage.

Seeds of *Paphiopedilum bellatum* and *P. wardii* were cultured starting from the induction stage on 25th December, 2022. Subsequently, on 21st October, 2023, a small number of seeds from one contaminated (fungal infection) culture bottle of *P. bellatum* and *P. wardii* were examined under a microscope in the Wood Anatomy Section in figure 38 (b) and (b_1). According to the observations, the seed coats had not yet ruptured; some seed coats were still intact. According to my practical records from the Biotechnology Laboratory of the Forest Research Institute (FRI), Forest Department, the fruits and seeds of this genus are smaller than those of other orchid genera such as *Dendrobium*, *Vanda*, and *Cymbidium*.

Based on these records, when 0.015 ± 0.01 g of *P. bellatum* and *P. wardii seeds* were inoculated into a single culture bottle for one treatment or medium, approximately 218 seeds were estimated to be present. During the induction stage of P. *wardii* and P. *bellatum*, the period from germination to protocorm formation required approximately eight months. Furthermore, the development from protocorm enlargement through multiplication to plantlet formation required approximately ten months.

According to my practical records from the Biotechnology Laboratory of the Forest Research Institute (FRI), Forest Department, *Dendrobium chrysotoxum* Lindl., D. devonianum Rchb.f., D. primulinum, *D. crystallinum* Rchb.f., *D. findlayanum* Parish & Rchb.f., *D. draconis*

Rchb.f., and *D. longicornu* Lindl. They require approximately four months from germination to protocorm formation during the induction stage. Some of these *Dendrobium* species required nearly five months from protocorm development and multiplication to plantlet formation.

In the case of *Vanda coerulea* Griff.ex Lindl., *Cymbidium tracyanum* Hort., and *Cymbidium aloifolium*, the period from germination to protocorm formation during the induction stage was approximately six months, while the development from protocorm enlargement and multiplication to plantlet formation required approximately eight months, according to my practical records from the Biotechnology Laboratory of the Forest Research Institute (FRI), Forest Department. Although KC and HY media contain coconut water and NAA, the low germination rate may be due to the low proportion of viable (healthy) seeds. In addition, the lower number of protocorms and the smaller maximum and minimum sizes of protocorms compared with 1/4 MS2 suggest that KC (Knudson, 1964) medium and Hyponex N0.6.5–6–19 media contain lower levels of macronutrients and micronutrients than Murashige and Skoog (MS) medium. Although the KC and HY media contain coconut water and NAA, the low germination rate may be due to the small proportion of well-developed (viable) seeds. In addition, the fact that the number of protocorms and the maximum and minimum sizes of protocorms were lower than those observed on 1/4 MS2 suggests that the KC (Knudson, 1964) medium and Hyponex N06.5–6–19 medium may contain lower levels of macronutrients and micronutrients than the Murashige and Skoog (MS) medium.

P. bellatum and *P. wardii*, compared with *Vanda coerulea* Griff. ex Lindl., *Cymbidium tracyanum* Hort., *Dendrobium chrysotoxum* Lindl., *D. devonianum* Rchb.f., *D. primulinum, D. crystallinum* Rchb.f., *D. findlayanum* Parish & Rchb.f., *D. draconis* Rchb.f., and *D. longicornu* Lindl., were found to be more difficult in vitro seed germination, to have smaller fruit and seed sizes, to require a longer time for in vitro seed culture than the orchid species mentioned above, and to show wide variation in the number of protocorms and plantlets obtained. The results from our investigation indicate that the frequency of in vitro seed germination and shoot organogenesis in Paphiopedilum species is lower than that of most other orchid species, even when in vitro techniques are used (Long et al., 2010). Therefore, it can be confirmed that the endangered status of Paphiopedilum species is closely and significantly associated with these observed factors.

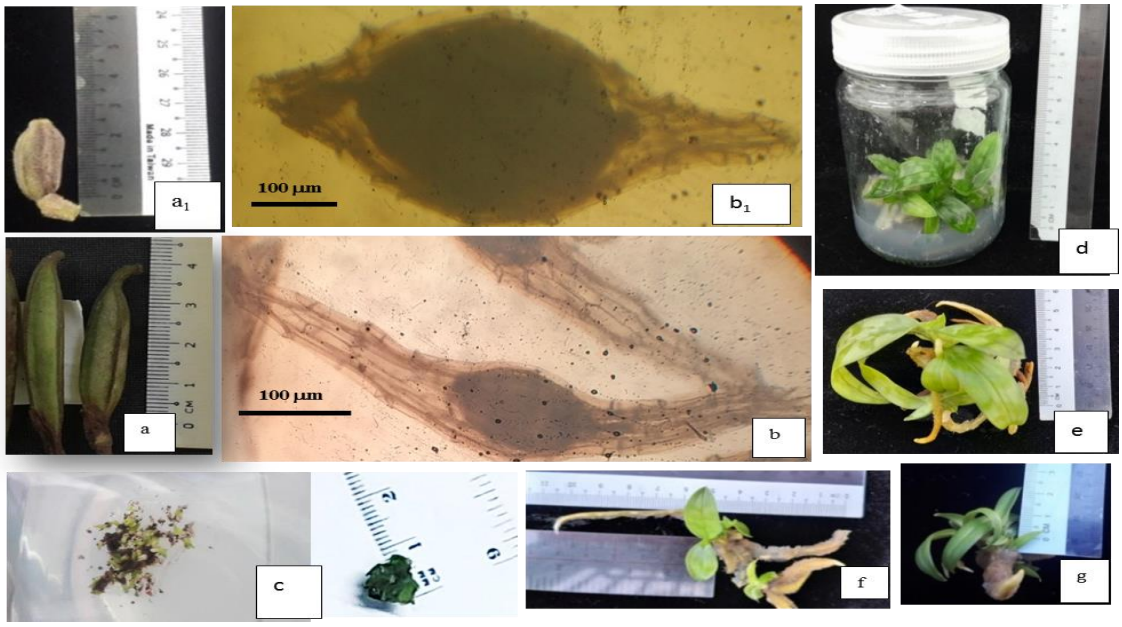


Figure 38: (*a)* and (a_1) show the fruits of *P. bellatum* and *P. wardii*, respectively. "Figures b1 and b2 show the seed morphology and the intact (unsplit) seed coat of some seeds during the induction stage within 10 months of culture. Figure 37: c illustrates the formation of

protocorms at the induction stage and the recording of protocorm size. Figure *d* shows proliferating plantlets of *P. wardii* in a culture bottle during the multiplication stage, grown on the most suitable medium (T2) among six media tested.

The highest multiplication rate was obtained on the media supplemented with Medium (T2) (Poniewozik et al.) and (microbiologyinfo.com). T2 or 1/4 MS Base**:** Reduced salt strength (one-quarter of normal MS) limits nutrient-induced browning and is ideal for delicate tissue growth.20% Coconut Milk**:** Acts as a rich source of natural cytokinins (cell division hormones), amino acids, and vitamins, often aiding in growth enhancement and embryo development NAA (0.5 mg/l): Naphthaleneacetic acid, an auxin used to induce rooting. Peptone (1 g/l): Provides organic nitrogen and amino acids, encouraging rapid growth. Activated Charcoal (1 g/l): A crucial antioxidant that adsorbs inhibitory compounds (phenolic exudation/browning), preventing necrosis. Sucrose (10 g/l): A low concentration of carbon source (1% instead of the standard 3%), which reduces osmotic stress. Figures *e, f,* and *g* show the measurement and recording of shoot height and root size of *P. wardii* plantlets.

6. Conclusions

It was confirmed that both 1/4 MS1 and 1/4 MS2 media have a significant combined effect on the germination of *P. bellutum*, while the 1/4 MS2 medium alone showed a significant impact on the maximum, minimum, and number of protocorms of *P. bellutum* and *P. wardii*, as well as on the germination of *P. wardii*. Although the stock solution type and concentration were the same, the use of NAA, which promotes cell growth and root formation, together with 10% coconut water, demonstrated that the 1/4 MS2 medium had a significant effect during the induction stage of both *Paphiopedilum* species. Among the media test three media with the same Murashige and Skoog (1962) stock solution type but two different concentrations, one medium with Knudson C (KC) stock solution type (Knudson, 1964), and two media with the same Hyponex 7–6–19 (HY1) stock solution type but different concentrations six media in total containing different types and concentrations of supplemented hormones and vitamins were evaluated. Only the T2 medium showed a significant effect during the multiplication stage of both species. Therefore, it was confirmed that the stock solution type, the ratio of the stock solution, the type of hormone used, and the hormone concentration had significant effects at both the induction and multiplication stages.

Consequently, this research approach on *Paphiopedilum bellutum* and *P. wardii* highlights the importance of reintroduction programs for orchid species in their natural habitats and can contribute to the restoration of targeted orchid species within their respective ecosystems. Therefore, further improvement of in vitro tissue culture techniques for Paphiopedilum orchids is crucial for their future development. It can be demonstrated that using both in situ and ex situ approaches to continue the conservation and support of *Paphiopedilum bellatulum* and *P.wardii* species will be valuable for preventing their extinction and ensuring their long-term sustainable development.

7. Recommendation

Researchers / thesis students conducting experiments and research studies on endangered orchid species need to use a Scanning Electron Microscope (SEM) to examine the number of orchid seeds and determine whether the seeds are well-developed and viable.

If sufficient data are obtained during the research on the light, temperature, moisture, and humidity preferred by these orchid species, the study can be continued as a successful research project. In the induction stage of tissue culture, an ultrasonic cleaner (40 kHz Bransonic 8210R-DTH, Danbury, CT) is required for the sterilization of organ explants.

8. **Acknowledgements**

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