IN VITRO SEED GERMINATION AND SEEDLING DEVELOPMENT OF THE ORCHID *PHOLIDOTA ARTICULATA* LINDL.

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Abstract

In this experiment, the plantlets were initiated from seeds of *Pholidota articulata* Lindl. (Kwyetmeee pan myokywe). The experiment was carried out at Plant Tissue Culture Laboratory of the Htone Bo Agricultural Research Farm, Taunggyi, Department of Agricultural Research from October, 2022 to March, 2023. This study was focusing on evaluating the effect of natural additive as plant growth regulators (PGR). The additive used in this study was 100 ml of coconut water (CW) that supplemented in Murashige and Skoog (MS) medium. Four treatments such as T_1 (MS only), T_2 (half MS), T_3 (Full MS and CW) and T_4 (half MS and CW). Each treatments of 6 replications and experimental design was set up using Completely Randomized Design (CRD). Murashige and Skoog (MS) and Half MS media were used to study their effects in seed germination and protocorm development. Maximum percentage of seed germination was observed in Half MS and coconut water (T_4) medium and minimum percentage of seed germination was in MS only medium (T_1). The best protocorms for plantlet development i.e MS media was supplemented with various combinations of Cytokinins (BAP) and Auxin (NAA). Therefore, *in vitro* developed protocorms were sub-cultured on the Murashige and Skoog (MS) medium, supplemented with coconut water and different concentrations of 6-benzylaminopurine (BAP) and α-naphtalene acetic acid (NAA). The highest number of shoot and number of leaves was observed in MS medium supplemented with 3mg.l^{-1} BAP.

Keywords: *Pholidota articulata*, coconut water, BAP, NAA

Introduction

Orchidaceae is a large family with 25,000-30,000 species and 600-800 general known from the world. (Backer and Ben Den Brink, 1968). The genus *Pholidota* are epiphytic herbs generally grown on rocks and trees. Most plants of the genus *Pholidota* found in India grow as epiphytes. Some are also found growing on moist, mass covered rock structures on large, hilly slopes. Sexual reproduction of orchids in nature is being very slow as only 2-5% of their seeds can germinate after symbiosis with a special mycorrhizal fungus (Pant *et al.*,2017). So, plant tissue culture technique is one of the alternative ways to propagate the plants through seeds without fungal association those cannot be reproduced sexually in nature easily (Pant, 2013). In vitro propagation protocol by plant tissue culture has not been developed yet in this orchid. In vitro propagation of this orchid could be an alternative to fulfill its demand on horticulture and traditional medicine as well as for its conservation.

The aims and objectives are to obtain the *in vitro* plants of *Pholidota articulata* Lindl. From seed culture, to obtain the plantlet production from PLBs potentially *in vitro*, to identify the best hormonal effect for in vitro regeneration of orchid and to establish an effective protocol for *in vitro* seed germination, protocorm and plantlet development of *Pholidota articulata* Lindl.

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Materials and Methods

Experimental Site

The research was carried out at the laboratory of Plant Tissue Culture of the Htone Bo Agricultural Research Farm, Taunggyi, Department of Agricultural Research during October, 2022 to March, 2023.

Collection and Identification of *Pholidota articulata* Lindl.

The specimens were collected from Maung Myanmar Orchid Garden, Taunggyi area. The collected plant specimens were studied by under using dissecting microscope in laboratory, Department of Botany, Taunggyi University. The morphological and taxonomical studies were made from the collected specimen by using available literature such as Backer, 1968 and Shi xiantaoshu, 2010.

Method of Media Preparation

According to the Murashige and Skoog (1962), method of 1000ml medium preparation applied in the present study is as follows;

1. 300ml distilled water was poured into 3000ml beaker.
2. Proper amount of stock solution were added.
3. Sugar 30g was added and stirred.
4. Agar powder 6 g was slowly added and stirred.
5. Before the addition of agar, the pH value was adjusted to be 5.7 with 0.1N NaOH and 0.1N HCl
6. The volume of solution was made up to 1000ml and the level was marked.
7. The solution was gently heated until it starts to boil.
8. Then medium was heated and stirred until agar was completely dissolved and becomes amber-colored.
9. The medium was dispensed into culture bottles.
10. The culture bottles were covered and autoclaved.
11. The media were sterilized in an autoclave at 120-121°C and 1.2 kg/cm^2 for 30 minutes.
12. Then the bottles were cooled and used.

Experiment 1 (Initial Seeds Culture)

Source of Plant Material

The green healthy capsule of *Pholidota articulata* Lindl. which is a native species of Myanmar was obtained from Maung Myanmar Orchid Garden, Taunggyi area.

Culture Bottles

All flasks were washed with soap detergent, then rinsed throughly with hot distilled water and dried.

All flasks with the capacity of 100 ml each containing 20 ml of nutrient medium were used.

Culture Medium

MS media used in this investigation were shown in Table 1. MS media consist of coconut, sugar (2%) and agar (1%).The pH value was adjusted to be 5.5 ± 0.1 with 0.1 N NaOH and 0.1 N

HCl. Sterilization was made in autoclave at 120°C-121°C and 1.0 – 1.5 kg/cm2 (260°F and 15 lb/in2) for 20 minutes.

Decontamination

The green capsules was washed with 10% Clorox solution by a soft brush, then it was dipped into 70% ethyl alcohol and flame rapidly.

Culture Condition

All culture were maintained at 25 ± 2°C under continuous illumination of about 120 foot candles from 4- feet white fluorescent tube and 25%- 35% relative humidity.

Figure 1. Procedures of Seeds Culture

Experiment 2 (Multiplication of shoots on the different nutrient medium)

Source of Plant Material

The protocorm like bodies (PLBs) of *Pholidota articulata* Lindl. were obtained from previous culture of seed germination. Protocorm explants were obtained through *in vitro* cultures, no sterilization was required.

Elongation and Multiplication of Shoots

Protocorms with first initiated leaf developed from the germinating seeds were used for shoots multiplication. MS medium with different treatments and different concentration of BAP and NAA (1 and 3 mg/l) were used for the elongation and multiplication of shoots from the seed derived-protocorms.

Experimental Design

Six treatments such as T_1 control (MS Only), T_2 (MS + Coconut water 200ml/liter), T_3 (M.S + 1 mg/l BAP), T_4 (M.S + 3 mg/l BAP), T_5 (M.S + 1mg.l^{-1}mg/l NAA) and T_6 (M.S + 3 mg/l NAA) with six replications. Each was set up using completely randomized design (CRD).

Culture Condition

All culture bottles were maintained in a culture room where light was supplied by 4 feet fluorescent tubes (1000-1200 lux) light intensity for 8/16 hours (light/dark). Throughout the culture period temperature ranged from 23°C to 25°C. Their experiment were carried out at the tissue culture laboratory.

Data Collection and Statistical Analysis

In this experiment, 6 treatments which six replications each were used for plantlets initiation and number of shoots and number of leaves recorded three months after culture. The treatment means of the plantlets from all replicates were calculated.

The data were analyzed using the IRRISTAT software, version 4, developed by International Rice Research Institute (IRRI), Philippines. The mean separation was calculated by Least Significant Different (LSD) (Gomez and Gomez, 1984).

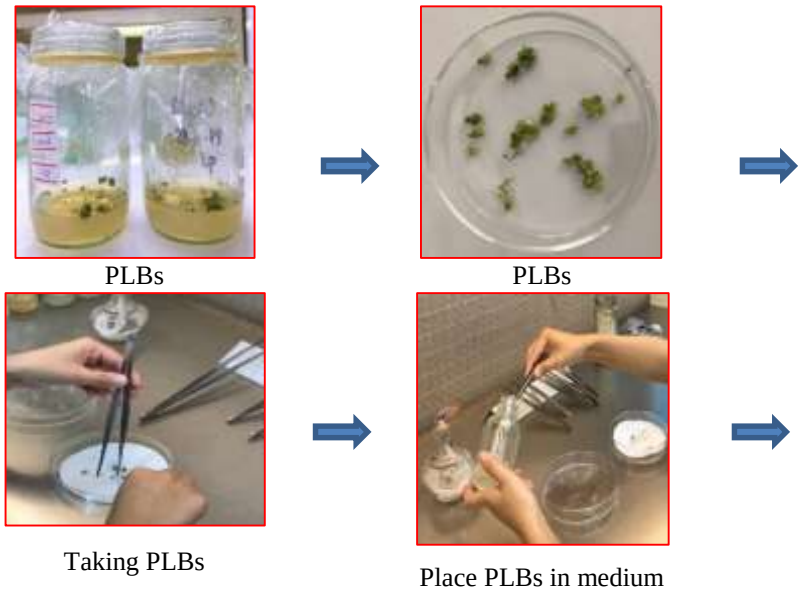


Figure 2. Multiplication of Shoots on the Different Nutrient Medium

Results

Morphological Characters of *Pholidota articulata* Lindl.

Scientific Name	:	*Pholidota articulata* Lindl.
Myanmar name	:	Kwyetmeee pan myokywe
Distribution	:	Kachin State, Kayin state, Mon State, Shan State
Flowering period	:	May – June
Characteristics	:	Flowers white with light
Size	:	0.8 – 1 cm
Number of flowers	:	10 – 20

Epiphytic on tree trunks in forest, lithophytic on shade rocks. Pseudobulbs connected to each other at both ends and stem like, subcylindric, sometimes slightly narrowed, branching or not, sometimes with very short rhizomes between then and producing a few roots. Leaves 2, at apex of new pseudobulb; leaf blade obovate-elliptic, oblong, or narrowly elliptic, veins plicate, subacute or obtuse. Inflorescence at apex of new pseudobulb, floral bracts deciduous during flowering, narrowly ovate-oblong. Flowers greenish white or white and slightly tinged with reddish. Dorsal sepal oblong or elliptic, dorsally carinate, 5-veined; lateral sepals ovate, oblique, slightly wider than dorsal sepal. Petals oblong-lanceolate or suboblanceolate, Column stout, apex winged; rostellum broadly ovate, pollinia 4, waxy. Capsule ellipsoid to obovoid-ellipsoid, slightly 3-ridged Epiphytic on trees in forests, lithophytic on shaded rocks.

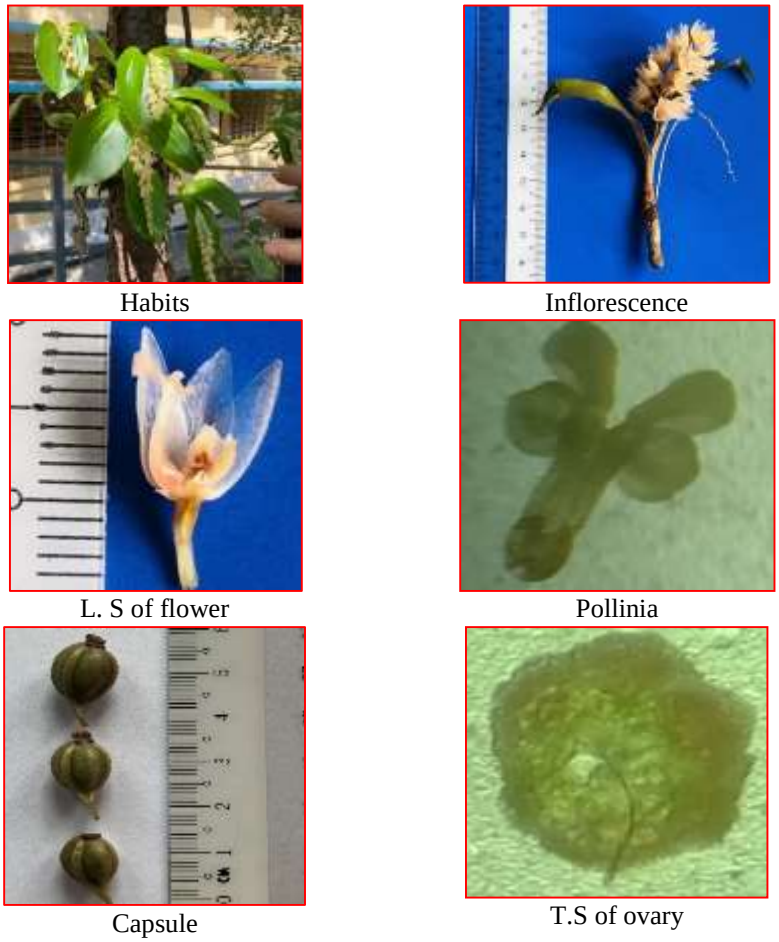


Figure 3. Morphological Characters of *Pholidota articulata* Lindl.

Experiment 1. Germination of Seeds on Different Nutrient Medium

Seeds were inoculated on the full- and half-, of MS medium supplement with coconut water. Sign of seeds germination is by turning them into light yellowish-green on 4th week, they became swollen and elongated spherules in 8th week on all the strengths of ½ MS medium with coconut water.

Protocorms formation was observed in 10th week of seeds culture on ½ MS medium (T_2) and ½ MS medium with coconut water (T_4). They were globular, light yellowish-green and hairy.

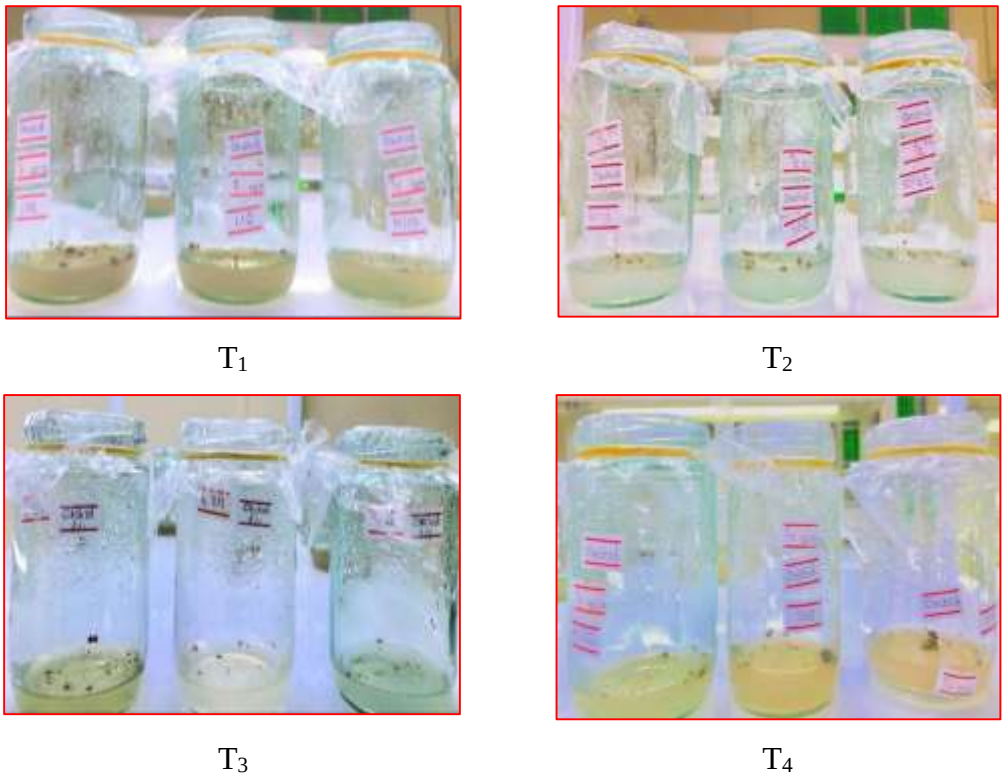
The green, globular hairy protocorms changed into pale yellow over time on these medium. No shoot proliferation or further growth of protocorms was observed in all the concentrations and combinations of the MS medium.

Table 1. *In vitro* Seeds Germination Resulted in Different Treatments of *Pholidota articulata* Lindl.

Treatments	Germination result	Observation
$T_{1\ (control)}$ = MS Only	-	-
T_2 = ½ MS	+	Good germination
T_3 = Full MS+ 100 ml Coconut water	+	Fair germination
T_4 = ½ MS+ 100 ml Coconut water	+	Good germination

- Germination did not occur

\+ Germination was observed



T_1 T_2

T_3 T_4

Figure 4. Development of Protocorms from Seeds on the Full- and Half-strength of MS Medium After12 Week Culture

Experiment 2 (Multiplication of Shoots on the Different Nutrient Medium) Number of Shoots

The new shoots formation were formed three months after culture in all treatments. When compared the various concentrations of treatments, the highest average number of shoots per explants (6.80) were observed at the concentration of (T_4) 3 $mg.l^{-1}$ BAP (Table 2 and Figure 5) showed the multiple shoots of *Pholidota articulata* Lindl.

Table 2. Mean Values of the Number of Shoots on Standard MS Solid Medium Supplemented with Different Treatments

Treatments	Number of Shoots					
	67 days	74 days	81 days	88 days	95 days	102 days
T1	0.00	0.60	1.10	1.70	1.80	2.10
T2	0.00	1.00	1.70	2.20	2.40	2.60
T3	0.00	1.20	1.30	2.10	2.20	4.50
T4	0.00	1.90	3.40	3.90	4.70	6.80
T5	0.00	2.30	3.10	4.80	5.10	5.20
T6	0.00	0.20	0.80	0.90	0.90	1.90
F-test	ns	**	*	**	**	**
5% LSD	0.00	0.69	1.35	1.40	1.40	2.37
CV %	0.0	37.1	50.4	48.4	37.3	40.4

Each value represented the mean from 6 replications. Each replicate consisted of 2 plants. Mean differences in each column was determined by LSD at 5% level of significant. * = Significant at 5%, ** = highly significant , ns = not significant.

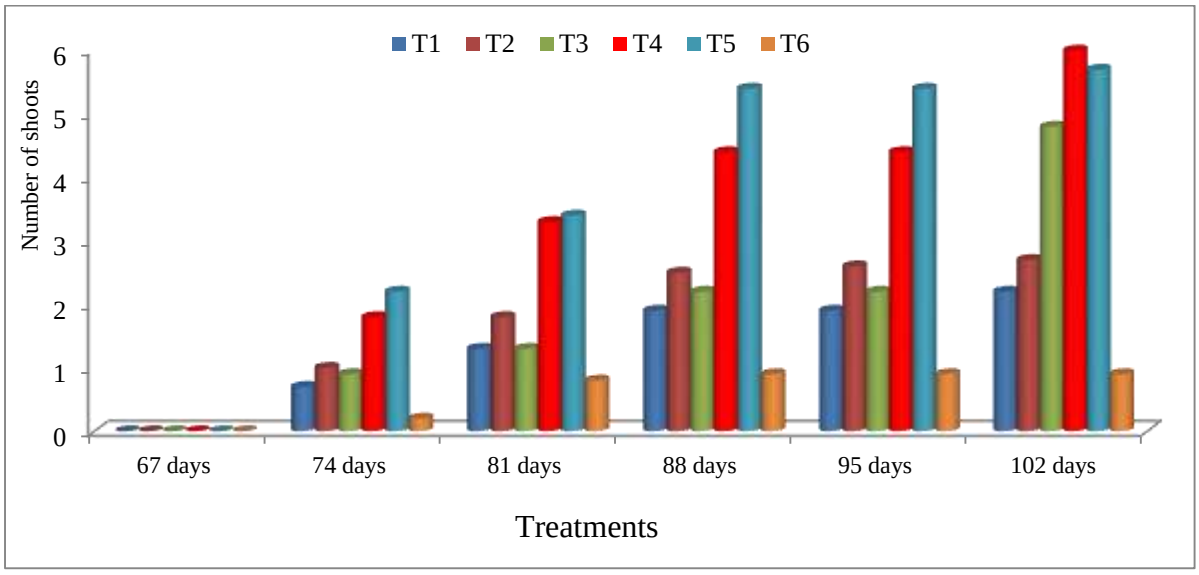


Figure 5. Mean Values of the Number of Shoots on Standard MS Solid Medium Supplemented with Different Treatments

Number of Leaves

The numbers of leaves formation were formed three months after culture in all treatments. When compared the various concentrations of treatments, the highest average number of leaves (9.40) were observed at the concentration of (T_4) 3 mg/l BAP (Table 3 and Figure 6).

Table 3. Mean Values of the Number of Leaves on Standard MS Solid Medium Supplemented with Different Treatments

Treatments	Number of leaves					
	67 days	74 days	81 days	88 days	95 days	102 days
T_1	2.10	4.20	4.40	4.60	4.70	5.20
T_2	3.40	4.00	4.10	4.30	4.40	5.10
T_3	0.65	1.80	2.00	2.10	2.20	4.10
T_4	2.20	3.90	4.90	5.20	5.40	9.40
T_5	2.20	3.40	4.40	4.50	4.70	7.40
T_6	2.40	3.60	4.10	3.90	3.90	4.20
F-test	ns	ns	*	*	*	ns
5% LSD	2.31	1.79	1.95	2.01	2.03	4.42
CV %	71.1	37.5	35.4	35.7	35.4	55.9

Each value represented the mean from 6 replications. Each replicate consisted of 2 plants. Mean differences in each column was determined by LSD at 5% level of significant. * = Significant at 5%, ns = not significant.

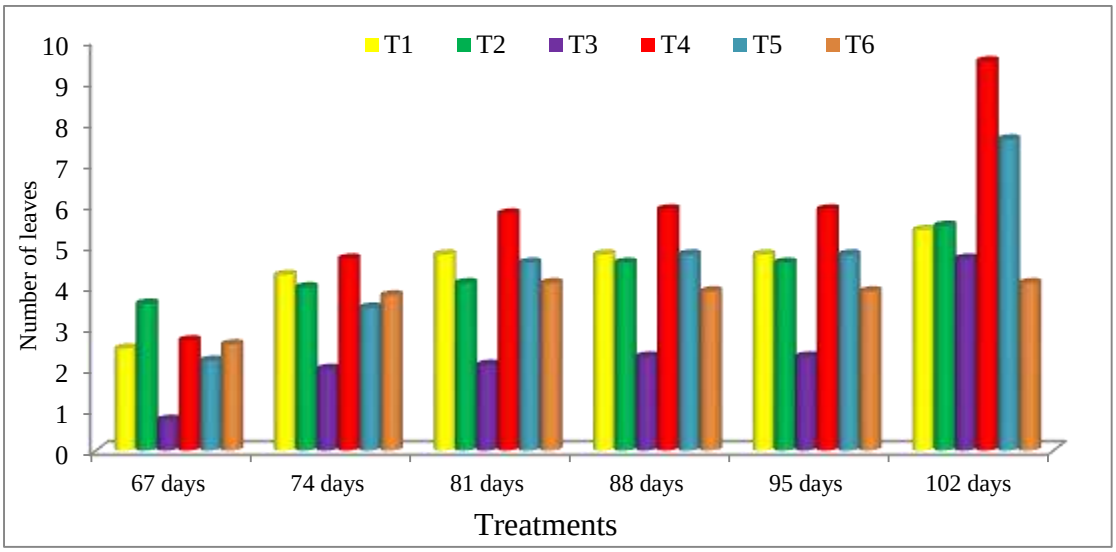


Figure 6. Mean Values of the Number of Leaves on Standard MS Solid Medium Supplemented with Different Treatments

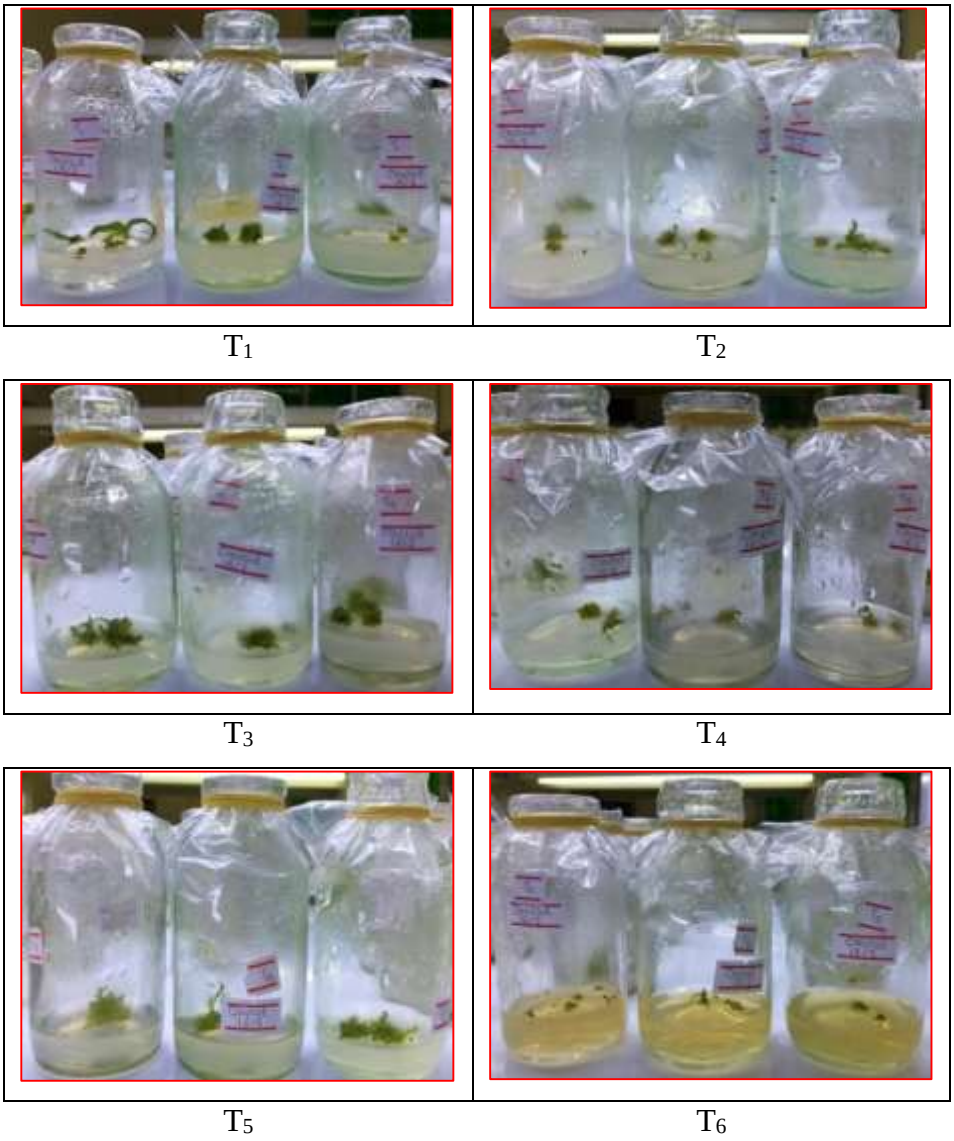


Figure 7. Effects of Different Treatments on Shoot Development of *Pholidota articulata* Lindl. From PLBs Subculture When Placed MS Solid Medium After 102 Days in Culture

Discussion and Conclusion

The morphological characters of *Pholidota articulata* Lindl. are epiphyte, perennial herb, pseudobulb connected to each other at both ends and stem like, subcylindric. Leaves narrowly elliptic, ovate, or oblong. Inflorescence terminal, emerging from apex of pseudobulb, distichous, pendulous, slender, racemose, laxly or densely many flowered. Capsule ellipsoid to ovoide-ellipsoid, slightly 3-ridged and a fruit with numerous minute seeds. This finding was in agreement with Backer and Ban Den Brink, 1968 and Shi xiantaoshu, 2010.

Seeds were germinated in four different treatments full MS and half MS. Seeds germination data were collected following 70 days of culture. Protocorm formation data were recorded at an interval of 7 days up to 70 days. On MS medium protocorm appears after 7 to 8 weeks of germination. Appearance of protocorm was 5 weeks slower in Half MS treatment (T_2) and 4 to 5 weeks in half MS with coconut water (T_4) as compared to MS medium. The process of germination started with the swelling of embryos after 3 weeks of inoculation of the immature yellowish seed. After 5 weeks spherules were observed which were subsequently transformed to protocorm like

bodies after 7 weeks. Addition of 100 ml coconut water to basal half MS medium gave the maximum proliferation. This result was in accord with the study of Peixe *et al*., 2007.

Protocorms were transferred on the MS medium supplemented with Coconut water 200 ml/liter, BAP and NAA (1 and 3 mg/l) for their elongation and multiplication. MS medium supplemented with 3 mg/l BAP was found to be the most effective condition for the multiplication of shoots from protocorms.

The results show that the seeds of *Pholidota articulata* Lindl. can be germinated and seedlings can be developed successfully under *in vitro* conditions.

According to the present study, there were statistically significant differences between nutrition media in terms of germination and seedling development parameters. Different components like macro elements, micro elements, vitamins, and organics showed significant differences for asymbiotic *in vitro* germination. Half-strength of MS medium was found to be the most appropriate for the non-symbiotic seeds germination and protocorms formation of *Pholidota articulata*. MS medium supplemented with 3 mg/l BAP was found to be the most effective condition for the multiplication of shoots from protocorms. Hence, this protocol might be useful for non-symbiotic germination, mass propagation and conservation of *Pholidota articulata* Lindl.

Acknowledgements

We are deeply indebted to Dr. Kay Thi Thin, Rector, Taunggyi University, Dr. Ko Ko Naing, Dr Aung Nay Myo. Pro-Rectors, Taunggyi University, for their permission and encouragement for this research paper. We wish to express our deep gratitude to Dr. Nilar, Professor and Head, Dr. Khin San Win and Dr San San Hlaing, Professors, Department of Botany, Taunggyi University, for their encouragement and suggestions.

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