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IN VITRO SEED GERMINATION AND PHYTOCHEMICAL SCREENING OF AN EPIPHYTIC MEDICINAL ORCHID, *PHOLIDOTA IMBRICATA* W. J. HOOK. OF BANGLADESH

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Abstract

Orchids are one of the immense ecological and evolutionary momentous plants and have effectively colonized almost every habitat on the earth. The seeds of *Pholidota imbricata* W. J. Hook. were aseptically grown on four nutrient media namely KC, MS, PM, MVW and these successfully germinated on all of the tested media. The highest percentage of seed germination (80.00%) was achieved on MS medium and greenish protocorms were obtained. During phytochemical screening, secondary metabolites *i.e.* alkaloids and ten other compounds namely, steroids, flavonoids, glycosides, phlobatannins, terpenoids, quinine, coumarin, anthroquinones, saponins, and tannin were recorded in both natural and *in vitro* developed plant parts. The comparative results of the alkaloid test revealed that *in vitro* developed plant parts showed better results than those of naturally grown ones. Comparative results of ten secondary metabolites tested in naturally grown plant parts were better than *in vitro* grown plantlets. The current investigation provides new inventions on the competency of *P. imbricata* and enhances the continued inquiry of medicinal orchids, in Bangladesh.

Introduction

ORCHIDS ARE one of the pleasing group of plants that flourish in a variety of habitats on the earth, but they are very sensitive to habitat switch. Due to their horticultural and medicinal value, the family is accomplishing many endeavors throughout the world to unroll the biology, evolution, taxonomy, cytology, phytochemistry, hybridization, and cultivation (Deb *et al*., 2009). Orchidaceae comprises 28,484 species in approximately 750 genera (Govaerts *et al*., 2017). The plant tissue culture technique is envisaged as a means for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large scale regeneration and for genetic manipulation studies (Sundaram *et al.,* 2011). However, seedlings developed by *in vitro* propagation are highly desirable especially for horticulture uses due to the long juvenile period before flowering (Decruse *et al*., 2003). Recently, *in vitro* asymbiotic seed germination has been reported in a few species by some authors (Anuprabha and Pathak, 2019; Bhowmik and Rahman, 2020; Lalduhsanga *et al*., 2021; Sunita *et al*., 2021; Thakur and Pathak, 2021; Vasundhra *et al*., 2021).

Phytochemical investigations of orchids were performed for alkaloid constituents (Luning, 1974) and identification and inheritance of flower pigments in orchids for ornamental value (Arditti and Fischer, 1977). Many orchids play a significant role in traditional systems of medicine because they are rich in alkaloids, flavonoids, glycosides, carbohydrates and other phytochemical contents (Hoque *et al.,* 2021; Rahman and Husen, 2003; Sanjana *et al.,* 2021). Orchids are commercially important for their glycosidal value.

Pholidota imbricata is known for ethnobotanical purposes and is used in ayurvedic practices and traditional medicine (Pant, 2013). It is an epiphytic orchid with hardy pseudobulbs and found on different tree species in the forest areas. Its biological status is Near Threatened (NT) and flowering time is from June to July (Huda, 2008). The extract of the plant is reported to have good antibacterial and antifungal properties against microorganisms like *Vibrio cholera* and *Staphylococcus aureus* (Kaushik, 2019; Marasini and Joshi, 2012). The paste of this plant species is used to treat dislocated bones with the addition of raw organic turmeric (Arditti *et al*., 1982). The whole plant is used as a tonic and the root powder is used to treat cancer; juice is used to treat skin ulcers and eruptions, and for relieving nerve, abdominal, and rheumatic pain (Arditti, 1992). The root powder is also used to induce sleep (Roy *et al*., 2007). Hence, keeping in view its medicinal importance, the present study was carried out to standardize the protocol for efficient mass propagation and to investigate the phytochemical constituents of *Pholidota imbricata.*

Material and Methods

Collection of Plant Material

In the present investigation, medicinal orchid species *Pholidota imbricata* was studied for *in vitro* germination

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and phytochemical investigation, especially for the determination of secondary metabolites such as alkaloids, phlobatannins, flavonoids, terpenoids, steroids, glycosides, quinine, coumarin, anthroquinones, saponins, and tannin content. The whole plant samples and capsules were collected from Naikhongchhari, Bandarban, Bangladesh. The seeds were used for *in vitro* germination and protocorms, callus, SPSs (shoot primordia like structures developed at the base of the shoots), shoot buds, and subsequently seedlings/plantlets were developed. Naturally grown leaf, pseudobulb, root and *in vitro* developed plant parts were used for the qualitative screening of alkaloids and other secondary metabolites.

Sterilization of Capsules

Green capsules were collected and scrubbed with Teepol (0.01%) and washed thoroughly under the running tap water for 10-15 min and then washed with sterile distilled water, two times. Capsules were dipped in 70% ethyl alcohol for 30 sec and washed thrice with double distilled water. Then the capsules were treated with $HgCl_2$ (0.1%) for 10 min for surface sterilization and thereafter rinsed three times with double distilled water. The sterilized capsules were then split open longitudinally with a sterilized blade to scoop out the seeds, under aseptic condition, in a laminar airflow cabinet.

Culture Medium and Culture Conditions

Four different media namely, MS (Murashige and Skoog, 1962) with 3% (w/v) sucrose, PM (Phytamax; Arditti, 1977), MVW (Modified Vacin and Went, 1949) and KC (Knudson, 1946) with 2% (w/v) sucrose were used for aseptic culture of seeds. The medium was gelled by using 0.8% (w/v) agar (Fluka, USA) and the pH of the media was adjusted at 5.8 in MS and 5.4 in KC, PM, and MVW media by using 0.1N NaOH or HCl. Agar was dissolved by boiling the mixture in the water bath and about 50 ml of medium was dispensed into 100 ml of each culture vessel and autoclaved (Hisense, South Korea) at 121˚C for 30 min at 15 psi pressure. The cultures were maintained at 25±2˚C temperature and exposed to 14 hrs illumination of 3500 lux intensity.

Methods for Phytochemical Screening

Preparation of Different Reagents for Qualitative Test

For the qualitative analysis of alkaloids, five alkaloid detecting reagents were used and prepared following the methods of Cromwell (1955). They were:

a) Dragendroff's reagent: Bismuth nitrate (8 g) was dissolved in Nitric acid (conc.) (20 ml). Then Potassium iodide (27.2 g) was dissolved in distilled water (50 ml). Two solutions were mixed and the mixer was allowed to stand when the Potassium nitrate was crystallized out. The supernatant was decanted off and made up to 100 ml with distilled water. The reagent was most widely used for alkaloid detection and it gives water-red turbidity or precipitation with most of the alkaloids in dilute solution.

b) Hager's reagent: Solid, yellow colored picric acid (2, 4, 6-trinitro phenol) was dissolved in distilled water up to saturation. This reagent generally produced yellow precipitates with most of the alkaloids.

c) Mayer's reagent: Mercuric chloride (1.36 g) was dissolved in distilled water (60 ml). Then it was added to a solution of Potassium iodide (5g) in 20 ml distilled water, mixed thoroughly and made up to 100 ml by addition of distilled water. This reagent is mostly used for detecting alkaloids. This reagent gave white or cloudy precipitate with the hydrochloride of most alkaloids in a very dilute solution.

d) Wagner's reagent: Iodine (2.27 g) and Potassium iodide (2g) were dissolved in 5 ml distilled water and then the solution was diluted to 100 ml. This reagent gave brown flocculent precipitates with most of the alkaloids.

e) Tannic acid reagent**:** Tannic acid (10 g) was dissolved in 100 ml distilled water. This reagent is very sensitive to most of the alkaloids and precipitates with most alkaloids. All these reagents were preserved separately in coloured reagent bottles.

Procedure for Qualitative Test

Qualitative tests for the following secondary metabolites was done

a) Alkaloids: For the qualitative tests of alkaloids, the most reliable and rapid testing method was developed by Webb (1949) and the method was slightly modified by Aplin and Canon (1971).

Procedure of Extraction and Test

Fresh finely chopped and pasted plant material (5 g) was mixed up to moisten with 10 ml HCl (2%) and heated in water bath at 60ºC for one hour. After cooling, the extract was filtered through Whatman No.1 filter paper. Two drops of extract were put on a groove slide with one drop of the alkaloid detecting reagent. The relative abundance of precipitate, if any, formed in the plant extract with the reagent was considered as an index of the quality of the presence of alkaloid and was expressed by "+", "++",'+++', and '++++' signs which mean slight , moderate, substantial and heavy amount respectively.

No precipitate was indicated by '–' (negative sign) and stood for the absence of alkaloid in the plant extract.

b) Phlobatannins: Deposition of a red precipitate when an aqueous extract of each sample was boiled with aqueous Hydrochloric acid (HCl) (1%) was taken as evidence for the presence of phlobatannins (Edeoga *et al*., 2005).

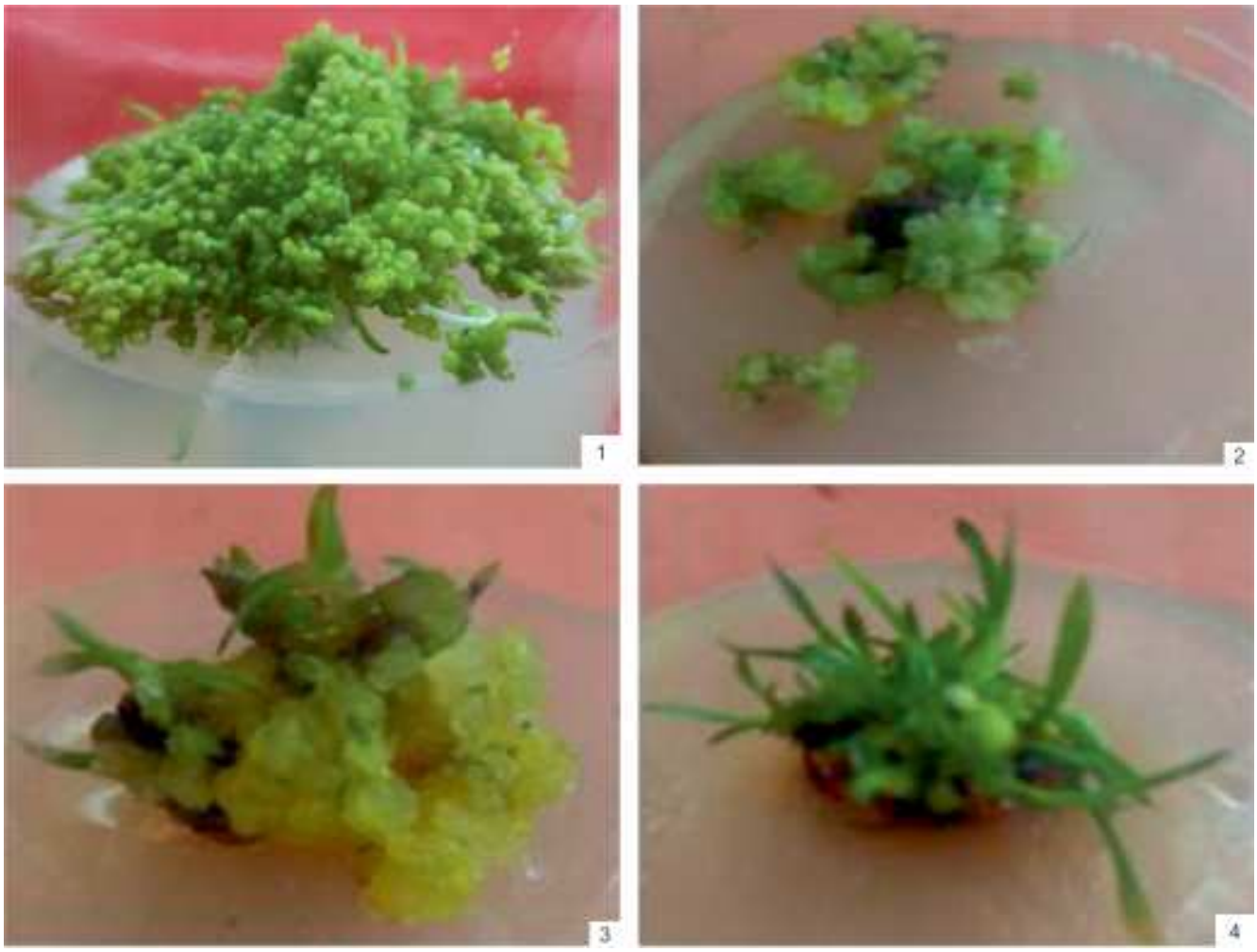
c) Flavonoids: A portion of the crude powdered plant sample was heated with Ethyl acetate (10 ml) over a steam bath for 3 min. The mixture was filtered and 4 ml of filtrate was shaken with dilute ammonia solution (1 ml). A yellow colouration was observed indicating a positive response for flavonoids (Edeoga *et al*., 2005).

d) Saponins: Crude powder (2 g) was boiled with 20 ml of distilled water in a water bath and filtered. 10 ml of filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The persistent of froth indicates the presence of saponins (Kapoor *et al*., 1969).

e) Tannin: Crude powdered (0.5 g) samples boiled in 10 ml of distilled water in a test tube and filtered. A few drops of Ferric chloride reagent added to the filtrate. A blue-black precipitate was taken as an evidence for the presence of tannins (Harborne, 1973).

f) Terpenoids: Crude powder (0.5 g) was dissolved in Methanol (5 ml). 5 ml of the extract was treated with chloroform (2 ml) in a test tube. Sulfuric acid (conc.) (7 ml) was carefully added to the mixture to form a layer. An interface with a reddish brown coloration formed if terpenoids constituent is present (Kolawole *et al*., 2006).

g) Steroids: Crude powder (0.5 g) was dissolved in methanol (5 ml). 1 ml of the extract was dissolved in Chloroform (10 ml) and equal volume of concentrated Sulfuric acid was added by sides of the test tube. The



Figs. 1-4. *In vitro* asymbiotic seed germination and seedling development in *Pholidota imbricata*: 1, Protocorm formation; 2, Callus formation; 3, Shoot bud development; 4, Seedling development.

upper layer turns red and Sulfuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids (Kolawole *et al.*, 2006).

h) Glycosides: Crude powder (0.5 g) was dissolved in Methanol (5 ml). HCl (50%) (10 ml) was added to Methanolic extract (2 ml) in a test tube. Then it was heated in a boiling water bath for 30 min. Fehling's solution (5 ml) was added to the mixture and the mixture was boiled for 5 min. A brick-red precipitate was taken as evidence for the presence of glycosides (Harborne, 1973).

i) Anthroquinones: 2 ml of solution was added with Magnesium acetate. Formation of pink color indicated the presence of Anthroquinones (Sofowara, 1993).

j) Quinine: 1 ml of extract, 1 ml of concentrated Sulfuric acid was added and was allowed to stand for some time to develop colour. Development of red colour confirmed the presence of Quinine (Sofowara, 1993).

k) Coumarin: 1 ml of extract, 1 ml of 10% NaOH was added and was allowed to stand for some time and development of yellow colour confirmed the presence of Coumarin (Sofowara, 1993).

Results and Discussion

In Vitro Seed Germination

The seeds of medicinal orchid, *Pholidota imbricata* were aseptically grown on four nutrient media namely KC, MS, PM, and MVW. The results have been presented in Table 1 and depicted in Figs. 1-4. The seeds germinated on all of the media but their germination percentage varied. The highest percentage of germination and the greenish protocorm were observed on MS (80.00%), followed by PM (73.34%), and MVW (53.34%) media. A poor germination rate was observed on the KC medium (46.67%). These results might be due to the chemical composition of MS medium which is highly enriched with macro and microelements with different vitamins that favoured seed germination and seedling development.

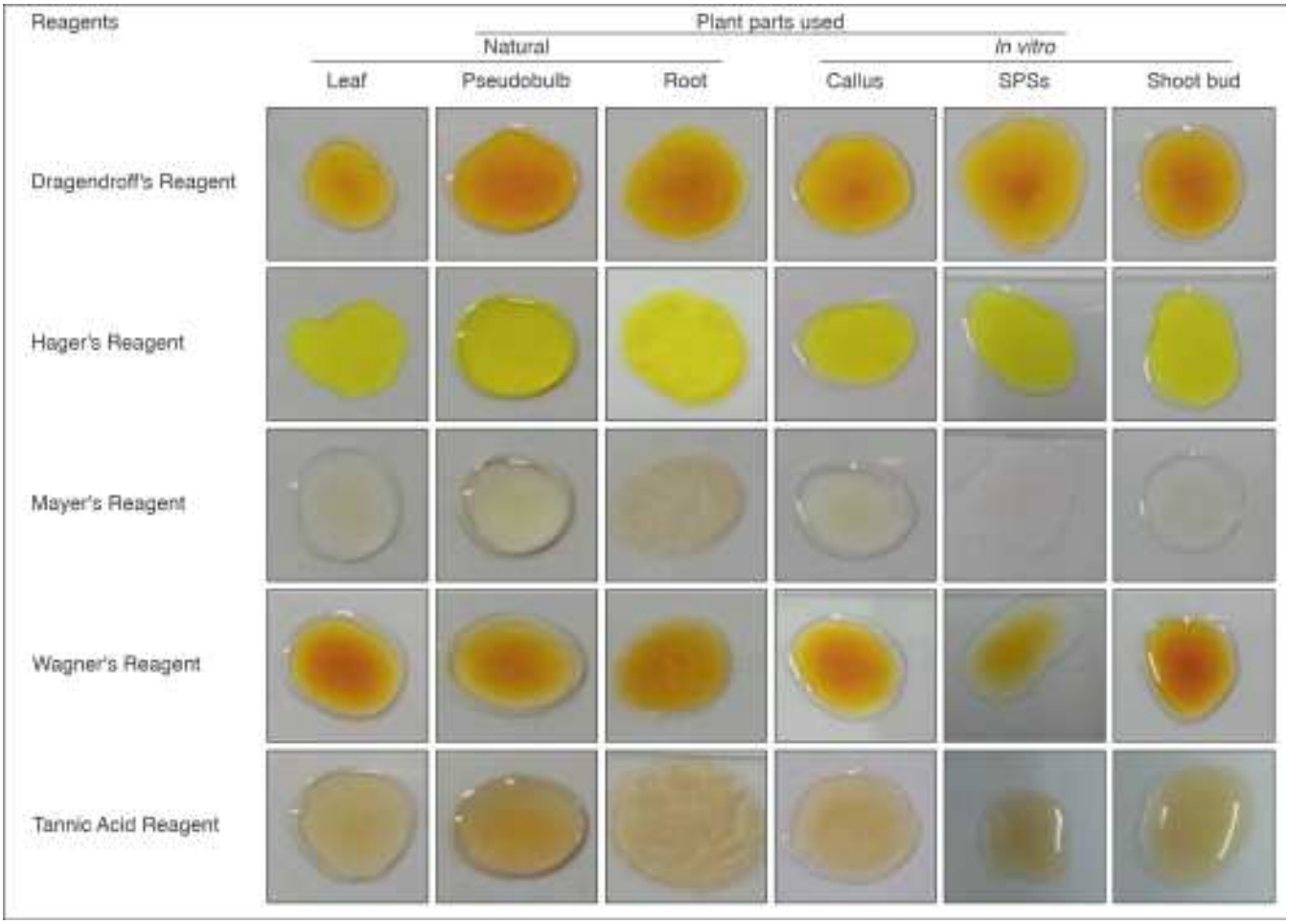


Fig. 5. Comparison of alkaloid contents of naturally grown plant parts leaf, pseudobulb, and root extract and *in vitro* developed callus, SPSs, shoot bud of *Pholidota imbricata.*

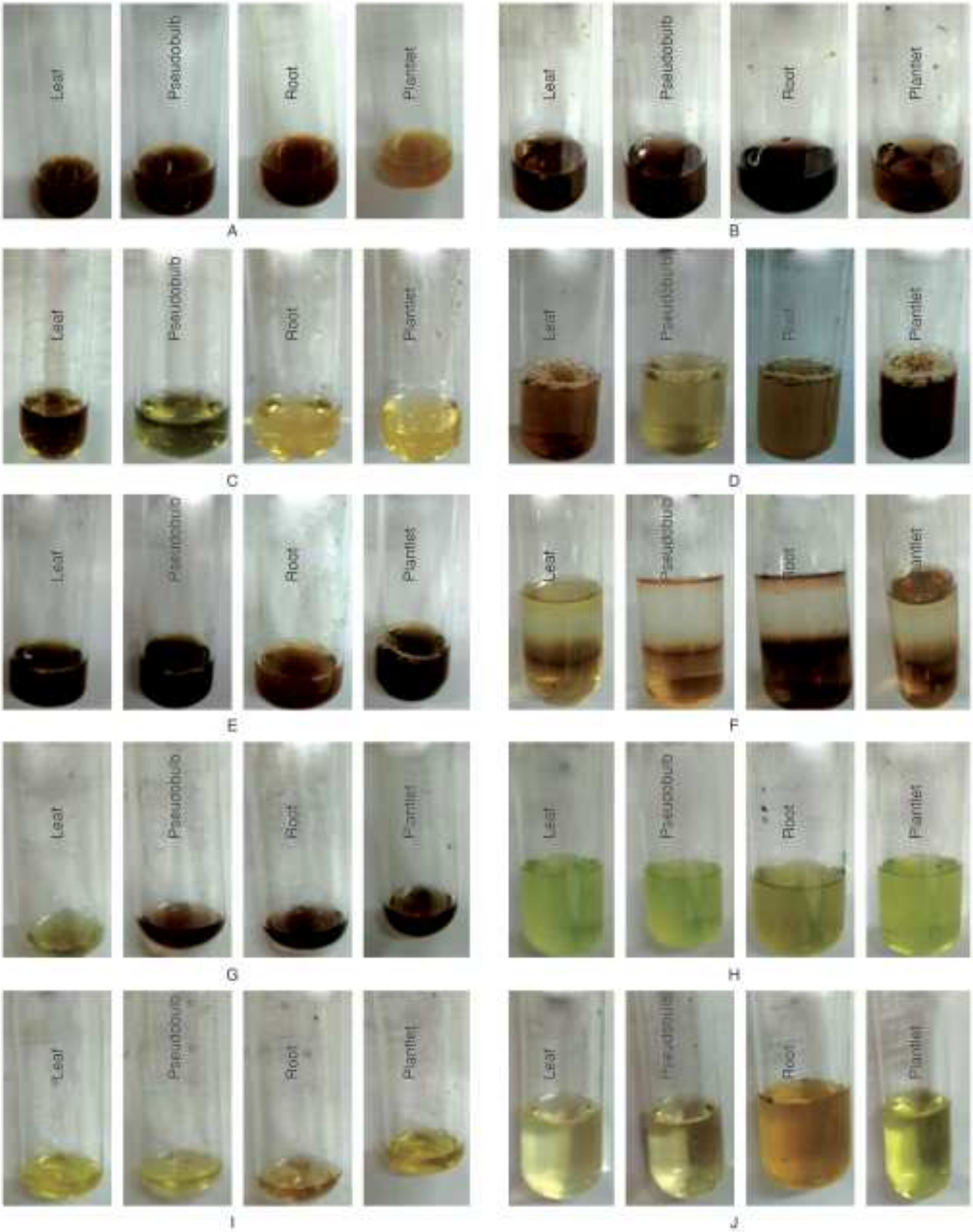


Fig. 6. A-J. Qualitative test for secondary metabolites of naturally grown plant parts leaf, pseudobulb, root, and *in vitro* developed plantlets of *Pholidota imbricata:* A, Phlobatannins test; B, Terpenoids test; C, Flavonoids test; D, Saponins test; E, Tanins test; F, Steroids test; G, Quinine test; H, Glycosides test; I, Anthroquinones test; J, Coumarin test.

A higher concentration of Nitrogen present in MS medium was required earlier for the optimal germination of seeds (Pradhan *et al*., 2013). The present observations are in conformity with the findings by Bhattacharyya and Banerjee (2020), Pant *et al.* (2011), Sunita *et al*. (2021), and Thokchom *et al.* (2017).

Table 1. *In vitro* seed germination of *Pholidota imbricata.*

Nutrient medium	Carbohydrate concentration	Number of culture vessels used	Number of culture vessels in which seeds germinated	Seed germination (%)	Protocorms colour	Remarks
KC	2% (w/v) sucrose	15	07	46.67	Yellowish white	+
MS	3% (w/v) sucrose	15	12	80.00	Greenish	+++
PM	2% (w/v) sucrose	15	11	73.34	Greenish yellow	++
MVW	2% (w/v) sucrose	15	08	53.34	Yellowish green	++

+, Minimum germination (0% d" + d" 49%); ++, Medium germination (50% d" ++ d" 74%); +++, Maximum germination (75% d" +++ d" 100%).

Qualitative Phytochemical Investigation of P. imbricata for Different Secondary Metabolites

In the present work, secondary metabolites like alkaloids, flavonoids, phlobatannins, steroids, tannins, saponins, terpenoids, and glycosides were considered for qualitative assessment. Alkaloids were assessed qualitatively with five different alkaloid detecting reagents. They were Dragendroff's (D), Wagner's (W), Mayer's (M), Hager's (H), and Tannic acid reagent (T). The results (Table 2 ;Fig. 5) indicate that plant parts of *in vitro* developed and naturally grown *P. imbricata* plants gave different responses for alkaloids test. *In vitro* developed callus, shoot buds and SPSs in *P. imbricata* revealed the highest (+++) performance in Tannic acid reagent (T). Shoot bud and SPSs also showed the highest (+++) results in Dragendroff's (D), Hager's (H), and Wagner's (W) reagent, medium (++) performance showed in Mayer's reagent (M). On the other hand, Callus showed the medium (++) responses in Dragendroff's (D) and Wagner's reagent (W), lowest (+) results in Hager's (H) and Mayer's (M) reagent.

Whereas, naturally grown plant parts *i.e.* leaf, pseudobulb, and root segments showed the highest (+++) effect in Tannic acid reagent (T). Pseudobulbs and root segments also gave the highest (+++) responses in Dragendroff's (D) and Hager's reagent (H), medium (++) amount in Wagner's reagent (W). Leaf segments gave medium (++) performance in Dragendroff's (D) and Wagner's reagent (W), lowest (+) results showed in Hager's (H) and Mayer's reagent (M). All plant parts gave positive results; based on overall results of alkaloids test, it may be concluded that *in vitro* developed plant parts showed better results than naturally grown plant samples of *P. imbricata*. Bhowmik *et al*. (2020a) noted that root and leaf sample of natural *Staurochilus ramosum* gave the highest precipitation followed by *in vitro* SPSs, callus or shoot buds, respectively. Bhowmik *et al*. (2020b) reported that naturally grown root and leaf sample of

Table 2. Qualitative phytochemical profiling (alkaloids) of naturally grown leaf, pseudobulb, root, and *in vitro* developed callus, SPSs and shoot buds of *Pholidota imbricata*.

Plant parts used		Qualitative estimation of alkaloids				
		D	H	M	T	W
Natural	Leaf	++	+	+	+++	++
	Pseudobulb	+++	+++	+	+++	++
	Root	+++	+++	++	+++	++
In vitro	Callus	++	+	+	+++	++
	Shoot bud	+++	+++	++	+++	+++
	SPSs	+++	+++	++	+++	+++

D, Dragendroff's reagent; H, Hager's reagent; M, Mayer's reagent; T, Tannic acid reagent; and W, Wagner's reagent; +++, highest result, ++, medium result, +, lowest result.

Table 3. Qualitative test of ten secondary metabolites of *Pholidota imbricata*.

Plant parts used		Secondary metabolites (% of colouration)									
		Phl.	Flv.	Sap.	Tan.	Ter.	Str.	Gly.	Ant.	Qui.	Cou.
Natural	Leaf	+++	+++	++	+++	+++	++	++	++	-	-
	Pseudobulb	+++	++	-	+++	+++	++	++	-	+++	-
	Root	+++	-	+	++	+++	+++	+++	-	+++	+++
In vitro	Plantlets	+	-	+++	+++	+++	++	++	++	+++	++

Gly., Glycosides; Flv., Flavonoids; Phl., Phlobatannins; Sap., Saponins; Tan., Tanins; Ter., Terpinoids; Str., Steroids; Ant., Anthroquinone; Qui., Quinine; Cou., Coumarin; Ph., Phenol; +++, highest response, ++, medium response, +, lowest response and -, absent.

Spathoglottis plicata gave the highest precipitation in alkaloid tests followed by *in vitro* developed SPSs, callus and shoot buds.

Different parts of *P. imbricata* gave different responses for secondary metabolite tests (Table 3; Fig. 6A-J). The naturally grown leaf samples revealed the highest (+++) results of phlobatannins, flavonoids, tannins, terpenoids; medium (++) performance of saponins, steroids, glycosides, anthroquinones and absence (-) of quinine and coumarin were observed. Pseudobulb segments showed the highest (+++) amount of phlobatannins, tanins, terpenoids, quinine; medium (++) responses of flavonoids, steroids, glycosides and absence (-) of saponins, anthroquinones and coumarin were seen. Root possessed the highest (+++) amount of phlobatannins, terpenoids, steroids, glycosides, quinines, coumarin; medium (++) results of tanins; the lowest (+) performance observed for saponins and while absence (-) of flavonoids and anthroquinones were observed. *In vitro* developed plantlets of the species showed the highest (+++) responses of saponins, tannins, terpenoids, quinines; medium (++) results of steroids, glycosides, anthroquinones, coumarin; the lowest (+) amount observed for Phlobatannins and absence (-) of flavonoids. Similar experiments were performed by Shrestha *et al.* (2015) on phytochemical screening of nepalese medicinal orchid, *Dendrobium amoenum*. They observed that plant extract showed the presence of positive results for alkaloids, terpenoids, flavonoids, tannins, and glycosides which support the present observations. Banerjee *et al.* (2018) worked to carry out pharmacognostical and phytochemical evaluation of individual root, stem and leaves of *Dendrobium ochreatum*. Bhowmik *et al.* (2020c) studied the phytochemical screening of a therapeutic orchid *Cymbidium aloifolium.* Comparative exploration of secondary metabolites of this species was done using leaf, root, and stem of naturally grown plants and *in vitro* developed plantlets.

Conclusion

MS medium proved to be the best as highest percentage of seed germination and greenish protocorms were observed followed by other nutrient media, PM, MVW, and KC. In case of alkaloid test, *in vitro* developed SPSs and shoot buds showed better results than naturally grown plant parts. Naturally grown root extract was found superior to others in terms of ten other secondary metabolites studied. The present observations provide new inventions on the competency of *P. imbricata* and enhances the continued inquiry of medicinal orchids, in Bangladesh.

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