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Comparative phytochemical investigation of natural and *in vitro* raised plant parts of *Spathoglottis plicata* Blume: A terrestrial medicinal orchid of Bangladesh

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Abstract
In the present investigation, asymbiotic germination of *Spathoglottis plicata* seeds were carried through *in vitro* methods for rapid propagation on KC, MS, PM and VW basal media. Amongst four basal media, PM medium gave the maximum response (86.87%) followed by MS (73.34%), VW (73.34%) and KC (46.67%) media. Minimum time was required for the initiation of germination on PM (10.30 ± 0.27^{a} weeks) medium, subsequently, MS (12.20 ± 0.32^{b} weeks), VW (14.73 ± 0.35^{c} weeks) and KC (16.30 ± 0.35^{d} weeks) media. The well-developed seedlings were acclimatized afterward transferred to pots in green house and watered regularly. Screening of secondary metabolites were conducted among the *in vitro* callus, shoot buds, SPSs and leaf, root, stem sample of naturally grown orchid to compare their occurrence. Naturally grown root and leaf sample of *Spathoglottis plicata* gave the highest precipitation in alkaloid tests followed by *in vitro* developed SPSs, callus and shoot buds. Anthroquinone is absent in only shoot buds and leaf while Phlobatannin is only absent in callus, shoot bud and stem. Root and SPSs possess all the tested metabolites. The occurrence of secondary metabolites in different plant parts is sporadic and uneven.

Keywords: medicinal orchid; PGRs; PLBs; secondary metabolites; *Spathoglottis plicata*; SPSs

1. Introduction
Orchids belong to the Orchidaceae family are distributed generally in the tropical parts of the world by 880 genera and 26567 species [1] and few are in the arctic region. Orchidaceae is rich with diversity of species in Asia, represented by more than 1300 species in India, 579 species in Bhutan [2], 450 species in Nepal [3] and 187 species in Bangladesh [4].
Orchids in nature regenerate through seeds but due to the deficiency of suitable hosts, the seeds do not germinate sufficiently. A single orchid capsule/pod contains millions of seeds which lacks of endosperm or functional storage foods. Because of the absence of true seed coat, they lack metabolic machinery [5] which does not let them to germinate; only 0.2- 0.3% gets germinated in environment [5], these troubles may be overcome by adopting *in vitro* tissue culture method [6-7]. The frequency of protocorm like body (PLBs) production in orchids is influenced by many factors, such as genotypes, type of explants and composition of media [8]. Nutrient composition is considered to be main source of distinction in plant tissue culture. Diverse culture media have been used for capable seed germination in orchid tissue culture [9]. Among them, PM medium was found to be able for PLBs development and leaflet regeneration [10].
Spathoglottis plicata is a terrestrial orchid species found from tropical and subtropical Asia to the western Pacific including Hawaii, Tonga and Samoa. It is the type species of the genus *Spathoglottis* [11]. It is commonly known as the Philippine ground orchid, Philippine orchid, or large purple orchid. It also occurs in Australia from Cooktown to the Jardine River on Cape York Peninsula and is listed as vulnerable [11]. It is found in seasonally inundated and other moist areas, in sunny areas near swamps, seepages and small streams. It flowers from September to April [11]. The leaves are extensively used for stypic properties in the treatment of boil and fevers by the local tribes [12]. The roots may be pounded with ginger and the mixture is extracted with water and used as medicine to cure paralysis and chronic illness. Local tribal people in the region use small seeds of plants for healing wounds [13]. In addition to this, the whole plant can also be used as tonic and in treatment of vertigo, weakness of eyes, burns and [14].
Chittagong, Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur and Sundarbans mangrove forest harbors orchid rich areas of Bangladesh; [15]. Loss of habitat, deforestation, destructive collection technique and over exploitation of orchids with medicinal and ornamental values in CHT has depleted day by day [16]. Many orchids are now at the verge of extinction, so it is high time to conduct valuable strategies to protect in nature. However, very little work has been done on orchid tissue culture and comparative phytochemical screening of both natural and *in vitro* plant parts in Bangladesh. Therefore, the present study was undertaken to expand an effective *in vitro* propagation protocol for *S. plicata* and comparative searching of the secondary metabolites in both natural and *in vitro* grown *S. plicata* to reduce the adverse pressure on natural environment.

2. Materials and Methods
Source and Collection of Materials
Mature seeds from undehisced capsules of *Spathoglottis plicata* were collected from hilly area of Khagrachari district, Bangladesh.

Sterilization of Capsules
The collected capsules were washed with running tap water for 10 min to wash away the dust and other external particles from the green capsules. The capsules were

transferred to laminar air flow cabinet; where they were surface sterilized with 3% (w/v) sodium hypochlorite (NaOCl) solution for 10 min followed by rinsing with sterile distilled water, subsequently they were treated with 70% ethanol (v/v) for 30 sec and washed with double distilled water. Then, the capsules were dipped in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 min followed by three times rinsing with sterile distilled water.

2.3. Culture medium and incubation

In the present investigation, 0.8% (w/v) agar supplemented KC [17], MS [18], PM [19] and VW [20] media were used for *in vitro* seed germination. Basal medium were fortified with 30g/l sucrose for MS (Murashige and Skoog) and 20g/l sucrose for KC (Knudson c), PM (Phytamax), VW (Vacin and Went). pH of the media was adjusted at 5.8 in case of MS and 5.4 in KC, PM and VW by using 0.1N NaOH or HCl. Agar was dissolved by boiling the mixture and about 50 ml of media was dispensed into 100 ml each culture vessel and autoclaved at 121 ^{0}C for 20 minutes at 15 lb/cm^2 pressure. All cultures were maintained at 25±2 ^{0}C under 350-500 lux illumination for 14h photoperiod using white fluorescent tubes and 10h dark.

Seed culture and Sub-culturing

Surface sterilized mature green capsules were kept on sterilized petri dish containing sterilized filter paper for drying. They were cut longitudinally with the help of sharp sterilized surgical blade. The mature seeds were scooped out with the help of sterilized spatula and transferred to and spread over the surface of different KC, MS, PM and VW basal media. Sub-culturing was carried out every 4-6 weeks into fresh medium till the protocorms grew and formed complete seedlings. The entire experiment was performed in aseptic condition under laminar air flow hood to prevent contamination.

Hardening and Transplantation

Well-developed rooted seedlings were transferred outside the environment by successive phases of adjustment. Plants were treated with auxins to induce *ex vitro* rooting and roots were treated with fungicide. Then the seedlings of *S. plicata* were transferred to plastic pots containing a potting mixture of sterilized Soil, Sand, Activated Charcoal and Pit Moss, Vermicompost at a ratio of 1 : 1 : 1 : 1 and kept in the green house (at 25-30 ^{0}C and RH 60-70%). Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well.

Computation and presentation of Data

The experiments were conducted thrice using 15 replicates per treatment. The data on different parameters were recorded after required days or weeks of culture.

Statistical analysis

Experiment was set up as a randomized complete design and all graphs were prepared with using Microsoft Excel 2013. The data were statistically analyzed, using SPSS software package. ANOVA and mean comparison were carried out by DMRT at 5% level of significance (P=0.05).

Phytochemical screening of secondary metabolites

In vitro developed plantlets and naturally grown orchids were used for the phytochemical screening of secondary metabolites. Preliminary qualitative phytochemical screening was carried out with the following methods.

Alkaloids

For qualitative test of alkaloids, the most reliable and rapid testing method was developed by Webb [21] and the method was slightly modified by Aplin and Cannon [22]. This method is known as spot test method.

Preparation of different reagents

For the qualitative test (spot test) of alkaloids, 5 alkaloid detecting reagents were used which were prepared following the standard methods [23]

a. **Dragendroff's reagent:** In 20 ml conc. Nitric acid (HNO_3), 8 g Bismuth nitrate was dissolved. Then 27.2 g Potassium iodide (KI) was dissolved in 50 ml distilled water. Two solutions were mixed and the mixer was allowed to stand when the Potassium nitrate was crystallized out. The supernatant was decants 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:** 1.36 g Mercuric acid chloride was dissolved in 60 ml distilled water. Then it was added to a solution of 5 g Potassium iodide 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 hydrochloride of most alkaloid in very dilute solution.
d. **Wagner's reagent:** 2.27 g iodine and 2 g Potassium iodide 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:** 10 g Tannic acid 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 preserved separately in colored reagent bottles.

Procedure of extraction and test

5 g fresh finely chopped and pasted plant material was mixed up to moisten with 10 ml 2% HCl and heated in water bath at 60ºC for one hour. After cooling the extract was filtered through Wathmann No.1 filter paper.

Two drops of extract were put on a microscopic 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 the lowest, moderate and the highest amount respectively. No precipitate was indicated by '-' (negative sign) and stood for the absence of alkaloid in the plant extract.

Flavonoids: 1 ml of extract was dissolved in diluted NaOH and then HCl was added. A yellow solution that turns colorless indicates the presence of flavonoids [24].

Saponins: 5 ml of extract will be mixed with 20 ml of distilled water and then were agitated in a graduated cylinder for 15 minutes. Formation of foam was indicating the presence of saponins [25].

Tannins: 2 ml of extract will be added to few drops of 1% lead acetate. A yellowish precipitate was indicating the presence of tannins [26].

Terpenoids: 2 ml of extract will be added to 2 ml of acetic anhydride and concentration of H_2SO_4. Formation of blue, green rings was indicating the presence of terpenoids [27].

Steriods: 1 ml of the extract was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids [27].

Phenols: phenols are tested by adding 2 ml of ferric chloride solution to 2 ml of plant extract. Appearance of bluish green colour solution indicates the presence of [28].

Leucoanthocyanins: 5 ml of aqueous extract will be added to 5 ml of isoamyl alcohol. Upper layer appears red in colour were indicate for presence of leucoanthocyanins [29-30].

Glycosides: 1 ml of the extract, 1 ml of alpha napthol was added to which chloroform was added along the sides and it was looked for the development of color and the result was recorded. Development of violet color indicates the presence of glycosides [31].

Anthocyanins: 2 ml of aqueous extract were added to 2 ml of 2N HCl and ammonia. The appearance of pink-red turned into blue-violet was indicating the presence of anthocyanins [29].

Coumarins: 3 ml of 10% NaOH was added to 2 ml of aqueous extract formation of yellow colour indicates the presence of coumarins [31].

Phlobatanins: 1 ml of extract was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl solution. Red precipitate indicates the presence of phlobatanins [24].

3. Results and Discussions

Table-1 represents the response of *Spathoglottis plicata* seed cultured on KC, MS, PM and VW media supplemented with 0.8% (w/v) agar. Remarkable differences were displayed by all the cultured media in terms of frequency of germination and quality of the protocorms. Overall results indicate that PM (86.87%, Fig. 1) was greater to MS (73.34%), VW (73.34%), KC (46.67%) media correspondingly, in respect of requisite time and the percentage of germination. Least time needed for *in vitro* germination on PM (10.30 ± 0.27^a weeks) medium followed by MS (12.20 ± 0.32^b weeks), VW (14.73 ± 0.35^c weeks) and KC (16.30 ± 0.35^d weeks) media. However, the percentage of seed germination is equal in MS and VW media but the minimum time is required in MS (12.20 ± 0.32^b weeks) than VW (14.73 ± 0.35^c weeks) medium. In the PM medium, the germinated protocorms continued normal growth and produced healthy seedlings after subsequent subcultures. Similar findings was noted in *Cymbidium aloifolium* [32]; *Dendrobium aphyllum* [33]; *Arundina graminifolia* [34]; *Calanthe densiflora* [35]; *Dendrobium transparens* [36] and *Micropera obtusa* [37] orchid species. PM media is enriched with vitamins and organic additives. Addition of vitamins and additives into the medium was reported to be enhanced for seed germination and seedling growth of many orchids. Peptone in media enhances the germination rate and also favours the healthy protocorm development. Our results indicate that selection of medium is an important aspect of success in asymbiotic germination of this orchid species.

The *in vitro* developed protocorms on the basal media produced callus (Fig. 2) or shoot buds (Fig. 3) on subsequent subculture in the same media. But when the tiny seedlings were grown on with various combinations, concentrations of PGRs (BAP, Kn, NAA and IAA) supplemented MS medium gave differential responses and produce SPSs (Shoot Primordia Like Structures, Fig. 4) at the base of the seedlings.

Table 1: *In vitro* germination of seeds of *Spathoglottis plicata* Blume

Nutrient medium	Carbohydrate concentration	Number of culture vessels used	Number of culture vessels in which seeds germinated		Time (weeks) required for germination (Mean ± SE)	Remarks
			No.	%		
KC	2% (w/v) sucrose	15	07	46.67	16.30 ± 0.35^d	Yellowish White PLBs
MS	3% (w/v) sucrose	15	11	73.34	12.20 ± 0.32^b	Greenish PLBs
PM	2% (w/v) sucrose	15	13	86.87	10.30 ± 0.27^a	Yellowish green PLBs
VW	2% (w/v) sucrose	15	11	73.34	14.73 ± 0.35^c	Yellowish PLBs

Values represent mean ± SE of each experiment consist of 15 replicates. Mean values followed by different superscript letters within a column are significantly different at p = 0.05 according to DMRT.

Rooted well developed plantlets were transferred from culture room to the green house during successive phase of acclimatization. For this purpose, the culture vessels were kept open for one day in the culture room and then kept outside of the culture room for 6h in the next day. On the third day, those were kept outside of the culture room for 12h. Finally, the seedlings were taken out of the culture vessels and rinsed with running tap water for removal of agar attached to the roots. Then the seedlings were transferred to plastic pots containing a potting mixture of sterilized Soil, Sand, Activated Charcoal and Pit Moss, Vermicompost at a ratio of 1: 1: 1: 1 and kept in the green house (at 25-30 ^{0}C and RH 60-70%). Transplanted seedlings were watered regularly for about 2-3 months where the seedlings standard and grew well.

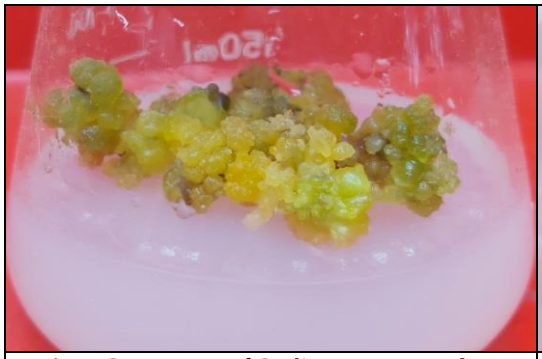

Fig 1: Germination of *S. plicata* on PM medium

Fig 2: Callus of *S. plicata*

Fig 3: Shoot buds of *S. plicata*

Fig 4: SPSs of *S. plicata*

Among the tested secondary metabolites, firstly alkaloids were tested in the *in vitro* and naturally grown plants with five alkaloid detecting reagents i.e. Dragendroff's reagent (D), Hager's reagent (H), Mayer's reagent (M), Wagner's reagent (W) and Tannic acid reagent (T). The presence of relative alkaloid contents in the extract of test plants or their organs were expressed by '+' sign ranging in the order of '+', '++' and '+++' signifying its presence in degrees ('+' minimum to '+++', the highest quantity). Absence of alkaloids was denoted by '-' sign.

SPSs of *Spathoglottis plicata*, indicated '+++' response in Hager's (H), Wagner's reagent (W), Tannic acid (T) whereas indicated '++' response in Dragendroff's (D), Mayer's reagent (M). In case of shoot buds the response was observed '+' in Mayer's reagent (M) '++' in Dragendroff's (D), Wagner's (W), Hager's (H), Mayer's reagent (M), Tannic acid (T). Subsequently, callus responded '+++' in Hager's reagent (H), '++' in Dragendroff's (D), Mayer's (M), Wagner's reagent (W), Tannic acid (T). In case of natural plant parts; Leaf sample responded '+++' in Hager's (H), Mayer's (M), Wagner's reagent (W), Tannic acid (T) '++' in Dragendroff's reagent (D); whereas, natural root sample gave '+++' response in Hager's (H), Mayer's (M), Wagner's reagent (W) and Tannic acid (T) and '++' response in Dragendroff's reagent (D). In case of stem samples, the response was '+++' in Hager's (H), Wagner's reagent (W) and Tannic acid (T); '++' in Dragendroff's reagent (D), '+' in Mayer's reagent (M). So, in both cases, the species gave positive response for alkaloid test. This result showed that, naturally grown root and leaf sample of *Spathoglottis plicata* gave the highest precipitation followed by *in vitro* developed SPSs, callus and shoot buds (Table 2).

Hossain *et. al.* [38] while screening the alkaloids in the methanolic extract of leaves R. retusa reported the highest response in Dragendroff's reagent and tannic acid; a moderate response showed in Wagner's reagent, while the lowest response was observed in Hager's reagent and Mayer's reagent. Similarly, the root extract of R. retusa showed the highest response in tannic acid and a moderate response in Dragendroff's and Wagner's reagent, while in other reagents it showed the lowest results. The methanolic extracts of leaves and stems of L. zeylanica showed the highest results in tannic acid and Wagner's reagent, a moderate result in Mayer's reagent and the lowest results in other reagents. In the case of P. teres, the methanolic extracts of leaves and stems showed the highest result in Dragendroff's reagent, Hager's reagent and tannic acid and a moderate result in Mayer's reagent. These findings indicated the degree of presence of alkaloids in different plant parts also. This finding is an agreement with the finding of the present investigation.

Table 2: Phytochemical profiling (alkaloids) of *Spathoglottis plicata.*

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

Notes: Name of the reagents- D- Dragendroff's reagent, H- Hager's reagent, M- Mayer's reagent, T- Tannic acid reagent and W- Wagner's reagent. Here, "+++" means highest result, "++" means medium result," +" means lowest result.

Other than alkaloids, qualitative assessment of ten other secondary metabolites, e.g. Flavonoids, Saponins, Tanins, Terpinoids, Steroids, Phenol, Leucoanthocyanins, Glycosides, Anthocyanins, Coumarin and Phlobatannins for both *in vitro* and naturally grown *Spathoglottis plicata*. In this experiment, *in vitro* grown *Spathoglottis plicata* gave three '-' response in Phlobatannin (callus, shoot bud); Anthroquinone (shoot bud) followed by natural leaf sample in Anthroquinone and stem sample of Phlobatannin test. All *in vitro* and natural sample gave low responses (-/+) in

Phlobatannin test and highest responded '+++' in Terpinoids test (Table 3). Naturally grown root sample was found best for responding positive against all tested metabolites. followed by leaf and stem samples. *In vitro* developed SPSs were more effective than *in vitro* developed callus and shoot bud in containing metabolites. So, there are little differences in the presence or absence of these secondary metabolites yet they have grown in different environment.

Similar data was also observed by Hossain *et. al.* [38] while screening the phytochemicals of three orchid species of Bangladesh. They observed that both the leaves and root extracts of *R. retusa* showed the highest result in steroid, tannin and xanthoproteic tests and a moderate result in cardiac glycoside, glycoside, phlobatannin, flavonoid, carbohydrate and terpenoid tests. In the case of L. zeylanica, the highest result was recorded by them in steroid and xanthoproteic tests and a moderate result in phytosterol, flavonoid, tannin, carbohydrate and terpenoid tests. On the other hand, *P. teres* showed the highest result in steroid and xanthoproteic tests and a moderate result in phytosterol, tannin, carbohydrate and terpenoid tests. This finding is substantial to the finding of the present investigation.

Table 3: Phytochemical profiling (other than alkaloids) of *Spathoglottis plicata.*

Plant parts used		Secondary metabolites (% of coloration)										
		Flv.	**Sap.**	**Tan.**	**Ter.**	**Str.**	**Ph.**	**Leu.**	**Gly.**	**Ant.**	**Cou.**	**Phl.**
In vitro	SPSs	++	+++	+++	+++	++	+++	+++	++	++	++	+
	Callus	++	++	++	+++	++	+++	+++	++	++	+	-
	Shoot buds	++	++	++	+++	++	++	++	++	-	+	-
Natural	Root	+++	++	+++	+++	++	+++	+++	++	++	+	+
	Leaf	+++	+++	+++	+++	++	+++	+++	++	-	+++	+
	Stem	++	++	+++	+++	+++	+++	+++	+++	+++	+	-

Notes: Flv. = Flavonoids, Sap. = Saponins, Tan. = Tanins. Ter. = Terpinoids, Str. = Steroids, Ph. =Phenol, Leu = Leucoanthocyanins, Gly. = Glycosides, Ant. = Anthroquinone, Cou. = Coumarin, Phl. = Phlobatannins. Here, "+++" means highest response, "++" means medium response, "+" means lowest response and "-" means absent.

4. Conclusions

PM was found better than KC, MS & MVW media for promoting germination of orchid seeds. Sucrose supplemented media was found to be best whereas lactose containing medium gave at least performance. For comparing the effectiveness in terms of enhancing seedling, liquid culture was better than agar solidified condition. Increased in root length and number of roots is higher in IAA supplemented full strength MS media. However, the *ex situ* conservation of this species is highly recommended not only for its conservation but also to best utilized its commercial demand. The present work makes the plant a candidate for bioprospecting for phytochemicals. Results also support the folkloric usage of this plant as a therapeutic agent. Further phyto-chemical studies were required to determine the type of compound responsible for different bioactivity, which could serve as a useful source for new bioactive agent.

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