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Tapash Kumar Bhowmik
Assistant Professor, Department of Botany, University of Chittagong, Chittagong, Bangladesh

Minhajur Rahman
Assistant Professor, Department of Botany, University of Chittagong, Chittagong, Bangladesh

Md. Mahbubur Rahman
Professor, Department of Botany, University of Chittagong, Chittagong, Bangladesh

Corresponding Author:
Tapash Kumar Bhowmik
Assistant Professor, Department of Botany, University of Chittagong, Chittagong, Bangladesh

Phytochemical screening of a therapeutic orchid *Cymbidium aloifolium* (L.) Sw. from its wild and *in vitro* origin: A comparative study

Tapash Kumar Bhowmik, Minhajur Rahman and Md. Mahbubur Rahman

Abstract
In the present investigation, *in vitro* seed germination of *Cymbidium aloifolium* (L.) Sw. was assessed on 0.8% (w/v) agar solidified KC, MS, PM, VW media. Here, 3% (w/v) sucrose was applied in MS medium while KC, PM, VW media were prepared with 2% (w/v) sucrose. MS medium proved to be best (93.34%) for early germination and greenish PLBs formation followed by PM (86.87%), VW (73.34%) and KC (73.34%) media. Minimum time needed for *in vitro* germination on MS (9.33 ± 0.33^{a} weeks) medium followed by PM (10.47 ± 0.31^{b} weeks), VW (11.34 ± 0.31^{c} weeks) and KC (12.37 ± 0.26^{d} weeks) media. However, the percentage of seed germination is equal in KC and VW media but the time is required minimum in VW compared to KC medium. The well-developed rooted seedlings were acclimatized and subsequently transferred to pots in green house with regular watering. Comparative exploration of secondary metabolites were done using *in vitro* callus, shoot buds, SPSs and leaf, root, stem sample collected from the nature. Natural leaf, root, stem sample of *Cymbidium aloifolium* exhibited the presence of maximum metabolites followed by *in vitro* developed SPSs, callus and shoot buds.

Keywords: *Cymbidium aloifolium*, phytochemical screening, PGRs, PLBs, SPSs

Introduction
Orchids exhibits a broad range of diversity in form, size, colour and texture of flowers beyond the mind's eye of human mind [1]. Due to their ornamental and medicinal significance they are highly precious in the international market. Orchidaceae is one of largest families of flowering plants, comprising 25,000-35,000 species to which more and more new ones are being added every year [2] of which Bangladesh comprises 187 species [3]. Chittagong, Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur and Sundarbans mangrove forest are orchid rich areas of Bangladesh [4]. Many orchids are now at the margin of extinction, so it is high time to carry out efficient strategies to conserve in nature.

Orchid seeds, distinct the seeds of other flowering plants are extremely small, dust like, produced in large numbers (1300-4,00,000/capsule) and with little or no stored food [5]. Orchid seeds have exceptional physiology of germination. Under natural condition, fungal aid mycorrhiza usually species of *Rhizotectonia* is required. In nature, the germination rate is very poor (5%) and takes long time [5]. Therefore highly exploited species instantly require *ex situ* conservation by tissue culture method. During the last few years tissue culture technique have been extensively exploited for the large scale propagation of many orchid species.

Several orchids growing in the region possess high ornamental and medicinal values even though many of them have been listed as endangered in Red Data Book. The alarming reduction in orchid population is due to extensive collection for illegal orchid trade and habitat destruction largely because of the "Jhum cultivation" an age old agricultural practice adopted by local tribes of the CHT region. Loss of habitat, deforestation and destructive collection technique [3, 6] and over exploitation of orchids with medicinal and ornamental values has depleted the orchid wealth of Bangladesh. Many orchids are now at the verge of extinction, so it is high time to conduct effective strategies to conserve this precious jewelry of nature.

Cymbidium aloifolium, the aloe-leafed *cymbidium*, is an orchid species of Bangladesh also available in Asia, especially China and Southeast Asia from Burma to Sumatra. It can be found growing between rocks or on another plant.

The word *Cymbidium* comes from the Greek *kumbos* meaning "hole, cavity" and the Latin specific name is just a translation of the English "aloe-leafed". *C. aloifolium* is one of the horticulturaly as well as medicinally important epiphytic orchid of CHT of Bangladesh. The plant is reported to have emetic and purgative properties [7]. Paste of pseudobulb and leaves is used as tonic and used over fractured or dislocated bones. The leaves are also extensively used in the treatment of boils, fever and other inflammatory conditions [7]. This orchid is highly demanded in floriculture market because of its exquisite highly intricate beautiful flowers. Indiscriminate collections by orchid lovers, habitat destruction and over exploitation for medicinal purposes are the main factors that have threatened the survival of this species. Therefore conservation of this orchid is now a matter of universal concern. Tissue culture technique has been widely used for the *in vitro* mass propagation of several commercially important orchids [8].

The plant derived natural products have been used as source of herbal drugs for over 40000 years for partial treatment of various diseases [9]. People since time immemorial have largely been dependent on locally available plants for cure and temporary relief from various forms of ailments as they possess compounds of enormous therapeutic values [10]. The local medicinal practitioners use the medicinally important indigenous plants for traditional healing and curing purposes. Though there have few instances of employing orchids by traditional healers for therapeutic treatment in CHT, the practice has not been widely adopted in spite of the region being bestowed with rich orchid resources. The limited use of orchids for medicinal and therapeutic purposes may stem from the ignorance of local people towards the usefulness of plants in indigenous system of treatment and remedies.

For *ex situ* conservation *in vitro* medicinal orchid plantlets production has a great value and also been used in commercial firm for various purposes [11-12]. Various bioactivity tests along with the screening of the secondary metabolites [13-14] of both *in vitro* and naturally grown medicinal orchids has been opened a new research avenue in Biological Science. Some research articles have been published on this area of research [15-19] but no systematic comparison of phytochemicals available in both *in vitro* and natural medicinal orchids of Chittagong Hill Tracts has been carried out yet.

In the present study *in vitro* seed germination and seedlings development method was conducted for the rapid propagation and comparative searching of the secondary metabolites in both natural and *in vitro* grown plant parts of medicinally and horticulturaly important orchid *C. aloifolium*.

Materials and Methods

Seed germination of indigenous orchid *Cymbidium aloifolium* was carried out in the Plant Tissue Culture and Biotechnology Laboratory, Department of Botany, University of Chittagong. The materials used for the *in vitro* propagation were the mature green capsules of *C. aloifolium* which were collected from Bandarban, Bangladesh.

The dust in the capsules collected from the naturally grown orchid was removed. The mature green capsules was washed with tap water containing few drops of teepol solution for few minutes and washed under running tap water for five minutes. The capsules were surface sterilized by immersing it in the solution of 0.1% $HgCl_2$ for 10 minutes followed by 70% ethanol for 30 second and finally rinsing three times with sterile distilled water.

Four basal media namely, KC [20]; MS [21]; PM [22] and VW [23] were used for seeds germination. 3% (w/v) sucrose used in MS medium, while 2% (w/v) sucrose were used in KC, PM and VW media. The pH of all media was adjusted to 5.8 in MS medium and 5.4 in KC, PM, VW media using 0.1N NaOH or HCl before mixing agar. 0.8% (w/v) agar was added as a gelling agent and dissolved by boiling. 100 ml of the media were dispensed into 250 ml culture bottles and autoclaved at 121^0C for 20 minutes at 15 lbs pressure. The experiment was conducted under aseptic condition and the cultures, incubated at 25 ± 2 ºC were subjected to 14 hr photoperiod at 4000-5000 lux intensity and 60% humidity level were maintained regularly [24]. For subsequent development of the seedlings, they were subcultured on respective media at different intervals.

For the inoculation of seeds, mature green capsule was put on sterile petridish containing sterile filter paper and cut longitudinally using a sharp sterile blade under laminar air flow cabinet. The very tiny seeds were scooped out with the help of sterile forceps and spread over the surface of the germination media. After germination seeds were turned to PLBs (Protocorn Like Bodies) and PLBs further differentiated into clumps and each developing into new callus or plantlets. In order to induce quick elongation and increase growth, germinated PLBs will be transferred to new media and produce a good number of callus or shoot buds. Callus was further subcultured at lower density in the same medium, produced plantlets. When the minute seedlings cultured on PGRs (Plant Growth Regulators) supplemented MS medium for healthy seedlings development and produce SPSs (Shoot Primordia Like Structures) at the base of seedlings. The well-developed seedlings were taken out of the culture vessels and successfully transferred to outside the culture room following successive phases of acclimatization. Transplanted seedlings were watered frequently for about 2-3 months where the seedlings recognized and grew well in the Orchidarium.

Phytochemical screening of secondary metabolites

In vitro and naturally grown plant parts of *C. aloifolium* were used for the qualitative screening of secondary metabolites. The 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 [25] and the method was slightly modified by Aplin and Cannon [26]. 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. These reagents were prepared following the standard methods [27].

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 60 ºC 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 [28].

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 [29].

Tannins: 2 ml of extract will be added to few drops of 1% lead acetate. A yellowish precipitate was indicating the presence of tannins [30].

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 [31].

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 [31].

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 [32].

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 [33-34].

Coumarins: 3 ml of 10% NaOH was added to 2 ml of aqueous extract formation of yellow colour indicates the presence of coumarins [35].

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 [33].

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 [28].

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 [30].

Results and Discussions

The seeds of *Cymbidium aloifolium* were aseptically grown on 0.8% (w/v) agar solidified KC, MS, PM and VW media (Table 1). Overall results proved that, MS (93.34%, Fig. 1) was superior to PM (86.87%), VW (73.34%), KC (73.34%) media respectively, in respect of required time and the percentage of germination. Minimum time required for *in vitro* germination on MS (9.33 ± 0.33^{a} weeks) medium followed by PM (10.47 ± 0.31^{b} weeks), VW (11.34 ± 0.31^{c} weeks) and KC (12.37 ± 0.26^{d} weeks) media. Though, the percentage of seed germination is equal in KC and VW media; the time was required minimum in VW (11.34 ± 0.31^{c} weeks) than KC (12.37 ± 0.26^{d} weeks) medium. Each plant species has specific nutritional requirements, for its seed germination and plant regeneration. Such species medium specificity for germination of orchid seeds has been noted in in *Erythrodes humilis* [36]; *Cymbidium aloifolium* and *Spathoglottis plicata* [37], *Arundina graminifolia* [38], *Phalaenopsis amboinensis* [39] and *Dendrobium officinale* [40]. MS media is enriched with macronutrients, micronutrients and vitamins which are enhanced for seed germination and seedling development of many orchids [41-42]. In MS medium, PLBs are deep green in colour whereas, in PM medium are produce yellowish green PLBs.

The *in vitro* developed PLBs on the same basal media produced callus (Fig. 2) or shoot buds (Fig. 3). 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 *Cymbidium aloifolium* (L.) Sw

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	11	73.34	12.37±0.26^{d}	Yellowish green PLBs
MS	3% (w/v) sucrose	15	14	93.34	9.33±0.33^{a}	Greenish PLBs
PM	2% (w/v) sucrose	15	13	86.87	10.47±0.31^{b}	Yellowish green PLBs
VW	2% (w/v) sucrose	15	11	73.34	11.34±0.31^{c}	Greenish yellow 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

Well-developed and rooted plantlets were transferred from culture room to the outside environment through 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 small brick, coal pieces, saw dust and peat moss at a ratio of 1 : 1 : 1 : 0.5 and kept in the green house (at 25-30 ^{0}C and RH 60-70%). Transplanted seedlings were watered frequently for about 2-3 months where the seedlings recognized and grew well.

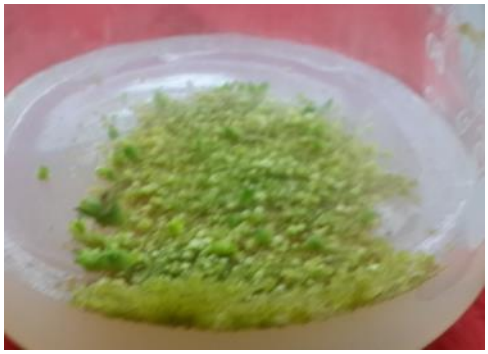

Fig 1: Germination of *C. aloifolium* on MS medium

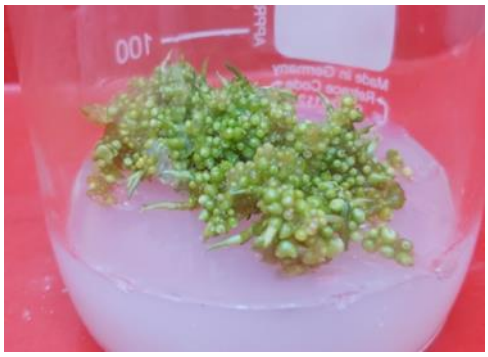

Fig 2: Callus of *C. aloifolium*

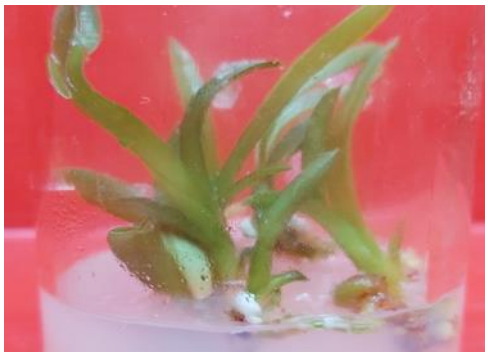

Fig 3: Shoot buds of *C. aloifolium*

Fig 4: SPSs of *C. aloifolium*

Secondary metabolites including alkaloids were tested in the *in vitro* developed and naturally grown plants of this species with five alkaloid detecting reagents such as 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 it's presence in degrees ('+' minimum to '+++', the highest quantity). Absence of alkaloids was denoted by '-' sign.

In alkaloid detecting tests (Table 2), the *in vitro* grown SPSs of *Cymbidium aloifolium* exhibited '+++' response in Tannic acid (T); '++' response in Dragendroff's (D), Hager's (H), Wagner's (W) and Mayer's reagent (M); while the shoot bud responded '++' in Tannic acid (T) and '+' in Mayer's (M), Hager's (H), Dragendroff's (D), Wagner's(W) reagents. On the other hand, callus responded '++' in Dragendroff's reagent (D), Tannic acid (T) and '+' in Mayer's (M), Hager's (H), Wagner's (W) reagents.

Naturally grown Leaf samples of this species responded '+++' in Dragendroff's (D) and Tannic acid (T); '++' in Hager's (H) and Wagner's reagent (W) and '+' in Mayer's reagent (M). On the other hand, root samples gave '+++' in Dragendroff's (D); '++' in Hager's (H), Mayer's reagent (M) and Tannic acid (T) and '+' in, Wagner's reagent (W). Subsequently, stem sample showed '++' response in Tannic acid (T) and Wagner's (W); '+' in Dragendroff's (D), Hager's (H) and Mayer's reagent (M). This finding corroborates with [43] who also found maximum response for alkaloid in *Pholidota pallida* leaf.

In the qualitative test of alkaloids (Table 2) Dragendroff's, and Tannic acid reagents appeared more effective to which plant extracts exerted their moderate (3+) response. While, Wagner's, Hager's and Mayer's reagents seemed to be less effective to which they responded mild (2+ and +).

Table 2: Phytochemical profiling (alkaloids) of *Cymbidium aloifolium* (L.) Sw

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

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

In addition to alkaloids, qualitative assessment of ten other secondary metabolites, e.g. Flavonoids, Saponins, Tanins, Terpinoids, Steroids, Phenol, Leucoanthocyanins, Coumarin, Anthocyanins, Phlobatannins and Glycosides from both *in vitro* developed and naturally grown *Cymbidium aloifolium* plant parts were done respectively (Table 3).

In this experiment, Phlobatannin was absent in leaf, stem and shoot buds (*in vitro*) while Glycosides was recoded absent in leaf and callus (*in vitro*). In contrast, both *in vitro* developed and naturally grown plant parts displayed presence of Flavonoids, Saponins, Tanins, Terpinoids, Steroids, Phenol, Leucoanthocyanins, Coumarin, Anthocyanins, Phlobatannin and Glycoside ranging from '+++' to '+' which indicted that the contents of phytochemicals in natural and *in vitro* derived plants were rich in bioactive compounds and the *in vitro* plantlets can be considered as the alternate for the wild plants in terms of therapeutic purposes [44].

The present experiment also substantiates with [43] who reported that flavonoids, tannins and quinine were present highly whereas saponins, steroids and coumarin were present moderately in the extract of *Eria tomentosa* leaves. On the other hand, saponins, tannins, steroids, glycosides and coumarin were found to be highly positive in the extract of bulbs. Furthermore, phlobatannins, tannins, terpenoids, steroids, glycosides, quinine and coumarin were remarkably present in the extract of roots of the studied orchid.

Table 3: Phytochemical profiling of *Cymbidium aloifolium* (L.) Sw. (other than alkaloids)

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

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

Conclusions

MS was found superior than KC, MS and VW media for promoting germination of orchid seeds. However, the *ex situ* conservation of this species is highly recommended not only for its conservation but also to best utilized its commercial demand. Naturally grown leaf, root, stem sample of *Cymbidium aloifolium* gave the highest precipitation followed by *in vitro* developed SPSs, callus and shoot buds, respectively. All *in vitro* and natural sample gave poor responses (-/+) in Phlobatannin test and highest responded '+++' in Tanins and Terpinoids test.

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