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# Exploration of phytochemical composition of *Staurochilus ramosum* (Lindl.) Seidenf. from nature and *in vitro* grown plant parts

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**Abstract**
Seeds of medicinally important orchid *Staurochilus ramosum* (Lindl.) Seidenf. cultured on four 0.8% (w/v) agar solidified KC; MS; PM and VW basal media. The highest seed germination (86.77%) was achieved on PM medium followed by MS (80.00%); VW (66.67%) and KC (60.00%) media. Time required minimum for seed germination was recorded on PM (10.30±0.24$^{a}$ weeks) followed by MS (11.20±0.33$^{b}$ weeks), VW (12.73±0.29$^{c}$ weeks) and KC (14.30±0.31$^{d}$ weeks) media. The well-developed rooted seedlings were transferred to outside environment by successive phases of acclimatization and watered regularly. *In vitro* developed callus, SPSs, shoot buds and root, stem, leaf of naturally grown orchid were used for comparative phytochemical screening. Root and leaf sample of natural *Staurochilus ramosum* gave the highest precipitation followed by *in vitro* SPSs, callus or shoot buds, respectively. All *in vitro* and natural sample gave mild responses (-/+) in Phlobatannin test and responded strong (+++) in Tanins and Terpinoids test. Naturally grown root exhibited best responses in secondary metabolite tests followed by stem and leaf sample.

**Keywords:** PGRs; PLBs; SPSs; secondary metabolites; *Staurochilus ramosum*

## 1. Introduction

Orchids with over 25,000 species represent one of the most advanced and largest families of angiosperms with innumerable; more than 17,000 hybrids and varieties [1]. Their distribution is found around the globe except the freezing Antarctic region and deadly hot desert areas [2]. In Bangladesh, it represents one of the largest groups of flowering plants and 177 species with a variety under 70 genera [3]; distributed throughout the country, especially in Chittagong, Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur and Sundarbans mangrove forest [4].

They have extremely high floricultural appeal because of their extraordinarily beautiful and highly enchanting flowers with incredible range of variation in floral shape, size, coloration and fragrance [5]. Besides their high ornamental values and expensive price, they command in the international floricultural market, orchids are of considerable importance in medicines as they have high contents of alkaloids, glycosides, phenols and useful phytochemicals [6]. Their therapeutic values have been extensively utilized in the indigenous system of medicine for the treatment of many ailments in different regions [7].

The orchids produce numerous, microscopic, non-endospermic and poorly developed seeds which lack proper metabolic machinery to directly utilize their own lipidaceous food reserves and require a suitable fungal stimulus for germination in nature [8]. Less than 1% seeds contact a suitable substrate to germinate in nature [8]. The technique for germinating the orchid seeds at the base of mother plant was in vogue, till in 1904 Bernard [9] suggested and successfully tested the possibility of germinating orchid seeds in the laboratory using an appropriate fungus. The technique of asymbiotic seed germination has added a new vista in orchid propagation and a large number of orchid species have been successfully germinated *in vitro* [10, 14].

The Chittagong Hill Tracts is one of the vast reserves of different species of flora and fauna including many medicinal orchids [15]. Medicinal orchids are highly valuable in respect to their healing capacity with low or zero side effects. A number researches of medicinal orchids have done or in progress. Most of these are related to plants contain different bioactive molecules of extra nutritional constituent's survey, collection and screening-based research. But in most cases of such research, it causes excessive an unregulated exploitation which often increase the risk of future availability and survival of them. Many medicinal orchid species of CHT become rare or threatened. Moreover, it is noted that, *in vitro* propagated orchid plantlets sometimes produce valuable secondary metabolites in comparison with natural orchids. So, it is prime requisite to comparative searching of screening of the secondary metabolites in both natural and *in vitro* grown medicinal orchid which may reduce the adverse pressure on natural population [15].

*Staurochilus ramosum* (Lindl.) Seidenf. is an epiphytic orchid and root portion mostly used for making herbal medicine. About 3-4g of root of this orchid and 2g of fresh leaf buds of *Pisum sativum* are made into paste with water. One gram of the paste is taken orally with water on an empty stomach twice a day for seven days to cure blood dysentery [16]. The plant is also used as emollient and leaf paste is applied externally to cure wounds [17]. *S. ramosum* is decreasing day by day in Bangladesh for indiscriminate collections by orchid lovers, habitat destruction and lack of public awareness [18]. For that, the species has detrimentally affected the size and frequency of its natural populations.

To cope up with alarming situation, a long term systematic programme and a unique technology must be outlined or executed to provide production of huge quantity of *S. ramosum* medicinal orchid of CHT, Bangladesh. The

present paper reports the germination of its seeds and development of seedlings with a view to developing a protocol for its mass multiplication and comparative searching of the secondary metabolites in both natural and *in vitro* grown *S. ramosum* to reduce the unfavorable stress on natural environment.

## 2. Materials and Methods

### *In vitro* seed germination and seedlings development

Capsules of *Staurochilus ramosum* (Lindl.) Seidenf. were collected from Manikchari at Khagrachori district of Bangladesh. Mature green capsules were washed in the running tap water for 5- 10 minutes to remove dust in the surface area. Then few drop of teepol solution was added for few minutes and washed under running tap water for five minutes. The capsules were surface sterilized by 0.1% $HgCl_2$ for 10 minutes followed by 70% ethanol for 30 second. The capsule washes carefully by sterile distilled water to remove any traces of the sterilizing agents.

The seeds were inoculated on agar gelled four basal media namely, KC [19]; MS [20]; PM [21] and VW [22] for germination. The pH of all media was adjusted to 5.8 in MS and 5.4 in KC, PM, VW prior 0.1N NaOH or HCl before mixing agar to autoclaving at $121^0$C for 20 minutes at 15 lbs pressure. Seed germination was carried out in the culture room at 25±2 $^o$C and under white fluorescent tubes at an intensity of 4000-5000 lux with a 14/10-h (day/night) photoperiod [10]. All The experimental manipulation was carried out under aseptic conditions and experiment is repeated thrice.

For the inoculation of seeds, mature green capsule was put on sterile tile and cut longitudinally using a sharp sterile blade under laminar air flow cabinet. The very minute seeds were scooped out with the help of sterile forceps and spread over the surface of the germination media. After germination orchid seeds produce protocorn like bodies (PLBs) and PLBs further differentiated into clumps and each developing into new callus or plantlets. In order to induce rapid elongation and enhance growth, germinated PLBs will be transferred to new media and produce a good number of Callus or shoot buds. Callus tissues, which were further subcultured at lower density in the same medium, produced plantlets. When the tiny seedlings cultured on PGRs (Plant Growth Regulators) supplemented MS medium for vigorous seedlings development and produce SPSs (Shoot Primordia Like Structures) at the base of seedlings. Rotted well developed seedlings were taken out of the culture vessels and successfully transferred to outside the culture room following successive phases of acclimatization and transplanted seedlings were watered regularly for about 2-3 months.

### 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 [23] and the method was slightly modified by Aplin and Cannon [24]. 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 were

- Dragendroff's reagent.
- Hager's reagent.
- Mayer's reagent.
- Wagner's reagent.
- Tannic acid reagent.

**These reagents were prepared following the methods [25].**

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$^o$C 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.

**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 [26].

**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 [26].

**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 [27].

**Tannins:** 2 ml of extract will be added to few drops of 1% lead acetate. A yellowish precipitate was indicating the presence of tannins [28].

**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 [29].

**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 [29].

**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 [28].

**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 [30].

**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 [30-31].

**Coumarins:** 3 ml of 10% NaOH was added to 2 ml of aqueous extract formation of yellow colour indicates the presence of coumarins [32].

**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 [33].

## 3. Results and Discussions

The seeds of *Staurochilus ramosum* germinated on 0.8% (w/v) agar solidified all the four-nutrient media like KC, MS, PM and VW (Table-1). 3% (w/v) sucrose was used in MS media whereas, 2% (w/v) sucrose were used in KC, PM and VW media. The highest seed germination was recorded on 2% (w/v) sucrose containing PM medium (86.77%; Fig. 1) and it was followed by that on MS (80.00%; Fig. 2), VW (66.67%) media and least in KC (60.00%) media. Minimum time required for seed germination was recorded on PM ($10.30\pm0.24^a$ weeks) followed by MS ($11.20\pm0.33^b$ weeks), VW ($12.73\pm0.29^c$ weeks) and KC ($14.30\pm0.31^d$ weeks) media. Similar result was also found in *Ponthieva* and *Geodorum densiflorum* [34]; *Arundina graminifolia* [35]; *Calanthe densiflora* [36]; *Dendrobium transparens* [37]; *Micropera obtusa* [38]; and *Cymbidium cyperfolium* [39] orchid species. PM medium also proved more effective for inducing early and better germination of seeds. 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. Peptones has significant role as excellent natural sources of amino acids, peptides and proteins in growth media. Peptone in media enhances the germination rate and also favours the healthy protocorm development.

The *in vitro* developed PLBs on the same basal media produced callus (Fig. 3) or shoot buds (Fig. 4). 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) at the base of the seedlings.

**Table 1:** *In vitro* germination of seeds of *Staurochilus ramosum* (Lindl.) Seidenf.

| 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 | 09 | 60.00 | $14.30\pm0.31^d$ | Greenish PLBs |
| MS | 3% (w/v) sucrose | 15 | 12 | 80.00 | $11.20\pm0.33^b$ | Greenish PLBs |
| PM | 2% (w/v) sucrose | 15 | 13 | 86.77 | $10.30\pm0.24^a$ | Greenish yellow PLBs |
| VW | 2% (w/v) sucrose | 15 | 11 | 66.67 | $12.73\pm0.29^c$ | Yellowish green 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.

Established rooted seedlings were transferred from culture room to the green house during successive phase of adjustment. 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 regularly for about 2-3 months and established in the Orchidarium.

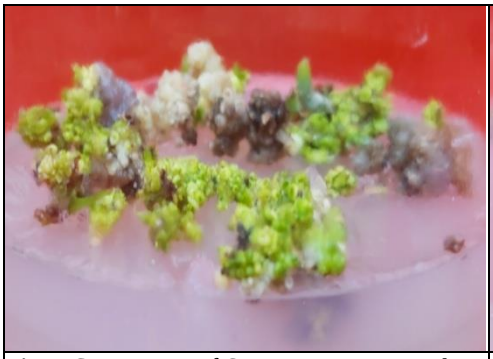

**Fig 1:** Germination of *S. ramosus* on PM medium

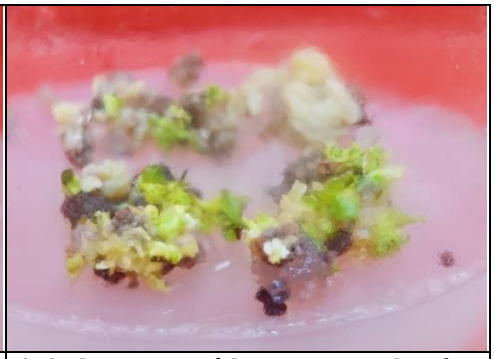

**Fig 2:** Germination of *S. ramosus* on MS medium

**Fig 3:** Callus of *S. ramosum*

**Fig 4:** Shoot buds of *S. ramosum*

Generally, phytochemicals induce the function of the immune system, act directly against harmful pathogens, reduce acidity, inflammation, active as antidiabetic, anticancer, antioxidants, antiseptic, antipyretic, antimalarial and used in the treatment heart & cardiovascular diseases, aging problems. 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 its presence in degrees ('+' minimum to '+++', the highest quantity). Absence of alkaloids was denoted by '-' sign.

The response of *in vitro* developed SPSs of *Staurochillus ramosum*, '+++' in Wagner's (W), Hager's (H), Mayer's reagent (M), Tannic acid (T); whereas '++' in Dragendroff's (D). In case of *in vitro* developed shoot bud, it responded '++' in Hager's (H), Wagner's reagent (W), Tannic acid (T) and '+' in Dragendroff's (D), Mayer's reagent (M). *In vitro* derived callus responded '+++' in Hager's (H), Wagner's reagent (W), '++' in Dragendroff's (D), Mayer's reagent (M) and Tannic acid (T). Both leaf and root sample of this natural species responded '+++' in Hager's (H), Mayer's (M), Wagner's reagent (W) and Tannic acid (T) '++' in Dragendroff's reagent (D), whereas, natural stem sample gave '+++' in Hager's (H), Wagner's reagent (W) and Tannic acid (T); '++' in Dragendroff's reagent (D) and '+' 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 *Staurochillus ramosum* gave the highest precipitation followed by *in vitro* developed SPSs, callus and shoot buds respectively (Table 2). This might be caused because of the accumulation of different substances by the additional plant growth regulators in culture media.

**Table 2:** Phytochemical profiling (alkaloids) of *Staurochilus ramosum.*

| 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 other than alkaloids, comparative qualitative assessment for ten other secondary metabolites, e.g. Phlobatannins, Flavonoids, Saponins, Tanins, Terpinoids, Steroids, Glycosides, Anthocyanins, Leucoanthocyanins, Coumarin and Phenol for both *in vitro* developed and natural *Staurochillus ramosum* were done. In this experiment, *in vitro* grown shoot bud of *Staurochillus ramosum* gave '-' test in Phlobatannin and Anthroquinone. All *in vitro* and natural sample gave low responses (-/+) in Phlobatannin test and gave highest response '+++' in Tanins and Terpinoids test (Table 3). Naturally grown root sample gave best responses followed by stem and leaf sample. So, there are little differences in the presence or absence of these secondary metabolites yet they have grown in different environment.

Table 3 illustrates that, Saponin and Tanin exhibited presence with 3+ responses; while the occurrence of both Leucoanthocyanins and phenol was also similar in each part of both *in vitro* and natural plants. Phlobatannins was present in both natural (leaf, root) and *in vitro* (callus, SPSs) samples while also absent in stem and shoot bud. Anthocyanin was found absent in both leaf (natural) and SPSs (*in vitro*) plants. This means that, distribution of phytochemicals is sporadic and uneven [40].

**Table 3:** Phytochemical profiling (other than alkaloid) of *Staurochilus ramosum.*

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

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

Similar types of sporadic distribution of secondary metabolites was observed by Akter *et. al.* [41] while the phytochemical profiling of *Pholidota pallida* Lindl from Bangladesh. They noted that, Flavonoids, Saponins, and Steroids were present in leaf and bulb but absent in root. They also noticed the presence of Terpinoids and Coumarins in leaf and root but absence in bulb. They found that, Quinine is absent in leaf but present in bulb and leaf while Glycoside is only present in bulbs.

This result denoted that the contents of phytochemicals in natural and in vitro derived plants were similar and the in vitro plantlets can be considered as the alternate for the seed grown plants in terms of therapeutic values which was also supported by the findings of Manivannan *et. al.* [42]. Naturally grown root sample gave best responses followed by stem and leaf sample. So, there are little differences in the presence or absence of these secondary metabolites yet they have grown in different environment.

## 4. Conclusions

In conclusion, the current endeavor has accomplished a widespread investigation of phytochemicals from natural and *in vitro* propagated plants in pharmaceutically important *S. ramosum*. Therefore, the present work can be contributed to the large-scale production of *S. ramosum* for germplasm conservation and commercial cultivation. In addition, the determined phytoconstituents can be further justified and utilized for the therapeutic purposes. Thus, the difficulty of acquiring the in vivo plants from seeds can be addressed using plant tissue culture approach established in the present study without compromising the medicinal value using *in vitro* propagated *S. ramosum* plants. And hence *in vitro* plantlets can be considered as the alternate for the seed grown plants in terms of therapeutic values.

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## 6. References

1. Chugh G, Rao IU. Micropropagation of orchids: A review on the potential of different explants. Sci. Hortic. 2009; 122:507-520.
2. Sazak A, Ozdener Y. Symbiotic and asymbiotic germination of endangered Spiranthes spiralis (L.) Chevall. and Dactylorhiza osmanica (Kl.) SoA3 var. Osmanica (Endemic). Pak. J Biol. Sci. 2006; 9:2222-2228.
3. Huda MK. An updated enumeration of the family orchidaceae from Bangladesh. The Journal of the Orchid Society of India. 2007; 21(1, 2):35-49.
4. Khanam M, Uddin MZ, Khan MS, Hasan MA. Our present knowledge on the terrestrial orchidaceous taxa from Bangladesh. Bangladesh Journal of Plant Taxonnomy. 2001; 8(2):35-49.
5. Nongdam P, Nirmala C. In vitro seed germination and mass propagation of Cymbidium dayanum Reichb. An important ornamental orchid of North-east India. Trends Hortic. Res. 2012; 2:28-37.
6. Duggal, C. Orchids in human affairs. Acta Phytother. 1972; 19:163-167.
7. Besra RC, Majhee L, Sharma J. Evaluation of phytochemical, antioxidant and hepatoprotective activity of tuber of Geodorum laxiflorum Griff. J Pharmacol. Toxicol. 2011; 6:610-623.
8. Chauhan S, Pathak P, Sharma S, Vij SP. In vitro asymbiotic seed germination of Satyrium nepalense D. Don, an endangered and medicinally important orchid. J. Orchid Soc. India. 2010; 24(1-2):61-66.
9. Bernard N. Recherches experimentales sur l'es Orchidees. Rev. Gen. Bot. 1904; 16:405-451.
10. Bhowmik TK, Rahman MM. Effect of Different Basal Media and PGRs on In Vitro Seed Germination and Seedling Development of Medicinally Important Orchid Cymbidium aloifolium (L.) Sw. Journal of Pharmacognosy and Photochemistry. 2017; 6(1):167-172.
11. Hossain MM, Sharma M, Teixeira da SJA, Pathak P. Seed germination and tissue culture of Cymbidium giganteum Wall. Ex Lindl. Sci. Hortic. 2010; 123:479-487.
12. Pathak P, Mahant KC, Gupta A. In vitro propagation as an aid to conservation and commercialization of Indian orchids: Seed culture. In: Orchids: Science and Commerce (eds. Pathak P, Sehgal RN, Sharma M, Sood A.) Bishen Singh Mahendra Pal Singh, Dehra Dun, India, 2001, 319-362.
13. Vij SP, Pathak P. Asymbiotic germination of the saprophytic orchid, Cymbidium macrorhizon: A study in vitro. J. Orchid Soc. India. 1988; 2(1-2): 25-32.
14. Vij SP, Pathak P, Mahant KC. Green pod culture of a therapeutically important species Dactylorhiza hatagirea (D. Don) Soo. J Orchid Soc. India. 1995; 9(1-2):7-12.
15. Bhowmik TK, Rahman MM. Effect of different basal media and PGRs on asymbiotic seed germination of Spathoglottis plicata Blume- a highly ornamental and medicinal orchid. Journal of Pharmacognosy and Phytochemistry. 2020; 9(5):2254-2259.
16. Das PK, Sahoo B. Ethnobotanical studies on orchids of Niyamgiri Hill Ranges, Orissa, India. Ethnobot. Leaft. 2008; 12:70-78.
17. Deorani C, Sharma GD. Medicinal Plants of Nagaland. Bishen Singh, Mahendra Pal Singh, New Delhi. ISBN: 9788121106016: 396-396, 2007.
18. Encyclopedia of Flora and Fauna of Bangladesh, Angiosperms, Asiatic Society of Bangladesh, Published

2008. 12:125-125.
19. Knudson L. For orchid seedlings in culture. Am. Orchid Soc. Bull. 1946; 15:214-217.
20. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum. 1962; 15:473-497.
21. Arditti J. Clonal propagation of orchids by means of tissue culture: A manual In: J Arditti (ed.), Orchid Biology: Reviews and Perspectives, I. University Press, Ithaca, New York, 1977, 114-1255.
22. Vacin E, Went F. Some pH change in nutrient solution. Botanic Gardens Conservation News. 1949; 110:605-613.
23. Webb LJ. An Australion Phytochemical survey. I. Alkaloids and Cyanogenic compound in the queensland plants. CSIRO. Bull. 260, Melborn, 1949.
24. Aplin THE, Cannon JR. Distribution of alkaloids in some Western Australian plants. Economic. Bot. 1971; 25(4):366-380.
25. Rahman M, Rahman A, Alamgir ANM. Phytochemical screening of some anticancer designate medicinal plants of Polypetalae. Journal of Pharmacognosy and Phytochemistry. 2017; 6(2):177-180.
26. Edeoga IHO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. Afr Boitech. 2005; 4:685-688.
27. Kapoor LD, Singh A, Kapoor SL, Srivastava SN. Survey of Indian plants for saponin, alkaloids and flavpnoids. Lloydia. 1969; 32(3):297-304.
28. Harbrone JB. Phytochemical Methods. Chapman and Hall. Ltd. London, 1973, 49-188 pp.
29. Kolawole OM, Oguntoye SO, Agbede O, Olayemi AB. Studies on the efficacy of Bridelia ferruginea Benth. bark extract in reducing the coliform load and BOD of domestic waste water. Ethnobotanical Leaflets. 2006; 10:228-238.
30. Paris R, Moyse H. Precis de matiere medicinale. Paris: Masson, 1969.
31. Savithramma M, Linga R, Suhrulatha D. Screening of Medicinal Plants for Secondary Metabolites. Middle-East Journal of Scientific Research. 2011; 8(3):579-584.
32. Sofowara A. Medicinal plants and Traditional medicine in Africa Spectrum Books Ltd. Ibadan, Nigeria, 1993, 289.
33. Soloway S, Wilen SH. Improved Ferric Chloride Test for Phenols. Anal. Chem. 1952.
34. Bhadra SK, Hossain MM. In vitro germination and micropropagation of Geodorum densiflorum (Lam.) Scheltr., an endangered orchid species. Plant Tissue Culture. 2003; 13(2):165-171.
35. Bhadra SK, Bhowmik TK. Axenic germination of seeds and rhizome-based micropropagation of an orchid Arundina graminifolia (D. Don) Hochr. Bangladesh J. of Botany. 2005; 34(2):59-64.
36. Bhowmik TK, Rahman MM. In vitro seed germination and rhizome based micropropagation of Calanthe densiflora Lindl: An indigenous terristerial orchid of Bangladesh. International J. of Botany Studies. 2017; 2(1):110-116.
37. Bhowmik TK, Rahman MM. In vitro mass scale propagation and seedlings development of medicinal orchid Dendrobium transparens Wall ex Lindl. Journal of Medicinal Plant Studies. 2020; 8(3):141-146.
38. Bhowmik TK, Rahman MM. Establishment of in vitro mass propagation protocol in Micropera obtusa (Lindl.) Tang & Wang. International Journal of Advanced Research and Development. 2020; 5(4):30-35.
39. Bhowmik TK, Rahman MM. Study of in vitro seed germination and micropropagation of an orchid Cymbidium cyperifolium Wall. of Bangladesh. European Journal of Biotechnology and Bioscience. 2020; 8(3):12-17.
40. Rahman M, Rahman A, Alamgir ANM. Screening of anticancer medicinal plants for secondary metabolites. Journal of Pharmacognosy and Phytochemistry. 2016; 5(4):100-103.
41. Akter S, Huda MK, Rahman M, Eva TA, Hoque MM. Phytochemical profiling and bioactivities of Pholidota pallida Lindl. International Journal of Advanced Research in Botany. 2019; 5(1):8-13
42. Manivannan A, Soundararajan P, Park YG, Jeong BR. In vitro propagation, phytochemical analysis, and evaluation of free radical scavenging property of Scrophularia kakudensis Franch tissue extracts. Bio. Med. Research International. 2015; 2015:480564. doi: 10.1155/2015/480564. Epub, 2015.