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PHYTOCHEMICAL SCREENING OF A MEDICINAL ORCHID, *ACAMPE PRAEMORSA* (ROXB.) BLATT. & MCCANN UNDER *IN VITRO* AND *IN VIVO* CONDITIONS

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Abstract

Acampe praemorsa (Roxb.) Blatt. & McCann is a monopodial epiphytic orchid which possesses eminent medicinal value. In the present study, four different nutrient media namely MS (Murashige and Skoog, 1962), PM (Phytamax; Arditti, 1977), MVW (Modified Vacin and Went, 1949), and KC (Knudson, 1946) were used for *in vitro* asymbiotic seed germination. PM basal medium proved the best percentage for seed germination (66.67%) followed by MS (58.34%), MVW (41.67%), and KC (33.34%) media. Phytochemical investigation was conducted for plant parts such as leaf, stem, root (procured from *in vivo* grown plants) and for *in vitro* raised plantlets, in *A. praemorsa*. Maximum amount of alkaloid content was found in leaf in Wagner's and Tannic acid reagent; in stem in Dragendroff's and Tannic acid reagent; in root in Tannic acid reagent; and in *in vitro* raised plantlets in Wagner's reagent. Amongst other secondary metabolites, the highest amount of tannin, flavonoid, steroid, and phenol was found in leaf, whereas, tannin, steroid, terpenoid, and phenol was found in stem; and tannin, flavonoid, steroid, protein, phenol, quinine, and coumarin were found in root. The highest quantity of saponin, flavonoid, and phenol was observed in *in vitro* raised plantlets.

Introduction

THE ORCHIDS, belonging to family Orchidaceae, are a group of flowering plants that are the nature's most extravagant and are well known for their distinctive, vibrant, and aromatic flowers and have always been interesting to evolutionary biologists because of their remarkable floral forms and diversity in pollination systems (Prakash and Pathak, 2020a, 2022). These are the most magnificent flowering plants of nature, found everywhere from the tropics to high altitudes and they are the most varied group of angiosperms (Lal *et al.,* 2021; Prakash and Pathak, 2019; White and Sharma, 2000). These plants have occupied top position amongst the flowering plants valued for cut flowers and as well as pot plants (Prakash and Pathak, 2020b). The family comprises 705 genera (POWO, 2023) with 29,481 species (WFO, 2023). According to Huda (2007), Bangladesh is a repository of orchids with 177 species categorized under 70 genera; twenty six of which are used by the tribal communities to treat various illnesses (Huda *et al*., 2006). They are found all over the nation but are particularly prevalent in the Chattogram hill tracts, Cox's bazar, greater Sylhet, Gazipur, and Sundarban (Huda, 2007). However, their conventional asexual reproduction is incredibly slow, producing only 2-4 plants per year (Nasiruddin *et al*., 2003).

Acampe praemorsa is an epiphytic orchid species with floricultural potential due to its evergreen, clustered foliage. It also serves as a common medicinal plant used to treat fever, throat problems, noxious infections, and eye and blood ailments (Huda and Kashem, 2021). This species has a range of secondary metabolites and can contribute as a significant source of various bioactive components, such as antioxidants, anti-inflammatory, and cytotoxic compounds. But the status of occurrence is conservation dependent (cd) and there are risks due to habitat degradation and over exploitation. Earlier, some scientists have worked on propagation of different orchid species (Anuprabha and Pathak, 2019, 2020; Anuprabha *et al.,* 2017; Arora *et al.,* 2016; Bhowmik and Rahman, 2017, 2020 a,b, 2022; Kaur *et al.,* 2017; Kumari and Pathak, 2021; Lalduhsanga *et al.,* 2021; Mutum *et al.,* 2022; Pathak *et al*., 2016, 2017, 2022, 2023; Sunita *et al.,* 2021; Thakur and Pathak, 2020, 2021; Tripura *et al.,* 2022; Vasundhra *et al.,* 2021) for *ex situ* conservation and these may be employed in commercial enterprises for a variety of applications. A new area of biological science research has been opened up by various bioactivity tests and the screening of the secondary metabolites of medicinal orchids grown naturally and *in vitro* (cf. Karthishwaran *et al*., 2010). For the purpose of rapid propagation and comparative analysis of secondary metabolites in both *in vivo* (naturally occurring) and *in vitro* grown plant parts of the medicinally significant orchid *A. praemorsa*, *in vitro* seed germination, seedlings development, and phytochemical screening methods were designed in the present study.

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Material and Methods

In Vitro Seed Germination

The immature seeds from green capsules of *A. praemorsa* procured from Bengchari, Kaptai, Rangamati, Bangladesh were used for *in vitro* propagation. In order to prevent contamination, sterilizing the capsule is a crucial step in *in vitro* cultivation. To get rid of dust, the capsules were first washed under running water, then three to four times in sterile distilled water. Following a savlon-soaked cotton rubdown, the fruits were rinsed in distilled water 2-3 times. These were then cleaned 2-3 times with double distilled water after being exposed to 0.2% (w/v) $HgCl_2$ for 5 min to sterilize their surface. Finally, the surface was disinfected in laminar airflow by treating these with 70% ethanol for 1 min and then washing it 2-3 times with double sterile distilled water.

For the aseptic culture of seeds, 2% (w/v) sucrose was added to four different basal media: MS (Murashige and Skoog, 1962), PM (Phytamax; Arditti, 1977), MVW (Modified Vacin and Went, 1949), and KC (Knudson, 1946). 0.8% (w/v) of agar was used to solidify the media. Before combining the dissolved agar, the pH of the media was adjusted to 5.8 in MS and 5.4 in KC, PM, and MVW media using 0.1N NaOH or HCl. Agar was dissolved in the mixture by boiling it in a water bath. Then, 50 ml of medium was poured into 100 ml of each culture vessel, and the vessels were autoclaved at 121°C for 30 min at 15 psi pressure. The seeds were then carefully inoculated on the nutrient medium inside the vessels in sterile conditions, under clean air laminar airflow. The cultures were consistently kept at an ambient temperature of 25±2°C and given a 14 hrs photoperiod with an intensity of 4000-5000 lux.

Phytochemical Screening

Alkaloids

For the qualitative screening of secondary metabolites, *in vivo* and *in vitro* grown plant parts of *A. praemorsa* were used. The samples were collected, dried by air drying, and then ground into powders, individually. For the preparation of the plant extract, the ground powdered elements were soaked in methanol. The most reliable and efficient method for evaluating alkaloids qualitatively was designed by Webb (1949), and was just slightly modified by Aplin and Cannon (1971). This procedure is known as the spot test method. Five alkaloid detecting reagents were utilized for the qualitative alkaloid test.

Freshly chopped and pasted plant material (5 g) was moistened with 10 ml of 2% HCl, then heated in a water bath for one hour at 60°C.The extract was filtered using Whatman No. 1 filter paper after cooling. One drop of the alkaloid detecting reagent and two drops of the extract were placed on microscopic slide. The relative abundance of precipitate, if any, formed in the plant extract with the reagent was thought to be a measure of the strength of the presence of alkaloids and was indicated by the signs "+," "++," and "+++," which denoted mild, moderate, and considerable to heavy amounts, respectively. The negative symbol (-) denoted the absence of precipitate and indicated that there were no alkaloids in the plant extract.

Phlobatannin

The presence of phlobatannin was determined by the formation of a red precipitate after boiling an aqueous extract of each plant sample with 1% aqueous hydrochloric acid (Edeoga *et al.*, 2005).

Flavonoid

The crude powdered plant sample was boiled for three min in the presence of 10 ml of ethyl acetate over a steam bath. After filtering the mixture, 1 ml of diluted ammonia solution and 4 ml of the filtrate were mixed together. A yellow colour was seen, confirming a positive flavonoid test (Edeoga *et al.*, 2005).

Saponin

Crude powder (2g) 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 firmly for stable persistent froth. The persistence of froth indicates the presence of saponin. 20 ml of distilled water was added to about 2 ml of filtrate with 5 ml of distilled water. The presence of saponin is indicated by persistent foam (Kapoor *et al.*, 1969).

Tannin

In a test tube with 10 ml of distilled water, 0.5 g of the crude powdered samples were boiled and then filtered. Ferric chloride reagent was applied in a few drops to the filtrate. The presence of tannin was identified through the appearance of a blue-black precipitate (Harborne, 1973).

Terpenoid

With 5 ml of methanol, 0.5 g of crude powder was dissolved. In a test tube, 5 ml of the extract was combined with 2 ml of chloroform. A layer was carefully formed in the mixture by adding 3 ml of pure sulfuric acid. If terpenoid ingredient is present, an interface with a reddish brown colouration is formed (Kolawole *et al.*, 2006).

Steroid

With 5 ml of methanol, 0.5 g of crude powder was dissolved. 1 ml of the extract was dissolved in 10 ml of chloroform, and an equivalent volume of strong sulfuric acid was added to the test tube from the sides. The upper layer turns red, and the layer containing sulfuric acid appears yellow with green fluorescence. This revealed the presence of steroids (Kolawole *et al*., 2006).

Anthraquinone

Magnesium acetate was added to a solution of 2 ml. Anthraquinone is present if pink colouration develops (Sofowara, 1993).

Quinone

Extract (1 ml) was combined with 1 ml of concentrated sulphuric acid, and the mixture was left to stand for some time to develop colour. The emergence of a red colour indicates the existence of quinone (Sofowara, 1993).

Coumarin

Extract (1 ml) was mixed with 1 ml of 10% NaOH and left to stand for a while to produce colour. Yellow colour development shows the presence of coumarin (Sofowara, 1993).

Phenol

While 10% aqueous ferric chloride is added to plant extract, the presence of phenol is detected by the appearance of a blue or green colouration (Soloway and Wilen, 1952).

Protein

Copper sulphate solution (2%) was used to treat the test solution. 1 ml of ethanol was added, followed by too many pellets of potassium hydroxide. The presence of protein is shown by the green colour (Gahan, 1984).

Cardiac glycoside

In the plant extract (5 ml), 2 ml of glacial acetic acid, 1 drop of ferric chloride solution, and 1 ml of concentrated sulfuric acid were added. The presence of cardiac glycoside is indicated by a brown ring or green colour (Kaffoor *et al*., 2016).

Results and Discussion

In Vitro Seed Germination

On all the nutrient media, the seeds showed germination response, but the germination rates varied depending upon the medium used (Table 1; Fig. 1a-d). On PM medium (66.67%), the maximum germination rate was observed and the greenish yellow protocorms were obtained (Fig. 1a-b). This was followed by in MS (58.33%) and MVW (41.67%) media. KC medium had the lowest percentage of seeds that germinated (33.33%). The numerous protocorm-like bodies (PLBs) that were formed from the multiplication of the protocorms were placed on new culture media for their further growth and development (Fig. 3).

After being transferred to the fresh nutrient media, well-developed plantlets were obtained (Fig. 4). Through successive stages of acclimatization, well-formed and rooted plantlets were transferred from the culture room to the outside environment. The seedlings were put into little plastic pots filled with sawdust, coal, dried wood, and wet coir, and they were left at room temperature for three to five days. For two to three months, transplanted seedlings were regularly watered, and subsequently, they established themselves under natural conditions.

Earlier, the technique for *in vitro* propagation of *A. praemorsa* was established by Roy (2014); he used *in vitro* asymbiotic seed germination method and used three different nutrient media (1/2 MS, MS, and KC). As the seeds showed very low frequency of germination

Table 1. *In vitro* asymbiotic germination of *A. praemorsa* seeds on different nutrient media.

Nutrient medium	Number of culture vessels used	Number of culture vessels in which seeds germinated		Colour of the protocorms	Remarks
		Number	%		
KC	12	04	33.33	Yellowish white	+
MS	12	07	58.33	Greenish	++
PM	12	08	66.67	Greenish yellow	++
MVW	12	05	41..67	Yellowish green	+

Values represent mean±SE of each experiment consist of 12 replicates.

on these basal media, auxin and cytokinin were utilized in various quantities and combinations so as to stimulate seed germination and seedling development. *Acampe rigida* seeds were cultivated by Bhowmik and Rahman (2021) on agar solidified MS, PM, MVW, and KC media supplemented with sucrose, glucose, and lactose individually; the seeds showed better germination response on PM medium with sucrose. Literature studies revealed that a few successful attempts have also been made for mass propagation of some orchid species of diverse habits and habitats (Anuprabha and Pathak, 2020; Bhowmik and Rahman, 2020b, 2022; Kumari and Pathak, 2021; Lalduhsanga *et al.,* 2021; Mutum *et al.,* 2022; Pathak *et al*., 2022, 2023; Sunita *et al.,* 2021; Thakur and Pathak, 2021; Tripura *et al.,* 2022; Vasundhra *et al.,* 2021).

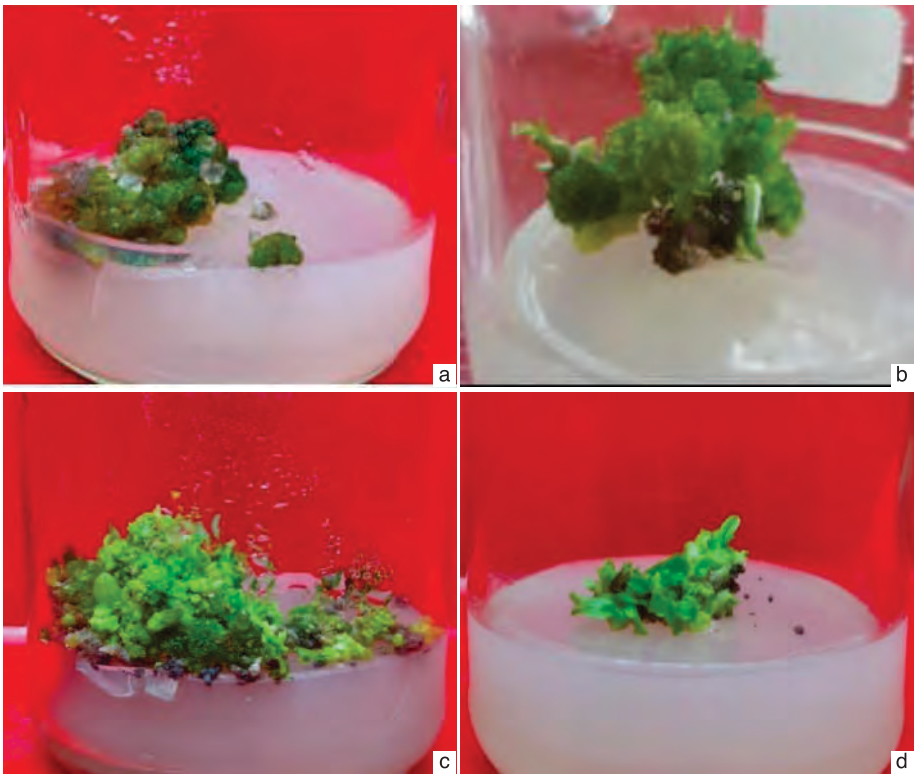

Fig. 1. a-d. *In vitro* seed germination in *A. praemorsa*: a, Development of protocorms; b, Rapid protocorm multiplication; c, Differentiation of leaves and roots; d, Seedling development.

For phytochemical studies, the qualitative evaluation of secondary metabolites including alkaloids, flavonoids, phlobatannin, steroids, tannin, saponin, terpenoid, anthraquinone, coumarin, quinone, phenol, protein, and cardiac glycoside was dono. Five different alkaloid detecting reagents were used to qualitatively measure alkaloids. These were Dragendroff's reagent (D), Wagner's reagent (W), Mayer's reagent (M), Hager's reagent (H), and Tannic acid reagent (T). Table 2 demonstrates that the alkaloid test for plant parts of naturally grown plants and *in vitro* plantlets of *A. praemorsa* gave different results. The highest (+++) response in the Tannic acid reagent (T) was found in plant parts of *in vivo* grown plants (leaf, stem, and root). Leaf responded to Dragendroff's reagent (D) with a medium (++) response. In Hager's reagent (H), the leaf, stem, and root exhibited a mild (+) response. *In vitro* raised plantlets indicated the strongest (+++) responses to Wagner's reagent (W), medium (++) response to Dragendroff's reagent (D) and Tannic acid reagent (T). In a similar type of experiment held by Marjoka *et al.* (2016), leaf extracts of *A. praemorsa* exhibited the greatest (+++) response to Dragendroff's reagent (D), a moderate response (++) to Tannic acid reagent (T) and the least (+) response to Hager's reagent (H), Wagner's reagent (W), and Mayer's reagent (M). Other secondary metabolites like flavonoid, phlobatannin, steroid, tannin, saponin, terpenoid, anthraquinone, coumarin, quinone, phenol, protein, and cardiac glycoside were considered for qualitative evaluation. The results have been depicted in Table 3. The highest amount of tannin, flavonoid, steroid, and phenol in leaf; tannin, steroid, terpenoid, and phenol in stem and tannin, flavonoid, steroid, protein, phenol, quinone, and coumarin in root were found. Medium quantity of protein and cardiac glycoside in leaf; saponin, protein, quinone, coumarin, and cardiac glycoside in stem; and saponin, terpenoid, and cardiac glycoside in root were observed.

Table 2. Qualitative phytochemical profiling (alkaloids) plant parts (leaf, stem, root) of *in vivo* grown plants and *in vitro* plantlets of *A. praemorsa.*

Plant parts used		Qualitative estimation of alkaloids				
		D	H	M	W	T
In vivo	Leaf	++	+	+	+++	+++
	Stem	+++	+	+	+	+++
	Root	+	+	-	++	+++
In vitro	plantlets	++	-	-	+++	++

Reagents: D, Dragendroff's reagent; H, Hager's reagent; M, Mayer's reagent; W, Wagner's reagent; T, Tannic acid reagent; +++, means the highest result; ++, means medium result; +, means mild result; -, means no alkaloid present.

Table 3. Qualitative test of twelve other phytochemicals of *A. praemorsa*.

Plant parts		Secondary metabolites (% of coloration)											
		Sap.	Tan.	Flv.	Str.	Ter.	Pro.	Phe.	Phl.	Qui.	Ant.	Cou.	Car.gly.
In vivo	Leaf	+	+++	+++	+++	-	++	+++	-	-	-	+	++
	Stem	++	+++	-	+++	+++	++	+++	-	++	-	++	++
	Root	++	+++	+++	+++	++	+++	+++	-	+++	-	+++	++
In vitro	*In vitro* raised plantlets	+++	++	+++	+	+	++	+++	-	++	-	++	+

Sap., Saponin; Tan., Tannin; Flv., Flavonoid; Str., Steroid; Ter., Terpenoid; Pro., Protein; Phe., Phenol; Phl., Phlobatannin; Qui., Quinone; Ant., Anthraquinone; Cou., Coumarin; Car. gly., Cardiac glycoside. Here, +++, means the highest response; ++, means medium response; +, means the lowest response; -, means absent.

In vitro raised plantlets showed the highest quantity of saponin, flavonoid, and phenol; moderate amount of tannin, protein, quinine, and coumarin with the least amount of steroid, terpenoid, and cardiac glycoside. No phlobatannin and anthraquinone were found in both plant parts obtained from *in vivo* grown plants and also *in vitro* raised plantlets. Rahman and Huda (2021) examined the secondary metabolites in *Acampe praemorsa* leaf. In the methanolic leaf extract of *A. praemorsa*, high amounts of flavonoids and tannins were observed with modest amounts of coumarin, glycoside, quinone, saponin, and terpenoid. The quantity of steroids and anthraquinone was minimal. Phytochemical studies have also been made in few other orchid species recently by some scientists (Hoque *et al.,* 2021a,b; Pathak *et al.*, 2023).

Conclusion

The present data revealed that PM medium proved as the most effective seed germination in *A. praemorsa*. Both *in vitro* raised plantlets and plant parts obtained from naturally grown plants showed the existence of alkaloid content. Several active secondary metabolites were also found in both plant parts obtained from naturally grown plants and *in vitro* raised plantlets of *A. praemorsa.* The present data would add the information to the ongoing research regarding medicinal orchids in Bangladesh and may lead to further discoveries regarding the efficacy of this medicinally important orchid, *A. praemorsa.*

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