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Growth patterns of *Azolla pinnata* R.Br. on artificial medium under *in Vitro* and *in Vivo* conditions

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Abstract

A special study on growth of the fern *Azolla pinnata* R.Br. was carried out with reference to the formation and continuation of life cycle and sex organ formation in two different artificial medium under *in vitro* and *in vivo* conditions. The sporophytes were grown in KC, Kn and MS medium without any carbon source or hormone and agar, and pH at 5.8. Liquid medium was preferred over solid medium since the plant itself was a water fern. The sporophytes of the fern showed different growth and reproductive phases in these media.

Keywords: *Azolla pinnata*, liquid medium, MS medium, sporophyte

INTRODUCTION

Pteridophytes are primitive, non-flowering plants well known for their tribal medicine and secondary metabolites. *Azolla* is an aquatic Pteridophyte widely distributed in the water bodies. It has been traditionally used as a biofertilizer for paddy fields owing to its potential to fix atmospheric nitrogen. In addition to this it has several other uses and Wagner (2010) referred it as "green gold mine". The plant system has the inherent capacity to synthesize several phytochemicals. Besides its utilization as biofertilizer on a variety of crops, *Azolla* can be used as animal feed, human food, medicine and water purifier. It may also be used for the production of hydrogen fuel and biogas, control of weeds, and mosquitoes, and reduction of ammonia volatilization, which accompanies the application of chemical nitrogen fertilizer.

Azolla plants have crowded mass like leaves borne on a fragile free-floating rhizome with submerged roots. The leaves are arranged in alternate rows and each leaf is divided into two lobes of which the upper lobe is aerial and lower lobe is submerged in water. The aerial lobe is more than one cell thick and is photosynthetic in nature with stomata on both surfaces (Vasishta, 1982).

Various studies on agriculture, hormonal, clinical and phytochemicals aspects have been worked out on this plant *Azolla pinnata* R.Br. Perusal of literature proved that works on various aspects on the growth pattern are scanty of various osmoticum and the plant response for the same. The present article deals with the growth of the sporophyte of the water fern, *Azolla pinnata* R.Br., and the effects of various artificial media used under *in vitro* and *in vivo* conditions, and the production of primary and secondary metabolites and quantification under various media composition.

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MATERIALS AND METHODS

Collection of plants

The fern, *Azolla pinnata* R.Br., was procured from the Agriculture College, Tiruchirappalli and maintained were in the department of Botany and also cultured on tanks and ponds. The water plant was also maintained in the artificial pond in the premises of Botany garden. The explants were collected for the experiment from 3 months old plants. The healthy plants were transferred to a glass container and experimental plots were maintained in plastic containers.

Medium preparation

Media such as Knudson's C medium (KC) (Knudson, 1946), Knop's medium (Kn) (Knops, 1865) and Murashige and Skoog's medium (MS) (Murashige and Skoog, 1962) were prepared by using the chemical nutrients as stock solutions and whenever needed diluted with distilled water. The pH was adjusted to 5.8 and the media were sterilized in autoclave.

Experimental set up

The preferred osmoticum KC, Kn and MS media were prepared in triplicates. The young *Azolla* plants were transferred and inoculated into the culture medium with the sterile needle. Five plants were introduced. The studies like growth area and production of reproductive structures were observed after 30 days of time.

Measurement of Growth area and observation on reproductive structures

The measurement of length and breadth at initial stage of inoculation was carried out and at the final stage after 30 days in the three osmoticum studied. Growth area was represented as mm. *Azolla* plants on three culture media were also observed for the reproductive structures like sporocarps after 30-45 days time. Microphotographs of the stages of sporophytic growth and reproductive structures of *Azolla* in different media

were taken by using Weswox and Digital Trinocular microscope.

Biochemical and phytochemical analysis

Azolla plant samples in triplicates was collected from the coffee cups cultured on different media after thirty days and the primary metabolites such as proteins (Lowry *et al.*, 1951) amino acids (Moore and Stein, 1948) and reducing sugar were analysed by standard procedures, whereas secondary metabolites like Phenol (Mahadevan and Sridhar, 1982), ascorbic acid (Mukherjee and Choudhary, 1983) and flavanoids were analysed by the respective procedures.

RESULTS AND DISCUSSION

Azolla pinnata plants cultivated *in vitro* and *in vivo* were observed after 30 days of transfer in to different osmoticum namely KC, Kn and MS medium. The plants cultured under *in vitro* condition showed poor growth and most of them died within 8-10 days after browning. The plants cultured *in vivo* showed prolonged growth and further branching occurred (Plate 1a & b), leaves emerged from a free floating rhizome with submerged roots, fronds are arranged in two rows (Plate 1c) upper lobe bend upwards and lower lobe was submerged in water as referred by Vashishta (1982). Mucilaginous cavities harbouring live colonies of *Anabeana azollae* Strasb. (Plate 1d) were observed in all the cultures studied. Another important observation is the formation of sporocarps on the abaxial side of the floating rhizome.

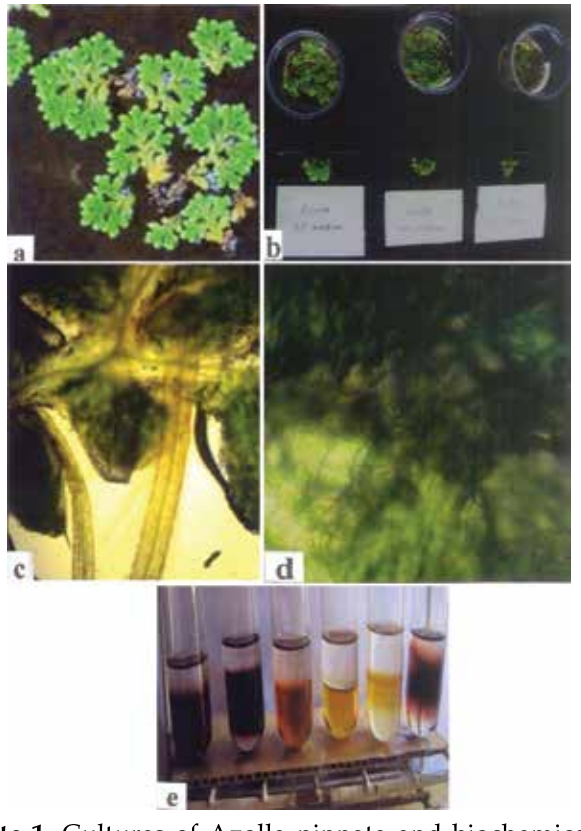

Plate 1. Cultures of Azolla pinnata and biochemical testing

1 a. Azolla pinnata plant morphology
1 b. Azolla pinnata in various medium in cups
1 c. Azolla fronds ventral surface with rhizoids
1d. Anabaena azolla on the ventral surface of the frond as filaments
1e. Primary and secondary metabolite biochemical test results

Formation of sporocarps on *Azolla* in different medium.

Sporocarps are the reproductive structures seen at the abaxial surface near the rhizome segments of the plant. Apart from the asexual reproduction fragmentation, sporocarp formation was seen in the cultures. According to the references, sexual reproduction in *Azolla* sets itself apart from other members of its group by producing two kinds of sporocarps. During the unfavourable conditions, numerous spherical

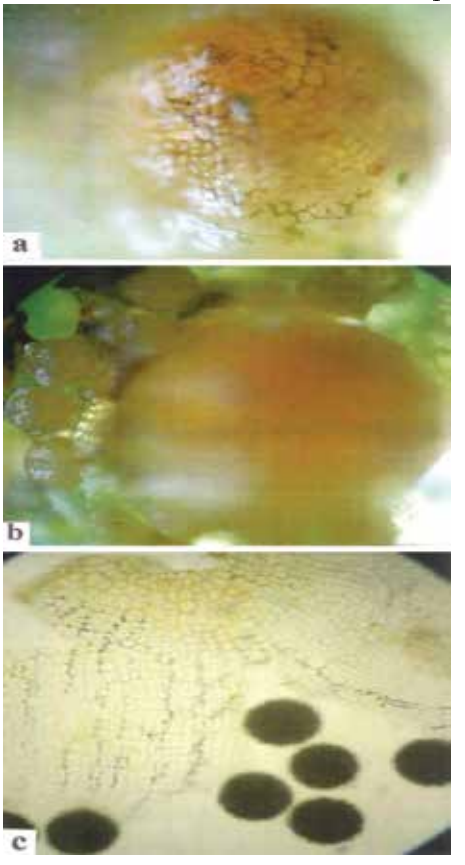

Plate 2. Azolla pinnata reproductive structures - close up views of sporocarps

2 a. Aerial view of Microsporocarp (spider web like or insect like)
2 b. Aerial view of Megasporocarp at the base of the microsporocarp
2 c. Crushed microsporopcarp showing masses of microspores in bundles

structures called sporocarps form on the undersides of the branches. The same structures are formed on the experimental plots of our study also (Table 1, Plate 2). The male sporocarp is greenish or reddish and looks like the egg mass of an insect or spider. It is two millimeters in diameter, and inside are numerous male sporangia (Plate 2a).

Female sporocarps are much smaller, containing one sporangium and one functional spore (Plate 2b). Since an individual female spore is considerably larger than a male spore, it is termed a megaspore (Plate 3d) (URL -1). Nearly six to eight (Plate 2b) small female sporocarps flanked by one larger, globose male sporocarp is observed in our studies.

Male spores (microspores) are small and are produced inside each microsporangium (Plate 2c) and microspores tend to adhere in clumps called massulae. Very curious observation about microspores is that they tend to stick together in little clumps or masses called massulae. (Plate 3a). Each spore mass (massula) is covered with minute hairs (barbed at the tips) called glochidia (Plate 3b) after 20 days of formation. Under high magnification the massulae look like strange space satellites with radiating antennae (Plate 3b). The barbed glochidia on the male spore clusters are assumed to cause them to cling to the female megaspores, thus facilitating fertilization (Armstrong, 1985). *Azolla* has

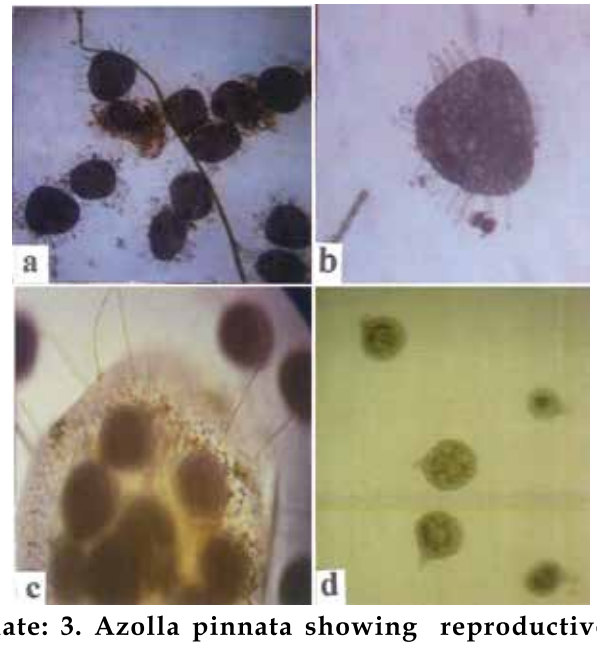

Plate: 3. Azolla pinnata showing reproductive structures - Sporocarps

3a. Clumps of massulae (Male sporocarps) with glochidia and filament of Anabaena

3b. Single clump of massula with antenna satellite like glochidia all over the microspores

3c. Microsporocarps with Anabaena azollae filaments on the outer walls

3d. Megaspores from megasporocarps ruptured.

microscopic male and female gametophytes that develop inside the male and female spores. The female gametophytes protrudes from the megaspore and bears a small number of archegonia, each containing a single egg. The microspore forms a male gametophyte with a single antheridium which produces eight swimming sperm.

In the present observation maximum microspores clusters of 25 in number could be seen in KC medium cultured *Azolla* plants whereas 10- 12 megaspores clusters were seen in the same medium next to knudson's medium. In knop's medium nearly 22 microspore clusters inside microsporocarp were evidenced and up to 11 microspores evidenced. Minimum number of 12 microsporocarp and 6 megasporocarp was observed in MS medium.

Vegetative growth of *Azolla* coincides with its reproductive growth in the cultures. From the present studies we can find out that growth area is higher in Kc medium followed by Kn and next the MS medium. In reproductive structure formation KC medium showed maximum sporocarp production and next to that Kn showed maximum sporocarp (Table 1)

Cyanobacterial partner ship and its precedence on life cycle

Unlike plant-cyanobacterial symbioses in vascular plants, the *Azolla-Anabaena* symbiotic system is sustained throughout the fern's life cycle, where the cyanobacterium and bacteria are always present, either in the dorsal lobe leaf cavities or in the sexual structures (sporocarps). The *Azolla* plants are never infected *de novo*, since *Anabaena* is transferred between generations as akinete inocula. This maintains continuity of the symbiosis during sexual reproduction.

During *Azolla's* sporulation, filaments of *Anabaena* are packaged into the developing sporocarps. As sporocarp gender is determined later in *Azolla's* development, *Anabaena* is present in both the megasporocarps and microsporocarps, but *Anabaena* is only maintained by the microsporocarps (Plate 3c), thus maintaining the symbiotic continuity.

Table 1. Number of sporophytes in 40 days old sporophytes and growth area of *A.pinnata* in different medium

Medium	Number of sporocarps		Growth area(mm)	Length of the rhizoid (mm)
	Microsporocarp	Megasporocarp		
Control	10	24	320±15.2	31±0.055
Knudson's medium (KC)	12	25	810±16.7	50±0.3082
Knops medium (KN)	11	18	240±10.4	22±0.0447
Murashige & Skoog (MS)	3	8	420±19.8	34±0.28

The exploitation of the symbiotic system is limited due to its sensitivity of high /low temperatures and high phosphorous requirements. Also the identity of endosymbionts variously identified as *Anabaena / Nostoc / Trichomonous*, has been one of the major reasons for lack of indepth classification and biotechnological intervention of these green gold mine. Although immunological and nif probes have been utilized to analyze the genetic nature of the symbionts, no clear picture has emerged so far.

Growth area of *Azolla* on different medium

Growth area of the sporophyte of heterosporous fern *Azolla* was calculated by observing the length and breadth of the sporophyte after 30 days of transfer or treatment with the medium studied. (Table 1) shows the growth area as well as rhizoid length of the plants grown on KC, Kn and MS medium. Maximum growth area of 810mm was recorded on KC medium, 420 mm on MS medium and minimum growth area on Kn medium (240 mm) . This shows that the optimum osmoticum for the growth and reproduction is Knudsons medium rather than the other two medium preferred.

The preference of simple medium (KC medium) over macro micro nutrients, Iron and vitamins stock (MS medium) shows that the plant prefers simple medium with ferrous and manganous sulphate components. Most of the homosporous ferns were grown under *in vitro* condition on knudson's medium luxuriantly (Sara *et al.*, 1998; Manickam *et al.*, 2003; Johnson *et al.*, 2005, Sara and Manickam, 2005 and Sara and Manickam. 2007). From the present work it is also evident that more amounts of micronutrients and vitamins and growth factors may inhibit the growth of the sporophyte. This experimental hetersporous fern *Azolla pinnata* also respond to the simple medium rather than complex medium like MS medium.

Primary and secondary metabolites of *Azolla* on different medium

The primary metabolites like protein, amino acid and reducing sugar was studied and it showed various values in *Azolla* plants. An increased content of protein (660 mg), amino acid 5.4 mg and reducing sugar (14.2 mg) was found to record on knudson's medium grown *Azolla* plants. *Azolla* plants grown in knop's medium showed 640 mg protein, 2.5 mg amino acid and 7.2 mg reducing sugar. Except reducing sugar (97 mg) the other two primary metabolite content decreased in the rest of the medium other than KC medium (Table 2).

Amino acid transfer between organs through xylem and phloem is critical for optimizing nitrogen allocation in the plant to the growth condition or developmental stage. Amino acid is the primary metabolite which shows the indication of stress. To overcome the stress the plants produce amino acids. In our study the plant showed maximum accumulation of amino acid in KC medium than Kn and MS medium. Hence it is known that the growth and amino acid production may be inhibited by high osmoticum and pH rather than knudson's medium under cultures.

Accumulation of secondary metabolites like phenol, ascorbic acid and flavanoids evidenced in all the medium grown *Azolla* plants rather than control. Maximum phenol content was evidenced in Kn medium and it declined with KC and MS medium in order as shown in (Table 2 and Plate 1e). Ascorbic acid in KC medium is maximum (0.9 mg), whereas Kn and MS medium showed diminished quantity of 0.7 and 0.6 mg respectively. Control also contains 0.8mg/gm ascorbic acid (Table 2). Flavanoid analysis also showed the same results.

Ascorbic acid an important antioxidant, which react not only with H_2O but also with OH and lipid hydroperoxidases (Reddy *et al.*, 2004). Bors *et al.* (1990) reported effective free radical capacity of flavanoids. Bolink *et al* (2001) observed increased tolerance to high light stress in pea and bean plants due to increase in flavanoid content. Brawn (1991) reported that the epidermal layer of oat seedling accumulated large

Table 2. Showing primary and secondary metabolites on *Azolla* plants cultured on different medium

Medium	Primary metabolite			Secondary metabolite		
	Protein	Amino acid	Reducing sugar	Phenol	Ascorbic acid	Flavanoid
Control	600±12.2	4.8±0.02	12.8±0.1	28.4±0.9	0.8±0.02	0.20±0.02
Knudson's medium (KC)	660±15.8	5.4±1.56	14±0.8	27.9±0.02	0.9±0.01	0.24±0.015
Knops medium (KN)	640±26.1	2.5±1.56	7.2±0.9	36±0.01	0.7±0.05	0.18±0.015
Murashie & Skoog (MS)	55±14.5	2.4±0.01	9.7±0.1	24±0.05	0.6±0.01	0.13±0.015

amount of UV absorbing pigment flavanoid and anthocyanin during early development which gave a better protection against UV-B. On the whole regarding the primary metabolites, enrichment of primary metabolites could be visualized apart from control in KC medium. This shows the normal growth is evidenced on KC medium than the other medium. Secondary metabolites except phenol the other two components say Ascorbic acid and flavanoids was high in KC medium grown *Azolla* plants rather than other culture medium.

CONCLUSION

From the work done so far the important results obtained include the following. The growth area was calculated on knudson's and knops medium and MS medium grown cultures. The growth area of sporophyte was high in Knudsons medium where as low in MS medium. Maximum number of sporocarps, mega and microspore carps could be seen in Knudson's medium rather than knops and MS medium. The continuity of symbiotic organisms like blue green algae especially *Anabaena azolla* was seen on the cultures studied.

The primary metabolites were get accumulated very high in Knudson's medium and it declines with the other medium. Regarding the secondary metabolite, except Phenol other secondary metabolite, ascorbic acid and flavanoid content was high in Knudson' medium grown *Azolla* plants. It shows that Knudson's medium was the optimum medium to grow *Azolla* plants and to carry out research works. Moreover symbiotic studies of fern algae could be motivated in future. Life cycle shows the dependence of water or liquid medium for their survival , growth and reproduction.

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