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RESEARCH ARTICLE

IN VITRO PROPAGATION OF *CYATHEA GIGANTEA* (WALL EX. HOOK) - A TREE FERN

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ABSTRACT

Cyathea is a genus of tree fern, Cyatheaceae is the scaly tree fern family and includes the World's tallest tree fern which reach heights upto 20m (Large & Braggins, 2004).According to Beniwal (1994) *Cyathea gigantea* is treated as one of the endangered plants of India. In this study an attempt was made to standardize the *in vitro* protocol for mass multiplication and conservation of threatened tree fern *Cyathea gigantea* Wall. ex. Hook, using spore as explants. Spores were found to germinate in Fern Propagation media and MS media. Spores cultured on MS medium showed 72% germination. Sporophytes were induced from the gametophytes on the MS media supplemented with 0.8mg/L IAA and 5mg/L kinetin with highest root length 20.29 cm. Period of germination, Fresh weight of prothallus and length of sporophytes were recorded. *In Vitro* raised shoots rooted on MS media supplemented with IBA. The well rooted micropropagated plants were transferred to hardening medium for acclimatization where 81% survival percentage was observed.

Abbreviations : MS – Murashige and Skoog, IAA – Indole 3- acetic acid, IBA - Indole 3- butyric acid.

INTRODUCTION

Ferns have been with us for more than 300 million years. Their diversity of forms is enormous and they thrive in many habitats. The fern and fern allies are the surviving members of earliest lineages of vascular plants. None of these primitive plants produce either seeds or flowers and they reproduce via single celled spores. Ferns during their life cycle, passes through two distinct phases, gametophyte, the small, simple haploid gamete producing phase and the large, diploid spore producing phase, sporophyte (Fernandez & Revilla,2003). Sporophytic phase is of commercial, ethno botanical and medicinal properties. Ferns are very ancient family of plants that are older than land animals. *Cyathea gigantea* is a giant fern. Jamir & Rao (1988) reported that it grows in all the states upto 1200m altitude, in North-eastern region. During the recent years, *C. gigantea* is depleting at an alarming rate and presently the species is nowhere abundant or frequent as reported by Baishya and Rao (1982) and this species is treated as one of the endangered plants of India (Beniwal,1994).

Tree ferns are all true ferns in that they are flowerless plants and reproduce by spores. The spores are developed in sporangia on the underside of the leaves (Braggings *et al*., 2004).Tree ferns are typical for rain forest of tropical and subtropical climate. Because of their ornamental beauty they are used for out and indoor decoration. The majority of tree ferns are illegally collected from natural sites, which resulted in decrease of their population, though they are listed on the International Red Book and protected by CITES(Etter,1993) In 2002, M.L. Khan et al., published an article "A plea of conservation of threatened tree fern (*Cyathea gigantea*)" in which they expressed their concern that in and around Itanagar (Arunachal Pradesh) especially this tree fern is on the verge of being extinct and they also reported that the spores of *Cyathea gigantea* becomes fertile in February when forest floor is dry due to absence of rain which hindered the spore germination in exposed and deforested area and which is a major constraint for regeneration of new plant. Keeping this in view, the investigation has been undertaken an investigation on formulating strategies for the conservation of *C. gigantea* through *in vitro* mass multiplication.

Cyathea is a genus of tree ferns.The genus name *Cyathea* is derived from Greek word, Kyatheion, meaning " little cup" refers to the cup shaped sori on the underside of the fronds.The genus *Cyathea* contains more than 470species.*Cyathea gigantea* (Wall.ex.Hook.) is a tree fern belonging to the family cyatheaceae. The Cyatheaceae is the scaly tree fern family and includes the World's tallest tree fern which reach heights up to 20m (Large & Braggings 2004).*Cyathea gigantea* grows in moist open areas at an altitude of 600 – 1000m. The trunk of this species is erect and as tall as 5m or more. Fronds are bi or tri pinnate and usually 2 – 3m long. The rachis is long, dark to black colouration and rough in appearance after the fall of scales. Scales are dark brown in colour and sori are round.

Cyathea gigantea (wall.ex.Hook.) have several active constituents like triterpenes, sterols,saponins,flavonoides, ß-sitostenone and whole plant contains oleanolic acid (Juneja et al.,1990). Flavonoid constituent in the genus *Cyathea* was carried out by Harda et al.,1995, Oleanolic acid having anti tumor, hepatoprotective and antiviral activity(Wolska et al 2010, Zhou et al., 2011). Oleanolic acid is found to exhibit

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strong anti HIV activity (Bhutani et al., 2010). ß- sitosterol component of *Cyathea gigantea* have anticancer activity.(Woyengo,2009). Traditionally the fresh rhizome of *Cyathea gigatea* mixed with black pepper seeds powdered and taken orally with milk twice a day for one week against white discharges (Rout et al., 2009).

By using tissue culture techniques many fern species have been successfully propagated such as *Asplenium nidus* (Khan et al.,2008), *Dryopteris affinis* (Soare et al.,2010), *Blechnum spicant* (Menendez et al., 2009). Only a few studies were reported on the genera *Cyathea* such as *C.dregei* (Finnie & Staden, 1987), and *C.spinulosa* (Shukla & Khare ,2012) and none on *Cyathea gigantea*. In this paper, we report regeneration of *C.gigantea* through *in vitro* culture using spore as explant.

MATERIALS AND METHODS

Spore Collection

Sporophylls were collected from wild plant of *Cyathea gigantea* Wall. ex Hook. The sori were separated from the pinnulus by scraping with a sterile blade and collected in glass vials stored at 4°C in a refrigerator.

Sterilization of Spores

Surface sterilization of spores were done with 30%(w/v) solution of sodium hypochlorite (4% active chlorine)for 7 minutes before filtering through autoclaved filter paper and washing several times with autoclaved double distilled water. The spores were then inoculated in medium with wet condition.

Germination Test

For the germination test about 5mg of sterilized spores were sown in 20 ml of liquid medium. The flasks were plugged with sterile cotton plugs wrapped with aluminium foil. All procedures was carried out inside Laminar Air Flow Cabinet.

Viability Test

Spores were taken on a slide and mounted with glycerine solution and observed under microscope in order to confirm that spores are fertile. Viable spores were round and dark brown in colour.

IN VITRO TECHNIQUE

Preparation of medium

The nutrient medium (MS and fern propagation media) was used . Before solidifying with agar power, the pH of the medium was adjusted with 1N HCl and 1N NaOH solutions between 5.8 to 6.0. After adjusting the pH agar (Himedia) powder was mixed with the medium and boiled for some time to obtain a clear solution. After that about 30ml of the medium was taken in each 100ml culture bottle and plugged properly. The culture bottles were then autoclaved under 121°C and 15lbs/sq inch for 20 mins to make the medium microorganism free. The culture bottles containing the nutrient medium were then allowed to cool for 24hrs in the culture laboratory.

Inoculation of spores

The surface sterilized spores were inoculated in the culture bottles under Laminar Air-Flow Cabinet.

The cultured spores were incubated in Growth Chamber of the Tissue culture Laboratory at 25°C ± 1°C, under 16 hours photoperiod under illumination 2500-3000 Lux. After every two weeks the cultured material were transferred into new medium for better growth of plants.

Standardization of Medium

Two media Murashige & Skoog (1962) medium and Fern Propagation medium without sugar was selected for spore germination. Different growth regulators selected for the study were IAA, KN. The different combinations of growth regulators were tried and observed its effect on growth of spores.

Acclimatization

Pots were filled with brick bats, leaf mold, charcoal, dried moss in 1:1:1:1 proportion.

Sterilization of the potting mixture

The earthen pots containing the potting mixture were autoclaved at 20lbs/sq inch, pressure for 30 minutes. After sterilization the pots were allowed to cool down for 48 hours and then kept in room temperature in a well ventilated room.

Transplantation

Plants were found to be ready for transplanting in hardening medium as the regenerated plants develop sufficient root like rhizoid. Rooted plants were removed from the culture bottle and rhizoids were washed thoroughly to remove the medium sticking to the plant. Then the plantlets were transferred to sterile small plastic cups containing vermiculite (a sterile inert medium for planting transferred plants) and kept inside the tissue culture room for acclimatization before exposing to the natural environments. During this period the plants were sprayed with liquid MS medium without agar and sugar, thrice in a week. After one month the plants were transferred to acclimatization media and medium comprised of pre sterilized Brick bats + Charcoal + Dried moss + leaf molds.

RESULTS

In vitro multiplication was investigated during this study and for this purpose, a series of experiments were performed.

Effect of sterilants for sterilization three different kind of surface sterilants were used for spore sterilization. 30% (w/v) sodium hypochlorite was found to be best for sterilization of spores as spores are very sensitive. Spores were treated for seven minutes with 30% sodium hypochlorite to sterilize. Below 30% the spores were not sterile and high mortality was observed when more than 30% concentration was used or treatment time was more.

Table 1 Average germination percentage

Media	Media composition	Period of germination (Days)	Germination percentage (%)
MS	MS + 0 mg/L sucrose + 0 mg/L IAA	25-27	72
Fern Propagation Media	FP+0 mg/L sucrose + 0mg/L IAA	12- 14	58

Prothallus formation After 25-27 days the spores on MS media (full strength) became swollen and a small heart like structure appeared called prothalli. Spores cultured on MS media showed 72% germination (table 1). Spores cultured on Fern propagation media (FPM) germinated within 12-14 days but germination percentage was not satisfactory only 58%. Observing both the parameter germination percentage and germination period MS media was selected for further multiplication of prothallus.

Table 2 Growth of gametophyte (prothallus) after 4 month in terms of fresh weight (mg) for 10 nos. of prothallus

Conc. of IAA in MS medium (mg/L)	Fresh weightb of prothallus (mg)
0.5mg/L	95
0.6mg/L	111
0.7mg/L	123
0.8 mg/L	136
0.9mg/L	110

Multiplication In this series of experiments 5 different media formulation was tried. After spore germination prothallus was transferred to the media containing different concentration of IAA (table 2) for growth of gametophytes. Fresh weight of the prothallus was observed which was 136mg in 0.8mg/L IAA (table 2). More concentration than 0.8mg/L reduce the growth of prothallus. MS media supplemented with kinetin and IAA showed profound effect on sporophyte initiation. MS media containing kinetin and IAA at concentration 5mg/L and 0.8mg/L respectively showed significant increase in multiplication of plant and length of the sporophyte was 20.29 cm (table 3) where high concentration of kinetin and IAA showed detrimental effect of the dose and low concentration reduce the number of plantlets.

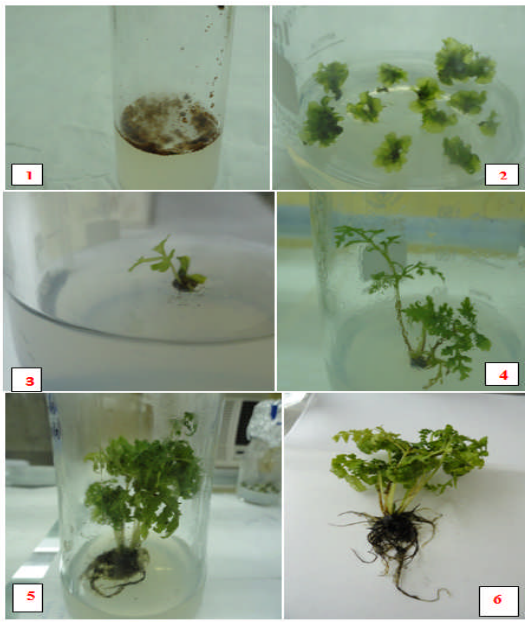

Fig. 1 Inoculated spores, **Fig. 2** Prothallus developed.
Fig. 3 Sporophyte developed from gametophyte.
Fig. 4 Sporophyte after 4 month,**Fig. 5** Full grown sporophyte.
Fig. 6 Length of sporophyte.

Rooting For root induction experiments were also done in order to optimize the rooting medium. Full strength MS medium supplemented with auxin (IBA) was tried for rooting (Data not shown).

Acclimatization cultured plants were transferred in the sterile vermiculite mixture for one month. After full growth of the cultured plants were transferred to potting mixtures containing brick bats + leaf mold + charcoal + dried moss (1: 1 : 1 : 1).Surviving rate 81% was observed after one month.

Table 3 Effect of Growth regulators on the length of sporophytes (Data collected after 10 month)

Treatment	IAA mg/L	KN mg/L	Mean Sporophyte Length (cm)	Sporophyte length ± SE
T_1	0.8	1	9.74	9.74± 0.20
T_2	0.8	2	11.44	11.44 ± 0.25
T_3	0.8	3	13.78	13.78±0.29
T_4	0.8	4	16.88	16.88±0.38
T_5	0.8	5	20.29	20.29±0.23

DISCUSSION

In this study spores were used as explant for *in vitro* culture. According to Linsay (1994) fern propagation by using spore is a reliable method and considered more advantageous than the vegetative propagation. To sterilize the spores 30% sodium hypochlorite for 7 minutes was used. Less concentration or more exposure to surface sterilants directly affect the germination rate of the spore. Fern propagation by using spore depends on many factors such as viability and storage of spores, media, sterilization of spores, temperature, pH range, gametophyte and sporophyte interaction (Kaur, 1991). Surface sterilize spores started to germinate within 25 – 27 days in MS medium (Full strength) and only 12 – 14 days required for germination in Fern Propagation medium (Full strength).Full strength MS medium was used during this study where half strength MS medium is preferred in previous experiment (Fernandez et al., 1997, Kyte & Kleyn , 1996).Spore germination period vary from few days to a year (Doughlas & Sheffield,1992). Smith & Yee (1975) observe *Nephrolepis* spores germinate in 3 - 4 days in culture while in ophioglossaceae germinate after 6 month of inoculation (Whittier,1981). *Helminthostachys* species are known to take 8 month to germinate (Hedge & Dsouza, 2000). According to Page (1979) in the majority of Cyatheaceae species the spore germination abilities decrease after a few weeks of storage. In *Cyathea delgadii* spores lost their viability for germination during 2 months at 12°c in presence of low humidity (Randi & Felippe, 1988). Spores of *C. gigantea* was germinate in full strength MS medium without sugar and 72% germination was observed in MS medium while in Fern propagation medium 58% germination was observed. Growth and proliferation needs sugar supplementation but germination of spore is generally inhibited by the presence of sugar in the medium (Randi & Renner, 2004) and the result of the present work was similar with the earlier reported work. Low concentrations of micro and macro salts was suitable for spore germination and gametophyte development as reported earlier in *Cyathea australis*(Goller&Rybczynski,1995),*Dicksoniasolviania*(Khoo & Thomas,1980).MS medium was taken for further culture of gametophyte and sporohyte because germination percentage was maximum in it. Knop and MS medium is best for growth and differentiation of fern as reported by Cheema (2005).

Media containing IAA at concentration 0.8mg/L and kinetin at 5 mg/L produced sporophyte with 20.29 cm (table 3) length. At higher concentration explants started to browning which confers detrimental effect of the dose (Higuchi & Amaki,

1989).For multiplication presence of auxin and kinetin in medium seems to be very important and the ratio between auxin and kinetin also very valuable for proper multiplication of plant (Bertrand et al.,1999, Fernandez et al ., 1999). Many reports are there which reveal that application of growth regulators enhance and suppress the growth of plant (Fernandez & Revilla, 2003). Media without auxin (NAA) produce much shoot and NAA is not essential for shoot production, only kinetin is necessary for shoot production of fishtail fern (Beck & Caponetti,1983). Result of the present study was totally different from previously cited work where in present work IAA and kinetin was essential for shoot proliferation.

A micropropagation protocol of *Cyathea gigantea* is successfully established in this work in which spores are used as explants. It is the fastest and most economic method for *in vitro* propagation and using this propagation technique tree fern can be regenerated in laboratory conditions which are vanishing from their natural habitat.

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