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In vitro study of effects of growth hormones on sporophyte development of *Cyathea spinulosa*

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***Cyathea spinulosa* Wall. ex Hook. is a type genus of Cyatheaceae family. It is listed in endangered category of IUCN Red Data Book and also placed in Convention on International Trade of Endangered Species of Flora and Fauna (CITES) Appendix II. The spore germination, gametophyte morphogenesis and sporophyte rooting of *Cyathea* in response to 2.0% sucrose and three major growth hormones: Indole–3 acetic acid (IAA), indole–3 butyric acid (IBA) and naphthalene acetic acid (NAA) was studied. The study was carried out at the tissue culture laboratory of Central Department of Botany, Tribhuvan University, Nepal. The spore of *Cyathea* was reared on Knop's Basal Medium (KBM) with 2.0% sucrose after surface sterilization. The sporophyte was sub-cultured on medium with growth hormones IAA, IBA and NAA, each containing 1, 2 and 3 ppm concentrations. The study suggested that *Cyathea* exhibited bipolar germination and the gametophyte development was Adiantum type. The mature gametophyte was observed as heart shaped. Sex organs were not observed up to 24 weeks which indicates the development of apogamous sporophyte. The growth hormones affect the development of leaves and roots. The sporophyte containing three leaves and two roots was the best result obtained in KBM containing 1 ppm IAA.**

Key words: Cyatheaceae, *Cyathea spinulosa*, morphogenesis, Knop's medium, growth hormones, apogamous sporogophyte.

INTRODUCTION

Cyathea spinulosa Wall. ex Hook. is a genus of Cyatheaceae family (Leptosporangiate of Pteridophyta), and well known for its excellent beauty for outdoor decoration. This plant prefers warm and humid atmosphere for its luxuriant growth along the streams side at 335 to 2000 m altitudes in East and Central Nepal (Gurung, 1991). It has great afforestation value due to well adoptability to push the rhizome into barren soil, act as colonal of disturbed soil especially in water logged areas, shade beneath the fronds help in the establishment of other grass, the root system efficiently binds top soil, can be easily propagated by rhizome cutting and splitting (Gurung, 1992). It is economically important and the wood log is much in demand in orchid cultivation and for starch extraction (Khare et al., 2005). It was listed in endangered category of IUCN Red Data Book in 1998.

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It is also included in the appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2013).

It has been possible to induce apogamous sporophytes and aposporous gametophyte without the intervention of fertilization and sporogenesis by suitably manipulating the cultural condition *in vitro*. Nutritional studies on fern have highlighted their response to cultural condition (Bir et al., 1982). Some of the studies carried out on tree fern were variation in performance across a successional mosaic (Arens, 2001), effect of sterilization and storage conditions on the viability of spores (Simabukuro et al., 1998), relationships of Papuasian Cyatheaceae (Raubeson et al., 1995), spore germination and gametophyte development (Rechenmacher et al., 2010), evidence for Gondwanan vicariance and limited transoceanic dispersal (Korall and Pryer, 2014), effects of light, temperature and pH on spore germination and early gametophytic development (Du et al., 2009), morphology of the gametophytes and young sporephytes (Huang et al., 2001). Some of the studies in which fern gametophytes have been used include investigation of growth correlations, regeneration and hormonal effects (Albaum, 1938a, b), the induction of sex organ formation (Naf, 1956, 1958), the effect of the inhibition of protein synthesis on growth (Hotta and Osawa, 1958), induction of apogamous sporophyte development (Whittier and Steeves, 1960, 1962; Mehra and Sulklyan, 1969; Kato, 1970), effect of sucrose on heteroblastic leaf development (Sussex and Cluttter, 1960; Caponetti, 1970), effect of storage temperature (Ballesteros et al., 2011) and many more.

The objectives of this research were to observe the effect of 2% sugar concentration on the gametophyte morphogenesis in the spore germination and to investigate the effect of different growth hormones on *in vitro* rooting, and micropropagation of *C. spinulosa*. *In vitro* studies of threathened species- *C. spinulosa*, so far has not been done in Nepal. Realzing the importance, it is very necessary to study *Cyathea in vitro*.

MATERIALS AND METHODS

Spores of *C. spinulosa* were collected from Godavari Botanical Gardens, Kathmandu, Nepal for micropropagation. Fresh spores were taken from May to October and dry spores were taken on remaining months.

Surface sterilization, spore culutre and preparation of explants

The mature fronds of *C. spinulosa* with spores on their ventral surface were washed serially with diluted detergent in running tap water for 30 to 45 min, and with distilled water for 3 to 4 times. The pinna was cut into small pieces and surface sterilized with 1% sodium hypochlorite solution for 12 min and finally rinsed with sterile distilled water for 5 to 6 times inside the laminar airflow chamber. Spores were taken in the sterilized Petri dish with the help of surgical blade pressing one side of the frond with the forceps. Then, spores were inoculated on 10 different cultrue tube containing slanting Knob's basal medium (KBM, 1884) containing 2% sucrose. Joshi et al. (2002) studied the effect of sucrose on spore germination of *C. spinulosa*. They found 91% as the highest percentage of spore germination in KBM with 2% sucrose. Similar protocol had been adopted in the study.

For the sub-culture of sporophytes, sporophytes were cut down into small pieces in Petri dish and inoculated in KBM containing different concentration (1, 2 and 3 ppm) of growth hormones– Indole–3 acetic acid (IAA), indole–3 butyric acid (IBA) and naphthalene acetic acid (NAA) by the help of forceps (Table 1). All the cultures were maintained at 25°C (±2) and eight-hour light daily. The responses were observed after one week of culture. Various stages of germinating spores and prothalli were observed under microscope. The slides were prepared by mounting in glycerine. Each culture was subcultured after three months on fresh media. The observations were taken in one week interval.

Acclimatization

The sporophytes were observed after 24 weeks of spore inoculation. After several sub-culture, sporophytes having developed roots were grown in soil containing soil, compost and sand in 2:1:1 ratio and high humidity was maintained covering by plastics. After few weeks the plastic cover were removed and the plants were planted in pots.

RESULTS

Spore germination and gametophytic morphogenesis

A spore, consisting of a single haploid cell, has simple requirements for differentiation. It is significant that no nutritional or hormonal gradients are imposed on the spore by the adjoining cell (Rasid, 1999). The spores did not germinate as soon as they were shed. They remained dormant for a period of time ranging from 3 to 4 weeks to 10 to 12 weeks. The rate of spore generation with respect to different period of year when spore collected is represented in Figure 1. The dormancy was found to be dependent on temperature of spore collection, maturity of spores and degree of hydration. At the lower temperature during winter, the spore took up to 12 weeks to germinate while during summer spore germinated within 3 to 4 weeks.

Swelling, expansion and greening of a spore (Figure 2) and rupture of exine were the basic pre-germination changes. That change was followed by the appearance of small protuberance like structure almost all over the spore surface. With the opening of the exine, the protoplasmic mass of the spores with intine appeared in the form of small protuberance, that is, protonemal filament. The germination of spore was bipolar resulting in the formation of small rhizoidal cell and a larger protonemal initial cell. A rhizoid was seen to be given off from the rhizoidal initial cell. The protonemal filament ultimately developed into prothalli after 8 weeks of spore inoculation (Figure 3) which contained few numbers of rhizoids.

Table 1. Culture of sporophyte on KBM containing various concentration of IAA, IBA and NAA.

Weeks of observation	IAA concentration			IBA concentration			NAA concentration		
	1 ppm	2 ppm	3 ppm	1 ppm	2 ppm	3 ppm	1 ppm	2 ppm	3 ppm
6 weeks	2 leaves + 2 roots	1 leaf + no root	2 leaves + no root	2 leaves + no root	1 leaf + no root	4 leaves + no root	2 leaves + no root	1 leaves + no root	2 leaves + 1 root
12 weeks	3 leaves + 2 roots	2 leaves + no root	3 leaves +1 root	Yellower and dead	2 leaves + no root	4 leaves + no root	3 leaves + no root	2 leaves + no root	3 leaves + 1 root
18 weeks	3 leaves + 2 roots	2 leaves + no root	3 leaves + 1 root	-	4 leaves + no root	6 leaves + no root	4 leaves + no root	3 leaves + no root	4 leaves + 1 root

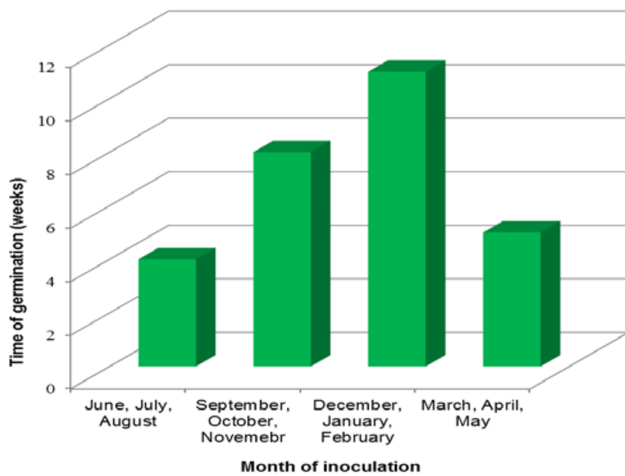

Figure 1. Effect of spore collection date on spore germination.

Figure 2. Sweeling, expansion and greening of spore.

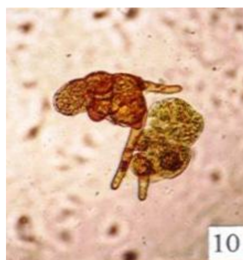

Figure 3. 8-Celled prathallus after 8 weeks (400x).

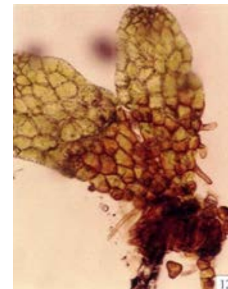

Figure 4. Gametophyte showing meristematic region at apical end and non-germinated spores below gametophye after 12 weeks (100x).

Structure of prothallus/gametophyte

Spatulate gametophytes with clearly visible meristematic cells at the apical end were observed after 12 weeks (Figure 4). At that stage, rhizoids were shorter than the length of the gametophyte. Numbers of rhizoids were more towards the distal end while no rhizoids were observed towards apical end. After 14 weeks, apical notch began to form at the anterior end below that laid the growing apex, consisting of meristematic cells. After 16 weeks, mature heart-shaped gametophytes were found. The growing apex was present in a deep notch at its anterior end, consisting of meristematic cells (Figure 5). The gametophytes were dorsoventrally flattened and numerous delicate rhizoids ascended from under surface. The rhizoids were unicellular, hyaline in colour, and double-layered wall. There were a single nucleus and a large number of chloroplasts in each cell and there were no intercellular spaces between the cells. Gametophytes of different age (20 and 22 weeks) grown on KBM + 2% sucrose are shown respectively in Figures 6 and 7. During entire developmental stages, the sex organs, that is, both antheridia and archegonia were not observed. This clearly indicated the development of apogamous sporophyte.

After three successive subcultures on KBM with 2% sucrose, the sporophytes initiation was observed only after 24 weeks of spore inoculation (Figure 8). After 26 weeks of culture, both gametophyte and apogamous sporophyte were observed in same medium (Figure 9).

Figure 5. Mature heart-shaped gametophyte clearly showing notched meristematic region and chloroplast affter 16 weeks (100x).

Figure 6. Gametophytes inside culture tube after 20 weeks.

Figure 7. Gametophytes inside culture tube after 22 weeks.

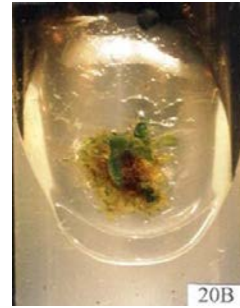

Figure 8. Development of sporophytes start after 24 weeks.

Figure 9. 26 weeks old culture showing both gametophyte and apogamous sporophyte.

Figure 10. 30 weeks old apogamous sporophyte.

After 30 weeks, the well-developed sporophyte was obtained (Figure 10). At that stage, roots were very few in number, unbranched and feebly developed. Rhizome were small, leaves were small and bipinnate or bifid.

Figure 11. Sporophyte on KBM + 1 ppm IAA after 12 weeks.

Figure 14. Sporophyte on KBM + 3 ppm IAA after 12 weeks.

Figure 12. Sporophyte on KBM + 1 ppm IAA after 18 weeks.

Figure 13. Sporophyte on KBM + 2 ppm IAA after 6 weeks.

Culture of sporophyte

The sporophyte explants were obtained from the *in vitro* grown spore on KBM with 2% sucrose. They were sub-cultured on KBM containing various concentrations of IAA, IBA and NAA.

On KBM with 1 ppm IAA, there was small protuberance like gametophyte on lower part of sporophyte with two roots observed after four weeks of culture. After six weeks, there was sporophyte with two leaves and two roots. One leaf was added and altogether formed three leaves and two roots after 12 weeks (Figure 11). This was sub-cultured on new media with same concentration of hormone. After 18 weeks of subculture, sporophyte with 3 leaves and 2 roots was observed (Figure 12). On KBM with 2 ppm IAA, there was only one leaf, root failed to form after six weeks (Figure 13). After 12 weeks, one leaf was added and two-leaved sporophyte was formed. After 18 weeks, still there was only two-leaved sporophyte, roots were not developed. On KBM with 3 ppm IAA, sporophyte only with three leaves was observed after six weeks. After 12 weeks, the sporophyte with three leaves and one root was developed (Figure 14). After 18 weeks the leaf and root number were same as 12 weeks and no further development was observed.

The sporophyte cultured on KBM supplemented with 1 ppm IBA showed two new leaves and no root after six weeks of culture (Figure 15). This remained as such for 10 weeks and after that ultimately became yellower and died. On KBM with 2 ppm IBA, after six weeks there was sporophyte with one leaf only and one gametophyte like body, but root was absent (Figure 16). After 12 weeks there was sporophyte with two leaves and no root (Figure 17). The gametophyte like body became brown and dead. After 18 weeks, sporophyte with four leaves and no root was observed (Figure 18). The sporophyte cultured on KBM supplemented with IBA 3 ppm showed four new leaves and no root after six weeks (Figure 19). Up to 12 weeks, the sporophyte remained as above. While after 18 weeks, two leaves were added and finally sporophyte with

Figure 15. Sporophyte on KBM + 1 ppm IBA after 6 weeks.

Figure 18. Sporophyte on KBM + 2 ppm IBA after 18 week.

Figure 16. Sporophyte on KBM + 2 ppm IBA after 6 weeks.

Figure 19. Sporophyte on KBM + 3 ppm IBA after 6 weeks.

Figure 17. Sporophyte on KBM + 2 ppm IBA after 12 weeks.

Figure 20. Sporophyte on KBM + 3 ppm IBA after 18 weeks.

with six leaves and no root was observed (Figure 20).

On KBM with 1 ppm NAA, the sporophyte with two leaves and no root was observed after six weeks (Figure 21). There was appearance of three leaves and no root after 12 weeks. After 18 weeks, there was sporophyte with four leaves and no root (Figure 22). When sporophyte was cultured on KBM with 2 ppm NAA, there was sporophyte with one leaf and no root after six weeks. After 12 weeks, two leaves sporophyte appeared where roots were to develop (Figure 23). After 18 weeks, sporophyte with three leaves were observed, root was not developed. On KBM supplemented with 3 ppm NAA, the sporophyte having two leaves and one root was observed after six weeks (Figure 24). After 12 weeks, three leaved and one-rooted sporophyte was found (Figure 25). The sporophyte with four leaves and one root was observed after 18 weeks (Figure 26).

When sporophytes were cultured on KBM with 2% sucrose, there were significant number of leaves but roots failed to develop. Roots were not observed up to 18 weeks (Figure 27).

Figure 21. Sporophyte on KBM + 1ppm NAA after 6 weeks

Figure 22. Sporophyte on KBM + 1 ppm NAA after 18 weeks.

Figure 23. Sporophyte on KBM + 2 ppm NAA after 12 weeks.

Figure 24. Sporophyte on KBM + 3 ppm NAA after 6 weeks.

Figure 25. Sporophyte on KBM + 3 ppm NAA after 12 weeks.

Figure 26. Sporophyte on KBM + 3 ppm NAA after 18 weeks.

Acclimatization

The rooted sporophytes were then transferred to soil and were kept at high humidity to avoid desiccation of the delicate fern fronds by covering with plastic from the upper side. The sporophyte with green leaves remained up to one week (Figure 28).

DISCUSSION

Sugar is an important factor for the spore germination. Kato (1973) observed that carbon compounds have a potent influence on protonema growth and secondary rhizoid formation. Parajuli and Joshi (2013) found 86% of spore germination of *Colysis latiloba* in 1% sucrocse. Similarly, in *Microsorium alternifolium* and *Microsorium scolopendria,* maximum germination was reported on media containing 1.0% sucrose and glucose while in *Melanophidium punctatum* and *Anisocampium cumingianum,* highest percentage of spore germination was reported in 0.25 and 1.5% sucrose respectively (Joshi, 1977). The findings of Miller and Miller (1961) showed that 1% sucrose for spore culture of *Onoclea sensibilis* growing under low intensities of light increased the growth rate of gametophyte. Whittier (1964, 1965) also found that vegetative growth of prothallus was accelerated by sucrose in *Cryptomium falcatum*, *Cheilanthes tomentosa* and *Cheilanthes farinose.*

Figure 27. Sporophyte on KBM after 12 weeks.

Figure 28. Acclimatization of rooted sporophyte.

The spore of ferns shows various germination pattern viz. bipolar, tripolar and amorphous germination (Rashid, 1999). Nayar and Kaur (1968) have considered bipolar pattern as most advanced where first division results in a small rhizoidal cell and large prothallial cell. *C. spinulosa* also exhibited bipolar germination in the study. The gametophyte of *C. spinulosa* was heart-shaped. Prothalli of homosporous fern follow a definite pattern of development and attain a characteristic adult form. Seven different patterns recognized (Nayar and Kaur, 1969) are: Osmuda-type, Marattia-type, Adiantum type, Drynaria-type, Kaulinia-type, Ceratopteris-type and Aspidum-type. The *C. spinulosa* gametophyte development was Adiantum-type.

Presence of sugar, carbon source for vegetative growth under sterile condition in the medium has been reported as one of the causal agents in the induction of apogamy (Kato, 1970; Mehra and Sulkalyan, 1969; Whittier 1964a, b). In the present work, sucrose at the concentration of 2% induced apogamous sporophytes in *C. spinulosa*. Similarly, Whittier and Steeves (1960, 1962) induced apogamy in *Pteridium aquilinum* by using 4.0% sucrose. They obtained greater response of apogamy at 5.0% sucrose. In *Ampelopteris* also 4.0% sucrose or 5.0% sucrose with 1 to 2 mg/l 2, 4-D was found optional for apogamous soprophyte initiation (Sulkalyan and Mehra, 1971). Earlier investigation of induced apogamy was done on soil grown gametophyte. Sulklyan and Mehra (1977) induced apogamy in the haploid and diploid gametophyte of *Nephrolepsis cordifolia* on Kundson's medium supplemented with 0.5 to 2% sucrose. Chopra and Loyal (1979) induced apogamy in four-months old culture of *Ceratopteris pteroides* containing 3-5% sucrose whereas the perennial gametophytes of *A. lepotophylla* exhibited apogamy in half year old cultures raised on sucrose free medium. Kawakami et al. (1995) has in-duced apogamous sporophyte in *Pteris multifida* asepti-cally in the dark but dark grown culture did not form apo-gamous sporophyte even when supplied with sugar (Whittier and Steeves, 1962). While, Elmore and Whittier (1974) has demonstrated the involvement of ethylene, a natural product of gametophyte metabolism as a true cause of apogamous bud induction.

In the present study, when the sporophytes of *C. spinulosa* were cultured on the KBM with different concentration of auxin, that is, IAA, IBA and NAA, best rooting was observed in KBM + IAA (1 ppm), KBM + IAA (3 ppm) and KMB + NAA (3 ppm), while no root was observed in IBA. Arockiasany et al. (2000) also found the regenerated shoots of *Pterocarpus santalinus* L. rooted on quarter strength of MS medium with IAA (1 mg/l). But IBA at 10 mg l^{-1} had a significant effect, both on rooting percentage and rooting system morphology of *Acacia tortilis raddiana* microcuttings, at NAA (1 to 5 mg l^{-1}) number of roots were low but strong (Sune et al., 2001). Copes and Madal (2000) has found 1 μM NAA treatment induced significantly greater rooting than the control in Douglas- fir cuttings. He also found 24.6 mM IBA produced greater rooting percentage. He further concluded that rooting decreased with both too little and too much auxin. Asami et al. (2000) found the rate of rooting from the adventitious shoots cultured in MS medium containing 0.1 mg l^{-1} NAA was higher than cultured in MS medium without NAA in the Japanese butterbur. Bonomo et al. (2013) noted that supplementing media with BAP affected the longiitudinal growth of the filamentous gametophytes, and the proliferation of ramification from early stage.

Conclusion

The result of the study suggested that *C. spinulosa* exhibited bipolar germination and the gametophyte development was Adiantum type. The mature gametophyte of *C. spinulosa* was observed as heart shaped. Sex organs were not observed up to 24 weeks which indicates the development of apogamous sporophyte. The sporophyte containing three leaves and two roots was the best result obtained in KBM containing 1 ppm IAA.

The knowledge on germination, morphogenesis and sporophyte development are very important to undersand the entire life cycle of *C. spinulosa* and to analyse the effect of sugar and various growth hormones. Moreover, these findings provide information to support the cultivation, management of conservation of this endangered species.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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