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Ex situ conservation of endemic fern allies, *Huperzia hilliana* (Spring) R.D. Dixit.

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Huperzia species are currently going to endangered category list of plant taxa. Most of the *Huperzia* species have been used for medicinal purposes. Huperzine A is derived from *Huperzia serrata*, which is currently clinically used for Alzheimer disease (AD). The present study has revealed in detailed about the development of the sporangia, spore germination, early stage of gametophytes and regeneration of *Huperzia hilliana* (Spring) R.D. Dixit. in its natural condition of Kodaiyar region, South India. The results of *H. hilliana* was microscopically observed for good spore germination within one month, after the matured stage of sporangia, spore germination stages, primary stage of tubercle and later heart shape gametophytes were also observed in natural conditions. Finally, it showed vigorous growth after the early stage of sporophytes and had a short period of six month life cycle which influence by environmental factors.

Huperzia /Conservation biology

Lycopods are one of the oldest primary vascular plants in the world. In the early Devonian period, Lycopods family dominated as large trees on landscape, but today it is found only in the reserve forest floor as common club mosses (Gensel, 1980; Chaloner, 1970).

Huperzia belongs to the family Huperziaceae, which covers about 415 species all over the world (Hassler and Swale, 2006) and out of them, 21 species are found in India (Dixit, 1984; 1987; Hassler and Swale, 2006). Many studies have been reported that the natural products of Huperziaceae, which contains various bioactive constituents including lycopodium alkaloids, triterpenes, flavones and phenolic acids possess pharmacological activities (Towers and Maas, 1965; Voirin and Jay,1978; Tong et al.2003; Shi et al.2005).

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About 211 *Lycopodium* alkaloids had been derived from various *Huprezia* species are Huperzine A, Huperzine B, N-methylhuperzine B, huperzinine, Lycoporine A and carinatumine A and B. Among these compounds, the lycopodium alkaloids, especially Huperzine - A (Hup A), were originally derived from *Huperzia serrata* investigated by several authors (Ma et al.2004; Ma et al.2005; Ma et al.2006). Huperzine A (Figure 1) was found the most powerful acetyl cholinesterase activity and exhibited positive effects on learning and memory *in vitro* and *in vivo* studies (Ma & Gang., 2004; Ma *et al.*, 2007).

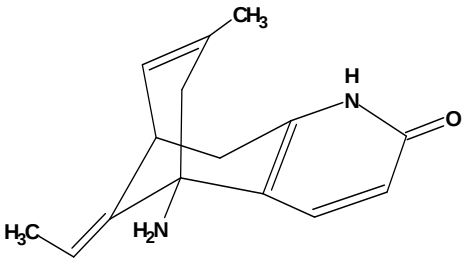

Fig.1. Chemical Structural formula of Huperzine A

Ex-situ conservation is one of the best conservation methods. Earlier studies on *Lycopodium* species conserved by *ex situ* method are reported (American Horticultural Society,1999). Fraser-Jenkins, (2008) reviewed on most of the *Huperzia* species were endemic to Southern Western Ghats and Western Himalaya. *Huperzia hilliana* (Nessel) Holub is going to an endangered category from Western Ghats region of South India (Benniamin et al. 2008). Fraser-Jenkins, (2008) reported that *H. hilliana* is endemic to South India, its synonym is *H. nilagirica* (Spring) R.D. Dixit. Recently, Maridass and Raju, (2009) have studied the phytochemical and antimicrobial activity of *H. hilliana* leaves and their leaf extract which was active against several human pathogens such as *Pseudomonas aeruginosa, Salmonella enteritidis, S. typhimurium, Staphylococcus aureus, Bacillus subtilis* and *Bacillus cereus.*

The present investigation observed the development of the sporangia, spore germination, early stage of gametophytes and regeneration of *H. hilliana* from the natural condition of Kodaiyar region, South India.

Photo.1. Natural habitats of mature fern allies of *Huperzia hilliana* occurs in Kodaiyar region, South India

Materials and Methods

Field evaluation and observation of *H. hilliana* were conducted at monthly intervals in the 3 selected sites of Southern Western Ghats region, South India during March 2009 to May 2011 having high altitudes ranging from 850-1200m. *H. hilliana* were collected and photographs were taken at each location. Field notes *viz.* ecological data, habit, habitat and some diagnostic characters of each location were recorded.

Spore collection

H. hilliana were collected and stored on clean paper in a dry environment with a constant temperature of 18^{o}C until the sporangia opened. Spores were collected and stored in cool and dry conditions.

Microscopical observation

Mature sporangia of *H. hilliana* were observed by microscopically. The spore germination and developmental stages of gametophyte on natural conditions were studied.

Propagation

As per the American Horticultural Society (1999) methods, 1 to 2 cm of rhizome attach with a root of *H. hilliana* was planted into the sand pots containing natural medium of equal parts of leaf moulds, sand, peat moss and loam. They are transplanted to the field conditions of low altitude. The rhizomes of *H. hilliana* were

kept in the moisture conditions and start in the growing stages was observed upto three months.

Results and Discussion

H. hilliana was very small and perennial herb, stem pendent, up to 10cm long, 0.8 - 1.3cm wide with leaves, 0.3 cm wide without leaves, dichotomously branched equally or unequally up to two to three times. Leaves are numerous, arranged in close spirals, adnate, ascending, up to 7 x 1.4 mm, oblong, slightly narrowed towards basal and distal halves, acute, entire, uninerval dark green, herbaceous, glabrous. Sporangia was borne on the axils of the leaves towards the distal most (up to 3-4cm) part of the branches, reniform, up to 1 x 0.8 mm, yellowish, dehisced in to two equal halves by vertical slit a along the outer edges; spores trilete, tetrahedral, 30-12µm in diameter, grayish - green, exine alveolate. Earlier studies, Parihar, (1973) reported that club mosses are small herbs, vascular plants, basically dichotomously branching and reproduced by spores . They were consisting of roots, an aerial stem and microphylls or scale-like leaves. These are small and spirally arranged on an elongated stem. The spores are small size, borne singly in the axils of specialised leaves (sporophylls) that are often aggregated into cone-like strobili similar to or different in shape and size to sterile leaves. The sporophylls may be aggregated into definite strobili or in zones on the stem (Parihar, 1973; Jermy and Camus, 1991).

Photo.2: Mature sporangia of *H. hilliana*

Spore morphology

The present study of spore morphology of *H. hilliana* was observed as smooth; trilete shaped, and size 3-12µm (Photo.3A). Most the *Huperzia* species found as monolete spores (Jones and Blackmore, 1988), and tetrad spores also produced in *H. australiana, H. beccarii, H. serrata* and *H. goliathensis* (Kongoi et al. 2007). But some earlier studies of some *Huperzia* species found as trilete spores (Chaloner, 1970; 1976; Gensel, 1980). Kongoi et al. (2007) reported that spore morphology was useful for identification, and classification of Lycopodiaceae (Kongoi et al.2007).

Spore germination

The present study about the complete life cycle of *H. hilliana* was observed by natural condition in every week of six month period from February, 2011 - July, 2011. It showed quick and vigorous growth and short period of life span within four months in the observed by period from May, 2011-July, 2011 (Photo.1). The observed results revealed that sporophytic stages within growth period of four months in natural condition. The sporangia were formed in one month sporophytes plants. They were yellow in colour, kidney-shaped (reniform), situated along the stem in the angle between the stem and attached leaf axils. The growth of sporangium was very short period (Photo.2&3). The germination of spore and developmental stages of early gametophytes recorded within in a one month. Huperziaceae are very slowly growing and normally requiring fifteen to twenty years of growth from spore germination to maturity (Ma et al.2004; Ma et al.2006). Recently, Singh and Singh, (2010) reported that the very slow growth of *H. serrata* develop from spore to mature plant needed 15- 20 years. The present study observed that *H. hilliana* was showed vigorous growth, spore formation, maturation and germination within three month period.

Development of gametophytes

The collected sporangium of *H. hilliana* was observed by microscopic and the early stage of germination of spore (Photo.3B) also noticed within a week after maturation of sporangium (Photo.2). Like that, Ernest and Adriance, (1989) reported that *Lycopodiella* species are hundreds to thousands of microspores ripen and disperse from openings in each sporangium during the late summer.

Gametophytes were developed upto eight cells within a week. The development of the primary tubercle was also observed in *H. hilliana* (Photo.3B). Earlier studies observed that germination stage of primary tubercle was formed in gametophyte of *Lycopodiella* at *in vitro* condition (Whittier and Renzaglia,2005). First stage of germination (Photo.3B) and fast growth of later stages of heart-shaped gametophyte stage were noticed (Photo. 3D). Our study reveals that *H. hilliana* spore germinate inside the sporangia when it is shed the developed sporophytes growing on rocks in the river banks of natural conditions. Previous, studies on some of the species of ferns have been shown to have spore banks (Schneller, 1988; Haufler and Welling, 1994). Haufler and Welling, (1994) observed that spores accumulated in the soil and may be in a state of dormancy until certain conditions initiate their germination. In some species, spores near the surface may germinate and produce a pheromone that promotes germination of spores in deeper soil (Haufler and Welling, 1994). Ernest and Adriance,1989, suggesting that they are in a state of dormancy. In addition, spore germination in the lab condition is very low for some species, such as *L. appressa* (Whittier, 1998), suggesting that specific conditions are needed to initiate germination that have not yet been determined. Hence, *L. subappressa* and *L. margueritae* have photosynthetic gametophytes, light is probably necessary for spore germination (Whittier,1998). If these species produce spore banks, germination may be initiated when spores are uncovered from a soil disturbance. The present study reported that *H. hilliana* has good germination and vigorous growth in the natural conditions.

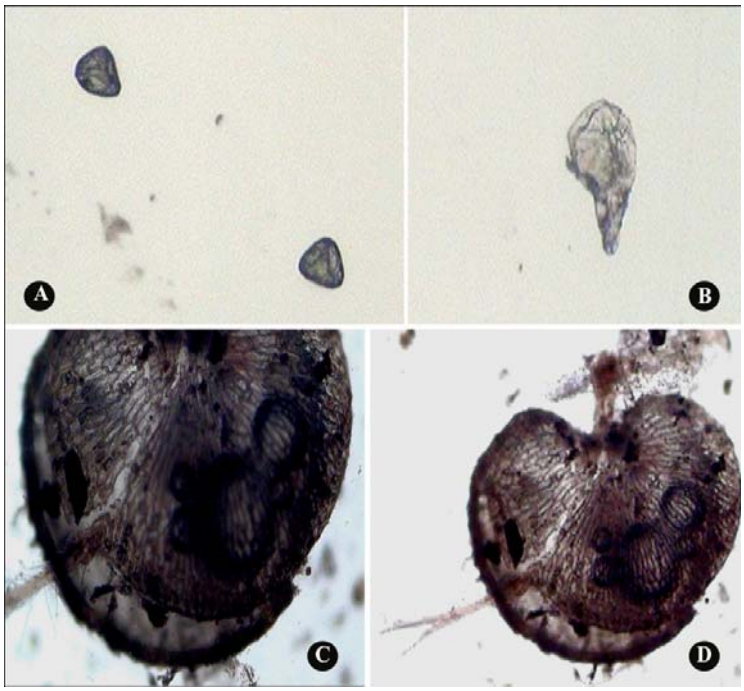

Photo 3. Four sequential stages in gametophyte development in *Huperzia hilliana.* A- spores; B- spore germinated stage of primary tubercle with young photosynthetic ; C- early gametophyte development; D- heartshaped prothallus

Propagation

H. hilliana has successfully established by vegetative propagation growing in it nature condition at lower altitude (Photo.4&5). Previous studies reveal that *Huperzia* species had the ability to reproduce through bulbils, which may reflect due to adaptation of specific environmental factors (Page, 1979; Neyland; 1986; Chuter et al.2008). But the life span of

Huperzia species can take several years in natural conditions (Whittier, 1998; Gola, 2008).

Photo 4: Mass propagation of *H. hilliana* through cutting

Photo-5: Vigorous growth of *H. hilliana* potted in low altitude

H. lucidula spores were germinated approximately three to five years in natural conditions (Whittier and Webster, 1986; Whittier,1998). *Huperzia serrata* was growing in shady habitats with sufficient rainfall and high relative humidity (LiPei-ling et al. 2005). In the present study, it was observed that vigorous growth of *H. hilliana* within six month period. The conclusion of the present observation that *H. hilliana* showed good spore germination and short period of life span. It has noticed that minimum one time rainfall necessary for the growth of *H. hilliana* within three month interval.

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