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In Vitro Multiplication and Ontogeny of Ferns *Tectaria zeylanica* (Houtt.) (Dryoptridaceae) from South India under Current Climate Conditions

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The oak leaf fern, *Tectaria zeylanica* (Houtt.) Sledge, is an endangered fern in India, native to the Southeast Asian region. For the ex-situ conservation of this endangered fern, spores were collected and cultured aseptically on full-strength KC Medium with sucrose at pH 5.8. The spores are monolete with a prominent winged perispore. The exine ruptures at the laesura, and three or four germ filaments grow out, following a Vittaria-type germination pattern. The prothallial development was of the Aspidium type. Adult gametophytes are cordate and much broader than their length. Antheridia develop between 120 and 140 days. They are characteristically 3-celled, with a basal cell, a median cell, and an opercular cell. The female sex organs, archegonia, develop between 150 and 170 days. They are superficial and arranged on the lower side of the prothallus near the midrib

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and the meristematic region. Fertilization and subsequent development of the sporophytes were observed after four months. The highest percentage of sporophyte formation was observed on KC medium fortified with Kin 0.5 mg/l. Sporophytes had uniseriate, unicellular, and multicellular hairs, similar to those found on adult prothalli. The highest percentage of root formation was observed in the medium with IBA + NAA (1.0 mg/l). The in vitro raised plants showed a 54% establishment during hardening in the field. The present study has established a reproducible protocol for the ex-situ conservation of this endangered oak leaf fern.

Keywords: Acclimation; gametes; ontogeny; tirunelveli.

1. INTRODUCTION

Tectaria Cav. is a pantropical genus within the Dryopteridaceae family. Approximately 23 species are known to occur in India [1], with 9 species found in South India [2]. Beddome initially reported 5 species from South India as a whole [3], while Benniamin documented 11 species from India's Western Ghats [4]. *Tectaria zeylanica* was first reported by Beddome from Tirunelveli and Travancore Hills at approximately 2,000 feet elevation [3]. After a 120-year absence, it was rediscovered [5]. Recently, this species was collected from Elumpilonthottam, Marthandam, in Tamil Nadu, South India, where it exhibits a patchy distribution. *Tectaria zeylanica* is distributed across Sri Lanka, Thailand, Vietnam, Southern China, and South India, with limited presence in each of these countries. It is primarily found in isolated patches within the Kanyakumari and Tirunelveli Hills of India's southern Western Ghats.

The current study is dedicated to the ex situ conservation of this endangered fern, along with detailed examination of its in vitro reproductive biology, including gametophyte and sporophyte ontogeny. Through this research, efforts are made to contribute to the preservation of *Tectaria zeylanica*, an important botanical species with a vulnerable presence in the ecosystems of South India.

2. MATERIALS AND METHODS

This endangered species' population was collected on the way to Inchukuzhi forest area, Karaiyar, Tirunelveli, Tamil Nadu, which served as the source of explants for the current study (Fig. 5. A-B). Mature *Tectaria zeylanica* (Houtt.) Sledge spores were collected from wild populations in South India, and sporophytes were also collected and established in the green house at the Research Centre for Plant Science, St. Mary's College, Thoothukudi as a source of explants for tissue culture. After drying the fronds on absorbent paper for 24 hours at room temperature, the liberated spores were passed through 40 µm nylon mesh to remove sporangial wall materials, and clean spores were collected and stored in a refrigerator at 5°C. The spores were surface sterilized for 5 minutes with 0.1 percent HgCl2 solution before being washed with sterile distilled water for 15 minutes. Surface sterilized spores were inoculated onto different media with sugar under aseptic conditions using sterile Pasteur pipettes and incubated at 25°C 2°C for 12 hours (1500 lux). The pH of the media was adjusted to 5.8 before adding agar (0.5 percent w/v) and autoclaving for 15 minutes at 121°C. For spore germination and sporophyte formation, both liquid and agar nutrient media were used. For sporophyte formation, gametophytes regenerated from spores were subcultured on various basal media such as Knops (Knops, 1885), Knudson C [6], Mitra [7], Moore's [8], Murashige, and Skoog's [9]. The percentage of spores that germinated, the growth area of the prothalli, and their development pattern were all studied.

3. RESULTS AND DISCUSSION

3.1 Development of the Prothalli

Tectaria zeylanica spores are monolete, planoconvex in the lateral view, and ovate in the polar view. The earliest spore germination was observed 95 days after sowing, and the majority of spores germinated 90-125 days after sowing. The exine ruptures at the laesura, allowing the germ filament to emerge, which is usually preceded by the first rhizoid. The spore coat remains attached to the basal end of the germ filament for a short time before shedding. The germ filament is typically short, with three or four cells (Fig. 6. D-H), barrel-shaped, and densely chlorophyllous. The basal cell is very similar to the other cells. Under crowded conditions, the cells of the germ filaments become greatly elongated, and the filaments frequently exceed four cells in length. Formation of a cell plate

begins with a vertical-longitudinal division of the terminal cell, which is usually three or four celled (Fig.6. D-H).The plane of cell division and the growth direction of the primary rhizoid and germinal filament follow a Vittaria-type [10] germination pattern. The penultimate cell may also divide soon after. One of the daughter cells of the terminal cell cuts off an obconical apical cell by means of a septum oblique to the median wall separating the two daughter cells of the terminal cell or may divide vertically one or two times by walls parallel to the median wall before an obconical meristematic cell is cut off from one of the daughter cells. The other daughter cell of the terminal cell generally produces an apical, unicellular, club-shaped hair and the terminal cell of the germ filament ends in a club- shaped apical hair before longitudinal divisions set in (Fig. 6. D-H). In such cases, the penultimate cell divides longitudinally, soon afterwards, and the daughter cells enlarge, squeezing the terminal cell into a broad wedge- shaped form. The meristematic cell soon cuts off daughter cells on either lateral surface, in alternate succession, and the prothallus soon becomes spathulate shape with a more or less flat apex (Fig.7. A-D). Unicellular, club- shaped hairs are continuously produced by marginal cells close to the apical meristematic cell, and the young prothalli often have a characteristic shape, being nearly triangular in outline as reported in Quercifilix (Nayar). The prothallial development is initiated on about day 60 when the terminal cell of the filament produces a hair at its apex (Fig. 7. E-F) and the intercalary cells of the filament begin to divide. The last cell of the germinal filament divides longitudinally giving rise to two cells. The prothalli develop hairs on both surfaces and on the margins, a pluricellular apical meristem is differentiated, and eventually, a thick midrib appears which is long in spathulate gametophytes, and short in the reniform and cordiform gametophytes. The adult phase is either spathulate-cordiform with wide wings, a shallow notch, and smooth prothallial plate margins, or cordiform-reniform with short wings, a shallow notch and a prothallial plate with slightly undulated margins. Cordiform gametophytes are with wide wings, marginal hairs on both surfaces and thin, elongate midrib upon which gametangia develop after 90–150 days. Spatulate gametophytes with a shallow notch, short iso-diametric wings, short marginal and superficial hairs, and a short, thin midrib were also found and the prothallial development was Aspidium Type [10]. The prothallial plate is one cell thick whereas the median midrib, which bears the sex organs in the adult gametophytes, is more than one cell thick (Fig. 7. G-I). Most of the prothallai were unisexual. Only very few prothalli produced bisexual gametophytes. Most of the unisexual prothalli are spathulate type and bisexual prothallai are cordate shape (Fig. 7. G-I). The male prothallai were relatively small in size and one cell thick.Thefemale sex organs (archegonia) develop between 150 and 170 days. They are superficial and arranged on the lower side of the prothallus near the midrib and the meristematic region. Fertilization and subsequent development of the sporophytes take place after four months (180–225 days). On sporophytes, unicellular and multicellular uniseriate hairs develop similar to those found on adult prothalli [11].

3.2 Influence of Season

Tectaria zeylanica spores were collected during the four seasonal months of January 2015 to January 2016 (January, April, July, and December) and cultured in full and half strength KC, KN, MS, and MI media. The highest percentage of spore germination (47%) was observed in spores collected in April, while the lowest percentage of spore germination was observed in spores collected in October. The difference in the percentage of spore germination in spores collected in October and April could be attributed to spore dormancy. There was no spore formation in the mother plants during the month of December (Table 1. & Fig. 1.).

Table 1. Influence of season on spore germination, gametophyte and sporophyte formation in *T. zeylanica*

Month of Collection	% of Germination ±S.D	% of prothalli formation ±S.D.	% of Sporophyte formation ±S.D
January	32.6±1.61	29.6±0.83	23.6±0.92
April	46.9±0.21	42.3±0.96	40.3±1.21
July	42.2±1.21	36.3±1.42	35.3±1.65
October	24.2±1.32	19.8±1.45	14.7±1.11

3.3 Influence of Media and pH on Spore Germination of *Tectaria zeylanica*

Tectaria zeylanica spores were cultured in hormone-free full, half strength Kundson (KC), Knudson (Kn), Mitra (Mi), and Murashige and Skoog (MS) basal media supplemented with 3% sucrose and 0.5 percent agar at various pH ranges (4.8,5.8 and 6.8). In full strength KC Medium at pH 5.8, the spores showed the highest germination percentage (14%), as well as the largest gametophyte size (length 224.55±2.33 µm, breadth 185.6±23.21 µm) (Table 2), whereas in other media the germination percentage was lower. (Table 2 and Fig. 2).

3.4 Influence of Sucrose on Gametophytes on *T. zeylanica*

The *In vitro* raised gametophytes of *T. zeylanica* were transferred to appropriate media with different concentrations and combinations of sucrose. The highest number of sporophytes (56.2) emerged in KC medium supplemented with 2% sucrose (Table 3). When the sucrose concentration exceeded 3%, the emergence of sporophytes was gradually reduced, and the sporophytes turned pale yellowish calli. The maximum sporophyte length was observed in KC medium supplemented with 3% sucrose. Beyond 3% sucrose, the number and length of sporophytes formed were reduced (Table 3 & Fig. 3).

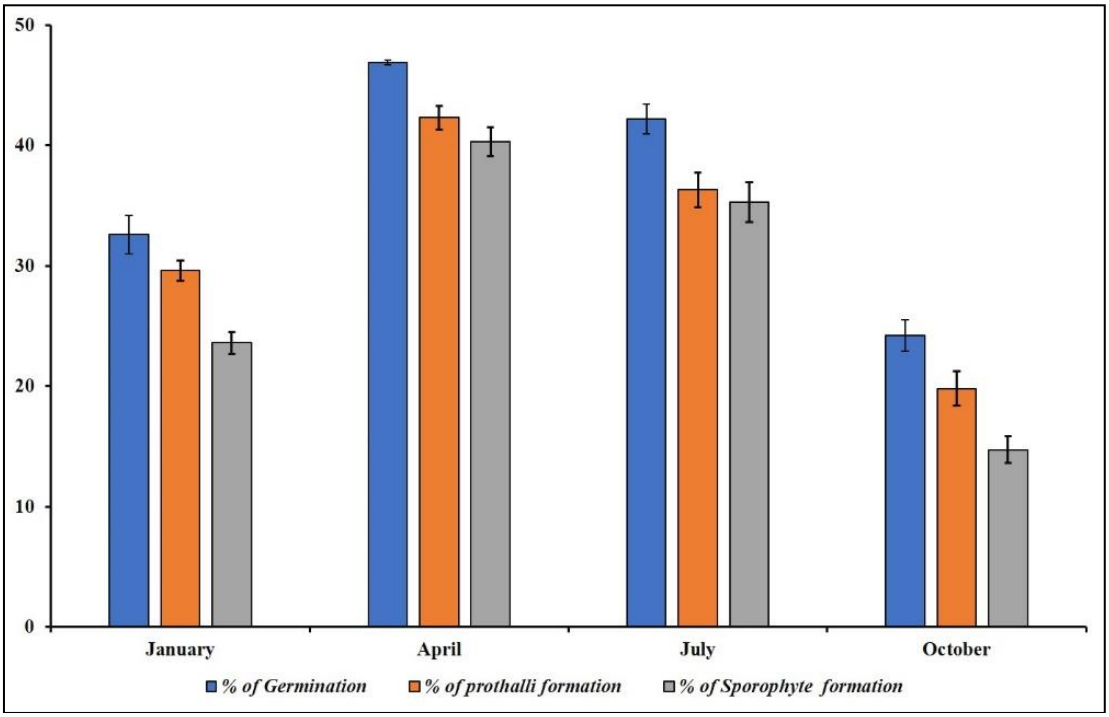

Fig. 1. Influence of season on spore germination, gametophyte and sporophyte formation in *T. zeylanica*

Table 2. Effect of medium and pH on spore germination in *Tectaria zeylanica* after 4 weeks of culture

Media	Spore Germinaton (%)		
	pH 4.8	pH 5.8	pH6.8
KC	06	14	10
1/2KC	05	12	12
KN	05	13	09
1/2KN	02	11	08
MS	-	-	-
1/2MS	-	-	-

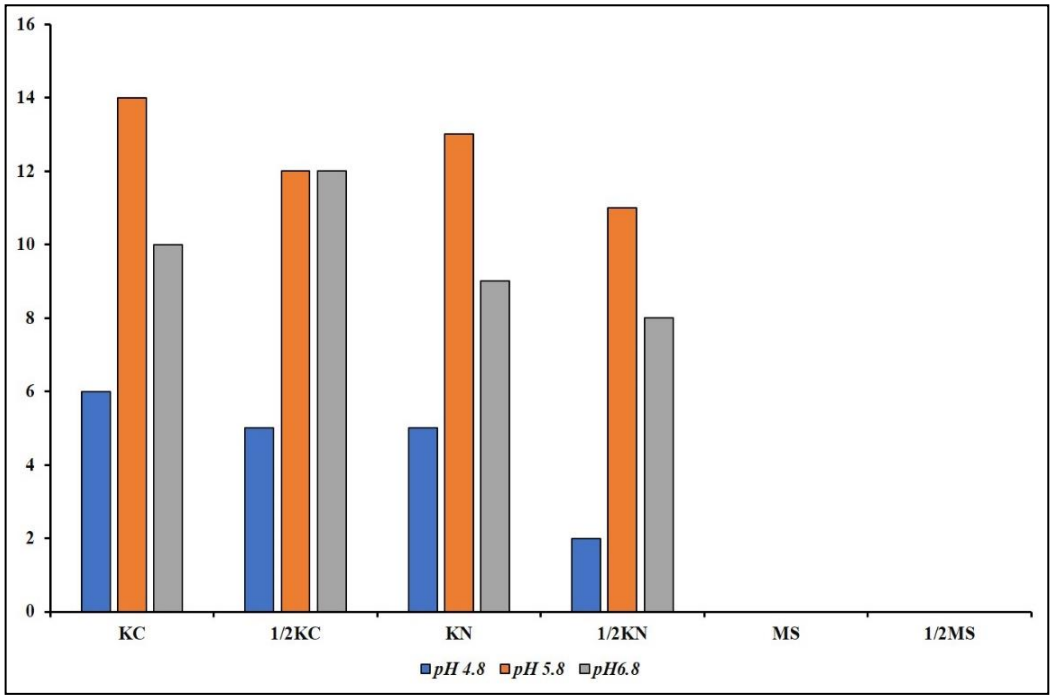

Fig. 2. Effect of medium and pH on spore germination in *Tectaria zeylanica* after 4 weeks of culture

Table 3. Influence of sucrose on gametophytes

Knudson C medium with diff. sucrose con.	% Sporophyte formation ± S.D.	Mean no of Sporophytes ± S.D.	% of Callus formation ± S.D.
Basal	32.5±0.52	19.6±0.24	NIL
1%	35. 4±0.43	20.3±0.65	NIL
2%	56.2±0.78	29.3±0.82	30.2±0.54
3%	44.5±0.67	24.5±0.54	28.5±0.76
4%	23,4±0.61	18.54±0.52	21.32±0.23
5%	14.6±0.21	10.6±0.48	14.3±0.42

3.5 Influence of PGRs on the Gametophytes

The *in vitro* raised gametophytes of *Tectaria zeylanica* were cultured on KC medium with different hormones such as Kinetin, BAP, NAA, 2.4-D, to observe the influence on gametophyte multiplication, callus formation and sporophyte formation. The highest percentage of gametophyte multiplication (23.4±1.43) was obtained on KC medium fortified with Kin 1.0mg/l, followed by (16.2±0.23) KC medium fortified with Kin 0.5mg/l. On KC medium fortified with 1.5mg/l, the highest percentage of sporophyte formation (18.2±1.7 1) was observed. The emergence of sporophytes was not observed in the medium supplemented with Kin (0.5mg/l). On KC medium supplemented with 2,4D (1.5mg/l), the highest percentage of callus formation was observed (29.3±0.3). A higher concentration of 2, 4 D inhibited the formation of callus. In the absence of auxin, compact and hard calli were formed in in vitro raised gametophytes. In KC medium supplemented with BAP (2.5mg/l), the lowest percentage of gametophytes (4.5±1.21) and sporophytes (2.3±0.21) was observed (Table 4 & Fig. 4).

3.6 Rooting of *in vitro* Shootlets

The *in vitro* raised shootlets were sub cultured in half strength MS medium supplemented with different concentration of auxins. Highest percentage (55.2±0.88) of root formation was observed in medium with IBA 1.0 +NAA 1.0 and the lowest percentage (12.3±0.32) was observed in medium with IAA 0.5 with the formation of basal callus. (Fig.8. G-H).

3.7 Hardening of *in vitro* Raised Plants

The regenerated plantlets, after root formation, were washed thoroughly with running tap water to remove the excess medium and transferred to plastic cups (10cm x 8 cm) containing a sterilized mixture of sand, garden soil, farmyard manure and were irrigated with liquid media once a week. The polycups were covered with polybags to maintain humidity and they were kept in the culture room for at least 15 days before transferred to the green house (Fig.8. J-L) under constant misting (R.H.80%). After 3 weeks the hardened plants were transferred to the field. The *in vitro* raised plant showed 54 % of establishment during hardening. The established plants did not show any variation in morphological or growth characteristics when compared to the mother plant. In the present study the protocol for *in vitro* multiplication of the rare Oak leaf fern, *Tectaria zeylanica* has been successfully established.

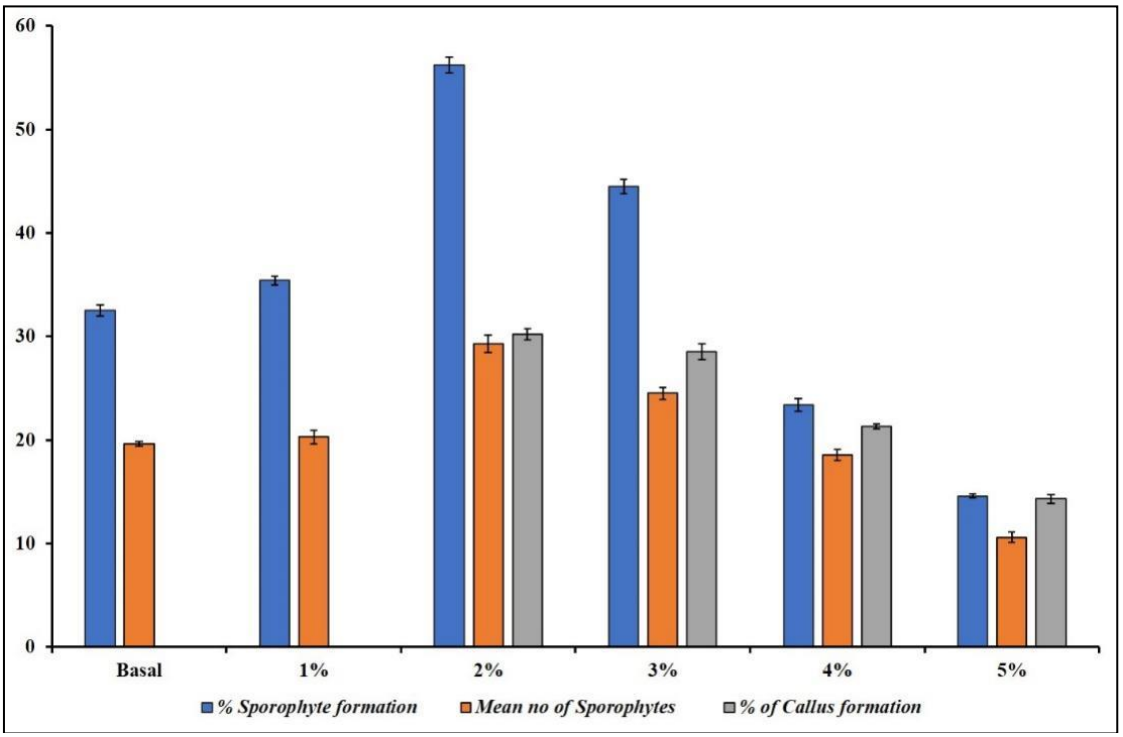

Fig. 3. Influence of sucrose on gametophytes

Table 4. Effect of PGRs on the gametophytes of *Tectaria zeylanica*

KC medium with PGR	% Gametophyte multiplication ±S.D.	% Sporophyte multiplication ±S.D.	% Callus multiplication ±S.D.	Mean no of Crozier + SE (cm)	Mean of Crozier length + SE (cm)
Basal	11.5±0.46	-	-	-	-
Kin 0.5	16.2±0.23	-	-	-	-
Kin 1.0	23.4±1.43	14.3±0.41	9.5±0.21	9.6±0.32	1.65±0.32
Kin 1.5	14.3±0.12	18.2±1.7 1	8.3±0.12	7.3±0.22	1.11±0.11
Kin 2.0	9.2±0.11	5.12±0.22	3.1±0.21	1.4±0.32	0.8±0.21
NAA 0.5	12.3±0.21	10.3±0.12	5.32±0.11	2.4±0.21	0.9±0.43
NAA1.0	18.3±0.22	14.2±0.43	7.76±0.23	5.3±0.87	1.01±0.22
NAA1.5	15.5±0.54	11.5±0.49	5.32±0.43	2.3±0.32	0.8±0.22
NAA 2.0	13.7±0.87	10.8±0.65	4.53±0.22	1.9±0.54	0.8±0.23
NAA 2.5	11.3±0.54	9.5±0.23	4.6±0.21	1.8±0.32	0.8±0.32
BAP 0.5	11.4±0.94	9.5±0.43	1.2±0.54	1.5±0.21	1.0±0.22
BAP 1.0	16.7±1.43	11.5±1.11	3.2±0.11	3.2±1.1	2.3±1.11
BAP 1.5	13.5±1.11	10.4±1.21	4.3±0.32	2.2±.2.1	1.5±0.7

KC medium with PGR	% Gametophyte multiplication ±S.D.	% Sporophyte multiplication ±S.D.	% Callus multiplication ±S.D.	Mean no of Crozier + SE (cm)	Mean of Crozier length + SE (cm)
BAP 2.0	10.7±0.54	7.9±0.41	5.6±0.21	1.1±0.92	0.6±0.11
BAP 2.5	4.5±1.21	2.3±0.21	6.5±0.32	1.4±1.21	1.3±0.43
2,4 D 0.5	15.4±2.4	-	7.5±2.1	-	-
2,4 D 1.0	19.5±2.1	-	11.2±0.9	-	-
2,4 D 1.5	16.4±1.1	-	29.3±0.3	-	-
2,4 D 2.0	-	-	7.3±0.6	-	-
2,4 D 2.5	-	-	5.2±0.21	-	-

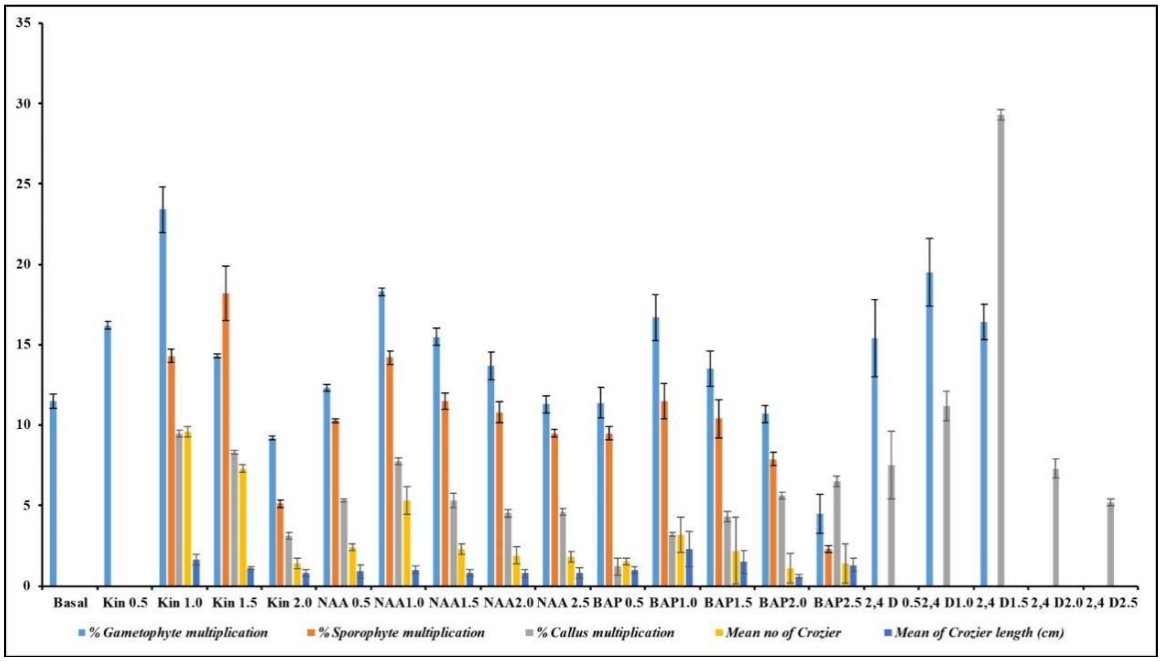

Fig. 4. Effect of PGRs on the gametophytes of *Tectaria zeylanica*

Fig. 5. Habit of *Tectaria zeylanica* (Houltt) Sledge

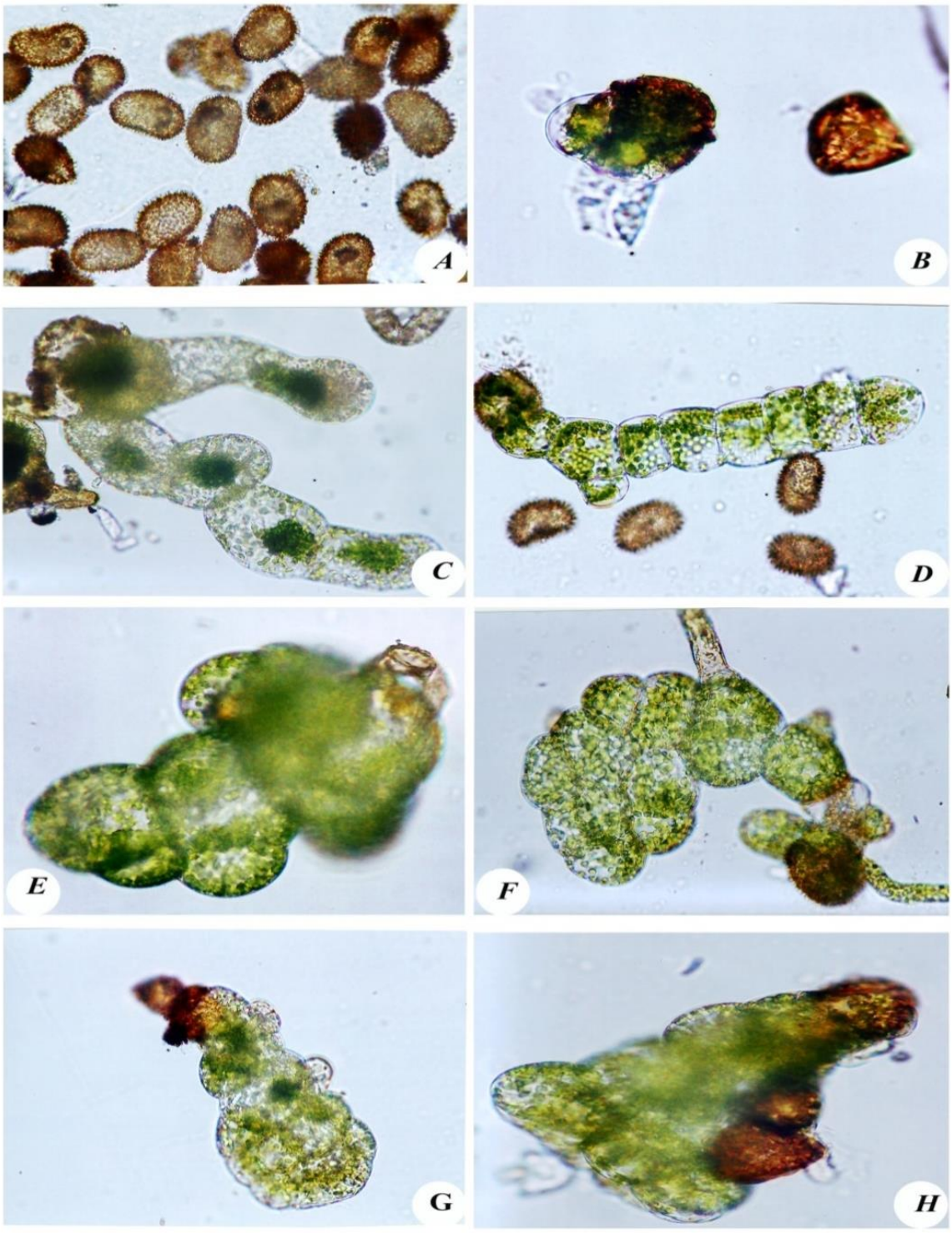

Fig. 6. A-H: Gametophyte developmental stages of *Tectaria zeylanica* (Houtt). Sledge

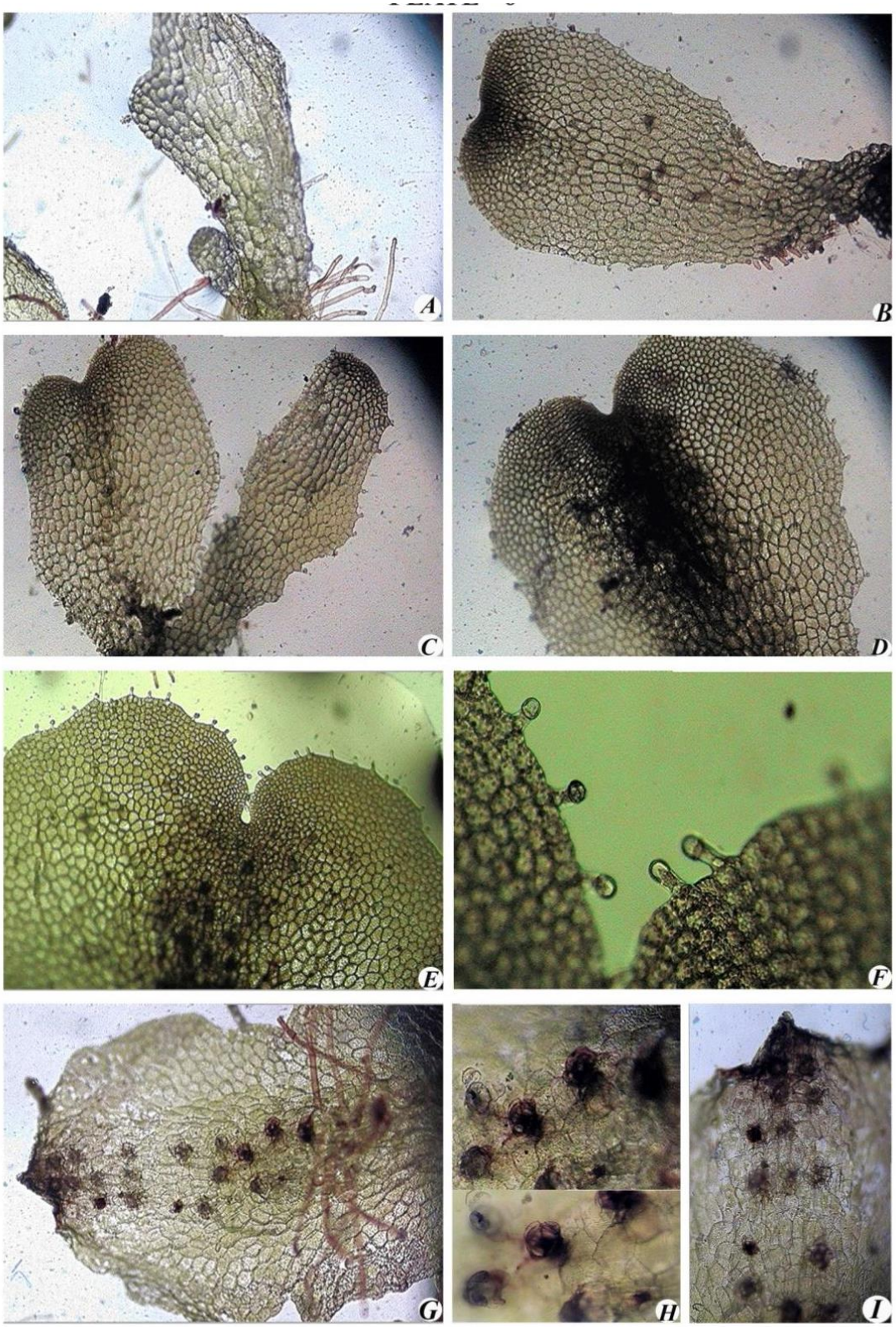

Fig. 7. Matured Gametophyte and Ontogeny in *Tectaria zeylanica. A*-C. *Aspidium* type of gametophytes development pattern, D-E. Matured cordate gametophytes with unicellular hairs formation, F- View of Unicellular hairs on gametophyte, G. Matured gametophytes with gametes, H- Gametophyte with Antheridia, I- Gametophyte with archegonia

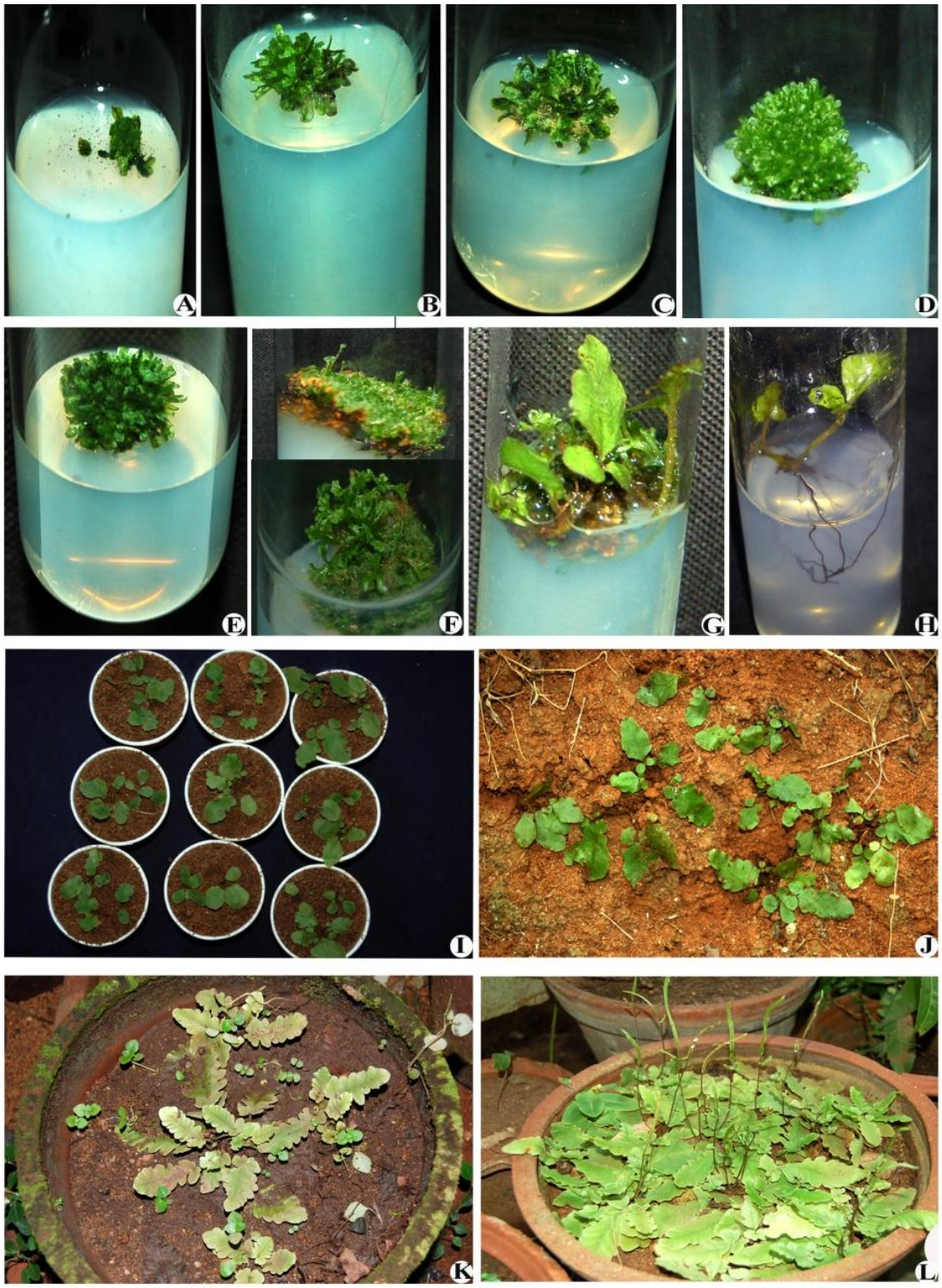

Fig. 8. Matured Gametophyte and Ontogeny in *Tectaria zeylanica*. A- *In vitro* Gametophyte from the matured spores of *T. zeylanica,* B-E. *In vitro* gametophyte formation, F-*In vitro* sporophyte formation, G-H, Sporeling formation from the *in vitro* raised gametophyte, I- Hardening of micropropagated plants on the polycups, J- Micropropagated plants reintroduced into the natural conditions, K- L Micropropagated plants hardened in the pots.

4. CONCLUSION

In conclusion, the study has successfully established a reproducible protocol for the ex situ conservation of the endangered oak leaf fern, *Tectaria zeylanica* (Houtt.) Sledge. Through meticulous collection and aseptic culture of spores on full-strength KC Medium with sucrose at pH 5.8, the researchers observed a distinct germination pattern characterized by monolete spores with a prominent winged perispore. Prothallial development followed the Aspidium type, with adult gametophytes exhibiting a cordate shape. Notably, antheridia and archegonia development occurred between 120-140 days and 150-170 days, respectively, with fertilization and sporophyte development observed after four months. The study identified the most favorable conditions for sporophyte formation on KC medium fortified with Kin 0.5 mg/l and for root formation in the medium with IBA + NAA (1.0 mg/l). Furthermore, the in vitro raised plants displayed a 54% establishment rate during hardening in the field, indicating the viability and adaptability of the propagated ferns. This comprehensive examination of the fern's reproductive biology and establishment in ex situ conditions provides valuable insights for conservation efforts aimed at preserving the endangered *Tectaria zeylanica* in its native habitat.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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