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SHORT PROTOCOL COMMUNICATION

In vitro propagation and developmental ontogeny of endemic fern *Anemia schimperiana* C. Presl subsp. *Wightiana* (Gardener) fraser-jenk. (Anemiaceae) and tree fern *Cyathea gigantea* (Wall. Ex Hook.) holtt (Cyatheaceae)

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Abstract

In vitro propagation protocol was optimized for the endangered fern *Anemia schimperiana* C. Presl subsp. *Wightiana* (Gardner) Fraser-Jenk and *Cyathea gigantea* (Wall. Ex. Hook.) Holtt. using *in vitro* spore culture. The matured spores that were collected and sterilized with 0.1% $HgCl_2$ for 15 min and washed with distilled water for 20 min showed less frequency of microbial contamination. The sterilized spores were inoculated on to Knudson C (KC) basal medium (Solid and Liquid) for germination. The germination pattern of *A. schimperiana* subsp. *wightiana* Fraser-Jenk is an anaemia type and was observed on the 10th day (d) and filamentous prothalli formed on 22nd d. The cordate-shaped gametophytes without sex organs were observed on 38th d after the inoculation. On 49th and 53rd d the male and female sex organs were formed. The sporophytes initiation was observed on 70th d. The spore germination of *C. gigantea* (Wall. ex Hook.) Holtt. is of the *vittaria* type and was observed on the 39th d; the cordate shape thallus was observed on 52nd d, and the sex organs were observed on 58th d after the inoculation. The sporophyte proliferation initiated after 74th d. The fully rooted plantlets were hardened successfully under greenhouse conditions.

Keywords *In vitro* · Spore · Germination · Filamentous · Cordate · Sex organs · Sporophytes

Introduction

Pteridophytes are vascular plants and, unlike most other members of the plant kingdom, pteridophytes do not reproduce through seeds, instead through spores. Globally, 11,916 species of Pteridophytes in 337 genera, 51 families, 14 orders, and two classes are reported (PPG 2016). Previously, Manickam and Irudayaraj (1992) reported 256 species of pteridophytes in Western Ghats and 320 Pteridophyte species (Sumesh *et al.* 2012). Benniamin and Sundari (2020) reported 351 species of pteridophytes from Western Ghats. The majority of Pteridophytes are terrestrial, and these plants prefer to grow in cool, moist, and shady places. Fern reproduction involves the formation of spores within sporangia, which subsequently germinate to produce the gametophytic generation (Gautam 2019). The life cycle of pteridophytes usually involves an alternation of free-living generation. Sporangia on mature sporophyte leaves produce spores that, upon release, germinate to avoid repetition.

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When the spore starts to germinate, the cells divide and form the filaments. Eventually it divides into heart-shaped gametophyte. On the sexual phase of the life cycle, the male gametangia spherical shaped antheridia formed at the base of the gametophytes along with rhizoids and archegonium was produced near the apical notch region. The neck canal cells of the archegonium spread, and the spermatozoid swims down the archegonial canal to fuse with the egg for fertilization and forming a zygote with double number of chromosomes (2n) as the gametophyte. This zygote grows and develops into a new sporophyte, completing the cycle.

In vitro spore culture increases the germination capability of spores due to the addition of adequate nutrition in the culture medium provided with a suitable environment condition, and it also reduces the competition of microbes (Ravi *et al.* 2014). Two rare pteridophytes, *Cheilanthes viridis* (Forrsk.) Swartz and *Diplazium cognatum* (Hieron.) Sledge, were multiplied using the spore culture method and *in vitro* organogenesis by Manickam *et al.* (2003). The normal and abnormal life cycle of *Metathelypteris flaccida* (Blume) Ching. was studied using *in vitro* spore culture by Knudson C medium (Johnson and Manickam 2006). Johnson and Manickam (2011) studied the protocol for the conservation of two endangered ferns, *Pronephrium triphyllum* and *Sphaerostephanos unitus* through *in vitro* spore culture. The alternative protocol for multiplication of an endemic fern *Elaphoglossum stigmatolepis* was studied by Johnson and Shibila (2018). Shibila and Johnson (2022) studied the mass multiplication protocol for *Lepisorus nudus* and *Elaphoglossum stelligerum* using various media. Previous observation confirmed the role of *in vitro* spore culture technique on large scale propagation and conservation of endangered ferns (Fernandez *et al.* 1997b; Fernandez *et al.* 1997a; Johnson *et al.* 2005; Johnson and Manickam 2006; Johnson and Manickam 2010b; Johnson and Manickam 2010a; Mazumder *et al.* 2010; Avila-Perez *et al.* 2011; Johnson and Manickam 2011; Bharati *et al.* 2013; Farida 2015; Aldea *et al.* 2016; Johnson and Shibila 2018). For taxonomic and phyletic studies, fern spore germination and its developmental characteristics are very helpful (Chandra et al. 2003).

Nayar and Kaur (1971) reported the spore germination pattern and gametophytic development of Anemiaceae. Pray (1971) studied the morphology and gametophyte development in *Anemia colimensis* John T. Mickel. Nester and Schedlbauer (1981) studied the gametophyte development in *Anemia mexicana.* Chambi and Martinez (2020) observed the gametophytes of *Anemia herzogii*, *A. tomentosa* var. *anthriscifolia*, and *A. tomentosa* var. *tomentosa.* But there is no report on *Anemia schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk.

Anemia schimperiana C. Presl subsp. *wightiana* (Gardner) Fraser-Jenk is endangered and endemic to the Eastern Ghats, South India (Fraser-Jenkins 2012). Historically, *A. wightiana* has been used to treat tuberculosis and exhibits anti-microbial activity (Sujatha *et al.* 2015) and supports antioxidant activity (Moorthy *et al.* 2021). *Cyathea gigantea* (Wall. ex Hook.) Holtt.is a tree fern found in humid places throughout India. According to Beniwal (1994), this species was treated as one of the endangered ferns in India. *C. gigantea* is a medicinally rich fern used to treat many illnesses like liver disease (Kiran *et al.* 2012), anti-cancer (Janakiraman and Johnson 2016), and anti-microbial agent (Nath *et al.* 2019). Due to the anthropogenic pressure, the population of *Cyathea gigantea* also decreased. The objective of this study was to produce a standardized *in vitro* spore culture protocol and to investigate the ontogenetic development of the endangered ferns *Anemia schimperiana* C. Presl. subsp. *wightiana* (Gardner) Fraser-Jenk and *Cyathea gigantea* (Wall. ex Hook.) Holtt (Fig. 1).

Materials and methods

The healthy fertile fronds of *Anemia schimperiana* C. Presl subsp. *wightiana* (Gardner) Fraser-Jenk n = 76 (Ghatak 1977; Fig. 2*A*) was collected from Shervarayon hills, and *Cyathea giantea* (Wall. Ex Hook.) Holtt—2n = 138 (Love 1977; Fig. 3*A*) was collected from Kothayar, Tirunelveli Hill, Tamil Nadu. The fronds were tightly packed in paper and kept in room temperature to liberate spores. The sporangia from the fertile frond were scraped using a dissecting needle, and the spores were separated from the sporangia through sieving using fine mesh cloth (60 µm). The collected spores were stored in the refrigerator (LG, India) at 4ºC for further inoculation.

The spores were surface sterilized with ethanol, various concentrations of sodium hypochloride (Himedia, Mumbai, India) (0.5, 1.0, 2.0, and 3%), and 0.05 and 0.1% $HgCl_2$ (Himedia, Mumbai, India) solution for 15 to 30 min and washed with sterile distilled water for 15 to 30 min. The sterilized spores were inoculated on Knudson C (Knudson 1946) solid and liquid media using sterile Pasteur pipettes and incubated at 25 ± 2ºC under 16 h photoperiod with 1500 lx. The media pH was adjusted to 5.8 before adding agar and autoclaved at 121ºC for 15 to 30 min. Periodical observations on spore germination, gametophyte formation, male and female sex organ formation, and sporophyte proliferation were made.

Result

The spores were sterilized with ethanol, which removed the pigments of the spores and reduced the spore germination percentage (less than 10% only germinated); the spores sterilized with sodium hypochloride solution

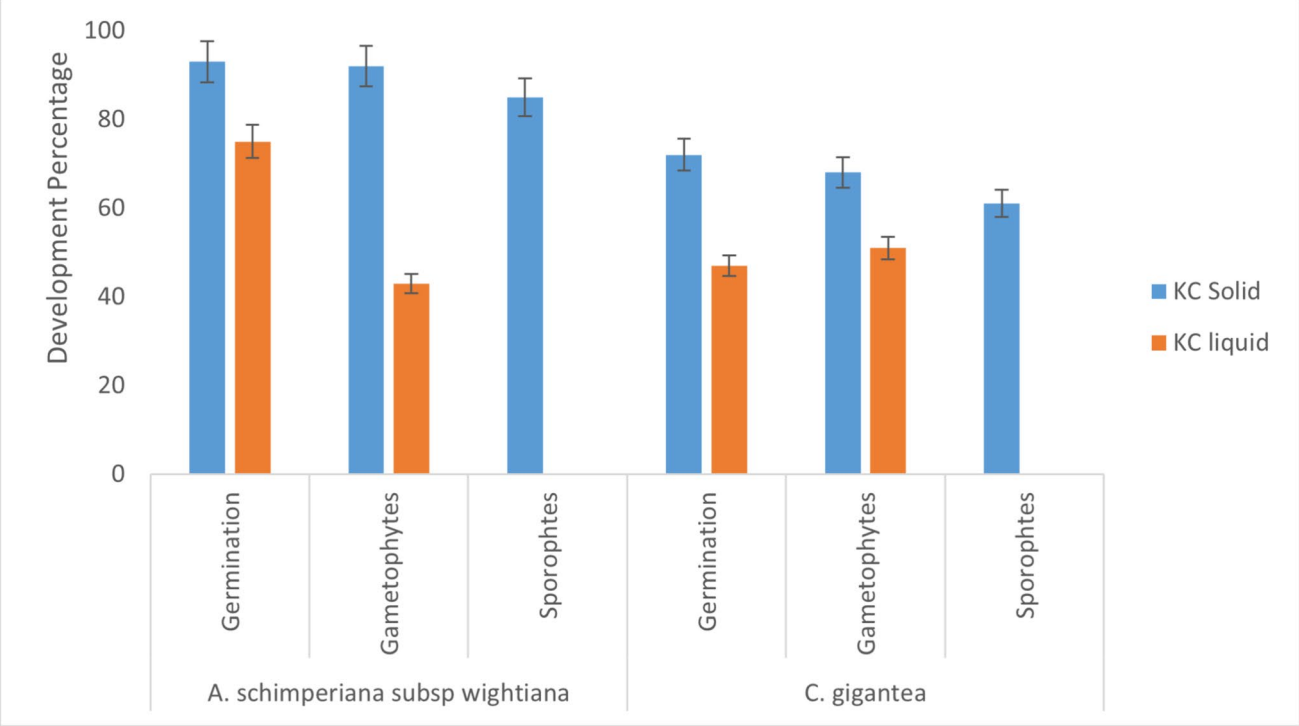

Figure 1. Developmental percentage of *Anemia. schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk and *Cyathea gigantea* (Wall. Ex Hook.) Holtte in Knudson C (KC) basal medium (Solid and Liquid).

concentration up to 3% and sterilization time up to 30 min did not provide microbe-free cultures. The spores of *A. schimperiana* subsp. *wightiana* and *C. gigantea* sterilized with 0.1% $HgCl_2$ for 15 min showed a high rate of survival and low percentage of microbial contamination. The spores with prolonged exposure at 0.1% mercuric chloride for 30 min resulted in a contamination-free culture, but the viability of the spore was affected. For *A. schimperiana* subsp. *wightiana,* Knudson C basal solid medium showed the highest percentage ($95 \pm 2\%$) of spore germination. The spore germination pattern is anemia type. For *C. gigantea* (Wall. ex Hook.) Holtt., Knudson C basal solid medium showed the moderate percentage of spore germination ($32 \pm 2\%$), and the germination pattern is *vittaria* type (Fig. 1*B* and 2*B*). The germination of *A. schimperiana* subsp. *wightiana* was observed on 10th d under the microscopic view; on the 15th d, the germination was able to be seen with the naked eye, and filamentous prothalli formed on the 22nd d (Fig. 1*C*, *D*). The cordate shaped gametophytes without sex organs were observed on 38th d after the inoculation (Fig. 1*E*). On the 49th and 53rd d, the male and the female sex organs were formed (Fig. 1*F*, *G*). The sporophytes initiation was observed on 70th d (Fig. 1*H*, *I*). The spore germination of *C. gigantea* was observed on 39th d; the filamentous, cordate shape porthallus was observed on the 52nd d (Fig. 2*C-E*), and the sex organs were observed on 58th d after the inoculation (Fig. 2*F*, *G*). The sporophyte proliferation was initiated after 74 d (Fig. 2*H*, *I*). The fully rooted plantlets were hardened successfully under greenhouse conditions. The highest percentage of gametophyte multiplication was observed in the solid medium than liquid medium. Both species showed sporophyte proliferation only in the KC basal solid medium. The developmental stage of two species at specific time points are presented in Table 1. The ontogeny of *A. schimperiana* subsp. *wightiana* and *C. gigantea* is illustrated in Figs. 3 and 4 (Table 2).

Discussion

Spores are the important reproductive structure in Pteridophytes. Spore viability length, rapidity of the germination, and gametophyte growth rate are the primary factors in the plant growth competition. These characteristics are based on two properties, including intrinsic (inherent, fixed chemical or pharmacological traits of plant compounds) and ex-intrinsic (variable external factors, for example environment—pH, temperature, media, light, humidity, processing—sterilant, sterilization time), affecting their efficacy (Robert and Edward 1970). The result of the present study validated the views of Robert and Edwards (1970)

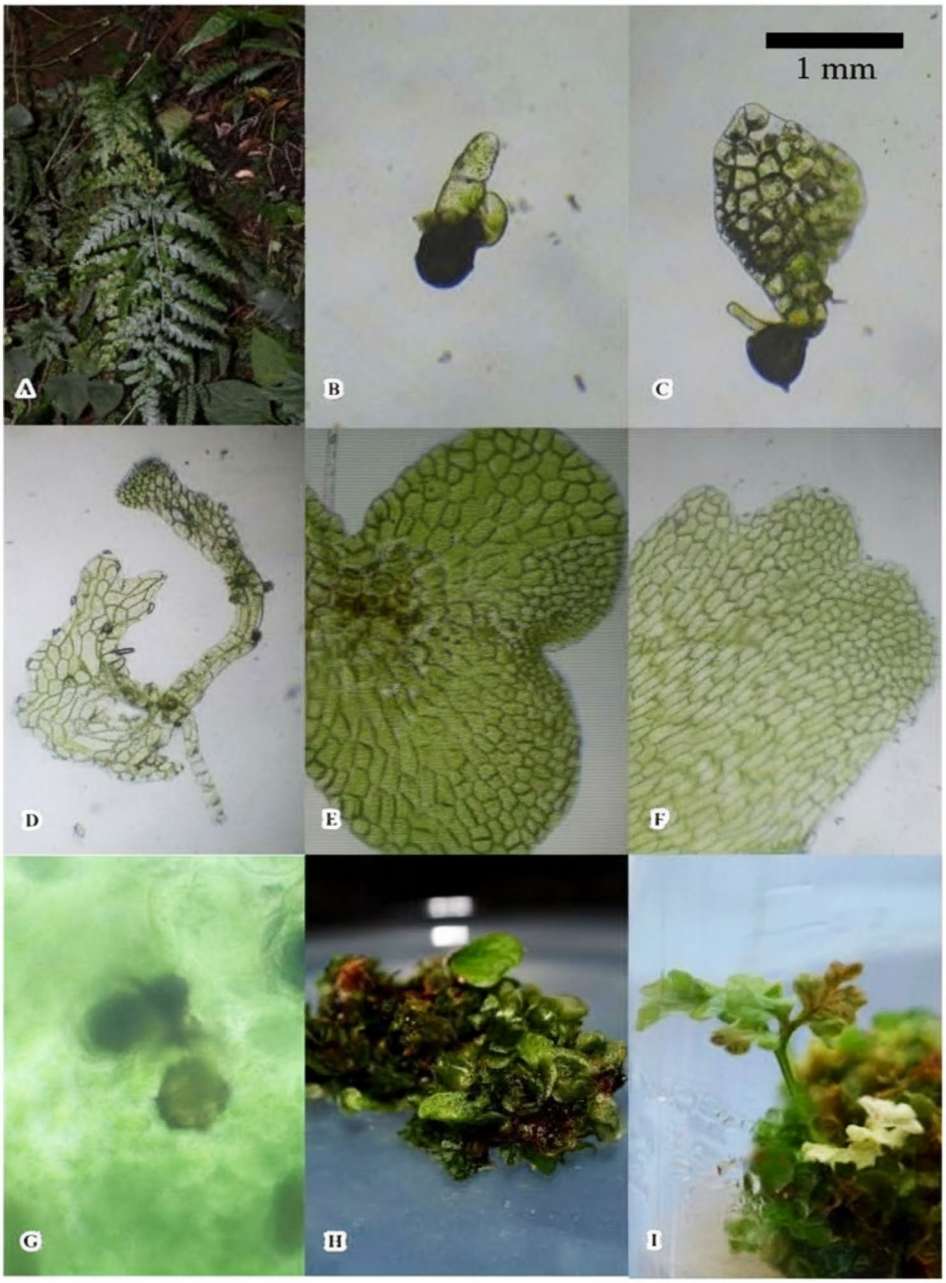

Figure 2. Different Developmental Stages of *Anemia schimperiana* C. Presl subsp. *wightiana* Gardner) Fraser-Jenk. (*A*) Matured fertile and sterile fronds; (*B*) Spore germination Initial stage; (*C*) and (*D*) Filamentous stage;(*E*) Cordate Prothallus; (*F*)and (*G*) Prothallus Male and Female Sex organs; (*H*) and (*I*) Sporophytes.

observations. The tissue culture method was used for the mass multiplication of plants; also, it provided the means to regenerate novel plants (Gaurav *et al.* 2015). Plant tissue culture is one of the fastest techniques that can reproduce several numbers of plantlets from single explants or spores (Bhaskaran and Prabhudesai1989; Fernandez *et al.* 1996; Johnson and Shibila 2018). By adopting an *in vitro* propagation method, a number of ferns were multiplied (Fernandez *et al.* 1996; Fernandez *et al.* 1997b; Fernandez *et al.* 1997a; Johnson *et al.* 2005; Johnson and Manickam 2006; Johnson and Manickam 2010b; Johnson and Manickam 2010a; Mazumder *et al.* 2010; Avila-Perez *et al.* 2011; Johnson and Manickam 2011; Bharati *et al.* 2013; Farida 2015; Aldea *et al.* 2016; Johnson and Shibila 2018; Shibila and Johnson 2022). In the present study, an optimized protocol was also established for the rare, endemic, and endangered ferns *A. schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk and *C. gigantea* (Wall. ex Hook.) Holtt (Fig. 5).

Many factors were responsible for spore germination, like color of spores, sterilization time, influence of media, and pH (Daniels 1978). In the present study, the spores of *A. schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk and *C. gigantea* (Wall. ex Hook.) Holtt showed good percentage of germination, gametophytes development, and sporophyte

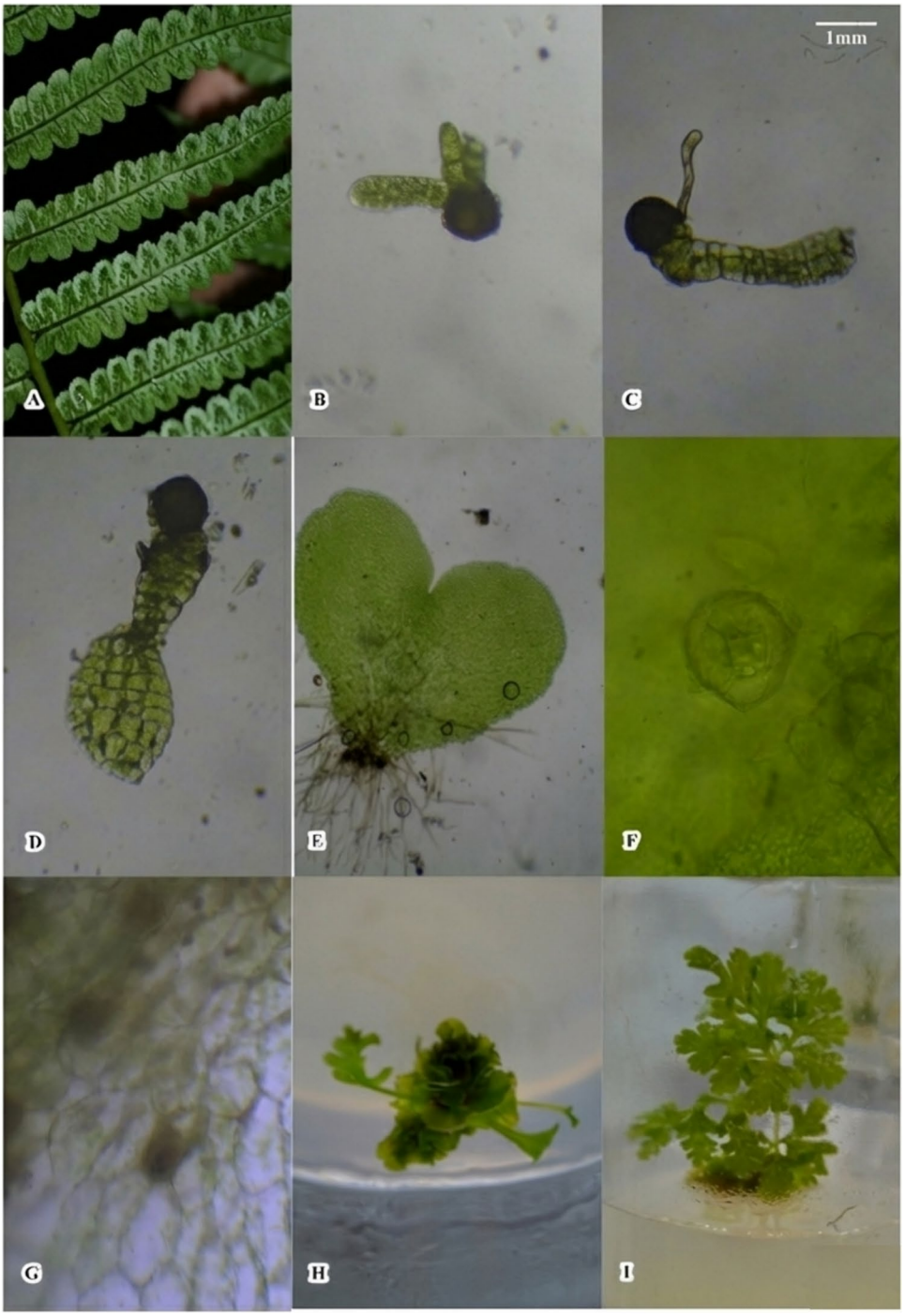

Figure 3. Different Developmental Stages of *Cyathea gigantea* (Wall. Ex Hook.) Holtt. (*A*) Matured fertile fronds; (*B*) Spore germination Initial stage; *(C)* and (*D*) Filamentous stage; *(E)* Cordate Prothallus; *(F)* and (*G*) Prothallus Male and Female Sex organs; (*H*) and (*I*) Sporophytes.

Table 1. Developmental stages of *Anemia schimperiana C. Presl.* subsp. *wightiana* (Gardner) Fraser-Jenk and *Cyathea gigantea* (Wall. ex Hook.) Holtt

Species	Knudson C medium	Initiation stage	Filamentous	Protonema	Cordate	Male sex organ	Female sex organ	Sporophyte
Anemia schimperiana subsp. *wightiana*	Solid	10th d	19th d	22nd d	33rd d	38th d	38th d	70th d
	Liquid	17th d	22^{nd}d	25th d	36th d	-	-	-
Cyathea gigantea	Solid	39th d	45th d	48th d	52nd d	58th d	62rd d	74th d
	Liquid	35th d	39th d	46th d	52nd d	-	-	-

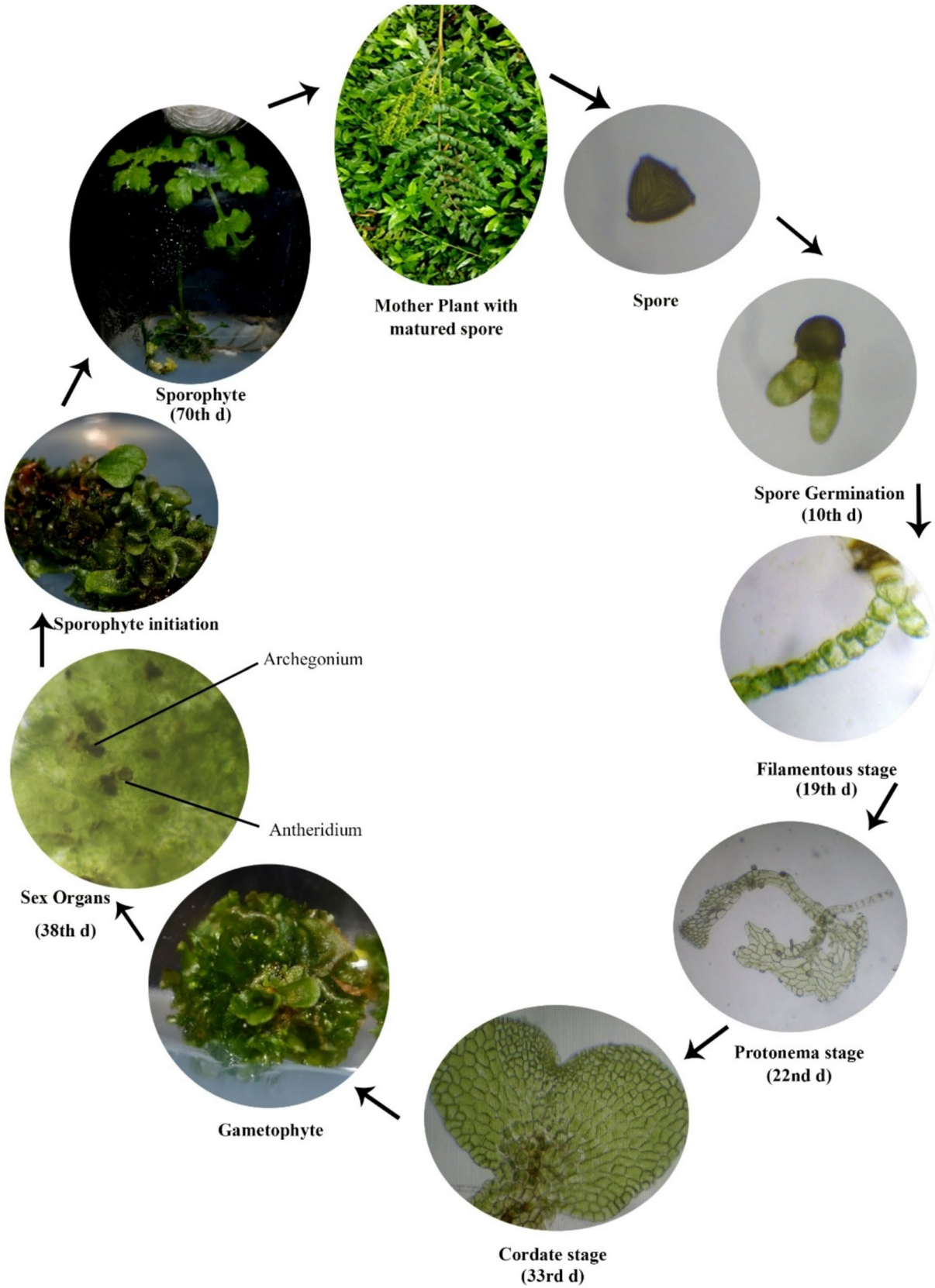

Figure 4. Developmental ontogeny of *Anemia schimperiana* C.Presl subsp. *wightiana* Gardner) Fraser-Jenk.

Table 2 Developmental percentage of *Anemia schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk and *Cyathea gigantea* (Wall. ex Hook.) Holtt

	A. schimperiana subsp wightiana (Gardner) Fraser-Jenk			*C. gigantea* (Wall. ex Hook.) Holtt		
	Germination	Gametophytes	Sporophytes	Germination	Gametophytes	Sporophytes
Knudson C Solid	93 ± 1.34	92 ± 0.34	85 ± 1.53	72 ± 1.45	68 ± 1.99	61 ± 1.09
Knudson C liquid	75 ± 1.04	43 ± 1.34	-	47 ± 1.77	51 ± 1.21	-

proliferation on Knudson C Solid basal medium with pH 5.8. A similar observation was observed by Amoroso and Amoroso (1998); Taha *et al.* (2011); Johnson and Shibila (2018); Shelikhan (2019); Shibila and Johnson (2022); Vallinayagam (2022). The color of the spore played an important role in their germination time. Lloyd and Klekowski (1970) compared the viability of chlorophyll-bearing spore and non-chlorophyll bearing spore germinations. The green spores germinated within three d, while the non-chlorophyll spores required a longer time. Also, green spores lost their viability very quickly, and these spores needed a controlled environment to maintain their viability (Ballesteros *et al.* 2011). The spores of *A. schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk and *C. gigantea* (Wall. ex Hook.) Holtt were yellowish brown in color and showed the germination after 15 to 40th d with good viability.

Johnson and Manickam (2011) used 0.1% mercuric chloride as a sterilant to sterilize the spores of *Pronephrium triphyllum* (Sw.) Holttum. and *Sphaerostephanos unitus* (L.) Holttum. for 3 to 5 min. In the *in vitro* culture of *Pteris vittata* L.*, Diplazium proliferum* (Lam.) Thouars., and *Rumohra adiantiformis* (G. Forst.) Ching., mercuric chloride was used to sterilize the spores (Ambika and Bir 1993; Winarto and Teixeira 2012; Golamaully *et al.* 2015). In the present study also, 0.1% mercuric chloride was used to sterilize the spores for 15 to 30 min to control the microbial contaminations. The observed results were validated by the previous observations of Ambika and Bir (1993), Johnson and Manickam (2011); Winarto and Teixeira (2012); Golamaully *et al.* (2015).

Johnson and Shibila (2018) propagated the endemic fern *Elaphoglossum stigmatolepis* (Fée) T. Moore using Knudson C basal medium. The endangered species *Elaphoglossum nilgiricum* Krajina ex Sledge was propagated using liquid and solid Knudson C basal media (Shibila and Johnson 2022). In *in vitro* spore culture of *Adiantum caudatum* L.*,* the germination percentage differed from Knudson C medium and Knop's medium. The maximum germination was observed in Knudson C medium with 95% (Gayathiri *et al.* 2018). In the present study, after the incubation period, spores cultured on Knudson C basal solid agar medium showed a significantly higher germination percentage than those grown in liquid medium with 93± 1.34% in *A. schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk and 72 ± 1.45% in *C. gigantea* (Wall. ex Hook) Holtt. on solid medium, compared to 75 ± 1.04% and 47 ± 1.77%, respectively, in liquid culture.

Likewise, Supriya *et al.* (2013) confirmed the spore germination of *Cyathea gigantea* (Wall. ex Hook.) Holtt. from 12 to 14th d after inoculation. Gayathiri *et al.* (2018) observed the spore germination of *Adiantum caudatum* L. spores after 15 to 20th d of inoculation. *Pronephrium triphyllum* (Sw.) Holttum and *Sphaerostephanos unitus* (L.) Holttum spores started to germinate after 38 and 35th d in Knop's medium (Johnson and Manickam 2011). In the present study, also the spores of *A. schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk and *C. gigantea* (Wall. ex Hook.) Holtt. showed germination on 15th and 39th d after inoculation. Contrary to present observation, Rechenmacher *et al.* (2010) observed the spore germination after 3th d of inoculation in *Cyathea atrovirens* Langsd. and Fisch. Similar to present observation on *A. schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk, the spores of *Anogramma chaerophylla* Desvaux (Desv.) (Maria *et al.* 2016) and *Drynaria bonii* H. Christ (Quyen *et al.* 2020) showed the germination within 2 wk after the inoculation.

The gametophytic development of *Cyathea atrovirens* (Langsd and Fisch.) Domin. was observed after 20 to 30th d of culture (Rechenmacher *et al.* 2010). Liliana *et al.* (2010) and Gayathiri *et al.* (2018) revealed that the gametophyte development of *Dryopteris affinis* (Lowe) Fraser-Jenk and *A. caudatum* L. took place after 45th d of inoculation. In this study, the gametophyte of *A. schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk was developed after 22nd d, and *C. gigantea* (Wall. ex Hook.) Holtt. was developed on the 52nd d after the initial stage formation.

Several studies have reported that sex organ formation in mature gametophytes occurs over different time periods. A number of studies reported that the sex organs formation in the matured gametophytes of different species varied in different period of times (Chang *et al.* 2007; Goller and Jan 2007; Johnson and Manickam 2011; Manonmani and Sara 2014). *In vitro* cultures of *Adiantum adulterinum* Milde. and *Adiantum cuneiolium* Stokes showed gametophyte with sex organ formation after 90th d of inoculation, and the sporophyte emerged after 4 mo (Jowita and Krystyna 2011). Shibila and Johnson (2022) observed the appearance of male and female sex organs of *Elaphoglossum nilgiricum* Krajina

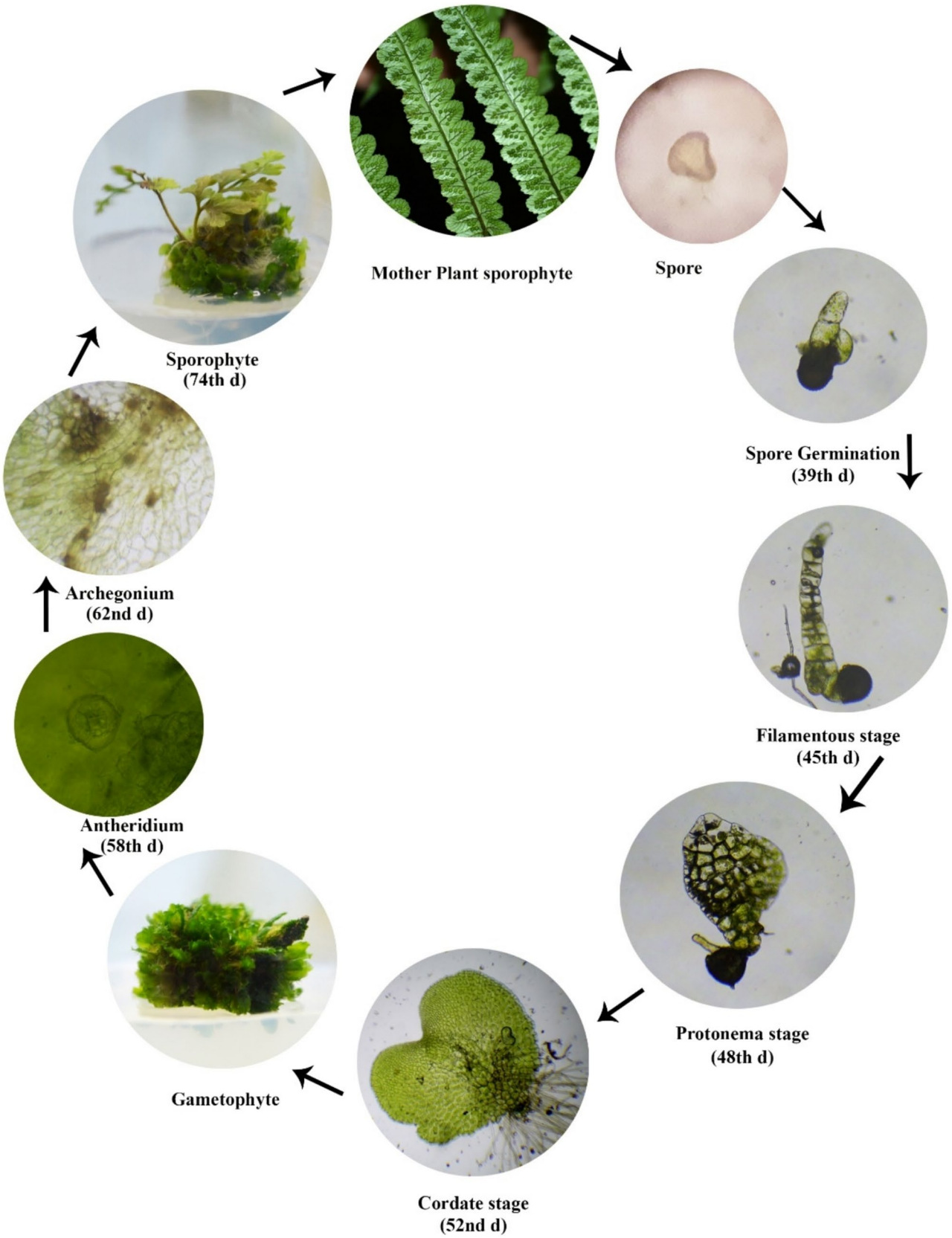

Figure 5. Developmental ontogeny of *Cyathea gigantea* (Wall. Ex Hook.) Holtt.

ex Sledge on the 41 st and 67th d, respectively. Similarly in this study, the male sex organ of *A. schimperiana* subsp. *wightiana* (Gardner) Fraser-Jenk was observed on the 49th d, and female sex organ was observed on the 53rd d. In *C. gigantea* (Wall. ex Hook.) Holtt., the male and female sex organs were observed on the 60th d.

The spore derived sporophytes of *Dryopteris affinis* (Lowe) Fraser-Jenk was observed after 45th d of spore inoculation (Liliana *et al.* 2010). In *Asplenium nidus* L. (Khan *et al.* 2008), *Dipteris wallichi* (R.Br.) T. Moore (Singha *et al.* 2013), *Platycerium bifurcatum* (Cav.) C. Chr. (Liao and Wu 2011), and *Diplazium esculentum* (Retz.) Sw., (Archana *et al.* 2014), the development of sporophytes on different d was reported. In this study, the sporophyte initiation in both species was observed on the 70th to 74th d after the inoculation.

Conclusion

The results of the present study indicated that Knudson C basal solid medium was significantly more effective than liquid medium for spore germination, gametophyte multiplication, and sporophyte proliferation in *A. schimperiana* subsp. *wightiana* (Lowe) Fraser-Jenk and *Cyathea gigantean* (Wall. ex Hook.) Holtt. Spore germination on solid medium reached $93 \pm 1.34\%$ and $72 \pm 1.45\%$ in *A. schimperiana* subsp. *wightiana* (Lowe) Fraser-Jenk and *C. gigantean* (Lowe) Fraser-Jenk, respectively, which was significantly higher than that observed in liquid medium. The results of the present study revealed the developmental ontogeny and produced the *in vitro* propagation protocol for *A. schimperiana* subsp. *wightiana* (Lowe) Fraser-Jenk and *Cyathea gigantea* (Wall. ex Hook.) Holtt. The optimized protocol may be used to isolate the metabolites with reference to gametophytes (filamentous, protonema, cordate prothallus) and juvenile sporophytes in large scale under *in vitro* conditions without affecting the natural population. This optimized protocol may be used for the further large-scale multiplication and conservation of the medicinally important ferns *A. schimperiana* subsp. *wightiana* (Lowe) Fraser-Jenk and *Cyathea gigantea* (Wall. ex Hook.) Holtt. and apply the developed protocol for other species also.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest. All authors have read and approved the final version of this manuscript.

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