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Protocol for successful sporophyte formation and development in *Pteris melanocaulon* Fée

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ABSTRACT

The purpose of this study was to develop a protocol for the formation and production of sporophytes of *Pteris melanocaulon*. Here we describe the procedure for the successful germination of spores and techniques of creating conditions that allow gametophytes to develop into sporophytes. The procedure described will allow for mass production of *Pteris melanocaulon* that will be used for phytoremediation as phytostabilizer in soil with high copper content in the mining areas.

1. Introduction

The black stemmed fern *Pteris melanocaulon* was recently collected in a mining area of Surigao del Sur, Philippines where the copper concentration in soil was high (De La Torre et al., 2015). The species was 1st collected in Corregidor, Cavite, Philippines by H. Cumming in 1836-1840 (http:// plants.jstor.org/ stable/10.5555/al.ap.specimen.mich1190957). The fern is relatively uncommon; even in mining areas where it was found. The plant's ash content indicated accumulation of copper from soil. *P. melanocaulon* was previously documented to thrive in soil with more than 300 µg/g copper in Cebu (Borja, 1986) and De La Torre et al. (2015) reported that the degree of clustering of this species was reported to be directly correlated to copper content in soil.

The fern, *P. melanocaulon* may have a potential use in phytoremediation due to its ability to accumulate large quantity of copper in roots and rhizomes. De La Torre et al. (2015) reported the bioaccumulation factor of 4.04 for this species, indicating that copper concentration is high in plants than in soil. However, for the species to be used for field scale phytoremediation, many seedlings are required. These are not readily available in the wild and furthermore, the fern easily withers when uprooted and separated; thus vegetative propagation may not be favorable. The large number of seedlings required therefore, can be provided through mass propagation using spores. The spores however need to develop into gametophytes and finally into sporophytes.

The formation of sporophyte is the crucial step for the mass production of planting material for phytoremediation. Sporophytes are young seedlings that are allowed to grow in size and acclimatized before field planting. But development of sporophytes from gametophytes is seldom successful owing to conditions required for successful union of male and female gametes. Nursery procedures that create conditions favorable for successful union of gametes and the ensuing development of sporophytes are absent. Thus, this study was aimed at developing protocols for the creation of conditions that will allow union of gametes in gametophytes for successful formation and development of sporophytes from spores for *P. melanocaulon*. The production of large number of sporophytes is deemed important in phytoremediation and revegetation projects for copper-gold mining areas.

2. Materials and methods

Mature fronds of *P. melanocaulon* were collected from a Copper-Gold mine in Surigao del Sur, Philippines (Fig. 1). It was then brought to the laboratory for air drying in an air conditioned room (22 ^{0}C) for 2 days. The fronds were never exposed to outside winds and sunlight to maintain internal moisture. With the sori of fronds facing down on a

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clean wax paper during the drying period, dehiscing spores were collected and cleaned by removing extraneous materials using 2.5 micron siever. Collected spores were then transferred to clean and dry test tubes with caps.

Propagation media taken from: 1) the collection site in the mining area (pH is 7.3), 2) nursery site in Cagayan de Oro (pH is 6.8) and 3) river sand in Cagayan de Oro (pH is 6.7), were pulverized, sieved and sterilized by heating at 70 ^{0}C for 1 hour.

Fig. 1: Sampling points of *Pteris melanocaulon* in a Copper-Gold mine in Surigao del Sur
Image produced through gpsvisualizer in the following coordinates: N 9.668055, E 125.589102; N 9.667946, E 125.589030; N 9.667819, E 125.588759; N 9.66769, E 125.588850; N 9.66756, E 125.588493.

Propagation trays made of transparent polypropylene plastic and with cover (similar to pasta trays) were sterilized with 10% chlorox® (Sodium hypochlorite) solution for 20 minutes to prevent development of disease in plants. These trays were filled with sterilized media up to ¾ full. Transparent trays were selected because it allows light to reach the spores on the media.

The protocol for *Pteris melanocaulon* is summarized in Fig. 2. Also shown are the stages of growth and physiological development of the plants.

3. Protocol

1. Extraction of spores: Air dried fronds with the sori facing down on the wax paper have spores that are dehiscing from the sori. Collect all the dehisced spores from wax paper and remove the indusium (the protective covering) using a 2.5 micron siever. Do this by gently scraping the spores with a finger. Shake the siever a few times to separate the spores from indusia and other extraneous materials.

2. Sowing of spores: Use a dry clean glass vial to spread about 16 mg of spores on a damp sterilized medium in the propagation tray (18cm x 10cm x 3cm). To spread uniformly, the mouth of the vial should be covered with fine nylon fiber. Apply approximately 50ml distilled water immediately after sowing; do it gently by misting, then cover.

NOTE: Use of distilled water is preferred since it does not contain chlorine that may prevent germination. To prevent dispersion and clustering of spores during watering, apply the water gently by misting with the nozzle of sprayer positioned at 30 cm distance from surface. Do not cover the spores with soil to allow light to reach the spores. Cover the propagation tray to trap the moisture inside and maintain a very humid condition.

3. Transfer of propagation trays: Transfer the propagation trays containing the newly sown spores to a condition that allows some amount of light to pass and reach the spores. This is in room condition with temperature ranging from 25 ^{0}C to 32 ^{0}C. NOTE: The spores must not be directly exposed to sunlight; however some amount of irradiance within photon flux density of 15µmol $m^{-2}s^{-1}$ to 70 µmol $m^{-2}s^{-}1$ is important. It was observed that it is within this range of irradiance that germination occurs most rapidly.

4. Maintaining conditions for germination and growth: High humidity and little irradiance should be maintained for 115 days. Frequent visual inspection for the presence of moisture on the cover side must be done. Apply water by misting when no moisture collects at the cover – this is the sign that humidity drops. Within 115 days duration, irradiance in daytime about 15µmol $m^{-2}s^{-1}$ to 70 µmol $m^{-2}s^{-}1$ intensity must be maintained by installing translucent plastic roofing in the nursery. NOTE: The key conditions for germination of spores and eventual growth are the humidity and light.

5. Assisting fertilization for sporophyte development: The critical period for fertilization is from 30 DAS to 60 DAS. To assist fertilization, a film of water on the surface of prothallus must be present all the time during this period, and thus misting with distilled water every 2 days and closing the propagation trays at all times to trap the evaporating moisture are important. Humidity during daytime however fluctuates even if the propagation trays are closed especially during summer, therefore more frequent visual inspection every 3 hours in daytime must be done. The presence of condensing vapor at

the underside of cover is an indication of high humidity inside the tray. If these condensing vapors are absent, misting must be done immediately. NOTE: This procedure should be observed within 30 DAS to 115 DAS, and when doing visual inspection there is no need to open the transparent tray because trapped moisture can readily be observed. It is important that this film of water on the surface of prothallus is present all the time to allow the sperm to move from the antheridium (male organ) to archegonium (female organ).

6. Transplanting with care and maintenance for sporophytes: Appearance time of sporophytes was from 77 DAS to 115 DAS, and sporophytes with split fronds were observed from 120 DAS to 140 DAS –it is in this period that the cover be removed to expose them to more oxygen, but frequent misting with distilled water must be done every 2 hours in the first day (this translates to 4X misting from 8:00 am to 4pm).

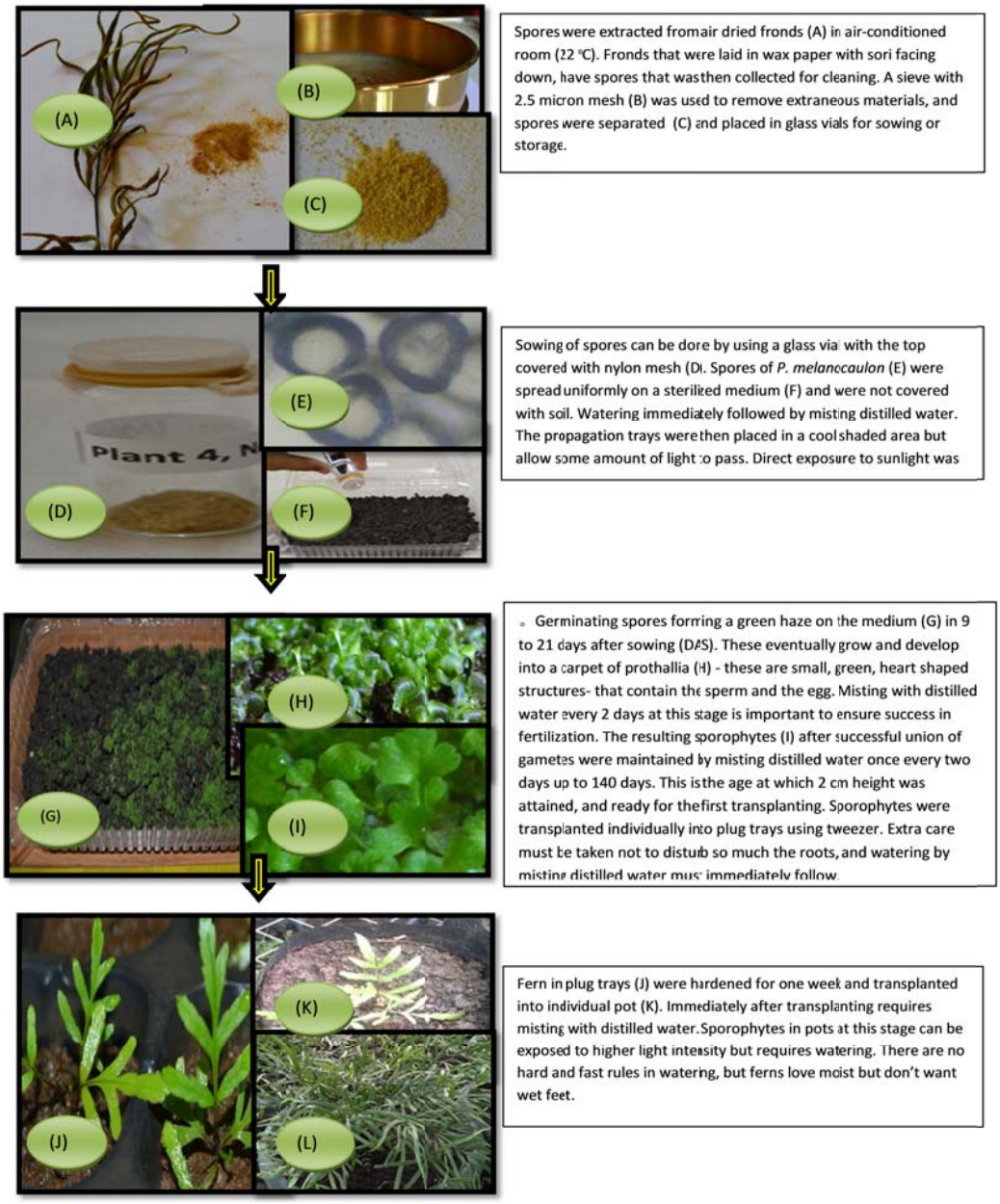

Fig. 2: Sequence of steps in propagating sporophytes of *Pteris m elanocaulon* from spores

Misting frequency can be reduced to 2 per day and this should be a routine for 1 week to prevent excessive loss of moisture. When sporophyte reaches 2cm in height, transplant them individually to plug trays containing sterilized soil medium, and apply water immediately by misting. A small pincers can be used in transplanting. Do not expose newly transplanted sporophytes to direct sunlight instead place them in a shaded area for six weeks. NOTE: Under shaded conditions, a minimum amount of irradiance of about 15µmol $m^{-2}s^{-1}$ to 70µmol $m^{-2}s^{-1}$ must be available. Transplanting must be done when temperature is low and humidity is high. In tropical condition, transplanting between 8:00 am to 3:00 pm is highly discouraged owing to high temperature especially when transplanting is done in the open.

3. Results and discussion

Sporophytes were successfully formed and developed after spores germinated and developed prolifically into gametophytes (Table 1) under humid condition at temperature ranging from 25 ^{0}C to 32 ^{0}C. Sporophytes appeared 77 DAS (Days after Sowing) to 115 DAS and reached a 2cm height at 140 DAS (Table 2) following the above protocol. The 140 days length of time for sporophyte development is a little longer to that observed by De Brum and Randi (2006) for *Rumohra adiantiformis* (133 days) but shorter than that of Ravi et al. (2015) for *Pteris tripartita* (about 150 days); which could be due to species differences. Both studies did not mention that their sporophytes at that age have reached the developmental landmark height of 2 cm.

Table 1: Descriptive performance of gametophyte and sporophyte formation in three different media

Development Stage	Media		
	On-site Soil	Sand	Top Soil
Gametophytes	Prolific	prolific	prolific
Sporophytes	prolific	Very few	prolific

Table 2: Time duration for every stage of development of *Pteris melanocaulon*

Developmental Stage	Length of Time (DAS*)
Germination	9 to 21
Gametophyte appearance time	68
Gametophyte formation time	68 to 94
Sporophyte appearance time	77 to 115
Age to reach to 2 cm	140

Ravi et al. (2015) recently developed a protocol for the development of sporophytes of the fern *Pteris tripartita*. The protocol developed however was in vitro; using MS medium with growth regulators and the plants were grown under controlled conditions in the laboratory. In contrast, the protocol described in this paper is under nursery conditions.

Humid condition inside the propagation tray in the nursery was intentionally created to facilitate successful union of male and female gametes in the gametophytes. In the fern *Drynaria fortunei*, Chang et al. (2007) supplied water daily by sprinkler-irrigation on the newly transplanted gametophytes, and such technique produced sporophytes. Srivastava and Uniyal (2013) developed a different technique, whereby they flooded the gametangia with sterile distilled water twice in a week to facilitate fertilization. In the proposed protocol by contrast, moisture was trapped by closing the cover of propagation tray. Distilled water was only applied to gametophytes in finer drops by misting when no visible water condensation is observed at the underside of cover. This was done before gametophytes were transplanted. Unlike sprinkling, misting may minimize damage and mortality of gametophytes owing to very fine droplets.

It should be noted that these three techniques of creating humid conditions, have so far been successful in producing sporophytes but their efficiency and discrepancy in number of sporophytes produced cannot be compared at present.

The decision to make the condition inside the propagation tray very humid was because the two gametes are physically separated in the gametophyte; further, they are held by special structures or organ: the antheridium for the male organ and the archegonium for the female organ (Chou et al., 2007; Y Galán et al., 2008). For union of two gametes to occur, the sperm must get out of the antheridium and move to the location of ova. Srivastava and Uniyal (2013) flood the gametophytes with water to burst the antheridium and release the male gametes and fertilized the female gametes.

It was assumed that maintaining elevated humidity in the propagation tray was necessary in order to facilitate sexual fertilization and eventual development of sporophyte. Although there are ferns such as *Asplenium nidus* that bypass fertilization (known as apogamous or asexual) and still produced sporophytes (Khan et al., 2008). Apogamous reproduction though, could yield only a small number of sporophytes such as the one observed by Srivastava and Uniyal (2013) in *Asplenium nidus*. Whether or not the sporophytes of *P. melanocaulon* were produce through sexual or asexual mechanisms, was not addressed in the current study.

The importance of irradiance in the germination of spores in ferns was reported by Nester and Coolbaugh (1986). They found that spores that were subjected to darkness did not germinate but for those exposed to 16 hours of light, as in the case of *Anemia mexicana* and *A. phyllitidis* ferns, germination was possible. Irradiance of about 5% to 20% was documented to stimulate the germination of the *Gleichenella pectinata* spores (Santos et al., 2010). The spores in step 2 of this proposed protocol were not covered with soil, instead the spores in the propagation trays were placed in open racks allowing light to pass.

In the current study, the irradiance to which the germinating spores, gametophytes and sporophytes were exposed was in the range of 15µmol $m^{-2}s^{-1}$ to 70µmol $m^{-2}s^{-1}$ photon flux density. Other study such as the in vitro propagation of *Pteris tripartita*, the fern was exposed to light intensity of 40 µmol $m^{-2}s^{-1}$ (Ravi et al., 2015). Srivastava and Uniyal (2013) used 47.3µmol $m^{-2}s^{-1}$ to 56.8µmol $m^{-2}s^{-1}$ for growing *Asplenium nidus* in vitro. The wider range of light in the present study was the actual irradiance during daytime under shaded nursery condition. Irradiance

used in studies of Ravi et al. (2015) and Srivastava and Uniyal (2013) are also within this range.

5. Conclusion

The protocol that we developed was under the assumption that *Pteris melanocaulon* follows sexual reproduction, yield success in the germination of spores including growth of gametophytes and the formation and development of sporophytes. The protocol described is for nursery condition that can be used for mass production of sporophyte seedlings for phytoremediation.

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