



## Propagation protocol of *Pteris vittata* L. using spores for phytoremediation

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### Abstract

*Pteris vittata* has been reported to be a hyperaccumulator of arsenic, and therefore useful in phytoremediation to clean-up arsenic-contaminated soils. Although factors for successful germination of spores have been identified, but information concerning the whole range of steps involved in mass propagating the fern is lacking. Thus, the study was aimed to develop a propagation protocol using spores to produce large number of seedlings. We implemented descriptive research methodologies such as observation and documentation of fern propagation activities in the nursery and conducted experiment following completely randomized designed with three replications. Here, we show that seedlings of *Pteris vittata* were successfully produced and protocol for its propagation is described.

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## Introduction

*Pteris vittata* L. (commonly known as Chinese brake fern) is a perennial evergreen species belonging to the family Pteridaceae; present in all tropical regions of Eurasia, Africa and America. Ma *et al.* (2001) and Chen *et al.* (2002) concurrently discovered that this fern species hyperaccumulated a carcinogenic metalloid arsenic (As). This plant can accumulate up to 6,805 ppm of arsenic in just six weeks in contaminated soils; and up to 93% of the arsenic extracted from soil is concentrated in the fronds (Ma *et al.*, 2001; Tu *et al.*, 2002; Chen *et al.*, 2002). The form of As in the leaves (with a concentration 25 times more than the roots and 142 times higher than soil) is inorganic As(III), and mostly confined in the upper and lower epidermis (Tu and Ma, 2002; Tu *et al.*, 2002; Lombi *et al.*, 2002; Zhang *et al.*, 2002). From the above observations as presented in various studies, *P. vittata* was identified to have the potential to remediate As-contaminated soil (Ma *et al.*, 2001).

Phytoremediation experiments on As-contaminated soils were done on field scale trials in Florida (Kertulis-Tartar *et al.*, 2006), China (Wei *et al.*, 2006) and lately in Australia (Niazi *et al.*, 2011). All of these field trials yielded positive results indicating feasibility for larger scale environmental clean-up projects with As-contaminated soil. A possible constraint to these projects will be the availability of sufficient quantity or stock of plants to be used in field applications. To increase plant availability, *P. vittata* can be propagated vegetatively, but such technique can pose a number of problems: 1) availability of substantial numbers of mother plants, 2) uniformity in age, 3) high transportation cost and handling, and 4) high stress and mortality during transport and transplanting. Sexual propagation using spores however can be an option to resolve these concerns thus can produce larger quantity of plants needed for large-scale phytoremediation.

A recent study on the sexual propagation of *P. vittata* investigated the effects of pH, calcium concentration and temperature (Wang *et al.*, 2010). In this study, the germination and growth of the plants were

favorable by higher pH and calcium availability at 25 °C. Unfortunately, similar studies were not pursued, and there were no attempts to establish a generic protocol in propagating *P. vittata* for mass production using spores. Thus, in this study, we aimed to describe in detail a procedure in germinating spores to produce gametophytes (haploid), as well as a technique that successfully produce sporophytes (diploid). Furthermore, the care and maintenance from sporophyte formation to the completion of its life cycle are discussed.

## Materials and methods

Spores of *P. vittata* were taken from mother plants collected from a mining site in Placer, Surigao del Norte, Philippines. Fronds with spores (light brown in color when mature) were selected from the mother plants, cut and secured in an envelope to capture the dehiscing spores. The fronds were then placed on a sheet of clean paper (with sori facing down) for air drying for two days to prevent development of disease and to prompt mature spores to dehisce. During the air drying process, the materials were not exposed to sunlight to prevent excessive loss of internal moisture, and were kept away from blowing wind. Extraneous materials were then separated using a 2.5 micron sieve, and the collected spores were stored in properly labeled test tube with cap.

Three different media were used: 1) Clay loam soil from the mining site (pH=7.3), 2) sandy loam soil from Manresa farm in Cagayan de Oro (pH=6.8), and 3) river sand (pH=6.7). These were pulverized, sieved and sterilized by heating at ca. 70 °C for one hour. These media were replicated three times to allow comparison for gametophyte appearance time (GAT) and sporophyte appearance time (SAT) following Completely Randomized Designed (CRD). F test was implemented for comparison using Paleontological Statistics (PAST) version 2.03.

Transparent polypropylene plastic containers (similar to those used in making pastries) were used as propagation trays. These trays were surface sterilized using 10% sodium hypochlorite Chlorox® solution to

prevent contamination and disease development in the developing plants. They were  $\frac{3}{4}$  filled with sterilized medium. Transparent containers were purposely used to allow light to reach the medium that contains the germinating spores.

The steps in the propagation of *P. vittata* are discussed in detail below and summarized in Fig. 1. Also shown are the stages of growth and physiological development of the plants.

1) Sowing of spores: Sow approximately 16mg of spores uniformly on the surface of a 18cm x 10cm x 3cm pastry container holding damp sterilized medium. A glass vial with very fine nylon fiber at the mouth end can be used in spreading (Fig. 1). Watering is supplied by spraying about 50ml of distilled water. NOTE: When applying mist, make sure the nozzle is positioned ~30cm away from surface. Too close a distance may cause dispersion of spores. Never use tap water because of high levels of chlorine that may hamper germination. The newly sown spores should not be covered with soil, since exposure to incident light facilitates germination of the spores. Immediately cover the container after sowing and watering. The purpose is to trap the evaporating water and maintain high humidity inside the container, and to prevent contamination.

2) Putting the propagation containers in open racks: The propagation containers with sown spores have to be placed in open racks at room temperatures (25C – 32C). NOTE: Ambient conditions must allow light within  $15\mu\text{mol m}^{-2}\text{s}^{-1}$  to  $70\mu\text{mol m}^{-2}\text{s}^{-1}$  but without direct exposure to sun. Germination is noted to occur most rapidly if the spores receive a fair amount of light.

3) Maintaining humid condition: Maintain a humid condition inside the propagation containers at all times by keeping the medium moist and the containers closed. This must continue up to 115 days from sowing. NOTE: Avoid overwatering as it encourages growth of algae that will hamper germination and also inhibit the growth of developing

gametophytes. In 5 to 7 days, a green mat or film appears across the surface of the propagation containers; this is a sign that spores are germinating. Average gametophyte appearance time ranges from 30 to 32 days (or 1 month from sowing) under this humid condition. Formation of gametophytes continues up to 54 days from sowing.

4) Facilitating sexual propagation among the gametophytes: The union of male and female gametes is facilitated by gently spraying very fine mist of distilled water over the gametophytes using an atomizer. This is especially critical at 30 days from sowing. Successful fertilization will produce sporophytes which will start to appear 54 days from sowing. Maintain the humid condition by misting once every two days up to 115 days from sowing – the age of sporophytes where most of them have attained a height of 2 cm.

5) Stabilizing the growth of sporophytes: Hardening of sporophytes is done by opening the cover of the propagation tray, and allowing the plants to grow in such condition for one week. In this procedure, sporophytes are acclimatized to low humidity conditions. NOTE: This is a critical period that may result in high mortality of sporophytes. It is therefore important that distilled water is applied by misting within 2 hours after uncovering the containers. The first day of exposure to low humidity air requires misting operations 4 times during daytime; and misting twice daily in the second day up to one week.

6) Transplanting the sporophytes to plug tray: Transplant the hardened sporophytes (about 122 days old) to plug trays, as this is the stage of growth with greater chance of survival. At this age, almost all sporophytes have reached about 2 cm height. Using a tweezer, prick the individual sporophyte and transfer it to the plug tray with sterilized soil medium. NOTE: This must be done late in the afternoon towards the evening, when the temperature is lower and relative humidity is high. Daytime transplanting between 8:00 a.m. and 3:00 p.m. is detrimental to plants

especially if done in an open condition, and thus discouraged.

7) Transferring the plug trays with transplanted sporophytes to shaded areas: Transplanted sporophytes in plug trays must be placed in shaded areas and not directly exposed to sunlight, and maintained for six weeks. Although shaded, ensure that the minimum amount of light is about  $15\mu\text{mol m}^{-2}\text{s}^{-1}$  to  $70\mu\text{mol m}^{-2}\text{s}^{-1}$ . NOTE: The surface of the medium must be damp, i.e visual sign of moisture is present but without film of water that signals excess.

8) Transplanting the sporophytes to plastic cups: The second transplanting must be done after six weeks. Transplant the sporophytes (164 days old from sowing) to 16 oz. plastic cups filled with garden soil. These transplanted seedlings should then be placed in an area of the nursery with sufficient light ( $100\mu\text{mol m}^{-2}\text{s}^{-1}$  –  $500\mu\text{mol m}^{-2}\text{s}^{-1}$ ), but still shaded with one layer of black nylon net. These are maintained for four weeks. NOTE: Transplanting must be done late in the afternoon when the temperature is low to minimize stress and excessive loss of moisture, and

the newly transplanted seedlings must be watered immediately with tap water. After transplanting, do not expose these seedlings directly to open area (more than  $1000\mu\text{mol m}^{-2}\text{s}^{-1}$  light) to maximize the survival percentage of the plants. Plants need hardening for 7 to 10 days. If the garden soil is not sterilized, it must be sourced from an area without a history of disease problems, nematodes and insects.

9) Transplanting the seedlings to polybags: The third transplanting of seedlings (192 days old from sowing) can be done directly on the field site or in a polybag 7cm x 12cm (if intended to be kept in the nursery as source of planting materials). Watering the plants (~200 ml/bag) should follow immediately after transplanting to minimize stress and to lower mortality. The seedlings can then be fully exposed to sunlight and can be maintained up to nine months in the nursery.

### Results and discussion

The described details in the propagation of *P. vittata* yielded prolific formation of gametophytes and sporophytes, except on spores germinating on the sand medium (Table 1).

**Table 1.** Qualitative description of germination performance of *P. vittata* spores in three propagation media.

Growing Media	Gametophytes	Sporophytes
On-Site Soil	Prolific	prolific
Sand	Prolific	very few
Top soil	Prolific	prolific

**Table 2.** ANOVA for Gametophyte appearance time.

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Treatment	.222	2	.111	.500	.630
Error	1.333	6	.222		
Total	1.556	8			

Comparing Gametophyte Appearance Time (GAT) of *P. vittata* spores among the three media yielded a non-significant result as shown in table 2 (df: 2,6; F:0.500; P:0.630), and Sporophyte Appearance Time (SAT) in table 3 was also non-significant at 5% (df: 2,6; F:1.301; P:0.3394). The on-site soil medium had a pH of 7.3, top soil was 6.8 and river sand medium

was 6.7. These were also the pH range observed by Wan *et al.* (2010) where spores of *P. vittata* germinated, though germination percentage was low compared to media with high pH. The same pH range was observed to be optimal in *Anemia mexicana* and *A. phyllitidis* (Nester and Coolbaugh, 1986).

It should be noted that in sowing (Step 1 of protocol), spores were not applied too thickly to avoid overcrowding. Ayrapetov and Ganger (2009) reported that the percentage of male gametophytes increased

with increasing density of gametophytes due to the cumulative effect of antheridiogen production. That is, overcrowded prothalli may lead to the absence of archegonia, making fertilization impossible.

**Table 3.** ANOVA for Sporophyte appearance time.

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Treatment	81.342	2	40.671	1.301	.339
Error	187.593	6	31.266		
Total	268.936	8			

**Table 4.** Germination performance of *P. vittata* spores in three different media.

Propagation Media	Total Surface Cover (%)	GAT (DAS)	GFT (DAS)	SAT (DAS)	SFT (DAS)	Age to reach 2cm (DAS)
On-Site Soil	62.67	30.17	45.17	75.33	39.67	115
Sand	76.93	30.17	49.83	80	36.67	116.67
Top soil	44.28	30.5	42.23	72.73	42.27	115

DAS – Days after sowing

GAT – Gametophyte Appearance Time

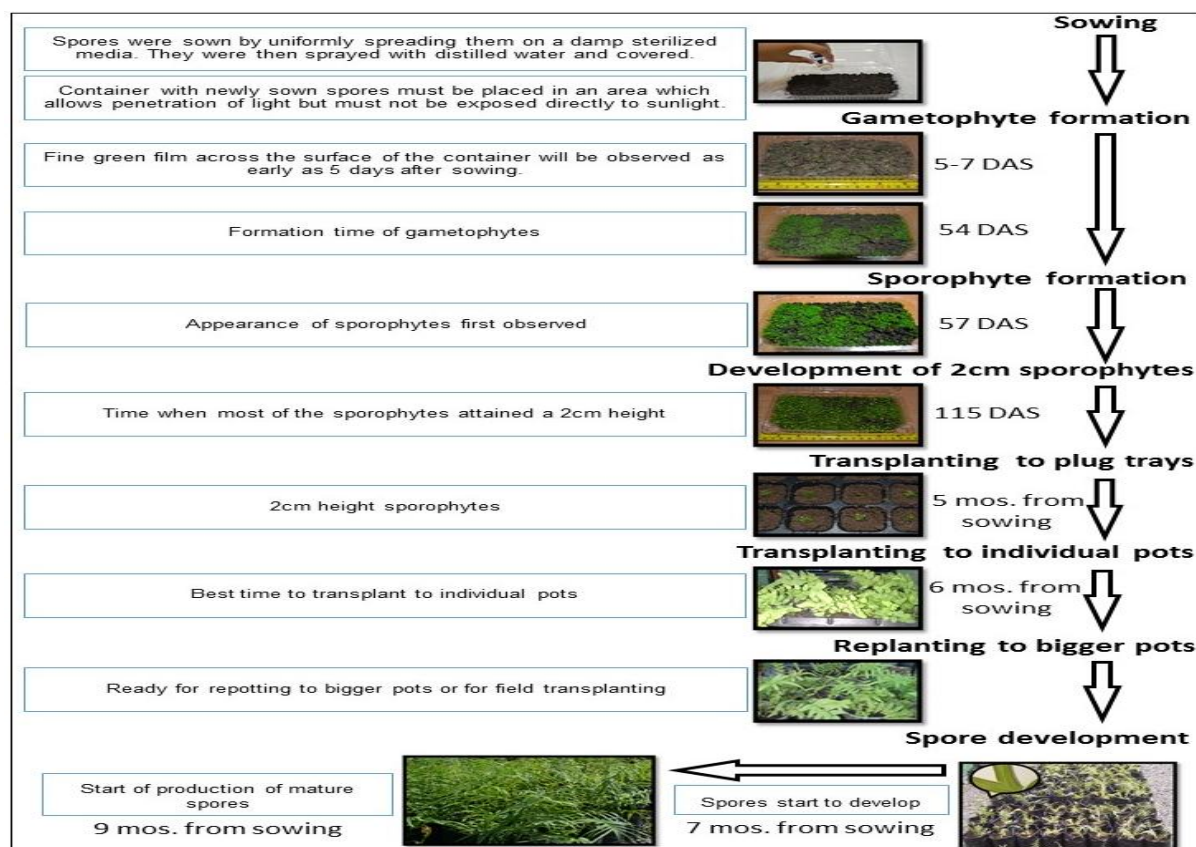
GFT – Gametophyte Formation Time

SAT – Sporophyte Appearance Time

SFT – Sporophyte Formation Time.

Spores were also not covered with soil and even the propagation trays were placed in open racks that allowed light to pass (Step 2 of protocol). In the

natural environment, mature spores are shed from mother plants to the ground and germinate on the surface where light can penetrate.



**Fig. 1.** Sequence of events, activities and plant development from day of sowing to the day of life cycle completion. *P. vittata* completes its life cycle in 9 months. DAS (Days After Sowing).

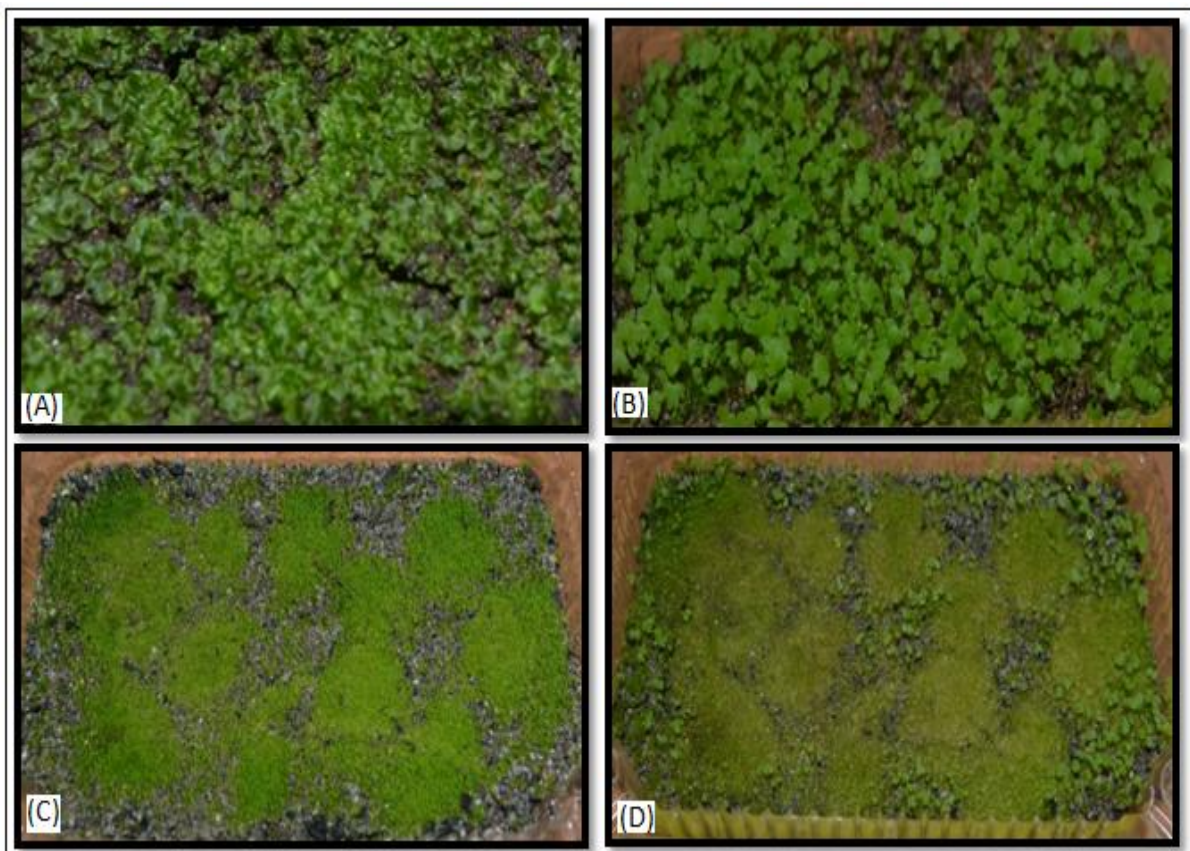




**Fig. 2.** The dense formation of prothalli, the first stage in the life cycle of brake fern 54 days after sowing.

This was confirmed when three replicates of each medium exposed under dark conditions had no spores germinating. In the species *Rumohra adiantiformis*, the spores subjected to darkness did not germinate (Brum and Randi, 2002), but those exposed to 16 hours of light, such as in the case of

*Anemia mexicana* and *A. phyllitidis* ferns, were stimulated to germinate (Nester and Coolbaugh, 1986). About 5% to 20% of light was shown to stimulate germination of *Gleichenella pectinata* spores (Gonçalves dos Santos *et al.*, 2010).



**Fig. 3.** (A) The dense formation of gametophytes 54 DAS in top soil, (B) prolific formation of sporophytes 115 DAS in top soil medium, (C) prolific formation of gametophytes 54 DAS in sand medium and (D) very few formation of sporophytes after 115 DAS in sand medium.

Germination of spores was first observed as a fine green film across the surface of the germination tray as early as 5 days after sowing (Fig. 1). Each tiny green speck slowly develops into a flat, heart-shaped prothallus (the first stage in the life cycle of the fern), usually 2-5 mm in width. The prothalli (Fig. 2) then begin to produce true fronds from the notch of the heart.

*P. vittata* gametophytes that successfully developed into sporophytes reached a height of 2 cm at 115 to 116 days after sowing (DAS) shown in Table 2 and Fig. 3. The growth therefore was faster because it reached such a height 21 days earlier compared to results obtained by Wan *et al.* (2010).

Sporophytes develop only when sperms successfully unite with the female gametes (Harvey, 1993), thus misting and maintenance of humid condition inside the propagation tray (Step 4 of protocol) allowed the sperm mobility to archegonia (female gametes).

Lack of nutrition may be a factor causing low production of sporophytes in the sand medium as shown in Table 1. This condition in the sand may have caused the gametophytes to be small. Sakamaki and Ino (1999) observed that archegonia are not formed when gametophytes are small. The formation of archegonia (female gametes) is important such that sporophytes are produced.

## Conclusion

The current study has developed and described a protocol in the production of new seedlings by sexual propagation through spores and was proven to be successful. Following the new protocol, we successfully produced sporophytes that continued to grow up to 9 months and complete the life cycle. In the new protocol, the following optimal conditions were necessary to achieve success:

1. High moisture and high pH are ideal conditions for the germination,
2. Misting with distilled water during the

3. gametophyte stage was necessary for sperm mobility, and thus facilitate fertilization of gametes and the eventual formation of sporophytes, and

4. Light intensity ranging from  $15\mu\text{mol m}^{-2}\text{s}^{-1}$  to  $70\mu\text{mol m}^{-2}\text{s}^{-1}$  is needed to stimulate germination.

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