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IN VITRO PROPAGATION OF *ANGIOPTERIS EVECTA* USING SPORES

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ABSTRACT

Techniques of establishing *Angiopleris evecta* plants *in vitro* were studied. Soaking of *A. evecta* spores in water for 24 hours markedly reduced spore contamination. Soaking of the spores in 1 -2 % of sodium hypochlorite for less than 5 minutes allowed satisfactory disinfestation without affecting spore viability. Lower concentration of minerals (1/4 MS), presence of charcoal in the medium and exposure of the spores to light were crucial for spore germination and gainetophytc development *of A. evecta.*

Keywords: *Angiopleris evecta I* King fern / spore / MS medium / bleach / light / tissue culture / sporophyte / gamctophytc

Abbrevations

BA - benzylaminopurine; BB - Bold's basal medium; pCPA - para-chloro-phenoxyacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indoleacetic acid; IBA - indolebutyric acid; Kinetin -6-furfurylaminopurine; MM - Moss's mineral medium; MS - Murashige & Skoog media; NAA - naphthaleneacetic acid; NOA - naphthoxyacetic acid.

INTRODUCTION

The fern and fern allies are the surviving members of the earliest lineages of vascular plants. None of these primitive plants produce either seeds or flowers; instead they reproduce via single-celled spores. Ferns are usually found in moist terrestrial or aquatic environments and their size may vary from a few centimeters to many meters.

Angiopteris evecta is known as King fern or Giant fern and it belongs to the family Marattiaceae. It is a magnificent fern, the fronds are reputedly largest in the world, and are about 5 m long, arching, semi-weeping, bi-pinnate, and glossy (Figure la). Lower pinnules have earlike lobes at the base. Sporangia always occur

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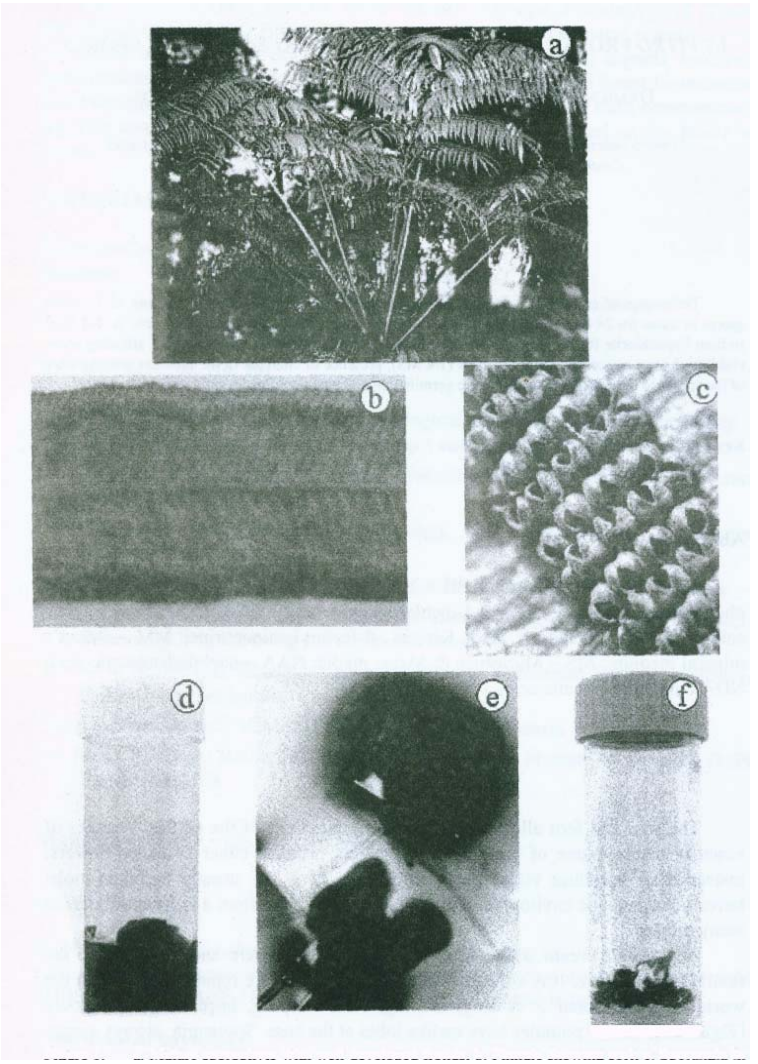

Figure 1. a. Mature sporophyte with well developed fronds; b. Pinnule showing sori; c. Sporangia in dense clusters; d&e. Developing gametophytes in MS Vi c medium; f. Germinating sporophyte from gametophyte.

in dense clusters of five to eight opposite pairs (Figure Ib & Ic). The King fern is found in Australia (Queensland and New South Wales), Malaysia, Polynesia and southern and northeastern parts of India. It also occurs in New Guinea and Indonesia (S. Marsterson; Department of E.P.A., Rockhampton, Pers. com.). In northeastern parts of India, the massive stem is cooked and eaten by the tribes. An intoxicating drink called 'Ruchshi' is also made out of it. The plant yields aromatic oil that is used for perfuming coconut oil in south sea islands (Manickam and Irudayaraj 1992). Conventionally, King ferns are almost impossible to propagate from spores but may be reproduced vegetatively from the fleshy earlike projections which are commonly known as auricles. Plant development is very slow and it may take 12 months or more before a small frond and sufficient roots develop to support the plant (Blomberry and Maloney 1994). Tissue culture can prove to be the fastest and the most effective method of multiplication of the King fern.

Studies on *in vitro* spore propagation and culture media requirements of different ferns have been conducted by Bernabe *et al*. (1999); Amoroso and Amoroso (1998); Fernandez *et al.* (1997); Goller and Rybczynski (1995) and Dong and Su (1993). Since spores remain the only source of fern propagation, considerable importance has been placed on spore disinfestation and culture media selection for different ferns. Fernandez *et al.* (1997) observed optimal growth of *Blechnum spicant* gametophytes in MS (Murashige and Skoog 1962) liquid medium spread over a 2% agar and exposed to 16 hours of light. Borelli *et al.* (1990) used both calcium and sodium hypochlorite solution for disinfesting spores and they obtained the best results with 2% sodium hypochlorite solution. Borelli *et al.* (1990) also observed better germination of the ferns *Cyathea schanschin* and *Dicksonia sellowiana* on John and Knop's media after 4-8 weeks, with the prothalli developing 30-40 days later. In a similar study, Goller and Rybczynski (1995) disinfested spores of *Cyathea australis* using 3% chloramine with Tween. They reported better germination of spores on Anderson's medium supplemented with 80 mg T^{1} of adenine sulfate solidified by 0.8% agar.

No studies are currently available for *A. evecta* either for spore disinfestation or for selecting suitable culture medium. The present study was carried out to optimize spore disinfestation procedures and to determine suitable culture medium and growth conditions for establishment of *A. evecta* in tissue culture.

MATERIALS AND METHODS

Collection and Storage of Spores

Recently, matured pinnules containing sori were collected fresh from the Rockhampton City Council Botanical Garden, Rockhampton, Australia. After a close microscopic examination, closed sori containing spores were isolated from the

fronds for disinfestation. Another set of pinnules was stored in a brown paper bag to allow drying without fungal infection.

Effect of Soaking Spores on Contamination

The sori were separated from the pinnulus by scraping with a scalpel. The released sporangia were then collected onto a piece of paper. These sporangia were exposed to three treatments. In Treatment 1, sporangia were filtered through a 106 um sieve using demineralised water and the filtrate was collected in a glass jar. The sporangia were allowed to soak in water for approximately 24 hours. Once soaked, the spore solution was microfuged (^{x} 10,000 rpm) for five minutes to concentrate the spores prior to sterilisation (sieved and soaked). The spore solution (1 ml) was inoculated onto agar plates to test for contamination and germination. In Treatment 2, the unsieved sporangia were used, and they were soaked in sterile water for 24 hours before microfuging as in Treatment 1 (not sieved but soaked). In Treatment 3, plant debris were removed by sieving and the spores were not soaked in water (sieved but not soaked). The rest of the operations were the same as for Treatment 1. In Treatment 4, spores were neither sieved nor soaked (neither sieved nor soaked).

Spore Disinfestation

Two concentrations (1% and 2%) of sodium hypochlorite were used for disinfestation, with the exposure times of 3, 5, 7 and 9 minutes. A known volume (1 ml) of solution containing sporangia was placed in an Eppendorf tube and 1.5 ml of 1% or 2% NaOCl was added. After 30 seconds of soaking, the Eppendorf tube was centrifuged for *3* minutes. One milliliter solution was removed from the top of the Eppendorf tube and a milliliter of sterile water was added. The contents were centrifuged again for 2 minutes. The washing with sterile water was repeated 4 times, to remove all traces of NaOCl prior to inoculation.

Inoculation

Using aseptic bench top tunnel technique, culture tubes (50 ml capacity, plastic) were inoculated with two drops of spore solution and the tubes sealed before being placed in a controlled environment room.

Media

Media with low nutrient content were selected, because the spores, like seeds, contain all required nutrients for early growth. Wide ranges of media were used. These include MS, MSc (MS media + 1 g/L charcoal), MS%c (MS medium with *V** nutrients +'/« sugar + 1 g/L charcoal), MS low (MS medium + low hormones*), MS'/z low (MS medium with *Vi* nutrients + low hormones*), MS% low (MS medium

with 1/4 nutrients + low hormones*), FN (fern normal), Bold's basal (Bischoff and Bold 1963) and Moss's mineral media.

*Here De Fossard's (1981) broad spectrum low hormones were used. Low hormones consisted of Auxins (IAA+IBA+NAA+NOA+2,4-D+pCPA; 0.1 uM each) and Cytokinins (BAP+Kinetin; 0.1 uM each).

Incubation

One set of inoculated tubes was randomly stacked in a clear plastic bag and incubated in a controlled environment room (CER) at 30 °C with a light intensity of 100 uEm~^{2}s~'. The CER was maintained at a photoperiod of 12 hours light and 12 hours dark. The second set of tubes was sealed within a black plastic bag to simulate dark environment that is often found in dense rainforest floor where mature ferns are usually found. Both sets of tubes were placed side by side in the CER to ensure that they were exposed to similar climatic conditions except for light.

RESULTS

Effect of Soaking on Contamination and Spore Germination

Pre-soaking of spores for 24 hours before disinfestation reduced contamination. The contamination rate could be reduced to as low as 3% with pre-soaking treatment alone. Most of this contamination was caused by fungi *(Penicillium* sp. and *Aspergillus* sp.) and rarely by bacteria. Contamination was as high as 75% when the spores were neither sieved nor soaked. The sieving and soaking reduced the contamination rate to 13% (Table 1). Sieving had very little effect on contamination, but the soaking markedly reduced the contamination rate.

Table 1. Effect of pre-soaking treatments on contamination and spore germination of *A. evecta*

	Pre-disinfestation treatments			
	Soaked and sieved	Soaked but not sieved	Not soaked but sieved	Not soaked Not sieved
Number of culture tubes inoculated	99	72	32	8
Contaminated tubes	12	2	20	6
% Contamination	13	3	63	75
No. of tubes showing spore germination	11	1	15	1
% germination	11	1	47	13

Response of *A. evecta* spores to NaOCl

The degree of contamination varied from 0% to 38%, with 1% NaOCl and from 0% to 9% at 2% NaOCl (Table 2). Exposure of King fern spores to 1 or 2% NaOCl for either 7 or 9 minutes resulted in complete disinfestation. However, none of these spores germinated when the exposure time exceeded 5 minutes. Thus, the treatment of King fern spores with 2% NaOCl solution for less than 5 minutes appear to provide the best results. The tubes that were inoculated with untreated spores were fully contaminated suggesting that disinfection is a must for King fern spores.

Table 2. Contamination status of *A. evecta* spores in response to naOCI concentration and time of exposure (number of tubes used per treatment varies from 8 to 38)

	Exposure time (minutes)							
	3		5		7		9	
NaOCl concentration (%)	1	2	1	2	1	2	1	2
% tubes contaminated	25	7	38	9	0	0	0	0

Effect of Culture Media on Spore Germination

The highest germination (69%) was observed in MSVic medium followed by Moss's mineral medium (25%), Hold's basal medium (17%), MS 'Alow (4%) and MS'/tlow (0%) (Table 3). Spores failed to germinate on full strength MS medium, either with or without hormones and charcoal. Reduced concentration of MS medium and the presence of charcoal maximized King fern spore germination (Figure 1d & 1e).

Table 3. Effect of culture media on spore germination of *A. evecta* (please see methods for description of acronyms)

	Culture media								
	MSc	MS0	MS¼ c	FN	MS low	MS½ low	MS¼ low	MM	BB
No. of culture tubes inoculated	41	42	16	34	16	26	26	12	12
No. of tubes showing germination	0	0	11	1	0	1	0	3	2
Percentage of tubes showing germination	0	0	69	3	0	4	0	25	17

Role of Light in Germination *of A. evecta* Spores

Light played a major role in 'the germination of *A. evecta* spores. Irrespective of soaking treatment, the length of exposure to NaOCl, or the type of medium used, almost all the spores failed to germinate in the absence of light (Table 4).

Gametophytes that grew well in tissue culture conditions produced sporophytes within 6 months (Figure If). The sporophytes were then successfully transplanted and raised in a potting mix.

Table 4 The role of light on spore germination of *A. evecta*

	Light	Dark
Number of tubes inoculated	170	49
Number of tubes showing spore germination	15	2
Percentage of tubes showing spore germination	9	2*

* Tubes germinated only after the cultures were exposed to light

DISCUSSION

Pre-disinfestation treatments are usually beneficial in reducing contamination, to a certain level, without harming the explant. This experiment gave a very useful clue for the use of pre-disinfestation treatment for decontaminating spores of *Angiopteris evecta*. Contamination percentage was reduced to as low as 3% when sporangia were soaked in water for 24 hours. It is likely that the bacteria and fungi that were adsorbed onto the fern spores become more susceptible to NaOCl treatment if they are soaked in water prior to disinfestation.

Prior to soaking of sporangia, the whole material was passed through 106 urn sieve (Treatment 1) with the aim of reducing the quantity of material to be disinfested, as it included a considerable amount of sori cases and dried leaf pieces. Sieving did not seem to make any difference to contamination rates both in soaked and unsoaked treatments.

Our results revealed that 2% NaOCl is highly effective in disinfesting *A. evecta* spores at both 3 and 5 minutes. These results are in agreement with those of Borelli *et al.* (1990) who obtained best results with 2% sodium hypochlorite for *Cyathea schanschin* and *Dicksonia sellowiana* spores. In contrast, Goller and Rybczynski (1995) obtained better results when they disinfested intact leaves containing the sori *of Cyathea australis* with 3% chloramine and Tween for 30 minutes.

The length of exposure of *A. evecta* spores to sodium hypochlorite also had a considerable bearing on the success of germination. Our results showed that three minutes exposure to sodium hypochlorite reduced contamination to a considerable

extent whereas, 7 and 9 minutes exposure proved to be lethal as the spores failed to germinate. Based on these results, we recommend the exposure of *A. evecta* spores to high concentration (1-2%) of NaOClfor short duration (<5 minutes).

The pattern of spore germination in various media compositions appears to reflect the need for low concentrations of mineral nutrients and the presence of charcoal for *A. evecta* spore germination. This observation is in consistence with the findings of Khoo and Thomas (1980), who found that high mineral salt concentrations tended to retard spore germination and sporophyte formation of *Adiantum raddianum* cv Tassel. However, the current findings contrast with those of Fernandez *et al.* (1997) who reported that MS full strength liquid media being optimal for growth of *Blechnum spicant* gametophyte. Apart from MS media, Andersen's media for *Cyathea australis* (Goller and Rybczynski 1995), Jone's media and Knop's solution, respectively, for *Cyathea schanschin* and *Dicksbnia solviana* (Borelli *et al.* 1990) and Knudson's media for *Cyathea spinulosa* (Agrawal *et al.* 1993) have also been advocated. Similar to our findings, Wardle *et al.* (1998) recommended the use of charcoal for the spore germination. Spores are analogous to seeds and they contain all the required nutrients for early growth, therefore it can be justified to use low nutrient media during initial stages of germination.

Light is an important factor and it plays a significant role in the germination of A. *evecta* spores. The results of this study concur with the field observations (S. Marsterson; Department of E.P.A., Rockhampton, Pers. com.) where the King fern seedlings usually occur in patches of rainforests that have been exposed to light possibly due to fire or other sources of damage to canopy. Light has been found to govern the development stage of whole gametophyte (Turnwald *et al.* 1999). Kiss and Kiss (1998) reported that light frequency in the red region is found to promote spore germination more than in far-red region. Light not only regulates the growth mechanism in haploid gametophyte, but it also affects development of sporophyte due to involvement of a photoreceptor phytochrome (Christensen *et al.* 1998). Presence of light has been shown to increase nuclear DNA and this increase is known to have an influence on fern spore germination (Raghavan 1993).

This study has demonstrated the importance of soaking of *A. evecta* spores to minimize contamination, sensitivity of King fern spores to high mineral concentration of the media and the need for light and charcoal for its spore germination. These data highlight the desirable conditions for disinfestation and germination of King fern spores.

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