Final Report to the Australian Flora Foundation on the project

The Biology and Cultivation of *Revwattsia fragilis* (Watts) D.L.Jones.

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7th August 2009

The Biology and Cultivation of a Rare, North Queensland, Epiphytic Fern, *Revwattsia fragilis*.

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INTRODUCTION

Australia has a rich and diverse fern flora, despite the relatively recent aridity of its modern climate. Ferns are an exceedingly attractive group of plants, a fact borne out by their popularity as both garden and indoor plants. Many Australian species of ferns are confined to the wet tropical north and many are rare. One of the more unusual ferns is the rare, endemic *Revwattsia fragilis* (Watts) D.L.Jones, a spectacular plant with fronds growing up to 1–2 m long. The narrow range and limited number of populations of this species, and its vulnerability to habitat disturbance, climate change and collecting predation make it an immediate priority for introduction into horticulture. Cultivation of this species would reduce these pressures and provide a sustainable source of material for translocation and population recovery if needed, and also act as a less damaging or low-impact source of material to collectors and the horticultural trade.

AIMS AND OBJECTIVES

The objectives of this project are:

- To establish *Revwattsia fragilis* as a viable horticultural commodity.
    - To investigate the developmental biology of *Revwattsia fragilis*, a monotypic tropical Australian endemic fern genus.
    - To determine optimum spore germination conditions using sterile media and axenic technique
    - To determine the optimum growth and horticultural conditions of *Revwattsia fragilis*

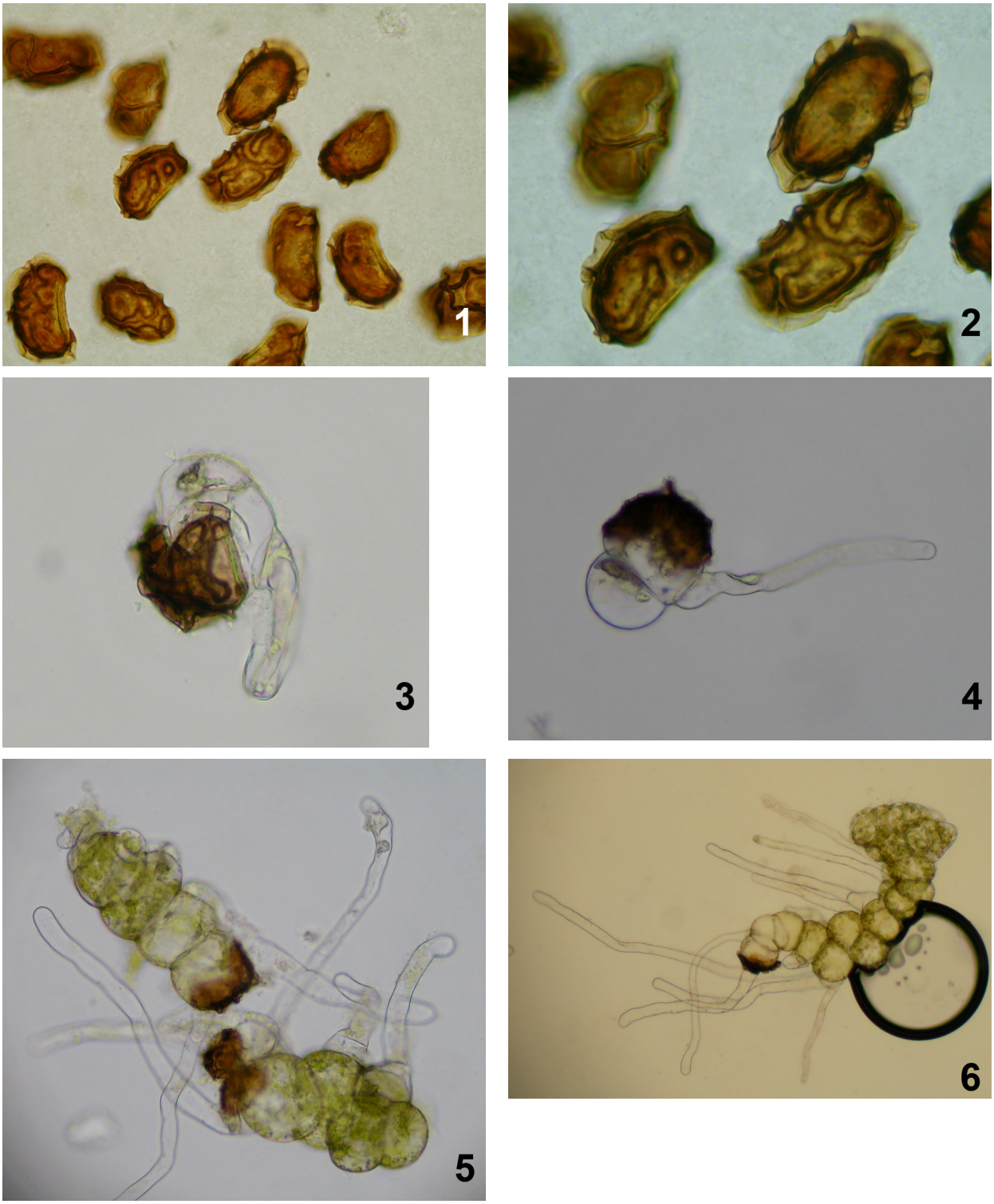
- To increase awareness and *ex situ* conservation of *Revwattsia* through its use as a horticulture and garden design element.

THE BIOLOGY OF *REVWATTSIA FRAGILIS*

Growing at intermediate altitude in rainforest in rotting tree hollows or within clumps of other epiphytic ferns, this enigmatic and little-known species was first collected in 1913. *Revwattsia fragilis* was described in 1914 (Watts, 1914) by the well-known New South Wales botanist Rev W.W.Watts, who specialised in the study of bryophytes (mosses, liverworts and hornworts) and pteridophytes (ferns). Originally placed into the genus *Polystichum*, it was not until 1998 that D.L.Jones recognised the unique features of this rare fern and placed it into its own monotypic genus. He named the genus *Revwattsia* in honour of the original author, Rev. Watts (Jones, 1998). At the time of Jones' publication of this new genus, it was only known from two locations. It is now known from six small populations, with a combined total of less than a dozen plants. It is restricted to the Atherton Tablelands in the wet tropical northeastern rainforests of Queensland.

THE SPORES

Revwattsia fragilis is a homosporous fern which produces non-chlorophyllous spores, i.e. all spores produced are of the same size and are darkly pigmented. Such spores are thought to be long-lived and able to tolerate desiccation (Lloyd and Klekowski, 1970). The spores are also bilateral or monolete. Monolete spores have a single scar along the flat side or surface of the spore, showing where the spore was joined to its sister spore during their formation by meiosis. The spores of *R. fragilis* are relatively small, at 47–58 µm long and 30–35 µm wide. They are ±bean-shaped and bear a sinuous lamellate pattern over the distal or curved surface of the spore. This patterning can be seen as a transparent wing around the circumference of the spores (Figs 1–2). Large numbers of spores are held within each of the sporangia and hundreds of sporangia are produced per sorus. (Figs 7–10).



Figures 1–6. Germination of spores (Fig. 3) and subsequent development of fern gametophyte (prothallus) from a hyaline rhizoid (Fig. 4) into a single row of chlorophyllous cells (Fig. 5) and then to a multicellular plate of cells with numerous rhizoids (Fig. 6).

THE GAMETOPHYTE

The spores of *Revwattsia fragilis* (Figs 1, 2) germinate readily under optimal conditions, usually within a week or two of being sown onto the medium. Germination of the species is not inhibited by light. Germination is characterised by the spore wall splitting along the scar or monolete mark, which is the point of least resistance of a spore casing. The internal cell of the spore bulges out of its spore wall (Fig. 3) and will elongate to produce a hyaline or colourless rhizoid which functions to anchor the gametophyte to its substrate (Fig. 4). The cells within the spore continue to divide and produce chlorophyllous cells in a single row (Fig. 5) which eventually becomes two rows and then multiple rows (Fig. 6) to form the typical flat heart-shaped prothallus or gametophyte typical of many ferns. Other structures are also produced by the gametophyte, including slime hairs and eventually the sex organs of the plant. These sex organs, called archegonia (female) and antheridia (male), produce the egg and the sperm respectively. Once fertilisation occurs, the young sporophyte develops from the fertilised egg and typically grows to form the first frond of the young fern. This is the sporophyte stage of the fern life cycle (Figs. 7–10) and is the most recognisable of all the stages.

Figures 7–10. The sporangia form within rows of round, green sori along the underside of the fern frond pinnules (Fig. 7). They change from a chestnut colour (Fig. 8) into darkly pigmented mature sporangia (Fig. 9). The mature sporangia are clearly visible on the underside of fertile fronds (Fig. 10).

CULTURING OF *REVWATTSIA FRAGILIS*

Figures 11–14 Axenic cultures of *Revwattsia* fragilis gametophytes and young developing sporophytes.

MATERIALS AND METHODS

Two gelling agents, and four different nutrient media were tested for their efficacy in promoting initial germination of *Revwattsia* spores and for producing the best growth of sporelings to the prothallus stage over the set time period. The components of all four nutrient solutions are listed in Table 1 (Appendix).

Very early on in our experimental work, agar was found to be an unsuitable gelling agent for both the fern culture work and bryophyte culture that we are also undertaking. Consequently the testing of the media was completed with the Phytagel agent only. The nutrient media used were:

(1) Medium A: Knop's Basic Mineral Mix (Dyer 1979: 282);

(2) Medium B: modified Hatcher's nutrient media (Hatcher 1965: 230–231)

(2) Medium C: modified Moore's media for pteridophytes (after Bell, cited in Purvis *et al.* 1966: 240); and

(4) Medium D: C-Fern nutrient media (Hickok & Warne 1998: B9, Table 3).

(See Appendix for recipes for these media.)

Spores removed from surface-sterilised sporangia were inoculated onto the media in sterile plastic petri plates. Twelve replicates of each of the four media were tested. The inoculated plates were placed on an open shelf in a controlled temperature room operating at a 12/12 hour, 25/15ºC day/night regime. Light was provided by a bank of two Gro-lux light tubes. The plates were arranged randomly and numbered 1–48. The identity of the medium in each plate was disguised to reduce any bias when scoring. Spore germination and subsequent development of the prothallus were scored over a 12 week period. Observations and scoring were carried out with a dissecting microscope.

RESULTS, KEY FINDINGS AND RECOMMENDATIONS

Optimal results were obtained from two of the media tested: B (Modified Hatcher's), and C (modified Moore's). At the end of the fifth week of observations and scoring, most of the Knop's (Medium A) plates still had wholly ungerminated spore populations, while in comparison all plates of the other three media had spore populations that had achieved high germination rates. This clearly indicated that Knop's was not a suitable medium for culturing *Revwattsia fragilis* spores.

Differences in the levels of macro- and micronutrients among all four media tested are a likely explanation for the failure of Knop's medium to promote germination of *Revwattsia* spores. In comparison to the other three media tested, Knop's medium contains a high proportion of both nitrogen and calcium and lacks many of the micronutrients. Nitrogen has been found to be inhibiting to gametophyte growth in some ferns, while promoting growth in others (Fernández & Revilla, 2003).

While *Revwattsia* is already being grown commercially (a fact we did not discover until collecting in Queensland for this project) from spores straight onto potting mix in pots with apparent high germination rates, there are advantages to growing under axenic conditions on a controlled medium (such as Phytagel plus a nutrient solution). These advantages would include an initial reduction in contamination from pathogens such as bacteria and fungi, and a subsequent reduction in competition from algae, mosses and non-target fern species which are difficult to control in a glasshouse/nursery environment. Early observations of spore cultures frequently found plates which contained a single fast-growing gametophyte amongst many less-advanced individuals. When allowed to grow to the stage of producing sporophytes, these advanced gametophytes were identified as a 'weedy' fern species growing in the glasshouse where *Revwattsia* spores were sourced. This obvious difference in growth rates between weedy ferns and *Revwattsia* may be one reason why *Revwattsia* is rare in the wild. It may simply be outgrown by weedy or faster-growing competitors.

Revwattsia is slow-growing, requiring a number of years and repotting before reaching the stage of producing the large mature fronds found in wild populations. However, developing juvenile plants are also attractive, and the grower should not be disappointed as with time and patience potted juveniles will grow to maturity if correctly nurtured. It should also be noted that spore-producing fronds are produced very early in the sporophyte life cycle, so there is potential to harvest spores continuously for culturing. In this manner there is a continuous supply of plants to compensate for any mortalities.

As an epiphyte, *Revwattsia* requires good drainage—the optimal growing conditions in the glasshouse were found to be a potting mix of one part decomposed pine bark to one part small pebbles, with regular watering to promote good steady growth. A trial of growing juvenile plants outside the glasshouse environment in more temperate climates is also planned for the future.

From a conservation point of view, research should also be directed towards the long-term storage of spores. Plants can produce several fertile fronds with many thousands of spores being produced per frond, yielding potentially millions of viable spores from each plant. Information is required on how long spores remain viable and the optimal conditions under which they should be stored. Investigations should also be carried out on possible cryopreservation of spores for long term storage of germplasm.

Figures 15–16. Young plants grown from spores in axenic culture transplanted successfully into pots and grown in a heated glasshouse.

Figures 17–18. Unfurling crozier of a new *Revwattsia* frond.

ACKNOWLEDGEMENTS

This work was supported by an Australian Flora Foundation Research Grant to Dr D.C.Cargill. We would also like to thank the Centre for Plant Biodiversity Research, in particular David Jones and Jim Croft, for their support and encouragement. Thanks are also extended to the staff at the Australian National Botanic Gardens, in particular Tim Mulcahy for his horticultural expertise, observations and care of *Revwattsia* plants in cultivation. Also, much appreciation to Dr Anna Monro for reading the draft copy of this report and helping with images.

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APPENDIX

TABLE 1. Concentrations of nutrients present in media (ppm)

	Medium Type			
	Knop's (A)	Hatcher's (B)	Moore's (C)	C-Fern (D)
Macronutrient				
N	225	26.5	17.5	46.6
P	50.7	37.2	35.6	114
K	138	62.4	89.8	144
Ca	317	44.1	36.1	7.08
Mg	50.3	725	40.4	12
S	63.4	144	6.78	19.0
Micronutrient				
Fe	3.51	0.376	0.301	5.58
Cl	6.7	28.5	63.9	12.5
Mn	-	2.22	1.11	0.813
B	-	0.262	0.131	0.325
Zn	-	0.341	0.171	0.118
Mo	-	0.0297	0.014	0.0201
Cu	-	0.0191	0.0095	0.0942
Co	-	0.0340	0.0170	-
Na	-	60.0	30.0	4.61

MEDIA RECIPES

A. Knop's Basic Mineral Mix

(Dyer 1979: 282)

Stock Solutions	Concentration
A. $MgSO_4.7H_2O$	51 g l^{-1}
KNO_3	12 g l^{-1}
B. $FeCl_3.6H_2O$	1.7 g l^{-1}
C. $Ca(NO_3)_2.H2O$	144 g l^{-1}
D. KH_2PO_4*	12.5 g l^{-1}
K_2HPO_4*	12.5 g l^{-1}

Culture medium

Stock solution A	10 ml
Stock solution B	10 ml
Stock solution C	10 ml
Stock solution D	10 ml
Distilled water	960 ml
Phytagel[#]	2.50 g

*if use this version of stock D then pH 5.8 (Dyer 1979: 282)

[#] Phytagel used instead of 5–10 g agar

Mycostatin omitted

B. Modified Hatcher's media (after Hatcher 1965: 230-1)

Stock solution A (x10)

$MgSO_4.7H_2O$	7.35 g l^{-1}
$Ca(NO_3)_2.4H_2O$	2.6 g l^{-1}
Na_2SO_4	2.0 g l^{-1}
$NaH_2PO_4.H_2O$	1.65 g l^{-1}
KNO_3	0.8 g l^{-1}
KCl	0.6 g l^{-1}
Distilled water	1 l

Stock solution B (x10)

H_2SO_4	0.5 ml
$MnSO_4.\ 4H_2O$	3.0 g l^{-1}
$ZnSO_4.7H_2O$	0.50 g l^{-1}
H_3BO_3	0.50 g l^{-1}
$CuSO_4.5H_2O$	0.025 g l^{-1}-1
$Na_2MoO_4.2H_2O$	0.025 g l^{-1}
$CoCl_2$	0.025 g l^{-1}
Distilled water	1 l

Stock Solution C (x10)

$FeC_6H_5O_7.7H_2O$	0.25 g
Distilled water	100 ml

Culture Media

Stock solution A	100 ml
Stock Solution B<	3 ml
Stock solution C >	1 ml
Distilled water	896 ml
Phytagel	2.50 g

stock solution ½ concentration of original recipe
quantity used is x 3 that of original recipe
quantity used is x ¼ that of original recipe
Tryptone and sucrose omitted

C. Modified Moore's medium for Pteridophytes

(after P.R. Bell in Purvis *et al* 1966: 240)

Culture medium

NH_4NO_3	0.50 g l^{-1}
$MgSO_4.7H_2O^+$	0.41 g l^{-1}
K_2HPO_4	0.20 g l^{-1}
$CaCl_2$	0.10 g l^{-1}
Fe citrate solution~	0.8 ml
Trace element solution^	1.5 ml
Distilled water	998.7 ml
Phytagel	2.50 g

+originally as "Magnesium sulphate" so not known if hydrous/anhydrous
~ used 0.8 ml of Hatcher's stock C, which is equivalent to 2.0 ml of 0.1 g l^{-1} Fe citrate solution in the original recipe
^used 1.5 ml of Hatcher's stock B; original recipe specified 1.0–2.0 ml trace element solution
Ribose omitted

D. C-fern medium (Hickok *et al.* 2004: B9, table 3)

Macronutrients stock solution

NH_4NO_3	1.25 g l^{-1}
KH_2PO_4	5.00 g l^{-1}
$MgSO_4.7H_2O^+$	1.20 g l^{-1}
$CaCl_2$	0.26 g l^{-1}

Micronutrients stock solution

$MnSO_4.H_2O$	0.0500g l^{-1}
$CuSO_4.5H_2O$	0.0740g l^{-1}
$ZnSO_4.7H_2O$	0.1040g l^{-1}
H_3BO_3	0.372 g l^{-1}
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0074 g l^{-1}

Chelated iron solution

$FeSO_4.7H_2O$	2.78 g l^{-1}
diSodium EDTA.$2H_2O$	3.73 g l^{-1}

Culture medium

Macronutrient stock solution	100 ml
Micronutrients stock solution	5 ml
Chelated iron solution	10 ml
Distilled water	885 ml
Phytagel$^{=}$	10.00 g

= does not set with either 2.50 g l^{-1}or 5.0 g l^{-1} Phytagel