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Title:
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Date:
2016-05-01

Citation:
Kodym, A., Lang, M. & Delpratt, J. (2016). Propagation by partial tissue culture of Austral Bracken (Pteridium esculentum) for revegetation. Ecological Management and Restoration, 17 (2), pp.159-163. https://doi.org/10.1111/emr.12209.

Persistent Link:
https://hdl.handle.net/11343/291264

Propagation by partial tissue culture of Austral Bracken (*Pteridium esculentum)* for revegetation

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/emr.12209

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Acknowledgements

Thanks to Michael Garrett, Bicheno Nursery in Tasmania, for sharing his knowledge in propagation of Austral Bracken from spores and to David Carew, Melbourne Water for sharing his ideas and visions. Their input was instrumental in developing a successful acclimatisation and sporophyte development technique. Thanks to John Thomson from the RBG Sydney for providing spores and information for initial germination studies. The authors highly appreciate the support by the Fern Society of Victoria for collecting Austral Bracken spores and for providing contact details. Thanks to the staff of Melbourne Water, Fulton Hogan Ecodynamics Joint Venture and Woods Environmental Services for their co-operation in regards to field plantings and to Australian Ecosystems for running acclimatisation experiments. Thanks also go to the staff of the Conservation Biotechnology Unit of the Royal Botanic Gardens, Kew for sharing their expertise on fern propagation and encapsulation. This project was in large part funded by Melbourne Water. The project was further supported by The Frank Keenan Scholarship and The James John Woodward Prize from the University of Melbourne awarded to Mirini Lang.

Received Date : 06-Oct-2015

Revised Date : 14-Mar-2016

Accepted Date : 14-Mar-2016

Article type : Short note

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Summary

Austral Bracken (Pteridium esculentum) is a native fern common in many Australian ecosystems and is needed in large numbers for revegetation projects. The main limiting factor for the propagation of locally sourced material is spore availability. A mass propagation system was developed by combining tissue culture and nursery based systems. Spores collected over the summer months from wild populations were germinated in vitro on ½ MS medium containing 0.15% w/v activated charcoal. Gametophytes were rapidly multiplied on the same medium. In vitro sporophyte development was unreliable although sometimes prolific. However, gametophytes transferred to a pine bark potting medium with added coir, on a capillary bed in a fog house, produced sporophytes reliably. Across different seasons and populations, 75 to 100% of the gametophyte explants developed sporophytes within about nine weeks. Three hundred propagated ferns planted into two field sites within their provenance origins had a survival of 92 and 95% respectively, three or four months after planting. This report delivers a ready to use and reliable protocol for the mass propagation of bracken fern of local origin to the revegetation industry.

Key words

In vitro, spores, micropropagation, bracken fern, revegetation

Introduction

Pteridium esculentum (G. Forst.) Cockayne, commonly known as Austral Bracken, is a temperate and tropical terrestrial fern that grows in a wide range of environments. It is one of the most common plants in Australia and found in all states (Jones and Clemeshea 1980). In the greater Melbourne area, Austral Bracken is the most frequently listed plant in north-eastern Ecological Vegetation Classes (EVCs) and the third most frequent in the south-eastern EVCs (D. Robertson, pers. comm., 2013). The plant has earned a reputation as a problematic species on farms because the fronds are poisonous to livestock when ingested in large quantities (Low and Thomson 1990) and because it invades cleared, disturbed and recently burned areas (Thomson et al. 1986; Smith et al. 1994). However it plays numerous roles by providing habitat to small native animals (Cardinia Shire Council 2015), preventing erosion, suppressing exotic grasses and having a capacity to survive drought, repeated grazing and fire (Jones and Clemesha 1980, McGlone et al. 2005). This makes it important in the rehabilitation of degraded and difficult sites (e.g. floodway channels, road verges) where maintenance levels need to be kept to a minimum or water quality requires protection.

While Austral Bracken is a very common and prolific plant in its natural environment, where it mainly spreads vegetatively by rhizomes, its propagation using vegetative nursery-based systems is slow and unreliable (Australian Plants Society Maroondah Inc. 2001). Vegetative propagation requires at least 30 cm long rhizome parts with young vigorous lateral buds and an apex shoot (Jones 1987). Although sexual propagation is straightforward (M. Garrett, pers. comm., 2013) it is difficult to source sufficient spores because Austral Bracken is erratically fertile in Australia. For these reasons, a project was initiated by Melbourne Water to develop effective propagation protocols for Austral Bracken by tissue culture.

Tissue culture has been used successfully for the propagation of many commercially available ferns (Fernandez and Revilla 2003) and ferns of interest for conservation (Barnicoat et al. 2011). The in vitro propagation of Austral Bracken from Western Australia has been published recently (Willyams and Daws 2014). Although they reported that the culture of gametophytes (prothallus) was prolific, the formation of sporophytes was unreliable and highly variable between spore lines and culture vessels.

The aim of this technical trial was to develop a reliable method for producing sporophytes in Austral Bracken to allow the large scale propagation of material of local origin for revegetation.

Methods

Spore collection and germination of spores in vitro

Spores of Austral Bracken were collected from five Victorian populations: 1) Leversha Reserve (Montrose) in December 2011; 2) Bunarong Park (Frankston) in December 2011; 3) Jamieson in January 2013; 4) Kurth Kiln Regional Park (Gembrook) in May 2013; 5) Anglesea in December 2013 (see App 1 for map) In addition to these Victorian collections, spores collected at the Wakehurst Parkway, NSW in May 2001 were provided by the Royal Botanic Gardens Sydney.

Spores were sterilised in 0.5% (w/v) sodium dichloroisocyanurate (Sigma) and a few drops of Tween 20 (Sarasan et al. 2006) on a shaker for 35 min and then rinsed three times in sterile deionised water. Spores were cultured in Petri dishes with basal medium (BM). The BM was ½ MS medium (Murashige and Skoog 1962), full MS vitamins, 2% (w/v) sucrose, 0.2% (w/v) gelrite (Sigma) and 0.15% (w/v) activated charcoal. Cultures were maintained at 22 ^{o}C +/- 2 ^{o}C under 40 µmol m^{-1} s^{-1} light with a 16 h photoperiod provided by fluorescent lamps (Sylvania Grolux T8 36W).

Propagation of gametophytic tissue in vitro

After about 5 weeks the plant material was subcultured. Gametophytes with occasional small sporophytes were macerated in a vial with sterile water using a glass rod. The suspension was evenly spread across new Petri dishes containing BM. The populations were maintained as separate lines at all times and subcultured regularly.

Sporophyte development

In mid-April 2013 a trial with gametophytes of Frankston origin was set up to induce sporophytes in the fog house. Sealed Petri dishes of gametophytes were placed in the fog

house to enable acclimatisation to the changed light and temperature conditions prior to deflasking. After one week the gametophytes were carefully cleaned of the tissue culture medium in warm water and then separated into small clumps and arranged in half-tray punnets (335 x 140 x 55 mm) (App. 2 a). Two Petri dishes with gametophytes were used to plant one half-tray with 4 explants per row, 10 rows per half-tray. The explants were grown in two types of potting mix: a) general potting/coir mix (1:1 v/v) or b) perlite/coir (1:3 v/v). Three half-trays were planted per treatment. The general potting mix was 4 parts medium pine bark to 1 part coarse sand by volume. Coir (Grodan coir peat, standard medium grade) and perlite (Exfoliators Premium Perlite, Coarse grade) were both supplied by Duralite Horticultural Supplies (Braeside, Vic, Australia). Both potting mixes contained 4 g/l Green Jacket slow release fertiliser (NPK: 16.5: 4.1: 9.6) by Debco (Tyabb, Vic, Australia).

The half-trays were placed on a portable capillary bed (CapPlus Tray by Autopot Systems) and watered in. Three hours later CitroFresh spray (CitroLife of GCM Technologies Pty Ltd, Melbourne, Australia) was applied to avoid mould. Its application continued once a week for the entire trial. The fog house conditions were 90% shade, automatic fog to create 90% relative humidity, 24 °C bench heating and hand watering. After 9 weeks sporophytes identified with the naked eye were recorded. The percentage of explants growing into sporophytes was calculated for each half-tray (based on 40 explants) and then averaged over the number of replications.

After 12 weeks in the fog house the half-tray punnets were moved from the capillary system to a glasshouse with 70% shading, overhead watering and bench heating at 24 °C. The glasshouse was ventilated when the ambient air temperature reached 25 °C. After one week the sporophytes were separated as much as possible and potted into forestry tubes using the general potting/coir mix. Tubes could contain one or more sporophytes depending on the plant size and how densely clustered they were. The number of tubes potted up from each half-tray was recorded.

Testing the reliability of the new protocol

In order to evaluate how reliable the above protocol is for inducing sporophytes over different seasons and for different populations, five more trials were set up in the fog house in May,

June, July and November 2013 using gametophytes from two populations (Frankston and Gembrook). Only general potting/coir mix was used.

Growing-on in the nursery

The sporophytes coming from various acclimatisation experiments were grown in forestry tubes (50 x 50 x 120 mm, 0.2 l) and round pots (130 mm, 1l), all from Garden City Plastics (Monbulk, Vic, Australia). The general potting/coir mix (1:1 v/v) with 4 g/l Green Jacket fertiliser was used. The plants were kept in the glasshouse for two weeks before being transferred to an outdoor shade area (70% beige shade cloth overhead and 50% black shade cloth on three walls) with overhead watering.

Field plantings

Two Melbourne revegetation sites with very different site and climate conditions were chosen: Baxter and Montrose (App.1). Austral Bracken was planted on the 13th May and 25th of June 2013 respectively using material of provenance origin (Frankston and Montrose populations respectively). The plants originated from pre-trials and were hardened off in the full sun for 1-2 weeks before the field planting occurred. Two hundred plants were planted at Baxter and 100 at Montrose by professional planting crews following their routine procedures. The plants were then tagged and a location stake placed beside each plant. They were not watered in after planting.

At Baxter (App. 2b), a newly constructed pond system was revegetated. Site preparation included herbicide weed treatment. Tube stock was planted at a distance of 1.7 m to 16 m from the water's edge. No weed mats or plant guards were used. However, spraying with Garlon (active ingredient triclopyr) was carried out regularly to control broadleaf weeds. The Montrose planting (App. 2c) was along a creek in a degraded forest, which includes Austral Bracken in the understorey. Weed mats and plastic tree guards (Suregro, Dingley, Vic, Australia) were used for each plant.

At both sites, the height of each Austral Bracken was measured from the ground to the distal end of the longest green frond part. The vigour of plants was assessed on the presence of croziers. Plant assessments were repeated in spring, at the end of September 2013 (Baxter approx. 4.5 months, Montrose 3 months after planting).

Results

Spore collection and germination of spores in vitro

In all six spore collections abundant germination occurred after 1 to 2 weeks, demonstrating high viability. Gametophytes could be seen with the naked eye as early as 6 days after sowing. Spores sown more densely developed smaller gametophytes than those sown sparsely. Gametophytes were ready for subculturing after 4 to 5 weeks.

Propagation of gametophytic tissue in vitro

In all six lines, macerated gametophytes regrew quickly and covered the Petri dish uniformly in a thin layer within 4 to 5 weeks. Sporophytes could cover the whole Petri dish but generally they appeared in very low numbers or were altogether absent.

Sporophyte development

The first sporophytes developed in the fog house after about 3 weeks. After 9 weeks, the average percentage of explants producing sporophytes was 99% for both potting media (Tab. 1; 22. April).

The trial was stopped by week 13, when the sporophytes had grown into such a dense mass that mould was beginning to cause sporophyte deaths. Trays should have been removed from the fog house earlier, as sporophyte numbers started to drop. The number of tubes obtained from the small explants on a general potting/coir mix was 121±16 SE and 88.3±4.4 SE on perlite/coir. Survival of plants transferred into tubes was 100% after 2 weeks in the greenhouse.

Testing the reliability of the new protocol

The developed protocol for the induction of sporophytes of Austral Bracken proved to be highly reliable across different seasons and populations with 75 to 100% of the gametophyte explants producing sporophytes (App. 2d and Tab. 1). The sporophytes were potted on before they grew into dense stands, as they were susceptible to fungal infections and could suddenly collapse. The time in the fog house could range from 55 to 84 days and the final evaluations were carried out after 61 to 89 days, both depending on the speed of development. Plants

were ready for potting after one week in the greenhouse. The total time required from in vitro transfer to greenhouse pots ranged from 9 to 13 weeks.

Growing-on in the nursery

Pot size and shape influenced the bracken's growth. In the narrow forestry tubes, the rhizomes were soon constricted and the containers filled with fibrous roots, which made them prone to drying out. Overhead watering was often insufficient, not penetrating throughout the potting mix. In comparison, plants in the wider, round pots continued to grow vigorously.

Field Plantings

High survival was recorded after the winter in both field sites. At the Baxter site, 95% of the plants had survived. Plants that had died were often located in small depressions that had been flooded at times. At the Montrose site survival was 92%. Some plants had been partially eaten leaving only the rachis and stipe remaining. Slugs and caterpillars were observed during monitoring and were probably the cause of the damage.

Plants were generally smaller in the spring review than at the time of planting. The old fronds had died back during winter and the new growth was still small. Their average height at planting was 288 and 270 mm for Baxter and Montrose respectively, and 203 and 232 mm in September. At the time of planting, new croziers were counted on 86% of the plants at Baxter (mid-May) and 62% at Montrose (late June). In September, plants at the open, sunny Baxter site were more prolific than at the tree covered and cooler Montrose site. New crosiers were counted on 91% of plants at Baxter and 42% at Montrose.

Discussion

A reliable and robust method was developed to propagate Austral Bracken populations with low availability of spores. This overcame a serious bottleneck to producing planting material of local origin for revegetation projects. To what extent provenance origin plays a role in such a widely distributed species is unknown, but research has shown that populations differ at least in regard to cyanogenic phenotypes and the content of ptaquiloside (Low and Thomson, 1990; Smith et al. 1994). The propagation method combined a tissue culture system for spore germination and gametophyte multiplication, then a fog house capillary bed nursery environment for sporophyte development. The protocol (as summarised in Appendix 3) worked well on several populations and all year round in Melbourne, having an average 70

to 100% sporophyte development success (based on the percentage of explants producing sporophytes). In vitro derived plants were healthy and vigorous and showed 95 and 92% field survival in the first spring in a wetlands project and in a creek-side erosion control project.

Spores from five Victorian populations were collected over two years, but only one collection (Gembrook) would have provided sufficient spores for regular nursery propagation. While locating sporulating plants in the field was rare, December and January were the most likely months in Victoria for successful collections. Austral Bracken spores could be germinated, and gametophytes multiplied easily, on a plant tissue culture medium. Spores from all collections were found to be viable. Storing spores at 4 ^{o}C over a desiccant was a very efficient technique because spores stored this way were still viable after 10 years. This result showed that Austral Bracken spores are long-lived, similar to P. aquilinum spores (Dyer 1989).

When using tissue culture for the propagation of ferns, they commonly progress from the gametophyte stage to the sporophyte stage in vitro. Acclimatisation occurs when ferns are already at the sporophyte stage (Fernandez and Revilla 2003; Somer et al. 2010; Menendez et al. 2011). The pathway from gametophytes to sporophytes in vitro is influenced by a wide range of culture conditions such as the frequency of transfer, nutrient levels and the maceration of gametophytes, but can be problematic in some species (Menendez et al. 2011; Fernandez and Revilla 2003). In this study the development of sporophytes in tissue culture was unreliable, although sometimes high (data not presented). Willyams and Daws (2014) also observed that sporophyte in vitro development in Austral Bracken from Western Australia varied between vessels and spore lines.

The approach of combining rapid gametophytic growth in vitro with the induction of the sporophytic stage in the nursery is based on Knauss (1976) who worked with maidenhair fern (Adiantum tenerum) and further adapted his method to ferns that produced only gametophytes in tissue culture. In vitro grown gametophytes were macerated in a blender and distributed onto potting mix in a mist house. This relatively simple method was later employed for the Southern African tree fern Cyathea dregei (Finnie and Van Staden 1987). In Austral Bracken however, this method resulted in poor sporophyte production (data not shown) which confirms the observations by Willyams and Daws (2014). As a consequence the system was altered by using small clumps instead of blended gametophytes thereby causing less

disturbance to the gametophytes when being transferred from the Petri dishes to ex vitro conditions.

As many as 121 tubes (with multiple sporophytes) were produced in 13 weeks from two Petri dishes, despite fungal infections. This resulted from a cautious approach. When trays with sporophytes from the other trials were given to a local nursery for potting on, they reported having harvested between 230 and 500 plants per half-tray (Australian Ecosystems, pers. comm., 2013). Repeated tests showed that the time needed for a tray to be ready for potting could vary from 9 to 13 weeks. The partial tissue culture system was more productive and, most importantly, more reliable than a propagation system that relied purely on in vitro culture (Willyams and Daws 2014).

Coir, in a blend with pine bark based potting mix, provided an environmentally sustainable alternative to peat. Plants grew more vigorously in pots that had a higher water holding capacity and allowed some horizontal growth of the rhizomes than in forestry tubes (usually favoured for field planting). Willyams and Daws (2014) also found that the larger the pot, the higher sporophyte survival during greenhouse acclimatisation of in vitro plants.

This paper further reports on the first field plantings of in vitro derived Austral Bracken. Monitoring of field plantings after the first few months showed high survival and active growth.

The partial tissue culture system described here allows for the reliable large-scale propagation of Austral Bracken when spores are scarce. The in vitro propagation of gametophytes by tissue culture laboratories is very fast and cultures can easily be shipped to ferneries for further production. Because of its simple design and low cost it has potential for the commercial production of this species. Future studies could investigate the use of gametophyte beads for direct sowing as is currently being trialled in England for restoring peat land habitat (Moors for the Future Partnership, 2012).

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Tab. 1: The reliability of the partial tissue culture system to produce Austral Bracken sporophytes over different seasons and from different populations is shown here: Mean percentage of gametophyte explants producing sporophytes from eight different trials

initiated in the fog house between April and November 2013 and the days elapsed from the initiation date to their transfer to the greenhouse and the final evaluation. (Replications = half trays as depicted in Appendix 2a.)

Date initiated	Population	Replications*	%Sporophyte Mean± SE	Transfer to GH (d)	Final evaluation (d)
22. April	Frankston	3	99.3 ±0.7	84	65
22. April	Frankston	3	99.3 ±0.7	84	65
28. May	Frankston	2	92.5 ±2.5	63 - 84	63
28. May	Frankston	4	84.6 ±1.1	84	63
28. June	Frankston	5	90.5 ±5.2	81	89
10. July	Frankston	7	99.6 ±0.4	55	68
6. Nov.	Frankston	7	74.9 ±6.7	61	61
6. Nov.	Gembrook	2	83.5 ±16.5	61	61