In vitro soil-less (IVS) rooting medium

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Summary

The principle hypothesis of this thesis is that hypoxia, in agar-based media, compromises rooting in vitro. From a practical point of view this is important because most plant tissue culture activities require the material to be successfully acclimatised in a nursery environment. Compromised rooting often results in excessive losses at this stage which are costly and inconvenient. In addition, many plants with commercial and/or scientific interest remain unavailable as they are not able to be rooted and acclimatised reliably. The use of agar as a rooting medium has limited the capacity of plant tissue culture to clonally propagate many plants.

The thesis begins by demonstrating how poorly some plants respond to agar rooting media. Juvenile Chamelaucium hybrid microcuttings were pulsed with IBA 40 µM and then placed for 3 weeks on either M1 (½ MS) or aerated in vitro soil-less substrate (IVS) (Chapter 2). IVS had 42–82% rooting at the end of Stage 3 compared with 0–1% in agar. Shoot survival for IVS-rooted microcuttings was significantly greater than M1-rooted shoots. Pulsed shoots placed in IVS showed root primordia after 7 days. In contrast, shoots placed in agar showed no root primordia after 21 days and formed callus but did not root when subsequently placed in IVS for a further 4 weeks. The agar medium almost totally and permanently inhibited the capacity of competent shoots to form root primordia and roots.

The effectiveness of different types of aerated and non-aerated media, including IVS, were tested to validate the hypothesis (Chapter 3). Microcuttings from shoot cultures of two Australian plants Grevillea thelemanniana and Verticordia plumosa x Chamelaucium uncinatum were pulsed for 7 days on a high auxin (40 µM IBA), agar-solidified medium in the dark. Rooting of the microcuttings was then compared
on five experimental substrates: a) standard agar M1 medium (½ MS, no hormones, 8 g agar L\(^{-1}\)), b) porous-agar medium (½ MS, no hormones, 30 g agar L\(^{-1}\), solidified then blended to provide aeration), c) white sand wet with liquid M1, d) white sand with M1 medium containing agar, and e) IVS. A separate experiment involved flushing the IVS soil profile with low or normal oxygen. Low and variable rooting percentages were recorded on the controls on M1 medium. Root induction and average total root length per microcutting at final harvest were significantly higher using the porous media including IVS, blended agar or white sand. The M1 medium and the addition of M1 medium to sand suppressed the percentage rooting and elongation. Flushing the IVS rooting medium with low oxygen also suppressed rooting. The experiments showed that increasing the air-filled porosity of the rooting medium has a positive effect on rooting and this is most likely due to the increased oxygen at the base of the microcutting. The role of ethylene, and the sugar and nutrients in M1 were not investigated.

The efficacy of the IVS protocol on a range of Australian herbaceous and woody species was investigated to determine whether the observed benefits were generic or plant specific (Chapter 4). Improved rooting in IVS compared to agar was shown for 28 Australian species and genotypes from the families Liliaceae, Haemodoraceae, Myrtaceae, Thymelaeaceae, Proteaceae, Goodeniaceae and Rutaceae. Twenty-seven of the 28 species rooted in IVS medium at equal or better rates than in M1. In three cases — Actinodium cunninghamii, one of the Pimelea physodes genotypes and one of the Eriostemon australasius genotypes — shoots did not root in M1 but showed good root development in IVS medium. With few exceptions average root length and number in microcuttings rooted in IVS was superior to those in agar medium.
To further test the resilience of the hypothesis, it was tested on nodal microcuttings of lentil which are recalcitrant to root in vitro (Chapter 5). The veracity of a published conclusion that inverted lentil microcuttings (with their base in the air) root better because of their altered polarity was also examined. It was found that, as is the case for many species, roots initiated and grew only at the proximal end of the microcutting regardless of its orientation. When the proximal end was in agar (a hypoxic environment) the rooting percentage was low (9–25%) even when the orientation of the microcutting was altered by inverting the culture tube. In contrast, when the proximal end of the microcutting was in an aerobic environment (from the shoot being placed upside down in agar medium or placed normally or upside down in an aerated medium) rooting percentages were higher (62–100%).

Given that Stage 2 microcuttings are prepared with the objective to root and acclimatise them to nursery conditions, the duration of this activity becomes important as it can impact on plant quality and costs. The pulsing protocol and the length of time that Stage 3 cultures remain in the culture room during the rooting phase is a component of the unit cost of production of each rooted microcutting. Initially a 7-day IBA pulse was used after which the pulsed microcuttings were transferred to IVS to root. Chapter 6 shows that the pulsing period can be shortened to one day or replaced with a single auxin dip while still achieving high rooting percentages and maintaining plant quality. These materials handling improvements go some way to realising the logistical benefits of ex vitro rooting but without compromising the positive influences of hygiene and a stable environment of the in vitro environment.
The respiration rates of roots grown either in agar or IVS was investigated using microcalorimetry and gas exchange methods (Chapter 7). Calorimetric measurements were made with a Differential Scanning Calorimeter (Calorimetry Services Corporation) operated in isothermal mode at 25°C. Gas exchange measurements were made with an infrared gas analyser (Qubit S151® IRGA) connected to an AC gas pump and flow meter and the rate of CO₂ production was recorded using a Lab Pro® interface and Logger Pro® software. Metabolic heat production (q) and respiration (R_{CO2}) were measured on roots grown in hypoxic (M1) and porous (IVS) rooting environments. Gas exchange (R_{CO2}) rates were significantly higher for roots (testing the entire root mass) in IVS compared with roots in agar. Metabolic heat production (q) from excised root tips did not give meaningful results possibly because of a carry-over carbohydrate effect from the agar medium or that root tips quickly regain normal respiration rates once removed from agar during preparation for testing.

The capacity of rooted microcuttings to acquire photosynthetic competence is integral to their survival in the nursery. Gas exchange measurements were made with an infrared gas analyser (Qubit S151® IRGA) connected to an AC gas pump and flow meter and the rate of CO₂ uptake or production (R_{CO2}) was recorded using a Lab Pro® interface and Logger Pro® software. These leaves were shown to have normal shade-adapted responses (Chapter 8) in the culture room and in the nursery. Plants were grown in the culture room at PAR 50 and 110 µmol m⁻² s⁻¹ PAR and shown to develop greater photosynthetic capacity when exposed to the higher light levels.

However, Chapter 9 shows that priming of Stage 3 microcuttings with high light and passive ventilation did not increase the likelihood of survival in Stage 4. No survival
benefits or positive physical effects were shown when rooted microcuttings were
grown in three Stage 3 environments with or without passive ventilation. This is
most likely due to CO₂ being limiting. There is no compelling reason to add higher
levels of CO₂ given the observed high survival rates. As a result, rooting percentage
should be maximised in the simplest Stage 3 environment and once normal rooting
has occurred, induction of photosynthetic competence and final acclimatisation can
be done in the nursery. For species which root fast (within 14 d) the whole Stage 3
rooting and Stage 4 acclimatisation processes can occur in the nursery.

The main conclusion from this thesis is that the real rooting potential of many plant
species in vitro can not be accurately determined using agar as a rooting medium.
The consequences of using agar-rooting media for many of the plants tested, are low
and variable rooting percentages which compromise the acclimatisation process. It is
likely that, for many species, rooting data collected using agar as a rooting media is
unreliable unless the media is aerated, mediating all other influences such as
genetics, media pH, sucrose and nutrient effects, light and temperature effects, the
effects of auxin types and concentrations and the gaseous composition of the culture
vessel. The results of using IVS or a similar aerated rooting substrate are
microcuttings with normal roots capable of normal adaptive physiology required in
the nursery.

This thesis presents a strong argument on the basis of example and thorough
physiological studies that the suppressed rooting performance using agar-based
rooting media is due to hypoxia-compromised physiology. Furthermore, the thesis
demonstrates that plants rooted in IVS acclimatise normally. The applications of this
work include opportunities to clonally propagate many plants previously deemed to be recalcitrant to conventional plant tissue culture practices.
Declaration

I declare that this thesis is my own account of my research and contains as its main content work, which has not previously been submitted for a degree at any tertiary education institution.

(signed) ……………………………………………………………………………

Several chapters from this thesis have been published. In each publication, co-author McComb was a PhD supervisor and Growns assisted in selecting plants and resources. As the material has been published there is some unavoidable repetition in the text of the Introductions, Materials and methods, and Discussions.


Material from Chapter 5 has also been presented in poster form as Newell C, Growns D, McComb JA (2005) The effect of oxygen and shoot orientation for rooting lentil (*Lens culinaris* Medik) in vitro. Conference of the Australian Branch of the International Association for Plant Tissue Culture and Biotechnology – ‘Contributing to a Sustainable Future’ Bold Park Western Australia.

Acknowledgments

I’d like to start by saying that undertaking this thesis has been a wonderful life experience and the challenges and learning opportunities have been very rewarding. The process has helped me solve an ongoing problem of poor *in vitro* rooting which has dogged my career in plant tissue culture. I’ve been encouraged to learn many new skills and useful ways of thinking that have helped to integrate my overall approach to Horticultural Science. I’d like to pay my respects to all of the dedicated scientists and horticulturalists that have gone before me in this field.

To my wife Rebecca and my family, a big thanks for your support. I owe you one.

My principle supervisor Jen McComb has been a wonderful mentor and colleague and continues to play an important part in the development of my career in the field of plant tissue culture. I am extremely grateful for having had this experience and for her valued support and input into this thesis. Bernard Dell has helped sharpen my scientific rigor and his input has helped focus the conclusions of this thesis.

I am extremely grateful to my previous employer, the Department of Food and Agriculture, for their interest and support in this project, in particular Digby Growns, Terry Hill, Tony Devitt, Joe Sherrard, Deb Pett and Gerry Parlivliet. To my project team Jan Hooper, Chris McMullan and David Imre, a big thanks for your help. Many others including Clare Hobson, Anne-Marie Scott and Jashenka Vuksic provided technical support for some of the larger experiments.

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References
List of Abbreviations and terms

AFP air-filled porosity
BAP 6-benzyl-aminopurine
DW dry weight
FW fresh weight
IAA indole-3-acetic acid
IBA indole-3-butyric acid
IVS in vitro soil-less system
MS basal medium (Murashige and Skoog 1962)
M1 ½ MS (Murashige 1962) with 10 g L⁻¹ sucrose and 8 g L⁻¹ agar, with the pH adjusted to 7 prior to autoclaving
NAA α-naphthalene acetic acid
PA photoautotrophic
PAR photosynthetic active radiation
PM photomixotrophic
Pn photosynthesis
PTC plant tissue culture
RCO₂ respiration
RH relative humidity
RM40 ½ MS with 40 µM IBA, 10 g L⁻¹ sucrose, and 8 g L⁻¹ agar with the pH adjusted to 7 prior to autoclaving
S2 multiplication stage
S3 rooting stage
S3.1 auxin pulsing phase of S3
S3.2 root elongation phase of S3
S3–4 combined activity of rooting and acclimatisation
S4 acclimatisation stage
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