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Biopharming *Carica papaya* compounds with antibacterial efficacy

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Abstract

Previous studies have reported field grown *Carica papaya* leaves to have antibacterial activity. However there have been no reports on root culture of *Carica papaya* or on the efficacy of *Carica papaya* extracts on autoimmune inflammatory diseases. The current study was undertaken to test the potential use of *Carica papaya* root extracts to block the microbial triggers of autoimmune inflammatory diseases and to use metabolomics fingerprint analysis to detect anti-inflammatory compounds. *Carica papaya* roots were grown in vitro under controlled conditions. The roots were extracted with solvents of varying polarity and investigated for their ability to inhibit the growth of several bacterial triggers of autoimmune inflammatory disorders. The most promising extract was further analysed by RP-HPLC coupled to high accuracy TOF mass spectroscopy. The root extracts displayed potent inhibitory activity against the bacterial trigger of rheumatoid arthritis (*P. mirabilis*). However, no inhibition of the growth of the bacterial triggers of any other autoimmune disease was detected. The ethyl acetate, chloroform and hexane extracts were the most potent *P. mirabilis* inhibitors. The growth inhibitory bioactivity of *Carica papaya* root extracts against *Proteus* species shows their potential to block the onset of rheumatoid arthritis.

INTRODUCTION

Plants engage in “chemical ecology” through the production, accumulation and excretion of secondary metabolites (Wink 1988). Secondary metabolites play a major role in plant adaptation to their environment (Bourgaud et al, 2001). They function as a mediator for pest and pathogen defences, plant reproduction, and symbiotic plant and microbial relationships (Katare et al, 2009). Plant roots are of ecological and pharmacological interest due to their direct interaction with the biologically abundant rhizosphere (Bachmann and Kinzel 1992). A complex array of physical, biological and chemical interactions occur between plant roots and the rhizosphere, including root-insect, root-microbe, and root-root interactions (Hirsch et al, 2003).

During the last decade, increasing demand for novel secondary metabolites has seen the emergence and growth of in vitro root production systems on a global scale (Sivakumar et al, 2005; Lee and Paek 2012). An excellent case study on the use of root bioreactors for production of secondary products is the large scale culture of *Panax ginseng* for ginsenosides (Lee and Paek 2012). In vitro production provides a means for rapid and efficient production of biomass.

This research has used *Carica papaya* L. to develop a model for the in vitro production of root biomass for plants of tropical origin. Extracts of differing polarity prepared from root biomass were examined for bacterial inhibition efficacy against a range of pathogenic bacteria including; *Proteus mirabilis*, *Klebsiella pneumonia*, *Acinetobacter baylyi*, *Proteus vulgaris*, *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Experiment 1 - Optimum IBA Concentration and Duration of Exposure

Plant material used for the experiment was obtained from a tissue cultured clone [2.001] that was originally established in 1985 and maintained at Griffith University, Nathan campus. The clone was originally obtained from a yellow fleshed breeding line in Southeast Queensland.

Basal media (BM) contained high-strength DS Drew and Smith (1986) minerals supplemented with MS Murashige and Skoog (1962) growth factors, 20 gL⁻¹ sucrose, 8 gL⁻¹ agar. Various IBA concentrations (0, 5, 10, 15, 20, 25 µM) were added and the pH was adjusted to 5.65 using sodium hydroxide (NaOH) and/or hydrochloric acid (HCl). Indole-3-butyric acid (IBA)-free BM containing 10 µM riboflavin was prepared for transfers after duration of exposure to IBA had been achieved. Media was decanted into 120 mL plant tissue flasks in 20 mL aliquots. Flasks were autoclaved at 121°C for 15 min.

Roots were excised from sterile tissue cultured shoots, rinsed with deionised autoclaved water and cut into pieces 0.5-2cm in length. For each treatment a small amount of root material (< 1g) was placed onto media in replicates of 10. Cultures were incubated in a plant growth room at 27±1°C under diffused cool fluorescent light for 14 hours every 24 hours.

Varying durations of exposure (0, 3, 7, 14, 21, 28 days) were used for each IBA concentration then root material was transferred to the IBA-free BM containing riboflavin. After 28 days culture, each replicate was rated for root growth, callus growth and total growth using an ordinal five-point rating system (0-4), zero represented no-growth through to 4 representing the most growth. All data was analysed using SPSS version 22 (SPSS IBM, New York, U.S.A).

Experiment 2 - Optimum Nitrogen Composition and Explant Material

Plant material used for this experiment was obtained from adventitious roots and callus developed in experiment 1. Root material was also excised from tissue cultured shoot stock of the same clone as in experiment 1.

BM containing 10 µM IBA was modified by addition of various ratios of ammonia to nitrate (NH₄⁺:NO₃⁻) - 0:0, 100:0, 75:25, 50:50, 25:75, 0:100 (%) - using ammonium chloride and potassium nitrate (NH₄Cl:KNO₃). Each treatment contained 60 µM nitrogen and 20 µM potassium. Potassium chloride (KCl) was used to supplement treatments containing low levels of potassium nitrate (KNO₃) so that potassium levels were consistent in all treatments. All media were adjusted to pH 5.65 using NaOH and/or HCl. IBA-free BM containing 10 µM riboflavin was prepared for transfers after IBA exposure. Media were decanted into 120 mL plant tissue flasks in 20 mL aliquots. Flasks were autoclaved at 121°C for 15 min.

Roots excised from sterile tissue cultured shoots together with adventitious roots grown in experiment 1 were rinsed with deionised sterile water and cut into pieces 0.5-2cm in length. For each treatment either a small amount of root material (<1g) or callus material (1-2g) was placed onto 11 replicate flasks and weighed. Cultures were incubated in a plant growth room at 27±1°C under diffused cool fluorescent light for 14 hours every 24 hours.

After 3 days exposure to IBA all plant material was transferred to IBA-free BM containing riboflavin. Each replicate was reweighed and root growth was rated after 28 days in culture using an ordinal five-point rating system (0-4). All data was analysed using SPSS version 22 (SPSS IBM, New York, U.S.A).

Experiment 3 - Optimum Nutrient and Sucrose Concentration

Plant material used for this experiment was obtained from adventitious roots developed in experiment 1 and 2. Root material was also excised from tissue cultured shoot stock of the same phenotype as experiments 1 and 2.

KNO₃ as sole source of nitrogen and 10 µM IBA were added to both BM and modified BM that contained medium-strength DS minerals without agar. Four concentrations of sucrose (0, 20, 40, 60 gL⁻¹) were added to the media. All media were adjusted to pH 5.65 using NaOH and/or HCl. Both IBA-free BM and modified BM containing 10 µM riboflavin were prepared for transfers after IBA exposure. Media was decanted into 250 mL Erlenmeyer flasks in 100 mL aliquots. Flasks were autoclaved at 121°C for 15 min.

Roots excised from sterile tissue cultured shoots together with adventitious roots grown in experiment 1 and 2 were rinsed with deionised sterile water and cut into pieces 0.5-2cm in length. For each treatment a small amount of root material (<1g) was individually weighed before being placed into liquid media in 10 replicates. Cultures were incubated in a plant growth room on an orbital shaker (100 rpm) at 27±1°C under diffused cool fluorescent light for 14 hours every 24 hours.

After 3 days exposure to IBA all plant material was transferred to IBA-free BM and modified BM containing riboflavin. Each replicate was cultured for 28 days.

After 28 days culture, root material was filtered through a Büchner funnel using Whatman® #1 filter paper and weighed. All data was analysed using SPSS version 22 (SPSS IBM, New York, U.S.A).

Upscale to 5 litre bioreactor conditions

Plant material used for this experiment was obtained from adventitious roots developed in experiment 3.

BM containing medium-strength DS minerals, KNO₃ as the sole nitrogen source, 10 µM IBA and 30 gL⁻¹ of sucrose was prepared in liquid culture. The medium was adjusted to pH 5.65 using NaOH and/or HCl and decanted into 250 mL Erlenmeyer flasks in 100 mL aliquots. IBA-free modified BM containing 10 µM riboflavin was also prepared in 1L Schott® bottles to be used in the bioreactors after exposure to IBA. Media was autoclaved at 121°C for 15 min.

All components of the 5L balloon-type bubble bioreactor vessel were unassembled and either placed in autoclavable bags or wrapped in aluminium foil before being autoclaved at 121°C for 15 min. After cooling the bioreactor vessel was fully assembled in a laminar flow cabinet. Three replicate bioreactors were used for this experiment.

Roots grown in experiment 3 were rinsed with deionised sterile water and cut into pieces 0.5-2cm in length. A portion of root material (≈10 g) was individually weighed before being placed into 250 mL Erlenmeyer flasks containing liquid media supplemented with IBA in triplicate. Roots were incubated in a plant growth room on an orbital shaker (100 rpm) at 27±1°C under diffused cool fluorescent light over 3 days for 14 hours every 24 hours.

After 3 days exposure to IBA each portion of root material was transferred to a bioreactor containing 3L of IBA-free modified BM containing riboflavin. Air was supplied to the bioreactor through a sterile 0.22 µM Millex® syringe filter at a flow rate enough to prevent root material settling on the internal glass filter. Each of the three replicates was reweighed after 28 days culture.

Field Grown Root Material

A tissue cultured clone of the genotype used throughout this experiment was acclimatised and grown in soil in Mt Gravatt, Brisbane, Australia for a period of 6 months. Roots were excised from the plant and rinsed thoroughly in a colander under running water for an hour to remove any excess soil material from roots.

Preparation of Extracts

Material used for this experiment was obtained from adventitious roots grown in the bioreactors and from preceding experiments. Roots were also obtained from the clone grown in soil. All root material was rinsed with deionised water prior to being dried in a food dehydrator. Media was sampled from the bioreactors after 28 days culture (spent media) and used to prepare extracts. Fresh media was also used as a control for the spent media extracts.

Dried root material was ground to a powder using a coffee grinder. An amount of ≈ 1 g of powdered root material was combined with 50 mL of five different solvent treatments: water, methanol, ethyl acetate, hexane, or chloroform. The extracts were placed on an orbital shaker for 7 days at 270 rpm and 30°C. Each extract was centrifuged at 3750 rpm for 5 minutes and filtered through a 0.22 μm Millex[®] syringe filter. The solvents were evaporated in a water bath at 60°C and the remaining residue was weighed and resuspended in 10 mL of 0.5 % dimethyl sulfoxide (DSMO) prior to sonication. Each root extract was refiltered through a 0.22 μm Millex[®] syringe filter.

Extracts were also made from the spent media of the bioreactors and from the fresh media by centrifuging aliquots of 50 mL at 3750 rpm for 5 minutes then filtering through a 0.22 μm Millex[®] syringe filter. The filtered media solution was evaporated in an oven at 70°C and the remaining residue resuspended in 50 mL of five different solvent treatments: water, methanol, ethyl acetate, hexane, or chloroform. The extracts were placed on an orbital shaker for 7 days at 270 rpm and 30°C. Each extract was then centrifuged at 3750 rpm for 5 minutes and filtered through a 0.22 μm Millex[®] syringe filter. The solvents were evaporated in a water bath at 60°C and the remaining residue was weighed and resuspended in 10 mL of 0.5 % DSMO prior to sonication. Each media extract was refiltered through a 0.22 μm Millex[®] syringe filter. All extracts were stored at 4°C.

Bacterial Inhibition Efficacy Assay

Microbial strains were obtained from Griffith University, Nathan, QLD, Australia. Stock cultures were grown in nutrient broth at 30°C, subcultured, and maintained in nutrient broth at 4°C. Strains used throughout this experiment are both clinical isolates and reference strains from American Type Culture Collection (ATCC) (Rockville, USA).

Media was prepared by the addition of 28 g of Nutrient Agar (#CM0003 - Oxoid Ltd, Australia) to 1 L of deionised water. The solution was brought to boil until completely dissolved. Media was autoclaved at 121°C for 15 min. Aliquots of 20 mL of media was poured onto petri dishes inside a laminar flow cabinet and allowed to cool. Plates were sealed and stored at 4°C until use.

Bacterial inhibition efficacy of each extract was determined using a modified disc diffusion method (Cock and Mohanty 2011). Test bacteria were grown in 10 mL of fresh media until they reached a count of approximately 10^8 cells/mL assessed by direct microscopic determination. An aliquot of 100 μl of bacterial suspension was spread onto nutrient agar plates.

Each extract was tested using 6 mm sterilised filter paper discs. An aliquot of 10 μl of each extract was used to impregnate discs and allowed to dry prior to being placed onto inoculated plates. Plates were allowed to stand at 4°C for 2 hours before incubation at 30°C for 24 hours. After incubation, the diameters of the inhibition zones were measured in

millimetres. All measurements were to the closest whole millimetre. Discs impregnated with ampicillin (2 µg) and chloramphenicol (10 µg) (Oxoid Ltd, Australia) served as positive controls for bacterial inhibition activity. Discs were also impregnated with 10 µl of 0.5% DMSO for a negative control. Each bacterial inhibition assay was performed in triplicate. Extracts that displayed bioactivity in the initial screening were confirmed by replication.

The minimum inhibitory concentration (MIC) of the *Carica papaya* crude extracts that displayed bioactivity was determined using the disc diffusion method across a range of concentrations. Each extract was serially diluted 1:2 using sterile deionised water for five dilutions. An aliquot of 10 µl of each diluted extract was used to impregnate discs and allowed to dry prior to being placed onto inoculated plates in five replicates. Plates were allowed to stand at 4°C for 2 hours before incubation at 30°C for 24 hours. After the incubation period the diameters of the inhibition zones were measured to the nearest millimetres. Discs were impregnated with 10 µl of 0.5% DMSO as a negative control. Graphs of the zone of inhibition versus the natural log (Ln) of extract concentration were plotted for each extract. The formula for the exponential line of best fit was used to determine the MIC for each extract using 6 mm as the base line for the minimum inhibitory zone.

RESULTS

Optimum IBA Concentration and Duration of Exposure

There was a significant difference in root growth rating between treatment concentrations. Root growth rating was significantly greater for 5 (1.8 rating) and 10 (1.6 rating) µM IBA than those of other concentrations (Fig. 1). There was also a significant difference in root growth rating between durations of exposure. Root growth rating was significantly greater for 3 (1.6 rating) and 7 (1.3 rating) days exposure than those of other durations (Fig.2).

Optimum Explant Material and Nitrogen Composition

There was a significant difference in total growth weight between nitrogen compositions. Total growth weight was significantly greater for 50:50 (36.70% increase), 25:75 (36.57% increase), and 0:100 (87.37% increase) ratios of NH₄⁺:NO₃⁻. There was also a significant difference in total growth weight between explants material used. Total growth weight was significantly higher using root material which had an 87.37% growth increase.

Optimum Nutrient Strength and Sucrose Concentration

There was a significant difference in total growth weight between media strengths. Total growth weight was significantly greater for medium-strength (#2) DS media which had an 81.60% growth increase. There was also a significant difference in total growth weight between sucrose concentrations. Total growth weight was significantly higher for 20 (87.86% increase), 40 (85.61% increase), and 60 (74.57% increase) g/l sucrose.

Upscale to 5 Litre Bioreactor Conditions

The mean growth for the three bioreactors after 28 days culture was 3.9168 g ±0.5135 SE. The largest increase in biomass was 4.9000g (Fig. 3, 4)

The total growth increase as a percentage of initial explant weight was calculated after 4 weeks culture. Bioreactor #1 increased by 29.31 % whilst bioreactors #2 and #3 increased by 20.71 % and 26.84 % respectively.

Field Produced Root Material

Root material was produced from a clone grown in the field. A total of 9.4886 g of dried root material was harvested.

Preparation of Extracts

Crude extract concentration ranged between 6.77 mg/mL and 176.66 mg/mL.

Bacterial Inhibition Efficacy Assays

Extracts prepared from in vitro cultured roots inhibited growth in 11 of the 45 assays. Minimum inhibitory concentrations were calculated by use of exponential regression. A minimum inhibitory zone of 6 mm was used to reflect the size of the filter discs. The MIC values for the clinical strain of *P. mirabilis* ranged from 0.113 mg/mL for the field grown roots extracted in ethyl acetate, to 7.052 mg/mL for the field grown roots extracted in chloroform. The MIC values for the ATCC strain of *P. mirabilis* ranged from 0.543 mg/mL for the field grown methanol extract, to 8.085 mg/mL for the in vitro cultured roots extracted in chloroform. The MIC values for the clinical strain of *K. pneumonia* were 42.967 mg/mL for the spent media water extract and 50.277 mg/mL for the spent media methanol extract. The MIC values for extracts producing an inhibitory zone of greater than 10 mm are represented in Table 1.

DISCUSSION

Adventitious roots of *Carica papaya* L. have been isolated and proliferated in vitro. Three days exposure to a medium containing 10 μ M IBA before transfer to plant growth regulator free medium was the most effective method to initiate and proliferate *C. papaya* roots in vitro. This was consistent to tissue culture protocols used in our current laboratory (Drew 1992). This is still a lower concentration and shorter duration of exposure than for other root culture experiments on different species as reported in the literature. Other reports of IBA concentrations for in vitro root production described the use of 25 μ M IBA continuously for the duration of culture in species, including *Panax ginseng* (Oh et al, 2009), *Scopolia parviflora* (Min et al, 2007), and *Morinda citrifolia* (Ahmed et al, 2008).

Roots that are grown *in situ* require oxygen for growth and this is consistent to growth of new in vitro cultured roots away from the media into a gaseous environment. Researchers have reported the absence of root hairs on roots that grow in solidified media in vitro (Kaity et al, 2007). Although there have been reports on the duration of exposure to IBA for conventional plant propagation, the literature lacks any evidence of experiments that examined duration of exposure to IBA for in vitro root cultures.

Doughari et al, (2007) conducted the only antimicrobial study solely focused on *Carica papaya* L. roots that has been reported in the literature. They reported MIC values for acetone and methanol extracts of *C. papaya* root material at 150 mg/mL and 100 mg/mL respectively when tested against a clinical isolate strain of *Proteus mirabilis*. The authors also reported growth inhibition of *Pseudomonas aeruginosa* by acetone and methanol extracts at MIC values of 150 mg/mL and 100 mg/mL respectively. These results in contrast to those obtained in this experiment. Methanol extracts prepared from both in vitro cultured roots and field grown roots did not inhibit the clinical isolate strain of *P. mirabilis*, nor did they display a zone of inhibition for either the clinical strain or the ATCC strain (#39324) of *P. aeruginosa*. However, the methanol extract prepared from the field grown roots did display a zone of inhibition greater than 10 mm at a concentration of 26.70 mg/mL when tested against the ATCC strain (#33044) of *P. mirabilis*. There were however, differences in the preparation of extracts and the assay methods used in this experiment compared to that of Doughari et al, (2007).

The optimum growth conditions that have been described have enabled the in vitro cultured roots to maintain production of their phytoalexins, which are plant defence compounds produced in response to infection or stress and are commonly associated with antimicrobial properties (Ahuja et al, 2012; Jeandet et al, 2013). As the in vitro cultured roots were produced in a sterile environment this suggested that either; 1) *Carica papaya* L. roots produced some antimicrobial phytoalexins without the trigger of infection, or 2) the conditions under which the in vitro roots were cultured has induced enough stress to produce a phytoalexin response. In vitro culture of plant tissue is considered to be under stress at all stages of culture.

This study has identified a method to produce bioactive compounds from *Carica papaya* L. roots under sterile laboratory conditions. This method has enabled rapid production of compounds that have displayed bioactive inhibition equal to, and in the case of the clinical isolate strain of *Proteus mirabilis*, exceeding that of roots grown conventionally in the field.

Adventitious root culture offers a non-destructive, environmentally friendly, energy efficient and sterile method for the production of plant secondary metabolites localised in plant roots. This technique offers researchers the opportunity to explore plant root exudates by examining both the culture medium and root extracts for novel compounds. Plant organelle culture technology provides a promising method of rapid production of valuable natural plant compounds which would otherwise be obtained through laborious, time consuming methods.

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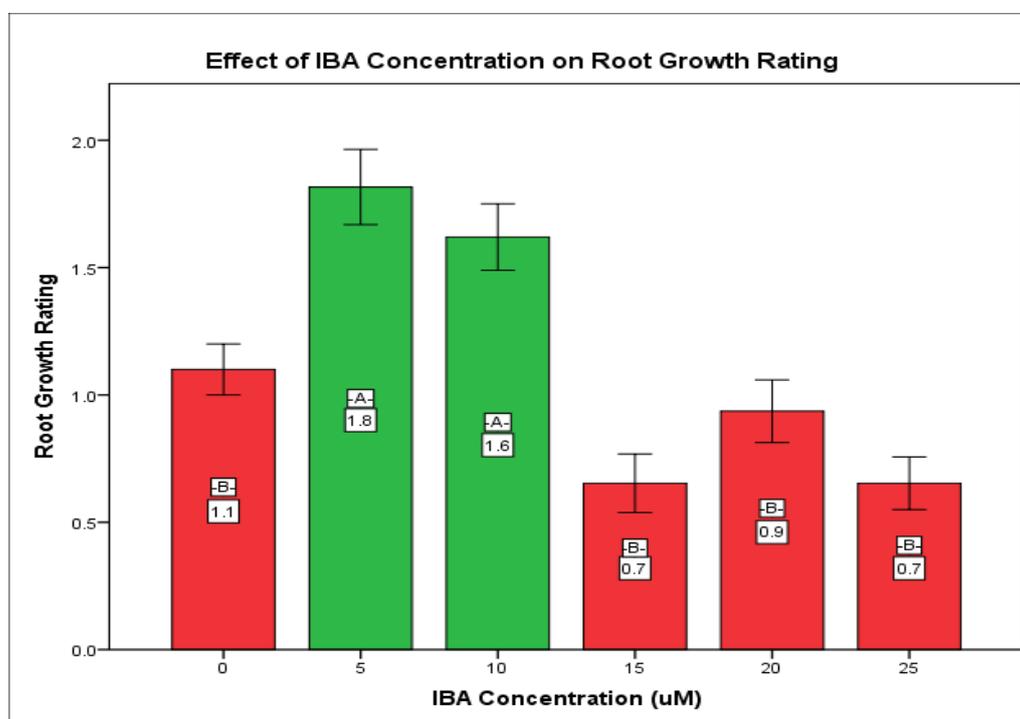


Figure 1 Effect of IBA concentration on mean root growth. Different letters above values represent a significant difference between treatments using Tukey HSD at $p < 0.05$. Error bars are \pm SE. Data are means of 10 replicates.

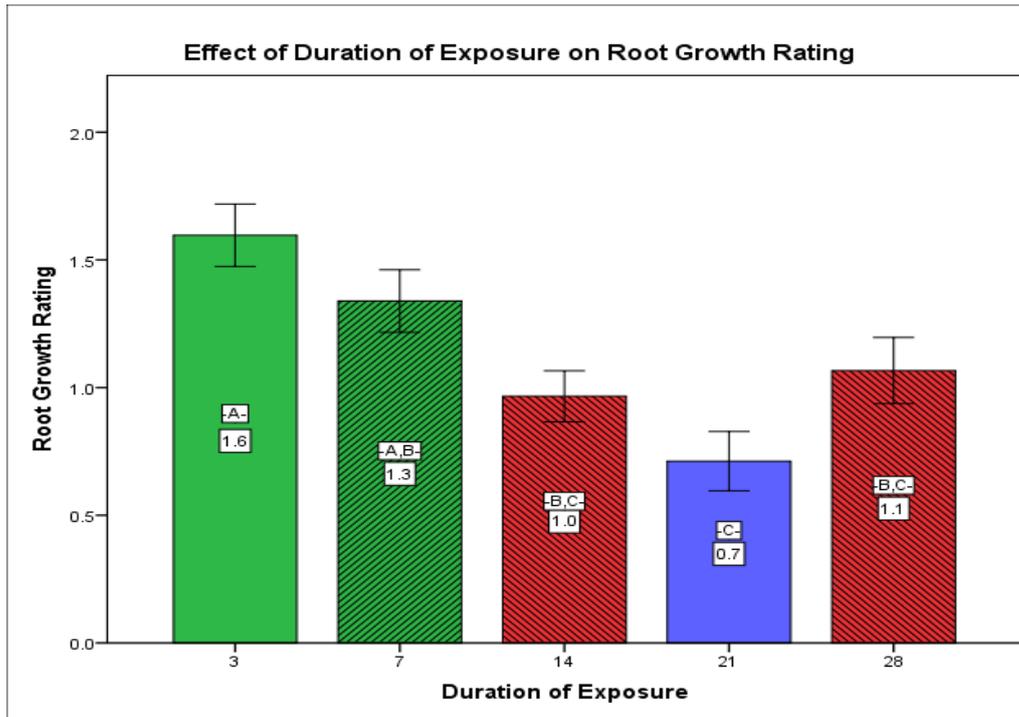


Figure 2 Effect of duration of exposure to IBA on mean root growth. Different letters above values represent a statistically significant difference between treatments using Tukey HSD at $p < 0.05$. Error bars are \pm SE. Data are means of 10 replicates.

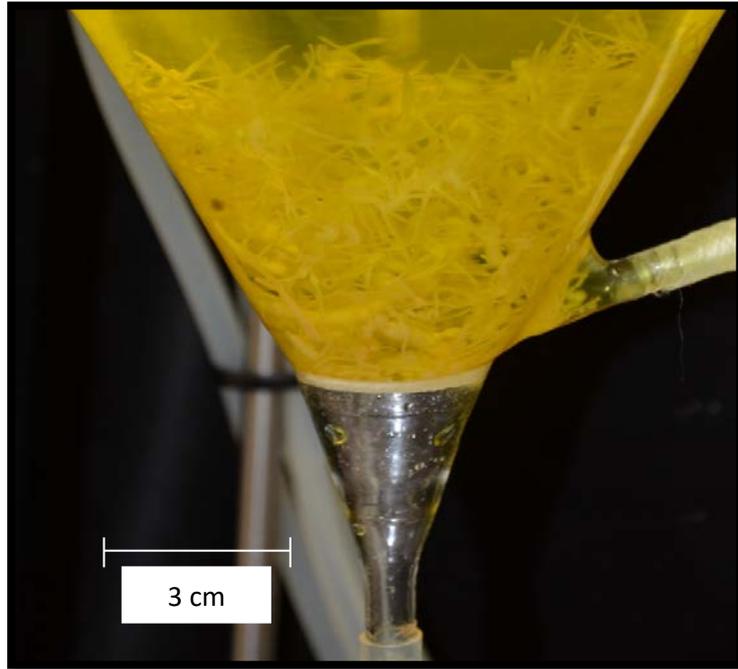


Figure 3 A close-up of settled root material prior to harvest after 28 days culture by Nathan Jamieson.



Figure 4 Total growth of root material after 28 days in bioreactor culture by Nathan Jamieson.

Table 1 Minimum inhibitory concentration (MIC) values for extracts that displayed a zone of inhibition greater than 10 mm when exposed to crude extract.

<i>Extract Source</i>	<i>Solvent Used</i>	<i>Bacterium</i>	<i>MIC (mg/mL)</i>
In vitro cultured roots	ethyl acetate	P. mirabilis	0.273 ^a
	chloroform	P. mirabilis	0.991 ^a
	chloroform	P. mirabilis – ATCC	8.085
Field grown roots	methanol	P. mirabilis – ATCC	0.543 ^a
	ethyl acetate	P. mirabilis	0.113 ^a
	ethyl acetate	P. mirabilis – ATCC	0.853 ^a
	chloroform	P. mirabilis	7.052
Spent Media	water	K. pneumonia	42.967
	methanol	K. pneumonia	50.277
	ethyl acetate	P. mirabilis – ATCC	2.608 ^b
^a Indicates concentrations considered of high-bioactivity according to standardised laboratory activity levels <1mg/mL.			
^b Indicates concentrations considered of moderate-bioactivity according to standardised laboratory activity levels <5mg/mL.			