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Selection of microbes for control of Rhizoctonia root rot on wheat using a high throughput pathosystem

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One sentence summary: A 3-phase *in planta* pathosystem was used to screen large numbers of plant-associated microbes resulting in the selection of candidate strains for control of Rhizoctonia root rot on wheat.

Key words: actinobacteria, bacteria, fungi, screening, *Rhizoctonia solani*, wheat *Triticum aestivum*

Running title: Selection of microbes for Rhizoctonia control

Abstract

The promise of microbial biological control of soilborne fungal pathogens of crops has yet to be fully realised with only a few strains commercialised and available to growers. One bottleneck is the availability of suitable methods to screen microorganisms for disease control efficacy relevant to controlling disease in the field. A 3-phase *in planta* pathosystem containing field soil was developed to screen 2,310 microorganisms for control of Rhizoctonia root rot on wheat. Test strains were added to seeds as a suspension at planting and plant growth assessed at two weeks. Strains increasing plant height and number of roots (185) were tested in a replicated Rhizoctonia pot bioassay with five wheat seedlings grown for four weeks and assessed for plant growth and root disease. Forty three strains (1.9% of strains tested) performed better than our benchmark strains and were reassessed in pot bioassays at three inoculation levels. These tested strains represented a wide diversity of microbial genotypes including fungi, (*Trichoderma*, *Aspergillus* and *Cylindrocarpon*) and bacteria encompassing four phyla (Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes). These results show that microbes can be successfully and rapidly screened directly for disease control on plants.

1. Introduction

The soilborne fungus *Rhizoctonia solani* (Kühn) is an important pathogen on many crops worldwide (Anees *et al.* 2010). *R. solani* AG8 is the most economically important root disease in southern Australia's dryland cropping systems, causing an annual loss of up to $77 million in yield in wheat and barley (Murray & Brennan, 2009a, Murray & Brennan, 2009b) and is also important in the Pacific northwest of the USA (Paulitz *et al.*, 2002, Weller *et al.*, 1986). In this work we use the term Rhizoctonia to refer to disease caused by *R. solani* AG8 on wheat unless otherwise indicated. *R. solani* AG8 causes root rot and stunting of seedlings resulting in reduced ability to access water and nutrients (Paulitz *et al.*, 2002). Rhizoctonia is difficult to control because it has a wide range of hosts used as rotation crops in cereal cropping systems (Cook *et al.*, 2002) and no genetic resistance is currently available to cereal growers although synthetic wheat lines are being developed (Mahoney *et al.* 2016, Okubara *et al.* 2009). Registered chemical controls with fungicides on wheat are now currently available in Australia, however their affect is limited (Bogacki *et al.* 2014). Rhizoctonia is also increased in direct-drill or minimal tillage and stubble retention farming systems and is a

significant constraint to the uptake of these practices (Paulitz *et al.*, 2002, Pumphrey *et al.*, 1987, Rovira, 1986).

The development of Rhizoctonia root rot is influenced by other soil microorganisms, and examples of microbial disease suppression have been reported for cereals in Australia (Barnett *et al.*, 2006, Roget, 1995), the USA (Mavrodi *et al.*, 2012a, Schillinger & Paulitz, 2006) and in sugar beet (Mendes *et al.*, 2011). Microbes have also been isolated and shown to be able to reduce Rhizoctonia-induced disease caused by *R. solani* AG8 and other anastomosis groups since 1971 (Broadbent *et al.*, 1971) with more recent work concentrating on developing strains as inoculants for wheat (Dua & Sindhu, 2012, Mavrodi *et al.*, 2012b, Yin *et al.*, 2013), lettuce and sugar beet (Faltin *et al.*, 2004) and potato (Grosch *et al.*, 2005). The potential for biocontrol agents to be commercially viable inoculants for controlling disease has been well documented (Berg, 2009, Dutta & Podile, 2010) and there are increasing social and economic drivers for the use of these agents (Bailey *et al.*, 2010). There are however numerous obstacles in the development of biocontrol agents for commercial use (Fravel, 2005, Köhl *et al.*, 2011, Schisler & Slininger, 1997). In order to be developed successfully into a commercial inoculant, a microorganism must have a number of characteristics, the paramount one being efficacy in the field. Other traits include survival during storage and when applied to seed, cost effective growth characteristics and ability to be formulated as a commercial product. Seed coating is currently the most likely application route in southern Australia until in-furrow liquid applications become more widely adopted by growers. Inoculants must also be compatible with the agrochemicals used and management practices applied (Fravel, 2005, Köhl *et al.*, 2011, Schisler & Slininger, 1997).

Schisler & Slininger (1997) succinctly state three common features of selection strategies for putative biocontrol agents that hamper the development of inoculant products: "(a) relatively few candidate microorganisms are tested; (b) microbes are selected based on the results of an assay that does not replicate field conditions; and (c) the amenability of microbes to commercial development is excluded as a selection criterion". Köhl *et al.* (2011) proposed a detailed screening program that uses a stepwise approach to screening for strains that possess all the features required for an inoculant. It is based on using the lowest cost screens for the largest number of organisms, progressively narrowing down the number of strains as costs for each progressively complex screen increases. The importance of screening for efficacy in a system replicating field conditions has been well documented (Campbell, 1994, Dunlap *et al.*, 2013, Knudsen *et al.*, 1997, Köhl *et al.*, 2011, Schisler & Slininger, 1997). However, because field-relevant bioassays can be costly and time consuming if

screening large numbers of organisms, many research groups still use *in vitro* inhibition assays as an initial step to screen for biocontrol agents (Dua & Sindhu, 2012, Mavrodi *et al.*, 2012b).

Screening efficacy can also be enhanced by using a combination of features in the initial process of isolating microorganisms to obtain isolates of interest. For instance, the diversity of isolates to be selected from can be increased by using different plant genotypes (Garbeva *et al.*, 2006, Kaewkla & Franco, 2013), isolating from multiple small sample sizes (Ranjard *et al.*, 2003) or from diseased plants (Barnett *et al.*, 1999). As well, chitinase producers can be selected for on baker's yeast agar (Christensen & Cook, 1978), biofilm producers can be selected for by the method of Fall *et al.* (2004) and heat treatment can be applied to samples to select heat resistant isolates (Kim *et al.*, 1997).

Due to the costs of bioassays, Köhl *et al.* (2011) placed screening for efficacy at step five of nine. We feel that efficacy in disease control *in planta* is of primary importance and should be tested for at an earlier stage if high throughput plant-pathogen-soil bioassays could be designed to minimize the time and cost for screening unknown isolates.

This paper reports the result arising from the use of a wheat-Rhizoctonia-field soil tube bioassay system designed for high throughput screening of newly isolated strains and strains from a culture collection for control of Rhizoctonia root rot on wheat.

2. Materials and Methods

2.1. Overview of screening procedure

Microbial strains were screened for control of Rhizoctonia root rot on wheat in a stepwise procedure (Figure 1). Strains were first assessed for disease control in a primary tube soil-pathogen-two week seedling bioassay, (see Section 2.7). Selected isolates that increased shoot and root growth were then evaluated in a more rigorous secondary soil-pathogen-four week seedling pot bioassay to confirm disease control efficacy (see Section 2.8). Isolates that reduced mean root disease or increased root or shoot dry weight to a similar or greater degree than our current best strains were assessed in a tertiary pot bioassay at different inoculum levels to assess required amount of inoculum for efficacy (Section 2.9). Strains that were rated as having efficacy in the above screens were identified to genus and characterised for survival on wheat seeds and for *in vitro* pathogen inhibition.

2.2. Fungal pathogen

The pathogen *Rhizoctonia solani* AG8 strain W19 used in all assays was isolated from diseased wheat roots from Waikerie GS soil (Table 1) on Ko and Hora (1971) medium and purified by subculturing to quarter strength potato dextrose agar (PDA/4, 6 g L^{-1} potato dextrose broth, Difco, 15 g L^{-1} agar, Becton Dickson). Strain W19 was identified as *R. solani* AG8 by SARDI Root Disease Testing Service (Ophel-Keller *et al.*, 1999). Strain W19 infects and causes disease on all crop plants tested, including wheat (bread and durum), barley, oats, triticale, peas, vetch, sub-clover, medic, canola and cotton. Strain W19 was added to bioassays as colonized millet seeds, prepared by incubating 20 g sterilised (autoclaved twice for 30 min at 121^{o}C) moist white millet seed with strain W19 in 500 ml polycarbonate tubs for two weeks at 25^{o}C. Colonised millet seed was dried aseptically and stored at -20^{o}C. *R. solani* W19 colonised millet seeds were added to pots within two hours of removal from -20^{o}C storage.

2.3. Source of biocontrol strains

Isolates screened for Rhizoctonia control came from two sources, (1) a culture collection of spore forming endophytic filamentous actinobacteria, referred to in this work as actinobacteria, from Flinders University (F strains F1, F2, etc), and (2) newly isolated strains from wheat roots and held in culture collections at the South Australian Research and Development Institute (SARDI, S strains S1, S2, etc). The endophytic actinobacteria are regarded as a good source of reliable biocontrol agents (Coombs & Franco, 2003, Franco *et al.*, 2007, Kaewkla *et al.*, 2013) and the collection was readily available. Isolation is discussed in detail below. Newly isolated strains were cultured on Baker's yeast agar (BYA, Christensen & Cook, 1978) containing baker's yeast, 4 g L^{-1} (Allied Mills, Rhodes, NSW, Australia) and Agar, 15 g L^{-1} (Amresco) for five days for non-filamentous bacteria or for 14 days for filamentous actinobacteria and fungi. Culture collection actinobacteria were cultured for 14 days on mannitol soy agar, (MS, 20 g L^{-1} mannitol, 20 g L^{-1} soya flour, 20 g L^{-1} Agar, Oxoid). All cultures were grown at 25^{o}C to 27^{o}C in the dark.

Our previous best performing biocontrol strains against Rhizoctonia root rot on wheat were used as positive 'benchmark' controls for tube and pot bioassays. *Streptomyces* strain EN16 (Coombs and Franco, 2003, Franco *et al.* 2007) was used when culture collection actinobacterial strains were assessed, or *Trichoderma* strain TB (Barnett, 2005) isolated from

wheat roots from Avon suppressive soil (Table 1, Roget 1995) when new bacterial and fungal strains were assessed.

2.4. Isolation of new strains

The strains designated as "new" were isolated from roots of cereal plants grown in one of five soils described in Table 1. Soils were collected from the top 10 cm following a wheat crop, sieved to less than two mm in a hand sieve and air dried and stored room temperature for one to two years prior to use. Soils were either untreated, heat treated at 80^{o}C for one hour to select for heat resistant organisms, or with addition of chitosan 10 g kg^{-1} (Sigma) (except for Netherton and Mudamuckla soils), and 300 g (dry weight) potted up in 300 ml non-draining pots at 60% water holding capacity. Three *R. solani* W19-infested millet seeds were placed in the center of each pot to encourage the proliferation of suppressive microbes on diseased roots (Barnett *et al*. 1999). Pots were planted with 10-15 seeds of bread wheat (*Triticum aestivum*) cultivar H45, durum wheat (*T. durum*) cultivar Yallaroi or Triticale (x. *Triticosecale*) cultivar Tahara. These cultivars were chosen as they had previously been shown to host a more suppressive microflora compared to five other wheat cultivars (Barnett, unpublished data). Seedlings were grown for three weeks at 15^{o}C in a controlled environment room with 12 hour day/night cycle. Shoots were removed and the soil air dried for one week then repotted as before to bioamplify wheat root-associated microflora. *R. solani* W19 was added at each replanting. The 80^{o}C treated soil was re-heated before every replanting. Chitosan was only added at the first planting.

Roots were either processed after air drying for one week to allow spores to form when targeting heat resistant strains or from fresh nine day old roots when targeting fungi or non-spore forming isolates. When isolating heat resistant strains, samples were heated to 80^{o}C for 30 min (Kim *et al*., 1997).

Strains were isolated from well washed root pieces, from macerated roots and from rhizosheath soil (soil closely adhering to roots). Isolation from root pieces was modified from the method of Fall *et al.* (2004) for isolating organisms forming biofilms and tightly adhering to roots. Roots were removed from pots, washed five times under high pressure spray in a kitchen sieve, five seconds at a time with turning of roots between sprays then rinsing in sterile reverse osmosis (RO) water, blotted dry with sterile tissues and placed in a sterile two ml tube. Roots were then rinsed three times in one ml phosphate buffered saline (PBS) with vortexing for 15 sec and blotting dry between rinsing with sterile tissues and transferring to

new sterile two ml tube. Washed roots were then cut into five mm lengths and four sections placed onto one agar plate.

When isolating from macerated roots, two to five mg of root was washed free of soil in sterile RO water, blotted dry with sterile tissues, macerated in a sterile mortar and pestle and 10-fold dilutions made in PBS and 0.1 ml spread plated onto each agar plate.

When isolating from rhizosheath soil, roots were shaken free of loose soil leaving only soil tightly adhering to roots and two cm root segments were placed in one ml PBS, vortexed, shaken for 30 min on orbital shaker (Ratex) at 200 rpm then 10-fold dilutions plated onto agar media as above.

Most isolation was on BYA, based on the medium of Christensen & Cook (1978) to detect chitinase producers. For plating of root pieces, agar was added at 10 g L^{-1} (BYA1% to identify biofilm formers, Fall *et al.*, 2004). Selective agents were used to select for particular groups. Cycloheximide (100 mg L^{-1}) was added to all bacteria selective media to inhibit fungal growth. Streptomycin 25 mg L^{-1} with penicillin G 20 mg L^{-1} (BYAsp) or crystal violet 5 mg L^{-1} (BYAcv, Elliot & Des Jardine 1999) were added to select for Gram negative bacteria. Colistin 10 mg L^{-1} (BYAc) or polymyxin B 32 mg L^{-1} and nalidixic acid 10 mg L^{-1} (BYApn) were added to select for Gram positive bacteria. All selective agents were sourced from Sigma-Aldrich.

Fungi were selected using a general fungal medium, BYA with streptomycin 50 mg L^{-1}, chloramphenicol 250 mg L^{-1} and metalaxyl 80 mg L^{-1} (BYAscm) and a *Trichoderma* selective medium using BYA with the selective agents used by Yang *et al.* (2005) in their *Trichoderma* selective medium (BYA-TSM) based on the medium of Elad and Chet (1983). Triton X-100 (Sigma) was used in all fungal media at 0.1% to limit the radial growth of fungal colonies.

All isolation plates were incubated at 25^{o}C. Plates with non-filamentous bacteria were grown for six to seven days, filamentous actinobacteria and fungi were grown for 14 days to allow spores to form.

Strains were selected for bioassay assessment based on evidence of rapid growth (greater than two mm after five days) or sporulation on isolation medium in order to eliminate slow growers and satisfying one or more of the following criteria: produced clearing zone on BYA indicating chitinase production, were mucoid indicating exopolysaccharide production (Amallal *et al*., 1998), were spreading indicating biofilm formation or were of a different morphology compared to other colonies on the plate.

Selection of microbes for Rhizoctonia control

2.5. Isolation of culture collection actinobacteria

The culture collection of actinobacteria used in these experiments were isolated as endophytes of crop plants and trees using the protocols described by Coombs and Franco (2003) and Kaewkla and Franco (2013) and from soil on humic acid vitamin agar (Hayakawa & Nonomura 1987). In general, cultures that sporulated within 10 days were selected to accommodate the need for high spore yield in subsequent commercialisation processes.

2.6. Enumeration of cell number

Enumeration of microbes in suspensions was carried out using a one in 10 dilution series in PBS and plating onto agar media using the drop plate method (four dilutions of three 20 µl spots, Chen *et al.*, 2003). Media were 1/10 strength Tryptic soy Agar (TSA/10, 3 g L^{-1} tryptic soy broth, Difco, 15 g L^{-1} agar, Amresco) for bacteria, ½ strength potato dextrose agar (HPDA, 19.5 g L^{-1} potato dextrose agar, Oxoid, 7.5 g L^{-1} agar, Oxoid) for Actinobacteria or ¼ strength potato dextrose agar (PDA/4, 6 g L^{-1} potato dextrose broth, Difco, 15 g L^{-1} agar, Amresco) for fungi. Two replicate dilution series were carried out for each suspension.

To enumerate microbes on seeds, five wheat seeds, were placed in a two ml tube, one ml PBS added, shaken on vortex mixer for 10 s at 3,000 rpm and shaken for 15 minutes at 200 rpm on an orbital mixer (Ratex) to remove cells. A one in 10 dilution series was plated onto media as described above, with two replicate extractions and dilution series for each sample. All cultures were incubated at 25°C in the dark. Bacterial colonies were counted after four days, fungal colonies were counted after seven days.

2.7. Primary high throughput Rhizoctonia *tube bioassay*

The primary assay to assess for biocontrol of Rhizoctonia root rot was carried out in sterile transparent polypropylene 50 ml centrifuge tubes (Sarstedt) containing field soil, pathogen, wheat seedlings and test isolate. Netherton soil (Table 1) was used at 9% moisture content (60% water holding capacity) with 45g added to each tube. Two *R. solani* W19-infested millet seed were added followed by 10 g soil and tamped down with a formed wooden tool to form a conical indentation (10 mm diam. at the soil surface, 8 mm deep) for later seeding. Each rack of 24 tubes was covered in aluminium foil and two racks placed in a sealable clear plastic tub (35 x 27 x 18 cm) to minimise evaporation. Tubes were incubated for two weeks at 15°C to allow *R. solani* W19 to colonize the soil (Roget, 1995, Barnett *et al.*, 2006). Wheat cultivar Yitpi was used for all bioassays. Seeds were germinated on a

sterile filter paper moistened with RO water for 24 hr and two seeds with an emerged radicle planted into the conical indentation in the soil. Seeds were not surface sterilised to replicate farmer practice. An inoculum suspension (150 µl) of the test organism or ½ strength PBS (PBS/2) was added to seeds, five gram soil added, tamped and soil covered with sterile alkathene beads (five mm diam. Poly Products, Australia) to reduce evaporation. Two ml of RO water was added to each tube after one wk. Plants were grown for two weeks at 15^{o}C, 12 h day/night and then assessed as described below.

For newly isolated strains, microbial suspensions were prepared in 96 well plates, with row A reserved for controls (either PBS/2 for uninoculated no added pathogen and pathogen added controls, and *Trichoderma* strain TB or *Streptomyces* strain EN16 for positive controls) leaving a 7x12 array for 84 test strains. One 10 µl loop was taken from the edge of a bacteria colony or spores taken from the centre of filamentous actinobacteria and fungi and suspended in 235 µl of 15% glycerol. Using a multi-channel pipette a one in 10 dilution was made in PBS/2 in a new plate for inoculation into the tube assay. The original glycerol plate was stored at -80^{o}C. For each tube, 150 µl of suspension was added to the two germinated seeds in the conical depression. There were two replicate tubes for each of the 84 strains plus eight tubes without added pathogen or microbial inoculum (no-pathogen control), eight tubes with *R. solani* only added (pathogen control) and eight tubes with either strain TB or EN16 (positive controls).

For inoculation of actinobacteria, spores from 14 d old cultures growing on MS agar were resuspended in PBS/2 at an absorbance at 550 nm (A_{550}) of 0.8 and 150 µl of suspension was added to the two germinated seeds as described above.

After two weeks growth, the height of each plant was measured from soil surface to tip of longest leaf and the number of roots visible at the bottom of the tube recorded before removal of the plants. Strains were selected for the next assay based on an increase in plant height and/or increased number of roots compared to disease control treatments in both replicate tubes.

2.8. Secondary Rhizoctonia *pot bioassay to confirm efficacy*

Candidate strains selected from the primary tube assay were tested in a pot assay to confirm disease control efficacy. Each secondary assay comprised 15 bio-control strains and three control treatments: no pathogen or microbial inoculum (no-pathogen control); *R. solani* only added (pathogen control); and positive bio-control strains TB or EN16. Waikerie GS soil

(Table 1) containing background levels of *R. solani* AG8 and conducive to disease expression was used for the assay. Soil was used at 8% moisture (60% water holding capacity). Soil was added to 300 ml non-draining plastic pots in two layers; 150 g soil, followed by the addition of two W19-infested millet seeds, followed by another 150 g soil. Each pot was covered with an opaque plastic lid and incubated at 15^{o}C for 2 weeks. Seven wheat seeds were planted per pot, covered with 24 g soil and alkathene beads added to reduce evaporation. Seeds were thinned to five seedlings per pot after emergence. Plants were grown in a controlled environment room at 15^{o}C, 12 h day/night cycle for four weeks. Pots were watered as needed to their original weight. There were four replicate pots per treatment arranged in a randomised complete block design.

For inoculation, two 10 µl loopfulls of bacterial cells or spores were suspended in 1.6 ml PBS, and diluted to an absorbance at 550 nm of 0.5 in 1/10 strength 'sticker' solution (0.05 g L^{-1} Na-alginate, Sigma; 0.3 g L^{-1} xanthan gum, Sigma) to a final volume of three ml. Twenty microlitre aliquots of suspension were taken to enumerate cells as described previously. Wheat seeds, 2.2 g of cultivar Yitpi were added to each tube of microbial suspension, gently mixed by inversion and soaked for one to two hours prior to use. Surplus microbial suspensions were drained from seeds and the seeds planted into the previously prepared pots.

Seedlings were assessed for disease and plant growth after four weeks. Roots were washed free of soil in running tap water and rated for disease severity on a 0 to 5 scale of root symptoms (Roget, 1995), where 0 = no disease and 5 = severe disease with all roots truncated close to the crown. Total length of seminal and crown roots per plant were measured by ruler. Shoots and roots were dried for four days at 60^{o}C and weighed. Strains showing similar or better disease control (mean root disease reduced, mean root or shoot dry weight increased) than positive control strain TB or EN16 were selected for further study.

2.9. Tertiary Rhizoctonia *pot bioassay to optimise effective cell number*

Candidate strains selected from the secondary bioassay were assessed at three different inoculation rates based on previous estimation of cfu ml^{-1} in the secondary bioassay to give a range between 10^4 and 10^6 cfu seed^{-1}. Five strains plus the three controls were assessed in each assay. Pot bioassays, microbial inoculations and assessments were carried out the same as for the secondary assay. Microbial populations in suspensions and on seeds were enumerated as described previously.

2.10. Identification of strains

Newly isolated bacteria were identified by partial sequencing of the 16S rRNA gene using primers 27F, AGAGTTTGATCMTGGCTCAG (Lane, 1991) and 907R, CCGTCAATTCCTTTRAGTTT (Muyzer *et al.*, 1995). Fungi were tentatively identified by partial sequencing of the ITS region using primers ITS-1F, CCTGGTCATTTAGAGGAAGTAA (Gardes & Bruns, 1993) and ITS-4, TCCTCCGCTTATTGATATGC (White *et al.*, 1990). For each strain, two replicate extractions and PCR reactions were carried out and products sequenced in both directions by Australian Genome Research Facility Ltd. Bacterial sequences were compared to type strains in Ribosomal Data Base 10 (RDP, http://rdp.cme.msu.edu/index.jsp). Fungal sequences were compared to National Centre for Biotechnology Information (NCBI) data base (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *Trichoderma* strains were identified to genus based on morphological features according to Rifai, 1969.

For the culture collection of actinobacteria, the 16S rRNA gene was PCR amplified separately in two segments using the primer pairs 27f and 765r (R1 amplicon) and the pair 704f and 1492r (R2 amplicon) (Coombs and Franco, 2003). The resultant sequences were compared to an online database using the BLAST algorithm at the NCBI website (www.ncbi.nlm.nih.gov). The standard blastn (nucleotide-nucleotide) algorithm was used with the default settings.

2.11. Survival of strains on seeds

Survival of candidate strains on wheat seeds was assessed following the inoculation of 20 g batches of wheat cultivar Yitpi. For each strain, 0.6 ml microbial suspension was added to 1.26 ml of full strength sticker solution (0.5 g L^{-1} Na-alginate, Sigma; 3.0 g L^{-1} xanthan gum, Sigma) and 18 µl Pillar Box Red food dye (Queen Fine food Pty. Ltd. Alderly, Qld. Australia). After mixing, 0.626 µl was added to each of two replicate lots of 20 g seeds in zip lock bags and mixed until the seeds were evenly coated. Addition of the red food dye aided visualisation of seed coverage by the inoculum suspension. After one hour, five seeds were taken for enumeration of colony forming units (t=0). Seeds were again sampled after one, two and seven days storage at room temperature (~20^{o}C). The concentration of cells on seeds was determined as described previously and percent survival of strains on seeds was calculated by the formula [(cfu $seed^{-1}$ at t=1, 2 or 7 d)/(cfu $seed^{-1}$ t=0)]x100.

2.12. In vitro *inhibition*

Ten newly isolated strains selected based on good disease control in pot assays and greater than 20% survival on seeds after one week, and 18 culture collection actinobacteria were assessed for *in vitro* inhibition of three fungal pathogens, *R. solani* AG8 strain W19, *Gaeumannomyces graminis* var. *tritici* (Walker) strain C3 isolated from wheat roots and *Fusarium pseudograminearum* (Aoki & O'Donnell) strain B4a isolated from wheat crowns and one oomycete root pathogen, *Pythium irregulare* (Buisman) strain 89 isolated from lucerne roots. These pathogens were selected as they are the most important root pathogens in terms of yield loss in southern Australia (Murray & Brennan, 2009a) and have previously been shown to cause disease on wheat. Fungi and *Pythium* were grown on PDA/4 for between two and seven days prior to use depending on strain. *In vitro* assays were performed on a medium containing TSA/10 + PDA/4 to facilitate growth of both bacteria and fungi.

The pathogens were added to the centre of nine cm agar plates as eight mm agar plugs taken from the edge of an actively growing fungal colony. Test strains were added as two 20 µl spots (10^7 cfu ml^{-1}) on opposite sides of the plate 30 mm from the centre. Test strains were added the same day as pathogens, except for *P. irregulare* which was added 24 h after the test strains applied due to its rapid growth. Control plates with pathogens only were included and cultures incubated at 25°C in the dark. Inhibition zones were recorded at two days for *P. irregulare*, four days for *R. solani* and seven days for *G. graminis* and *F. pseudograminearum* at which stage the pathogen colony had overgrown the inoculation site in the no test biocontrol strain controls or reached the edge of the plate. Inhibition zones were measured from the edge of bacterial or fungal colony to the edge of pathogen colony. There were three replicate plates for each pathogen-test strain combination in a randomised complete block design.

2.13. Statistical analysis

All statistical analysis was performed using GenStat version 14 (VSN International Ltd.) or later. Data from pot bioassays were analysed as ANOVA randomised complete block design (RCBD). Fisher's protracted least significant difference (lsd) was used to compare treatment means. Controls for the secondary and tertiary assay experiments were analysed as a split plot design fitting experiment as whole-plot with the three controls as sub-plots. There was no interaction between experiments. The efficiency of the different isolation methods for newly isolated strains were compared using Chi-squared contingency tables to test for

independence using the number of strains which passed the secondary pot bioassay and the total number of strains tested in the primary assay less the number of strains passing the secondary pot bioassay.

3. Results

3.1. Number of strains assessed in each assay

The total number of newly isolated and culture collection strains assessed in each assay system is shown in Table 2. Of the 2310 microbial strains assessed in the primary tube assay system, 185 progressed to the secondary assay based on similar or increased shoot height and root number compared to our best current biocontrol options (TB or EN16). An example of results after two weeks growth for the controls and two test strains are shown in Figure 2. For the no-pathogen control treatment, shoot height was 10 to 16 cm, with six to 10 roots reaching the bottom of the tube. For the pathogen only control, shoot height was reduced to between two and 10 cm, but usually (>90%) less than eight cm, with rarely (<10%) any roots reaching the bottom of the tube. For the positive control strains TB and EN16, seedlings were usually (>90%) between four and 10 cm with one to two roots occasionally (10%) reaching the bottom of the tube. Strains were selected for the second assay if one or more roots reached the bottom of the tube and plants were >10 cm in height in both replicate tubes.

Of the 185 strains assessed in the secondary assay, 43 strains were selected for assessment in the tertiary assay as they showed better disease control (mean root disease reduced, mean root or shoot dry weight increased) than either TB or EN16. There was no significant difference between newly isolated or culture collection strains in the percentage of strains selected for the tertiary assay (P=0.180), with 1.9% of strains from the combined cohorts selected for further assessment (Table 2).

The number of non-filamentous bacteria, actinobacteria and fungi assessed is given in Table 3. The percentage of each microbial type assessed in the tertiary assay was 1.3% for bacteria, 2.6% for actinobacteria and 4.4% for fungi. Chi-squared test for independence indicated that the percentage of strains proceeding to the tertiary assay was greater than expected for fungal strains compared to actinobacteria and bacteria (P=0.029, Table 3).

3.2. Efficacy of strains in secondary and tertiary assays

A total of thirteen separate assays were conducted to assess the 185 strains in the secondary assay. The mean values for the three control treatments (no pathogen, pathogen only, TB or EN16) for shoot and root dry weights, total root length and diseased root rating are shown in Tables 4a and 4b. The Waikerie GS soil had background levels of *R. solani* producing low levels of disease, resulting in a mean root rating score between 0.4 and 0.8. Addition of *R. solani* infested millet seed increased root disease score to between 3.0 and 3.7, with shoot growth being reduced to between 75 and 51% of the no pathogen controls. Root dry weight was reduced to between 42 and 32% of no pathogen controls (Tables 4a and 4b).

On average, strains TB and EN16 reduced root disease scores by 22 and 16%, respectively. Strain TB increased shoot and root dry weight and root length by 15, 27 and 42%, respectively (Table 4a). Strain EN16 increased shoot and root dry weight and root length by 5, 16 and 21%, respectively (Table 4b). A total of 24 new strains and 19 culture collection strains were selected from the secondary assay to progress further based on greater disease control (mean increase in shoot of dry weight, root length or mean decrease in root disease rating) than either strain TB or EN16. The percentage change in measured parameters compared to the pathogen only controls for new strains and culture collection strains are shown in Tables 5a and 5b respectively. The 24 newly isolated strains reduced diseased root rating between 21 and 50% in the secondary assay. Four strains increased root weight or length by over 100%, e.g. strains S8, S10, S14 and S17 (Table 5a). Most of the selected culture collection strains had a similar reduction in root disease score as the newly isolated strains, however the increase in root length and weight was generally less than with the new strains (Table 5b). Five strains in one secondary assay, (F1, F2, F3, F13 and F17) decreased root length compared to disease control, however, they were selected as they performed better (increased dry weight and root length or decreased diseased root rating) than the positive control strain EN16. This inconsistency was due to reduced disease levels in the disease control treatment in this assay compared to the other assays. Four of these strains (F1, F2, F3 and F17) subsequently showed good control efficacy (34 to 52 % reduction of root disease) in the tertiary assay (Table 5b).

The 43 strains selected from the secondary assay were assessed in the tertiary assay at three inoculation levels. Results from the most efficacious inoculum level (greatest mean shoot or root dry weight, longest mean root length, lowest mean root disease score) in the

tertiary assay are shown in Tables 5a and 5b for new and culture collection strains, respectively.

For the newly isolated strains in the tertiary assay, most strains reduced disease and increased plant growth, confirming efficacy measured in the second assay, and 18 of 24 strains were effective at reducing disease at less than 10^6 cfu seed^{-1}. Three strains (S3, S8 and S13) gave inconsistent results between experiments, with little disease control in the tertiary assay. The two *Microbacterium* strains (S7 and S10) and the Gram negative strains (S14, S15, S18 and S19) required greater than 10^6 cfu seed^{-1} for maximum disease control. The three fungal strains (S20, S21, and S22) required less than 10^4 cfu seed^{-1} for maximum efficacy out of the three inoculation levels (Table 5a).

For the culture collection strains in the tertiary assay, all strains provided greatest disease control at less than 10^6 cfu seed^{-1}. Five strains (F7, F11, F13, F14, and F15) did not reduce disease severity in the tertiary assay in contrast to their disease control performance in the second assay (Table 5b).

3.3. Identification of strains

Candidate strains assessed in the tertiary assay were identified by 16S rRNA or ITS sequencing. The newly isolated bacterial strains covered a range of genera within five phyla (Table 6a). There were six *Bacillus*, two *Paenibacillus*, *Microbacterium*, *Streptomyces* and *Pandoraea*, and one each of *Brevibacterium*, *Chryseobacterium*, *Phyllobacterium* and *Pseudomonas*. Similarity to type strains in the RDP database was generally greater than 0.97. The three fungi were identified as *Aspergillus*, *Cylindrocarpon* or *Trichoderma*. The culture collection strains were all identified as *Streptomyces* spp. with a sequence similarity of greater than 0.97, except for strain F16 which had 0.94% similarity to *Streptosporangium* (Table 6b). Two of the newly isolated strains (S23 and S24) were not identified as these were rejected from further development at this point due to inconsistent growth on agar media when subcultured from -80^{o}C storage, i.e. small and variable colony size compared to the initial isolation.

3.4. Survival on seeds

Strains assessed in the tertiary assay were evaluated for survival when applied to wheat seeds, with cfu measured at application and at one, two and seven days after application (Table 7). For the newly isolated strains, strains in the order Bacillales and the genus *Streptomyces* had 46% or more surviving cells after seven days. The *Microbacterium*, S7 and

S10 had 3.9 and 39% remaining, respectively, after seven days. All the Gram-strain negative strains had poor survival on seeds after seven days with less than 3% remaining, except for S15 (*Pseudomonas*) with 14% remaining at seven days (Table 7). For the culture collection actinobacteria, percentage survival ranged from 0.3% (F16) to 167% (F2, Table 7). Strains S3, S5, S11, S16, F2 and F18 had an increased numbers of cells at seven days compared to the initial populations (Table 7).

3.5. In vitro *antifungal activity*

The activity of the 10 newly isolates strains varied from strong inhibition of all four pathogens (S4, S17, F2 and F16) to no evidence of pathogen inhibition (S7, S8, S9, S10 and S16) (Table 8). All *Streptomyces* strains could inhibit *R. solani* and 14 out of 15 strains inhibited *G. graminis* but varied in their inhibition of *Fusarium pseudograminearum* and *Pythium irregulare*. *Streptosporangium* strain F16 inhibited all pathogens. *Bacillus* strains either had strong inhibition (>3mm inhibition zone) of all four pathogens (S17) or no inhibition of any of the four pathogens (S8, S9 and S16). The *Trichoderma* (S20) and *Aspergillus* (S21) fungi inhibited all four pathogens. The *Microbacterium* (S7, S10) did not inhibit any of the pathogens (Table 8).

3.6. Source of strains assessed

The source and isolation method for the newly isolated strains is given in Table 9a and the culture collection strains in Table 9b. The source and isolation methods used for the newly isolated strains were assessed for independence using Chi-squared statistics. There was no significant difference in the percentage of strains assessed in the tertiary assay for the source soil (P=0.473), plant cultivar used (P=0.154), location on the roots for isolation (P=0.423) or selection for chitinase activity on BYA (P=0.796). The only method where isolation of efficacious strains was increased was from preheating the incubation soils to 80^{o}C prior to planting (P=0.042), with 2.4% of strains from 80^{o}C heated soil being assessed in the tertiary assay compared to 1.0 and 1.4% for chitosan treated soil and no treatment, respectively. The frequency of isolating strains with the ability to reduce Rhizoctonia root rot was around 1.6%, regardless of treatment or method used.

4. Discussion

Rhizoctonia solani AG8 is the major fungal root pathogen causing economic loss in dryland cereal cropping systems in southern Australia, with current management and chemical control methods providing only partial control (Bogacki *et al*, 2014, Gupta *et al*., 2015). Rhizoctonia root rot is known to be influenced by soil microorganisms (Roget, 1995, Schillinger & Paulitz, 2006) and isolated microbes can reduce disease under controlled conditions (Barnett *et al.*, 2006, Dua & Sindhu, 2012, Mavrodi *et al*., 2012b, Yin *et al.*, 2013). However, the challenge is to select microbial strains which can produce consistent and economic reductions of disease in the field. One major obstacle in identifying potential strains is the ability to screen large numbers of strains for disease control in a system that is relevant to field application (Campbell, 1994, Dunlap *et al.*, 2013, Knudsen *et al.*, 1997, Köhl *et al.*, 2011, Schisler & Slininger, 1997). In the current work we were able to screen 2310 strains using a rapid, low cost, plant bioassay system as an initial screen to identify potential biocontrol strains for further testing and selection as commercial inoculants for control of Rhizoctonia root rot on wheat. The result of this work was the identification of 43 strains that when applied to wheat seeds planted into soils with *R. solani* AG8 resulted in a 19% increase in shoot dry weight, 36% increase in root dry weight, 44% increase in root length and 20% reduction in root rot disease. Individual strains increased shoot growth by 66%, increased root growth by 131%, increased root length by 146% and reduced root disease by 50% (Tables 5a&b).

The primary bioassay consisted of four of the components required to identify strains with the potential to control disease in the field. These components were: a disease conducive soil sourced from wheat fields with a continuing Rhizoctonia root rot problem; an aggressive pathogen strain; susceptible host plant (wheat); and the microbes applied to the seed. The assay was relatively quick to set up, inoculate with the test strains and assess, compared to the pot bioassays, taking a total of 10 person hours to test 84 strains and would be comparable to the person hours taken to set up *in vitro* inhibition assays. The results could also be evaluated two weeks after planting, providing a quick turnaround time for each assay. This was matched with a methodology to rapidly take an unknown strain from isolation plates directly into the assay system using the 96 well plate format. The rational for this was to minimise the time taken from isolation plate to assessment when the majority of strains were likely to be ineffective in controlling disease. The combination of these approaches enabled 84 microbial strains to be isolated and inoculated into the assay system in six hours by one person. Assessment of the assay was also rapid, with paired replicates with increased growth

compared to diseased plants without microbial inoculation easily identified (see Figure 2). Selected strains then can be recovered from -80^{o}C storage, purified and assessed in the secondary and tertiary bioassays and for characterisation. Although in the initial isolation strains were not purified and there was a cross contamination risk in using the 96 well plates, single colonies were selected and cross contamination was minimal as when strains were purified for the second assay it was rare for there to be multiple strains in the one culture. Using this system, 2310 strains were tested in the primary assay, 185 of these were selected and assessed in the secondary assay. Of these, 43 showed increased plant growth and reduced disease compared to our current best biocontrol strains, *Trichoderma* strain TB or *Streptomyces* strain EN16 and were assessed in the tertiary assay at 3 inoculation levels. Strains were required to perform better than strains TB and EN16 as these strains were not able to provide significant disease control in the field, even though they could consistently reduce disease in bioassays.

Our approach to screen large numbers of strains, as suggested by Campbell (1994) and Schisler and Slininger, (1997), was justified by the results, with only 1.9% of the strains assessed in the primary assay shown to have better disease control than our current best performing strains (Table 2). There was no significant difference in the percentage of efficacious strains from the culture collection (2.4%) compared to newly isolated strains (1.6%, P=0.180), with both sources providing strains for further development. For the newly isolated strains we used five different field soils with three different cereal cultivars, each with three different pre-treatments, and isolated from well washed root pieces to select for biofilm producers that tightly adhere to roots (Fall *et al.*, 2004), macerated root pieces, or from the rhizoplane. The purpose was to extend the diversity of strains isolated and not to restrict ourselves to a target group of interest.

The aim of this research was to develop commercial biocontrol agents, not to rigorously assess isolation methods, so soils, cultivars and methods which were producing strains with a disease control response were revisited in subsequent isolations. Nonetheless, it is of interest to understand which isolation methods were most productive in producing candidate biocontrol strains so as to inform future work. There was no significant difference between source of strains or isolation method, except for heat treatment of the pre-incubation soils which increased the success rate to 2.4%, compared to no heat treatment (1.4%) or chitosan amendment (1.0%). These results indicates that heat treatment slightly favours the isolation of candidate biocontrol agents effective in reducing disease, but is not exclusive of other methods. Heat resistance is an important property for a potential inoculant to survive in

storage and seeding stress from modern air-seeders. Park *et al.*, (2013) also used Chi-squared statistics to assess different sampling methods to isolate potential biocontrol agents for foliar pathogens and found little relationship between isolation source and method and the number of efficacious strains; except for plant species affecting the number of efficacious phyllosphere strains, with efficacious strains being isolated at a frequency of less than one percent. Of note is that there was no difference between source soils in our study, whether the well-studied disease suppressive Avon soil (Roget, 1995) or soils highly conducive to Rhizoctonia root rot (Netherton and Mudamuckla soils). These results support our approach to isolate from a diversity of environments to enable the testing of a greater range of strains as not one environment or method appeared to be significantly better than the other. From Tables 9a and 9b it can be seen that efficacious strains came from a variety of environments and methods.

The diversity of isolated strains reducing Rhizoctonia root rot suggests a variety of taxa could be contributing to natural disease control in the field. Suppressive soils are already known to have a diversity of taxa considered to be contributing to suppression (Barnett *et al*., 2006, Mendes *et al*., 2011, Weller *et al*., 2002,). Of the 22 new strains that were selected from the secondary assay and identified, there were nine bacterial genera from five Phyla, and three fungi genera from the phylum Ascomycota (Table 6a). Some of these genera are well known as biocontrol agents, e.g. *Bacillus* (S1, S3, S8, S9, S16 and S17), *Paenibacillus* (S4 and S5), *Streptomyces* (S12 and S13), *Pseudomonas* (S15) and *Trichoderma* (S20) (Druzhinina *et al*., 2011, Fravel, 2005, Mavrodi, *et al*., 2006, Raaijmakers, *et al*., 2010, Rybakova *et al*., 2016). Others, such as *Microbacterium* (S7 and S10) are less well known but have been reported by various authors as being able to reduce plant diseases (Barnett *et al*., 2006, Fukui *et al.*, 1999, Pereira *et al.*, 2007, Sturz *et al.*, 2005,). *Chryseobacterium* (S6) which has been reported as being able to reduce Fusarium seedling blight on wheat (Khan *et al.*, 2006), was recently identified from a pyrosequencing study as being involved in suppression of Rhizoctonia root rot on wheat (Yin *et al.*, 2013) and identified by Park *et al.* (2013) as a potential biocontrol agent of foliar pathogens. *Phyllobacterium* (S14) strains appear to be common in roots (Swings *et al.*, 2006) with previous reports indicating their potential as biocontrol agents against *R. solani* on potato and bean (Donmez *et al.*, 2015), and *Fusarium* on cotton (Chen *et al.*, 1995). *Aspergillus* (S21) strains have not previously been reported to control root diseases, although there are two products, AF36 (Arizona Cotton Research and Protection Council) and Afla-guard (Syngenta), based on atoxigenic strains of *Aspergillus* that are currently available in the US for control of aflatoxin-producing

Aspergillus on cotton and peanuts (Fravel 2005). *Pandoraea apista* (S18 and S19) and *Cylindrocarpon destructans* (S22) have not previously been reported as being able to reduce Rhizoctonia root rot on wheat. *Pandorea apista* is a known human pathogen associated with cystic fibrosis (LiPuma, 2010) and *C. destructans* is a well-known plant pathogen (Lamichhane & Venturi, 2015) and so we consider these strains unsuitable for further development as biocontrol agents due to downstream problems in satisfying government registration requirements. These results show that there is a diversity of taxa with the ability to reduce Rhizoctonia root rot, from both disease suppressive and conducive soils, and supports our strategy not to target a particular group of interest for testing.

Efficacy in reducing disease is only one characteristic important for a commercial inoculant. Other characteristics include the ability to be integrated into current methodologies for applying microbial agents (Fravel, 2005, Köhl *et al.*, 2011, Schisler & Slininger, 1997). In Australian cereal cropping systems the most likely route for introducing microbial inoculants is through seed coatings. Efficacious strains selected from the secondary assay were assessed for survival on wheat seeds up to seven days at room temperature using a simple xanthan gum-ascorbic acid sticker solution (Table 7). There were marked differences between strains, with all the newly isolated Gram negative strains having poor survival over this time. There were also seven strains, either *Bacillus*, *Paenibacillus* or *Streptomyces,* which appeared to increase in number in the seven days after inoculation, e.g. strain S3, S5, S11, S22, F2, F10 and F18, possibly due to growth on the seeds. Choi *et al* 2013 also found that *Paenibacillus polymyxa* strain E681 could increase over time on cucumber seeds using a xanthan gum based sticker solution. There were also marked differences within the same genus, with five *Streptomyces* maintaining populations over seven days (e.g. strains F2, F5, F6, F10 and F18) and nine having a marked decline (e.g. strains F3, F4, F7, F8, F9, F11, F13, F15 and F17). Xanthan gum has been reported to increase survival of rhizobia in peat formulations (Lorda *et al*., 2007) and to increase disease control efficacy by *Paenibacillus* (Schoina *et al*., 2011) and of yeast (Lima *et al* 2005). For future field trials we will use the same seed coat formulation and only strains with greater than 20% survival over seven days will be considered for further development. We note that although development of more sophisticated formulations for each strain can improve survival of Gram negative bacteria (Bashan *et al.* 2014), it was not possible to undertake this for a large number of strains within this project. Optimisation of formulations will be undertaken after proof of field trial efficacy.

In vitro inhibition of pathogens by microbes on agar plates is a common method used for preliminary screening of potential biocontrol agents, however, there often is a poor

correlation between *in vitro* inhibition and disease control (Castejón-Muñoz & Oyarzun, 1995, Elsherif & Grossmann, 1994, Knudsen *et al*., 1997, Reddy *et al.*, 1994,). Even so, *in vitro* inhibition studies are still used as initial selection criteria (Dua & Sindhu, 2012, Mavrodi *et al*., 2012b). In our work we used a plant based disease assay for the initial screening of unknown strains and assessed candidate strains for pathogen inhibition on agar after selection for disease control efficacy on plants for information of potential mechanisms of disease control. There are limited examples of screening directly into a plant pathosystem. Knudsen *et al.* (1997) review of the Nordic screening program for control of seed and soilborne diseases in cereals noted that *in planta* testing in a non-sterile field soil was more comparable to field results than using a sand substrate and that their seven best strains for control of disease by *Fusarium* and *Bipolaris* did not inhibit the pathogens *in vitro*, however, results probably depend on the pathosystem system. Pliego *et al.* (2011) compared three screening methods, *in vitro* inhibition, root colonization and *in planta* control of white root rot on avocado and identified different strains by each method, but all selected strains had *in vitro* inhibition of the pathogen on agar.

Our results again highlight the use of these *in vitro* assays as a poor predictor of disease control on plants, with five out of the 10 new strains showing no inhibition of *R. solani*, *Fusarium*, *Pythium* or *G. graminis* on the medium we used, even though these strains were able to reduce Rhizoctonia root rot on wheat grown in a field soil in three bioassays. We recognise that the media used for inhibition assays can affect the production of antifungal metabolites, however, screening assays are usually undertaken on a single medium (Dua & Sindhu, 2012, Mavrodi *et al*., 2012b, Yin *et al*., 2013). In our case we used to combination of a general bacteria growth medium, TSA/10, and general fungal medium, PDA/4, to ensure media did not inhibit the growth of the test isolates or pathogens.

Another easily applied *in vitro* test is for chitinase production using Baker's yeast agar (Christensen & Cook, 1978). Although clearing zones on BYA were not predictive of disease control, this was still a useful medium to use as all bacterial and fungal strains we have plated onto this medium have grown and it is much cheaper (Aus$6 for 500g) compared to proprietary microbial media (e.g. >AUS$250 for 500g of tryptic soy broth or potato dextrose broth), resulting in substantial cost savings when large numbers of agar plates are required and having an opaque medium is not an issue.

To be a successful commercial inoculant, microbial strains not only have to be efficacious in plant-soil systems and survive on seed but also need to be able to be grown to high cell densities, formulated and survive storage, be genetically and phenotypically stable

and compatible with other seed treatments (Fravel, 2005, Köhl *et al.*, 2011, Schisler & Slininger, 1997). Further characterization of strains for these properties is ongoing such that strains selected for field trials will have properties that will not discount them being further developed as inoculants.

5. Conclusions

We have identified a number of candidate strains that appear to be suitable for further development as inoculants for the grains industry. In the process we have also generated useful information to progress future screening efforts, namely: there is a wide diversity of fungi and bacteria that have the potential to reduce Rhizoctonia disease; strains that can reduce Rhizoctonia disease are rare, around 1.6%, irrespective of the source of strains and isolation methodology; preheating soils prior to incubating plants to capture root microbes improves the success rate in selecting efficacious strains; and efficacious strains vary considerably in their ability to survive on seeds so this should be tested for earlier in the screening process.

Selecting appropriate strains for control of fungal root diseases is difficult given that strains with the appropriate properties for an inoculant are rare (Campbell, 1994, Schisler & Slininger, 1997) and procedures to screen large numbers of strains in a relevant system that is also quick and cheap are limited. In this work we present a simple, cheap and rapid method for assessing unknown bacterial and fungal strains directly into a tube bioassay containing many of the components required to assess for the function of interest, i.e. control of Rhizoctonia root rot on wheat in a soil with an intractable Rhizoctonia problem with the microbes applied to the seed. Similar rapid high throughput systems could be developed for other pathosystems of interest and bypass the use of less informative *in vitro* screens.

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Selection of microbes for Rhizoctonia control

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Selection of microbes for Rhizoctonia control

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Figure legends

Figure 1. Stepwise approach for screening of microbial strains for control of Rhizoctonia root rot on wheat.

Figure 2. Example of primary assay results after two weeks growth of wheat showing replicate tubes for no pathogen control (A, B), pathogen (*R. solani* AG8) only control (C, D), test strain 1 (E, F) and test strain 2 (G, H). Test strains are examples of bacterial strains of previously unknown efficacy in controlling Rhizoctonia root rot that were selected for further assessment.

Table 1. Soils used for pre-incubation of cereal root microflora and for Rhizoctonia root rot bioassays on wheat. All soils collected over summer (between crops) with the previous crop being wheat.

Soil	Character	Comment
Avon	Brown sandy loam 70% sand, 20% clay	Previously reported to be suppressive to Rhizoctonia root rot (Roget 1995). Used for pre-incubation of root microflora.
Waikerie AB	Red calcareous sand 90% sand, 7% clay	From long term trial site with different cropping sequence. Soil taken from most suppressive treatment to Rhizoctonia root rot. Used for pre-incubation of root microflora.
Netherton	Grey siliceous sand. 93% sand, 6%clay	Disease conducive soil with continuing Rhizoctonia root rot problem. Used for primary tube bioassay and for pre-incubation of root microflora.
Minnipa N12	Brown sandy loam 79% sand, 16% clay	Disease conducive soil that has become more suppressive with long term retention of residues. Used for pre-incubation of root microflora.
Mudamuckla	Brown sand, 85% sand, 12% clay	Disease conducive soil with continuing Rhizoctonia root rot problem, 13% $CaCO_3$ content. Used for pre-incubation of root microflora.
Waikerie GS	Red calcareous sand 90% sand, 7% clay	Disease conducive soil with continuing Rhizoctonia root rot problem. Used for secondary and tertiary bioassays.

Table 2. Source of strains. Number of newly isolated strains and strains from a culture collection of actinobacteria assessed in the primary (1^0), secondary (2^0) and tertiary (3^0) assays for biological control of Rhizoctonia root rot on wheat. The percentage of strains assessed in the 3^0 assay relative to the 1^0 assay is given as % in 3^0, P=0.180 from Chi-squared test for independence.

Source of strains	1^o	2^o	3^o	% in 3^o
New isolates	1512	100	24	1.6
Culture collection	798	85	19	2.4
Total	2310	185	43	1.9

Table 3. Type of microorganism, bacteria, mycelial actinobacteria (Actino) or fungi, assessed in the primary (primary (1^0), secondary (2^0) and tertiary (3^0) assays for biological control of Rhizoctonia root rot on wheat. The percentage of strains assessed in the 3^0 assay relative to the 1^0 assay is given as % in 3^0, P=0.029 from Chi-squared test for independence.

Type of microbe	1°	2°	3°	% in 3°
Bacteria	1434	79	19	1.3
Actino	808	93	21	2.6
Fungi	68	13	3	4.4
Total	2310	185	43	1.9

Table 4a. Mean results for control treatments in combined secondary (2^0, 7 assays) and tertiary (3^0, 5 assays) Rhizoctonia root rot bioassays on wheat for newly isolated strains. Measured parameters are shoot and root dry weight (DW, mg pot^{-1}) total root length (cm $plant^{-1}$) and root disease rating (0-5 scale, 0 = no disease, 5 = max disease severity). TB is positive control *Trichoderma* strain TB. Percentage change = [(strain TB/Pathogen only control)x100]-100.

Assay	2^0 Shoot DW mg	2^0 Root DW mg	2^0 Root Length cm	2^0 Root Rating (0-5)	3^0 Shoot DW mg	3^0 Root DW mg	3^0 Root Length cm	3^0 Root Rating (0-5)
No Pathogen control	376*	330*	55*	0.8*	297*	247*	52*	0.7*
Pathogen only control	236	147	23	3.6	152	80	12	4.1
TB	270*	175	31*	2.9*	180*	96	17*	3.9
% change with TB inoculation	14	19	30	-20	18	20	39	-6

*significantly different from Pathogen only control at P=0.05 by Fisher's LSD.

Table 4b. Mean results for control treatments in combined secondary (2^0, 6 assays) and tertiary (3^0, 5 assays) Rhizoctonia root rot bioassays on wheat for culture collection strains. Measured parameters are shoot and root dry weight (DW, mg pot^{-1}) total root length (cm $plant^{-1}$) and root disease rating (0-5 scale, 0 = no disease, 5 = max disease severity). EN16 is positive control *Streptomyces* strain EN16. Percentage change = [(strain EN16/ Pathogen only control)x100]-100.

Assay	2^0 Shoot DW mg	2^0 Root DW mg	2^0 Root Length cm	2^0 Root Rating (0-5)	3^0 Shoot DW mg	3^0 Root DW mg	3^0 Root Length cm	3^0 Root Rating (0-5)
No Pathogen control	228*	178*	41*	0.4*	278*	175*	43*	0.4*
Pathogen only control	171	99	19	3.0	167	77	16	3.3
EN16	179	115*	23*	2.5	188	92	18	2.8
% change with EN16 inoculation	5	16	21	-16	13	19	15	-16

*significantly different from Pathogen only control at P=0.05 by Fisher's LSD.

Table 5a. Percentage change in measured parameters, shoot and root dry weight (DW), total length of roots and diseased root rating (0-5 scale), compared to pathogen only control for newly isolated strains selected from secondary (2^0) and tertiary (3^0) Rhizoctonia root rot bioassays on wheat. Results for tertiary assay from most efficacious inoculum level is given as Log_{10} (cfu $seed^{-1}$). Percentage change = [(strain/ Pathogen only control)x100]-100.

Assay	2^0	2^0	2^0	2^0	3^0	3^0	3^0	3^0	3^0
Strain	Shoot DW	Root DW	Root Length	Root Rating	Log_{10} (cfu $seed^{-1}$)	Shoot DW	Root DW	Root Length	Root Rating
S1	34**	56	68	-34**	5.7	1	4	17	-3
S2	-7	36	22	-23*	5.7	20	47	75*	-14
S3	-4	37	22*	-30**	4.9	-9	2	-15	10
S4	-1	42**	22*	-20*	4.5	55**	60**	105**	-27**
S5	3	22	14	-20	3.5	25	16	38	-5
S6	48**	59	30	-26**	ND[a]	35**	16	57*	-8
S7	35*	54	54	-32**	7.3	31*	8	30	-5
S8	43**	107**	141**	-37**	4.6	5	-1	38	-8
S9	49**	41	71	-26**	4.9	28*	16	50*	-8
S10	46**	131**	146**	-34**	6.9	11	33	123**	-29**
S11	23	41	50	-32**	5.0	10	15	48*	-8
S12	28	32	49	-30**	5.1	24**	16	54**	-14*
S13	42**	44	62*	-33**	5.3	-6	-2	49*	-13
S14	44**	93	124**	-50**	6.9	18	19	13	-9
S15	28**	77	74	-26	6.1	14	24	28	-11
S16	15	20	27	-25**	5.3	66**	104*	106*	-29**
S17	13	103**	92**	-40**	5.0	19	25	62	-17
S18	14	66	58	-25	7.6	18	40	55	-13
S19	24	74*	45	-23	6.4	48**	62**	72**	-16
S20	22	14	27	-25**	3.7	55**	105*	113**	-26**
S21	9	39**	44**	-31**	4.1	77**	138**	134**	-29**
S22	19	30	41**	-31**	3.0	65**	100	87	-24**
S23	43**	62	29	-21	3.4	23	19	59*	-13
S24	2	59**	23*	-25**	4.2	9	10	10	-2
TB[b]	15	27	42	-22	4.3	18	20	39	-6

*significantly different from Pathogen only control at P=0.10 by Fisher's LSD.
**significantly different from Pathogen only control at P=0.05 by Fisher's LSD.
[a] Not Determined

[b] mean of 7 (2^0) or 5 (3^0) experiments

Table 5b. Percentage change in measured parameters, shoot and root dry weight (DW), total length of roots and diseased root rating (0-5 scale) compared to pathogen only control for culture collection strains selected from secondary (2^0) and tertiary (3^0) Rhizoctonia root rot bioassays on wheat. Results for tertiary assay from most efficacious inoculum level is given as Log_{10} (cfu $seed^{-1}$). Percentage change = [(strain/ Pathogen only control)x100]-100.

Assay	2^0	2^0	2^0	2^0	3^0	3^0	3^0	3^0	3^0
Strain	Shoot DW	Root DW	Root Length	Root Rating	Log_{10} (cfu $seed^{-1}$)	Shoot DW	Root DW	Root Length	Root Rating
F1	6	3	-15	-8	5.6	20	42	58	-52
F2	5	1	-23	0	5.5	29	40	37	-41
F3	-1	-15	-10	-8	4.9	9	31	39	-38
F4	20	36*	23	-32	4.8	24	44	31	-44
F5	-5	27*	25	-41**	4.8	14	38	63	-42
F6	22	40	34	-17	3.5	23	7	56	-13
F7	7	-6	9	-10	5.4	1	-4	16	0
F8	4	11	27	-29	ND[a]	17	9	28	-12
F9	21	7	38	-26	5.2	24	4	22	-7
F10	23	55	64	-31	4.9	17	30	63*	-28
F11	20	11	14	-25	2.8	5	-16	-13	4
F12	27	36	80**	-47**	5.4	8	9	12	-6
F13	1	2	-12	10	2.8	-3	-20	-12	28
F14	-6	19	58	-31	5.8	10	-4	2	-6
F15	3	6	18	-25	5.9	10	-7	8	6
F16	3	39**	22	-7	5.6	7	75*	25	-27
F17	11	3	-18	5	4.5	19	108**	74**	-34**
F18	1	33*	23	-19	5.3	14	88**	65	-29
F19	-8	30**	26	-27	3.2	6	84*	45*	-24
EN16[b]	5	16	21	-16	6.3	13	19	15	-16

*significantly different from Pathogen only control at *P*=0.10 by Fisher's LSD.
**significantly different from Pathogen only control at *P*=0.05 by Fisher's LSD.
[a] Not Determined
[b] mean of 6 (2^0) or 4 (3^0) experiments

Table 6a. Identification of newly isolated bacterial strains by 16S rRNA sequencing and comparison with type strains in the Ribosomal Data Base Project. Fungi were identified by morphological characteristics (*Trichoderma*) or ITS sequencing and comparison to NCBI data base. Similarity scores are for the closest match in database. Mucoid strains indicating extra cellular polysaccharide production on BYA indicated by +. Chitinase production on BYA indicated by: -, none; + <1mm clearing zone, ++ 1-5 mm clearing zone; +++ > 5mm clearing zone.

Strain	Phylum	Closest match in database and 16S rRNA accession no.	Similarity Score	Mucoid	Chitinase
S1	Firmicutes	*Bacillus acidiceler* DQ374637	0.995	-	++
S2	Firmicutes	*Brevibacillus formosus* AB112712	0.976	-	++
S3	Firmicutes	*Bacillus simplex* AJ439078	0.998	-	++
S4	Firmicutes	*Paenibacillus peoriae* AJ320494	0.999	+	+
S5	Firmicutes	*Paenibacillus castaneae* EU099594	0.988	+	+
S6	Bacteroidetes	*Chryseobacterium formosense* AY315443	0.737	+	-
S7	Actinobacteria	*Microbacterium paraoxydans* AJ491806	0.944	-	-
S8	Firmicutes	*Bacillus simplex* AJ439078	0.999	-	++
S9	Firmicutes	*Bacillus simplex* AJ439078	0.973	-	-
S10	Actinobacteria	*Microbacterium thalassium* AM181507	0.917	-	-
S11	Firmicutes	*Paenibacillus peoriae* AJ320494	0.978	-	-
S12	Actinobacteria	*Streptomyces microflavus* DQ445795	0.988	-	+++
S13	Actinobacteria	*Streptomyces canus* AY999775	1.000	-	++
S14	α Proteobacteria	*Phyllobacterium ifriqiyense* AY785325	0.999	+	-
S15	β Proteobacteria	*Pseudomonas koreensis* AF468452	0.997	+	++
S16	Firmicutes	*Bacillus megaterium* D16273	0.97	+	++
S17	Firmicutes	*Bacillus vallismortis* AB021198	0.974	-	++
S18	β Proteobacteria	*Pandoraea apista* AF139173	0.979	-	+++

Selection of microbes for Rhizoctonia control

S19	β Proteobacteria	*Pandoraea apista* AF139173	0.974	-	-
S20	Fungi: Ascomycota	*Trichoderma*		-	++
S21	Fungi: Ascomycota	*Aspergillus ustus* FJ878630	0.990	-	++
S22	Fungi: Ascomycota	*Cylindrocarpon destructans* var. *destructans* AM419062	1.000	-	++

Table 6b. Identification of culture collection strains by 16S rRNA sequencing and closest match in NCBI data base and accession number. Similarity scores are for the closest match in database.

Strain	Phylum	Closest match in database and 16S rRNA accession no.	Similarity Score
F1	Actinobacteria	*Streptomyces chartreusis* AB184839	0.98
F2	Actinobacteria	*Streptomyces violaceusniger* NC015957	0.99
F3	Actinobacteria	*Streptomyces avermitilis* BA000030	0.99
F4	Actinobacteria	*Streptomyces avermitilis* BA000030	0.98
F5	Actinobacteria	*Streptomyces prasinosporus* AB184390	0.98
F6	Actinobacteria	*Streptomyces avermitilis* BA000030	0.97
F7	Actinobacteria	*Streptomyces avermitilis* BA000030	0.98
F8	Actinobacteria	*Streptomyces flavogriseus* AJ494864	0.99
F9	Actinobacteria	*Streptomyces flavogriseus* AJ494864	0.99
F10	Actinobacteria	*Streptomyces coelicolor* AB184196	0.99
F11	Actinobacteria	*Streptomyces lysosuperficus* SAMN02471827	0.97
F12	Actinobacteria	*Streptomyces flavogriseus* AJ494864	0.99
F13	Actinobacteria	*Streptomyces flavogriseus* AJ494864	0.97
F14	Actinobacteria	*Streptomyces flavogriseus* AJ494864	0.99
F15	Actinobacteria	*Streptomyces flavogriseus* AJ494864	0.99
F16	Actinobacteria	*Streptosporangium roseum* CP001814	0.94
F17	Actinobacteria	*Streptomyces chartreusis* AB184839	0.98
F18	Actinobacteria	*Streptomyces coelicolor*	0.99

		AB184196
F19	Actinobacteria	ND*

*ND = not determined

Table 7. Percentage of cells surviving on wheat seeds after 1, 2 and 7 days at room temperature from an initial population at t=0 given as Log10(cfu seed^{-1}). Mean of two replicates.

Strain	*Genus*	Log10 (cfu seed^{-1}) t=0	%survival t=1d	%survival t=2d	%survival t=7d
S1	*Bacillus*	5.87	120	77	46
S2	*Brevibacillus*	5.67	100	100	67
S3	*Bacillus*	4.87	139	184	157
S4	*Paenibacillus*	4.50	116	63	65
S5	*Paenibacillus*	2.86	63	30	137
S6	*Chryseobacterium*	5.81	20	14	2.9
S7	*Microbacterium*	5.48	111	62	3.9
S8	*Bacillus*	5.62	112	62	50
S9	*Bacillus*	5.06	133	114	97
S10	*Microbacterium*	6.77	118	104	39
S11	*Paenibacillus*	3.89	153	68	134
S12	*Streptomyces*	5.21	118	124	77
S13	*Streptomyces*	4.73	141	88	61
S14	*Phyllobacterium*	5.86	60	51	1.4
S15	*Pseudomonas*	5.73	81	43	14
S16	*Bacillus*	5.01	131	84	110
S17	*Bacillus*	5.73	75	48	62
S18	*Pandoraea*	6.23	17	4	0.1
S19	*Pandoraea*	6.14	12	4	2.1
S20	*Trichoderma*	2.44	104	95	41
S21	*Aspergillus*	4.27	162	142	108
S22	*Cylindrocarpon*	3.12	67	80	137
F1	*Streptomyces*	5.85	95	50	71
F2	*Streptomyces*	5.58	57	72	167
F3	*Streptomyces*	1.92	40	18	28
F4	*Streptomyces*	4.01	108	79	18
F5	*Streptomyces*	3.54	124	75	148
F6	*Streptomyces*	4.62	84	88	96
F7	*Streptomyces*	2.43	50	21	13
F8	*Streptomyces*	4.45	94	46	9

F9	*Streptomyces*	2.43	38	28	33
F10	*Streptomyces*	2.88	104	102	113
F11	*Streptomyces*	2.75	14	2	1
F12	*Streptomyces*	6.31	93	79	81
F13	*Streptomyces*	3.33	97	33	23
F14	*Streptomyces*	6.40	85	60	78
F15	*Streptomyces*	2.82	70	70	32
F16	*Streptosporangium*	3.64	16	1	0.3
F17	*Streptomyces*	5.20	46	71	36
F18	*Streptomyces*	6.27	125	138	150

Table 8. *In vitro* inhibition profile of 10 newly isolated strains and 19 culture collection strains against wheat root pathogens *Rhizoctonia solani* AG8, *Fusarium pseudograminearum*, *Pythium irregulare* and *Gaeumannomyces graminis tritici*. Responses of fungal pathogen to test strains are given as: - no sign of inhibition; + hyphal avoidance but no clear zone of inhibition; ++ inhibition zone 1-2 mm; +++ inhibition zone >3mm.

Strain	Genus	*R. solani*	*F.pseudogram.*	*P.irregulare*	*G. graminis*
S4	*Paenibacillus*	+++	+++	+++	++
S7	*Microbacterium*	-	-	-	-
S8	*Bacillus*	-	-	-	-
S9	*Bacillus*	-	-	-	-
S10	*Microbacterium*	-	-	-	-
S12	*Streptomyces*	++	++	++	+++
S16	*Bacillus*	-	-	-	-
S17	*Bacillus*	+++	+++	+++	+++
S20	*Trichoderma*	++	++	+	++
S21	*Aspergillus*	++	+++	+++	++
F1	*Streptomyces*	+++	-	-	++
F2	*Streptomyces*	+++	+++	+++	+++
F3	*Streptomyces*	+++	-	-	+++
F4	*Streptomyces*	+++	-	-	+++
F5	*Streptomyces*	+++	+++	-	+++
F6	*Streptomyces*	+++	+++	-	++
F7	*Streptomyces*	+++	-	-	+++
F8	*Streptomyces*	+++	-	-	-
F9	*Streptomyces*	+++	-	-	+++
F10	*Streptomyces*	+++	+++	-	+++
F11	*Streptomyces*	+++	+++	-	+++
F12	*Streptomyces*	+++	-	-	+++
F13	*Streptomyces*	+++	-	+++	+++
F14	*Streptomyces*	+++	-	+++	+++
F15	*Streptomyces*	+++	-	++	+++
F16	*Streptosporangium*	+++	+++	+++	+++
F17	*Streptomyces*	+++	-	+++	-
F18	*Streptomyces*	+++	-	-	+++
F19	*Streptomyces*	+++	-	+++	-

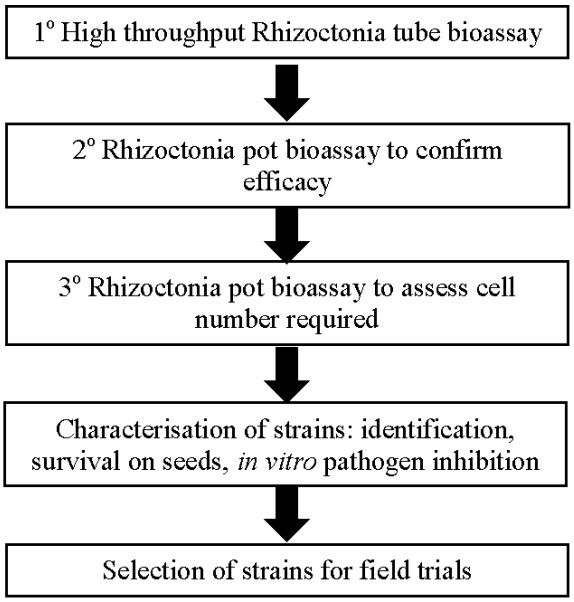
Table 9a. Source and isolation method for newly isolated strains selected for assessment in tertiary assays. Source of strains includes soil, soil pre-treatment and host plant, either wheat cultivars H45 or Yallaroi or triticale cultivar Tahara. Strains were isolated from either well washed root pieces plated onto media or from dilution series from the rhizosheath, subjected to none or 80°C heat treatment prior to plating and the agar medium strains were isolated from.

Strain	Soil	Soil pre-treatment	Host	Isolation location	Pre-plating heat treatment	Agar medium
S1	Waikerie	80°C	H45	Root pieces	80°C	BYA1%
S2	Waikerie	80°C	H45	Root pieces	80°C	BYA1%
S3	Waikerie	80°C	H45	Root pieces	80°C	BYA1%
S4	Waikerie	80°C	H45	Root pieces	80°C	BYA1%
S5	Waikerie	80°C	H45	Rhizosheath	80°C	BYAc
S6	Waikerie	Chitosan	H45	Root pieces	None	BYAc1%
S7	Waikerie	Chitosan	H45	Root pieces	None	BYAc1%
S8	Mudamuckla	80°C	Triticale	Rhizosheath	80°C	BYAc
S9	Mudamuckla	80°C	Triticale	Rhizosheath	80°C	BYAc
S10	Mudamuckla	80°C	Triticale	Root pieces	80°C	BYA1%
S11	Netherton	80°C	H45	Rhizosheath	80°C	BYAcl
S12	Mudamuckla	80°C	Triticale	Rhizosheath	80°C	BYAc
S13	Mudamuckla	80°C	H45	Rhizosheath	80°C	BYAcl
S14	Avon	Chitosan	H45	Root pieces	None	BYAsp1%
S15	Minnipa	80°C	H45	Root pieces	80°C	BYA1%
S16	Netherton	80°C	H45	Root pieces	80°C	BYA1%
S17	Minnipa	80°C	H45	Rhizosheath	80°C	BYA
S18	Netherton	80°C	Yallaroi	Root pieces	80°C	BYA1%
S19	Avon	80°C	Triticale	Root pieces	80°C	BYA1%
S20	Waikerie	none	H45	Rhizosheath	None	BYA-TSM
S21	Mudamuckla	none	H45	Rhizosheath	None	BYA-TSM
S22	Mudamuckla	none	H45	Rhizosheath	None	BYA-TSM
S23	Minnipa	80°C	H45	Root pieces	80°C	BYA1%
S24	Waikerie	80°C	H45	Root pieces	80°C	BYA1%

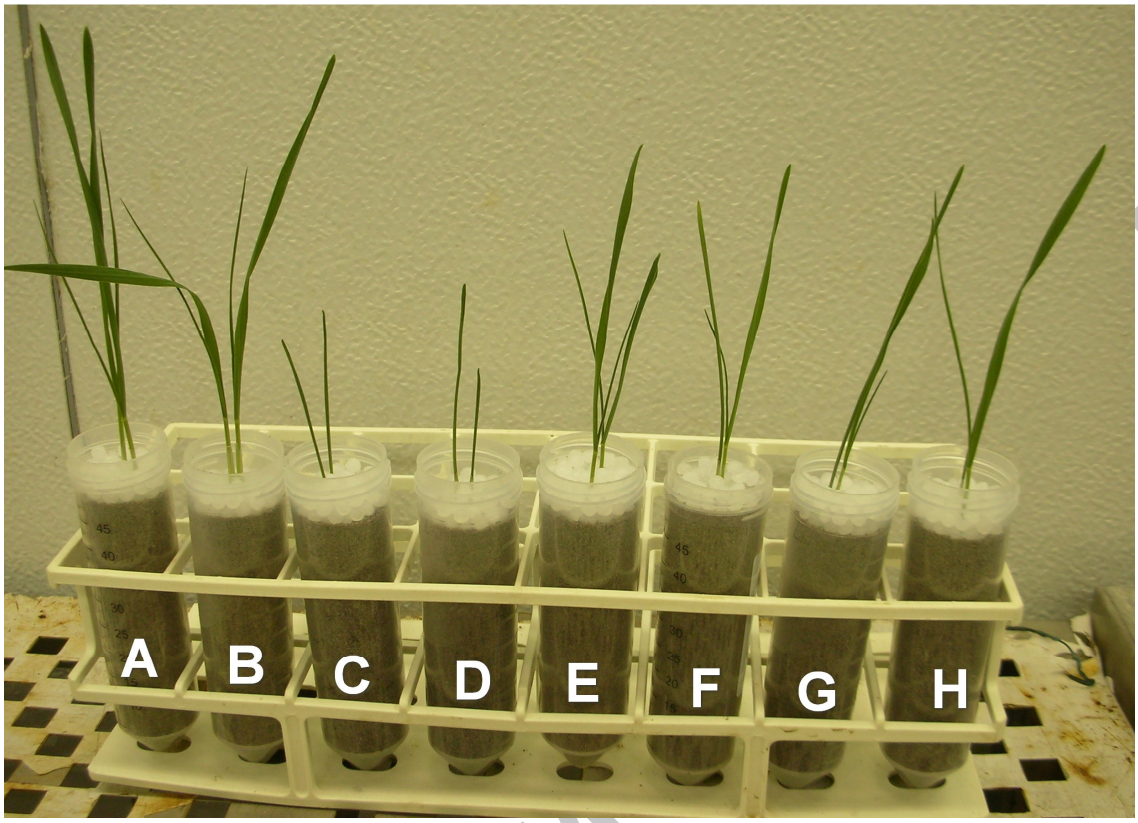
Table 9b. Source and isolation methods for culture collection strains. Strains isolated from root, tuber or leaf were isolated as endophytes. Media ingredients are given in Kaewkla and Franco, 2013: TWYE, tap water yeast extract agar; VL70 gellan gum medium with Pec, pectin; AA, amino acids: GGXA D-galacturonate, D-glucuronate, L-ascorbate and D-gluconate; GGXA, D-glucose, Dgalactose, D-xylose and L-arabinose; HV, Humic acid vitamin B medium; HVG, Humic acid vitamin B medium with gellan gum.

Strain	Source location	Host or soil location	Isolation location	Agar medium
F1	Pinnaroo	Potato	Tuber	TWYE
F2	Bedford Park	*Callitris preissii* (native pine)	Root	VL70 Pec
F3	Bedford Park	*Eucalyptus microcarpa* (Grey Box)	Root	VL70 AA
F4	Bedford Park	*Eucalyptus camaldulensis* (red gum)	Root	VL70 Pec
F5	Peterborough	Soil	Bulk soil	HV
F6	Peterborough	Soil	Bulk soil	HV
F7	Bedford Park	*Eucalyptus microcarpa* (Grey Box)	Root	VL70 GGAG
F8	Peterborough	Soil	Bulk soil	HV
F9	Bedford Park	*Pittosporum phyliraeoides* (native apricot)	Root	VL70 GGXA
F10	Peterborough	Soil	Bulk soil	HV
F11	Bedford Park	*Callitris preissii* (native pine tree)	Root	VL70 Pec
F12	Bedford Park	*Callitris preissii* (native pine tree)	Root	VL70 AA
F13	Bedford Park	*Pittosporum phyliraeoides* (native apricot)	Root	VL70 Xyl
F14	Pinnaroo	Potato	Leaf	TWYE
F15	Peterborough	Soil	Bulk soil	HV
F16	Peterborough	Soil	Bulk soil	HV
F17	Peterborough	Wheat	Root	HV
F18	Peterborough	Soil	Bulk soil	HV
F19	Bedford Park	*Eucalyptus microcarpa* (Grey Box)	Root	HVG

Selection of microbes for Rhizoctonia control



Selection of microbes for Rhizoctonia control



Selection of microbes for Rhizoctonia control

Highlights

- Screening for biocontrol of Rhizoctonia root rot on wheat in a 3-phase system
- High throughput *in planta* screening method developed
- 2310 strains assessed directly in a plant-pathogen-soil system
- 185 strains assessed in replicated pot bioassay to confirm efficacy
- 43 strains showed greater efficacy compared to our current best biocontrol strains

Selection of microbes for Rhizoctonia control