Brachypodium distachyon is a pathosystem model for the study of the wheat disease Rhizoctonia root rot

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***Brachypodium distachyon* is a pathosystem model for the study of the wheat disease Rhizoctonia root rot**

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Key words: Brachypodium, *Rhizoctonia solani*, plant-pathogen interaction

Abstract

Brachypodium distachyon (Bd) is increasingly being used as a model for cereal diseases and to study cereal root architecture. *Rhizoctonia solani* AG 8 is a necrotrophic root pathogen that infects wheat soon after germination resulting in reduced plant growth and yield loss. Genetic resistance to *R. solani* AG 8 is not available in commercial wheat cultivars, although some quantitative levels of resistance have previously been found in mutant lines and grass relatives. Resistance mechanisms in cereals remain unknown. The ability to use Bd as a model to study the wheat - *R. solani* AG 8 pathosystem was investigated. The results presented show that Bd is susceptible to *R. solani* AG 8 and that the pathogen infects both species to a similar degree, producing comparable disease symptoms. Root length reduction was the primary indicator of disease, with shoots also affected. The second objective was to develop a repeatable phenotyping method to screen Bd populations for resistance to *R. solani* AG 8. Results of a preliminary experiment provide evidence for variation in resistance

between Bd inbred lines. This is the first report showing the potential of Bd as a model plant for discovery of quantitative genetic variation in resistance to a necrotrophic cereal root pathogen.

Introduction

Brachypodium distachyon (Bd), a grass in the Pooideae subfamily of the Poaceae, originated in and around the Fertile Crescent, the region where other members of the Pooideae (wheat, barley and rye) were domesticated (Salamini et al., 2002; Vogel et al., 2006; Watt et al., 2009). Bd was proposed as a model plant for the cereals by Draper et al. (2001) because it has characteristics required to be a successful model. The species is self-fertile and inbreeding; has a short life-cycle of approximately 3 – 4 months; is small in size, reaching approximately 20 cm; and can be transformed. At 272 Mb, the genome size of Bd is only slightly larger than that of Arabidopsis, but far smaller than the approximately 17,000 Mb hexaploid bread wheat genome (International Brachypodium Initiative, 2010). Draper et al. (2001) also recognised the value of the presence of a range of susceptibility and resistance phenotypes in the natural accession population to two fungal diseases, rice blast (*Magnaporthe grisea*) and wheat/barley yellow stripe rust (*Puccinia striiformis*). Since being proposed as a model, the Bd genome has been sequenced (International Brachypodium Initiative, 2010) and several Bd resources have become publically available, including sequenced inbred natural accessions and T-DNA insertion mutant lines (Bragg et al., 2012).

Bd is now being used to study several foliar fungal diseases of cereals. Genetic variation in resistance has been found in the Bd natural accession collection for the hemibiotrophic pathogens eyespot (*Oculimacula* spp.), ramularia leaf spot (*Ramularia collo-cygni*) and head blight (*Fusarium* spp.) (Peraldi et al., 2013); biotrophic stem, leaf and stripe rusts (*Puccinia* spp.) (Ayliffe et al., 2013), and rice blast (Draper et al., 2001). Genetic variation in resistance

to necrotrophic fungal diseases in Bd accessions has not yet been demonstrated, although Bd is known to be susceptible to *Pythium* root rot (Vogel & Bragg, 2009) and is being used to study crown rot (*Fusarium pseudograminearum*) (Kazan et al., 2012). While there is also no reported evidence of genetic variation in Bd resistance to root diseases, the species has been shown to be a useful model for the study of root traits in monocots (Watt et al., 2009; Chochois et al., 2012). The small stature of Bd allows it to be grown in pots to a later stage than wheat, before roots become crowded and unsuitable for analysis. The internal root structure and root system architecture of Bd is similar to other cereals, and markedly different from that of the common dicot model Arabidopsis.

The wheat disease known as Rhizoctonia barepatch or Rhizoctonia root rot is caused by the basidiomycete fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* [Frank] Donk), evident in the field as patches of stunted wheat. The necrotrophic pathogen invades seedling roots, leading to disintegration of the root cortex and characteristic spear tipping (MacNish & Neate, 1996). It was only in 1985 that *R. solani* isolates responsible for disease in wheat were allocated the specific anastomosis group *R. solani* AG 8 (Wall et al., 1994). The pathogen is still being discovered in wheat fields of different countries and was recently found in Turkey (Ünal & Dolar, 2012), the origin of many characterized Bd natural accessions (Chochois et al., 2012).

Incidence and severity of Rhizoctonia root rot in Australia has fluctuated over the past century, largely due to changes in tillage practices and machinery design, with soil cultivation reducing disease (MacNish & Neate, 1996). In the Pacific Northwest of the USA the increase in Rhizoctonia root rot concomitant with reduced tillage leaves farmers reluctant to adopt direct seeding, even though the benefits include reduced soil erosion, fuel use and labour costs (Schroeder & Paulitz, 2008). Some control of Rhizoctonia root rot can be achieved by

using fungicidal seed treatment, fertilizer and by ensuring a two to three week weed-free fallow prior to sowing.

Availability of *R. solani* AG 8 disease resistance in wheat would be of particular benefit to farmers in marginal regions where tillage is not a suitable management practice; however there is little evidence of resistance in wheat or Pooid cereals, such that no *R. solani* resistance traits are available for commercial breeding programmes. Recently a mutant wheat line with heritable increased tolerance to *R. solani* AG 8 was generated by Okubara et al. (2009), while Okubara and Jones (2011) demonstrated the ability to transfer a partial increase in resistance to *R. solani* AG 8 to Chinese Spring wheat, in addition lines with chromosome 4 from *Thinopyrum* spp., speculating that the shortening and thickening of diseased roots could indicate the involvement of the hormones jasmonic acid and ethylene in the defence response. Ethylene signalling, mediated by ethylene responsive transcription factors, is involved in the defence response of the legume *Medicago truncatula* to *R. solani* AG 8 (Anderson et al., 2010). However the mechanisms controlling *R. solani* AG 8 disease resistance in grasses are still largely unknown.

Finding quantitative phenotypic variation in resistance to *R. solani* AG 8 in Bd natural accession or mutant collections would allow investigation into genetic regions linked to resistance in Bd and in orthologous regions in wheat, or the discovery of regions not present in cultivated wheat genomes that could be introduced via marker-assisted breeding or transformation (Chochois et al., 2012).

We tested the hypothesis that Bd is susceptible to *R. solani* AG 8, as would be expected given the broad host range of the pathogen (MacNish & Neate, 1996). We developed a quantitative phenotyping method to compare resistance to *R. solani* AG 8 between Bd accessions and show that Bd can be successfully used as a model for this necrotrophic root pathogen.

Materials and methods

Brachypodium distachyon seed sources

Brachypodium distachyon accessions were donated by Dr Iain Wilson (CSIRO Plant Industry, Canberra, ACT, Australia), from the collections of Drs David Garvin (USDA-ARS, University of Minnesota, St Paul, MN, USA) and John Vogel (USDA-ARS, Albany, CA, USA). *Triticum aestivum* cv. Janz is a commercial Australian spring wheat available from the Australian Winter Cereals Collection. In order to synchronise germination, seeds were surface sterilized following the method of Alves et al. (2009). The procedure was performed at the bench. Seeds were moved from the final rinse water onto 3% water agar plates and incubated in a sealed container in the dark at 22°C. Seeds germinated overnight and were planted the day after surface sterilization. Wheat seeds were surface sterilized by the same method.

Fungal isolate and inoculum preparation

The isolate of *Rhizoctonia solani* AG 8 ZG1-1 (WAC10335) used in this study was donated by Dr Jonathan Anderson (CSIRO Plant Industry, Perth, WA, Australia). Millet inoculum was prepared according to the method of McDonald and Rovira (1985) and ground after air-drying to reduce propagule size. White millet seed (90 g, approximately 18 000 seeds; Kialla Pure Foods Pty Ltd) was soaked overnight in Milli-Q water in a 500 mL Erlenmeyer flask. Excess water was tipped off, the flask capped with aluminium foil, then autoclaved for 1 h at 121°C on three successive days. Millet was well shaken after each autoclaving to ensure grains did not clump. *Rhizoctonia solani* was grown on potato dextrose agar (PDA), of which 3 cm^2 surface area was cut into small squares and added to a flask of sterile millet. Millet inoculum was incubated at 25°C for 10 d and dried in a laminar flow hood for 2 d. The dry millet inoculum was ground in a coffee mill, then sieved through screens, retaining

the 0.5 – 2.0 mm fraction. Millet inoculum was stored for several years at -20°C in sealed 50 mL Falcon tubes without affecting pathogenicity, as evidenced by the same level of inoculum resulting in similar levels of root reduction over the years. Stored *R. solani* millet inoculum viability was initially calculated to be approximately 4000 propagules per gram. Propagule number was measured by shaking 200 mg inoculum in 0.1% water agar (100 mL) for 15 min. An aliquot (1 mL) was transferred to a ¼ PDA plate using a wide-bore pipette tip and spread evenly across the plate. Plates were incubated overnight at 22°C and colonies counted the following day.

Soil inoculation and growth conditions

The soil used for these experiments was a blend of 80% compost and 20% perlite, prepared by the CSIRO Plant Industry potting shed. This potting mix was used as it promotes good growth of Bd and wheat in control treatments, as well as allowing development of Rhizoctonia root rot in infested treatments. While the potting mix is not really comparable with Rhizoctonia root rot-producing field soils, its properties are more suitable for pot experiments, e.g. allowing good drainage, less likely to contain soil-borne pathogens, ease of washing out roots for scanning. The compost was prepared from a mix of recycled soil, leaf mulch, river loam, peat moss, perlite, vermiculite, river sand, straw and fertilizers. Potting mix was sieved to remove coarse bark and stones, then steam-sterilized.

As Bd is known to be susceptible to a common pathogen of potting mix, *Pythium* spp. (Vogel & Bragg, 2009), soil was tested for contamination using a modified method of *Pythium* enumeration from soil on VP3 *Pythium*-selective medium (Pankhurst et al., 1995). Briefly, 1 g of soil was shaken for 15 min in 0.1% water agar (100 mL). An aliquot (1 mL) was transferred onto VP3 medium using a wide-bore pipette tip and spread evenly across the plate. Plates were incubated at 22°C for 3 d. No *Pythium* colonies grew from two replicates

of soil, each with five internal plate replicates. The presence of *Rhizoctonia* spp. was tested using a toothpick re-isolation check based on the method of Paulitz and Schroeder (2005) to ensure that there was no contamination with the fungus in control treatments, as described below.

The cone inoculation system for studying *R. solani* disease on cereal roots is based on the method of Okubara et al. (2009). Plants were grown in narrow cone-shaped pots composed of low density polyethylene (LDPE, 21 cm x 3.8 cm diameter, 164 mL) placed in a 7 x 14 hole tray within a medium flow tray (Stuewe & Sons). Metal plates manufactured by the CSIRO workshop were used to raise the tray by 2 cm, to ensure the bases of cones did not contact drained water. Cones were plugged with a cotton ball, prepared and inoculated on the day of planting.

Millet inoculum was added to soil at an approximate concentration of 0.1 propagules per gram (ppg) dry soil and mixed manually for 10 min to distribute the inoculum evenly. Each cone was filled with inoculated soil (~116 g) and tapped down lightly to give a bulk density of approximately 0.85 g cm^{-3}. All cones were watered to near saturation with tap water (25 mL). A germinated seed was then placed at the soil surface and gently covered with uninoculated potting mix (~20 cm^3) as shown in Figure 1a. Control pots were prepared as for *R. solani* infested treatments, except no inoculum was added. In one experiment a second, higher, inoculum level of 1 ppg was also used.

Cones were incubated in a growth cabinet (Conviron CMP 2023) programmed with 12 h days with cool white fluorescent light (approximately 200 µE m^{-2} s^{-1}) at a constant temperature of 16°C. Day length and temperature conditions were chosen to approximate field conditions near Harden, NSW, during the wheat sowing period. These conditions gave good growth of Bd in uninoculated treatment and strong *R. solani* infection at low levels of inoculum. The shorter day length ensured that all Bd lines remained in the vegetative growth phase for the

duration of experiments (Vogel et al., 2006). Cones were watered with tap water the day after sowing and then every two to four days, as required. All cones received the same volume of water. Plants were removed from pots at harvest and roots rinsed gently, before being stored with intact roots and shoots in ethanol (50% v/v) in plastic containers.

Median emergence date was between three and five days after planting (DAP) for all genotypes. Plants that did not emerge from the soil surface within seven days of planting were excluded from analysis.

Re-isolation of *Rhizoctonia* spp. from soil

A modified *Rhizoctonia* toothpick bait method was used to detect *Rhizoctonia* spp. in the top layer of soil, and check levels of *R. solani* AG 8 in treated and control cones. White birchwood splinter toothpicks (approximate length 57 mm, Alpen Products) were soaked in tap water and autoclaved. After watering at 8 DAP, a toothpick was inserted into each cone to a depth of 4 – 5 cm, with a centimetre left exposed to grip with forceps (Figure 1b). After 24 h the toothpick was removed and placed onto Ko and Hora selective media, modified after Gill et al. (2000). Four toothpicks were placed onto each plate, as shown in Figure 1c. Plates were placed into a sealed box and incubated in the dark overnight at 22°C. Each toothpick was scored for *Rhizoctonia* hyphal growth, with 0 = no hyphae; 1 = sparse hyphae, < 10% coverage; 2 = definite, but patchy hyphae, 10 - 80% coverage; 3 = dense hyphae with agar browning, >80% coverage. Control treatments with toothpick scores of 1, 2 or 3 were excluded from analysis, due to possible *R. solani* contamination. Inoculated treatments with toothpick scores of 0 or 1 were excluded from analysis, due to low or no inoculum present near the base of the plant.

Phenotype measurements

Root length measurements and disease severity ratings are the best indicators of resistance to *R. solani* in wheat experiments (Okubara et al., 2009). As Bd generally produces only one primary root (Watt et al., 2009), a visual disease score is limited to a single root and may thus be a less effective disease severity measure for Bd than wheat, which produces three to five primary roots. Root length was chosen as the major indicator of *R. solani* disease severity.

For total root length measurement, roots were removed from ethanol (50% v/v) and adhering particles gently removed in water using plastic forceps and by gently brushing with gloved fingers. Roots were stained to enhance contrast for 3 min in 0.05% toluidine blue solution (pH 4.4), rinsed well in water and scanned at 400 dpi on an Epson Expression 1680 flatbed scanner. WinRHIZO™ software (Regent) was used to calculate total root length. Emerged nodal roots were counted from root scans.

Leaf number was measured on the day prior to harvest using Haun's scale, which describes the length of an emerging main stem leaf as a decimal fraction of the length of the previous fully emerged leaf (Haun, 1973).

Length of leaves 1 and 2 were measured using a ruler on the day prior to harvest. Leaf length was measured from the crown, which forms at the soil surface, to the leaf tip (Supplemental Figure 1).

Leaf area measurements were made on leaves that had been softened by storing for over a week in ethanol (50% v/v). After removal from ethanol, each blade was cut at the ligule, patted dry and smoothed out onto the surface of a sheet of transparency film with a small amount of petroleum jelly. Blades were scanned immediately at 200 dpi on an Epson Expression 1680 flatbed scanner. Images were converted to binary and leaf area calculated using ImageJ 1.43u software (National Institutes of Health), as described in Supplemental Figure 2.

225 **Experimental design**

226 Three independent experiments were set up in randomized block designs to test different
227 hypotheses.

228 The first experiment compared phenotypic responses to *R. solani* AG 8 between Bd and
229 wheat. Five plants were sown into inoculated (0.1 ppg *R. solani* AG 8) or uninoculated
230 control treatments in adjacent positions within a flow tray, with treatments replicated across
231 three flow trays. After removal of unemerged plants and those that failed the *Rhizoctonia* re-
232 isolation check, between two and five plants per treatment remained in each tray for analysis,
233 i.e. a total of eight to 15 plants per treatment. Wheat was harvested at 14 days after planting
234 (DAP) at around the 2.8 leaf stage and Bd harvested at 20 DAP at around 3.0 leaves. Leaf 1
235 and leaf 2 were fully expanded at harvest for both species.

236 In the subsequent experiments three replicate pots were sown per treatment in adjacent
237 positions in a flow tray. Each treatment was randomly allocated once per flow tray, with two
238 trays in total. A total of five to six plants per treatment remained for analysis. Treatments for
239 the experiment measuring the effect of *R. solani* over time were days to harvest (10, 14, 18,
240 22 and 26 days) and inoculum (control and 0.1 ppg *R. solani* AG 8). Treatments for the
241 experiment measuring genetic variation in resistance of Bd to *R. solani* were Bd accession
242 and inoculum (control, 0.1 ppg and 1 ppg *R. solani* AG 8).

243

244 **Statistical analysis**

245 Statistical analysis was carried out using a residual maximal likelihood (REML) model in
246 GenStat (VSN International). The REML model was chosen as it is appropriate for analysis
247 of unbalanced data sets (Robinson, 1987). Data sets could become unbalanced when samples
248 were removed from analysis, either because the plant did not emerge within seven days or if
249 the toothpick re-isolation check failed.

In some cases the variance component of the random model term was negative. This generally occurred when variation for a treatment between samples within a tray was greater than the variation between trays (Fletcher & Underwood, 2002). In these cases the random factor was set to zero.

Total root length was square root transformed prior to analysis for the experiment investigating genetic variation in resistance in Bd to improve distribution of residual variance resulting from large differences in root growth between control and inoculated treatments. Shoot measurements did not require transformation, as the residual variance was constant. Standard errors and least significant differences at 5% were calculated in Genstat. Bd and wheat were analysed separately, due to large differences in magnitude between measurements in the two species.

Results

Bd and wheat have a similar phenotypic response to *R. solani* AG 8

The disease phenotypes of five genotypically diverse accessions of Bd were compared, along with that of bread wheat cv. Janz. Means of untransformed root and shoot measurements in *R. solani* inoculated and control treatments were predicted using a linear regression model. These measurements and *R. solani*/control ratios are presented in Table 1. The isolate of *R. solani* AG 8 infected all five lines of Bd and wheat. Amplification of Bd root DNA extracts using primers specific for *R. solani* AG 8 5.8S rDNA (Foley et al., 2013) confirmed the presence of the pathogen within infected roots (data not shown). At an inoculum level of 0.1 ppg, total root length was reduced to an average 49% of control in infected Bd ($P < 0.001$) and 39% of control in wheat ($P < 0.001$). Leaf number was reduced to an average 96% of control in infected Bd ($P = 0.023$) and 95% of control in wheat ($P = 0.007$). Leaf 1 length was not significantly affected by *R. solani* in either Bd or wheat. A difference between

the two species was that wheat leaf 2 length in *R. solani* treatment was significantly reduced to 89% of the control ($P = 0.001$), whereas mean Bd leaf 2 length was not significantly reduced by *R. solani*, despite both species being harvested when leaf 2 was fully expanded. Overall, *R. solani* AG 8 has a similar effect on root and shoot measurements in Bd and wheat, with *R. solani*/control root and shoot ratios for wheat cv. Janz falling within the observed range of ratios for the five Bd lines tested. The level of root truncation and reduction in shoot growth by *R. solani* at 0.1 ppg in both species can be seen in Figure 2.

R. solani AG 8 affects root and shoot growth over time

Root and shoot measurements were taken at five time-points for line Bd 21-3 between 10 and 26 days after planting (Figure 3). Reduced root length in the inoculated treatment was significant from 10 DAP ($P = 0.013$), becoming highly significant ($P < 0.001$) from 14 DAP onwards. The difference in leaf number due to inoculum became significant at 18 and 22 DAP ($P < 0.05$) and highly significant at 26 DAP ($P < 0.001$). Leaf area was also measured in this experiment, with the difference between the *R. solani* and control treatments becoming significant at 18 DAP ($P = 0.004$) and then highly significant from 22 DAP ($P < 0.001$) onwards. In summary, total root length, leaf number and leaf area continue to increase and become more significantly different between control and *R. solani* treatments over time. These measurements all became effective indicators of *R. solani* damage from 18 DAP onwards. On the other hand, leaf 1 and 2 lengths were significantly different ($P < 0.05$) between treatments only at 22 DAP in this data set. Leaf 1 had fully elongated by 10 DAP, while leaf 2 was fully elongated by 14 DAP. Once leaves had reached their maximum length, there was little change in leaf length over time. Thus, a difference in length of leaf 1 or leaf 2 in *R. solani* treatment compared with the corresponding leaf length in the control treatment,

would indicate an effect of the fungus prior to 10 DAP and 14 DAP, respectively, under these conditions.

In wheat cv. Janz the difference in root length increase and leaf appearance rate between inoculated and control treatments was similar to Bd (Figure 4). The endogenous growth of wheat was greater than Bd, with total root length of the control wheat treatment four times greater than that of Bd by 18 DAP. The rate of development of wheat was also slightly faster than for Bd, with leaf numbers at 18 DAP for wheat and Bd control treatments at 3.7 and 3.3, respectively.

Plotting the relationships between leaf area and total root length of Bd (Figure 5) reveals a higher shoot-to-root ratio in *R. solani* treatments than in the control. The allometric relationship in control and *R. solani*-infested conditions between more and less resistant lines of Bd may provide clues towards root and shoot responses to disease.

Overall, nodal roots were more abundant in inoculated treatment than control treatment ($P < 0.001$) but data was too variable to find significant differences at single time points (Figure 3e).

Preliminary evidence of genetic variation in resistance to *R. solani* AG 8 in the Bd natural accession collection

An experiment to test the effect of two levels of inoculum (0.1 and 1 ppg) revealed a significant ($P < 0.05$) variation in the plant-pathogen interaction between Bd accessions (Table 2). The ratio of *R. solani*/control root length is used as a comparative measure of resistance to the fungus, with values square root transformed prior to analysis. Line Koz-3 had the highest *R. solani*/control root length ratio, indicating the greatest level of pathogen resistance. The root length ratio of Koz-3 was significantly ($P < 0.05$) greater than the ratio for lines Bd 21, BdTR 13c and Koz-1 at both levels of inoculum. Some *R. solani*/control

shoot measurement ratios were also reduced compared with Koz-3, but to a lesser degree than root length.

At the higher level of inoculum toothpick colonisation at 8 DAP was noticeably increased, with hyphae often visible at the junction between the toothpick and the soil surface. The higher inoculum level revealed a greater number of significant ($P < 0.05$) plant-pathogen interactions between Koz-3 and other Bd lines than the 0.1 ppg treatment, but the number of plants available for analysis was reduced with two plants dying during the course of the experiment. Pre-emergent damping off was never observed with this inoculation method, with no significant differences between numbers of emerged plants in control and *Rhizoctonia* treatments.

Using untransformed values, at the lower level of inoculum the most resistant line in this experiment, Koz-3, maintained 55% of control root length in *R. solani* treatment, whereas the most susceptible line, Bd 21, maintained only 19% of control root length.

Discussion

The aims of this study were to learn whether *R. solani* AG 8 has the same pathogenic effect on Bd as on wheat and to develop a phenotyping method to compare the response of different lines of Bd to *R. solani* AG 8, in order to find lines differing in resistance to the pathogen. Visual symptoms of Rhizoctonia root rot in Bd were similar to those described in wheat, including primary root truncation and rotting of cortical tissue leading to spear tips (MacNish & Neate, 1996). A visual disease score is commonly used in wheat and other species to describe the level of primary root truncation and root system necrosis with *R. solani* (McDonald & Rovira, 1985). Despite its wide use however, the sensitivity of visual disease scores in place of quantifiable measurements has been questioned (Smith et al., 2003). With

only one primary root and a smaller root diameter than wheat (Watt et al., 2009), scoring visual disease symptoms in Bd is more difficult than in wheat.

Total root length is considered a suitable phenotypic measurement of disease severity (Okubara et al., 2009), as the primary effect of *R. solani* is root rot leading to root length reduction. Of the root and shoot measurements taken from Bd accessions and wheat cv. Janz harvested at a similar stage of development, at respective average leaf stages 3.0 and 2.8, root length reduction was the largest effect produced by *R. solani* treatment, as expected. Root length reduction was comparable between Bd accessions and wheat. The range of *R. solani*/control root length ratios (0.30 – 0.68) for Bd grown at 0.1 ppg *R. solani* AG 8 overlaps with the ratio of 0.4 recorded by Kirkegaard et al. (1999) for wheat cultivar 'Dollarbird' in high *R. solani* AG 8 inoculum, and the ratio range of 0.53 – 0.92 seen across different levels of *R. solani* AG 8 inoculum for wheat cultivars 'Scarlet' and 'Scarlet-Rz1' by Okubara et al. (2009).

A smaller but still significant ($P < 0.05$) effect of *R. solani* was seen on the leaf appearance rate (measured as leaf number at harvest), with the fungus reducing leaf number in Bd and wheat to 0.96 and 0.95 of control, respectively. Masle et al. (1989) showed that temperature and day length are major determinants of leaf appearance rate in wheat. These factors also have a strong effect on leaf appearance rate in Bd (Watt et al., 2009). Abiotic root stresses, including high soil strength and nutrient deficiency, can reduce leaf appearance rate in wheat and barley (Masle et al., 1989; Prystupa et al., 2003), while infection with *R. solani* AG 8 in wheat can affect final leaf number and relative leaf expansion rate (Wall et al., 1994; Kirkegaard et al., 1999).

Looking at root and shoot growth of Bd over time, total root length was the most sensitive indicator of *R. solani* disease, followed by leaf number and leaf area. Root length was significantly ($P < 0.05$) lower in infested treatments at all harvest time-points between 10 and

26 days after planting, with the difference increasing over time, while a significant ($P < 0.05$) reduction in leaf number and leaf area was only seen from 18 DAP onwards, two harvest time-points after the first observed significant reduction in root length. Shoot effects can occur in response to reduced root system length, systemic wounding responses or pathogen effectors that directly affect shoot growth, with disease responses seen in leaves expected to be smaller than root effects (Kirkegaard et al., 1999). Knipfer and Fricke (2011) speculated that in conditions where reduction of root system size does not affect transpiration, the explanation for reduced leaf growth rate and leaf lengths may be either due to cumulative effects of reduced solute supply or hormone-driven regulation of root to shoot biomass ratios. Leaf length measurements can potentially give information on early infection. Length of leaf 2 was more affected than length of the first true leaf, indicating that the pathogen had less impact at the stage when plants were growing on seed reserves (James et al., 1997). In fact, even though *R. solani* AG 8 is a disease of seedlings and may cause damping off (Ünal & Dolar, 2012), reduced leaf 1 length is not generally regarded as a disease symptom. Under these experimental conditions soil is inoculated on the day of sowing and seeds have already germinated at sowing. At 16°C *R. solani* hyphae extend from the inoculation point at a rate of approximately 5.5 mm d^{-1} (Refshauge, 2007), so it is expected that there would be some lag between sowing and roots contacting *R. solani* hyphae.

The ratio of root to shoot biomass varied between treatments, but remained constant over the measured range. Plants in *R. solani* treatment maintained over 50% more shoot biomass per unit of root biomass than plants in control treatments. The greater allocation of biomass to the leaves in the diseased treatment could be due to a physical stress (Poorter et al., 2012) or may simply illustrate that roots are rotted away faster than they can be replaced to regain the allometric balance of the control treatment.

An increase in nodal root emergence was seen in *R. solani* inoculated line Bd 21-3 across all time-points, from 10 to 26 DAP. This trend of increased nodal root emergence with *R. solani* AG 8 disease has previously been observed by Schroeder and Paulitz (2008) in barley, but the mechanism driving nodal root emergence remains unknown. The role of nodal roots in a plant's ability to survive Rhizoctonia root rot and maintain yield would be an interesting area for further research, e.g. by carrying out Rhizoctonia inoculation experiments with Bd in larger 0.5 m tall pots used by Chochois et al. (2012), in which plants are grown to maturity to measure the final distribution of primary and nodal roots.

A major aim of using this Bd pathosystem is to find QTL for increased resistance to *R. solani* AG 8 that can lead the improvement of wheat germplasm. In order to compare root growth between Bd accessions we developed a phenotyping method using a level of inoculum (0.1 ppg) that did not reduce emergence rates or cause post-emergent damping off. In a preliminary experiment with a small subset of the Bd natural accession collection we found quantitative levels of genetic variation in resistance to *R. solani* AG 8. Further screening of the collection may reveal a greater range of reproducible quantitative resistance that can be used to discover genetic traits linked to resistance.

Links between *R. solani* AG 8 disease resistance phenotype and genotype have been made in *Medicago truncatula* and *Arabidopsis* (Anderson et al., 2010; Foley et al., 2013). With Bd already a useful model for the study of several fungal diseases, we now have an opportunity to explore durable genotypic resistance to *R. solani* AG 8 in a grass with a high level of synteny to small-grain crop cereals (Peraldi et al., 2013). Approaches to gene discovery in this model system are discussed by Chochois et al. (2012). Lines differing in resistance to *R. solani* AG 8 could be crossed to generate a recombinant inbred line population for further screening and mapping of QTL. Known genes of interest may be screened directly using T-DNA lines, followed by re-transformation with the disrupted gene to confirm the trait.

This is the first report of a Bd pathosystem with a root-infecting necrotroph. Observation of variation in quantitative genetic resistance between accessions of Bd to *R. solani* AG 8 in this study indicates that this model species may, with further research, provide clues to genes and mechanisms involved in resistance.

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Figures

Figure 1 Cone preparation and re-isolation of *Rhizoctonia* from soil. a) Cones are plugged with cotton, filled with infested soil and watered. A germinated seed is placed at the soil surface and covered with uninfested soil. b) Placement of toothpick in cone for re-isolation of *Rhizoctonia*. c) Placement of toothpicks on selective media. The two toothpicks at left were from control cones. The two toothpicks at right have *Rhizoctonia* growth scores of 3, with dense hyphal growth and agar browning (white arrows).

Figure 2 Effect of *R. solani* AG 8 on root and shoot growth of a) *B. distachyon* line Bd 3-1 and b) wheat cv. Janz, at 18 days after planting. All wheat roots and some Bd roots in control treatment have reached the base of the 21 cm deep cones. Scale bars, 2 cm.

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Supplemental Figure 1 Anatomical features of a Bd plant at 4.5 leaf stage (Zadoks 14, 22). Orange asterisks indicate the position of leaf ligules. Scale bar, 2 cm.

Supplemental Figure 2 Calculation of leaf area. a) Leaves were scanned at 200 dpi on a flatbed scanner; b) Using ImageJ software, greyscale images were converted to binary; c) The "Analyze Particles…" command was used to calculate the area of objects with area greater than 50 pixels.

Tables

Table 1 Phenotype measurements for Bd natural accessions and wheat cv. Janz. Predicted means of total root length, leaf 1 length, leaf 2 length and leaf number in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani*/control treatment values. Reduction of root and shoot measurements in wheat cv. Janz fall within the range *R. solani*/control ratios for the Bd accessions tested; n = 8-15 plants.

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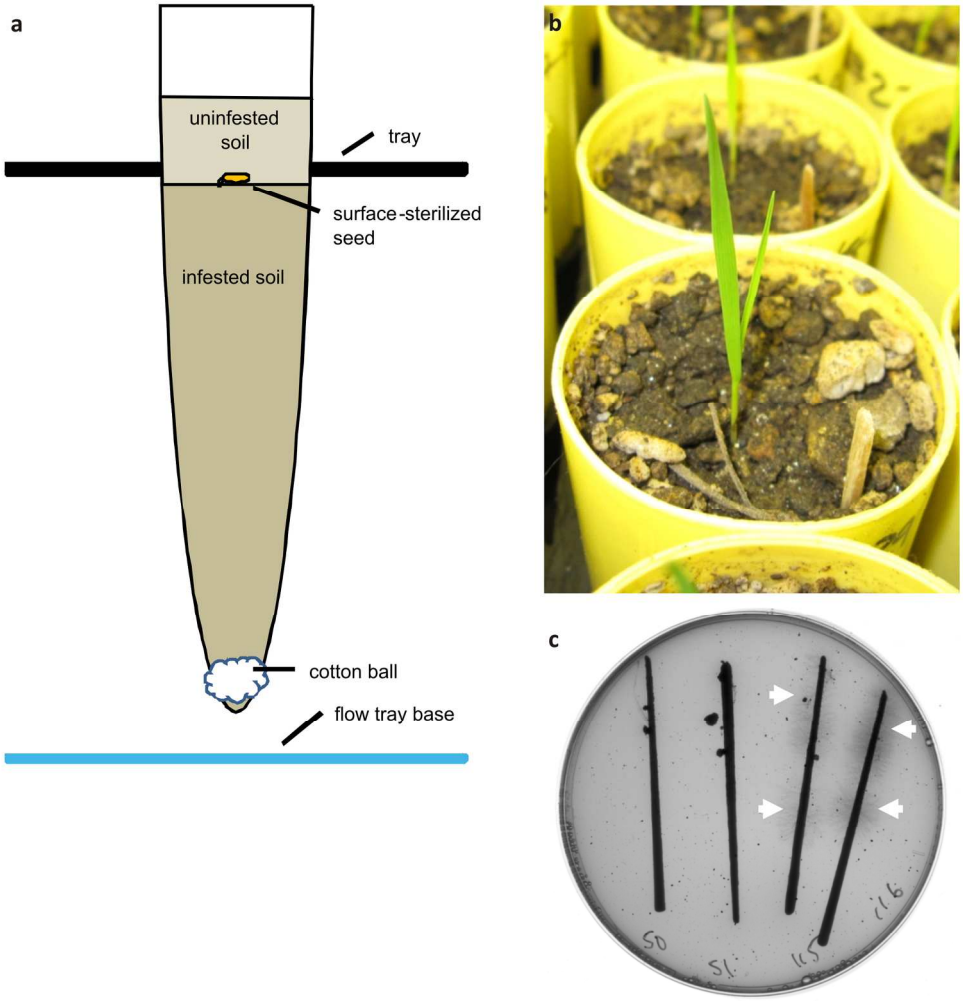

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189x200mm (300 x 300 DPI)

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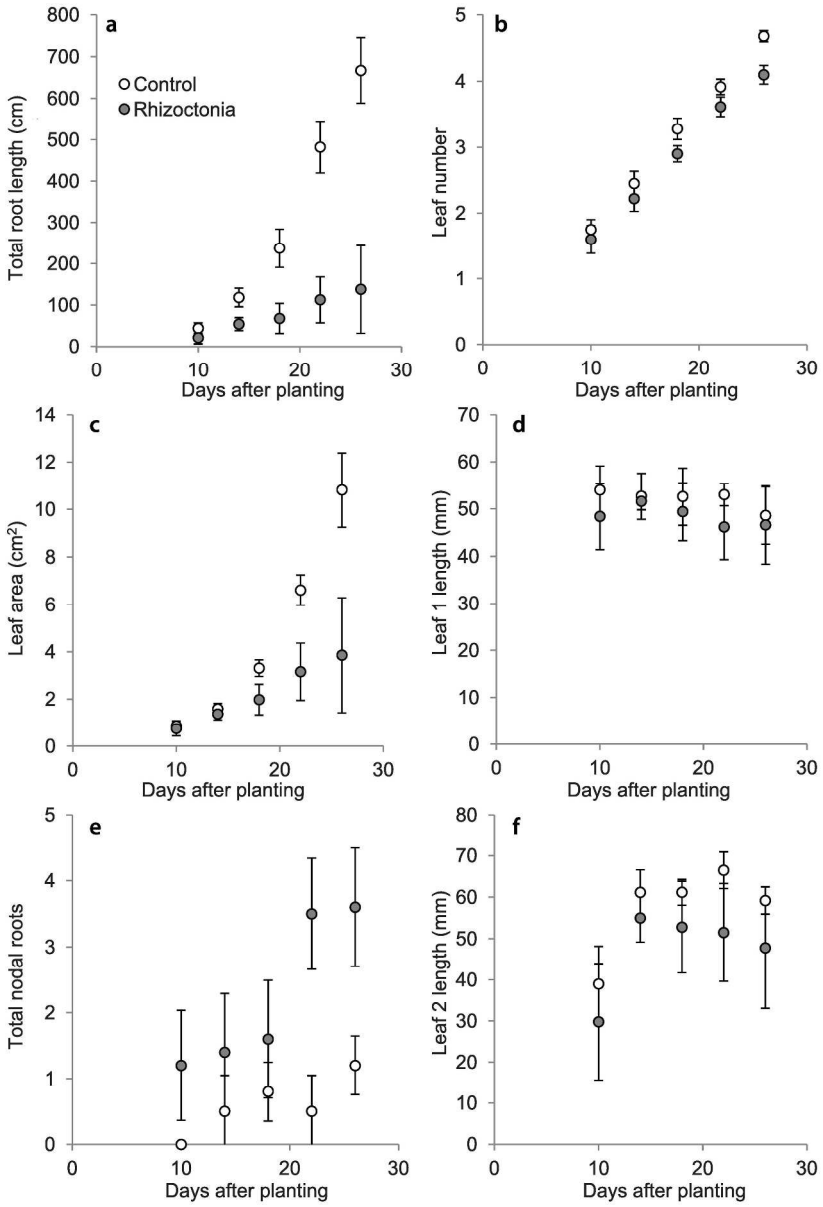
127x95mm (300 x 300 DPI)



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233x337mm (300 x 300 DPI)

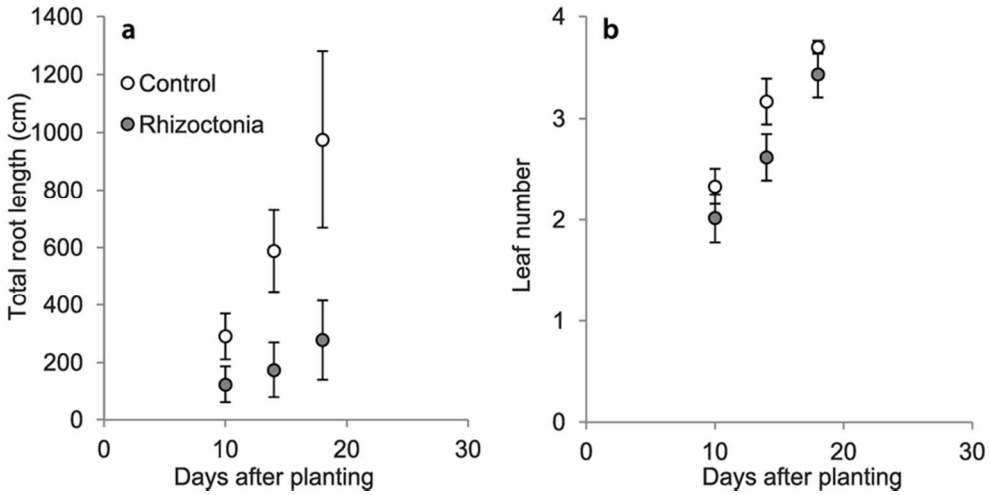

Figure 4 Wheat cv. Janz a) mean total root length, and b) mean leaf number at 10, 14 and 18 days after planting; n = 5-6, SD.
77x37mm (300 x 300 DPI)

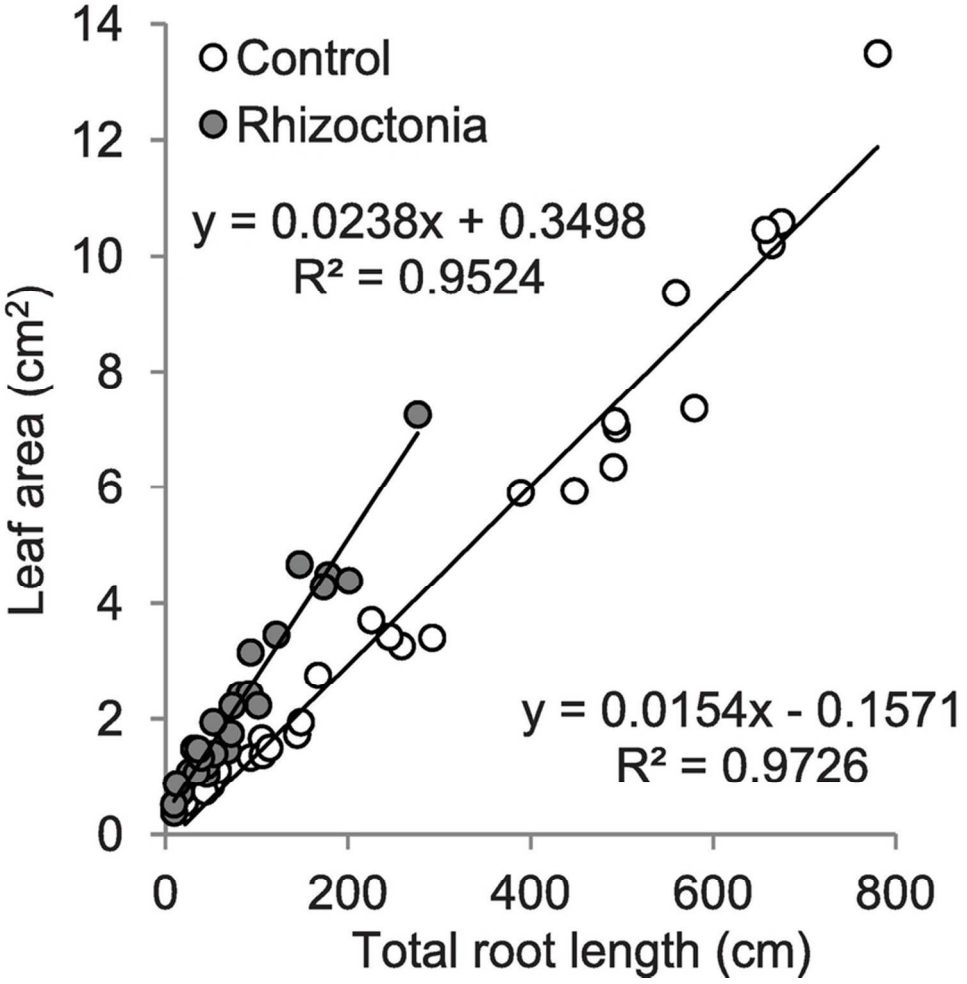

Figure 5 Correlation of total root length with leaf area over time for individual plants of line Bd 21-3. Plants were harvested at 10, 14, 18, 22 and 26 days after being sown in control (white) and *R. solani* (grey) treatments.
77x75mm (300 x 300 DPI)

Table 1 Phenotype measurements for Bd natural accessions and Janz wheat. Predicted means of total root length, leaf 1 length, leaf 2 length and leaf number in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani*/control treatment values. Reduction of root and shoot measurements in Janz fall within the range *R. solani*/control ratios for the Bd accessions tested; n = 8-15 plants.

Host	Total root length (cm)			Leaf number			Leaf 1 length (mm)			Leaf 2 length (mm)		
	Rs	C	Ratio	Rs	C	Ratio	Rs	C	Ratio	Rs	C	Ratio
Adi 10	74	162	0.46	3.1	3.3	0.94	48	48	1.01	62	65	0.96
Bd 21	91	133	0.68	2.8	2.9	1.00	49	46	1.07	64	61	1.06
Bd 21-3	62	144	0.43	2.8	3.0	0.94	55	55	1.08	66	67	0.98
Bd 3-1	46	151	0.30	2.9	3.1	0.95	40	46	0.88	54	60	0.89
BdTR 10o	84	150	0.56	3.1	3.2	0.99	44	45	0.97	63	63	1.00
Janz wheat	277	709	0.39	2.7	2.8	0.95	141	145	0.98	213	239	0.89

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Table 2 Phenotype measurements for seven Bd accessions in 0, 0.1 and 1 ppg *R. solani* AG 8 inoculated treatments. Predicted means of total root length, leaf 1 length, leaf 2 length and leaf number for seven Bd accessions. Means are followed by the ratio of *R. solani*/control treatment values in parentheses; n = 5-6 plants.

Inoculum	Host						
(ppg)	Bd 21	Bd 21-3	BdTR 13a	BdTR 13c	BdTR 3c	Koz-1	Koz-3
Total root length (√ cm)							
0	23[a]	19	19	21[a]	18	22[a]	18
0.1	10[a] (0.44)[b]	11 (0.58)	10 (0.54)	11 (0.51)[b]	12 (0.64)	11 (0.51)[b]	13 (0.74)
1	5.2 (0.23)[b]	4.9 (0.26)	3.5 (0.18)[b]	3.9 (0.18)[b]	4.1 (0.22)	4.4 (0.20)[b]	6.3 (0.36)
Leaf number							
0	3.8	3.7	4.1	4.2	3.6	4.1	3.9
0.1	3.3[a] (0.87)[b]	3.4[a] (0.91)	3.7 (0.92)	3.6 (0.87)	3.6 (0.99)	3.4[a] (0.83)[b]	3.8 (0.97)
1	3.0 (0.80)	3.0[a] (0.79)	3.1 (0.75)	3.1 (0.73)[b]	2.8[a] (0.78)	3.0[a] (0.73)[b]	3.3 (0.84)
Leaf 1 length (mm)							
0	51[a]	50[a]	42	45	50[a]	54[a]	44
0.1	44 (0.85)	45[a] (0.90)	37 (0.89)	39 (0.88)	47[a] (0.94)	45[a] (0.84)	40 (0.90)
1	34 (0.66)[b]	42[a] (0.84)	30[a] (0.73)	34 (0.77)	37 (0.74)	34 (0.62)[b]	36 (0.81)
Leaf 2 length (mm)							
0	62	63	55	61	59	62	58
0.1	44[a] (0.72)[b]	51 (0.82)	47 (0.85)	49 (0.80)	56 (0.94)	53 (0.85)	52 (0.90)
1	32 (0.52)[b]	41 (0.65)	30[a] (0.54)	33[a] (0.54)[b]	37 (0.62)	31[a] (0.49)[b]	40 (0.69)

[a] Significantly different from Koz-3 measurement at the same level of inoculum (5% least significant difference); [b] Significantly lower *R. solani*/control ratio from Koz-3 at the same level of inoculum ($P < 0.05$).

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