Genetic variation in resistance of *Brachypodium distachyon* to *Rhizoctonia solani* AG8

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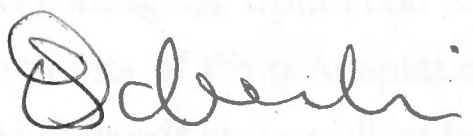
Declaration

This thesis contains my original work and has not been submitted for any other award. Any contributions made by others are acknowledged and cited in the thesis.



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Abstract

Wheat is one of the three major cereal crops, alongside rice and maize, which together supply half of the world population's food energy requirement. Wheat production is affected by climatic conditions and biotic factors including weed competition and pathogen attack. Around 18% of yield loss in Australian wheat is attributable to root diseases. Plant defence against root-invading pathogens often involves multiple quantitative resistance genes.

Rhizoctonia root rot, caused by *Rhizoctonia solani* AG8 (teleomorph *Thanatephorus cucumeris*), costs Australian farmers around $60 million per annum. The necrotrophic fungus attacks seedling roots, resulting in patches of severely stunted plants. During root invasion *R. solani* AG8 secretes a range of enzymes; however specific requirements for pathogenesis are not yet understood. Effective resistance to Rhizoctonia is not available in wheat cultivars, so farmers must rely on management techniques to control the disease.

This research aimed to discover genetic resistance to Rhizoctonia root rot in *Brachypodium distachyon*, a grass developed in recent years as a model for cereals. The species has shown strong potential as a model for wheat shoot and crown diseases, as well as cereal root architecture. Two *B. distachyon* germplasm resources were used in this project: natural accessions collected from Turkey and Spain, and a T-DNA insertional mutant collection.

Rhizoctonia solani AG8 produced similar disease phenotypes and severity in *Brachypodium distachyon* and wheat. A method developed to screen for disease resistance in the *B. distachyon* collections incorporated toothpick baits to check for the presence of inoculum and contamination in pots. The major indicator of disease severity was reduced root length, with leaf lengths and plant development rate being secondary symptoms. Resistance of different lines was ranked based on the ability of plants grown in infested soil to maintain root and shoot measurements similar to uninfested control plants.

Twenty-six genetically diverse natural accessions and 25 selected T-DNA lines of *B. distachyon* were included in replicated experiments to screen for variation in resistance to *R. solani* AG8. Greatest variation in resistance to was identified in the natural accession collection. Root length of the least resistant line was reduced to 19% of the control in *R. solani* infested soil, while the most resistant line maintained 53% of

control root length. This difference is similar to quantitative resistance levels in other grasses and wheat mutant lines described in the literature.

Exploring potential resistance mechanisms, nodal root emergence in response to infection correlated with greater resistance in an experiment with natural accessions. Increased endogenous seedling vigour was linked with lower resistance, but this factor alone did not explain all variation in resistance. Further work is required to validate increased resistance associated with a T-DNA tagged gene, *Bradi3g14370*, that encodes a putative beta-1,3-galactosyltransferase.

Brachypodium distachyon was found to be a useful model pathosystem for wheat root diseases. The variation in genetic resistance to *R. solani* AG8 described in the natural accession collection provides a basis for further work to discover genes involved in resistance to this pathogen in *B. distachyon* and subsequently in wheat.

Abbreviations

$\sqrt{RLR_A}$	square-root transformed *R. solani*/control TRL ratio (*Method A*)
$\sqrt{TRL}$	square-root transformed TRL
ABA	abscisic acid
AG	anastomosis group
Arabidopsis	*Arabidopsis thaliana*
BC_2F_4	backcrossed twice , 4th filial generation
Bd	*B. distachyon*
bp	base pair
CNR	coleoptile nodal root
DAP	days after planting
DW	dry weight
Em	emission
EMS	ethyl methanesulfonate
ET	ethylene
Ex	excitation
f. sp.	*forma specialis*
ff. spp.	*formae speciales*
FITC	fluorescein isothiocyanate
FW	fresh weight
HR	hypersensitive response
HST	host-selective toxin
ISR	induced systemic resistance
JA	jasmonic acid
kb	kilo base pairs, 1 kb = 1000 bp
LNR	leaf nodal root
LP	long pass
Mb	mega base pairs, 1 Mb = 1,000,000 bp
p.a.	per annum
PCD	programmed cell death
PCR	polymerase chain reaction
PG	polygalacturonase
PGPR	plant growth-promoting rhizobacteria
ppg	propagules per gram
PR protein	pathogenesis-related protein
qPCR	quantitative real-time PCR
QTL	quantitative trait locus/loci
rDNA	ribosomal DNA
REML	residual maximum likelihood
R-gene	resistance-gene
RIL	recombinant inbred line
RLR_A	*Rhizoctonia solani*/control root length ratio (*Method A*)
RLR_B	*Rhizoctonia solani*/control root length ratio (*Method B*)
ROS	reactive oxygen species
Rs	*R. solani* AG8
SA	salicylic acid
SAR	systemic acquired resistance
SSR	simple sequence repeat
T-DNA	transfer DNA
TNR	total nodal root
TRL	total root length
UV	ultraviolet
WGA	wheat germ agglutinin
ZG	zymogram group

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Chapter 1

Introduction

Summary

Wheat is one of the world's major cereal crops. In Australia wheat is grown over an area of around 13 million hectares. Grain yield is impacted by many factors, including root diseases. The extent of crop loss attributable to root pathogens can be difficult to measure as disease symptoms may not always be easily diagnosed.

This chapter describes two root diseases of wheat that infect the plant soon after germination and pave the way for secondary infections. *Rhizoctonia solani* AG8 and *Pythium* spp. are both necrotrophic pathogens with broad host ranges. Varieties of wheat with resistance to these pathogens are not available, so the only means of controlling disease is with management strategies, including tillage and fungicide application. Mechanisms that could potentially confer resistance to these pathogens in wheat are discussed.

Since the turn of this century a grass relative of wheat, *Brachypodium distachyon*, has become a widely used model for the cereals, with its genome sequenced in 2010. Several *B. distachyon* germplasm collections are available and have previously been found useful in studying foliar diseases and root traits.

This chapter proposes *B. distachyon* to be a suitable model for studying resistance to the wheat root pathogens *Rhizoctonia solani* AG8 and *Pythium* spp., and outlines the aims of this thesis to develop and apply methods to screen *B. distachyon* collections for resistance to these diseases.

1.1 Wheat production

The modern wheat crop is the result of around 12,000 years of domestication of ancient Fertile Crescent grasses (Salamini *et al.*, 2002). The unique character of wheat flour, with its high gluten protein content, is irreplaceable in baking. Around the world wheat-based products, including breads, cakes and pasta, hold strong cultural significance, as well as being a staple source of dietary energy (Shewry, 2009).

About half of the world population's dietary energy is supplied by cereals (WHO, 2003). Wheat, maize and rice are by far the world's most important cereal crops, with over 650 million metric tonnes of each grain produced every year (FAOSTAT, 2013). Hexaploid bread wheat *(Triticum aestivum aestivum)* makes up over 90% of global wheat production, with tetraploid durum wheat *(Triticum turgidum durum)* comprising around 5% of total production (Dixon *et al.*, 2009).

Wheat is the major cereal grown in Australia, with around 23 million tonnes harvested from over 13 million hectares annually. Barley is the country's second largest cereal crop, with approximately 8 million tonnes produced annually, followed by sorghum, oats, triticale, maize and rice (ABARES, 2013).

The prevalence of wheat in the Australian agricultural production system and its wide geographical range is the result of several factors, including climate, seasonal temperature and rainfall conditions, and soil type. These factors also affect the distribution and severity of cereal diseases (Murray and Brennan, 2009).

1.2 Root diseases of wheat

After weeds, microbiological pathogens cause the greatest worldwide crop loss in wheat (Oerke and Dehne, 2004). On average; around 18% of the Australian wheat harvest is lost to diseases caused by fungi, bacteria, viruses and nematodes, with root diseases considered to be responsible for almost half the loss. The actual impact is difficult to estimate, as root diseases are not always easily diagnosed (Raaijmakers *et al.*, 2009).

Root diseases in decreasing order of importance to the Australian wheat crop are root lesion nematodes (*Pratylenchus* spp.), crown rot (*Fusarium pseudograminearum*), Rhizoctonia barepatch (*Rhizoctonia solani*), cereal cyst nematodes (CCN, *Heterodera avenae*), common root rot (*Cochliobolus sativus,* anamorph *Bipolaris sorokiniana*), take-all (*Gaeumannomyces graminis* var. *tritici*) and damping off/root rot (*Pythium* spp.) (Table 1.1) (Murray and Brennan, 2009).

Commercial wheat varieties are available with resistance to cereal cyst nematode (CCN) and some fungal shoot diseases, including flag smut (*Urocystis agropyri*) and leaf, stem and stripe rusts (*Puccinia* spp.). Apart from common root rot, resistant varieties are not available to fungal and oomycete root pathogens (Matthews *et al.*, 2013). This is partially due to the nature of root diseases. Shoot pathogens are often obligate biotrophs, requiring the living host for survival and reproduction, while root pathogens tend to be necrotrophic, killing host tissue before consuming it essentially saprophytically (Raaijmakers *et al.*, 2009). Some pathogens, known as hemibiotrophs, infect plant tissues initially biotrophically before causing necrosis (Oliver and Ipcho, 2004). Although the categorisation of pathogens as biotrophs and necrotrophs is fraught with exceptions, genetic disease resistance mechanisms generally follow this division. In biotrophic interactions, variation of a single plant gene can often be the difference between susceptibility and resistance. Resistance to necrotrophic pathogens usually requires multiple plant genes, each providing a small increase in resistance. Thus resistance to necrotrophic pathogens can be more difficult to identify and select for breeding (Poland *et al.*, 2009).

Biotrophic pathogens have an obligatory association with their hosts that results in co-evolution of resistance, described as a resistance 'arms race'. This often refers to *R*-gene resistance, which is a highly specific gene-for-gene interaction between plant and pathogen (Poland *et al.*, 2009). For example, resistance to sedentary endoparasitic root nematodes *Heterodera* spp., obligate biotrophs, is conferred by a major gene (Trudgill, 1991). This form of resistance is known as qualitative disease resistance.

In necrotrophic disease the *R*-gene resistance strategy is seen less often than in biotrophic interactions and is limited to host-specific necrotrophs that produce host-selective toxins (Mengiste, 2012).

Host-selective toxins and the avirulence factors recognised in biotrophic *R*-gene resistance are known as effectors. The term 'effector' is increasingly used to encompass all molecules produced by pathogens that affect the host cell, allowing easier invasion by the pathogen or triggering a defence response (Kamoun, 2006). Hogenhout *et al.* (2009) define effectors as "all pathogen proteins and small molecules that alter host-cell structure and function".

Table 1.1 Summary of major root diseases of wheat in Australia. [1](Murray and Brennan, 2009), [2](GRDC, 2011), [3](MacLeod *et al.*, 2008), [4](Murrumbidgee CMA, 2008), [5](GRDC, 2010).

Disease and disease organism	Crop Loss (%)[1]	Field symptoms[2]	Resistant varieties?[2]	Recommended control practices[2,3]
Root lesion nematodes; *Pratylenchus* spp.	2.9	Poor plant vigour, Patches developing from mid-tillering or no patches, Yellowing of lower leaves, Loss of lateral roots	Yes	Sow resistant varieties, Grass-free break crops, Weed control, Fertilize
Crown rot; *Fusarium pseudograminearum*	1.7	Whiteheads on single tillers, Brown discolouration of lower stem, Pinking on nodes or stems, Root cysts & knotting	Partial	Grass-free break crops, Control grass weeds, Fertilize, Deep cultivation, Sowing rate & inter-row sowing, Sow less susceptible varieties
Rhizoctonia barepatch; *Rhizoctonia solani*	1.3	Early severe bare patches with distinct edges, Brown spear-tipped roots, Severely reduced root system	No	Deep cultivation, Weed-free fallow prior to sowing, Fungicide seed dressing, Fertilize, Retain stubble to build up suppressive microorganisms[4]
Cereal cyst nematodes (CCN); *Heterodera avenae*	1.3	Early larger bare patches with indistinct edges	Yes	Sow resistant varieties, Grass-free break crops, Control grass weeds, Fertilize
Common root rot, Bipolaris leaf spot; *Cochliobolus sativus*, anamorph *Bipolaris sorokiniana*	0.7	Poor plant vigour, Patches developing later or not at all, Sub-crown internode & crown browning	Partial	Grass-free break crops, Sow less susceptible varieties, Fertilize
Take-all; *Gaeumannomyces graminis* var. *tritici*	0.3	Patches of whiteheads, Black lower stems, sub-crown internode, and 1° & 2° roots	No	Grass-free break crops, Control grass weeds, Fungicide seed dressing, Fertilize, Sowing time
Damping off/root rot; *Pythium* spp.	0.2	Poor emergence & seedling death from waterlogging damage, Short stubby main roots	No	Fungicide seed dressing, Avoid weed incorporation, Fertilizer placement[5], Sowing time

While qualitative resistance strategies are often used with biotrophic pathogens, plants generally rely on quantitative disease resistance to withstand broad host-range necrotrophic pathogens, which tend to attack using a range of toxins and cell-wall degrading enzymes (Mengiste, 2012). Quantitative disease resistance is considered to be a reduction of disease severity, race non-specific and thus more durable than qualitative resistance (Poland *et al.*, 2009). For example, two quantitative resistance loci (QTL) were found together to contribute approximately 30% of the resistance to the major root pathogen in Australia, the migratory endoparasitic nematode *Pratylenchus* spp., in a synthetic hexaploid wheat (Zwart *et al.*, 2006).

Two necrotrophic pathogens, *Rhizoctonia solani* and *Pythium* spp., are amongst the first invaders of wheat roots after sowing. As typical necrotrophs, they are non-obligate and have a wide host range (Okubara and Paulitz, 2005). With no genetic resistance currently available in commercial wheat cultivars to *R. solani* or *Pythium* spp., farmers must rely on field management practices to reduce inoculum levels and disease severity.

Rhizoctonia solani and *Pythium* spp. are often studied together as they both cause poor seedling emergence, and make way for secondary pathogens and saprophytes to invade young roots. While the effects of these organisms are similar, phylogenetically they are vastly different. *Rhizoctonia solani* belong to the kingdom Fungi, while *Pythium* spp. are oomycetes belonging to a nominally variable group known as, in one example, the Straminipila-Alveolata-Rhizaria super-kingdom (Lévesque *et al.*, 2010).

1.3 Rhizoctonia root rot

The wheat disease known as 'Rhizoctonia barepatch' or 'Rhizoctonia root rot' is caused by the fungus *Rhizoctonia solani*. The genus name *Rhizoctonia* was derived from Greek in 1815, aptly meaning 'death of roots' (Menzies, 1970). The fungus infects roots at germination and the early seedling stage, becoming evident in the field from as early as two weeks after sowing. Root rot in wheat manifests as circular patches of stunted or dying plants, with a characteristically distinct boundary (Figure 1.1). Plants inside bare patches may recover towards the end of the season, but usually yield less than the surrounding crop (MacNish and Neate, 1996). In some regions, *R. solani* damage is also evident as uneven growth in the crop (Davis *et al.*, 2008; GRDC, 2012).

In Australia Rhizoctonia root rot is most severe in the cooler southern latitudes. In South Australia, Victoria, Tasmania and southern New South Wales disease is seen in half of the wheat crop area in 76% of years. Root rot occurs in around 61% of years in

Western Australia, but not at all in Queensland and northern New South Wales (Murray and Brennan, 2009). This distribution is consistent with research showing that *R. solani* is most pathogenic on wheat at lower temperatures, in the range of 10 to 20°C (Smiley and Uddin, 1993; Gill *et al.*, 2001a).

Figure 1.1 Rhizoctonia disease in a two metre wide plot of wheat near Harden, NSW.

Rhizoctonia solani is also a major problem in wheat in lower rainfall areas of the Pacific Northwest region of the United States of America, where the increase in Rhizoctonia root rot seen following conversion to no-till has prevented farmers from adopting this principle of conservation agriculture (Paulitz *et al.*, 2009). In some cases, a strong rise in *R. solani* disease in the years following conversion to no-till is followed by a fall in disease. This phenomenon, known as 'Rhizoctonia decline', was reported by Australian farmers and researchers in the 1980s and 1990s (Rovira, 1986; Roget, 1995). The reduction in disease has been attributed to biological suppression of *R. solani* by soil micro-organisms (Smiley *et al.*, 1996; Wiseman *et al.*, 1996; Barnett *et al.*, 2006) and possibly an increase in earthworm numbers (Stephens *et al.*, 1993). Members of the Pseudomonadaceae are coming to the fore as plant growth-promoting

rhizobacteria (PGPR) that can suppress Rhizoctonia root rot through biological control (Mendes *et al.*, 2011; Mavrodi *et al*, 2012).

1.3.1 *Rhizoctonia solani*

Rhizoctonia solani is a basidiomycete fungus. The species is subdivided into anastomosis groups (AG), in which hyphal fusion is usually only possible between members of the same AG (Vilgalys and Cubeta, 1994). It was only in 1985 that the specific anastomosis group *R. solani* AG8, responsible for disease in wheat, was first described (Neate and Warcup, 1985). The disease is still being described for the first time in wheat fields of different countries, for example in Turkey (Ünal and Dolar, 2012).

Hyphal fusion allows gene exchange between compatible fungal isolates. Sexual recombination by way of generation of the perfect state of multinucleate *R. solani* (teleomorph *Thanatephorus* spp.) and formation of basidiospores is rarely observed and can be extremely difficult to achieve in the laboratory (Phillips, 1993). Survival structures of *R. solani* are bundles of melanised hyphae, known as sclerotia (Figure 1.2).

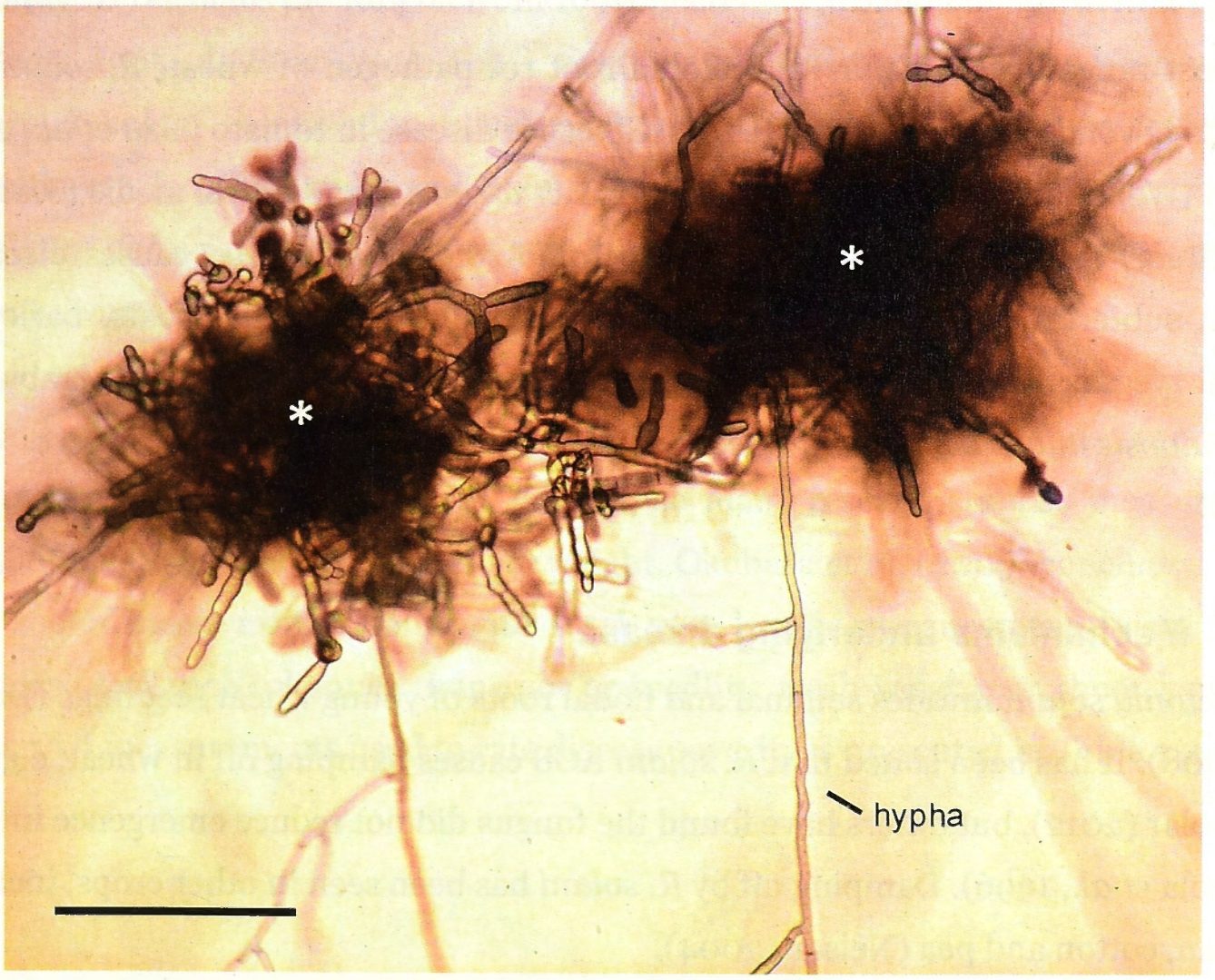

Figure 1.2 *Rhizoctonia solani* sclerotia (*) developing on agar medium. Bundles of hyphae become melanised, forming the hard brown lumps that are the fungus' survival structures. Scale bar, 200 µm.

Chromosome number varies both within and between anastomosis groups of *R. solani*, with between 11 and 16 chromosomes making up a genome of around 40 Mb in size (Keijer *et al.*, 1996). Further heterogeneity exists in the nuclear number of *R. solani*, which can be in excess of 15 nuclei per cell. As cells age nuclei move towards the cell wall. The youngest cells at the hyphal tips contain higher numbers of nuclei than more mature cells in the mycelium (Shatla and Sinclair, 1966). In bare patches of wheat, it was found that multinucleate isolates of *R. solani* were associated with disease, whereas binucleate isolates of *Rhizoctonia* spp. were not pathogenic (Yang *et al.*, 1994). McCabe *et al.* (1999) reported that segregation of nuclei during hyphal tipping can lead to incompatibility with the parent line. This flexibility in chromosomal arrangement is considered to be advantageous to fungal evolution and adaptation to new hosts and environments.

Rhizoctonia solani produces a range of enzymes that degrade pectin, which allow the further subdivision of anastomosis groups into zymogram groups. The enzymes produced by *R. solani* are visualised by separating proteins with gel electrophoresis followed by a pectin-agarose overlay to detect pectinase activity. Yang *et al.* (1994) attributed Rhizoctonia root rot sampled in Western Australian wheat to *R. solani* AG8 ZG1-1. Zymogram groups are often not reported.

The host range of the major Rhizoctonia root rot pathogen of wheat, *R. solani* AG8, extends beyond the cereals. Isolates of AG8 cause disease in tomato (Gao *et al.*, 2006), potato (Woodhall *et al.*, 2008), onion (Wicks *et al.*, 2011) and barrel medic (*Medicago truncatula*) (Streeter *et al.*, 2001). *Rhizoctonia solani* AG8 ZG1-1 causes disease in lupins, subterranean clover, Indian mustard, canola, wild radish, oats, barley and annual ryegrass (*Lolium rigidum*) (Khangura *et al.*, 1999). Surprisingly, Arabidopsis (*Arabidopsis thaliana*) resists infection to *R. solani* AG8 ZG1-1 (Perl-Treves *et al.*, 2004).

1.3.2 Mechanisms underlying disease

Rhizoctonia solani invades seminal and nodal roots of young wheat seedlings (Davis *et al.*, 2008). It has been stated that *R. solani* AG8 causes damping off in wheat, e.g. Ünal and Dolar (2012), but others have found the fungus did not reduce emergence in wheat (Mazzola *et al.*, 1996). Damping off by *R. solani* has been seen in other crops, including soybean, cotton and pea (Nelson, 2004).

Murray (1982) studied penetration of barley roots and coleoptiles by *R. solani* AG1 to AG5, isolated from a wide range of plants. He found that the specialized infection

structures (appressoria or infection cushions) were only formed during coleoptile invasion, whereas root entry was by narrow infection pegs. With severe disease, root lesions develop into necrosis that spreads throughout the root cortex and the stele causing root truncation in a characteristic 'spear tip' (Ogoshi *et al.*, 1990). Kirkegaard *et al.* (1999) asked whether a reduced ability to acquire water and phosphorus by roots was responsible for reduced shoot growth in wheat. Their experimental results indicated that *R. solani* AG8 directly affected shoot growth, possibly through the release of effectors (described in §1.5) or by inducing a plant wounding response. As disease severity increased plants also experienced difficulty in transporting water from the soil to leaves.

Root disease can be more difficult to measure than shoot diseases, as damage occurs below ground, with the shoot phenotype being a secondary effect. A rating scale, measuring root truncation and necrosis, developed by McDonald and Rovira (1985) is often used to measure disease severity in wheat under Australian field conditions. The authors found that increasing levels of *R. solani* inoculum added to soil reduced seminal root length in wheat. Levels of *R. solani* in field soil have been measured by baiting and plating onto selective media (Paulitz and Schroeder, 2005) and by DNA quantification using real-time PCR (Okubara *et al.*, 2008; Ophel-Keller *et al.*, 2008). The ability to reisolate the fungus from roots decreases around shoot tillering (Harris and Moen, 1985; de Boer *et al.*, 1991). Quantitative real-time PCR has been used to compare *R. solani* colonization between control and mutant *Medicago truncatula* (Anderson *et al.*, 2010) and was demonstrated to be a useful scoring technique in a *Verticillium dahlia*-potato resistance screen (Dan *et al.*, 2001), but has so far not been applied to wheat screening experiments .

A visual root disease rating may not always be the most quantifiable and reproducible assessment of disease (Smith *et al.*, 2003a), so many researchers also measure root and shoot length and/or root and shoot weight. Okubara *et al.* (2009) concluded that a visual root disease rating along with total root length measurements were better indicators of disease tolerance than root or seedling fresh weight, or shoot length. A summary of measurements used to rate disease severity is presented in Table 1.2.

Table 1.2 Measurements used to assess *R. solani* disease severity in pot experiments with wheat seedlings.

Root measurements	Shoot measurements	Visual root disease rating	Reference
		✓	(Blair, 1942)
DW, shoot:root ratio progression to 40 DAP	Leaf area, DW		(Harris and Moen, 1985)
Seminal root length	Shoot height	✓	(McDonald and Rovira, 1985)
		✓	(Rovira, 1986)
	DW	✓	(Roget *et al.*, 1987)
	Shoot length	✓	(Ogoshi *et al.*, 1990)
DW	DW	✓	(Stephens *et al.*, 1993)
DW	Leaf & tiller number, DW, FW, leaf nutrients	✓	(Wall *et al.*, 1994)
TRL, DW	Leaf area, leaf 1 length, DW	✓	(James *et al.*, 1997)
TRL, DW	Leaf area, DW, leaf P, relative leaf expansion rate	✓	(Kirkegaard *et al.*, 1999)
	Plant FW	✓	(Duffy, 2000)
TRL, DW	DW		(Gill *et al.*, 2000)
		✓	(Smith *et al.*, 2003a)
Number of root tips, TRL, FW	Plant FW	✓	(Okubara *et al.*, 2009)
TRL, FW		✓	(Okubara and Jones, 2011)

DW, dry weight; FW, fresh weight; TRL, total root length; DAP, days after planting.

Rhizoctonia root rot is an important early disease of wheat and makes way for invasion by secondary pathogens. Harris and Moen (1985) argued that the invasion of secondary pathogens produces a 'disease complex' to increase Rhizoctonia disease severity. The disease complex theory was supported by Roberts and Sivasithamparam (1987), who showed that Rhizoctonia root disease was greater in wheat co-inoculated with other root rot fungi and oomycetes. The ability to recover *R. solani* from affected plants using selective media decreases as plants mature, and thus the initial cause of the disease may not be identifiable later. James *et al.* (1997) go further to suggest that yield decline in direct-drilled wheat often attributed to *R. solani*, may in fact be due to other deleterious micro-organisms.

Research into the factors that promote *R. solani* growth and virulence has gone on for many decades. Studies looking at the growth of *R. solani* mycelium through soil and

sand found that the rate of spread of *R. solani* in soil is variable, but in the order of roughly one centimetre per day (Blair, 1943; Gill *et al.*, 2001b; Schroeder and Paulitz, 2008). Increasing soil moisture was found by Blair (1943) to reduce *R. solani* spread due to the concomitant effect of reduced soil aeration, however Gill *et al.* (2001b) saw no effect of soil moisture on the rate of spread of *R. solani* AG8. There is a general assumption in rural media that cultivating soil reduces *R. solani* disease by breaking up hyphal networks, e.g. (GRDC, 2013), but this is not reflected in the scientific literature. The role of cultivation in reducing disease appears to be mainly due to the effect of incorporating carbon-rich stubble into the soil, making it available for degradation by soil micro-organisms. Blair's (1943) experiments suggested that cellulose-degrading micro-organisms flourish after the addition of wheat straw or dried grass and quickly assimilate available nutrients, making them unavailable to *R. solani.* A further hypothesis is that this increase in bacterial metabolism creates a flush of carbon dioxide, which inhibits *R. solani* growth. While *R. solani* can live as a saprophyte for a short time, it does not survive well without a living host and a fallow period of two weeks is generally sufficient to reduce disease levels prior to sowing (Cook, 2000).

Otten and Gilligan (1998) hypothesized that the fungus' faster growth along soil surfaces allows *R. solani* to rapidly colonize biopores and to colonize young seedlings before they develop resistance. The development of circular patches of stunted plants, with distinct edges, suggests that *R. solani* hyphae spread from a central point infecting plants until such time that the plants become resistant. Active hyphae may still be detected in the soil surrounding the patch, although at a lower frequency (Paulitz and Schroeder, 2005). This apparent change in the ability of plants to become infected with time is reminiscent of adult plant resistance, a complex widespread phenomenon that has rarely been studied in *R. solani* disease (Develey-Rivière and Galiana, 2007).

1.3.3 *Rhizoctonia solani* effectors

The poor state of understanding of effectors produced by *R. solani* is summed up by Bent and Mackey (2007), who ask, "Are important broad host-range necrotrophic pathogens such as *Rhizoctonia* or *Botrytis* successful because they carry a few particularly effective effectors, and/or an unusually broad suite of effectors?" This question remains to be answered.

Enzymes are thought to be major effectors in *R. solani* pathogenicity. Sweetingham *et al.* (1986) created five zymogram groups (ZG) of multinucleate *R. solani* isolates based

on the different enzyme patterns produced in pectic zymograms. In heterokaryotic multinucleate isolates of *R. solani* AG8 ZG1-1, single spore progeny were shown to produce different enzyme activity patterns. The pectic enzymes produced in the assay were mainly polygalacturonase and pectin lyase. As yet there is no clear correlation between any particular pectin degrading enzyme and *R. solani* AG8 pathogenicity (O'Brien and Zamani, 2003). In addition to pectinase, *R. solani* also produces laccase, cellulase and xylanase. Laccase enzymes can break down phenolic defence compounds and lignin (Bora *et al.*, 2005).

Sheath blight, caused by *R. solani* AG1-IA, is a major problem in rice. Research into disease and resistance mechanisms in this system can provide clues about *R. solani* AG8 root infections. Publication of the draft genome sequence of *R. solani* AG1-IA has allowed researchers to look for predicted effectors in rice sheath blight (Zheng *et al.*, 2013). Following infection of rice, carbohydrate-active enzyme expression in *R. solani* peaked in a sequential order of glucoside hydrolases, followed by hemi-cellulose degrading enzymes and then cellulose and pectin degrading enzymes. Xylanase and laccase genes were also predicted from the genome, but not cutinases. Three classes of effectors, including glycosyltransferase family 2, caused necrosis symptoms in rice, maize and soybean in a pattern redolent of host-specific toxins (Zheng *et al.*, 2013). Previously, endo-polygalacturonase (endo-PG) produced by *R. solani* AG1-IA was shown to be involved in pathogenicity in rice (Yang *et al.*, 2012). Endo-PG is a cell wall degrading enzyme that hydrolyses a component of pectin.

In addition to protein effectors, *R. solani* can also produce metabolite toxins. Brooks (2007) used the term 'phytotoxin' to describe those compounds acting as virulence factors across a broad range of hosts, in contrast with host-selective toxins (HST) that are genotype specific. In the rice-sheath blight pathosystem a carbohydrate-based HST produced by *R. solani* AG1-IA causes necrosis in rice lines that carry an allele for toxin sensitivity (Vidhyasekaran *et al.*, 1997; Costanzo *et al.*, 2011). Host-selective toxins have not been discovered in *R. solani* pathosystems other than rice. Described by Deacon (1996) as a Poaceae family-specific pathogen, *R. solani* AG8 is more likely to produce phytotoxins. Sherwood (1965) patented a method to produce a phytotoxin, tentatively identified as *o*-nitrophenyl-β-D-glucoside, from an isolate of *R. solani* collected from alfalfa root canker. *Rhizoctonia solani* AG3, AG4 and AG1-IA release phenylacetic acid (PAA) and its derivatives, phytotoxic compounds that are auxinic plant-growth regulators (Mandava *et al.*, 1980; Bartz *et al.*, 2013).

1.3.4 'Resistance' versus 'tolerance'

The terms 'resistance' and 'tolerance' are often used interchangeably to describe plant disease responses, but they refer to effectively mutually exclusive concepts. Roy and Kirchner (2000) summarised the different mechanisms:

> "...we use resistance to refer to traits that prevent infection or limit its extent, and we use tolerance to refer to traits that do not reduce or eliminate infection, but instead reduce or offset its fitness consequences. Thus, resistance and tolerance can both improve host fitness; resistance does so by reducing infection, whereas tolerance does so by reducing the fitness loss under infection."

When a plant-pathogen interaction is not well characterised, the appropriate term to use can be unclear. In a study of oat cultivar yields after infection with crown rust, Politowski and Browning (1978) concluded that true disease tolerance was rare and suggested that cultivars should therefore be presumed resistant until evidence to the contrary emerged. On the other hand, Okubara *et al.* (2009) preferred to use the term 'tolerance' to describe the unknown mechanism that led to reduced *R. solani* disease severity in a mutant wheat line, later moving to the term 'resistance' in work with *Thinopyrum* spp. (Okubara and Jones, 2011).

The effects of resistance and tolerance on a plant population were modelled by Roy and Kirchner (2000), who argued that tolerance is the more favourable phenotype as it does not create the same evolutionary pressure on pathogens as resistance. However, their model shows that the incidence of infection increases over successive generations of tolerant plants, whereas a decrease is seen over time with resistant plants (Figure 1.3). In terms of crops, reduction of inoculum levels in the field is a key component of managing disease (Strange and Scott, 2005). Crop rotations are often used as a management practice to reduce inoculum load when resistant cultivars are not available and pathogen host range is limited.

Durable quantitative resistance is arguably the most desirable phenotype, leading to lower inoculum level in the field but without creating a strong selection pressure on the pathogen. Thus, the term 'resistance' will be used here to describe reduced disease severity where the mechanism is unknown.

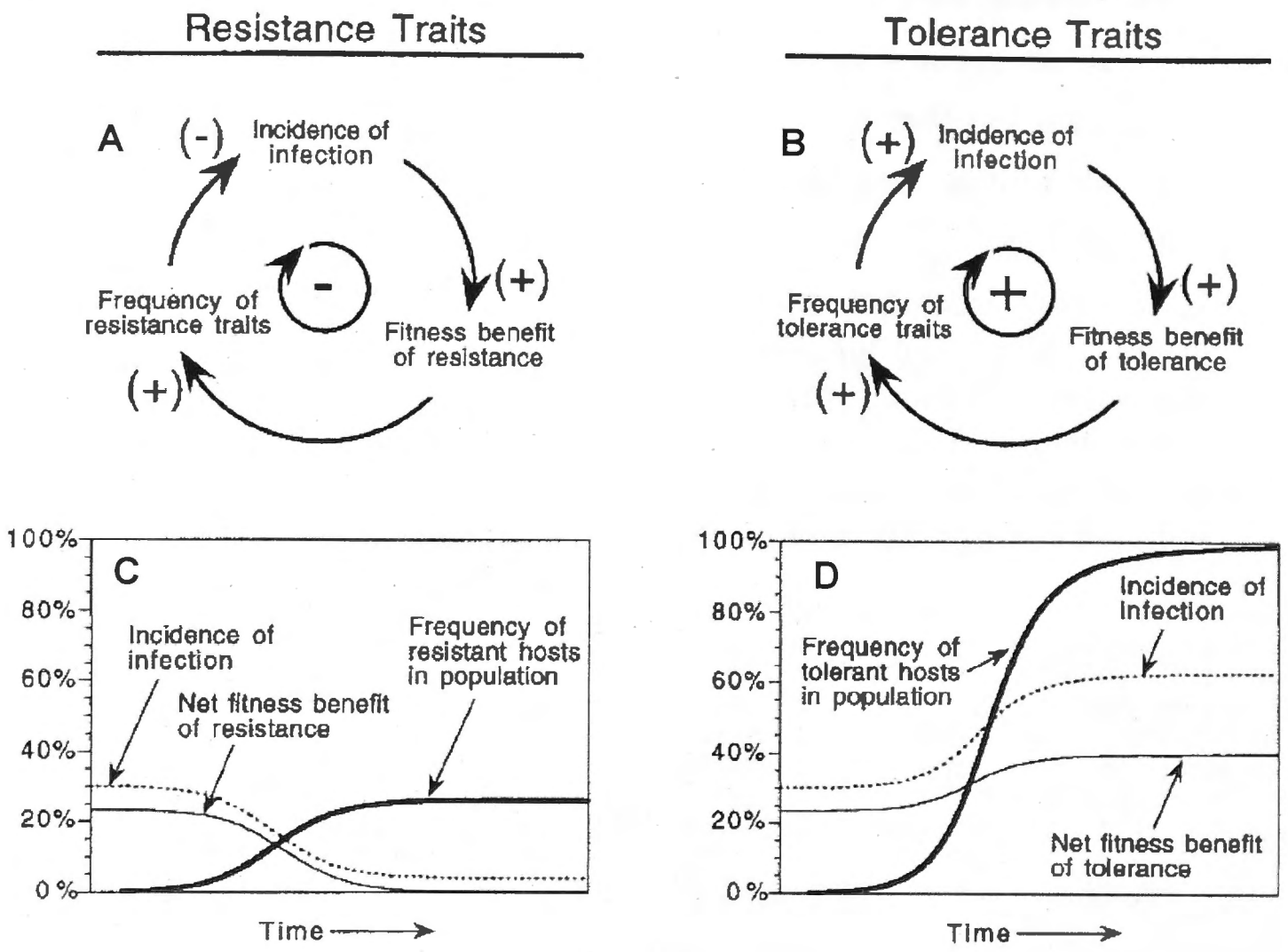

Figure 1.3 The modelled effect of resistance and tolerance traits on the inheritance of each trait in the host population and incidence of disease over time. Resistance and tolerance traits both have a fitness benefit and are therefore inherited and increase in frequency in the host population (a - d). Resistance traits lead to a decrease in infection (a, c), whereas tolerance traits allow infection to increase over time (b, d). Modified from Roy and Kirchner (2000).

1.3.5 Resistance to *Rhizoctonia solani* in close relatives of wheat

There is little evidence of resistance to *R. solani* in the wheat or Pooideae cereals, such that no *R. solani* resistance traits are available for commercial breeding programmes. Neate (1989) found little overall difference between disease severity ratings across cultivars of wheat, barley, oats, rye and triticale, with cereal disease susceptibility rankings changing between years and between pot and field experiments.

Other research has proven more positive and is summarised in Table 1.3. Some varietal resistance was found in wheat by Smith *et al.* (2003a). Okubara *et al.* (2009) generated a wheat mutant with heritable increased tolerance to *R. solani*, but the genes and mechanisms involved are still to be determined.

Despite the broad host range of *R. solani* AG8, varying levels of resistance are found in the wild Triticeae relatives of wheat. A number of genotypes of *Dasypyrum villosum* (mosquitograss, Pooideae subfamily) are classified as resistant, although they can develop some lesions following inoculation with *R. solani* AG8. *Dasypyrum villosum*

was considered a potential donor of Rhizoctonia resistance genes for cereal crops, but *D. villosum*/wheat amphidiploids remained susceptible (Smith *et al.*, 2003b).

Okubara and Jones (2011) demonstrated the ability to transfer a partial increase in resistance to *R. solani* AG8 to Chinese Spring wheat, in addition lines carrying chromosome 4E or 4J from *Thinopyrum elongatum* and *Th. bessarabicum,* respectively. They speculated that the shortening and thickening of diseased roots could indicate the involvement of the hormones jasmonic acid and ethylene in the defence response. The mechanisms controlling disease resistance in wild relatives of wheat are still unknown.

Table 1.3 Genetic resistance to *R. solani* AG8 in relatives of wheat.

Plant	Nature of resistance	Reference
Thinopyrum elongatum	*Th. elongatum* (tall wheatgrass) had 13 – 19% greater root length ratio* than 'Chinese Spring'	(Okubara and Jones, 2011)
'Chinese Spring' wheat addition line	Chromosome 4E addition from *Th. elongatum* increased root length ratio* by 14 – 23% compared with 'Chinese Spring'.	
'Chinese Spring' wheat addition line	Chromosome 4J addition from *Th. bessarabicum* increased root length ratio* by 10 – 50% compared with 'Chinese Spring'.	
'Scarlet-Rz1' wheat mutant	BC_2F_4 EMS mutants of 'Scarlet' ('Scarlet-Rz1') had up to 39% greater root length ratio* compared with 'Scarlet'.	(Okubara *et al.*, 2009)
Wheat	Varietal differences were found for grain yield in the field, as a measure of resistance to *R. solani.* Differences in resistance were not evident in a pot assay.	(Smith *et al.*, 2003a)
Dasypyrum villosum Barley Wheat	Moderate host resistance in *D. villosum* (mosquitograss) based on root disease scores. Wheat synthetic hexaploids, barley and *D. villosum* / wheat amphidiploids did not show increased resistance.	(Smith *et al.*, 2003b)

*Increase in inoculated/control root length ratio at 14 days after planting

1.4 Pythium root rot

Pythium root rot in wheat is caused by a number of *Pythium* species. In the field, the disease manifests as pre-emergent seed loss (damping off) and post-emergent loss of seedling vigour (Ingram and Cook, 1990). The direct impact of *Pythium* spp. on cereal production is difficult to assess. When disease is present, it generally causes a widespread reduction in wheat growth, rather than obvious patches (GRDC, 2010). Thus, Pythium root rot is often referred to as the 'common cold' of wheat (Cook and Veseth, 1991).

In Australia *Pythium* disease occurs in all wheat growing regions, but is most severe in the southern cropping zone (South Australia, Victoria, Tasmania and southern New South Wales), where disease is seen in 63% of the wheat crop area in 68% of years. In Western Australia and in the northern region (Queensland and northern New South Wales) *Pythium* affects a quarter of wheat crops in 49% and 36% of years, respectively (Murray and Brennan, 2009).

Pythium root rot is also a problem in the Pacific Northwest of the United States of America, where it is more prevalent in the higher rainfall regions (Paulitz *et al.*, 2009).

1.4.1 *Pythium* spp.

The broad host range of the oomycete *Pythium* is not to be underestimated. Species of *Pythium* destroy across the kingdoms, including tropical trees (Augspurger and Wilkinson, 2007), seaweed (Hwang *et al.*, 2009), mushrooms (Godfrey *et al.*, 2003), mosquito larvae (Scholte *et al.*, 2004), tigers (Buergelt *et al.*, 2006) and even humans (Krajaejun *et al.*, 2006). Infection in mammals generally occurs in warmer climates after exposure to swamps or ponds. The oomycete generally invades opportunistically through open wounds in skin or, in the case of mosquito larvae, through mechanical damage.

Many species of *Pythium* are found to colonize wheat embryos and roots, but not all cause disease. The most pathogenic species of *Pythium* recorded are *P. aristosporum, P. volutum, P. ultimum, P. sylvaticum* and *P. irregulare* (Chamswarng and Cook, 1985), *P. irregulare* and *P. ultimum* var. *sporangiiferum* (Ingram and Cook, 1990) and *P. debaryanum* = *P. irregulare* and *P. ultimum* (Higginbotham *et al.*, 2004b). These isolates were all collected from the USA states of Washington and Idaho. Pythium root rot of wheat has also been attributed to *P. arrhenomanes* and *P. graminicola* in Great Britain, *P. arrhenomanes* and *P. volutum* in Canada, and *P. graminicola* in India

(Waller, 1979). In Australia *P. irregulare* is reported to be the most common species associated with wheat disease (Pankhurst *et al.*, 1995; Harvey *et al.*, 2008).

Oospores are the survival structures of *Pythium*, allowing the oomycete to survive in the soil for lengthy periods (Figure 1.4). Traditionally Pythium root rot has been considered a disease of cold wet soils, but recent evidence shows that the disease can also occur during drought conditions (GRDC, 2010). This is supported by studies into the viability of oospores under different soil moisture conditions. Oospores remain viable and pathogenic for longer under dry soil conditions (-10 kPa) than moist conditions (0 and -1 kPa) (Mondal and Hyakumachi, 2000) and are reported to have survived in soil for several years (Martin and Loper, 1999).

Pythium spp. can cause disease in a complex with other pathogens, such as *Fusarium* spp. (Harvey *et al.*, 2008). Some species of *Pythium*, including *P. irregulare*, may stimulate the growth of fungi, such as *R. solani*, while other *Pythium* spp. are mycoparasites (van der Plaats Niterink, 1981).

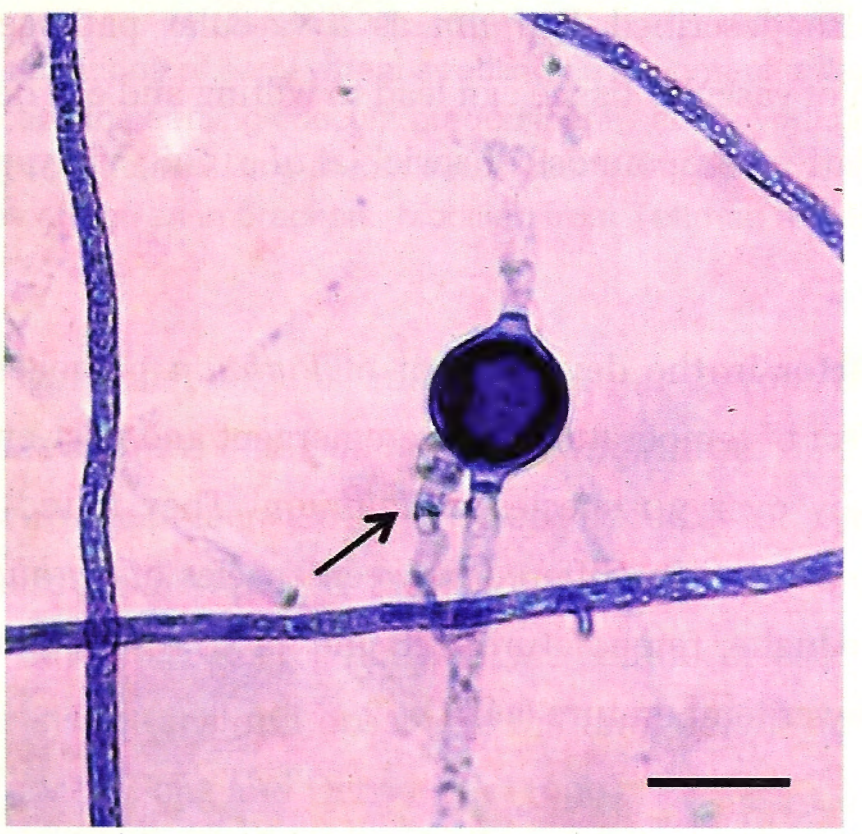

Figure 1.4 An oospore of *Pythium irregulare* forming on agar medium, stained with Trypan blue. The arrow points to the antheridium, the male part of the reproductive structure. The antheridium has fertilised the oogonium to produce an oospore. Hyphae on either side of the oospore are no longer viable. This oospore could either germinate directly or produce zoospores which will encyst and then germinate on roots. Scale bar, 20 µm.

1.4.2 Mechanisms underlying disease

In young seedlings, *Pythium* hyphae quickly invade the root by penetrating breaks in the epidermis, but may also enter the epidermis by forming appressoria (Figure 1.6). During zoospore infection appressoria enter root hairs, but this rarely occurs with mycelial appressoria. Infection in two-day-old wheat seedlings is highest behind the root cap, 1 - 2 mm from the root tip, in the region of root hair formation. An increase in root hair length and density in this region is observed within a day of *Pythium* infection. Resistance to penetration is markedly increased for mature tissue further than 5 mm from the tip (Mojdehi *et al.*, 1991).

Hyphal invasion of the root is generally limited to the cortex, with extensive physical and enzymatic tissue destruction as the mycelium extends intracellularly. The endodermis provides a barrier to invasion of the stele in all but the youngest tissue within 2 mm of the tip. Severe infection causes roots to cease growing, becoming brown and rotted. This is followed by reproductive oospore formation in the root tip (Mojdehi *et al.*, 1991) .

Researchers have also described *Pythium* as a vascular pathogen in Arabidopsis, finding that invasion of vascular tissue can lead to wilting and cell death in the absence of detectable degradative compounds (Staswick *et al.*, 1998; Vijayan *et al.*, 1998; Adie *et al.*, 2007).

Temperature is a factor in the development of *Pythium* pathogenicity. Abad *et al.* (1994) tested the effect of temperature on pre-emergent and post-emergent disease on creeping bentgrass by over 30 species of *Pythium*. They found that the effect of temperature on disease severity differed between species of *Pythium*. Generally, and for *P. irregulare*, the higher temperatures (28 and 32°C) resulted in greater incidence of disease than the lower temperature (16°C).

Ingram and Cook (1990) found that colonisation of germinating wheat embryos by *P. irregulare* in soil occurred at all temperatures in the range 5, 10, 15, 20 and 25°C. Emergence was not significantly reduced by *P. irregulare* at any temperature, but there was a significant reduction in leaf 1 length at all temperatures except 20°C. The authors concluded that while *P. ultimum* is generally more pathogenic to wheat than *P. irregulare*, the ability of *P. irregulare* to grow at low temperatures can make it an important pathogen in soils where the temperature is close to freezing. Illustrations of germination and early growth stages of wheat are reproduced from Tottman and Broad (1987) in Figure 1.5.

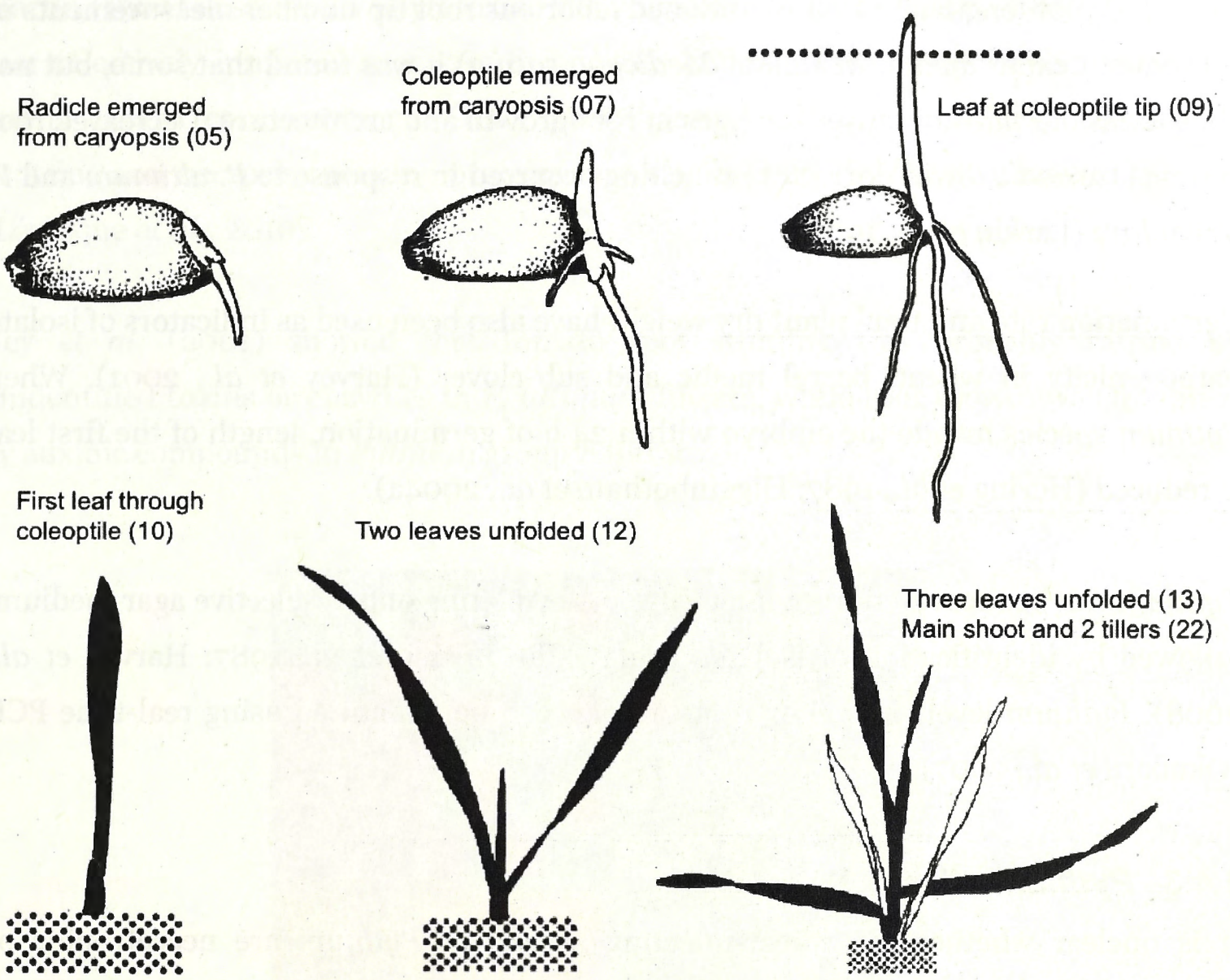

Figure 1.5 Illustrations of early wheat seedling development, after Zadoks *et al.* (1974). *Pythium* infection in seedlings occurs predominantly at the youngest root tissue behind the extending root tip, resulting in reduced first leaf length. Zadoks decimal codes for growth stages are given in brackets. Modified from Tottman and Broad (1987).

Pythium infection can cause lesions and degradation of root tissue. However, the presence of disease symptoms and isolation of *Pythium* from field-grown wheat is not always associated with the presence of root lesions (Cook *et al.*, 1980). A lack of visible root damage has also been reported for Pythium disease in lettuce, tomatoes and turfgrass (Abad *et al.*, 1994; Rey *et al.*, 2001). Pythium disease in turfgrass can be diagnosed by foliar symptoms and observing *Pythium* colonisation of roots through the presence of oospores (Feng and Dernoeden, 1999).

In field-grown wheat the presence of oospores within the root tip can be observed in *Pythium* infected plants, along with root tip browning. Disease symptoms include distortion of the first leaves, loss of fine roots and reduced plant vigour (Paulitz and Adams, 2003). Damage to fine roots is associated with reduced plant vigour and delayed maturity (Cook and Haglund, 1982).

Total root length and number of root tips are indicators of Pythium disease severity in wheat. Higginbotham *et al.* (2004a) found that the number of root tips was correlated

with total root length and thus abandoned laborious root tip number measurements in subsequent experiments. In alfalfa (*Medicago sativa*) it was found that some, but not all, species of *Pythium* caused changes in root growth and architecture. Decreased root system size and a lower degree of branching occurred in response to *P. ultimum* and *P. irregulare* (Larkin *et al.*, 1995).

Germination rate and total plant dry weight have also been used as indicators of isolate pathogenicity in wheat, barrel medic and sub-clover (Harvey *et al.*, 2001). When *Pythium* species invade the embryo within 24 h of germination, length of the first leaf is reduced (Hering *et al.*, 1987; Higginbotham *et al.*, 2004a).

Pythium can be recovered from infected roots by plating onto a selective agar medium, followed by identification of isolates using PCR (Hering *et al.*, 1987; Harvey *et al.*, 2008). *Pythium* levels in soil or root samples can be quantified using real-time PCR (Okubara *et al.*, 2007).

1.4.3 *Pythium* effectors

It is unclear whether the wheat-infecting species of *Pythium* are necrotrophic or hemibiotrophic. In Arabidopsis infection Oliver and Ipcho (2004) described *P. irregulare* as a necrotroph, while Adie *et al.* (2007) observed hemibiotrophic disease progression and haustoria-like structures typical of biotrophs (Figure 1.6). Cheung *et al.* (2008) and Lévesque *et al.* (2010) concluded that the absence of RLXR effector sequences in the *P. ultimum* genome was consistent with its more necrotrophic nature than the related oomycetes *Phytophthora* spp. Effectors carrying the RXLR amino acid motif are abundant in *Phytophthora* spp. The motif is thought to be involved in translocation of the protein into the host cell via haustoria (Cheung *et al.*, 2008). The *P. ultimum* genome does encode proteins expected to be capable of host translocation. These include secreted proteins of the *Crinkler* gene family and a newly discovered YxSL[KR] family of RXLR-like proteins that is present across the oomycetes (Lévesque *et al.*, 2010).

Cell-wall degrading enzymes, including pectinases, cellulases and glycoside hydrolases, are predicted in the *P. ultimum* genome (Lévesque *et al.*, 2010). An absence of cutinase genes in the *P. ultimum* genome was also seen in the *R. solani* 1-IA genome (Zheng *et al.*, 2013). Furthermore, *P. ultimum* lacks xylanase and pectin methylesterase genes. This is expected to be why pathogen penetration of roots is limited to unsuberized regions near the root tip or damaged areas (Lévesque *et al.*, 2010).

Martin (1964) described a phytotoxin, likely proteinaceous, produced by *P. irregulare* that selectively inhibited root growth of beets and other members of the Amaranth family at low concentrations. Other proteinaceous effectors, including elicitin-like proteins and necrosis-inducing proteins, are also encoded by the *P. ultimum* genome (Lévesque *et al.*, 2010).

Rey *et al.* (2001) showed that tomato root stunting was probably caused by unidentified toxins or enzymes in *P. ultimum* filtrate, while root growth was inhibited by auxinic compounds in *Pythium* group F filtrate.

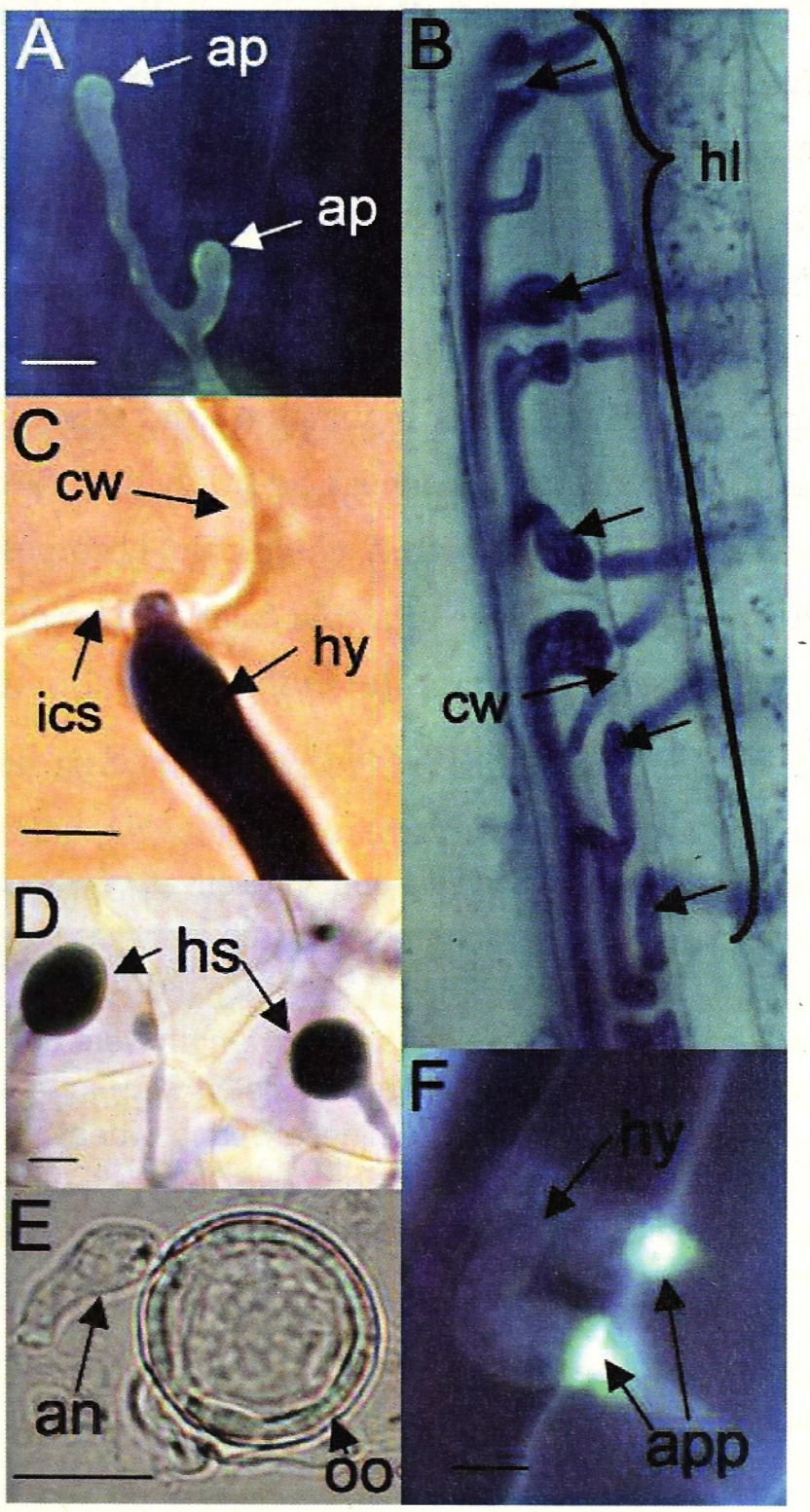

Figure 1.6 Infection structures of *P. irregulare* (stained) on Arabidopsis root (a, b, f) and leaf (d). Scale bar, 10 µm (a, b, d, e), scale bar 5 µm (c, f); an, antheridium; ap, appressoria; app, apposition; cw, cell wall; hl, haustoria-like structure; hs, hyphal swellings; hy, hyphae; ics, intracellular space; oo, oogonium. Reproduced from Adie *et al.* (2007).

1.4.4 Resistance to *Pythium* spp. in close relatives of wheat

Genetic resistance to Pythium root rot is not available in commercial wheat varieties (Okubara and Jones, 2011) and there are few reports of resistance in other cereals (Table 1.4). Raftoyannis and Dick (2006) did not observe zoospore encystment on roots of oat or maize by any of the ten species of *Pythium* tested, while zoospores of seven species encysted on wheat roots. The different species of *Pythium* caused varying levels of root length reduction in oat, maize and wheat, with oats consistently the most resistant of the cereals. This increased resistance in oats may be due to the release of a toxin, possibly avenacin, which lyses zoospores (Deacon and Mitchell, 1985). Higginbotham *et al.* (2004a) measured varying levels of tolerance to *P. ultimum* and *P. debaryanum* (= *P. irregulare*) across thirty diverse lines of wheat. The ratio of inoculated/control total root length ranged from 27% to 81% with *P. ultimum*, with least significant difference of 23%. The authors did not find any correlation between tolerance and other varietal traits. Okubara and Jones (2011) showed that *Thinopyrum elongatum* was more resistant to *P. ultimum* than Chinese Spring wheat. The inoculated/control total root length ratios of wheat averaged 44%, while *T. elongatum* ratios were around 71%. This higher level of resistance was present in a wheat-*Thinopyrum* amphidiploid and a wheat addition line carrying *T. elongatum* chromosome 4E. A wheat addition line with *T. elongatum* chromosome 4J had the low resistance level of wheat. Rice genotypes in a cold tolerance breeding program ranged from susceptible to moderately resistant to *Pythium* spp. in the field and to *P. arrhenomanes* in pot experiments (Rothrock et al., 2009).

Table 1.4 Genetic resistance to *Pythium* spp. in relatives of wheat.

Plant	Nature of resistance	Reference
Thinopyrum elongatum	First leaf length, root weight and root length were not significantly lower in seedlings inoculated with *P. ultimum* than in control. 'Chinese Spring' wheat has significantly lower values for these measurements in inoculated treatments than in control.	(Okubara and Jones, 2011)
'Chinese Spring' wheat addition line with *Th. elongatum* chromosome 4E		
'Chinese Spring' wheat – *Th. elongatum* amphidiploid	As above for *P. ultimum*. This line also showed some increased resistance to *P. irregulare*.	
Wheat	Thirty genotypes of wheat were tested for resistance to *P. debaryanum* (= *P. irregulare*) and *P. ultimum*, measuring the inoculated/control ratio of number of root tips, root length and first leaf length. Variation in resistance was found across the genotypes.	(Higginbotham *et al.*, 2004a)

1.5 Disease resistance mechanisms

Our knowledge about plant disease resistance mechanisms is steadily growing. This section gives a broad description of disease resistance mechanisms, focussing on those that are relevant to the root diseases *Rhizoctonia solani* and *Pythium* species. Studies that have delved into resistance mechanisms and signalling in response to these pathogens are summarised in Tables 1.5, 1.6 and 1.7, and Figure 1.7. A diverse range of plant and *Pythium* species, along with different anastomosis groups of *R. solani*, are included in this summary. Not all mechanisms will necessarily be relevant to wheat and *B. distachyon*.

1.5.1 Genetic resistance

Genetic resistance mechanisms fall into two groups. Qualitative resistance confers immunity to a specific pathogen via a plant *R*-gene. Quantitative resistance does not confer complete resistance, but is considered to be the more durable form of defence (Poland *et al.*, 2009).

Relatively few *R*-gene resistance mechanisms are seen in necrotrophic disease (Poland *et al.*, 2009). Sensitivity to a carbohydrate-based *R. solani* AG1-IA host-selective toxin (HST) is conferred by the *Rsn1* locus in rice (Costanzo *et al.*, 2011). The authors speculate that the enzymatic product of the *R*-gene breaks down the HST, releasing α-glucose molecules that interfere with the plant's cytokinin signalling, thereby causing necrosis. Nair and Thomas (2013) recently characterised a putative *R*-gene in wild ginger, the presence of which protects the plant from *P. aphanidermatum*. The gene has the classic qualitative resistance CC-NBS-LRR (coiled coil-nucleotide binding site-leucine rich repeat) amino acid sequence motif.

Resistance to necrotrophic disease is generally expected to involve multiple quantitative disease resistance genes. Quantitative resistance genes may be pleiotropic, also involved in resistance to multiple pathogens or in plant growth and development, or in other instances they may act as weak *R*-genes (Poland *et al.*, 2009). Quantitative trait loci (QTL) have been found for resistance to *R. solani* in rice and sugar beet (Lein *et al.*, 2008; Eizenga *et al.*, 2013).

1.5.2 Defence proteins

A wide array of proteins is involved in pathogen defence, not only in plants, but across kingdoms. These proteins can be divided into 1) pathogenesis-related (PR) proteins, which are induced in response to biotic attack, but may also protect plants from abiotic damage such as cold stress and wounding, and 2) other proteins that are upregulated

during the defence response, but are otherwise present in normal cell functioning (van Loon *et al.*, 2006).

Van Loon *et al.* (2006) summarise pathogenesis-related proteins, the production of which is induced by jasmonic acid, ethylene and salicylic acid signalling. Several PR-proteins are shown to be involved in the plant defence response to *R. solani* and *Pythium* spp. Lectins play multiple roles in plant defence, symbiosis and in animal innate immunity (De Hoff *et al.*, 2009). Cammue *et al.* (1990) showed that wheat germ agglutinin production is increased in the presence of pathogens, including *R. solani* and *P. ultimum*, but concluded that the lectin probably does not directly inhibit pathogens. Likewise, the protective nature of pectin lyase inhibitor protein (PNLIP) produced by sugar beet was thought to be due to three-way binding of PNLIP with *R. solani* pectin lyase and the pectin substrate (Bugbee, 1993).

A range of proteins produced by wheat has been shown to inhibit the growth of *R. solani* and *Pythium* spp. *in vitro*. This offers the possibility of selecting for lines with high expression of genes encoding defence compounds or of introducing a novel gene by transformation. Liu *et al.* (2009) showed that a wheat β-1,3-glucanase gene expressed *in vitro* inhibited the fungi *R. solani* and *R. cerealis*, as well as the oomycete *Phytophthora capsici*. Inhibition of *R. solani* growth *in vitro* by wheat xylanase inhibitors (TAXI) was suggested to be simply due to binding of fungal β-D-glucans and not degradation of the fungal cell wall (Dornez *et al.*, 2010).

1.5.3 The transgenic approach

The use of transgenic gene expression, primarily of defence proteins, to control fungal diseases has been investigated since the early 1990s, beginning with the over-expression of a bean chitinase gene in tobacco (Broglie *et al.*, 1991). Barley seedlings expressing a chitinase from *Trichoderma* were more resistant to *R. solani* AG8 in pot assays (Kogel *et al.*, 2010). Recently a rice xylanase inhibitor protein was shown to degrade *R. solani* fungal cell wall via chitinase activity (Wu *et al.*, 2013a).

Stefani and Hamelin (2010) summarised the studies that used transgenic methods to target fungi. Over twenty research articles showed a decrease in response to *R. solani* in species of *Nicotiana* (12 articles), *Oryza* (7), *Solanum* (2), *Brassica* (1) and *Gossypium* (1). The genes effective against *R. solani* expressed chitinases, glucanases, thaumatin-like proteins, ribosome inactivating proteins, wheat puroindolines, bacterial and plant anti-fungal toxins, an osmotin promoter binding protein and a zinc-finger protein. Genes that were also effective against *Pythium* spp. included a

toxic bacterial peptide expressed in tobacco (Mitsuhara *et al.*, 2000) and thaumatin expression in tobacco (Rajam *et al.*, 2007).

Where endogenous gene expression is tissue-specific, the use of traditional breeding methods to develop disease resistant plants is difficult. For example, puroindoline expression is endosperm-specific. To achieve increased resistance to *R. solani*, wheat puroindoline synthesis genes were transformed into rice under control of an ubiquitin promoter to extend expression into leaf tissue (Krishnamurthy *et al.*, 2001).

To date no crop species carrying transgenic fungal or oomycete resistance genes have been approved for commercialization (ISAAA, 2013). This may partly be due to the complex nature of resistance signalling, meaning that measurable increases in resistance in pot assays may not translate to field experiments. In future, a nuanced approach to incorporation of resistance genes into crop plants is needed, such as rewiring of hormone signalling pathways to prevent hijack by pathogens and the stacking of multiple resistance genes (Grant *et al.*, 2013).

1.5.4 Secondary metabolites

Secondary metabolites synthesised by plants for pathogen resistance fall into two categories. Phytoanticipins are defence compounds expressed even in the absence of attack, while phytoalexins are synthesised in response to pathogen invasion. Some compounds may be expressed constitutively, but upregulated in response to pathogen attack, in which case they would fall into both categories (Dixon, 2001). The major defence secondary metabolites produced by cereals are benzoxazinoids, terpenoids, flavonoids, cyanogenic glycosides and, only in oats, saponins (Du Fall and Solomon, 2011).

Flavonoids and other secondary metabolites are released into the rhizosphere by plants as a means of communicating with other plants, symbionts and pathogens (Hassan and Mathesius, 2012). Glyceollin is an isoflavonoid derivative that is upregulated in soybean roots by *R. solani* (Wyss *et al.*, 1991). Rosmarinic acid, a caffeic acid ester, is exuded by roots of sweet basil in response to *P. ultimum* and inhibits mycelial growth *in vitro* (Bais *et al.*, 2002). The shikonins, napthoquinone derivatives, of *Lithospermum erythrorhizon* inhibit growth of *R. solani* and *Pythium* spp. *in vitro* (Brigham *et al.*, 1999).

A fascinating split-pot study by Jousset *et al.* (2011) demonstrated the ability of barley to release vanillic acid, *p*-coumaric acid and fumaric acid from uninfected roots, when

other roots of the same plant were infected with *P. ultimum*. Furthermore, these phenolic acids were shown to induce *in vitro* expression of an antifungal gene in the rhizosphere bacterium *Pseudomonas fluorescens*.

1.5.5 Changes in gene expression and signalling

Changes in gene expression during infection provide clues to genes and pathways required for defence. The major drivers of the plant defence response are the phytohormones jasmonic acid (JA), ethylene (ET) and salicylic acid (SA). These signalling molecules modulate defence mechanisms to halt invading pathogens with an innate immune response and to prime defences against future invasion (van Loon *et al.*, 2006; Dodds and Rathjen, 2010). Necrotrophic pathogens are generally met with a JA–ET mediated response, while the response to biotrophic and hemibiotrophic pathogens relies on SA signalling (Glazebrook, 2005). *Rhizoctonia solani* and *Pythium* spp. are considered to be necrotrophic pathogens, although there is a suggestion that some *Pythium* spp. may be hemibiotrophic (Latijnhouwers *et al.*, 2003).

Plant defence priming mediated by JA–ET, known as induced systemic resistance (ISR), occurs in response to biotic factors, such as plant growth-promoting rhizobacteria (PGPR) or pathogenic organisms, while defence priming mediated by SA, known as systemic acquired resistance (SAR), is induced by both biotic and abiotic factors (Vallad and Goodman, 2004; van Loon *et al.*, 2006).

With these distinct roles, the JA–ET and SA pathways often, but not always, act antagonistically. The hormones and transcription factors that modulate these major defence hormones can affect each pathway differently (Pieterse *et al.*, 2012). Auxin (indole acetic acid) has an antagonistic effect on SA signalling while acting synergistically with JA–ET in response to necrotrophic pathogens (Llorente *et al.*, 2008; Kazan and Manners, 2009). Abscisic acid (ABA) positively or negatively modulates the SA response, but tends to induce JA signalling (Fan *et al.*, 2009). Ethylene signalling, mediated by ET responsive transcription factors, is involved in the defence response of several species to *R. solani* (Oñate-Sánchez *et al.*, 2007; Anderson *et al.*, 2010; Guerrero-González *et al.*, 2011).

Many of these defence hormones play separate roles in root development. The hormones JA and ET modulate root development by inhibiting elongation and altering lateral root formation (Okubara and Paulitz, 2005). Abscicic acid and auxin are involved in lateral root initiation (Nibau *et al.*, 2008; Zhang *et al.*, 2008).

Defence signalling is closely linked with the production of reactive oxygen species (ROS), including hydrogen peroxide. Plants increase production of ROS in response to pathogens or effectors, leading to localized cell death. This hypersensitive response is an effective means of halting the invasion of biotrophic pathogens. Necrotrophic pathogens are able to obtain nutrients from dead host tissue and tolerate higher levels of ROS, so the hypersensitive response may in fact aid these pathogens (Mayer *et al.*, 2001).

On the other hand, it does appear that there is a role for the production of ROS in combating necrotrophic infection, as plants that are defective in ROS production are more susceptible to *R. solani* (Foley *et al.*, 2013; Nikraftar *et al.*, 2013). Production of ROS stimulates the production of flavonoids, which act as antioxidants (Agati *et al.*, 2012). This may seem counterintuitive, but flavonoids can also have antifungal properties (Hassan and Mathesius, 2012). Furthermore, hydrogen peroxide activity, mediated by peroxidases, is required for cell wall strengthening, lignification of xylem vessels and suberisation of the epidermis (Almagro *et al.*, 2009). Taheri and Tarighi (2010) described an example of increased lignification in response to *R. solani* sheath blight in rice leading to decreased lesion length.

It is worth noting that *R. solani* and *Pythium* spp. attack seedlings and have the greatest impact at or soon after germination. At this early stage of a plant's life it is heterotrophic, relying on energy stored in the endosperm to grow and develop. In the first few days after germination seedlings are tolerant to dehydration. Signals associated with sucrose metabolism are thought to cause a transition to dehydration intolerance and then to autotrophy (Bogdan and Zagdańska, 2009). Thus, defence signalling in young seedlings takes place in a constantly changing background signalling environment, which may be quite different to that experienced in older plants.

Table 1.5 Studies into plant defence mechanisms with both *Rhizoctonia solani* and *Pythium* spp.

Plant	Mechanism	Reference
Wheat	**Defence protein (lectin)** Wheat germ agglutinin (WGA) in wheat roots rose when incubated in solutions containing polygalacturonic acid fragments, *R. solani* cell wall extract, S-glucan from the extracellular mucilage of *Schizophyllum commune* or R-glucan from cell walls of *S. commune*. The lectin was found to be released into the nutrient solution. WGA also increased upon inoculation with *R. solani, Fusarium culmorum, P. ultimum* or non-pathogenic *Neurospora crassa*. WGA accumulation had previously been demonstrated in response to drought and osmotic stress. The authors disputed previous findings that WGA has an anti-fungal role related to chitin-binding.	(Cammue *et al.*, 1990)
Lithospermum erythrorhizon hairy root cultures	**Secondary metabolite** *Rhizoctonia solani* elicitor increased shikonin production and altered the ratio of shikonin derivatives produced by the roots on media, possibly related to pH variation. Shikonin inhibited growth of *R. solani* above 50 µg/mL *in vitro*. *R. solani* sequestered shikonin from the medium. *Pythium ultimum* and *P. aphanidermatum* were inhibited from 5 µg/mL and 50 µg/mL, respectively.	(Brigham *et al.*, 1999)

Table 1.6 Studies into plant defence mechanisms with *Rhizoctonia solani*.

Plant	Mechanism	Reference
Arabidopsis	**Antioxidant activity / Defence gene expression** Differences in Arabidopsis gene expression were studied, in response to infection with non-pathogenic *R. solani* AG8 compared with pathogenic *R. solani* AG2-1 at 7 days after inoculation. Lines with single mutations in the auxin, camalexin, salicylic acid, abscisic acid and ethylene/jasmonic acid pathways did not respond differently to *R. solani*. Gene expression changes were of greater interest in oxidative stress, cell wall associated protein, transcription factor and heat shock protein pathways. An NADPH oxidase double mutant (A*trbohD AtrbohF*) allowed greater colonisation by *R. solani* AG8, while the single mutant lines did not. *Rboh*-NADPH oxidases produce ROS in response to infection.	(Foley *et al.*, 2013)
Arabidopsis	**Antioxidant activity** A glutathione-S-transferase promoter (*GSTF8*) is induced in Arabidposis after infection with less pathogenic isolates of *R. solani*. The promoter was not induced by strains that caused severe disease.	(Perl-Treves *et al.*, 2004)
Soybean Maize	**Antioxidant activity** Differences were measured in the enzyme activity of soybean and maize plants at 10 days after inoculation with *R. solani* AG2-2 IIIB. Disease symptoms were more severe on soybean than maize. Reactive oxygen species (ROS; superoxide anion $O2^-$ and hydroxyl radical ^-OH) production increased in soybean after infection, but not in maize. Superoxide dismutase (SOD) activity increased only in infected maize roots. Guaiacol peroxidase (GPX) and pyrogallol peroxidase (PPX) activity significantly increased in infected leaves, except for a significant decrease for PPX in maize leaves. Lipid peroxidase (LP) increased in infected soybean leaves and roots, but decreased in maize leaves. Phenylalanine ammonia-lyase (PAL) activity increased in infected soybean roots and in both maize tissues. Polyphenol content increased in infected roots of soybean and maize, but decreased in soybean leaves. Flavonoids and carotenoids were measured in leaves only. Flavonoids increased in infected soybean and maize leaves, while carotenoids decreased. Glutathione (GSH) content increased only in infected soybean leaves. DPPH-free radical scavenging activity increased only in infected leaves of soybean and maize. The authors concluded that the enhanced non-enzymatic antioxidant system (eg, GSH, phenolics, flavonoids) in maize was more effective at preventing ROS increase than the enzymatic antioxidant system (eg. SOD, LP, GPX, PPX).	(Kiprovski *et al.*, 2012)

Table 1.6 continued Studies into plant defence mechanisms with *Rhizoctonia solani*.

Plant	Mechanism	Reference
Tomato	**Antioxidant activity** Twelve hours post inoculation with *R. solani* AG3, a partially resistant variety of tomato had higher accumulation of H_2O_2 than a more susceptible variety. Inhibiting H_2O_2 production reduced resistance to the pathogen. Peroxidase and ascorbate peroxidase activity increased after inoculation. Ascorbate peroxidase reduces H_2O_2 to water. The partially resistant variety accumulated a higher level of phenolics in response to pathogen inoculation than the more susceptible variety or control treatments. Phenolic accumulation reached a maximum following the peroxidase maximum, leading the authors to speculate that peroxidase may be involved in phenolic production. Phenolics are involved in cell wall strengthening and detoxification of mycotoxins.	(Nikraftar *et al.*, 2013)
Tomato	**Antioxidant activity** *Rhizoctonia solani* AG3, AG1-IA and AG4 produced an auxinic plant growth regulator phenylacetic acid (PAA) and its hydroxyl and methoxy derivatives via the shikimate pathway. In uninfected tomato, PAA (0.1 mM and above) reduced root system length an increased necrosis. Quinic acid (QA) is a cyclic polyol released from lignin in decomposing plant tissue. QA was found to increase the *in vitro* growth of *R. solani* AG3, AG1-IA and AG4. The concentration per mycelial weight of PAA and its derivatives produced *in vitro* by *R. solani* decreased following addition of QA. There was an improvement in tomato survival of *R. solani* AG3 when grown on medium containing QA.	(Bartz *et al.*, 2013) following on from (Bartz *et al.*, 2012) and (Liu *et al.*, 2003)
Phaseolus vulgaris (common bean)	**Defence gene expression** Inoculation of bean roots with *R. solani* increased expression of early defence genes *ERF* (ethylene response factor), *PvRK20-1* (receptor-like kinase) and *PA* (acid phosphatase), and subsequent increased expression of *PGIP* (polygalacturonase inhibitor protein), *PR1* (pathogenesis-related protein) and *α-DOX* (alpha-dioxygenase). The greatest induction was seen with *α-DOX*. Alpha-dioxygenases are involved in the synthesis of oxylipins, such as JA, from polyunsaturated fatty acids.	(Guerrero-González *et al.*, 2011)
Rice	**Defence gene expression** *Rhizoctonia solani* AG1-IA growing along the plant surface induced expression of the pathogenesis-related genes *PR1b* and *PRZ1*. Twenty-five genes were found to be induced by *R. solani* and at least one other pathogen.	(Zhao *et al.*, 2008)

Table 1.6 continued Studies into plant defence mechanisms with *Rhizoctonia solani*.

Plant	Mechanism	Reference
Rice	**Defence gene expression** Over-expression of a WRKY transcription factor (*WRKY30*) in rice reduced *R. solani* and *Magnaporthe grisea* lesion length. Expression of WRKY30 could be induced by JA, SA and infection with *R. solani* or *M. grisea.* WRKY30 was found to be localized in the nucleus. Greater induced transcription of lipoxygenase (*LOX*), allene oxide synthase (*AOS2*), pathogenesis related *PR3* and *PR10/PBZ1* in *WRKY30* over-expressing plants in response to *R. solani* infection suggests that WRKY30 is involved in the upstream regulation of these genes. WRKY30 over-expressing plants accumulated greater levels of JA 24 hours after inoculation with either pathogen; however SA increase was not greater than in wild type.	(Peng *et al.*, 2012)
Rice	**Defence gene expression** Gene expression comparison of a moderately resistance rice cultivar, 'Jasmine 85', with and without *R. solani* inoculation. Sense and antisense transcripts were found to be expressed only in inoculated treatments for genes encoding endochitinase, lipid transfer protein, phenylalanine ammonia-lyase, glutathione-S-transferase, WRKY transcription factor, Ras-related protein ARA-3, NAC domain, LRR protein, ubiquitin-conjugating enzyme, serine/threonine kinase, lipoxygenase 8 chloroplast precursor, metallothionein-like protein 1 and hypersentive-induced response protein. Copy number was generally much higher for sense than antisense. The role of antisense transcripts is not known, but the authors speculate that they may be involved in the RNA interference pathway by forming double-stranded RNA.	(Venu *et al.*, 2007)
Barley (transgenic)	**Defence protein (chitinase)** Expression of chitinase from *Trichoderma* reduced *R. solani* AG8 disease in barley in a pot assay at one week after inoculation. Presence of the transgene was found to have a negligible effect on the background transcriptome of field-grown barley.	(Kogel *et al.*, 2010) following on from (Wu *et al.*, 2006)
Rice-derived	**Defence protein (chitinase)** A chitinase-like protein (OsCLP) produced by rice had chitinase activity and degraded *R. solani* cell wall *in vitro.* Although it was phylogenetically classed as a putative xylanase inhibitor protein (XIP) it did not exhibit xylanase activity. *OsCLP* expression was induced following infection with *Magnaporthe oryzae.*	(Wu *et al.*, 2013a)

Table 1.6 continued Studies into plant defence mechanisms with *Rhizoctonia solani*.

Plant	Mechanism	Reference
Sugar beet	**Defence protein** A pectin lyase inhibitor protein (PNLIP) extracted from sugar beet inhibited *R. solani* derived pectin lyase *in vitro*. Colonisation by *R. solani* AG2-2 increased the pH of above-ground tissues from 6.5 to as high as 8 and decreased pH of below-ground tissues to as low a 4. Pectin lyase activity increased with increasing pH.	(Bugbee, 1993)
Wheat-derived	**Defence protein** A lipid transfer protein (LTP) cloned from *Triticum aestivum* inhibited growth of *R. solani in vitro*. Earlier, over-expression of an *Allium cepa* non-specific LTP was shown to reduce *R. solani* sheath blight in *indica* rice.	(Patkar and Chattoo, 2006; Kirubakaran *et al.*, 2008)
Arabidopsis	**Ethylene signalling** Application of exogenous ET to wild type Arabidopsis increased expression four of ET response factor (ERF) genes, defensin (*PDF1.2*) and basic chitinase (*ChiB*). *AtERF14* was required for *PDF1.2* induction following ET treatment and, to a lesser extent, induction of *ChiB, ERF1, AtERF2* and *AtERF15*. *AtERF14* loss-of-function mutants were more susceptible to *Fusarium oxysporum*, but not to *R. solani* ZG 3 & ZG 5. Wild type Arabidopsis was partially resistant to the *R. solani* strains.	(Oñate-Sánchez *et al.*, 2007)
Medicago truncatula	**Ethylene signalling** An ET insensitive mutant *(MtSkl1*, orthologue of *AtEin2*) was more susceptible to *R. solani* AG8 ZG1 and *Phytophthora medicaginis*. The mutant had reduced levels of ET and 1-amino-cyclopropane-carboxylic acid (ACC) oxidase following inoculation with *Ph. medicaginis* and was unable to convert added ACC to ET. Previously, *MtSkl1* had been found to allow hyperinfection by the nodule-forming symbiotic bacteria *Sinorhizobium meliloti*. This study found increased early infection by *Glomus* spp.	(Penmetsa *et al.*, 2008)
Medicago truncatula	**Ethylene signalling** Application of ET reduced damping off in barrel medic due to *R. solani* AG8 ZG1-1, but had less impact on reducing disease symptoms in established seedlings. Over-expression of an ET response transcription factor (ERF) increased resistance to *R. solani* AG8 and *Phytophthora medicaginis*, but not root rot nematode, in barrel medic, independent of nodulation. The authors conclude that ET is more important than SA and JA in defence signalling pathways during the first 24 h after infection with *R. solani*.	(Anderson *et al.*, 2010)

Table 1.6 continued Studies into plant defence mechanisms with *Rhizoctonia solani*.

Plant	Mechanism	Reference
Rice (transgenic)	**Ethylene signalling** Over-expression of ET increased expression of defence genes and reduced lesion size of *R. solani* and *Magnaporthe oryzae*. Expression was controlled by a pathogen-inducible *PBZ1* promoter. Suppression of ET biosynthesis increased susceptibility to *R. solani.* In rice, flooding can lead to hypoxia-induced ET production.	(Helliwell *et al.*, 2013)
Phaseolus vulgaris (common bean)	**Induced resistance / Biocontrol** Inoculation of one week old seedlings with binucleate *Rhizoctonia* (BNR) 48 hours prior to pathogen challenge reduced the severity of disease caused by *R. solani* or *Colletotrichum lindemuthianum.* BNR protection resulted in increased peroxidase activity, increased levels of 1,3-β-glucanase in bean hypocotyls and increased levels of chitinase in cotyledons. Only peroxidase and 1,3-β-glucanase was correlated with resistance to *R. solani.*	(Xue *et al.*, 1998)
Rice	**Induced resistance / Lignification** Rice plants sprayed with riboflavin or JA had increased lignin formation and reduced *R. solani* lesion length. Suggestion that riboflavin priming effect was related to increased H_2O_2 production in leaves. Treatment with the lipoxygenase (LOX) inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA) blocked the IR response to riboflavin. *LOX* is involved in the biosynthesis of JA. Riboflavin treatment increased expression of *LOX* and *PAL* (phenylalanine ammonia-lyase) more than in the control. Induction of *PAL* was independent of JA. ETYA inhibited *LOX* and, to a lesser extent, *PAL.*	(Taheri and Tarighi, 2010)
Rice	**Metabolic pathway changes** *Rhizoctonia solani* activated the glycolytic, OPPP, TCA, shikimate and phenylpropanoid pathways in rice. Increase in H_2O_2 production and glycolytic enzyme activity.	(Mutuku and Nose, 2012)
Rice	**Quantitative resistance** Found several QTL for resistance to *R. solani* in a backcross population of *Oryza sativa* and *O. nivara*.	(Eizenga *et al.*, 2013)
Sugar beet	**Quantitative resistance** Found three major QTL that explained 71% of resistance to *R. solani* AG2-2 IIIB in sugar beet.	(Lein *et al.*, 2008)

Table 1.6 continued Studies into plant defence mechanisms with *Rhizoctonia solani*.

Plant	Mechanism	Reference
Rice	***R*-gene resistance** Mapping of the *Rsn1* locus. The dominant allele conferred sensitivity to a *R. solani* AG1-IA host-selective toxin (HST), resulting in necrosis. Leaves of recessive *rsn1* plants only exhibited chlorosis at the site of toxin infiltration. Two candidate genes at the locus are predicted to encode a cytokinin-*O*-glucosyltransferase. The authors suggest that the enzyme may directly degrade the carbohydrate-based HST. Genotypes of wheat and barley were tested and not found to be sensitive to the toxin.	(Costanzo *et al.*, 2011) following on from, (Brooks, 2007) and (Vidhyasekaran *et al.*, 1997)
Potato	**Secondary metabolite** Gene expression comparison of potato sprouts with and without *R. solani* AG3 inoculation. Infection increased production compounds including the aglycon solasodine, sesquiterpene phytoalexins, oxidised unsaturated fatty acids, fungitoxic colneleic and colnelenic acids, the majority of carboxylic acids, β-alanine, 4-aminobutyric acid (GABA), pipecolic acid, ferulic acid, α-tocotrienol and phenylacetic acid.	(Aliferis and Jabaji, 2012)
Soybean	**Secondary metabolite** *Rhizoctonia solani* infestation of soil increased production of glyceollin in soybean roots. Glyceollin production was not increased by infection with the mycorrhizal fungus *Glomus mosseae*.	(Wyss *et al.*, 1991)

Table 1.7 Studies into plant defence mechanisms with *Pythium* spp.

Plant	Mechanism	Reference
Arabidopsis	**Defence gene expression** *PEN2* (glucosyl hydrolase gene) was involved in avoiding penetration by *P. irregulare* and *ERECTA* (receptor-like kinase gene) was involved in PAMP recognition. Following invasion ABA was involved in upregulation of a third of genes induced by the pathogen, including activation of JA biosynthesis. ABA putatively primes callose biosynthesis. *P. irregulare* was seen to have biotroph-like infection structures, but had gene expression responses more similar to necrotrophs. Differential expression of JA/SA/ET independent defence genes was similar to expression in response to abiotic stresses.	(Adie *et al.*, 2007)
Ginger	**Defence gene expression** Differential expression of 41 genes in response to SA, JA, ET and *P. aphanidermatum* in lines tolerant or susceptible to the pathogen. Resistance of *Z. zerumbet* to *P. aphanidermatum* appeared to be independent of these signalling molecules.	(Kavitha and Thomas, 2008)
Physcomitrella patens (moss)	**Defence gene expression** Infection with *P. irregulare* or *P. debaryanum* led to ROS production and cell death. The pathogen induced synthesis of JA and its precursor 12-oxo-phytodienoic acid (OPDA), as well as expression of *CHS* (chalcone synthase), *LOX* (lipoxygenase) and *PAL* (phenylalanine ammonia-lyase). Chloroplasts were moved closer to the cell wall nearest the infected area. Cell walls were reinforced with phenolic compound accumulation and callose deposition in infected plant cells.	(Oliver *et al.*, 2009)
Tobacco Arabidopsis (transgenic)	**Ethylene signalling** ET insensitive mutants of tobacco and Arabidopsis were more susceptible to *P. irregulare, P. sylvaticum, P. jasmonium and P. aphanidermatum*. Greater colonisation of plant tissue by *Pythium* in mutants, including stele, stem and leaf tissues. Speculated inability of ET insensitive plants to recover from disease due to impaired adventitious root production.	(Geraats *et al.*, 2002)
Arabidopsis	**Induced resistance** *Pythium oligandrum* is a non-pathogenic species used for biocontrol. Treatment with a cell wall protein fraction of *P. oligandrum* induced resistance to two bacterial pathogens. CWP is made up of two glycoproteins, structurally similar to class III elicitins. *SGT1* and *RAR1* (co-chaperones of *HSP90*), *NPR1* (activation of SA signalling and SAR; cross-talk between SA and JA/ET signalling) and *JAR1* (JA signalling) were all required for defence response.	(Kawamura *et al.*, 2009)

Table 1.7 continued Studies into plant defence mechanisms with *Pythium* spp.

Plant	Mechanism	Reference
Arabidopsis	**Innate immune response** Wounding, MeJA and ET induced the expression of *PROPEP1*, which produces the precursor to *At*Pep1. The *At*Pep1 peptide acts as a plant-derived elicitor of innate immune response. Constitutive over-expression of *PROPEP1* increased root growth, caused over-expression of *PDF1.2* (*defensin*) and conferred resistance to *P. irregulare.* *At*Pep1 application induced the production of H_2O_2 and *PDF1.2* expression.	(Huffaker *et al.*, 2006)
Arabidopsis	**Jasmonic acid signalling** Increased susceptibility to *P. irregulare* in a *jar1* (jasmonate signalling) mutant of Arabidopsis. The mutant also exhibits increased sensitivity to ABA.	(Staswick *et al.*, 1998)
Barley	**Root exudate / Biocontrol** *Pythium ultimum* infection increased exudation of vanillic, *p*-coumaric and fumaric acid at a distance. These phenolic acids were shown to induce the expression of *phlA* in *Pseudomonas fluorescens*. *phlA* is involved in synthesis of the compound 2,4-diacetylphloroglucinol (DAPG), an inhibitor of *Pythium* growth. Infection of barley roots with *Pythium ultimum* increased *phlA* expression at a distance.	(Jousset *et al.*, 2011)
Ginger	***R*-gene resistance** Induced expression of the *ZzR*1 resistance gene in response to *P. aphanidermatum* infection. *ZzR1* is a CC-NBS-LRR type *R*-gene, which recognises an oomycete avirulence factor.	(Nair and Thomas, 2013)
Ocimum basilicum (Sweet basil) hairy root cultures	**Secondary metabolite** Suggestion that *P. ultimum* induced production of rosmarinic acid in sweet basil hairy root cultures grown on solid medium. Rosmarinic acid inhibited *in vitro* growth of *P. aphanidermatum, R. solani*, other fungi and oomycetes. Chitosan, JA and SA inhibited root growth, while *Phytophthora* cell wall extract increased root growth *in vitro*.	(Bais *et al.*, 2002)

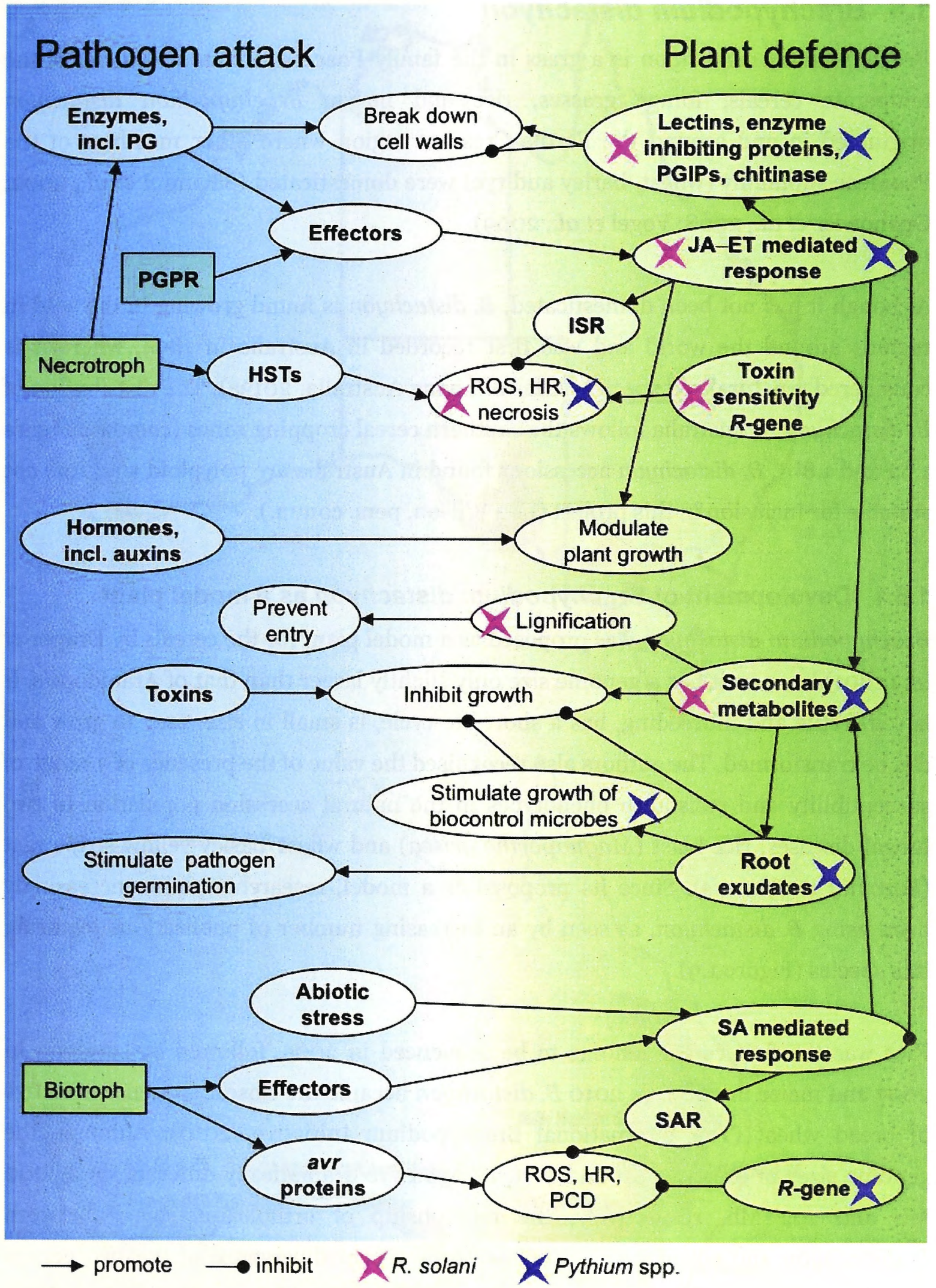

Figure 1.7 Plant-pathogen defence pathways in response to necrotrophs and biotrophs. Stars indicate stages that have been previously studied in *R. solani* or *Pythium* spp. disease. ET, ethylene; JA, jasmonic acid; HR, hypersensitive response; HSTs, host-selective toxins; PCD, programmed cell death; PG, polygalacturonase; PGIP, polygalacturonase inhibitor protein; PGPR, plant growth-promoting rhizobacteria; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance.

1.6 *Brachypodium distachyon*

Brachypodium distachyon is a grass in the family Poaceae, which also includes the temperate cereals, forage grasses, rice and maize. *Brachypodium distachyon* originated in and around the Fertile Crescent region where other members of the Pooideae subfamily (wheat, barley and rye) were domesticated (Salamini *et al.*, 2002; Opanowicz *et al.*, 2008; Vogel *et al.*, 2009).

Although it has not been domesticated, *B. distachyon* is found growing in the wild in regions around the world and was first recorded in Australia in 1894, where it is considered a naturalised species (Atlas of Living Australia, 2013a). The distribution of *B. distachyon* in Australia follows the southern cereal cropping zones (compare Figure 1.8a and 1.8b). *B. distachyon* accessions found in Australia are polyploid and thus not suitable for inclusion in this project (Iain Wilson, pers. comm.).

1.6.1 Development of *Brachypodium distachyon* as a model plant

Brachypodium distachyon was proposed as a model plant for the cereals by Draper *et al.* (2001) because it has a genome size only slightly larger than that of Arabidopsis, it is self-fertile and inbreeding, has a short life-cycle, is small in size, easy to grow and can be transformed. The authors 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 its proposal as a model, researchers have increasingly been using *B. distachyon*, as seen by an increasing number of publications featuring this species (Figure 1.9).

Rice was the first cereal genome to be sequenced in 2005, followed by sorghum in 2007 and maize in 2009. In 2010 *B. distachyon* became the closest sequenced relative of bread wheat (The International Brachypodium Initiative, 2010). Although the genome sizes of bread wheat and *B. distachyon* are dramatically different, at 17 000 Mb and 272 Mb, respectively, the relationship of orthologous genes between *B. distachyon* and wheat reflects the relatively recent divergence of the two genera around 32 to 39 million years ago (Figure 1.10). The high quality *B. distachyon* genome sequence is now being used to annotate the sequenced bread wheat genome (Brenchley *et al.*, 2012) and to help with fine-mapping of disease resistance genes, e.g. Zhang *et al.* (2013). Draft genomes of *Triticum urartu* and *Aegilops tauschii*, from which the wheat A- and D-genomes are derived, respectively, were published in 2013 (Jia *et al.*, 2013; Ling *et al.*, 2013).

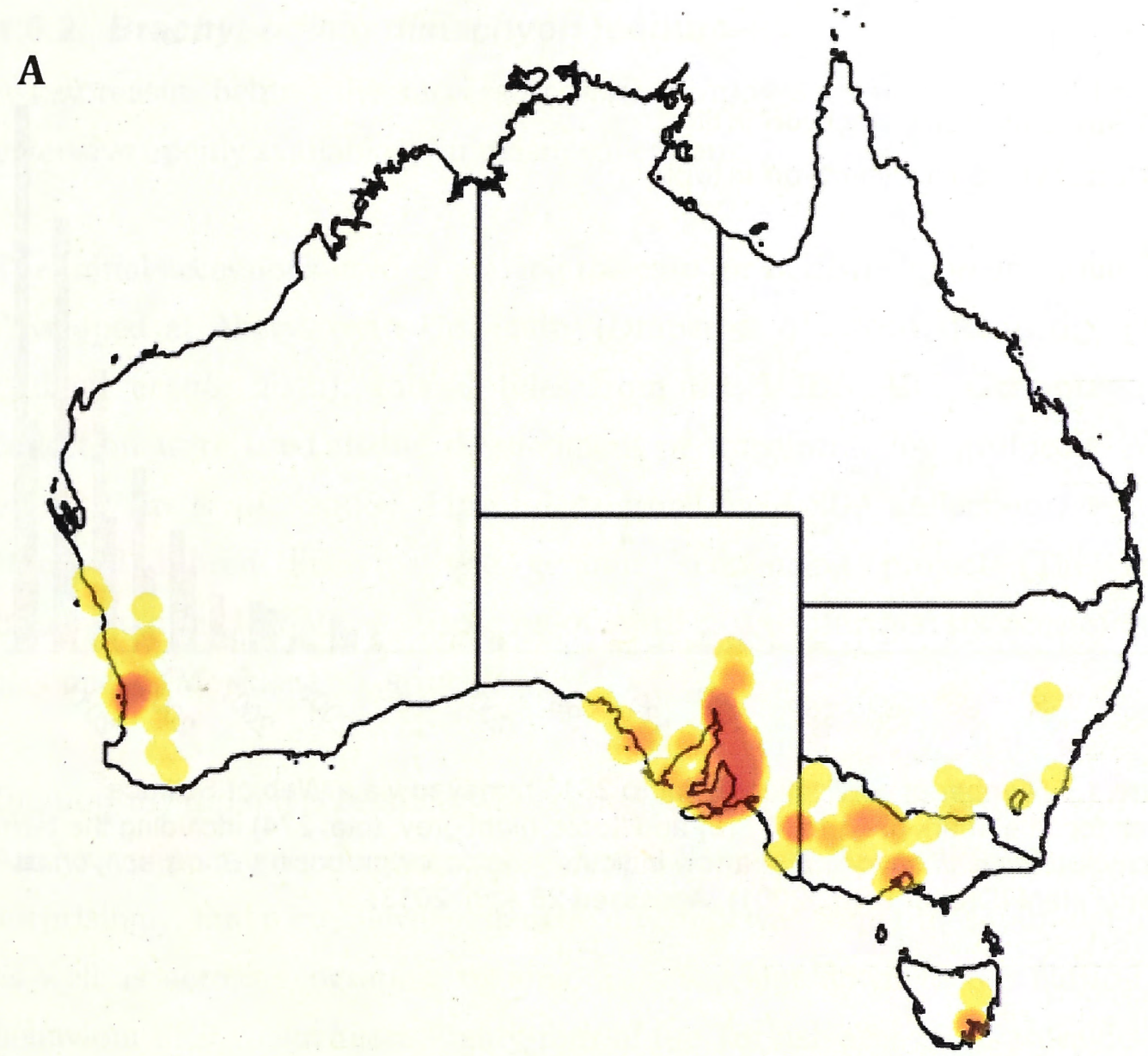

Figure 1.8 a) Distribution of 1,372 occurrence records of *Brachypodium distachyon* in Australia (Atlas of Living Australia, 2013a), b) Australian wheat crop agro-ecological zones (Murray and Brennan, 2009).

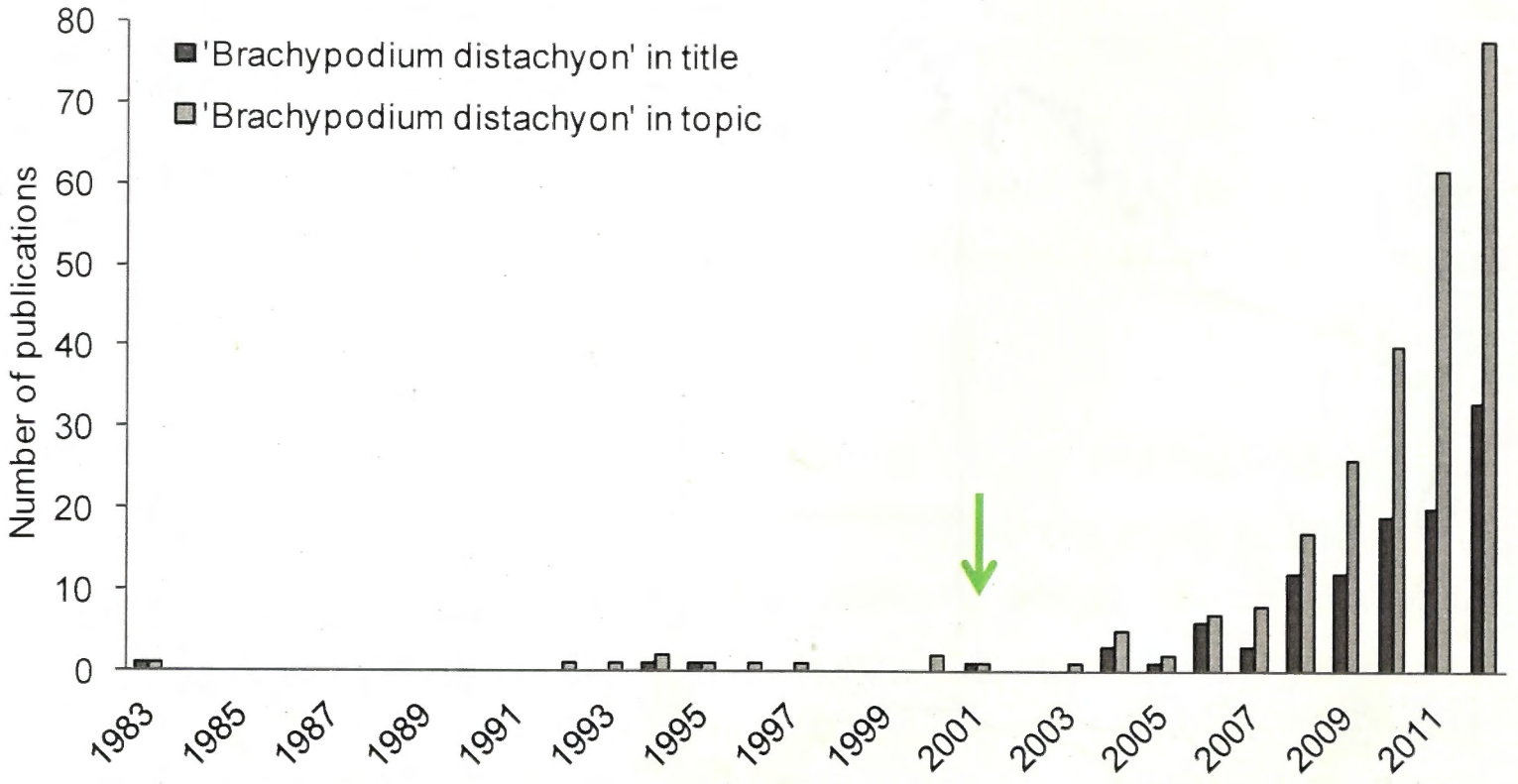

Figure 1.9 Number of publications prior to 2013 retrieved via a Web of Science® search for 'title' (dark grey, total 118) and 'topic' (light grey, total 274) including the term '*Brachypodium distachyon*'. The arrow indicates the paper proposing *B. distachyon* as a model plant (Draper *et al.*, 2001). Accessed 26 April 2013.

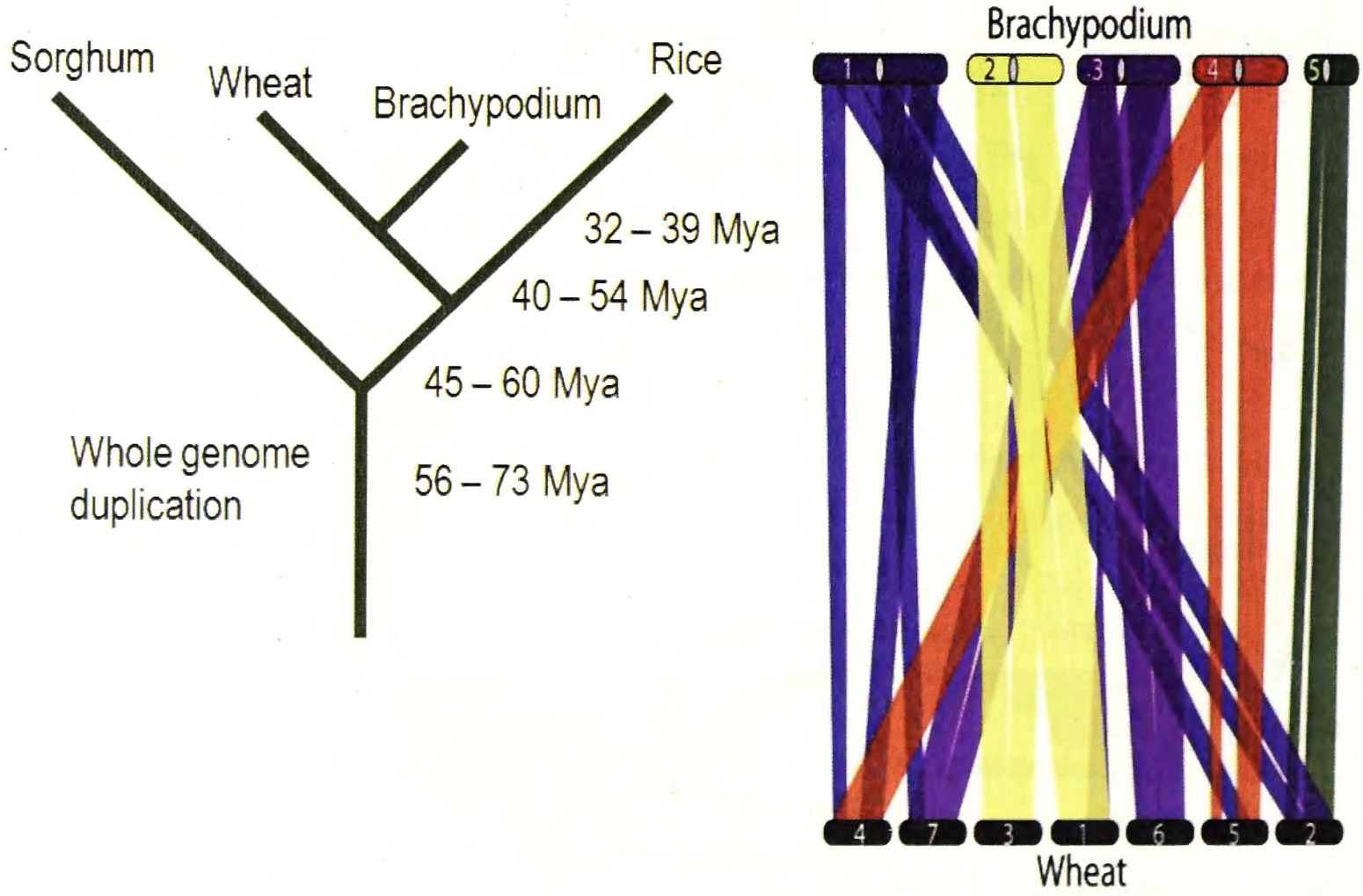

Figure 1.10 a) Divergence of the Poaceae species, following whole genome duplication 56 – 73 million years ago, b) Location of orthologous genes on the five chromosomes of *B. distachyon* and the seven chromosomes of bread wheat. Reproduced from The International Brachypodium Initiative (2010).

1.6.2 *Brachypodium distachyon* resources

A key reason behind the success of *B. distachyon* as a model is the development of extensive openly available germplasm collections.

The initial accessions used in making the case for *B. distachyon* as a model plant were developed at Aberystwyth University (Draper *et al.*, 2001; Routledge *et al.*, 2004; Catalán *et al.*, 2012). Inbred lines from the USDA ARS Germplasm Resources collection were used in the development of transformation protocols (Vogel *et al.*, 2006; Vain *et al.*, 2008). Line Bd 21 from the USDA collection was used as the standard inbred line for the genome sequencing project (The International Brachypodium Initiative, 2010). As of April 2013 a further six accessions had been sequenced (Mockler Lab, 2013).

In 2006 187 diploid accessions were collected from a range of locations around Turkey. Analysis of genotypic diversity using Simple Sequence Repeat (SSR) markers revealed, surprisingly, that a high level of genetic diversity was sometimes found at one location, as well as across geographic regions. This was put down to the natural inbreeding behaviour of *B. distachyon*. Eighty-four of the Turkish accessions were developed into inbred lines, boosting the natural phenotypic diversity in the available *B. distachyon* germplasm (Vogel *et al.*, 2009).

With the aim to create a model plant as useful as Arabidopsis, researchers went about developing genomic resources including mapping populations and T-DNA insertion mutant lines. By 2012 eight institutions had developed many thousands of T-DNA lines (Thole *et al.*, 2012). The two leading openly available collections were BrachyTAG, using line Bd 21, and the Western Regional Research Center (WRRC) collection, using line Bd 21-3 (Bragg *et al.*, 2012). Recombinant inbred line (RIL) mapping populations have been created to hone in on quantitative trait loci (QTL) for phenotypic traits including resistance to *Puccinia brachypodii* (leaf rust) and a *Barley stripe mosaic virus* resistance gene (Barbieri *et al.*, 2012; Cui *et al.*, 2012).

1.6.3 *Brachypodium distachyon* as a model to study cereal disease resistance

The common geographical origin of *B. distachyon* and wheat would suggest that the two species faced similar biotic and abiotic stresses under similar climatic and soil conditions. It is only with its domestication, that wheat has been taken to environments with different selection pressures.

Brachypodium distachyon has lent itself to the study of several fungal and viral diseases of cereals, as summarised in Table 1.8. Many of the studies report varying levels of pathogen resistance in the *B. distachyon* natural accession collection.

In using a model plant to study disease in a crop species, it must be kept in mind that, in comparison with the whole genome, the evolution of resistance genes can occur at a higher rate (Leister *et al.*, 1998). This means that the inheritance of resistance genes cannot necessarily be deduced from phylogenetic trees. For example, orthologues of the *Lr34* disease resistance gene can be found in a wide range of the Poaceae, including sorghum and rice. In Pooideae this gene has been lost from the *B. distachyon* and barley genomes, but retained in wheat (Krattinger *et al.*, 2011). Drader and Kleinhofs (2010) noted that a highly diverged barley stem rust resistance gene (*Rpg1*) orthologue could be found in *B. distachyon*, but not in wheat. There was a high level of synteny between barley and *B. distachyon* disease resistance genes in some regions of the genome. While this rapid evolution may pose a problem if orthologues of resistance genes found in *B. distachyon* are missing in wheat, it also demonstrates the advantage of having several model cereals.

1.6.4 *Brachypodium distachyon* as a potential model for root diseases

While *B. distachyon* has been used to study a range of cereal shoot diseases, the same is not true for root diseases. This probably reflects the generally lower level of interest in root diseases, rather than any deficiency in the ability of *B. distachyon* to be useful as a root disease model. *Brachypodium distachyon* has been used to study roots traits including gene expression in iron homeostasis (Yordem *et al.*, 2011), the effect of nitrogen and phosphorus on root system architecture (Ingram *et al.*, 2012) and in comparisons of nodal to primary root ratios in mature plants (Chochois *et al.*, 2012). *Brachypodium distachyon* interactions with root fungi are being studied in the comparison of phenotypic and expression differences during symbiosis with different arbuscular mycorrhizae (Hong *et al.*, 2012).

Table 1.8 Previous research into crop pathogens, using *B. distachyon* as a model.

Pathogen	Notes	Reference
Oculimacula spp. (eyespot), *Ramularia collo-cygni* (ramularia leaf spot)	Quantitative difference in severity of cereal diseases *O. acuformis* and *O. yallundae* between two *B. distachyon* inbred lines. The line more resistant to *Oculimacula* spp. also developed less necrosis and chlorosis after infection with *Ra. collo-cygni*, with the effect stronger under high light conditions.	(Peraldi *et al.*, 2013)
Puccinia graminis ff. spp. *tritici avena and phalaridis* (stem rust; natural hosts wheat, oat and phalaris grass, respectively), *Pu. triticina* (wheat leaf rust) *Pu. striiformis* (stripe rust)	Varying susceptibility to *Pu. striiformis* f. sp. *tritici* and *Pu. graminis* f. sp. *tritici* across *B. distachyon* natural accessions, with lines more susceptible to one rust species tending to be less susceptible to the other species. Differences in susceptibility followed the major phylogenetic grouping in *B. distachyon* (Garvin *et al.*, 2010). Little variation in *Pu. triticina* resistance between lines. *Pu. graminis* ff. spp. *avena and phalaridis* infected most lines, but not fully susceptible. *Pu. striiformis* f. sp. *tritici* sporulation only occurred under cooler temperatures. Lines were crossed to study the inheritance of non-host resistance and callose deposition.	(Ayliffe *et al.*, 2013)
Puccinia graminis ff. spp. *tritici, lolii* and *phlei-pratensis*	Varying levels of susceptibility found across eight *B. distachyon* inbred lines to *Pu. graminis* ff. spp. with natural hosts wheat, perennial ryegrass and timothy grass. None were fully susceptible to these isolates.	(Figueroa *et al.*, 2013)
Fusarium pseudo-graminearum (crown rot)	*B. distachyon* was found to be susceptible to the wheat pathogen *F. pseudograminearum*. Mutant lines are being used to look for disease resistance genes.	(Fitzgerald *et al.*, 2012a)
Puccinia brachypodii (leaf rust), *Pu. striiformis*	Three QTL for resistance to *Pu. brachypodii* were found in a *B. distachyon* recombinant inbred line (RIL) population. Most accessions were found to be immune to three isolates of *Pu. striiformis* with natural hosts wheat, barley and California brome grass.	(Barbieri *et al.*, 2012) following on from (Barbieri *et al.*, 2011)
Barley stripe mosaic virus	The resistance gene to BSMV, *Bsr1*, was mapped in a *B. distachyon* RIL population.	(Cui *et al.*, 2012)
Panicum mosaic virus and its satellite virus (SPMV)	Gene expression studies on *B. distachyon* infected with PMV or both PMV and SPMV. These viruses cause disease in turfgrasses.	(Mandadi and Scholthof, 2012)
Magnaporthe oryzae (rice blast)	*B. distachyon* was found to be a possible alternate host to barley and rice to look for resistance to *M. oryzae*.	(Wang *et al.*, 2012)
Fusarium spp. (head blight)	Variation in resistance was found between two *B. distachyon* inbred lines to *F. graminearum* and *F. culmorum*, necrotrophic pathogens of wheat.	(Peraldi *et al.*, 2011)

Table 1.8 continued Previous research into crop pathogens, using *B. distachyon* as a model.

Pathogen	Notes	Reference
Pythium spp.	Observation of Pythium root rot in *B. distachyon*.	(Vogel and Bragg, 2009)
Magnaporthe grisea (rice blast)	Segregation of resistance to a rice-adapted strain of *M. grisea* in a cross between *B. distachyon* ecotypes suggested a single dominant resistance gene. Ecotypes were generally distinctly resistant or susceptible to strains of *M. grisea* adapted to St Augustine grass, crabgrass and perennial ryegrass.	(Routledge *et al.*, 2004)
Magnaporthe grisea *Puccinia striiformis* *Blumeria graminis* (powdery mildew), *Pu. recondita* (brown rust)	The *B. distachyon* accessions tested were resistant to *Bl. graminis* f. sp. with natural hosts oats, barley and wheat, and to *Pu. recondita* ff. spp. with natural hosts barley and wheat. Varying levels of resistance were found towards *M. grisea* and to *Pu. striiformis* ff. spp. with natural hosts barley and wheat.	(Draper *et al.*, 2001)

While there are many conserved genes and functions between the dicotyledonous and monocotyledonous plants, the use of the dicot Arabidopsis as a model for grass plants has its limitations (Watt *et al.*, 2009). Root development and internal root structure differ markedly between monocots and dicots. Monocots produce one or several primary roots from the base of the embryo, followed by nodal roots, which emerge from leaf nodes on the stem. Dicots produce a single tap root from the radicle. Xylem and phloem vessels within the stele are arranged differently, and monocots lack vascular cambia and a periderm (Chochois *et al.*, 2012). These divergent root anatomies appear to be linked to fundamental differences in water uptake between monocots and dicots (Bramley *et al.*, 2009). In the model species, recent experiments with *B. distachyon* T-DNA mutant lines showed that auxin signalling pathways differ between *B. distachyon* and Arabidopsis (Pacheco-Villalobos *et al.*, 2013).

Looking to plant-pathogen co-evolution, the distribution of *R. solani* and *Pythium* spp. is widespread. *Pythium irregulare* has been recorded in all continents of the world except Antarctica, including in the Fertile Crescent region (CABI/EPPO, 2011). *Rhizoctonia solani* AG8 was recovered from fields in Australia, Scotland and the USA soon after it was first described (Ogoshi, 1987). In 2012 *R. solani* AG8 was first reported in Turkey, the country in which most natural accessions in the *B. distachyon* collection originated (Ünal and Dolar, 2012). Since the divergence of wheat and *B. distachyon* over 30 million years the region of present-day Turkey has experienced warmer and cooler climate conditions, and corresponding shifts in vegetation (Ivanov

et al., 2011). Thus it is possible that *R. solani* AG8 and *P. irregulare* have long interacted with the Poaceae under conditions favourable for pathogenesis.

At present there is no evidence that *B. distachyon* is susceptible to *R. solani* AG8. It has been observed that *Pythium* root disease occurs in *B. distachyon*, but the interaction has not been investigated further (Vogel and Bragg, 2009).

1.7 Rationale and objectives

The wild population of *B. distachyon* so far has been shown to be divergent for susceptibility to a number of fungal and viral cereal diseases. Therefore it is quite possible that these genotypes will respond differently to *Rhizoctonia solani* AG8. As of November 2013, there are no reported studies into the interaction of different *B. distachyon* accessions with oomycete or bacterial pathogens. Nevertheless, the *B. distachyon* population may vary in resistance to *Pythium irregulare*.

It is however unlikely that a complete resistance phenotype will emerge from the population to either *R. solani* or *P. irregulare*. There is no known complete resistance to these diseases in the cereals or their wild relatives. Disease resistance screening will focus on discovery of quantitative resistance, as this is the typical form of resistance found towards necrotrophic pathogens and has the advantage of being more durable than qualitative resistance (Poland *et al.*, 2009).

The aim of this thesis is to discover variation in resistance to wheat root diseases in the *B. distachyon* population, to pave the way towards elucidating markers for genetic regions involved in resistance in *B. distachyon* and, by virtue of their close relationship, in wheat. It is hoped that the small genome, diverse population and the petite stature of *B. distachyon* will reveal root disease resistance secrets hidden within the complex wheat genome.

1.7.1 Research objectives

1) To determine whether *Brachypodium distachyon* is susceptible to *Rhizoctonia solani* AG8 and *Pythium irregulare*.
2) To develop methods to screen a population of *B. distachyon* for differing levels of resistance to these root diseases.
3) To discover and quantify variation in resistance to these root diseases across the *B. distachyon* natural accession and T-DNA mutant collections.
4) To discover possible mechanisms contributing to variation in root disease resistance.

Chapter 2

Developing a phenotypic screen for *Brachypodium distachyon* resistance to *Rhizoctonia solani* AG8

Summary

This chapter describes a repeatable phenotyping system to screen *Brachypodium distachyon* for resistance to the fungal root pathogen *Rhizoctonia solani* AG8. Four experiments identified the following:

- An isolate of *R. solani* AG8, previously used in wheat studies that infects *B. distachyon* roots in a similar pattern to that of wheat;

- A method for soil inoculation and growth conditions to achieve repeatable infection patterns of *B. distachyon* roots by *R. solani*, that also included a 'tooth-pick' assay system to remove contaminated controls and standardise soil inoculation levels;

- That root length was the most robust phenotype for measuring *R. solani* impact on *B. distachyon*, with shoot phenotypes and DNA quantitation also evaluated as measures of disease resistance; and

- Preliminary evidence for variation in resistance to *R. solani* AG8 among *B. distachyon* accessions.

The results of this chapter provide the foundation for wider screening of *B. distachyon* accessions and T-DNA lines in Chapters 3 and 4, respectively.

2.1 Introduction

This chapter describes the development of a phenotyping method to screen *B. distachyon* genotypes for resistance to *R. solani* AG8. Although *R. solani* AG8 has a broad host range, its effect on *B. distachyon* had not been reported prior to this thesis.

Brachypodium distachyon accessions and *Triticum aestivum* cv. Janz bread wheat were used to compare the effect of *R. solani* on root and shoot growth between the two species, and to establish the phenotyping conditions to identify resistance in *B. distachyon* that would be relevant to wheat. *Triticum aestivum* cv. Janz is an Australian cultivar known to be susceptible to *R. solani* AG8 from other experiments within this thesis (Appendix B.1), and publications (Yang *et al.*, 1994; Kirkegaard *et al.*, 1999). The *B. distachyon* accessions included the two commonly studied reference lines, Bd 21 and Bd 21-3. Line Bd 21 was the first genotype sequenced (Vogel et al., 2010), and Bd 21-3 is the genotype used most widely for transformation, and is the parent line of the T-DNA lines used in this thesis in Chapter 4 (Vogel and Hill, 2008; Bragg *et al.*, 2012). Eight additional lines from distinctly divergent groups of a collection of 187 Turkish inbred, diploid *B. distachyon* accessions were also included (Adi-10, Bd 3-1, BdTR 3c, BdTR 10o, BdTR 13a, BdTR 13c, Koz-1 and Koz-3; see Figure 2.1). The Turkish collection was assembled to maximise geographic and climatic origins by Vogel *et al.* (2009). Phylogenetic analysis of the collection using 43 simple sequence repeat (SSR) markers shows a high level of genetic diversity in the population (Figure 2.1). This supported the large range of phenotype diversity seen across the lines, including vernalization requirements, shoot architecture and seed size observed when the lines were being grown for seed at CSIRO.

For these phenotyping activities, an isolate of *R. solani* AG8 ZG1-1 was obtained from Jonathan Andersen of CSIRO. This isolate has been used to study *R. solani* AG8 disease in Arabidopsis, a resistant host, and *Medicago truncatula* (Anderson *et al.*, 2010; Foley *et al.*, 2013). *Rhizoctonia solani* AG8 ZG 1-1 also causes disease in wheat (Sweetingham *et al.*, 1986; Neate *et al.*, 1988) and is thus an appropriate isolate to test against *B. distachyon*.

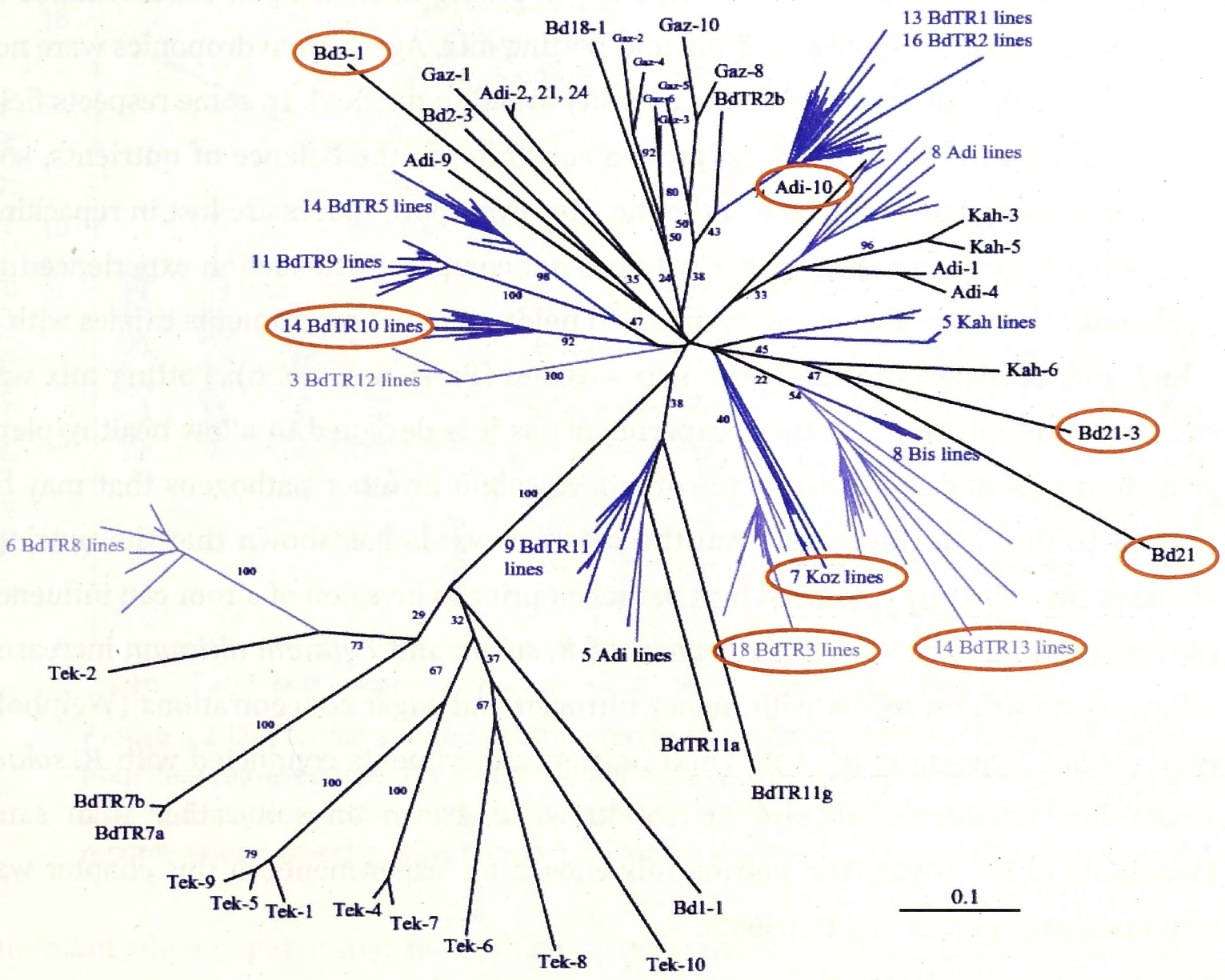

Figure 2.1 Phylogenetic comparison of 187 lines in the Turkish population using 43 SSR markers. Circles indicate those lines used in experiments in this chapter: Adi-10, Bd 3-1, Bd 21, Bd 21-3, BdTR 3c, BdTR 10o, BdTR 13a, BdTR 13c, Koz-1 and Koz-3. Reproduced from (Vogel *et al.*, 2009).

A large number of experimental conditions have been used to test responses of wheat to *R. solani*, and a wide survey of the literature was conducted to arrive at the seed preparation, inoculum and growth substrate for the phenotyping (summarised in Table 2.1). To achieve a consistent high level of emergence *B. distachyon* seeds were dehusked, surface-disinfected and germinated overnight prior to sowing, consistent with previous studies. This method allows seed viability to be checked before sowing to achieve a high rate of emergence. Potential pre-germination damping off is also avoided so that the measured effect of *R. solani* is only on root growth. Inoculum was prepared using millet seeds due to its stability over time (it was able to be stored at -20°C for over a year without affecting pathogenicity), and its ability to be incorporated into growth substrate immediately prior to sowing, in order to be able to more accurately calculate propagule number at sowing. To standardise level of inoculum across pots and ensure no contamination of controls, a toothpick re-isolation method was adapted to ensure the fungus consistently colonised the region of soil at seed level within a week after sowing.

Four growth substrates were considered for screening *B. distachyon* for resistance to *R. solani*: agar, hydroponics, field soil and potting mix. Agar and hydroponics were not used because they do not provide the porosity found in the field. In some respects field soil would be considered the ideal growth substrate, as the balance of nutrients, soil particles and organic matter are authentic. However, pore spaces are lost in repacking field soil into pots and suction in a pot does not compare with suction experienced in deep soils. Therefore, the use of unamended field soil in pot experiments carries with it a high risk of hypoxia in the bottom 10 – 15 cm (Passioura, 2006). Potting mix was chosen as the substrate for these experiments as it is designed to allow healthy plant growth in pots and is less likely to contain *R. solani* or other pathogens that may be present in the field. Research from the 1960s onwards has shown that the nutrient status of the substrate colonised by a pathogen prior to invasion of a root can influence the severity of disease. The pathogenicity of *R. solani* and *Pythium ultimum* increased following growth on media with higher nitrogen and sugar concentrations (Weinhold *et al.*, 1969; Johnson *et al.*, 1981) and nutrient experiments conducted with *R. solani* resulted in greater lesion size on cotton when grown on soil rather than sand (Weinhold *et al.*, 1972). The potting mix chosen for experiments in this chapter was rich in organic matter and nutrients.

Experimental temperature and light conditions were chosen to resemble conditions experienced by wheat during sowing and seedling development, the time when Rhizoctonia root disease develops in the field. Field sites near the town of Harden NSW have been regularly affected by Rhizoctonia root rot. Recommended wheat sowing times for this region fall within the months March to June (McRae *et al.*, 2010). The mean temperature range for Harden in March is 12 – 27°C, decreasing to 2 – 14°C by June. Over this period day lengths decrease from 13 h to 10 h (Figure 2.2) (Bureau of Meteorology, 2010; Geoscience Australia, 2013). Growth cabinet conditions were set at 16°C with 12 h day length. While it was possible to approximate temperature and day length conditions experienced in the field, field-level irradiance could not be achieved in these growth cabinets. An advantage of using day lengths below 14 h is that photoperiod-sensitive lines of *B. distachyon* remain in the vegetative growth phase (Vogel *et al.*, 2006).

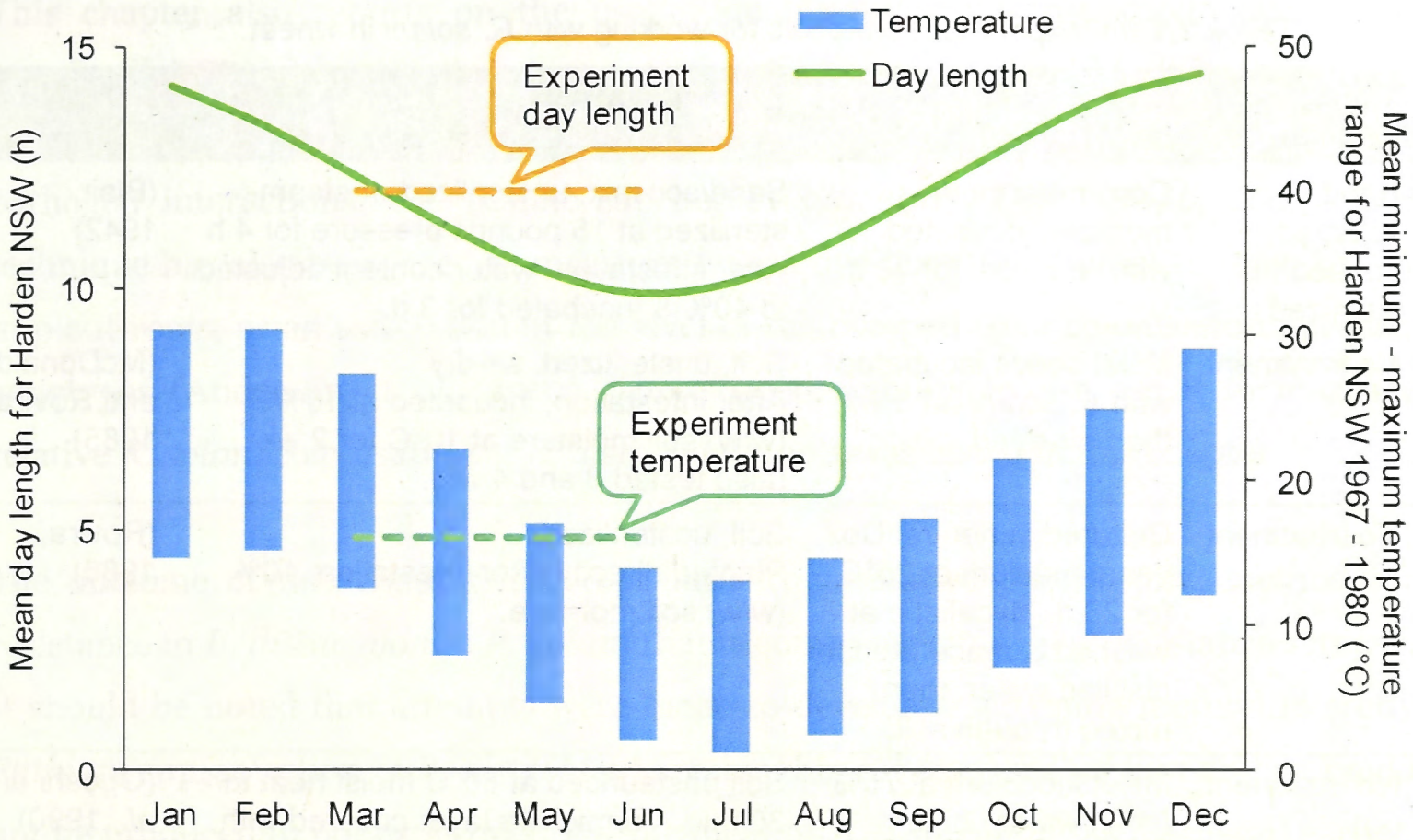

Figure 2.2 Day length and temperature range statistics for Harden, NSW. Calculated from data retrieved from Bureau of Meteorology (2010) and Geoscience Australia (2013). Experiment day length (12 h) and temperature (16°C) are indicated over the recommended wheat sowing period from March to June.

The plant phenotypes tested to establish a resistance screen for *B. distachyon* were based on those previously described in the literature on work with *R. solani* disease in wheat. Many plant phenotypes have been used to quantify or qualify *R. solani* AG8 root disease of wheat (reviewed in Chapter 1 and presented in Table 1.2, §1.3.2). Root measurements include total root length (TRL), dry weight and fresh weight. Shoot measurements include leaf area, length, number, fresh weight and dry weight. These quantitative measures are tested in this chapter, based on their use in wheat systems to identify resistance responses (Kirkegaard *et al.*, 1999; Okubara *et al.*, 2009), and their potential to be subjected to quantitative analyses. The visual disease score widely used in wheat and other species describes the level of primary root truncation and root system necrosis (McDonald and Rovira, 1985). Despite wide use however, the sensitivity of visual disease scores in place of quantifiable measurements has been questioned (Smith *et al.*, 2003a). In this chapter the visual score was not used so as to avoid subjectivity of a qualitative score and, further, because *B. distachyon* generally produces only one primary root (Watt *et al.*, 2009). This means that a visual score would be limited to a single root and may be less effective in this species than in wheat, which produces 3 to 5 primary roots.

Table 2.1 Pot experiment protocols for working with *R. solani* in wheat.

Wheat seed preparation	Inoculum preparation	Soil preparation	Reference
Hand selected, surface sterilized	Corn-meal sand medium, incubated with *R. solani* for 18 d.	Sand/soil mix, unsterilized or steam-sterilized at 15 pounds pressure for 4 h. After infestation, water content adjusted to 40% & incubated for 3 d.	(Blair, 1942)
No treatment	Millet seeds incubated with *R. solani* for 3 wk, then air-dried.	Soil, unsterilized, air-dry. After infestation, incubated at 10% (w/w) soil moisture at 10°C for 2 wk (also tested 0 and 4 wk).	(McDonald and Rovira, 1985)
No treatment	Cultured in neutral Dox yeast medium at 25°C for 28 d. Mycelial mat washed & macerated in distilled water, then mixed through soil.	Soil, unsterilized. Planted directly after infestation, 10% (w/w) soil moisture.	(Rovira, 1986)
No treatment	Millet inoculum at 75% (w/w) water content after (McDonald and Rovira, 1985).	Soil pasteurised at 80°C moist heat for 30 min. Vermiculite layer covered with inoculated soil. Watered to near-saturation after infestation & incubated in the dark at 10 or 20°C for 2 wk.	(Ogoshi *et al.*, 1990)
Surface sterilized with sodium hypochlorite	Millet inoculum after (McDonald and Rovira, 1985). Inoculum placed at depths of 5 and 10 cm.	Soil, air-dried, sieved, rehydrated with modified Hoagland's solution to (17% w/w) soil moisture, fumigated with methyl bromide 48 h, aired for 3 d. Incubated at 15°C for 2 wk after infestation.	(Kirkegaard *et al.*, 1999)
No treatment	Millet inoculum after (McDonald and Rovira, 1985).	Different soil types and nutrient levels. Incubated for 3 wk after infestation.	(Gill *et al.*, 2000)
Treated with fungicide, *P. fluorescens* or both	Plugs of 10 d old dilute potato dextrose agar cultures, placed 2-3 cm below seeds.	Vermiculite, autoclaved. Planted directly after infestation.	(Duffy, 2000)
Seeds pre-germinated	Whole oats incubated with *R. solani* for 2-3 wk, then air-dried and ground.	Soil pasteurised at 60°C moist heat for 30 min. Vermiculite layer covered with inoculated soil after (Ogoshi *et al.*, 1990). Watered to near-saturation after infestation & incubated for 1 wk, humidity maintained at 95%.	(Smith *et al.*, 2003a)
No treatment	Whole oats incubated with *R. solani* at 23°C in darkness for 3-4 wk, then ground & sieved.	Soil pasteurised at 60°C moist heat for 30 min. May add anti-oomycete drench at sowing (Okubara, pers. comm., 24 Sept 2010). Watered to near-saturation after infestation & incubated at 16°C for 1 wk.	(Okubara *et al.*, 2009)
Seeds pre-germinated	Oat inoculum after (Okubara *et al.*, 2009).	Soil prepared after (Okubara *et al.*, 2009). Planted directly after infestation.	(Okubara and Jones, 2011)

This chapter also reports on the use of quantitative real-time polymerase chain reaction (qPCR) alongside the plant growth measures as a possible plant phenotypic screen of resistance. Quantitative PCR is often used to study gene expression in plant pathogen interactions, e.g. (Oñate-Sánchez *et al.*, 2007; Foley *et al.*, 2013). The technique has also been used to measure the relative quantity of plant to fungal DNA in plant roots, as an estimation of the level of relative pathogen colonisation between genotypes (Anderson *et al.*, 2010). Therefore the ability to use qPCR to measure relative *R. solani* colonisation of *B. distachyon* roots was tested in this chapter.

The outcome of this chapter is a repeatable, quantitative phenotyping method for resistance to *B. distachyon* to *R. solani* that is comparable to wheat resistance screens. It should be noted that attempts were made to develop a screening method to study *Pythium* spp. infection of *B. distachyon* for this thesis, however *Pythium* disease could not be produced in pot experiments with wheat or *B. distachyon*. As discussed earlier, experiments using potting mix are more relevant to studying field crops than more simple substrates. Details of experimental work with *Pythium* are presented in Appendix B.

2.2 Materials and methods

2.2.1 Seed sources and preparation

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, and is available from the Australian Winter Cereals Collection.

In order to synchronise germination, seeds were surface disinfected following the method of Alves *et al.* (2009). The procedure was performed at the bench. *Brachypodium distachyon* seeds were imbibed in tap water for 2 h at room temperature in plastic containers. Husks were removed using forceps. Water was removed using a 20 mL plastic syringe. Ten to 20 mL of the following solutions were added to containers for the specified length of time, swirled occasionally and removed using the syringe: ethanol (70% v/v), 20 s; tap water, short rinse; sodium hypochlorite (1.3% v/v), 4 min; sterile Milli-Q water, rinsed three times. Seeds were moved from the final rinse water onto 3% water agar plates (Appendix A) using forceps wiped with 70% ethanol. Plates were incubated in a sealed container in the dark at 22°C. Seeds germinated overnight and were planted the day after surface disinfection, unless indicated otherwise. Wheat seeds were surface disinfected by the same method.

2.2.2 *Rhizoctonia solani* inoculum

The isolate of *Rhizoctonia solani* AG8 ZG1-1 (WAC10335) was donated by Dr Jonathan Anderson, CSIRO Plant Industry, Perth, WA, Australia. The isolate was initially collected from lupins in the Avon Wheatbelt region of Western Australia (Atlas of Living Australia, 2013b). Inoculum was prepared according to the method of McDonald and Rovira (1985). White millet seed (90 g, approximately 18 000 seeds; Kialla Pure Foods Pty Ltd, Greenmount, QLD, Australia) was soaked overnight with 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 (Appendix A). Using a sterile blade, small cubes of agar totalling around 3 cm^2 surface area were cut from the growing edge of a *R. solani* colony and mixed into a flask of sterile millet. The 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 with a coffee mill (CG-2, Breville, Botany, NSW, Australia), then sieved through brass screens, retaining the 0.5 – 2.0 mm fraction. The coffee mill and screens were wiped with 70% ethanol before use. Millet inoculum was

stored for over a year at -20°C in sealed 50 mL Falcon tubes without affecting pathogenicity.

Rhizoctonia solani millet inoculum concentration was approximately 4000 propagules per gram (ppg). The method used for propagule enumeration is based on the *Pythium* isolation method described in §2.2.3. Inoculum (200 mg) was shaken for 15 min in 100 mL of 0.1% water agar. An aliquot (1 mL) was spread evenly across a quarter-strength potato dextrose agar plate. Plates were incubated overnight at 22°C and colonies counted the following day. Inoculum density was estimated from two replicates, each with five internal plate replicates. Media preparation details are given in Appendix A.

2.2.3 Soil

The soil used for these experiments was a blend of 80% compost and 20% perlite, named 'Barley Mix', prepared by the CSIRO Plant Industry potting shed. The compost was prepared from a mix of recycled soil, leaf mulch, river loam, peat moss, perlite, vermiculite, river sand, straw and fertilizers. The potting mix was sieved to remove coarse bark and stones, and then steam-sterilized with aerated steam at 65°C for 45 minutes, timed from when the sterilization temperature is reached. Soil was supplied at approximately 16% (w/w) moisture content, with bulk density of 0.9 g/mL.

Soil was tested for *Pythium* contamination using a method first developed by Ali-Shtayeh *et al.* (1986), modified by Pankhurst *et al.* (1995) and by Rosemary Warren (pers. comm., 2010). Agar media preparation details are given in Appendix A. One gram of potting mix was shaken for 15 min in 0.1% water agar (100 mL). An aliquot (1 mL) was transferred onto VP3 *Pythium*-selective media 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 potting mix, each with five internal plate replicates. The presence of *Rhizoctonia* spp. was tested using a toothpick re-isolation check (§2.2.6) to ensure that there was no contamination with the fungus in control treatments.

2.2.4 Cone preparation and sowing

Plants were grown in narrow cone-shaped pots (Figures 2.3 and 2.4). "Cones" were prepared and inoculated on the day of planting. The cone inoculation system for studying *R. solani* disease on cereal roots is based on the method of Okubara *et al.* (2009). Low density polyethylene (LDPE, 21 cm x 3.8 cm diameter, 164 mL) cones were plugged with a cotton ball and placed in a 7 x 14 hole tray within a medium flow tray (Stuewe & Sons, Corvallis, OR, USA). 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 (Figure 2.3).

Millet inoculum was incorporated into potting mix for 10 min at an approximate concentration of 0.09 propagules per gram (ppg) of wet soil. Each cone was filled with inoculated potting mix (~116 g) and tapped down lightly. In order to minimise potential contamination, control cones were filled before *R. solani*-infested cones. Infested cones were transferred into trays before control cones were moved into position. 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 potting mix (~20 mL), as shown in Figure 2.4.

Control pots were prepared as for *R. solani* infested treatments, except no inoculum was added. In one experiment a second, higher inoculum level of 0.9 ppg was also used.

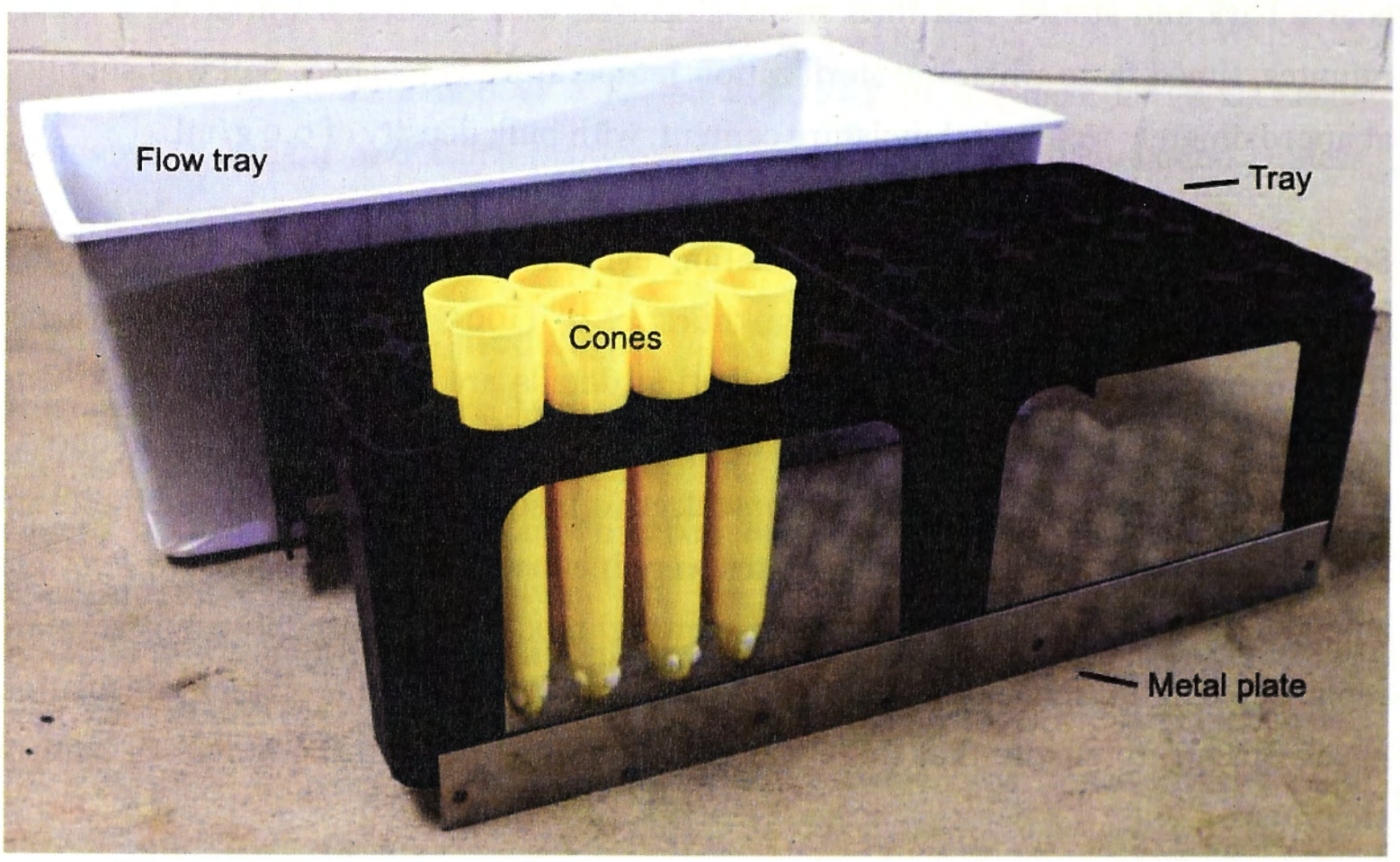

Figure 2.3 Components of the cone inoculation system. Cones are arranged in a tray, which is placed into a flow tray. Metal plates raise the tray to ensure cone bases do not contact drained water in the flow tray.

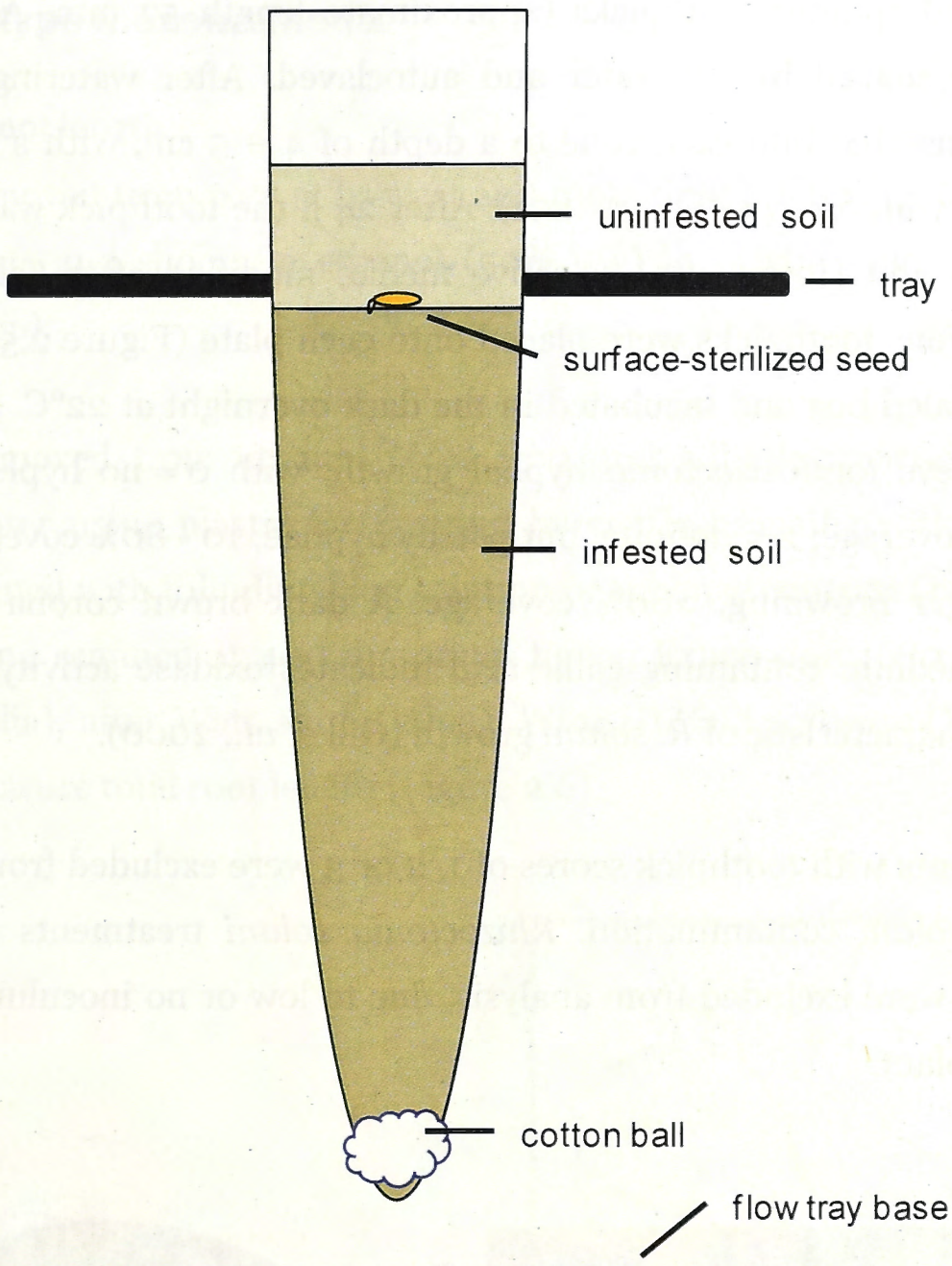

Figure 2.4 Cone preparation. Cones are plugged with cotton, filled with infested soil and watered. A germinated seed is placed at the soil surface and covered with uninfested soil.

2.2.5 Growth conditions

Cones were incubated in a growth cabinet (Conviron CMP 2023; Winnipeg, MB, Canada) programmed with 12 h days with cool white fluorescent light (approximately 200 $\mu E.m^{-2}.s^{-1}$) at a constant temperature of 16°C. 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. 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.

2.2.6 Re-isolation of *Rhizoctonia* from soil

A modified *Rhizoctonia* toothpick bait method (Paulitz and Schroeder, 2005) was used to detect *Rhizoctonia* in the top layer of soil, and check levels of *R. solani* in treated and control cones.

White birchwood splinter toothpicks (approximate length 57 mm, Alpen Products, Australia) 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 2.5a). After 24 h the toothpick was removed and placed onto Ko and Hora (1971) selective media, modified after Gill *et al* (2000) (Appendix A). Four toothpicks were placed onto each plate (Figure 2.5b). Plates were placed into a sealed box and incubated in the dark overnight at 22°C. Each toothpick was scored by eye 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. A dark brown corona formed under mycelium on medium containing gallic acid indicates oxidase activity (Bavendamm, 1928) and is a characteristic of *R. solani* growth (Gill *et al.*, 2000).

Control treatments with toothpick scores of 1, 2 or 3 were excluded from analysis, due to possible *R. solani* contamination. *Rhizoctonia solani* 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.

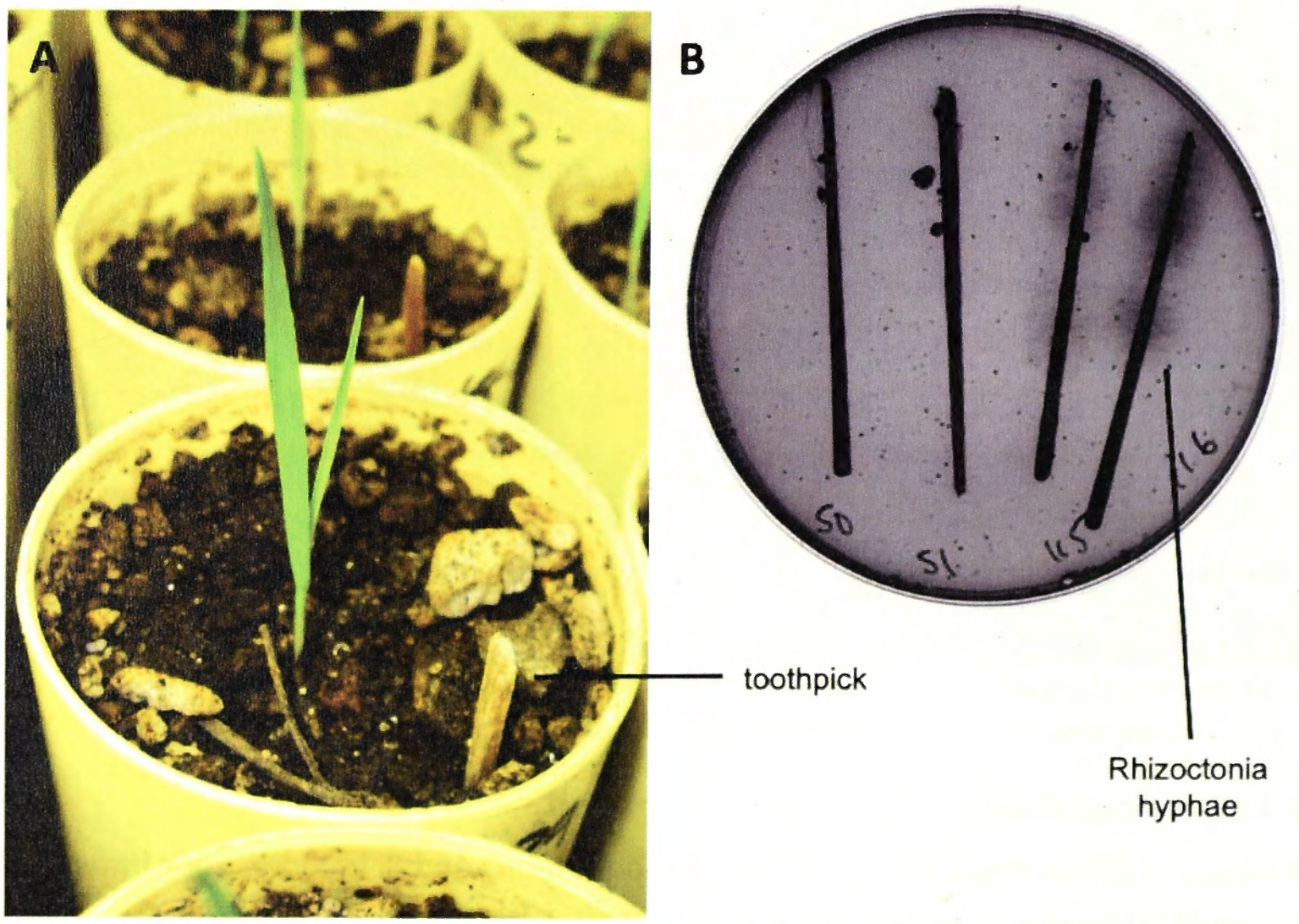

Figure 2.5 Re-isolation of *Rhizoctonia* from soil using toothpicks. a) Placement of toothpick in soil, b) Placement of toothpicks on selective media. The two toothpicks at left were from control cones. The two toothpicks at right have *Rhizoctonia* growth scores of 3, with dense hyphal growth and agar browning.

2.2.7 Phenotype measurements

2.2.7.1 Total root length

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 sauce containers (WF Plastic, Australia).

Roots were removed from ethanol (50% v/v) and adhering particles were gently removed in water using plastic forceps and by gently brushing with gloved fingers. Roots were stained with toluidine blue solution to enhance contrast (method details in Appendix A) and scanned at 400 dpi on an Epson Expression 1680 flatbed scanner (Epson, Australia), after Watt *et al.* (2005). WinRHIZO™ software (Regent, Quebec) was used to measure total root length (Figure 2.6).

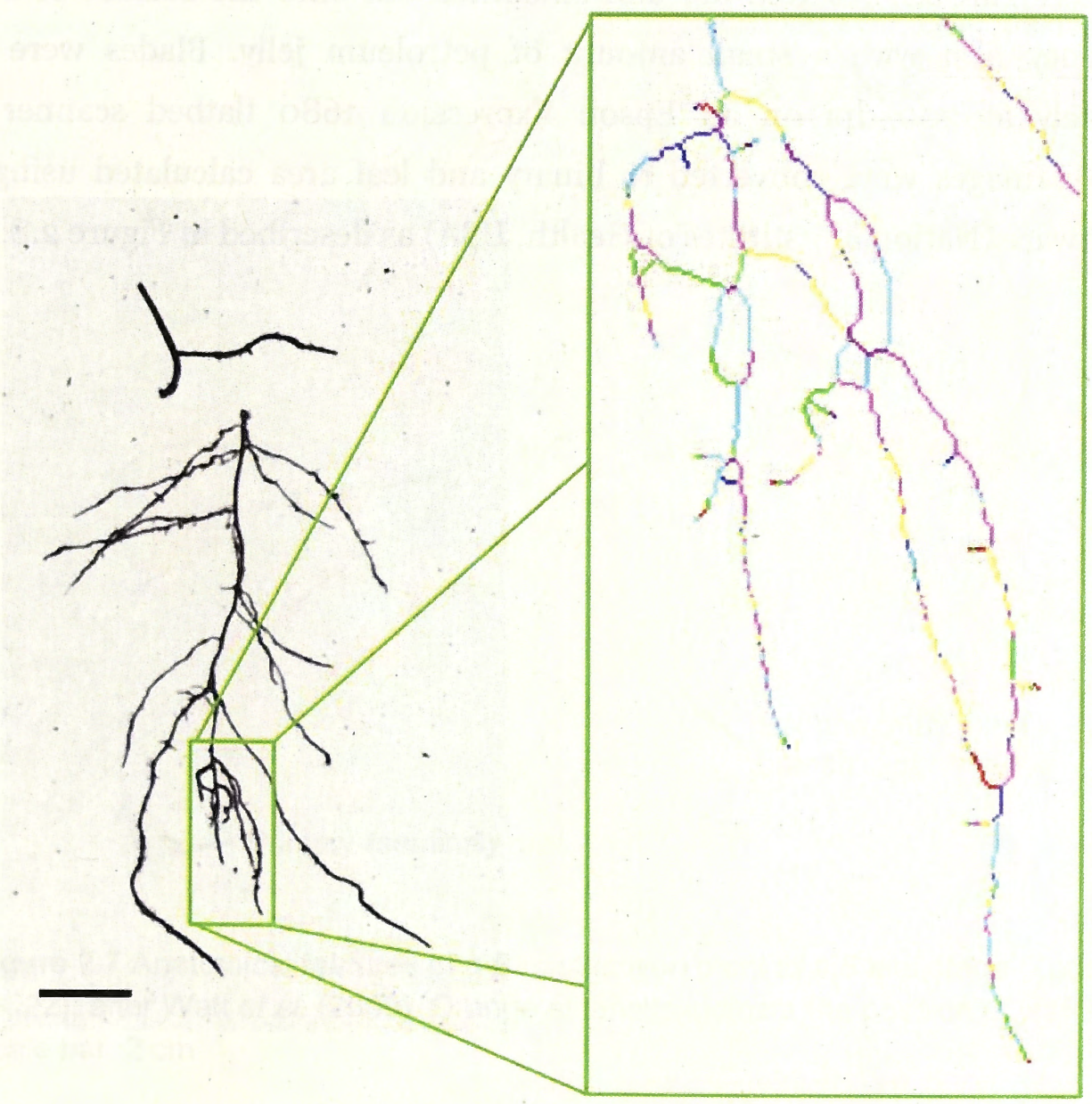

Figure 2.6 A toluidine blue stained *R. solani* infected root system (original plant shown in Figure 2.24). A section of the WinRHIZO skeleton is magnified, with different colours indicating variations in root diameter. Scale bar, 1 cm.

2.2.7.2 Leaf number

Leaf number was measured on the day prior to harvest using the decimal code described by Zadoks *et al.* (1974) (Figure 1.5). Zadoks' seedling growth scale is commonly sub-divided to give a leaf number describing the length of the last emerging leaf as a fraction of the previous fully emerged leaf length, as described by Haun (1973).

2.2.7.3 Leaf length

Length of leaves 1 and 2 were measured using a ruler on the day prior to harvest. Leaf length was measured from the crown to the leaf tip (Figure 2.7).

2.2.7.4 Leaf area

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 (Figure 2.7), 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 (Epson, Australia). Images were converted to binary and leaf area calculated using ImageJ 1.43u software (National Institutes of Health, USA) as described in Figure 2.8.

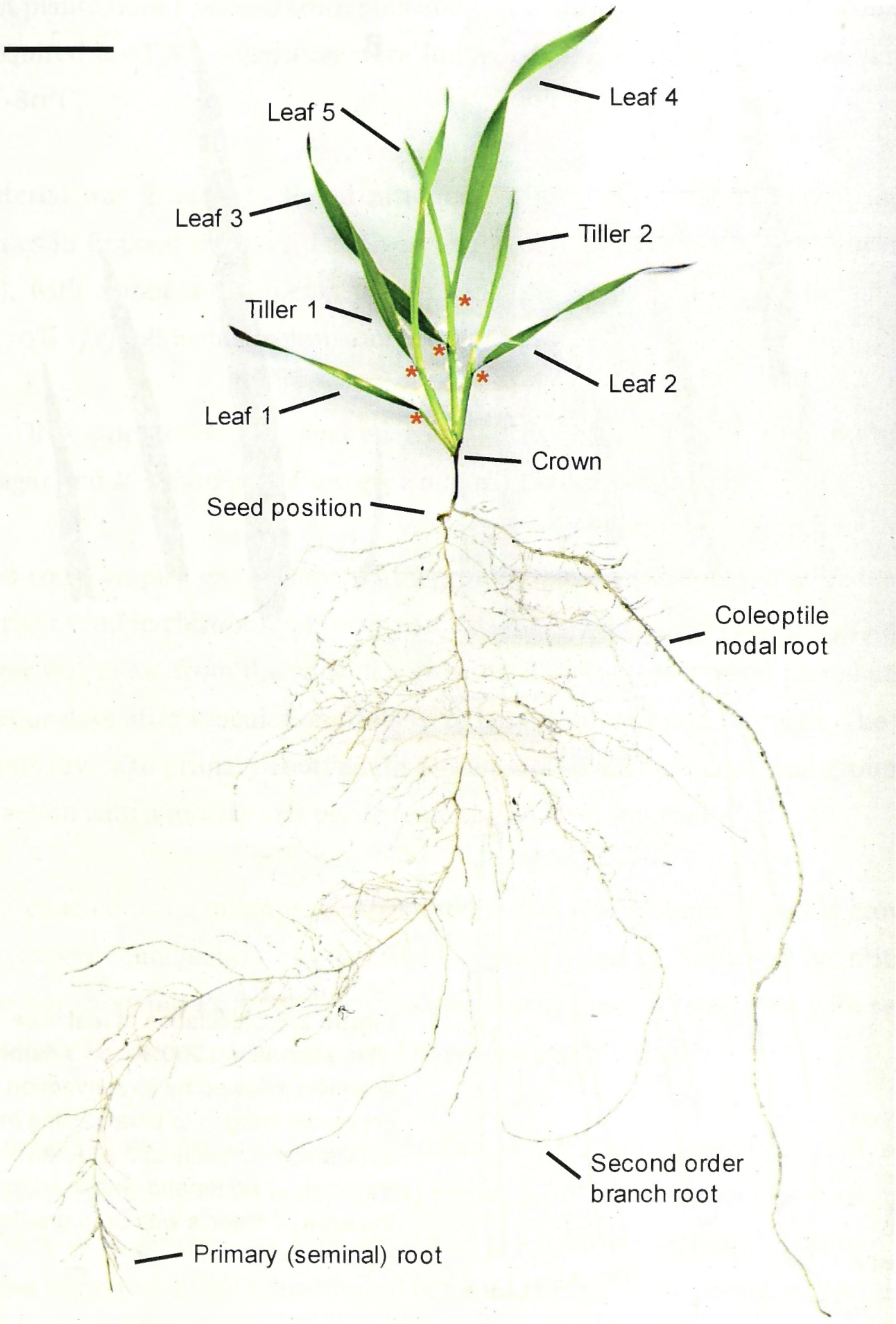

Figure 2.7 Anatomical features of a *B. distachyon* plant at 4.5 leaf stage (Zadoks scale 14, 22), after Watt *et al.* (2009). Orange asterisks indicate the position of leaf ligules. Scale bar, 2 cm.

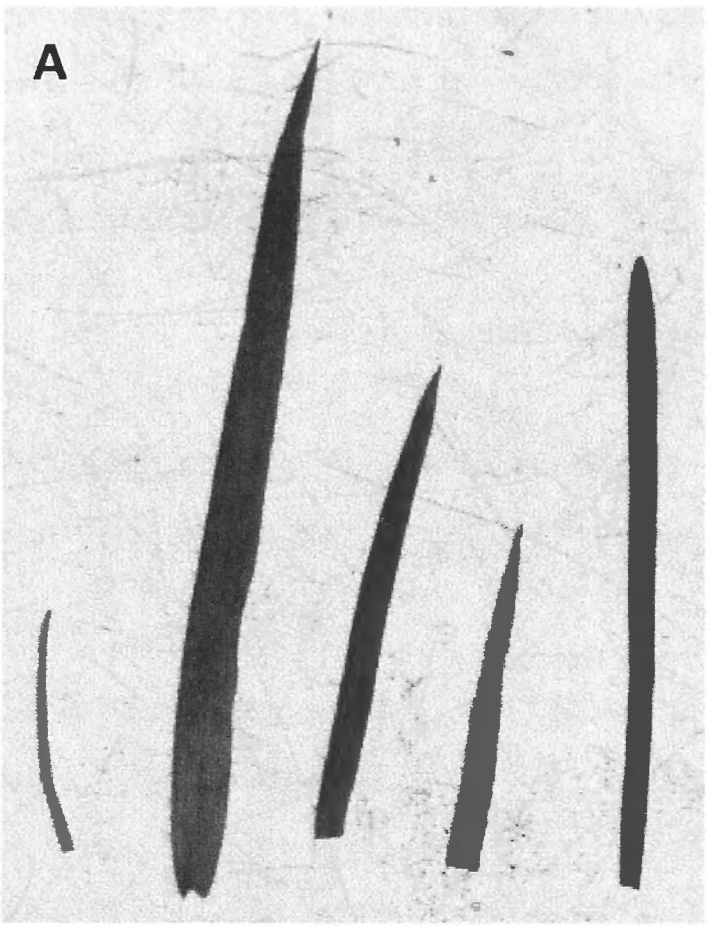

Figure 2.8 Calculation of leaf area. a) Leaves were scanned at 200 dpi on a flatbed scanner; followed by b) conversion of greyscale images to binary using ImageJ software; and finally c) the "Analyze Particles..." command was used to calculate the area of objects with area greater than 50 square pixels.

2.2.7.5 qPCR

At harvest plants were removed from pots and roots were rinsed out gently. Roots and shoots required for DNA extraction were immediately frozen in liquid nitrogen and stored at -80°C.

Plant material was ground in liquid nitrogen, either using a mortar and pestle or micropestles in Eppendorf tubes. DNA was extracted using the method of Edwards *et al.* (1991), with minor adjustments to the timing of steps and an additional wash (ethanol, 70% v/y) following isopropanol precipitation.

Standard DNA samples were prepared from *B. distachyon* roots grown on potato dextrose agar and *R. solani* mycelium grown in potato dextrose broth.

Inoculated root samples were prepared by growing surface-disinfected *B. distachyon* in sieved river sand in rhizoboxes (300 x 10 x 240 mm). Seven days after germination a plug of agar was taken from the edge of a growing *R. solani* colony and placed at each root tip. Four days after inoculation roots were harvested in liquid nitrogen. The roots of ten plants (average primary root length 85±23 mm) were combined and ground for DNA extraction with a mortar and pestle (sample 'Bd root, infected').

DNA was extracted using micropestles from individual root systems of plants grown to 26 days in experimental conditions described in §§2.2.4 and -5. Sufficient quantities of DNA were extracted, but PCR amplification was mostly unsuccessful, even with several attempts to modify the extraction protocol to reduce contaminants.

The PCR primers used for these experiments amplify a 200 bp length of the *R. solani* ribosomal internal transcribed spacer (ITS) region, a 201 bp length of wheat 18s rDNA (J. Anderson, pers. comm., 20 Sept 2011) and a ~1000 bp length of genomic DNA flanking the region of T-DNA insertion JJ3794 (J. Bragg, pers. comm., 3 Oct 2012). Further primer details are in Appendix A.

Primer amplification was checked in genomic DNA using a PCR program with an annealing temperature of 58°C (Figure 2.9). Primers for *R. solani* were specific for the fungus, with some non-specific bands appearing in *B. distachyon* root and shoot samples. The *B. distachyon* and wheat primers both amplified bands of the stated length in *B. distachyon* DNA and did not amplify fungal DNA. The *B. distachyon* primers did not amplify wheat DNA.

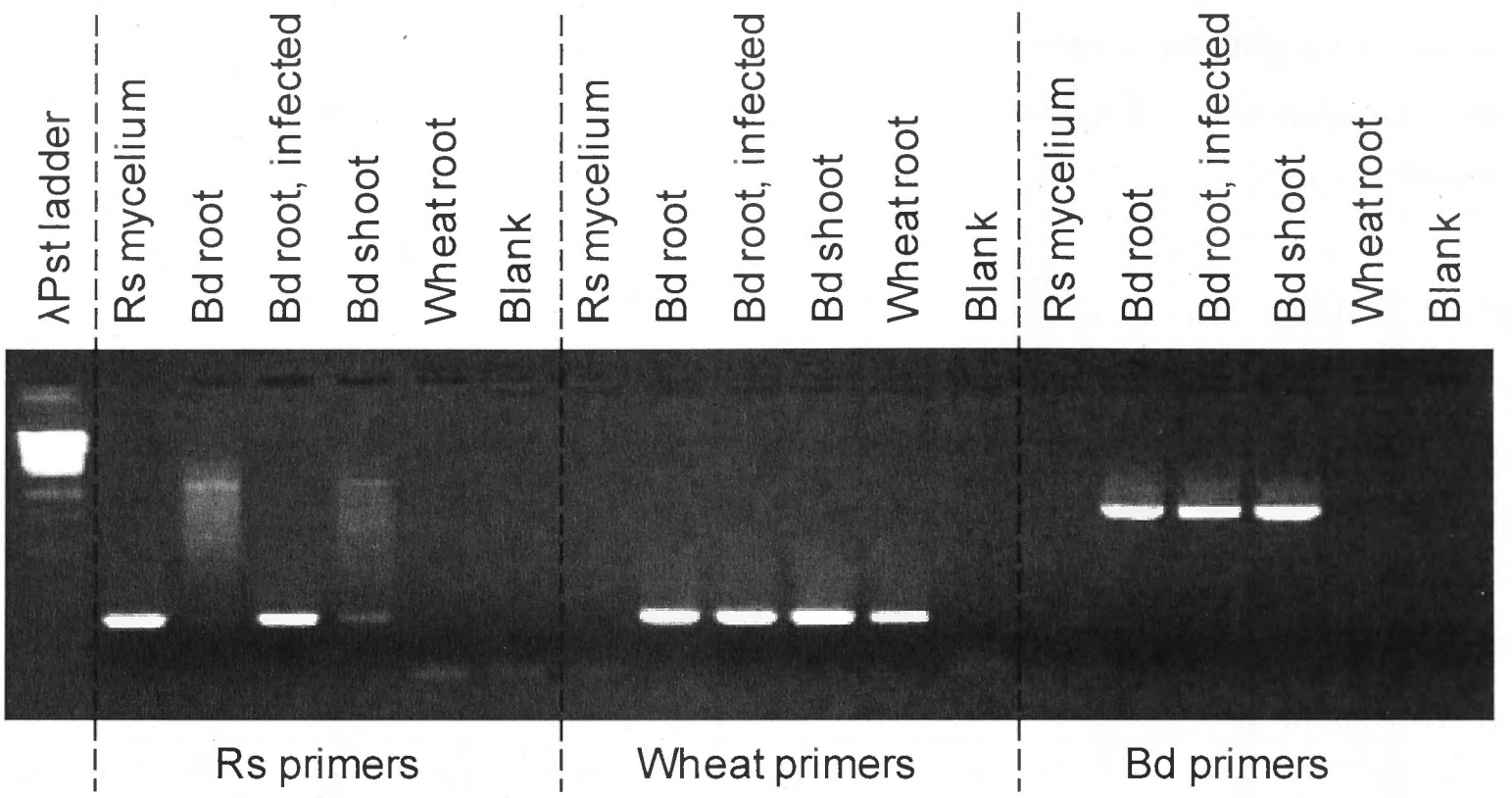

Figure 2.9 Amplification of DNA extracted plant and fungal tissues using primers designed to target *R. solani* (Rs), wheat or *B. distachyon* (Bd) DNA. Samples were *R. solani* mycelium (Rs mycelium), *B. distachyon* root (Bd root), *B. distachyon* root infected with *R. solani* (Bd root, infected), *B. distachyon* leaves (Bd shoot), wheat root and water blanks. These samples correspond to samples 2 – 6 in Figure 2.19.

Reactions for qPCR were prepared using SYBR® Green fluorescent dye and performed on a Rotor-Gene 6000 real-time cycler (Corbett Research). Three internal replicates were prepared for each sample.

2.2.7.6 General observations

Colonized roots were observed with a Leica DMR microscope (Leica Microsystems), using bright field and UV excitation with fluorescence filters.

Emergence of nodal roots was counted in Experiment 4 root scans. All emerged coleoptile and leaf nodal roots were counted, even if they had been severely truncated by infection.

2.2.8 Statistical analysis

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

Experiments were set up as randomized block designs to evaluate the interaction between different plant hosts (genotypes) and the presence of *R. solani* AG8 inoculum on various phenotypic measurements. A significant difference in this interaction indicated that lines differed in resistance to the pathogen. The blocking factor was different flow trays. Thus, in GenStat the REML model was written as

Fixed Model: *Host*Inoculum*

Random Model: *Tray*

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 and Underwood, 2002). In these cases the random factor was set to zero.

Total root length was square-root transformed prior to analysis, except in the preliminary investigation alongside wheat in Experiment 1. Square-root transformation improved residuals resulting from differences in growth of control and inoculated treatments. The purpose of root length transformation is described in further detail in §3.2.9.2 using a large data set collected in Chapter 3 experiments. Shoot measurements did not require transformation.

Standard errors and least significant differences at 5% were calculated in Genstat. *Brachypodium distachyon* and wheat were analysed separately, due to large differences in magnitude between measurements in the two species.

2.2.9 Experiments

Four experiments were conducted in this chapter to answer six questions towards development of a screening method. During this method development phase several block designs were tested in an attempt to overcome variation in growth within treatments, as well as spatial variation in physical conditions. All experiments were set up in randomized block designs across two or three flow trays with the treatments *Host* (genotype) and *Inoculum*. An overview of experiments and questions is shown in Table 2.2.

Experiment 1 yielded data to answer both Questions i and ii. In this experiment each *Host*Inoculum* treatment was randomly allocated once per flow tray. To test the variability in plant growth and cabinet spatial conditions, the experiment was set up in three blocks, with five plants sown per treatment. Every treatment was made up of five replicate pots sown in adjacent positions. After removal of unemerged plants and cones that failed the Rhizoctonia re-isolation check, 2 – 5 plants remained for analysis.

Wheat was harvested at 14 days after planting (DAP) at around the 2.8 leaf stage and the *B. distachyon* accessions harvested at 20 DAP at around 3.0 leaves. Leaf 1 and leaf 2 were fully expanded at harvest for both species. For the purpose of testing spatial variation, the blocking term was included in the fixed model as *Host*Inoculum*Tray*.

In Experiment 2 three replicate pots were sown per treatment in adjacent positions. Each treatment was randomly allocated once per flow tray, with two trays in total. Treatments for Experiments 2 were *Host*Inoculum*. This experimental design was subsequently used for the Screening activity in Chapter 3. Statistical analysis for Experiment 2 frequently required the blocking factor (*Tray*) to be constrained positive in REML, as described in §2.2.8.

In Experiment 3 two replicate pots were sown per *Host*Inoculum*Days sown after disinfection* treatment. Every treatment was sown five times across three flow trays. In contrast with the other experiments described in this chapter, all three flow trays were considered to be one block.

The experimental design for Experiment 4 was the same as for Experiment 2, except that treatments were only *Inoculum* and *Days to harvest*, with *B. distachyon* and wheat analysed separately. As in Experiment 2, the random model term *Tray* had to be constrained positive for the 18, 22 and 26 DAP harvest time-points for analysis.

Samples for the experiment to test Question vi were obtained from the Chapter 3 confirmation activity described in §3.2.8.2.

Table 2.2 Experiments described in this chapter.

Broad aims	Experiment number	Questions of experiments
Experiments towards development of a robust screening method using *B. distachyon*	1	i. Do *B. distachyon* and wheat have a similar response to *R. solani* AG8?
	1	ii. Can a toothpick re-isolation check improve screening robustness?
	2	iii. What is suitable level of inoculum for screening experiments?
	3	iv. Does sowing at different days after disinfection reduce root length variation?
Phenotype measurements	4	v. How does *R. solani* AG8 affect root and shoot measurements over time?
	*	vi. Can qPCR be used to measure differences in disease severity?

*This experiment was carried out in the Chapter 3 confirmation activity.

2.3 Results

2.3.1 Experiments towards development of a robust screening method using *B. distachyon*

2.3.1.1 Do *B. distachyon* and wheat have a similar response to *R. solani* AG8?

In order for *B. distachyon* to be a useful model plant for Rhizoctonia root rot in wheat, both species should be affected by the fungus in a similar manner. This was tested in Experiment 1 by comparing phenotypes of five accessions of *B. distachyon* with Janz bread wheat under control and *R. solani* AG8 inoculated conditions.

Means of root and shoot measurements in *R. solani* and control treatments of *B. distachyon* accessions and Janz wheat were predicted using a linear mixed model. These measurements and *R. solani*/control ratios are presented in Table 2.3.

The isolate of *R. solani* infected all five lines of *B. distachyon* and wheat. Total root length was reduced to an average 49% of control in infected *B. distachyon* ($p<0.001$) and 39% of control in wheat ($p<0.001$). Leaf number was reduced to an average 96% of control in infected *B. distachyon* ($p=0.023$) and 95% of control in wheat ($p=0.007$). Leaf 1 length was not significantly affected by *R. solani* in either *B. distachyon* 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 *B. distachyon* leaf 2 length was not significantly reduced by *R. solani*, despite both species being harvested when leaf 2 was fully expanded.

Overall, *R. solani* AG8 has a similar effect on root and shoot measurements in *B. distachyon* and wheat. *Rhizoctonia solani*/control root and shoot ratios for Janz wheat fell within the observed range of ratios for the five *B. distachyon* lines tested. The level of root growth reduction by *R. solani* in both species can be seen in Figure 2.10.

Comparing the disease response of the five *B. distachyon* accessions included in this experiment, a significantly different response to *R. solani* was not seen for any of the four phenotypic measurements. This result suggests that there was no genetic variation in susceptibility to *R. solani* within these lines.

It was noted that variation in measurements was sometimes quite high between replicates. The sample size left for analysis after removing unemerged plants and those

that failed the *R. solani* re-isolation check was as low as two in some cases. Further, there was a significant blocking effect of different trays on *B. distachyon* root length and leaf number measurements, indicating spatial variability of localised conditions in the growth cabinet. This variation may have obscured any plant-pathogen interactions between lines. Subsequent experiments in this chapter were designed to address these issues of growth variation and treatment replication.

Table 2.3 Comparison of *R. solani* infection on phenotypic measurements of five *B. distachyon* natural accessions and Janz wheat. Predicted means of total root length, leaf 1 length, leaf 2 length and leaf number for *B. distachyon* and wheat in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values.

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

Figure 2.10 Effect of *R. solani* AG8 on root and shoot growth of a) *B. distachyon* line Bd 3-1 and b) Janz wheat, at 18 DAP. All wheat roots and some *B. distachyon* roots in control treatment have reached the base of the 21 cm deep cones. Scale bars, 2 cm.

2.3.1.2 Can a toothpick re-isolation check improve screening robustness?

The amount of *R. solani* inoculum needed to cause disease was quite low, with 0.09 ppg equivalent to approximately 11 propagules per cone. At this low level there was a possibility that, with a poor mixing technique, some cones may occasionally not have received many or any *R. solani* propagules. In Experiment 1 a toothpick re-isolation method was adapted from the literature to check the consistency of inoculation and to pick up cross-contamination in *B. distachyon* and wheat.

Toothpick re-isolation checks showed that inoculum was generally well distributed through the *R. solani* treatments, with very little contamination of control treatments (Figure 2.11). Plants with a zero score in *R. solani* treatment had lower average root length than plants with zero score in control treatment, suggesting that false negative scores can occur in the infested treatment. Non-*Rhizoctonia* spp. fungi that occasionally grew out from control toothpicks had different morphology and did not cause the KHF medium to brown. To err on the side of caution, when identification was uncertain, fungi growing from control treatment toothpicks were generally assumed to be *R. solani*.

The toothpick re-isolation score at 8 DAP was significantly correlated ($p<0.001$) with total root length at 20 DAP for *B. distachyon* accessions and bread wheat. It was concluded that the toothpick re-isolation check was a useful component of the screening protocol.

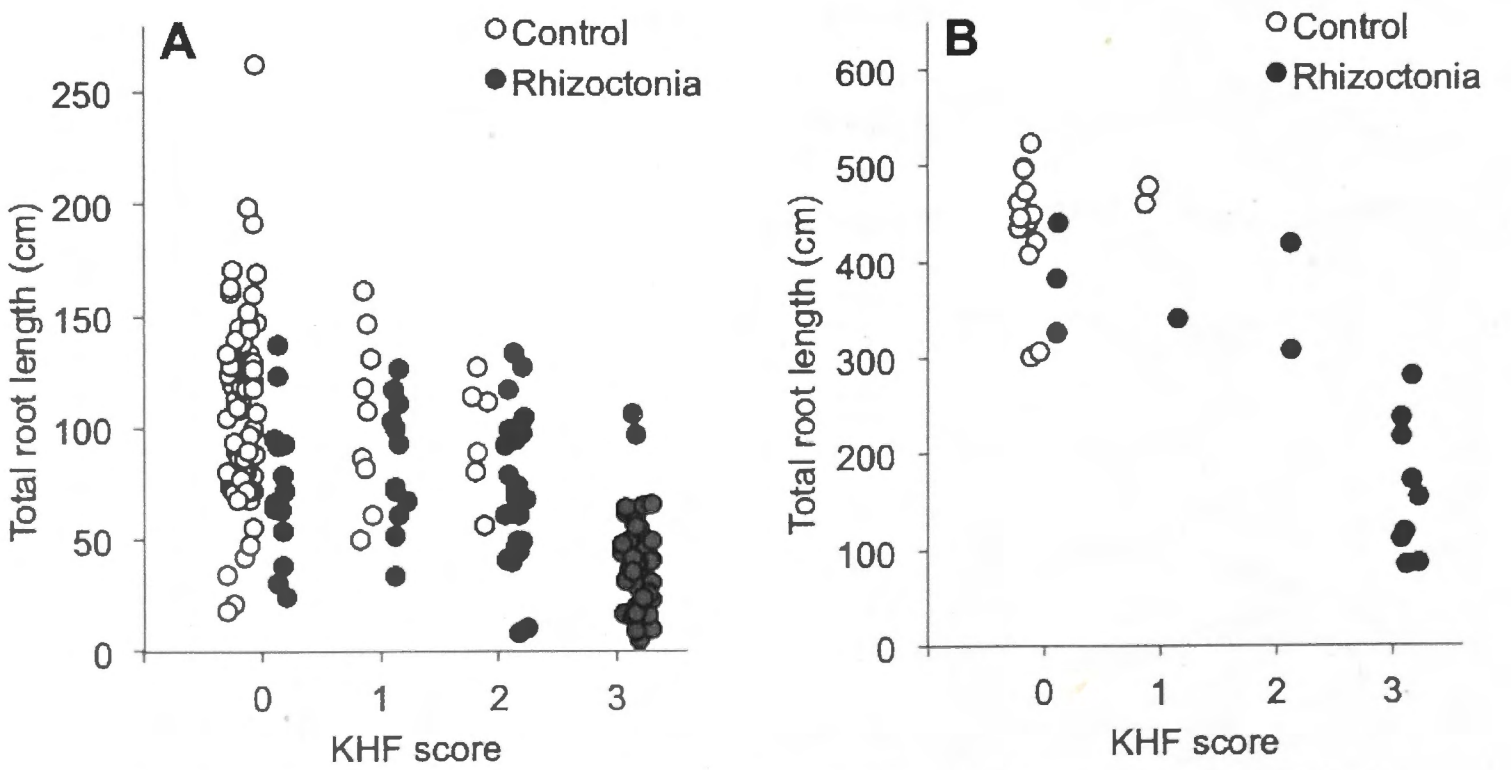

Figure 2.11 Evaluation of *R. solani* re-isolation from soil to check for consistent inoculation and lack of contamination in the control. Individual root length at 20 DAP for control and *R. solani* treatments in a) seven *B. distachyon* accessions, and b) Janz wheat, ordered by toothpick re-isolation score at 8 DAP.

2.3.1.3 What level of inoculum is suitable for screening experiments?

In Experiment 1 a low level of *R. solani* inoculum, 0.09 propagules per gram (ppg), was found to cause moderate disease symptoms. In this experiment (Experiment 2) the effect of a higher level of inoculum was tested using seven *B. distachyon* accessions from a narrow range of the SSR tree.

Inoculum was incorporated into soil at 0.09 and 0.9 ppg. Root systems of plants growing in soil containing 0.9 ppg of inoculum were severely reduced and fragile, with two plants dying during the course of the experiment; however the higher inoculum level did not have a significant effect on days to emergence. Toothpick colonisation was noticeably higher at the higher level of inoculum, with hyphae often visible at the junction between the toothpick and the soil surface.

Total root length measurements are reported here as the square-root transformed means (√TRL), used to normalize the data over the large range of values. There was some difficulty including the blocking factor in the statistical analysis, expected to be due to the greater variation of growth within a treatment than between trays (see Methods §2.2.8).

Rhizoctonia solani inoculated treatment measurements were significantly reduced ($p<0.001$) from control at both levels of inoculum for all phenotypes measured (√TRL, leaf number, leaf 1 length and leaf 2 length).

A significant variation in the plant-pathogen interaction was found between the accessions for the measurements √TRL ($p=0.019$), leaf number ($p=0.042$) and leaf 1 length ($p=0.036$; variation only significant in the 0.9 ppg treatment). Average root and shoot measurements of control and *R. solani* treatment phenotypes are presented in Figures 2.12 and 2.13, with values and ratios given in Tables 2.4 and 2.5. Line Koz-3 was considered the most resistant accession in this experiment, with the highest *R. solani*/control √TRL ratios at both levels of inoculum. At 0.09 ppg Koz-3 was significantly ($p<0.05$) more resistant than lines Bd 21, BdTR 13c and Koz-1. At 0.9 ppg Koz-3 was significantly ($p<0.05$) more resistant than these three lines, as well as BdTR 13a. Several shoot measurement ratios were also significantly reduced ($p<0.05$) in these lines, compared with Koz-3.

A notable difference between this and the previous experiment was that line Bd 21 had the lowest *R. solani*/control √TRL ratio at 0.09 ppg inoculum in Experiment 2, while

in Experiment 1 the line had the highest TRL ratio at the same level of inoculum. In both experiments, however, there was no significant difference in resistance between the two lines common to both experiments, Bd 21 and Bd 21-3, except for a lower leaf 1 length ratio for Bd 21 at 0.9 ppg.

The 0.09 ppg inoculum level was chosen for future experiments as it caused disease without plant death, thereby allowing the effect of *R. solani* on root system growth to be studied.

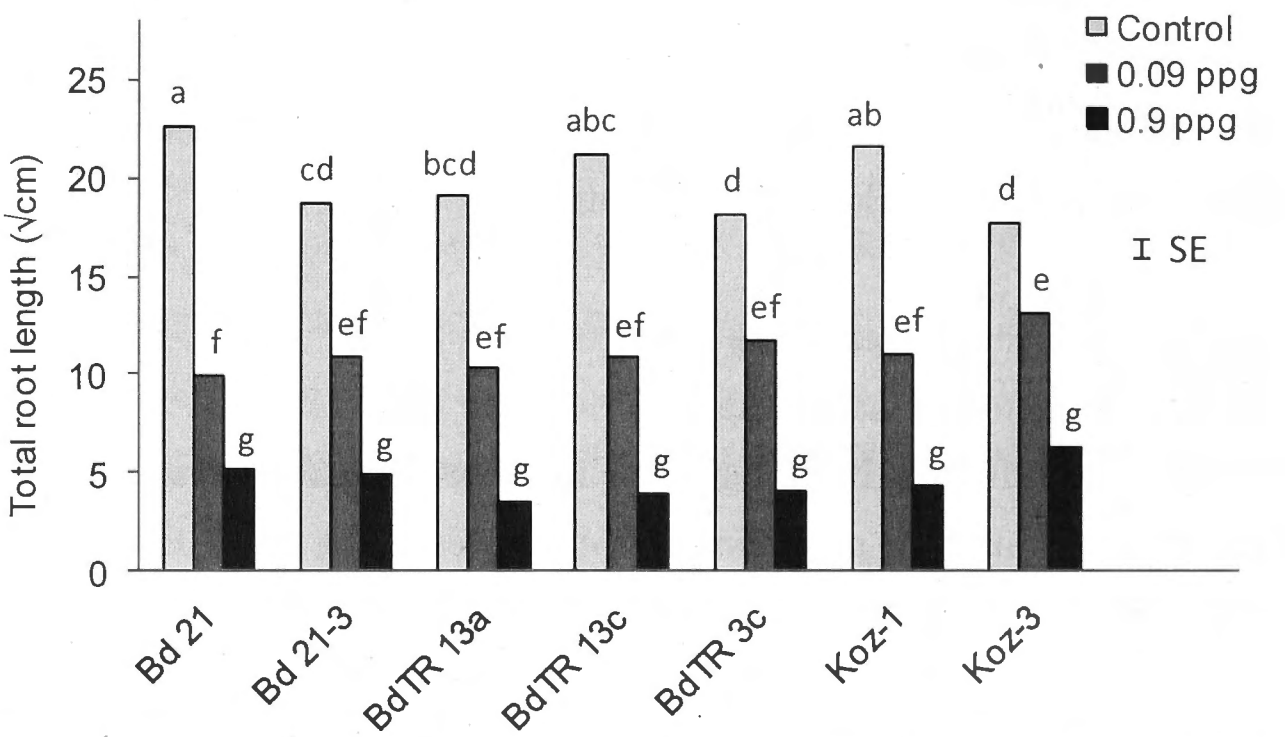

Figure 2.12 Effect of two levels of *R. solani* inoculum on root growth. Predicted means for square-root transformed total root length for seven *B. distachyon* accessions grown in soil infested with *R. solani* inoculum at 0.09 ppg and 0.9 ppg; n≤6; columns with the same letter are not significantly different at 5% LSD. Values are given in Tables 2.4 and 2.5.

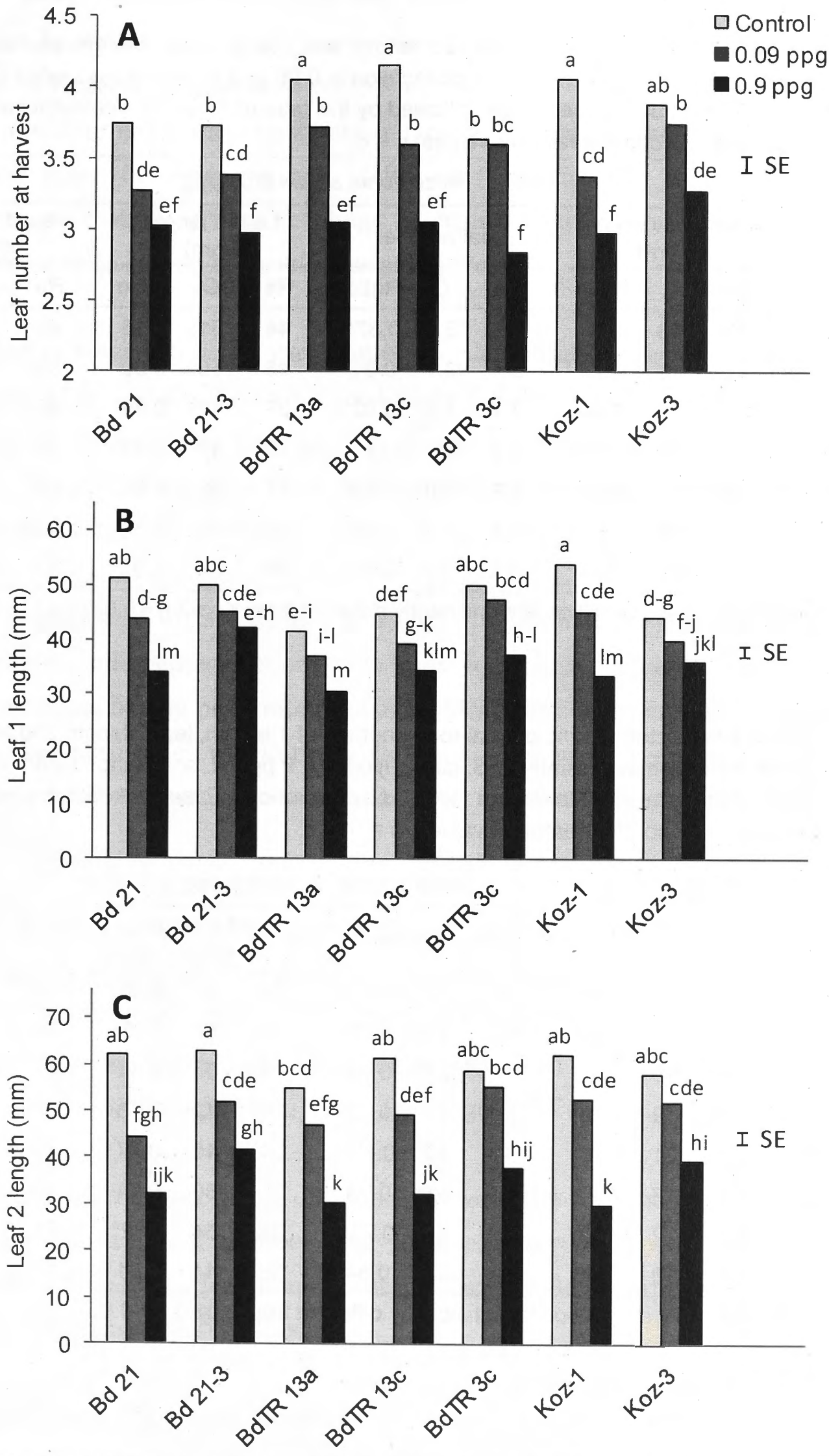

Figure 2.13 Effect of two levels of *R. solani* inoculum on shoot growth. Predicted means for a) leaf number, b) leaf 1 length, and c) leaf 2 length, for seven *B. distachyon* accessions grown in soil infested with *R. solani* inoculum at 0.09 ppg and 0.9 ppg; n≤6; columns with the same letter are not significantly different at 5% LSD; average SE. Values are given in Tables 2.4 and 2.5.

Table 2.4 Predicted means of total root length, leaf 1 length, leaf 2 length and leaf number for seven accessions of *B. distachyon* in 0.09 ppg *R. solani*-inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values; n = 6.

	Rhizoctonia solani 0.09 ppg											
	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
Bd 21	10	23	0.44*	3.3	3.8	0.87*	44	51	0.85	44	62	0.72*
Bd 21-3	11	19	0.58	3.4	3.7	0.91	45	50	0.90	51	63	0.82
BdTR 13a	10	19	0.54	3.7	4.1	0.92	37	42	0.89	47	55	0.85
BdTR 13c	11	21	0.51*	3.6	4.2	0.87	39	45	0.88	49	61	0.80
BdTR 3c	12	18	0.64	3.6	3.6	0.99	47	50	0.94	56	59	0.94
Koz-1	11	22	0.51*	3.4	4.1	0.83*	45	54	0.84	53	62	0.85
Koz-3	13	18	0.74	3.8	3.9	0.97	40	44	0.90	52	58	0.90

*Plant-pathogen interaction is significantly different from Koz-3 ($p<0.05$).

Table 2.5 Predicted means of total root length, leaf 1 length, leaf 2 length and leaf number for seven accessions of *B. distachyon* in 0.9 ppg *R. solani*-inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values; n = 5 to 6.

	Rhizoctonia solani 0.9 ppg											
	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
Bd 21	5.2	23	0.23*	3.0	3.8	0.80	34	51	0.66*	32	62	0.52*
Bd 21-3	4.9	19	0.26	3.0	3.7	0.79	42	50	0.84	41	63	0.65
BdTR 13a	3.5	19	0.18*	3.1	4.1	0.75	30	42	0.73	30	55	0.54
BdTR 13c	3.9	21	0.18*	3.1	4.2	0.73*	34	45	0.77	33	61	0.54*
BdTR 3c	4.1	18	0.22	2.8	3.6	0.78	37	50	0.74	37	59	0.62
Koz-1	4.4	22	0.20*	3.0	4.1	0.73*	34	54	0.62*	31	62	0.49*
Koz-3	6.3	18	0.36	3.3	3.9	0.84	36	44	0.81	40	58	0.69

*Plant-pathogen interaction is significantly different from Koz-3 ($p<0.05$).

2.3.1.4 Does sowing at different days after disinfection reduce variation?

In earlier Experiments 1 and 2 there was a high level of variation in root length at harvest. In Experiment 3 a variation to the sowing procedure was tested to see if early effects would influence measurements at harvest. The environment at germination can influence plant development and yield, e.g. Highkin and Lang (1966). It was hypothesised that disturbing seedlings at critical times soon after germination by transferring from agar to soil could also affect growth.

The effect of sowing time on variation of root length at harvest was tested with two accessions at 0, 1 and 2 days after seed surface disinfection. Lines Bd 3-1 and Bd 21 were chosen as they have been used to generate inbred lines for SSR-based linkage mapping (Garvin *et al.*, 2010) and subsequently to fine map a *Barley stripe mosaic virus* resistance gene (Cui *et al.*, 2012).

At 0 days after seed surface disinfection (DAD), seeds had not germinated. By 1 DAD embryos were enlarged, with roots up to 3 mm long. At 2 DAD roots were 10 – 15 mm long, with some having penetrated the agar. Mean days to emergence of the first leaf from the soil surface fell within the range 4.9 – 5.9 days after surface disinfection for all treatments. Plants were harvested at 22 DAP, i.e. at 22, 23 and 24 days after surface disinfection.

Rhizoctonia solani inoculum significantly ($p<0.001$) reduced TRL, leaf number and leaf 2 length, but not leaf 1 length (Figures 2.14 and 2.15).

Day of sowing had a significant influence on TRL ($p<0.001$), leaf number ($p<0.001$), leaf 1 length ($p=0.002$) and leaf 2 length ($p=0.036$).

The only significant phenotypic difference between the two lines was leaf number at harvest ($p<0.001$). Leaf number was positively correlated with time sown after disinfection. Line Bd 3-1 leaf number was greater than Bd 21 leaf number for any given treatment by on average 0.25 leaves.

While significant differences were between root and shoot measurements for different sowing days, there was no significant difference in the host-pathogen interaction between lines Bd 21 and Bd 3-1 for any measurement. Further, the effect of time of sowing was not found to influence the plant-pathogen interaction. Days to sowing was not an important factor in the variability, measured as standard deviation, of

phenotypic measurements at 22 DAP. Thus, subsequent experiments continued to be sown at the original time point of one day after disinfection.

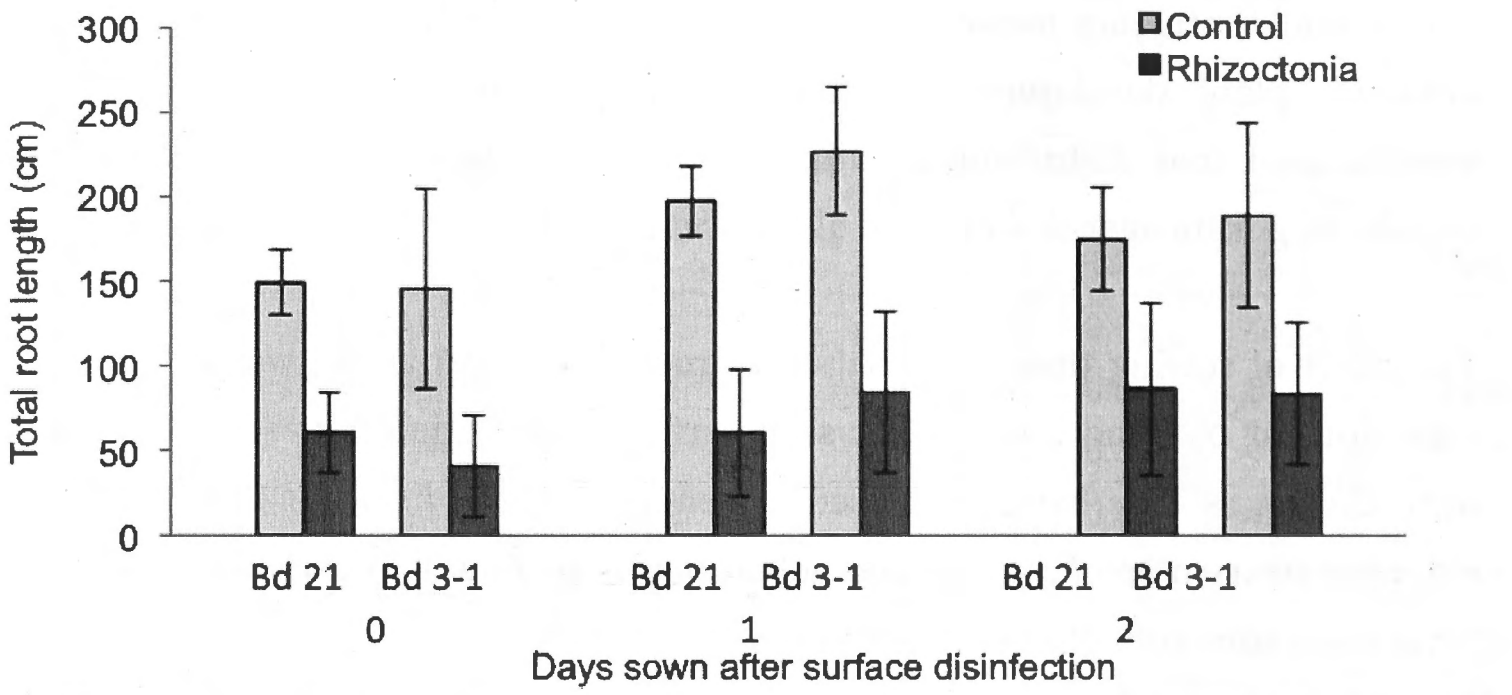

Figure 2.14 Influence of time of sowing after disinfection on variation in root length at 22 DAP. Mean total root length for control and *R. solani* treatments of *B. distachyon* accessions Bd 21 and Bd 3-1. Seeds were sown at 0, 1 and 2 days after surface disinfection. Variation in root length measurements was similar for all sowing time-points; SD, n = 5 to 10.

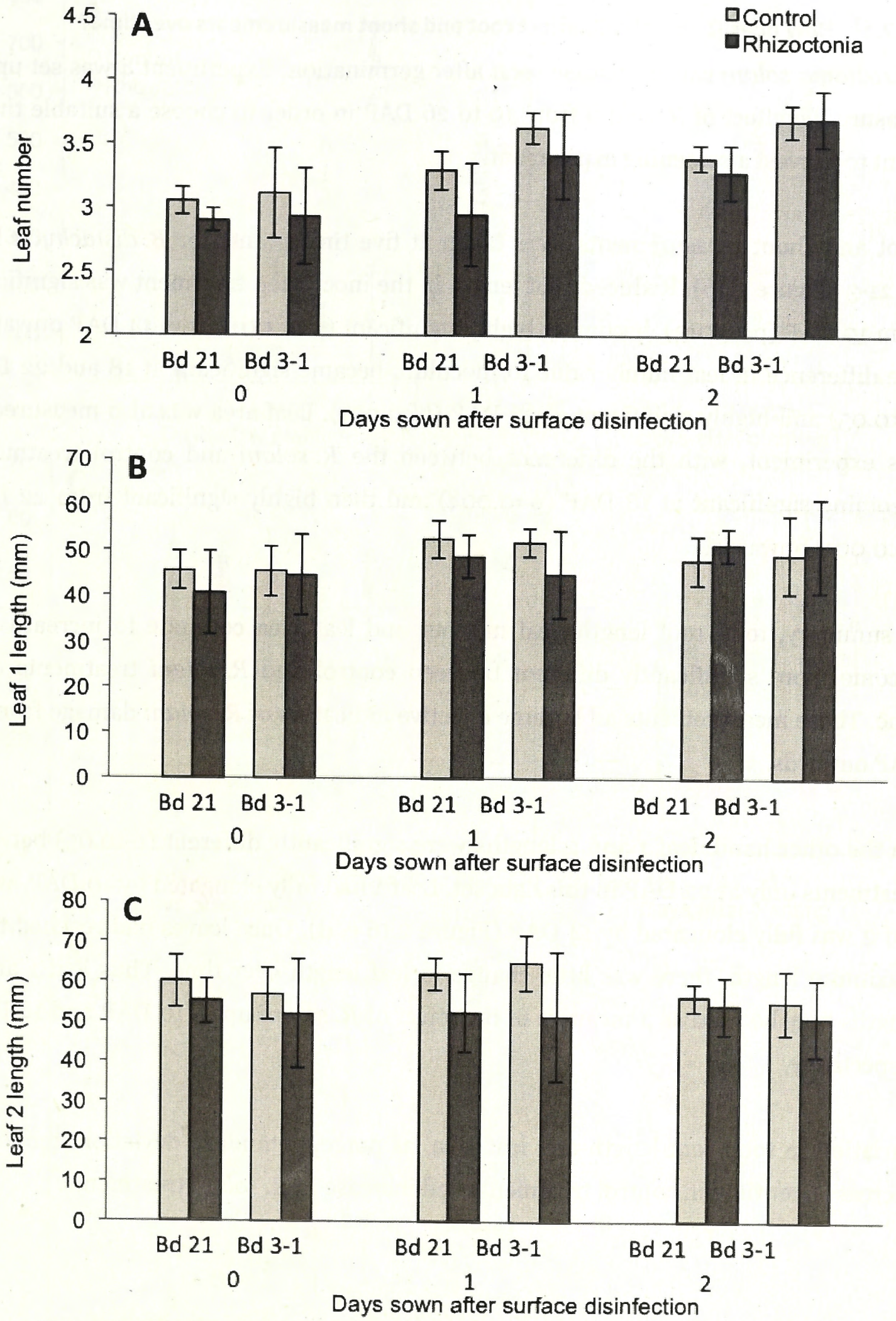

Figure 2.15 Influence of time of sowing after disinfection on variation in shoot measurements at 22 DAP. Mean a) leaf number, b) leaf 1 length, and c) leaf 2 length, for control and *R. solani* treatments of *B. distachyon* accessions Bd 21 and Bd 3-1. Seeds were sown at 0, 1 and 2 days after surface disinfection. Variation in shoot measurements was similar for all sowing time-points; SD, n = 5 to 10.

2.3.2 Phenotype measurements

2.3.2.1 How does *R. solani* AG8 affect root and shoot measurements over time?

Rhizoctonia solani causes disease soon after germination. Experiment 4 was set up to measure the effect of *R. solani* from 10 to 26 DAP in order to choose a suitable time-point to harvest a screening experiment.

Root and shoot measurements were taken at five time-points for *B. distachyon* line Bd 21-3 (Figure 2.16). 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 (Figure 2.16 c, d). Once leaves had reached their maximum length, there was little change in leaf length over time. Thus leaf 1 and 2 lengths may be used as a measure of the effect of *R. solani* up to 10 DAP and 14 DAP, respectively.

Variation in total root length and leaf area, as percent standard deviation, tended to decrease over time in control treatments and increase in *R. solani* treatments.

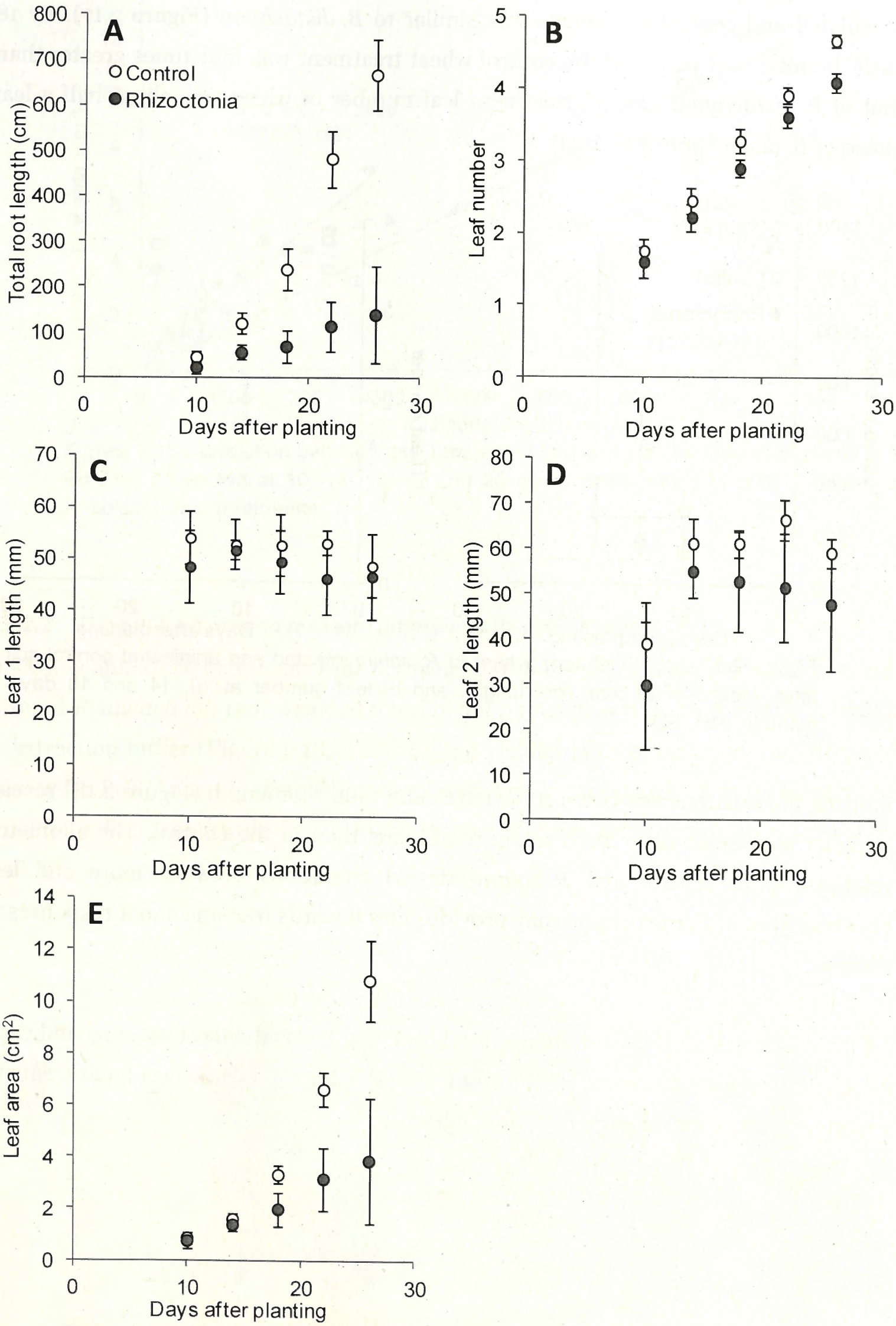

Figure 2.16 Growth of line Bd 21-3 in *R. solani* infested and uninfested control soil over time. Means of a) total root length, b) leaf number, c) leaf 1 length, d) leaf 2 length and e) leaf area, at 10, 14, 18, 22 and 26 days after planting; n≥5, SD

In Janz wheat the difference in TRL increase and leaf appearance rate between inoculated and control treatments was similar to *B. distachyon* (Figure 2.17). By 18 DAP the total root length of the control wheat treatment was four times greater than that of *B. distachyon*. Control treatment leaf number of wheat was about half a leaf ahead of *B. distachyon* at 18 DAP.

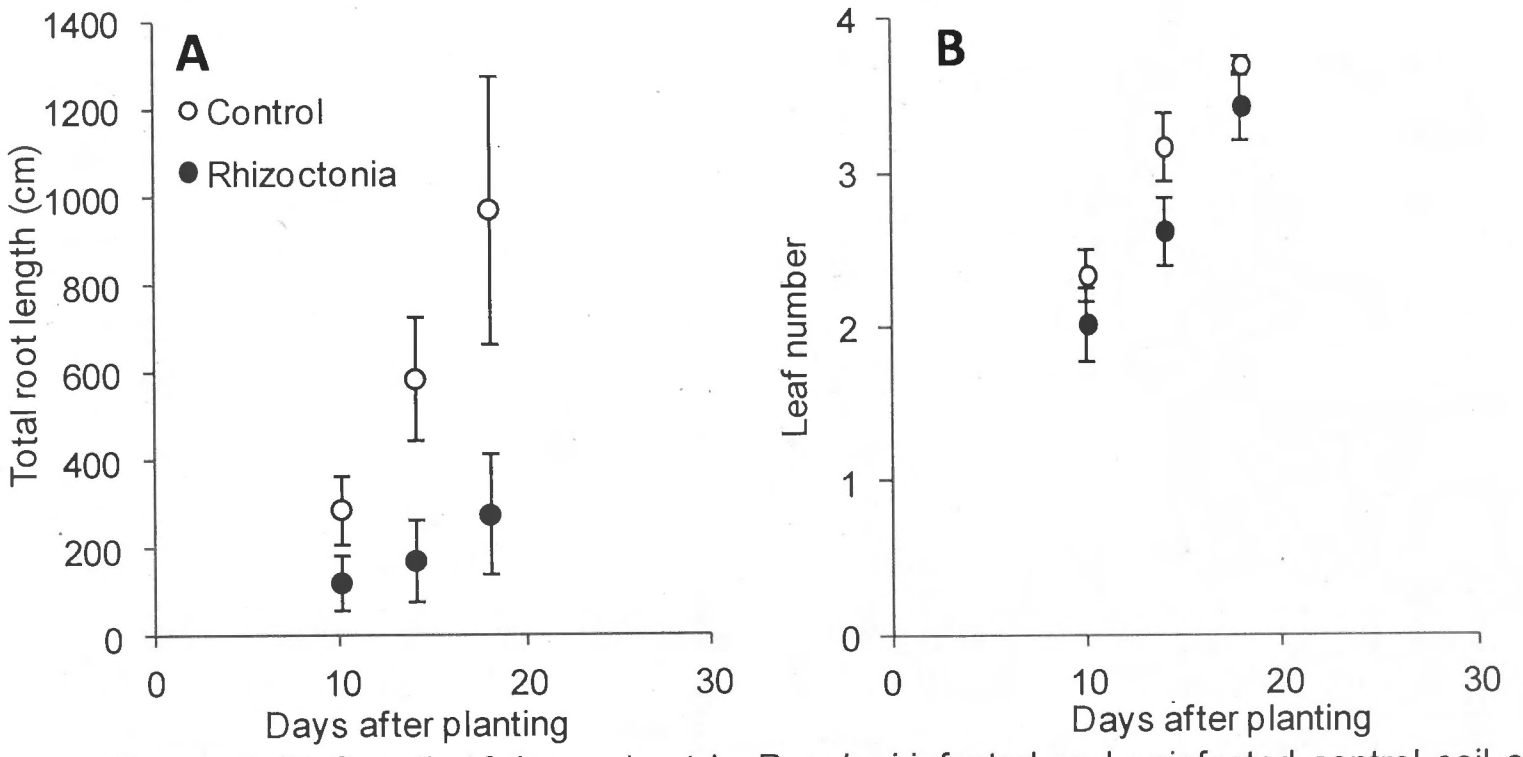

Figure 2.17 Growth of Janz wheat in *R. solani* infested and uninfested control soil over time. Means of a) total root length, and b) leaf number at 10, 14 and 18 days after planting; n≥5, SD

Plotting the relationships between leaf area and total root length (Figure 2.18) reveals a higher shoot: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 *B. distachyon* may provide clues towards root and shoot responses to disease.

Of the root and shoot measurements, total root length was the most sensitive indicator of *R. solani* disease, followed by leaf number and leaf area. Leaf length measurements can give information on early infection impacts.

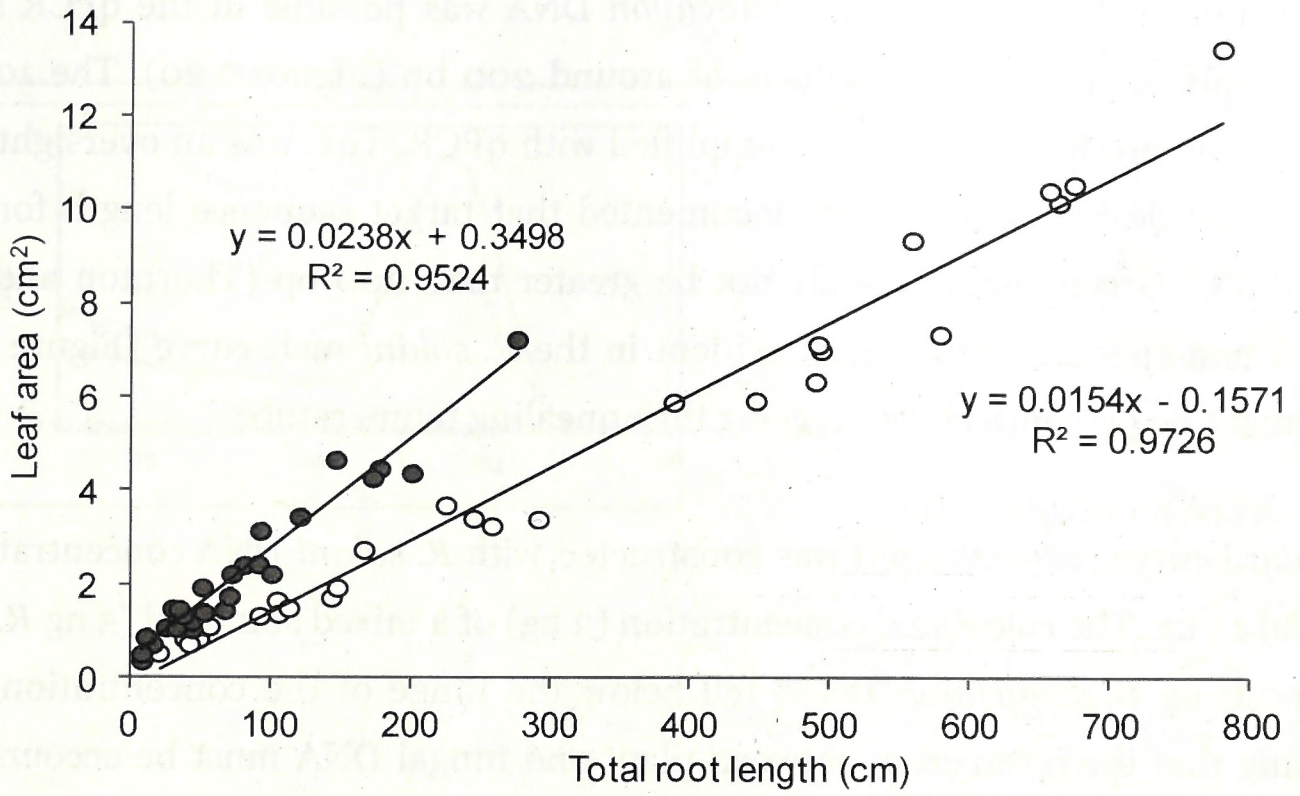

Figure 2.18 Correlation between total root length and leaf area for individual plants of line Bd 21-3 harvested at 10, 14, 18, 22 and 26 days after planting in control (white) and *R. solani* (grey) treatments.

2.3.2.2 Can qPCR be used to measure differences in disease severity?

DNA was able to be extracted from *B. distachyon* roots using a simple rapid method. Ground *B. distachyon* root tissue of ten-day-old plants grown in sand was dark brown in extraction buffer (Figure 2.18, 3 and 4), but yielded DNA of sufficient quantity and quality for PCR. When *B. distachyon* roots were grown on agar, the ground extract was a lighter colour, slightly darker than the extract of wheat roots grown on agar (Figure 2.18, 6).

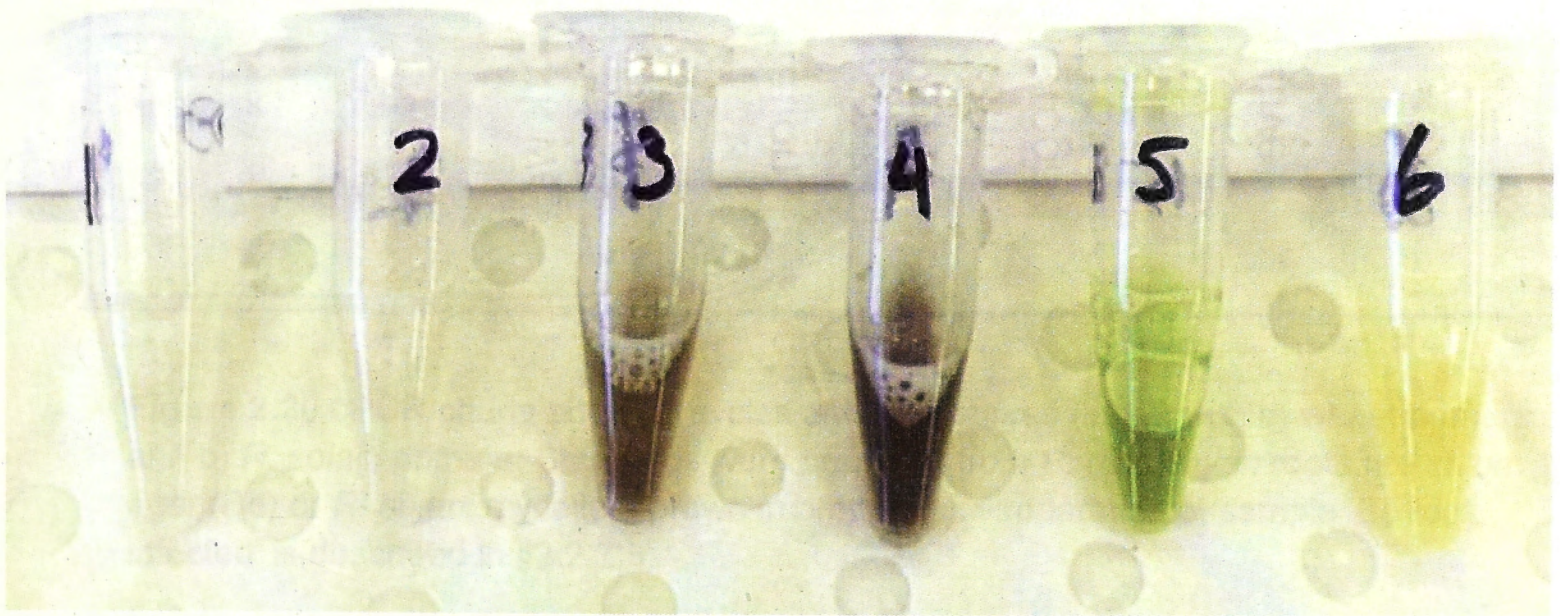

Figure 2.19 Ground tissues in extraction buffer. Samples are 1 and 2) *R. solani* mycelium, 3) uninfected *B. distachyon* roots grown in soil, 4) *R. solani* infected *B. distachyon* roots grown in soil, sample 'Bd root, infected', 5) *B. distachyon* leaves, and 6) wheat roots grown on agar.

Amplification of *R. solani* and *B. distachyon* DNA was possible in the qPCR system using the primer pairs with products of around 200 bp (Figure 2.20). The 1000 bp *B. distachyon* product could not be amplified with qPCR. This was an oversight in the experimental design, as it is well documented that target sequence length for qPCR using SYBR® Green should ideally not be greater than 200 bp (Thornton and Basu, 2011). A non-specific second peak evident in the *R. solani* melt curve (Figure 2.20 b inset) might be prevented by increasing the annealing temperature.

A standard curve (R^2=0.98295) was constructed with *R. solani* DNA concentrations of 5, 10 and 15 ng. The calculated concentration (3 ng) of a mixed standard (5 ng *R. solani* DNA + 90 ng *B. distachyon* DNA) fell below the range of the concentration curve, indicating that the interaction between plant and fungal DNA must be accounted for when measuring *R. solani* DNA concentration in *B. distachyon* roots. This can be achieved by using a set of standards containing a fixed amount of total DNA with varying proportions of fungal:plant DNA.

In a separate analysis a standard curve (R^2=0.97532) was constructed with *R. solani* DNA concentrations of 5, 10 and 15 ng. The calculated concentration of *R. solani* DNA in an infected young root sample ('Bd root, infected') was 8 ng per 100 ng DNA.

While qPCR was shown to be possible using DNA extracted from young sand-grown roots, attempts to extract amplifiable DNA from roots of plants included in a pot assay described in §3.2.8.2 proved unsuccessful.

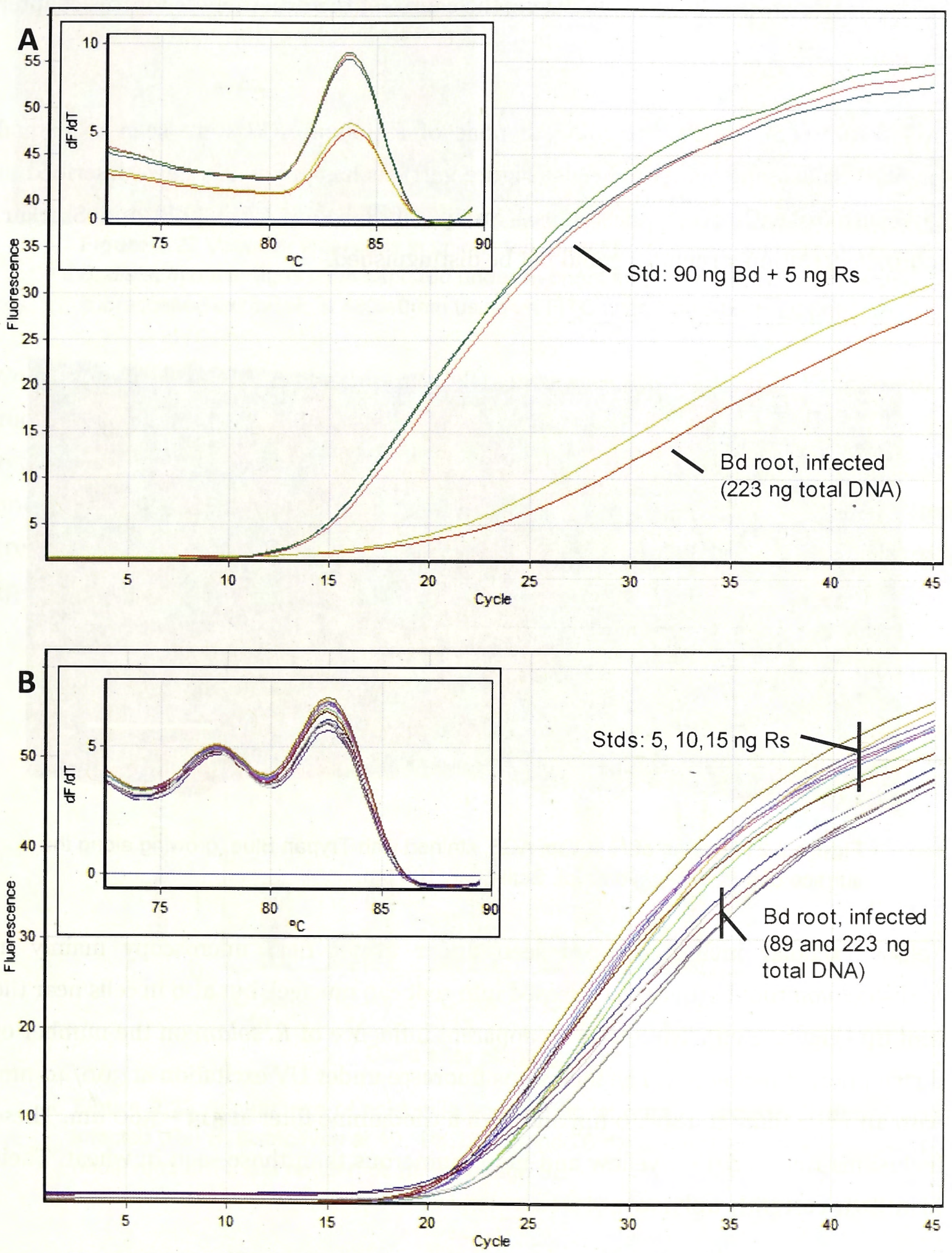

Figure 2.20 qPCR charts showing cycles and melt curves (inset) with a) wheat primers and b) *R. solani* primers. Standards were prepared from *B. distachyon* roots grown on agar (Bd) or *R. solani* mycelium grown in broth (Rs). Preparation of sample 'Bd root, infected' is described in §2.2.7.5.

2.3.2.3 General observations

Some general observations made during the course of the experiments for this chapter are noted here.

In infected roots of *B. distachyon*, hyphae of *R. solani* AG8 were seen to extend longitudinally along epidermal cells (Figure 2.21), as has previously been described in pathosystems with other anastomosis groups of *R. solani* (Weinhold and Sinclair, 1996). Penetration structures could not be distinguished.

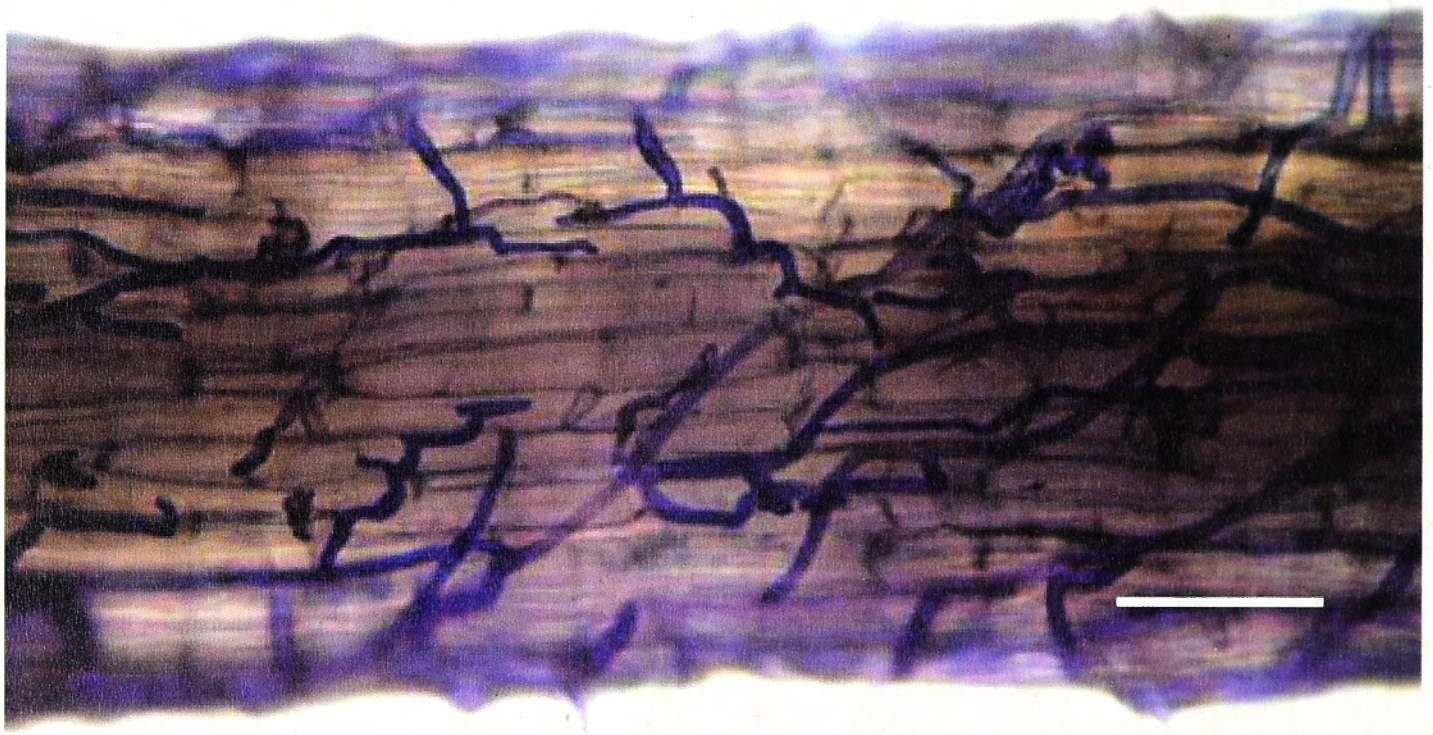

Figure 2.21 Hyphae of *R. solani* AG8, stained with Trypan Blue, growing along the surface of a *B. distachyon* root. Scale bar, 100 µm.

Yellow vacuolar inclusions were seen under bright field microscopy mainly in *B. distachyon* root border cells released into root cap mucigel, but also in cells near the root tip (Figure 2.22). There was no apparent influence of *R. solani* on the number or distribution of inclusions. The inclusions fluoresce under UV excitation at 360/40 nm, with an FITC filter at 480/40 nm and with a rhodamine filter at 515 – 560 nm. These inclusions were a darker yellow and more numerous than those seen in wheat. Their identity is currently unknown.

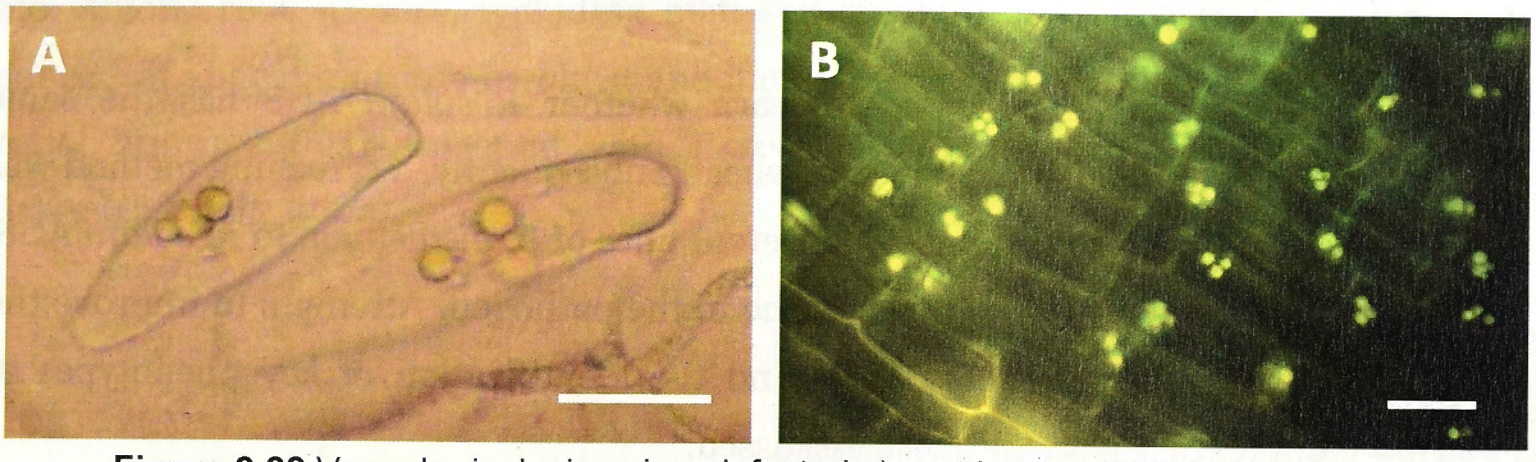

Figure 2.22 Vacuolar inclusions in uninfected a) root border cells, and b) root cells of *B. distachyon*. Images were captured under a) bright field microscopy, and b) with fluorescence excitation at 480/40 nm using an FITC filter. Scale bars, 20 µm.

Coleoptile (CNR) and leaf nodal root (LNR) emergence was counted in Experiment 4 root scans for *B. distachyon* line Bd 21-3 (Figure 2.23). Nodal roots could not be retrospectively distinguished from primary roots in wheat scans. Appearance of CNR and LNR tended to increase over time in both treatments, with *R. solani* inoculated treatments having significantly greater numbers of nodal roots than control treatments ($p<0.001$).

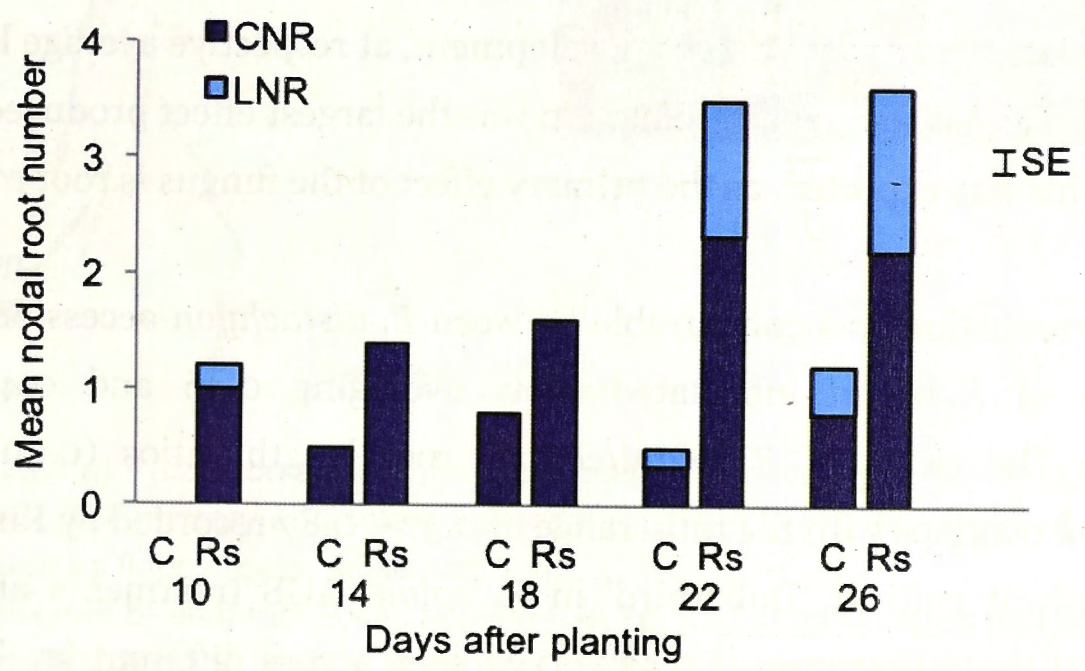

Figure 2.23 Appearance of coleoptile and leaf nodal roots in control (C) and *R. solani* infected (Rs) *B. distachyon* at 10, 14, 18, 22 and 26 days after planting. CNR, coleoptile nodal root; LNR, leaf nodal root; n≥5; average SE

2.4 Discussion

The first aim of this chapter was to learn whether *R. solani* AG8 has the same pathogenic effect on *B. distachyon* as on wheat. Subsequently, a screening method was developed to compare the response of different lines of *B. distachyon* to *R. solani* AG8, in order to find lines differing in resistance to the pathogen. Attempts to improve the repeatability of the screening protocol and the identification of key phenotypes to measure plant disease resistance are discussed.

2.4.1 Experiments towards development of a robust screening method using *B. distachyon*

2.4.1.1 Response of B. *distachyon* and wheat to *R. solani* AG8

Visual symptoms of Rhizoctonia root rot in *B. distachyon* were similar to those described in wheat, including primary root truncation and rotting of cortical tissue leading to spear tips (Figure 2.24).

The root and shoot measurements taken from *B. distachyon* accessions and Janz wheat harvested at a similar stage of development, at respective average leaf stages 3.0 and 2.8, showed that root length reduction was the largest effect produced by *R. solani* treatment. This was expected, as the primary effect of the fungus is root rot.

Root length reduction was comparable between *B. distachyon* accessions and wheat, with length of *R. solani* inoculated roots averaging 0.46 and 0.44 of control respectively. The range of *R. solani*/control root length ratios (0.30 – 0.68) for *B. distachyon* overlaps with the ratio range of 0.37 – 0.87 recorded by Kirkegaard et al. (1999) for wheat cultivar 'Dollarbird' in *R. solani* AG8 treatments at atmospheric pressure, and the ratio range of 0.53 – 0.92 seen across different levels of *R. solani* AG8 inoculum for wheat cultivars 'Scarlet' and 'Scarlet-Rz1' by Okubara et al. (2009). In experiments conducted with 'Scarlet' and '*Lr34*' wheat lines (Appendix C), *R. solani* AG8 reduced root length to 0.42 – 0.49 and 0.35 – 0.69 of control, respectively.

A smaller but still significant effect of *R. solani* was seen on the rate of leaf appearance, measured as leaf number. It is expected that leaf number has a linear relationship with leaf appearance rate (the inverse of phyllochron) during the early vegetative growth phase of *B. distachyon*, as this has been shown to occur in *Triticum* spp. (Cone *et al.*, 1995; Slafer and Rawson, 1997). The fungus significantly reduced leaf number in *B. distachyon* 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. Abiotic root stresses, including high soil strength and nutrient deficiency, can reduce leaf appearance rate in wheat and barley (Masle and Passioura, 1987; Prystupa *et al.*, 2003), as can infection with *R. solani* AG8 in wheat (Wall *et al.*, 1994; Kirkegaard *et al.*, 1999).

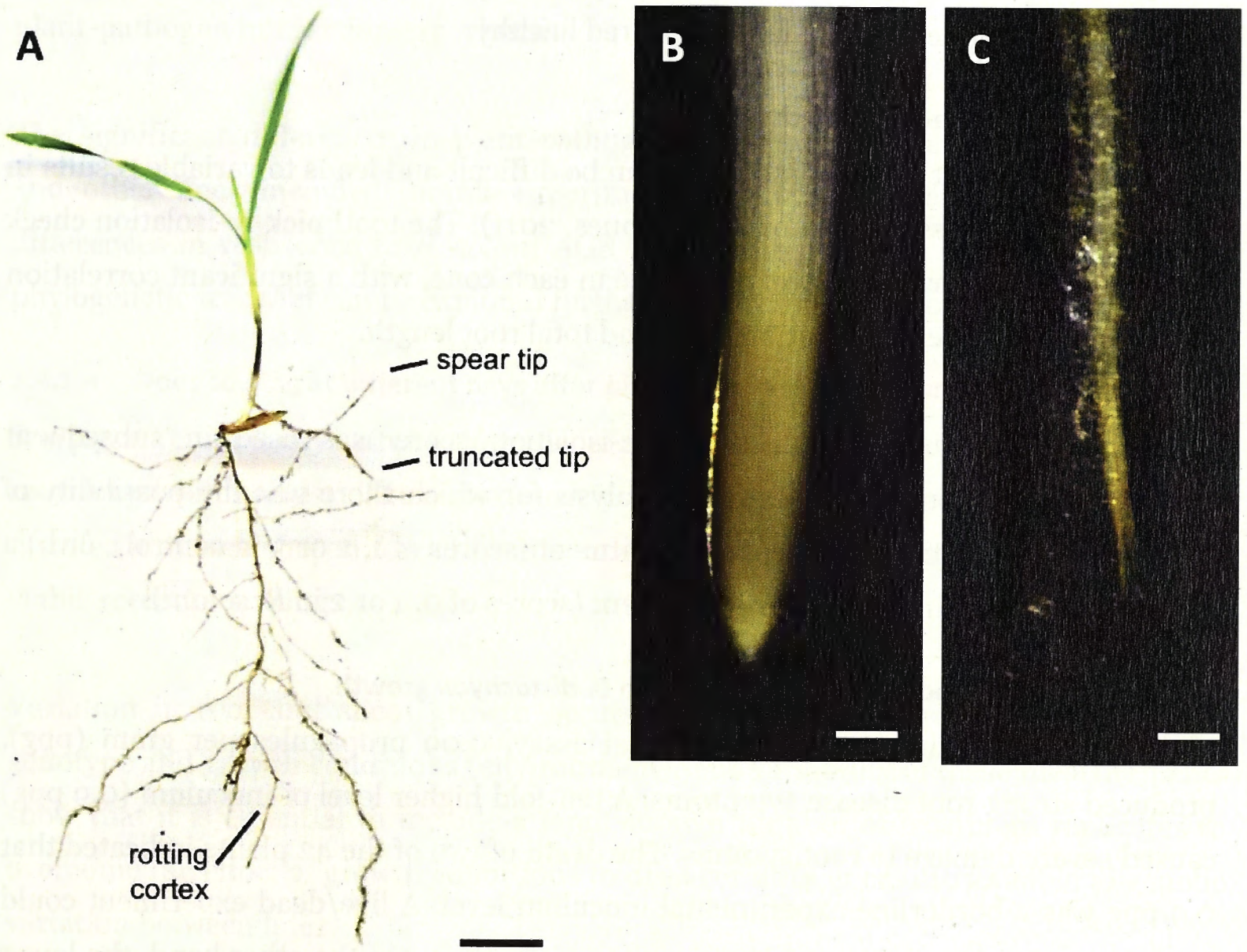

Figure 2.24 a) Rhizoctonia root rot symptoms in a *B. distachyon* seedling (line Bd 21-3) at 18 DAP. The primary and coleoptile nodal roots are truncated. Areas of rotting cortex are evident as floppy roots; scale bar, 1 cm.
Comparison of b) an intact *B. distachyon* root tip, and c) a truncated root tip, with the central stele extending beyond the cortex to form a spear tip; scale bars, 100 µm.

Leaf 1 length was not affected by *R. solani* in either wheat or *B. distachyon*. In experiments with *Pythium* spp, another early pathogen of wheat, first leaf length is considered to be an indicator of embryo colonisation by the pathogen (Higginbotham *et al.*, 2004a). *Rhizoctonia solani* AG8 is reported to reduce first leaf length in barley (Lee *et al.*, 2012). In these experiments leaf 1 length reduction may not have occurred due to seed pre-germination.

The significant reduction in leaf 2 length in wheat, but not in *B. distachyon*, suggests that the root disease affected wheat shoots by a slightly earlier stage of plant development than in *B. distachyon*.

In terms of random effects, spatial variation of the environment within a growth cabinet was found to affect disease expression, particularly on root length and leaf number of *B. distachyon*, but not relative disease resistance between lines.

The growth conditions used produced measureable *R. solani* disease symptoms in infected plants, while control plants appeared healthy.

2.4.1.2 Toothpick re-isolation check

Achieving consistent levels of inoculum can be difficult and leads to variable results in disease resistance assays (Okubara and Jones, 2011). The toothpick re-isolation check was a good indicator of the level of disease in each cone, with a significant correlation between the toothpick re-isolation score and total root length.

Based on this result, the toothpick re-isolation score was used in subsequent experiments to exclude samples from analysis for which there was the possibility of either a) contamination in the control treatment (scores of 1, 2 or 3 in control), or b) a low levels of inoculum in *R. solani* treatment (scores of 0, 1 or 2 in *R. solani*).

2.4.1.3 Effect of inoculum concentration on *B. distachyon* growth

The level of inoculum used in the initial assay, 0.09 propagules per gram (ppg), produced strong root disease symptoms. A ten-fold higher level of inoculum (0.9 ppg) caused severe damage to root systems. The death of two of the 42 plants indicated that 0.9 ppg was a borderline experimental inoculum level. A live/dead experiment could be developed using an even higher amount of inoculum. On the other hand, the lower level of inoculum (0.09 ppg) allows all plants to survive, thus providing as complete a set as possible for comparison of root and shoot traits. For this reason the lower level was chosen for subsequent experiments.

The *R. solani* inoculum level of 0.09 ppg lies within a vast range of concentrations used in previous research. It is apparent that comparison of inoculum levels between experiments described in the literature is uncertain, likely due to differences in inoculum particle size, incubation time, growth conditions and isolate pathogenicity. Calculating initial propagule densities in Kirkegaard *et al.* (1999) gives approximately 0.004 – 0.011 ppg for low inoculum (for which little root damage was measured) and 0.008 – 0.023 ppg for the high inoculum level, with further inoculum development during a two week incubation prior to sowing. Okubara *et al.* (2009) challenged mutant wheat lines with inoculum levels starting at 80 ppg, followed by 20, 100 and 200 ppg, of *R. solani* AG8 with one week of incubation prior to sowing. Neate (1989)

measured increasing root disease from 0.004 – 0.016 ppg *R. solani* AG8 in a pot experiment, with 26 days incubation prior to sowing.

The variation in resistance ratios seen between Bd 21 and Bd 21-3 in Experiments 1 and 2, although not significant, again highlighted the importance of replication in plant-pathogen interaction experiments.

The significant differences in plant-pathogen interactions measured between Koz-3 and other lines included in this experiment indicates that there may be genetic differences in resistance to *R. solani* AG8 within this relatively narrow range of the phylogenetic tree that can be explored further in a natural accession screen.

2.4.1.4 Does sowing at different days after disinfection reduce variation?

Sowing at 0, 1 or 2 days after surface disinfection did not have an effect on variation in root length at harvest. Thus, the most convenient sowing time (1 d after surface disinfection) was chosen for all future experiments. At 1 DAD it is possible to select viable seeds for sowing.

Variation in root and shoot growth occurs as a result of interactions between the genotype and growth conditions (environment). The variability of these measurements show that it is essential to include a large enough number of treatment replicates to overcome the effect of growth conditions to discover differences attributable to genetic variation between lines.

2.4.2 Phenotype measurements

2.4.2.1 Root and shoot measurements over time

The best quantitative measure to assess resistance of *B. distachyon* to *R. solani* was found to be root length. Total root length was significantly lower in infested treatments over all harvest time-points (10 to 26 days after planting), with the difference increasing over time. This result agrees with the observation of Okubara *et al.* (2009) that total root length measurements, along with disease severity ratings, are the most reliable measurements of *Rhizoctonia* disease.

Leaf number and leaf area were also good indicators of disease. A significant reduction in these shoot measurements in *R. solani* treatment was seen from 18 DAP onwards, two time-points after root length was significantly reduced. Shoot effects can occur in response to reduced root system length, systemic wounding responses or pathogen effectors that directly affect shoot growth. Disease responses seen in the leaves are

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 strict root to shoot biomass ratios.

Leaf 1 and 2 lengths were only significantly different between treatments at one time-point, when leaves were already fully expanded. Variation in first leaf length is a associated with early plant vigour in wheat (Richards and Lukacs, 2002). First leaves of *B. distachyon* were fully expanded by 10 DAP. Reduction in leaf 1 length would indicate that *R. solani* had impacted seedling vigour before this time-point.

Under the conditions in this assay, leaf 2 reached full expansion between 10 and 14 DAP. As with leaf 1, leaf 2 length does not vary markedly after it has reached its maximum length. Thus, reduction in leaf 2 length is a measure of disease impact in the first two weeks after germination.

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, such as waterlogging (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.

2.4.2.2 qPCR

The level of colonization of 11-day-old *B. distachyon* roots infested for four days with *R. solani* was measured to be approximately 8 ng *R. solani* DNA per 100 ng DNA. This is in the range seen in previous studies of pathogen colonization of roots. Harrach *et al.* (2013) measured around 6 – 8 ng *Fusarium culmorum* DNA in 100 ng DNA from infected barley roots. In *Phytophthora medicaginis* infection of alfalfa, Vandemark and Barker (2003) measured between 0.2 – 4 ng of oomycete DNA in 100 ng of DNA from infected roots, depending on disease susceptibility.

Extraction of DNA from infested soil-grown *B. distachyon* roots of a suitable quality for PCR and qPCR was more successful with younger root tissue than with plants harvested at 26 DAP. Mommer *et al.* (2011) discuss several problems that can arise with DNA extraction from roots for PCR, including lower DNA quality in roots than

shoots, a decrease in DNA yield as roots age and inhibition of PCR reactions by humic acid binding magnesium chloride. Better quality DNA might be achieved by developing screening experiments using sand as the growth medium and harvesting plants earlier. Harrach *et al.* (2013) also cautioned that extraction of DNA from necrotrophic plant-pathogen interactions can potentially overestimate the amount of fungal DNA in a root, if plant DNA is degraded during necrosis.

The potential of qPCR as a measure of the degree of pathogen colonisation of plant tissue is of interest, particularly as a means of determining whether differences in response between *B. distachyon* lines are due to tolerance or resistance mechanisms. As discussed in §1.3.4, the ability of a plant to reduce pathogen colonisation points to a 'resistance' mechanism, while a genotype with reduced disease symptoms under the same level of pathogen colonisation exhibits a 'tolerance' phenotype. The difficulties with developing a simple protocol for qPCR of infested roots, however, forced this method not to be used subsequently for phenotypic measurements in the *B. distachyon-R. solani* pathosystem.

2.4.2.3 General observations

Root border and cortical cells of uninfected *B. distachyon* had pigmented vacuolar inclusions (Figure 2.19), expected to contain phenolic compounds (R. White, pers. comm., 16 Feb 2011). Some phenolic acids are involved in plant defence response against soil pathogens (Jousset *et al.*, 2011). Phenolics can also inhibit DNA extraction for PCR. These inclusions are strongly reminiscent of yellow maize vacuolar inclusions found by Grotewold *et al.* (1998) to be accumulated with the expression of P, a Myb-related transcriptional regulator involved in flavonoid biosynthesis. The maize vacuole accumulated *C*-glycosyl flavones, luteoforol, ferulic acid and auto-fluorescent compounds, which have not yet been identified. Lin *et al.* (2003) observed that the yellow fluorescent bodies resemble anthocyanic vacuolar inclusions (AVIs), while Agati *et al.* (2012) suggest that the unknown compounds may be highly hydroxylated flavonoids. Future experiments could include mass spectrometric identification of compounds and confirmation using microspectrofluorometry.

An increase in nodal root emergence was seen in *R. solani* inoculated *B. distachyon* line Bd 21-3 across all time-points, from 10 to 26 DAP. This trend of increased nodal root emergence with *R. solani* AG8 disease has previously been observed by Schroeder and Paulitz (2008). The capacity of barley seedlings to compensate for loss of primary root length by increasing the number and length of nodal roots has also been demonstrated in solution culture experiments (Crossett *et al.*, 1975).

2.5 Summary of findings

An isolate of *R. solani* AG8 was demonstrated to be pathogenic to both wheat and *B. distachyon*, producing a similar level of disease severity and symptoms in both species.

The best phenotypic measure of disease severity at harvest was found to be total root length, followed by leaf number and leaf area. The impact of disease on these measurements increased over time. Leaf 1 and 2 lengths allowed the effect of *R. solani* in the first two weeks after sowing to be measured.

A repeatable screening method was developed to compare the relative resistance of different *B. distachyon* lines to *R. solani* AG8. Preliminary indications point to differences between the natural accession genotypes for response to *R. solani*. The application of this method to screen *B. distachyon* natural accessions and T-DNA lines for disease resistance is presented in Chapters 3 and 4, respectively.

Chapter 3

Rhizoctonia solani AG8 resistance among genetically diverse *Brachypodium distachyon* natural accessions

Summary

The phenotyping system to identify resistance to *Rhizoctonia solani* AG8, described in Chapter 2, was applied to twenty-six diverse accessions of *Brachypodium distachyon*. The aim was to identify a low level of repeatable resistance to *R. solani* AG8. Complete resistance was not expected, as the pathogen is a broad host-range necrotrophic fungus that infects all grass genotypes tested to date. In Chapter 3, ten experiments led to the identification of repeatable variation in root length resistance to *R. solani* between Koz-3 and BdTR 13a ($p<0.001$). The following activities associated with this finding are reported here:

- Selection of twenty-six *B. distachyon* accessions from ecotypes collected from the Middle East by John Vogel and collaborators, USDA, based on widest genetic relationships, diverse geographic and climatic origins, and reported variation in resistance to shoot pathogens;

- Wide screening of 26 accessions to identify four accessions with repeated variation in resistance to *R. solani* across three independent replications;

- Confirmation that the widest and most repeatable variation in resistance was between Koz-3 and BdTR 13a ($p<0.001$). Koz-3 had 33% more total root length when infected with *R. solani* compared with uninfected roots, compared with BdTR 13a infected and uninfected roots;

- Analyses of root and shoot responses of *B. distachyon* to *R. solani* across accessions confirmed that total root length is the largest and most repeatable indicator of infection for phenotyping;

- Correlation studies to understand mechanisms of resistance indicated that seed size and endogenous seedling vigour is associated with lower resistance, but that other factors are also involved. Evidence is presented that resistance is positively associated ($p=0.019$) with tendency to make nodal roots in response to infection.

3.1 Introduction

The aim of the experiments described in this chapter was to discover and confirm variation in resistance to *R. solani* AG8 among the *B. distachyon* natural accession collection obtained from Vogel et al. (2009) at the USDA, using the phenotyping methods developed in the previous chapter.

These *B. distachyon* natural accessions have shown great promise for discovering resistance mechanisms for different shoot pathogens. Peraldi *et al.* (2011) showed that *B. distachyon* lines Bd 21 and Bd 3-1 responded differently to necrotrophic *Fusarium* spp. and the mycotoxin deoxynivalenol (DON), with 32 – 53% more conidia produced on florets of Bd 3-1 than Bd 21. Subsequently the group measured quantitative differences in resistance to two hemibiotrophic leaf pathogens with these lines (Peraldi *et al.*, 2013). Line Bd 3-1 had up to a 40% higher visual necrosis score than Bd 21 for *Ramularia collo-cygni* leaf spot. Line Bd 3-1 also had a 30 – 44% higher visual severity score than Bd 21 after infection with *Oculimacula acuformis* or *O. yallundae* eyespot. Barbieri *et al.* (2012) discovered three QTL for the biotrophic leaf rust fungus, *Puccinia brachypodii*, by crossing a less susceptible (Bd 3-1) with a more susceptible (Bd 1-1) line. Earlier, these researchers found that lines Bd 1-1, Bd 2-3, Bd 21, Bd 18-1 and Bd 3-1 had different quantitative levels of resistance to four rust isolates of *P. brachypodii* (Barbieri *et al.*, 2011). Varying levels of non-host resistance were found in *B. distachyon* to *Puccinia graminis* ff. spp. *tritici*, *lolii* and *phlei-pratensis*, the natural hosts of which are wheat, perennial ryegrass and timothy grass, respectively. Lines Bd 2-3, Bd 18-1 and Bd 21-3 were generally most susceptible, Bd 21 and Bd 30-1 were moderately susceptible, while Bd 1-1 and Bd 3-1 were least susceptible to the stem rust strains (Figueroa *et al.*, 2013). Ayliffe *et al.* (2013) noted differences in susceptibility of *B. distachyon* lines to *Puccinia striiformis* f. sp. *tritici* and *Pu. graminis* f. sp. *tritici*, with lines in the TEK group (collected from near Tekirdağ, Turkey; Figure 2.1) generally more susceptible to *Pu. striiformis* and less susceptible to *Pu. graminis*. Non-TEK group susceptibility towards these two species tended to be the inverse to that of the TEK group. An atypical response to these pathogens was seen in the BdTR 10 group. The lines BdTR 13k and Bd 21, along with BdTR 10h and Tek-4, were crossed to study inheritance of non-host resistance to *Pu. striiformis* f. sp. *tritici*. Further, Cui *et al.* (2012) mapped the location of the *Bsr1 Barley stripe mosaic virus* (BSMV) resistance gene using inbred lines with Bd 3-1 as the resistant parent and Bd 21 as the susceptible parent. Variation in resistance to BSMV was found in the natural accessions included in these experiments: Adi-10, Bd 3-1 and Tek-4 were resistant, while Adi-12, Bd 21, Bd 21-3, Bd 2-3, BdTR 10c, BdTR 11i, BdTR 12c, BdTR 13c, BdTR 2g, BdTR 3c, BdTR 5i, BdTR 9k and Koz-3 were susceptible.

There was no published data on variation in root disease resistance or resistance to *R. solani* in *B. distachyon* prior to this thesis. However, the published evidence above on shoot and crown pathogens suggests that the *B. distachyon* accessions could be a source of resistance to *R. solani*. Chapter 2 also presented preliminary evidence for variation in resistance between lines Koz-3, Bd 21, BdTR 13c and Koz-1, suggesting that phenotyping the collection more widely could reveal larger and more repeatable variation to this root pathogen. The aim was to identify quantitative resistance, as multiple genes are usually involved in plant defence against broad host-range necrotrophs like *R. solani* AG8 (Mengiste, 2012).

The *B. distachyon* accessions selected for phenotyping in this Chapter are presented in Table 3.1. These diploid inbred lines were selected for maximum diversity, based on the SSR tree published by Vogel *et al.* (2009) (see phylogenetic tree in Chapter 2, Figure 2.1), and to include diverse climatic locations (Table 3.1). It is possible that the different accessions had experienced selection pressure to different pathogens. In addition, all but three accessions chosen were included in the US Department of Energy Joint Genome Institute resequencing project, such that the complete genomes of the accessions would be available by the end of this thesis, for future association of phenotypes against genomic information. The lines included in the resequencing project had been chosen on genetic and phenotypic diversity (pers. comm, Dr Ludmila Tyler, University of California, Berkeley CA, USA), confirming that the selection here was diverse. Three non-resequenced lines were included in these experiments, as they had been included earlier in method development experiments for *R. solani* AG8 and *Pythium* spp. (Chapter 2 and Appendix B).

This chapter describes two broad sets of activities: accession screening (experiments 1 to 8) and confirmation (experiments 9 and 10). Total root length, fully expanded lengths of leaves 1 and 2, and leaf number were taken as measures of root, shoot and plant developmental responses to *R. solani*, respectively. The impact of early plant vigour, measured as seed size and growth under control conditions, was also investigated, as was nodal root development in response to disease. Different analysis strategies were considered for ranking accession disease resistance based on the total root length (TRL) in *R. solani* treatment as a proportion of either the accession control TRL or *R. solani* treatment TRL of a reference line.

Table 3.1 Origin of *B. distachyon* natural accessions chosen for testing with *R. solani* to identify resistance.

Line	Genome reseq?[a]	Collection area	Latitude	Longitude	Elevation (metres above sea level)	Prior studies with this line?[b]
Abr 2	Yes	Octon, France[4]	N 43.604262	E 3.262939	212	
Adi-10	Yes	Adiyaman, Turkey[1]	N 37.770694	E 38.352278	510	Yes
Adi-12	Yes	Adiyaman, Turkey[1]	N 37.770694	E 38.352278	510	Yes
Arn 1	Yes	Arens, Spain[4]	N 42.25651	E 0.72985	681	
Bd 1-1	Yes*	Soma, Turkey[2,3,4]	N 39.190956	E 27.607942	na	Yes
Bd 18-1	Yes	Turkey[2,3]	N 39.367847	E 33.730253	~914	Yes
Bd 21	Yes*	Salah ad Din, Iraq[2,3,4]	N 33.760883	E 44.403075	na	Yes
Bd 21-3	Yes*	Salah ad Din, Iraq[1,4]	N 33.760883	E 44.403075	na	Yes
Bd 2-3	Yes	Iraq[2,3,4]	N 33.760883	E 44.403075	na	Yes
Bd 30-1	Yes*	Dilar, Spain[3]	N 36.990489	E 3.558733	1220	Yes
Bd 3-1	Yes*	Iraq[2,3,4]	N 33.760883	E 44.403075	na	Yes
BdTR 10c	Yes	Turkey[1]	N 37.778233	E 31.884911	1288	Yes
BdTR 10o	No	Turkey[1]	N 39.738164	E 28.040197	363	
BdTR 11i	Yes	Turkey[1]	N 39.738164	E 28.040197	363	Yes
BdTR 12c	Yes*	Turkey[1]	N 39.748181	E 34.650319	1035	Yes
BdTR 13a	No	Turkey[1]	N 39.756486	E 32.43235	787	
BdTR 13c	Yes	Turkey[1]	N 39.412856	E32.988122	1192	Yes
BdTR 1i	Yes	Turkey[1]	N 38.093064	E 28.583061	841	
BdTR 2g	Yes	Turkey[1]	N 40.393647	E 32.985367	1596	Yes
BdTR 3c	Yes	Turkey[1]	N 36.783033	E 32.962975	1957	Yes
BdTR 5i	Yes	Turkey[1]	N 40.393647	E 32.985367	1596	Yes
BdTR 9k	Yes	Turkey[1]	N 39.75295	E 30.788631	932	Yes
Cas 2	No	Candasnos, Spain[5]	N 41.46508	E 0.01766	207	
Koz-1	Yes	Kozluk, Turkey[1]	N 38.152294	E 41.609667	853	
Koz-3	Yes*	Kozluk, Turkey[1]	N 38.152294	E 41.609667	853	Yes
Tek-4	Yes	Tekirdağ, Turkey[1]	N 41.011139	E 27.519111	20	Yes

[a]Included in the Joint Genome Institute (JGI) resequencing project at time of this thesis; *Resequencing completed in 2011.

[b]Line used in prior plant-pathogen interaction studies? Refer to text for details.

Collection references: [1](Vogel *et al.*, 2009), [2](Vogel *et al.*, 2006), [3](Garvin, 2010), [4](USDA-ARS Western Regional Research Center, 2013), [5](Luis Mur via Iain Wilson, pers. comm., 28 May 2010).

3.2 Materials and methods

3.2.1 Seed preparation

The 26 natural accessions of *B. distachyon* used for the experiments were donated by Dr Iain Wilson (CSIRO Plant Industry, Canberra, 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) (Table 3.1). Seed increase was carried out at CSIRO Plant Industry, Black Mountain, by Dr Vincent Chochois. Seeds were dehusked, surface disinfected and germinated on agar plates overnight, as described in §2.2.1.

3.2.2 *Rhizoctonia solani* inoculum

Rhizoctonia solani inoculum was prepared with millet seed, as described in §2.2.2. Millet seed was inoculated with an isolate of *Rhizoctonia solani* AG8 received from Dr Jonathan Anderson, CSIRO Plant Industry, Perth, Australia.

3.2.3 Soil

The soil used for the screening activity was a steam sterilized mix of compost and perlite, prepared by the CSIRO Plant Industry potting shed, known as 'Barley Mix'. This is the same soil that was used for experiments in Chapter 2 (see §2.2.3).

The soil used for the confirmation activity was a blend of 50% river sand and 50% 'Special' potting mix prepared by the CSIRO Plant Industry potting shed. 'Special' potting mix was a composted mixture of recycled soil, straw and fertilizers. Soil was sieved to remove coarse gravel and steam-sterilized.

3.2.4 Cone preparation and sowing

Cone preparation and sowing conditions developed in §2.2.4 were used for the experiments in this chapter. Soil was inoculated with millet inoculum, watered to saturation and sown with germinated *B. distachyon* seeds.

3.2.5 Growth conditions

Plants were grown in growth cabinets in 12 h day length at a constant temperature of 16°C (§2.2.5). The screening activity was carried out in Conviron® CMP 2023 cabinets (Winnipeg, Manitoba, Canada). The confirmation activity was carried out in a Conviron® PGW40 cabinet (Winnipeg, Manitoba, Canada).

Cones were watered with tap water the day after sowing (5 mL) and then with five or ten mL every two to three days, as required.

3.2.6 Re-isolation of *Rhizoctonia* from soil

Rhizoctonia was re-isolated from soil to check for contamination in the control treatment and to ensure the presence of an adequate level of inoculum in the *R. solani* treatment. *Rhizoctonia* was re-isolated from cones at 8 days after planting (DAP) (§2.2.6). Control cones with toothpick scores of 1, 2 or 3 and *R. solani* inoculated cones with toothpick scores of 0, 1 or 2 were excluded from analysis.

3.2.7 Phenotype measurements to identify resistance

At 22 DAP plants were removed from pots and roots rinsed out gently. Plants were stored in plastic sauce containers in ethanol (50% v/v).

Total root length (TRL) was measured by staining roots with toluidine blue, separating them in a water-filled tray and scanning at 400 dpi on an Epson Expression 1680 flatbed scanner (Epson, Australia). The WinRHIZO™ system (Regent, Quebec) was used to calculate total root length (§2.2.7.1). During the course of experiments it was found that, by adjusting filters and background analysis parameters, roots could be measured just as well without staining. Thus, root systems were not stained for confirmation activity root length measurement.

Leaf number and length of leaves 1 and 2 were measured from the crown (§§2.2.7.2 and 2.2.7.3) at 21 DAP.

Nodal root number was measured by counting roots emerging from the coleoptile (coleoptile nodal root, CNR) and the stem leaf nodes at the crown (leaf nodal root, LNR) on root scans (Watt *et al.*, 2008). All emerged nodal roots were counted, including those severely truncated by *R. solani.*

To measure root fresh weight, soil was first gently removed from roots by rinsing in tap water and brushing with a gloved finger. Roots were blotted between a paper hand towel (Kleenex® Executive Hand Towel, Kimberly-Clark Professional, Australia), dried for 5 s and immediately placed onto the scale. The large root surface area promotes water evaporation, so mass was consistently measured 10 s after being placed on the scale and was rounded to the nearest milligram.

Shoot fresh weight was measured immediately after cutting the leaves off at the base of the plant. Shoot FW readings stabilised within 10 s of being placed on the balance.

Seed size was measured by averaging the mass of 16 seeds from the experimental seed source, with consistent larger seeds chosen in the same manner as for experiments. Seeds were not dehusked prior to weighing. Average seed masses are given in Appendix A.4.

3.2.8 Experiments

3.2.8.1 Screening activity (experiments 1 to 8)

A large experiment was set up to screen 26 diverse *B. distachyon* inbred lines from the natural accession collection for resistance to *R. solani* AG8 (Table 3.1). Each line was sown at three time-points (i.e. in three separate experiments) into two levels of inoculum: *R. solani*-inoculated and control. Three plants were sown per *Host*Inoculum* treatment in adjacent cones of a flow tray, with two flow trays (blocks) per experiment. Line Bd 21-3 was included in every experiment. An example of the tray layout in an experiment is shown in Figure 3.1.

	1	2	3	4	5	6	7	8	9	10	11	12	
A				Bd 21-3, Control			Bd 21-3, *R. solani*						
	Line 5, *R. solani*			Line 8, *R. solani*			Line 4, *R. solani*			Line 2, *R. solani*			
	Line 1, Control			Line 3, *R. solani*			Line 9, Control			Line 3, Control			
	Line 2, Control			Line 5, Control			Line 10, *R. solani*			Line 10, Control			
	Line 4, Control			Line 9, *R. solani*			Line 7, *R. solani*			Line 1, *R. solani*			
	Line 6, *R. solani*			Line 6, Control			Line 8, Control			Line 7, Control			

	1	2	3	4	5	6	7	8	9	10	11	12	
B				Bd 21-3, *R. solani*			Bd 21-3, Control						
	Line 1, Control			Line 5, *R. solani*			Line 9, Control			Line 6, *R. solani*			
	Line 6, Control			Line 2, Control			Line 7, Control			Line 4, *R. solani*			
	Line 3, Control			Line 1, *R. solani*			Line 8, Control			Line 9, *R. solani*			
	Line 10, Control			Line 2, *R. solani*			Line 3, *R. solani*			Line 8, *R. solani*			
	Line 4, Control			Line 7, *R. solani*			Line 10, *R. solani*			Line 5, Control			

Figure 3.1 Arrangement of cones across two trays in one screening experiment. Each tray holds 7 x 14 cones. Line Bd 21-3 was included in every experiment. The ten genotypes included in an experiment were randomly allocated as Lines 1 to 10.

Eight experiments were sown on 25 October 2011 (1), 1 November 2011 (2, 3), 22 November 2011 (4), 30 November 2011 (5, 6), 31 January 2012 (7) and 7 February 2012 (8), with several issues arising over this time period (Table 3.2).

Each of the 26 lines included in the experiments was repeated in three experiments. The exception was Bd 21-3, which was included as a reference line in every experiment. None of the lines included in experiment 2 was repeated in experiment 3, sown on the same date. Likewise, no lines included in experiment 5 were repeated in experiment 6. Adi-12, Bd 21-3, BdTR 13a, BdTR 3c and Koz-1 were included both in experiment 7 and 8.

On the morning of December 13, the growth cabinet was found broken down with the temperature at 36°C. Trays of experiments 5 and 6 were immediately moved to an adjacent growth cabinet, set at the same conditions, for the final week of the experiment. Experiments 7 and 8 were subsequently carried out in the second growth cabinet.

Median emergence date was between three and six days after planting (DAP) for all lines. Plants were harvested at 22 DAP.

Table 3.2 Experiments run for natural accession screen. Each accession, represented by a letter a – z, was included in three experiments sown on different dates.

Expt	Lines included	Date sown	Problems
1	b d ghi kl o r u y	25 Oct 2011	
2	a e **h** j m o q st v x	1 Nov 2011	Potting mix appeared to contain a higher than usual level of unidentified fungus.
3	c fghi k n p w yz		
4	ab def **h** j m q w z	22 Nov 2011	
5	b efghijk o yz	30 Nov 2011	Cabinet broke down on Dec 13 and trays moved to adjacent 2nd cabinet.
6	a d **h** l n qrs uvw		
7	c **h** l n p rstuv x	31 Jan 2012	Experiments grown in 2nd growth cabinet.
8	c **h** m p t x	7 Feb 2012	

Lines included: a, Abr 2; b, Adi-10; c, Adi-12; d, Arn 1; e, Bd 1-1; f, Bd 18-1; g, Bd 21; **h, Bd 21-3**; i, Bd 2-3; j, Bd 30-1; k, Bd 3-1; l, BdTR 10c; m, BdTR 10o; n, BdTR 11i; o, BdTR 12c; p, BdTR 13a; q, BdTR 13c; r, BdTR 1i; s, BdTR 2g; t, BdTR 3c; u, BdTR 5i; v, BdTR 9k; w, Cas 2; x, Koz-1; y, Koz-3; z, Tek-4.

3.2.8.2 Confirmation activity (experiments 9 and 10)

Based on results from the screening activity, four lines were chosen for inclusion in an experiment to confirm differences in resistance to *R. solani* AG8. A different soil mix (§3.2.3) was used in this experiment as *B. distachyon* root systems grew better and were more easily cleaned for scanning with this mix. Nodal root number and type were also more easily counted from scans of roots grown in this mix. Crucially, the pathogenicity of *R. solani* AG8 was not reduced, even though less organic material was present in the soil.

Experiments were designed to allow a subset of plants to be harvested for qPCR analysis of *R. solani*-infested roots, with a second subset harvested for total root length and leaf area measurements. Leaf number and fresh weights of roots and shoots were measured for all plants. Quantitative RT-PCR analysis could not be achieved for these samples (§2.3.2.2).

Two experiments were sown on different days. Eight replicates of each *Host**Control treatment and 16 replicates of each *Host***R. solani* treatment were sown in randomly allocated pots in each experiment. An example of the tray layout of one experiment is shown in Figure 3.2.

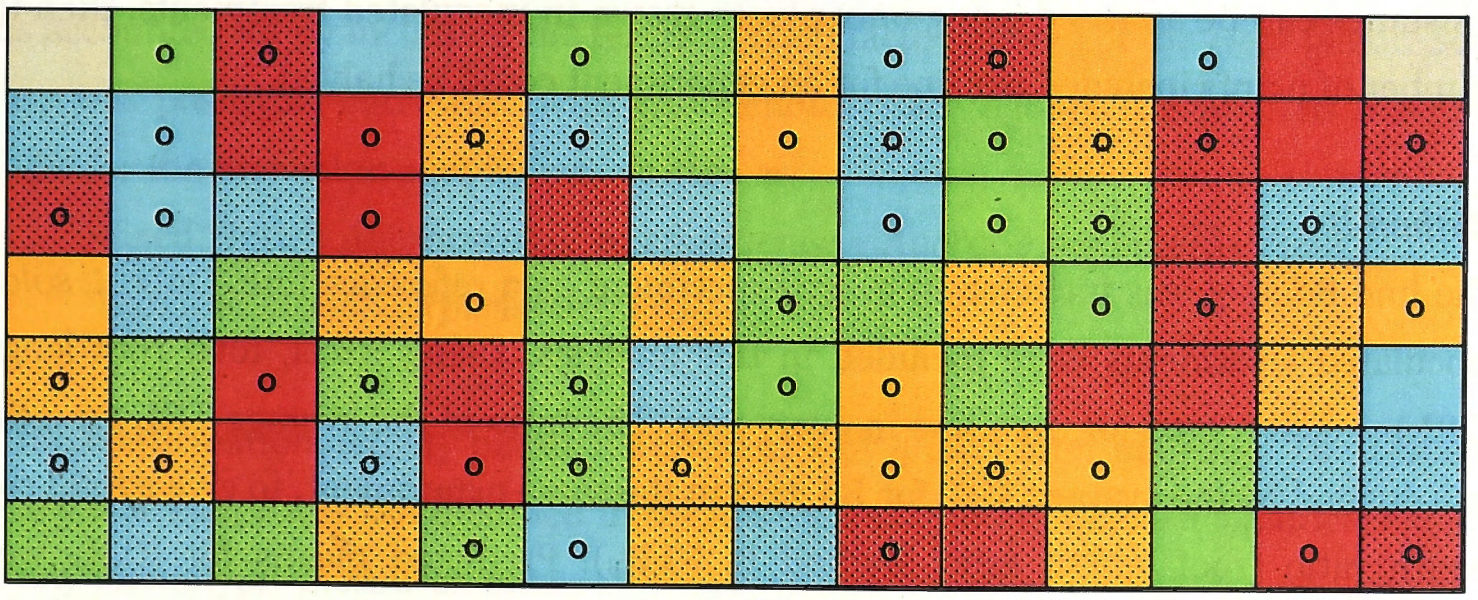

Figure 3.2 Arrangement of cones in one confirmation experiment. Each tray holds 7 x 14 cones. Four colours represent the random allocation of four genotypes. Patterned squares represent cones with *R. solani* inoculum. Root and shoot weight measurements were taken for all samples. Total root length and leaf area measurements were only measured for squares marked with '**o**'.

3.2.9 Statistical analysis

3.2.9.1 Methods for assessing resistance

Two methods were considered for ranking *B. distachyon* accessions with increasing resistance to *R. solani*.

Method A

A way to rank the resistance of different lines to *R. solani* is to compare the ability of infested plants to maintain root length similar to that of plants grown under control conditions, e.g. (Okubara *et al.*, 2009). This method compares ratios of *R. solani*/control measurements (root length ratio, RLR), with lines having higher ratios considered more disease resistant. In GenStat (VSN International, UK) total root length was square-root transformed before calculating predicted means for each line in control and *R. solani* treatment using a REML linear mixed model, as described in §3.2.9.2. The *Method A R. solani*/control square-root transformed total root length ratio ($\sqrt{RLR_A}$) was calculated in Excel (Microsoft), to give a resistance ranking for each line. Shoot measurements were not transformed prior to REML analysis.

An alternative strategy for assessing resistance to *R. solani* was considered, in which only the root growth values in *R. solani* inoculated treatments were used. If *R. solani* resistance can be assessed using only plants grown in inoculated treatments, then the number of plants included per line for an experiment could be halved.

Method B

The line Bd 21-3 was included in every experiment as a reference line. Only *R. solani* inoculated treatments were included in this analysis. Using Excel, total root length (TRL) was averaged for each line in every tray. The ratio of the average TRL for a line was divided by the average TRL for Bd 21-3 in the same experiment, to give a total root length ratio (root length ratio *Method* B, RLR_B). Predicted means for RLR_B were calculated for each line, using REML in GenStat to account for variation across experiments and trays within experiments. Using *Method B*, lines with higher RLR_B were considered more resistant than those with lower ratios. Root lengths were not square-root transformed for this analysis.

3.2.9.2 Statistical analysis

Plants were excluded from analysis if 1) they did not emerge from the soil surface within six DAP, or 2) were in cones that failed the toothpick re-isolation check (§3.2.6).

Root length data was skewed by the variation in growth due to growth chamber conditions, as well as the greater rate of increase for uninoculated root length than *R. solani* affected roots. Data analysis for total root length using *Method A* (§3.2.9.1) was carried out on square root transformed measurements, as residual plots showed an improved distribution for the normalized values (Figure 3.3). Shoot measurements did not require transformation prior to analysis (data not shown). Predicted means for phenotypic measurements were calculated using a REML linear mixed model in GenStat.

In the screening activity the number of days to plant emergence (3 – 6 DAP) was found to have a small but significant negative effect on phenotypic measurements. To account for the variation due to *Days to emergence* this factor was included in the fixed model (Dr Alec Zwart, pers. comm., 31 July 2013). Thus, in GenStat the REML model (*Method A*) for the Screening activity was written as

Fixed Model: *Days to emergence* + *Host*Inoculum*

Random Model: *Experiment/Tray*

The *Host*Inoculum* interaction for pairs of accessions was calculated by including only the data for those accessions in the analysis.

In analysis *Method B* the variation due to days to emergence and average seed mass was accounted for by including the terms as fixed factors ahead of the *Host* factor. Seed mass was positively correlated with endogenous plant vigour. The *Method B* REML model for the Screening activity was written as

Fixed Model: *Average days to emergence* + *Average seed mass*+ *Host*

Random Model: *Experiment/Tray*

In the confirmation activity plants emerged at 3 or 4 days after planting. The *Days to emergence* factor was not included in the analysis as it did not have a significant effect on any phenotypic measurement.

The REML model for the confirmation activity was written as

Fixed Model: *Host*Inoculum*

Random Model: *Experiment*

Root/shoot biomass ratios were calculated by dividing either TRL by leaf area or root FW by shoot fresh FW for individual plants, prior to analysis in GenStat. Total root length was not transformed for biomass ratio calculations. Standard errors and least significant differences at 5% were calculated in GenStat.

The significance of average seed size as an explanatory variable for the predicted means of the screening activity was estimated using a simple linear regression analysis in GenStat.

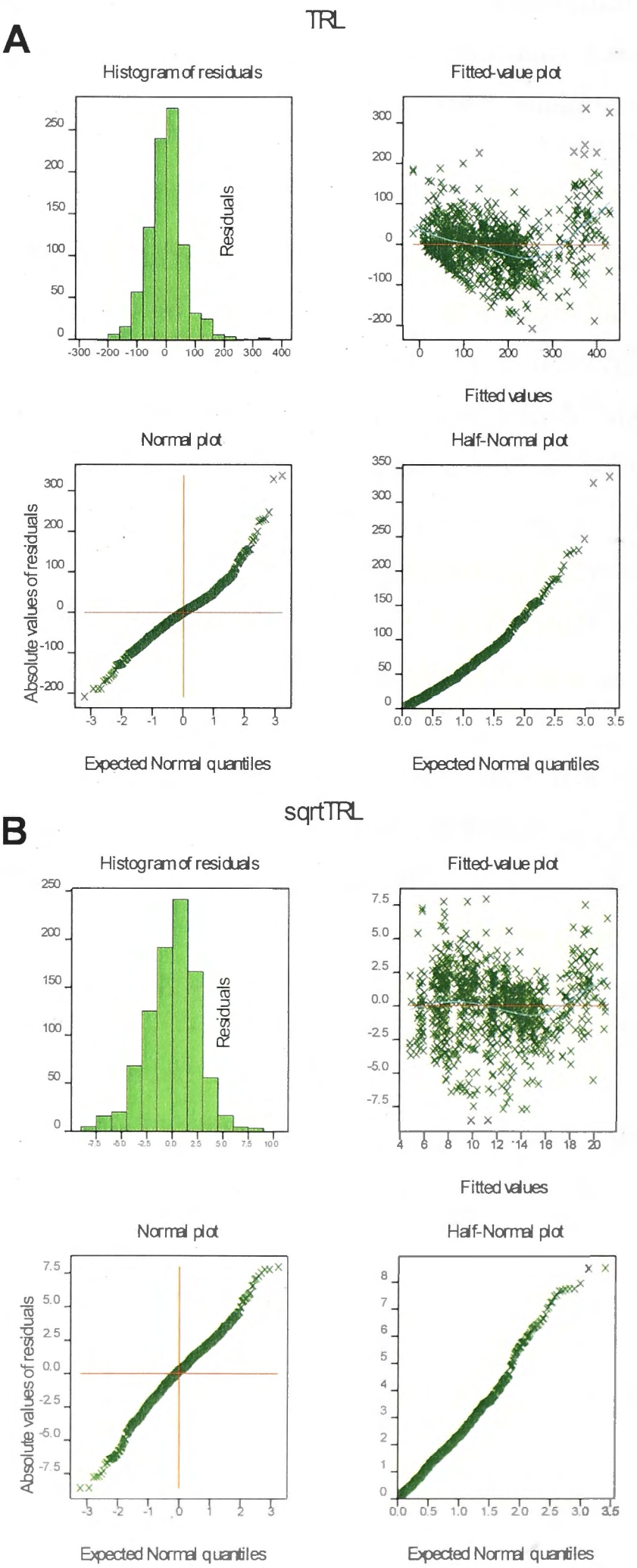

Figure 3.3 Residual plots for the 914 total root length (TRL) data points included in the natural accession screen analysis. Both control and *R. solani* inoculated treatments for the 26 accessions are included. Skewness in the fitted-value plot for untransformed TRL (a) is reduced by square root transforming the values before analysis (b).

3.3 Results

3.3.1 Screening activity

Variation in plant growth occurred due to growth conditions and genotype over the ten experiments conducted in the screening activity.

3.3.1.1 Spatiotemporal variation in plant growth

Experiments for the screening activity were conducted over a period of 18 weeks in two growth cabinets (Table 3.2). Although both cabinets were of the same design and set to the same program, a general increase in root growth was seen when experiments were conducted in the second cabinet. Mean total root length of control plants increased slightly in experiments 5 and 6, compared with the earlier experiments (Figure 3.4a). In experiments 7 and 8, root length of control plants was around 245% that of the first six experiments, while root length of the *R. solani* treatments increased to around 165% of the earlier experiments. It is also possible that unexpected variation between batches of potting mix was responsible for part of the difference in growth. Mean root lengths of individual lines in each experiment are presented in Figure 3.5.

Root growth variability due to growth conditions, particularly in the control treatment, was much greater than variability of any of the shoot measurements. Mean leaf number at harvest followed the same trend as total root length, increasing from 3.4 leaves at harvest in the first six experiments to 4.0 leaves in experiments 7 and 8 for control plants, and an increase from 3.2 to 3.6 leaves with *R. solani* (Figure 3.4b). Leaf lengths changed only slightly, with a 4% increase in leaf 1 length and a 3% decrease in leaf 2 length in the latter experiments (Figure 3.4c-d).

In addition to growth cabinet conditions, a batch of soil may have affected disease expression. Soil used in experiments 2 and 3 appeared to contain a higher than usual level of an unidentified fungus that grew at a similar rate to *R. solani* on selective medium. Unlike *R. solani*, the fungus sporulated and did not cause the media to brown. During the toothpick assay it was found that this fungus grew from toothpicks inserted into both control and *R. solani* inoculated cones. Only hyphae growing from toothpicks in *R. solani* inoculated cones caused the agar to brown, indicating that there was no *R. solani* contamination of control cones and that *R. solani* was growing in the inoculated cones. The *R. solani* to control ratios for total root length (RLR_A) and the three shoot measurements (Figure 3.4) were greater in experiments 2 and 3 than the remaining experiments.

Variations in growth cabinet and soil conditions were taken into account by including *Experiment* as a random factor during the analysis of *B. distachyon* genotype responses to *R. solani* AG8.

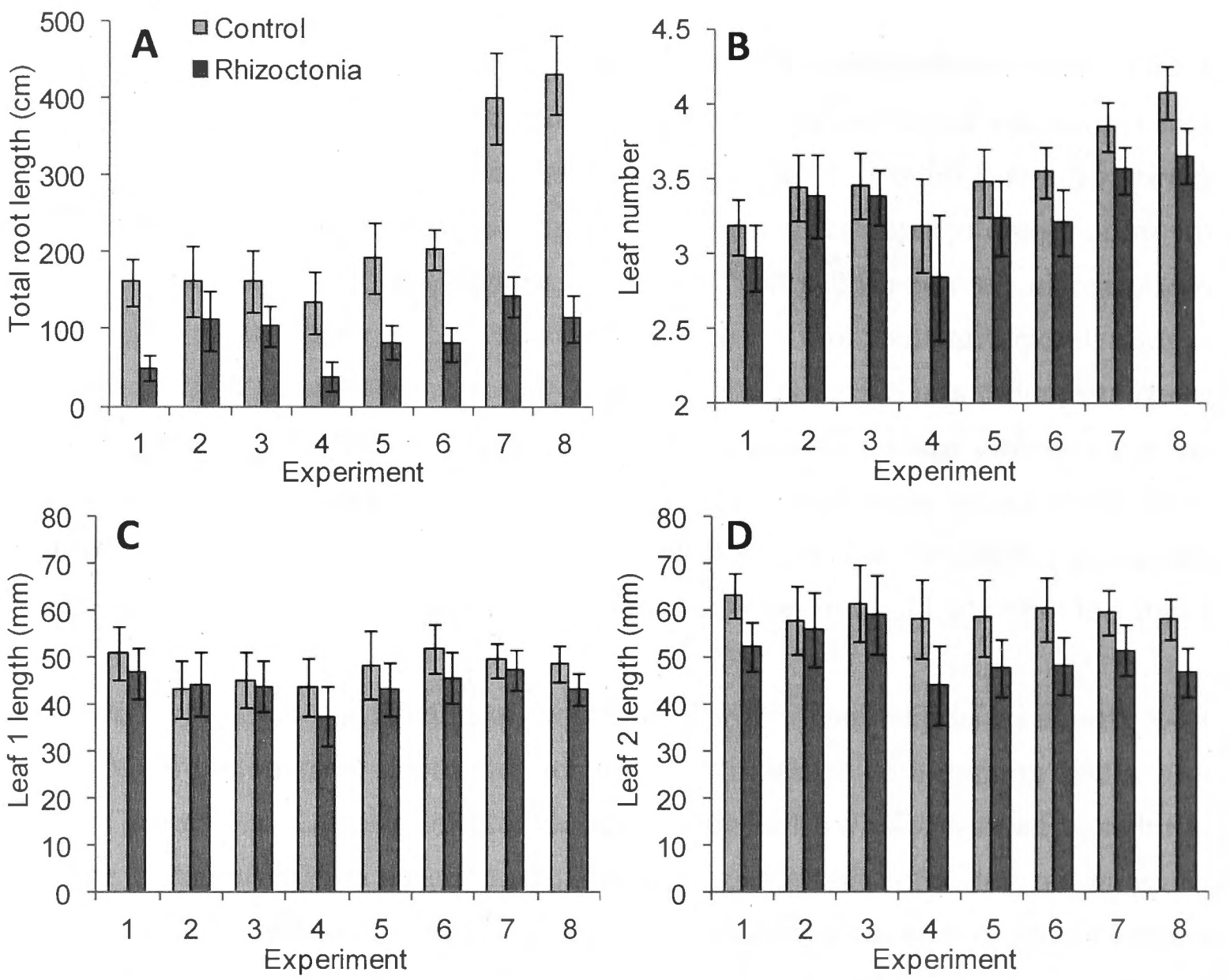

Figure 3.4 Overall variation between experiments for a) total root length, b) leaf 1 length, c) leaf 2 length, and d) leaf number. Each bar represents the mean of the means for n=11 lines in an experiment, except experiment 8 with n=6. Every n is the mean of up to 3 plants. Experiments 2 & 3, and 5 & 6 were sown on the same date. Experiments 7 and 8 were grown in a different growth cabinet. Control treatment (light bars), *R. solani*-inoculated treatment (dark bars); SD.

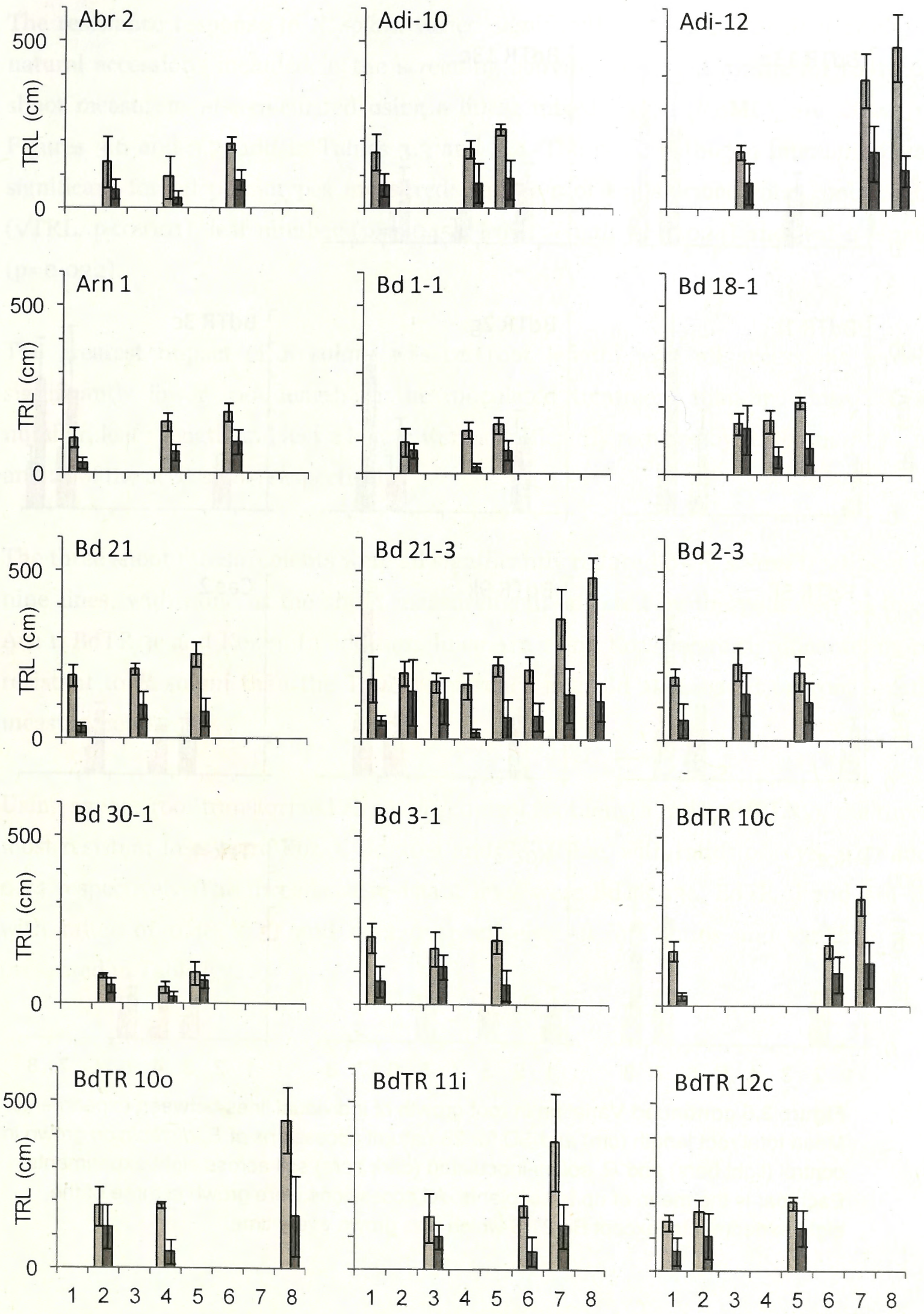

Figure 3.5 Variation in root growth of individual lines between experiments. Mean total root length (cm) and SD for 26 natural accessions of *B. distachyon* grown in control (light bars) and *R. solani*-inoculated (dark bars) soil across eight experiments. Each bar is the mean of up to six plants. All accessions were grown in three of the eight experiments, except Bd 21-3 which was grown every time.

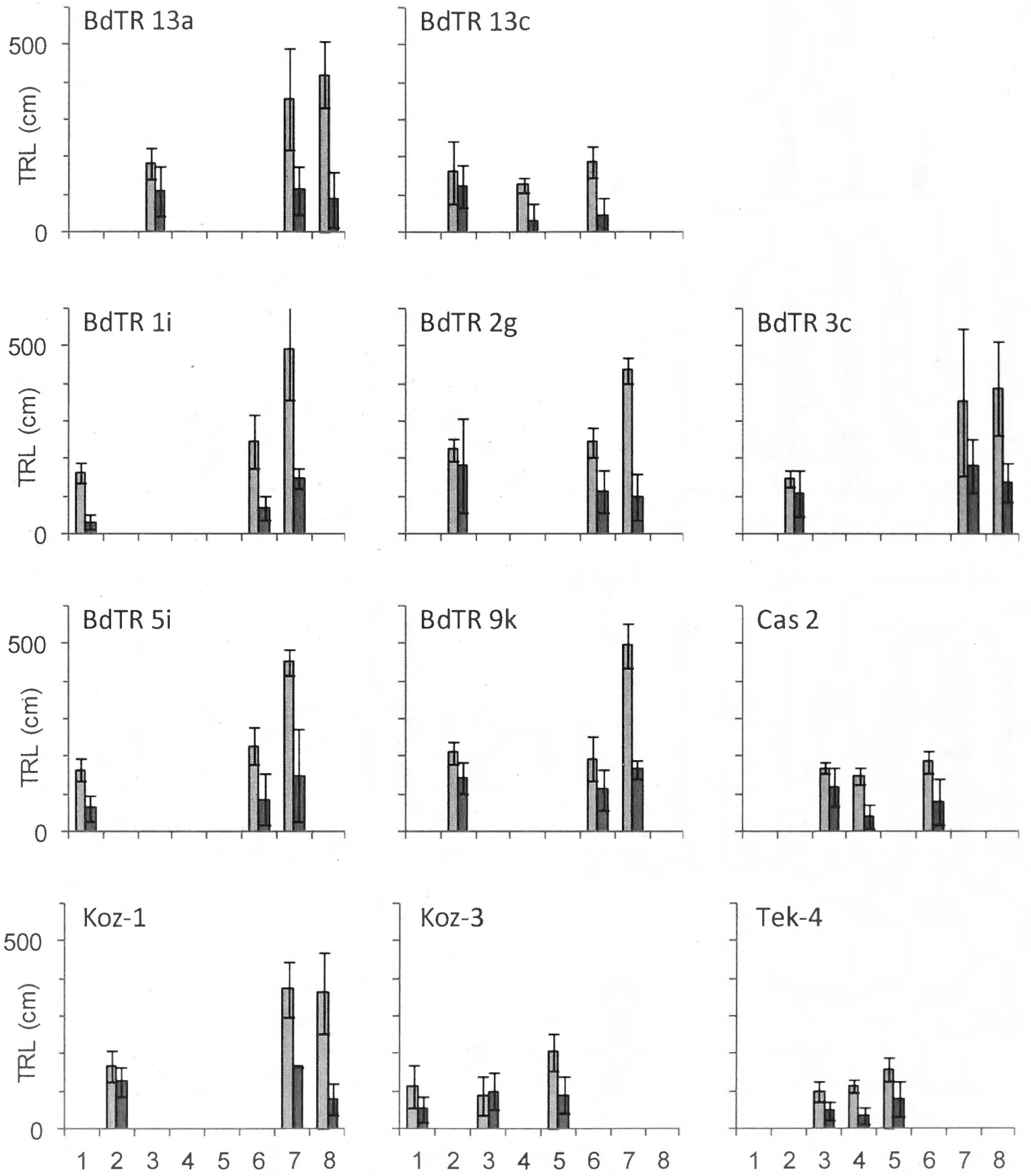

Figure 3.5 continued Variation in root growth of individual lines between experiments. Mean total root length (cm) and SD for 26 natural accessions of *B. distachyon* grown in control (light bars) and *R. solani*-inoculated (dark bars) soil across eight experiments. Each bar is the mean of up to six plants. All accessions were grown in three of the eight experiments, except Bd 21-3 which was grown every time.

3.3.1.2 Variation in resistance across accessions

The resistance response to *R. solani* varied significantly across the 26 *B. distachyon* natural accessions included in the screening activity. Predicted means for root and shoot measurements, calculated using a linear mixed model (REML), are shown in Figures 3.6 and 3.7, and in Tables 3.3 and 3.4. The plant-pathogen interaction was significant for all phenotypes measured: square-root transformed total root length ($\sqrt{TRL}$, $p<0.001$), leaf number ($p=0.045$), leaf 1 length ($p=0.024$) and leaf 2 length ($p=0.022$).

The greatest impact of *R. solani* was on root length, with all accessions having significantly lower root length in the inoculated treatment than in control. Leaf number, leaf 1 length and leaf 2 length were significantly reduced by *R. solani* in 20, 13 and 21 of the accessions, respectively.

The three shoot measurements were all significantly reduced by *R. solani* treatment in nine lines, with none of the shoot measurements affected by the pathogen in lines Arn 1, BdTR 3c and Koz-3. In addition, lines Arn 1 and Koz-3 were significantly more resistant to *R. solani* than the T-DNA reference line, Bd 21-3, based on root length measurements.

Using square-root transformed *R. solani*/control root length ratios ($\sqrt{RLR_A}$), the three most resistant lines were Koz-3, Bd 30-1 and BdTR 12c, with ratios of 0.78, 0.77 and 0.74 respectively. The three least resistant lines were BdTR 13a, BdTR 1i and Adi-12 with ratios of 0.48, 0.51 and 0.52, respectively. All line ratios and rankings are presented in Table 3.5.

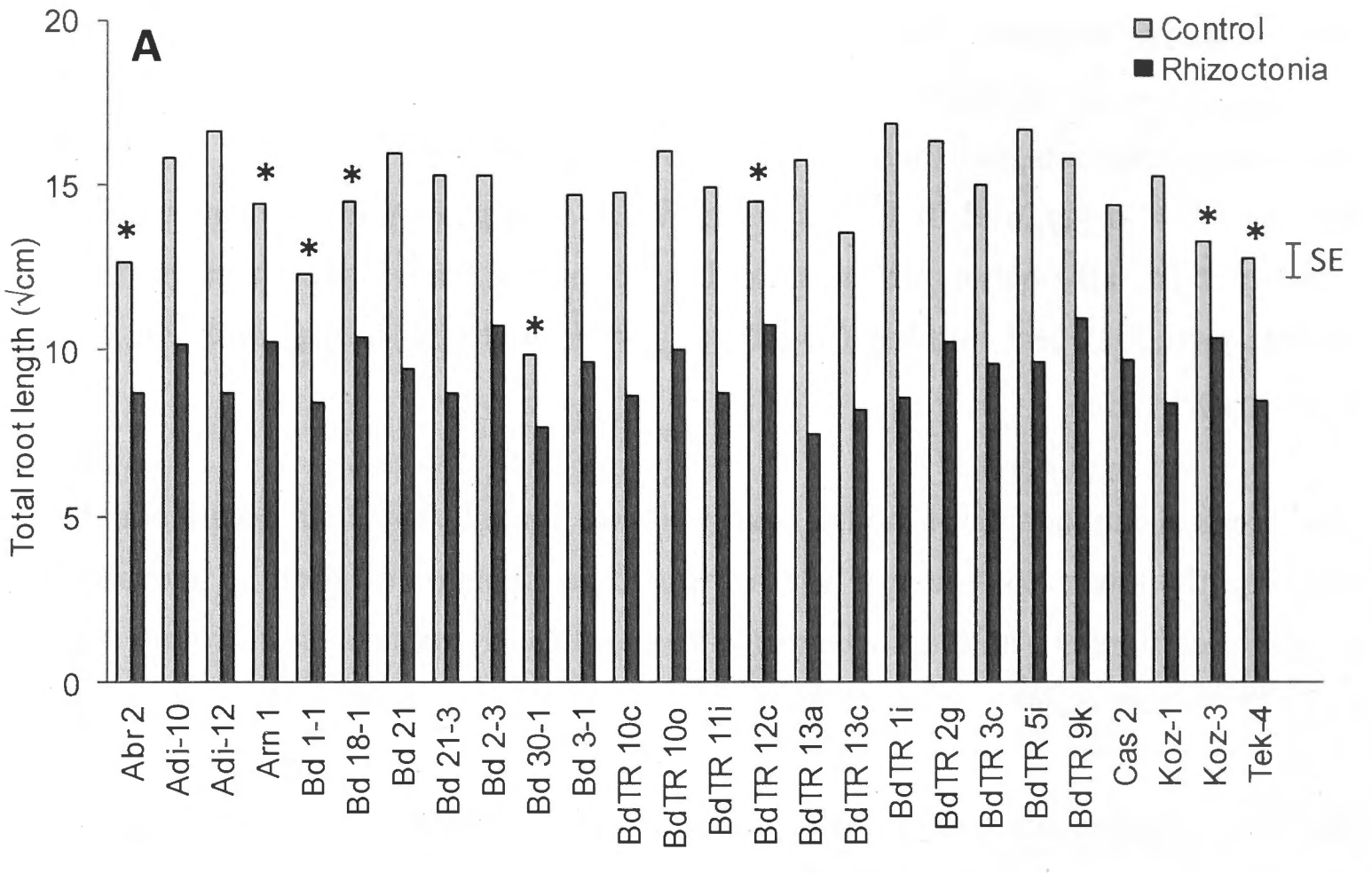

Figure 3.6 Screening activity phenotype measurements. Predicted means for a) total root length (square-root transformed prior to analysis), and b) leaf number, for 26 accessions, each included in three experiments. Values are presented in Table 3.3; n≤18, except Bd 21-3 (n≤47); average SE.
*Genotype response to *R. solani* is significantly different ($p<0.05$) from Bd 21-3, the reference line used in Chapter 4 T-DNA experiments, for total root length.
NS, no significant difference between *R. solani* and control treatments at 5% LSD.

Table 3.3 Means of total root length and leaf number at harvest for 26 accessions, each included in three experiments; n≤18, except Bd 21-3 (n≤47).

Line	Total root length (√cm)[1]				Leaf number[1]			
	Control		*R. solani*		Control		*R. solani*	
Abr 2	12.69	hij	8.70	mnopqrs	3.13	tuv	2.92	wx
Adi-10	15.82	abcde	10.15	lmnopq	3.74	bc	3.51	defghijk[2]
Adi-12	16.60	abc	8.69	mnopqrs	3.64	cdefg	3.43	ijklmno
Arn 1	14.44	efgh	10.23	lmnop	3.66	bcdefg	3.48	fghijklm
Bd 1-1	12.33	ijk	8.45	opqrs	3.33	lmnoqpr	3.12	uv
Bd 18-1	14.51	efgh	10.37	lm	3.84	ab	3.63	cdefgh
Bd 21	15.94	abcde	9.46	lmnopqr	3.45	hijklmn	3.25	opqrstuv
Bd 21-3	**15.33**	**bcde**	**8.70**	**pqrs**	**3.55**	**efghi**	**3.26**	**pqrstu**
Bd 2-3	15.27	abcdef	10.76	kl	3.52	efghijk	3.32	mnopqrstu
Bd 30-1	9.91	lmnopq	7.68	rs	3.11	uv	2.82	x
Bd 3-1	14.71	defg	9.63	lmnopq	3.71	bcd	3.56	cdefghi[3]
BdTR 10c	14.81	cdefg	8.61	mnopqrs	3.59	cdefgh	3.25	opqrstuv
BdTR 10o	16.05	abcde	10.05	lmnopq	3.67	bcdef	3.36	jklmnopqr
BdTR 11i	14.90	bcdefg	8.72	mnopqrs	3.53	defghij	3.15	stuv
BdTR 12c	14.52	efgh	10.73	kl	3.49	fghijklm	3.59	cdefghi
BdTR 13a	15.70	abcde	7.47	s	3.94	a	3.52	efghijk
BdTR 13c	13.56	fghi	8.23	qrs	3.73	bc	3.39	jklmnop
BdTR 1i	16.84	a	8.54	mnopqrs	3.69	bcde	3.28	nopqrstu
BdTR 2g	16.31	abcd	10.24	lmno	3.74	bc	3.51	efghijkl
BdTR 3c	15.01	bcdefg	9.58	lmnopq	3.52	efghijk	3.38	jklmnopq
BdTR 5i	16.70	ab	9.64	lmnopq	3.33	lmnopqrs	3.08	vw
BdTR 9k	15.81	abcde	10.98	jkl	3.53	defghij	3.35	klmnopqr
Cas 2	14.40	efgh	9.76	lmnopq	3.38	jklmnopq	3.16	rstuv
Koz-1	15.31	abcdef	8.38	mnopqrs	3.61	cdefgh	3.30	mnopqrstu
Koz-3	13.29	ghi	10.36	lmn	3.53	defghij[4]	3.47	ghijklmn
Tek-4	12.81	hi	8.50	nopqrs	3.20	qrstuv	3.11	uv

[1]Numbers followed by the same letter are not significantly different at 5% LSD
[2]Adi-10 (*R. solani*) is not significantly different from Koz-1 (*R. solani*)
[3]Bd 3-1 (*R. solani*) is not significantly different from Cas 2 (control), BdTR 3c (*R. solani*) and BdTR 13c (*R. solani*)
[4]Koz-3 (control) is not significantly different from BdTR 9k (*R. solani*)
[5]BdTR 13c (*R. solani*) is not significantly different from BdTR 10c (control)

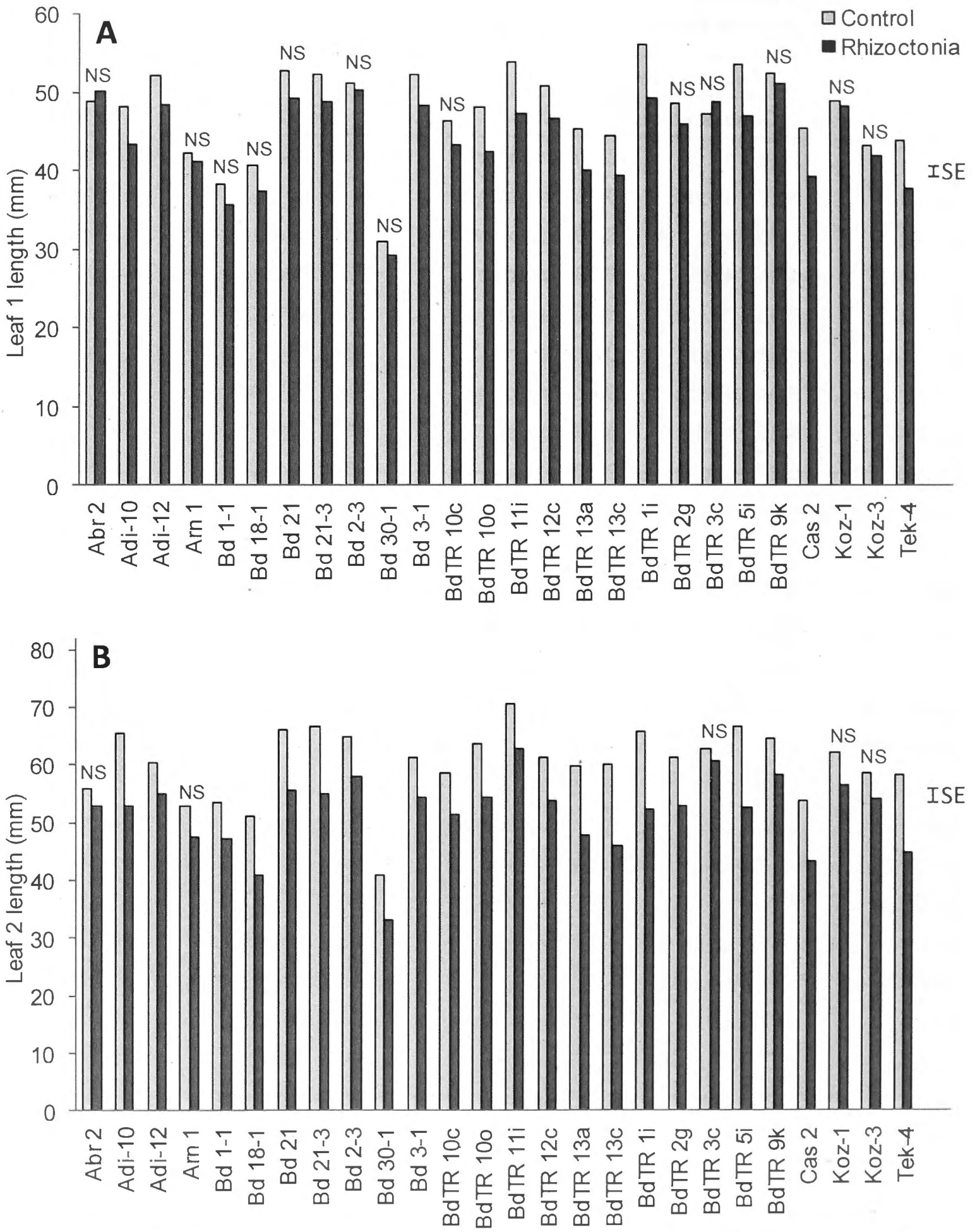

Figure 3.7 Screening activity phenotype measurements. Predicted means for a) leaf 1 length, and b) leaf 2 length, for 26 accessions, each included in three experiments. Values are presented in Table 3.4; n≤18, except Bd 21-3 (n≤47); average SE.
NS, no significant difference between *R. solani* and control treatments at 5% LSD.

Table 3.4 Means of leaf 1 and leaf 2 lengths for 26 accessions, each included in three experiments; n≤18, except Bd 21-3 (n≤47).

Line	Leaf 1 length (mm)[1]		Leaf 2 length (mm)[1]	
	Control	*R. solani*	Control	*R. solani*
Abr 2	48.9 defghijklm	50.1 bcdefghij	55.8 ijklmno[4]	52.9 lmnop
Adi-10	48.3 ghijklm	43.2 opqrstu	65.5 abcd	52.9 lmnopq
Adi-12	52.2 bcdef	48.4 ghijklm	60.6 defghi	55.0 jklmno
Arn 1	42.2 qrstu	41.1 rstuv	52.9 mnopq	47.4 pqrs
Bd 1-1	38.2 vw	35.7 w	53.7 lmno	47.4 qrs
Bd 18-1	40.6 stuv	37.4 vw	51.0 opqr	40.8 t
Bd 21	52.8 abc	49.4 cdefghijk	66.0 abc	55.5 ijklmno
Bd 21-3	**52.3 bcde**[2]	**48.8 ghijklm**[3]	**66.8 ab**	**55.0 lmno**
Bd 2-3	51.3 bcdefg	50.4 bcdefghi	65.0 abcde	57.9 ghijklmn
Bd 30-1	31.0 x	29.3 x	40.8 t	33.2 u
Bd 3-1	52.4 bcde	48.5 ghijklm	61.4 cdefgh	54.3 lmno
BdTR 10c	46.4 jklmno	43.3 opqrst	58.6 fghijkl[5]	51.4 opqr
BdTR 10o	48.1 ghijklm	42.4 pqrstu	63.8 bcdef	54.4 klmno
BdTR 11i	53.9 ab	47.3 hijklmn	70.6 a	62.7 bcdefg
BdTR 12c	50.8 bcdefgh	46.5 jklmno	61.4 cdefgh	54.0 lmno
BdTR 13a	45.4 mnopq	40.1 tuv	60.0 efghijk	47.8 pqrs
BdTR 13c	44.4 nopqr	39.3 uvw	60.2 defghij	46.0 rst
BdTR 1i	56.1 a	49.3 cdefghijk	65.9 abc	52.5 noqp
BdTR 2g	48.5 ghijklm	46.0 klmnop	61.3 cdefgh	53.1 lmnop
BdTR 3c	47.3 hijklmn	48.9 defghijklm	62.8 bcdefg	60.6 cdefghi
BdTR 5i	53.8 ab	47.0 ijklmno	66.9 ab[6]	52.8 mnopq
BdTR 9k	52.5 bcd	51.3 bcdefg	64.5 bcde	58.3 ghijklm[7]
Cas 2	45.6 lmnopq	39.4 uv	53.8 lmno	43.2 st
Koz-1	48.9 efghijkl	48.4 fghijklm	62.3 cdefgh	56.6 hijklmno
Koz-3	43.3 opqrst	42.0 qrstu	58.7 fghijkl	54.3 lmno
Tek-4	44.0 nopqrs	37.8 vw	58.4 ghijklm	44.7 st

[1]Numbers followed by the same letter are not significantly different at 5% LSD.
[2]Bd 21-3 (control) is significantly different from Abr 2 (control), BdTR 3c (*R. solani*) and Koz-1 (control).
[3]Bd 21-3 (*R. solani*) is significantly different from BdTR 13a (control) and Cas 2 (control).
[4]Abr 2 (control) is not significantly different from Bd 3-1 (control).
[5]BdTR 10c (control) is significantly different from Abr 2 (*R. solani*).
[6]BdTR 5i (control) is not significantly different from Koz-1 (control).
[7]BdTR 9k (*R. solani*) is significantly different from Abr 2 (*R. solani*) and BdTR 5i (*R. solani*).

3.3.1.3 The effect of endogenous vigour on disease resistance

Seed and embryo size is linked with vigorous early shoot growth (Richards and Lukacs, 2002). With a large range of seed weights across the genotypes in this data set, the effect of seed size was able to be measured. Using a simple linear regression, increasing seed mass was found to correlate significantly with greater total root length and leaf number in control treatments, but not in the *R. solani* treatment (Figure 3.8). An influence of seed size was seen on predicted means for leaf lengths in both treatments.

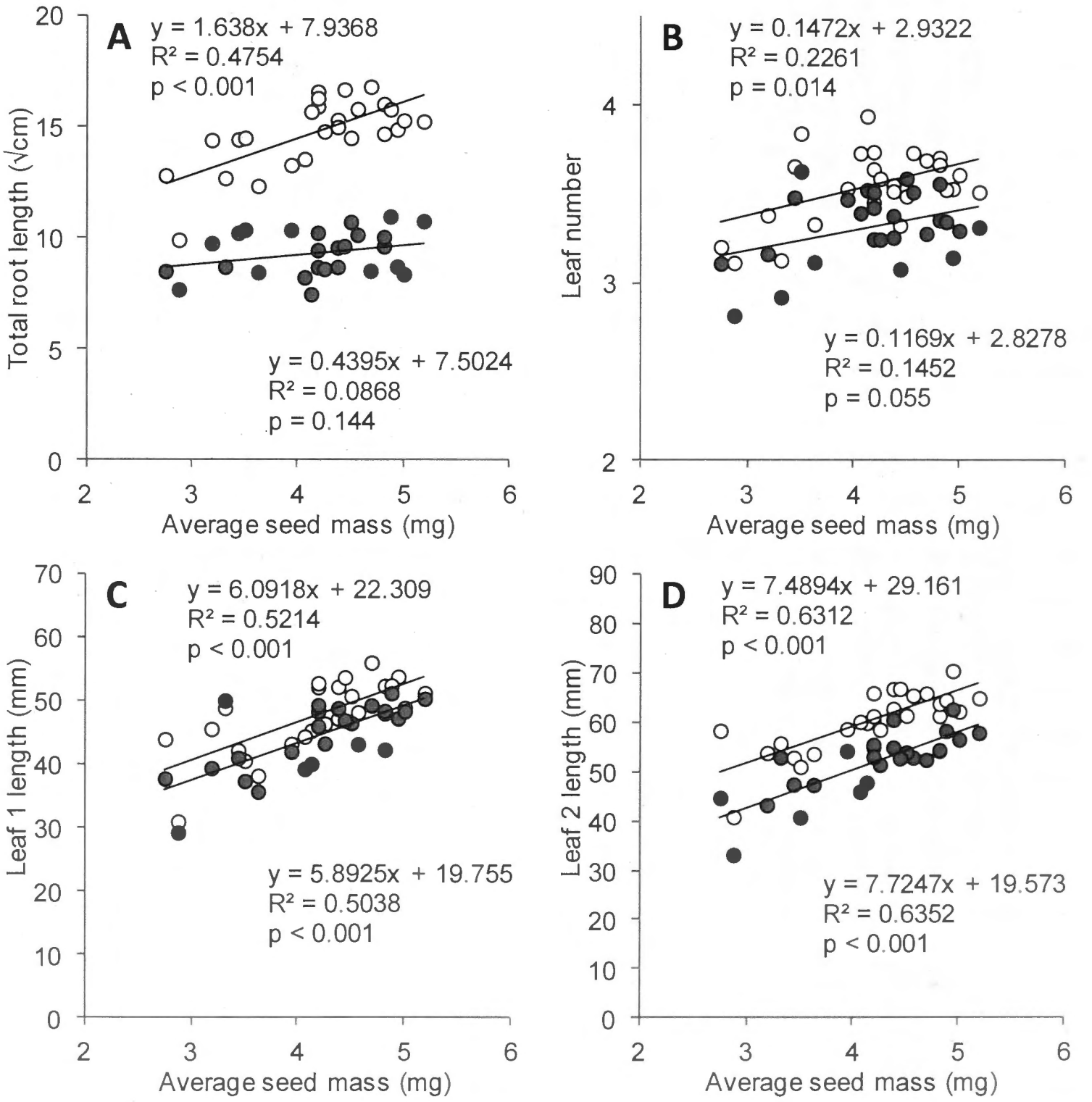

Figure 3.8 Correlations of root and shoot measurements with seed mass. Predicted means for a) total root length, b) leaf number, c) leaf 1 length, and d) leaf 2 length, plotted against average seed mass for 26 accessions grown in control (white circles) and *R. solani* infested soil (grey circles).

Germination rate is another indicator of seedling vigour (Maguire, 1962). The increase in early endogenous root and shoot growth was reflected in a small but significant ($p<0.001$) correlation between seed size and days to emergence, with a decrease of 0.3 days to emergence for every extra milligram of seed mass.

There was a small negative correlation between seed size and square-root transformed *R. solani*/control root length ratios ($\sqrt{RLR_A}$, $p=0.046$, Figure 3.9), but not with any shoot ratios, as is evident from the parallel lines of best fit in Figures 3.8b-d. Likewise, there was a negative correlation between root length in control treatment and $\sqrt{RLR_A}$ ($p<0.001$), but not between endogenous shoot growth measurements and their corresponding *R. solani*/control ratios.

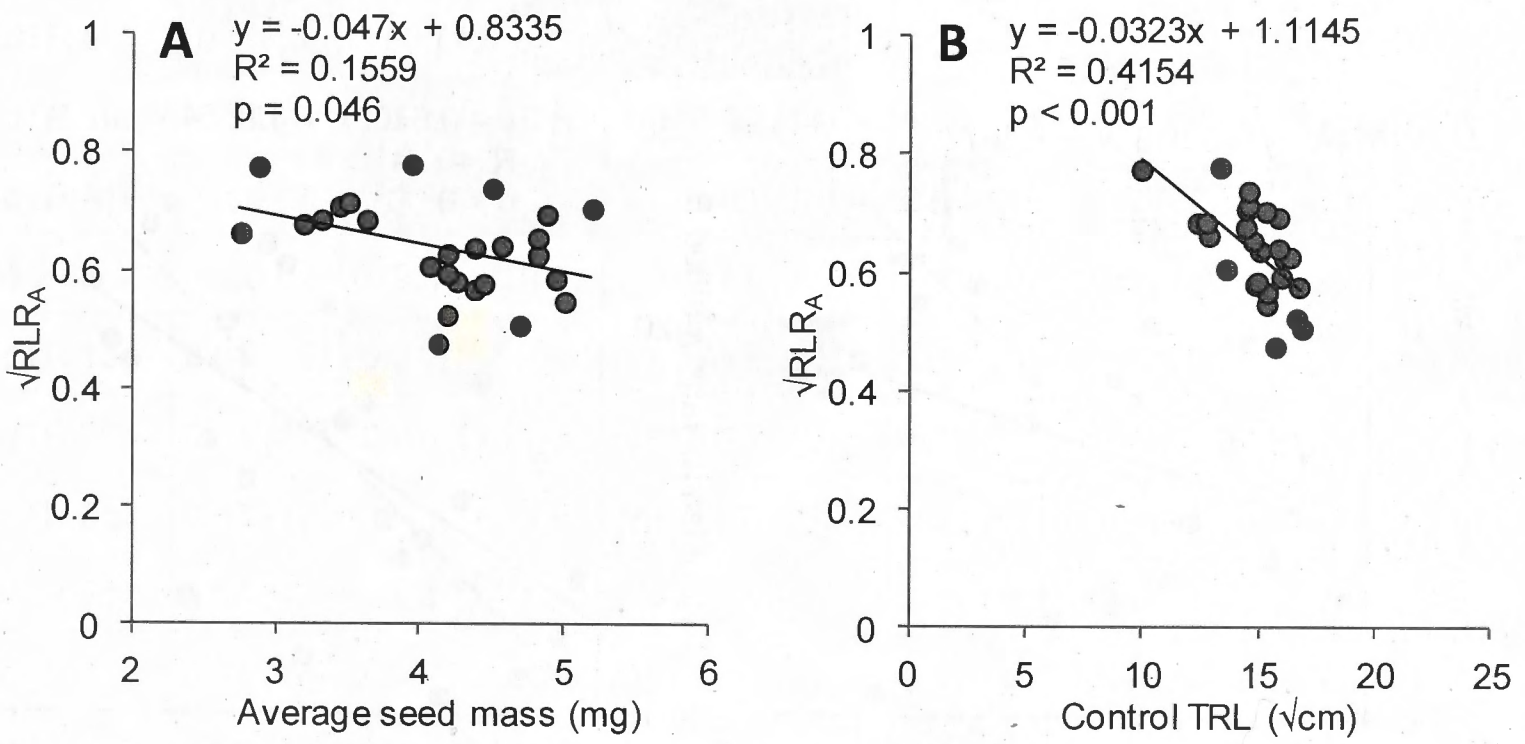

Figure 3.9 Correlations with *Method A* square-root transformed *R. solani*/control root length ratios ($\sqrt{RLR_A}$) using two different measures of endogenous seedling vigour: a) average seed mass, and b) predicted square-root transformed total root length in control treatment.

3.3.1.4 An alternative resistance ranking method using only *R. solani* treatment

The relative resistance of different accessions to *R. solani* was calculated using *Method A*, described in §3.2.9.1, with rankings based on ratios of transformed *R. solani*/control means ($\sqrt{RLR_A}$). *Method B*, a second ranking method using ratios of line mean/Bd 21-3 mean for *R. solani* treatment only (RLR_B), was also evaluated. The aim was to be able to use this alternative method to rank resistance of T-DNA lines in Chapter 4 screening experiments. *Method B* does not require plants to be grown in a control treatment, so it potentially increases the number of lines that can be screened.

The strong correlation between seed mass and endogenous root growth (Figure 3.8a, $p<0.001$) can be used to improve correlations between root length resistance ranking *Method A* and *Method B* (Figure 3.10).

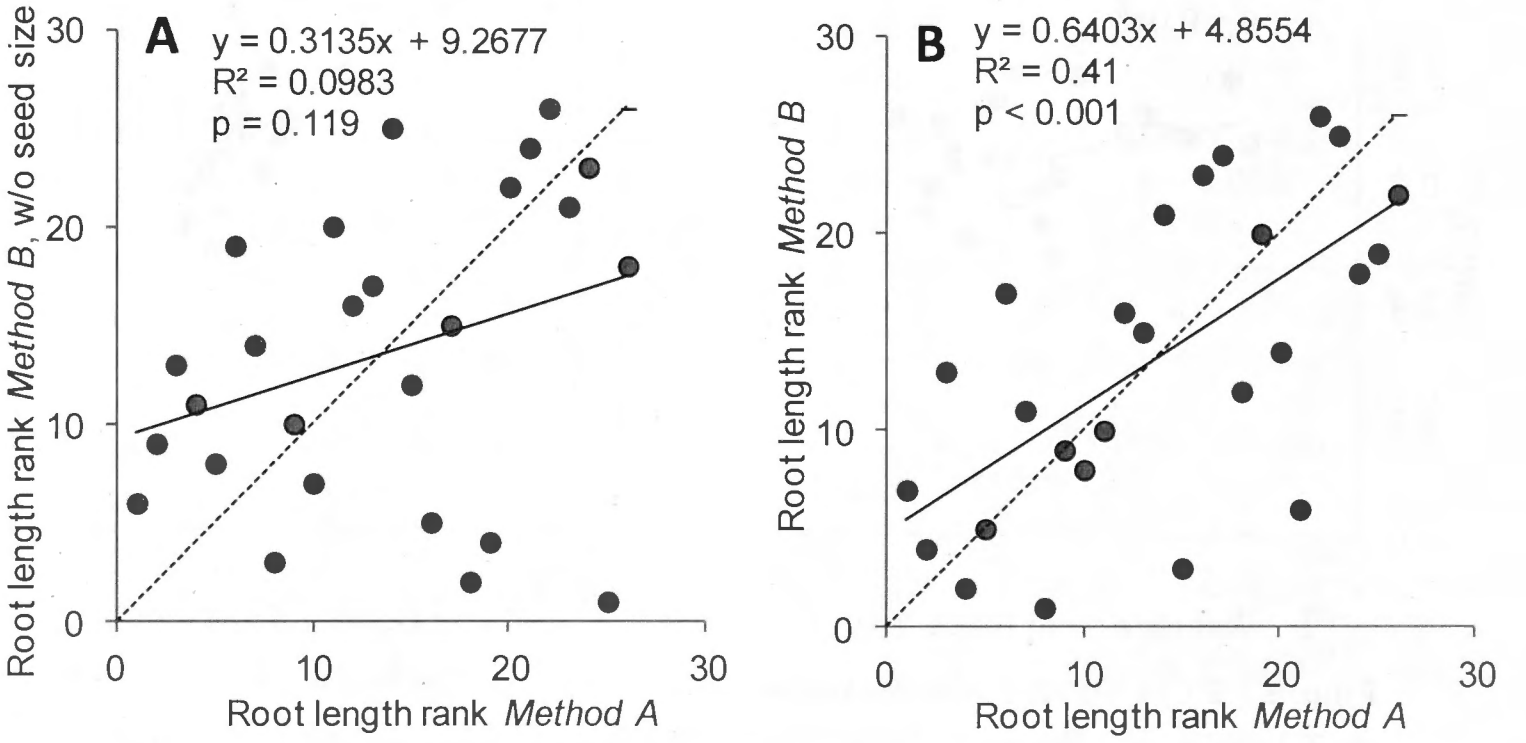

Figure 3.10 Correlation between resistance rankings based on total root length using *Method A* and *Method B* improved from a) to b) when seed size was included in the *Method B* analysis; rankings are from 1, least resistant, to 26, most resistant; dotted line, y=x.

Using simple linear regression analysis of resistance ranks in Table 3.5, with *Method A* root length was positively correlated with leaf number ($p<0.001$) and leaf 1 length positively correlated with leaf 2 length ($p<0.001$). Using *Method B* these same two pairs of resistance ranks were also positively correlated ($p<0.001$).

The *Method B* resistance ranking using root length (RLR_B) gives the three most resistant lines as Arn 1, Bd 18-1 and Cas 2, with ratios of 1.99, 1.76 and 1.69, respectively. The three least resistant lines with this method were BdTR 11i, Koz-1 and Bd 3-1, with ratios of 0.47, 0.59 and 0.71, respectively (Table 3.5).

Table 3.5 Summary of *R. solani*/control ratio rankings with different methods to measure resistance to *R. solani*. Rankings 1 to 26 (in parentheses) are from least to most resistant; green, more resistant; orange, less resistant. $\sqrt{RLR_A}$, *Method A* root length ratio; RLR_B, *Method B* root length ratio; L#, leaf number; L1, leaf 1 length; L2, leaf 2 length.

Line	*Method A* - ratios of *R. solani*/control treatment from linear mixed model predicted means; (*rank*)				*Method B* – ratios of *R. solani* treatment line mean/Bd 21-3 mean from linear mixed model predicted means; (*rank*)			
	$\sqrt{RLR_A}$	L#	L1	L2	RLR_B	L#	L1	L2
BdTR 13a	0.48 *(1)*	0.89 *(3)*	0.88 *(7)*	0.80 *(5)*	0.96 *(7)*	1.07 *(20)*	0.79 *(7)*	0.83 *(4)*
BdTR 1i	0.51 *(2)*	0.89 *(1)*	0.88 *(5)*	0.80 *(4)*	0.71 *(4)*	0.91 *(6)*	1.06 *(23)*	0.96 *(17)*
Adi-12	0.52 *(3)*	0.94 *(16)*	0.93 *(13)*	0.91 *(22)*	1.05 *(13)*	1.03 *(15)*	0.99 *(17)*	0.95 *(14)*
Koz-1	0.55 *(4)*	0.91 *(7)*	0.99 *(24)*	0.91 *(23)*	0.59 *(2)*	0.86 *(3)*	1.07 *(24)*	0.95 *(13)*
Bd 21-3	**0.57 *(5)***	**0.92 *(9)***	**0.93 *(15)***	**0.82 *(10)***	**0.83 *(5)***	**0.96 *(8)***	**1.01 *(19)***	**0.97 *(19)***
BdTR 5i	0.58 *(6)*	0.93 *(10)*	0.87 *(3)*	0.79 *(3)*	1.21 *(17)*	0.89 *(5)*	0.98 *(15)*	0.96 *(15)*
BdTR 10c	0.58 *(7)*	0.90 *(4)*	0.93 *(14)*	0.88 *(14)*	1.02 *(11)*	0.97 *(9)*	0.87 *(10)*	0.94 *(12)*
BdTR 11i	0.59 *(8)*	0.89 *(2)*	0.88 *(4)*	0.89 *(18)*	0.47 *(1)*	0.81 *(1)*	1.03 *(22)*	1.08 *(26)*
Bd 21	0.59 *(9)*	0.94 *(15)*	0.93 *(17)*	0.84 *(11)*	1.01 *(9)*	0.98 *(10)*	1.01 *(20)*	0.99 *(22)*
BdTR 13c	0.61 *(10)*	0.91 *(6)*	0.89 *(8)*	0.76 *(1)*	0.99 *(8)*	1.06 *(19)*	0.81 *(8)*	0.85 *(5)*
BdTR 10o	0.63 *(11)*	0.91 *(8)*	0.88 *(6)*	0.85 *(12)*	1.02 *(10)*	0.93 *(7)*	0.91 *(11)*	0.92 *(10)*
BdTR 2g	0.63 *(12)*	0.94 *(14)*	0.95 *(19)*	0.87 *(13)*	1.20 *(16)*	1.05 *(18)*	0.91 *(12)*	0.91 *(9)*
BdTR 3c	0.64 *(13)*	0.96 *(23)*	1.03 *(26)*	0.96 *(26)*	1.11 *(15)*	0.99 *(11)*	1.01 *(18)*	1.05 *(25)*
Adi-10	0.64 *(14)*	0.94 *(17)*	0.90 *(9)*	0.81 *(8)*	1.34 *(21)*	1.03 *(14)*	0.94 *(14)*	0.98 *(21)*
Bd 3-1	0.65 *(15)*	0.96 *(22)*	0.92 *(12)*	0.88 *(17)*	0.71 *(3)*	0.99 *(12)*	1.02 *(21)*	0.93 *(11)*
Tek-4	0.66 *(16)*	0.97 *(24)*	0.86 *(1)*	0.77 *(2)*	1.65 *(23)*	1.20 *(25)*	0.65 *(2)*	0.86 *(6)*
Cas 2	0.68 *(17)*	0.94 *(12)*	0.87 *(2)*	0.80 *(7)*	1.69 *(24)*	1.13 *(23)*	0.71 *(5)*	0.81 *(3)*
Bd 1-1	0.69 *(18)*	0.94 *(13)*	0.93 *(16)*	0.88 *(16)*	1.04 *(12)*	1.05 *(17)*	0.68 *(3)*	0.87 *(7)*
Abr 2	0.69 *(19)*	0.93 *(11)*	1.02 *(25)*	0.95 *(25)*	1.34 *(20)*	1.03 *(13)*	0.94 *(13)*	0.98 *(20)*
BdTR 9k	0.69 *(20)*	0.95 *(20)*	0.98 *(22)*	0.90 *(21)*	1.05 *(14)*	0.88 *(4)*	1.10 *(25)*	1.00 *(23)*
Bd 2-3	0.70 *(21)*	0.94 *(18)*	0.98 *(23)*	0.89 *(19)*	0.94 *(6)*	0.83 *(2)*	1.13 *(26)*	1.01 *(24)*
Arn 1	0.71 *(22)*	0.95 *(21)*	0.97 *(21)*	0.90 *(20)*	1.99 *(26)*	1.20 *(24)*	0.79 *(6)*	0.91 *(8)*
Bd 18-1	0.71 *(23)*	0.94 *(19)*	0.92 *(11)*	0.80 *(6)*	1.76 *(25)*	1.24 *(26)*	0.69 *(4)*	0.73 *(2)*
BdTR 12c	0.74 *(24)*	1.03 *(26)*	0.92 *(10)*	0.88 *(15)*	1.27 *(18)*	1.04 *(16)*	0.98 *(16)*	0.96 *(16)*
Bd 30-1	0.77 *(25)*	0.90 *(5)*	0.94 *(18)*	0.81 *(9)*	1.28 *(19)*	1.08 *(21)*	0.47 *(1)*	0.63 *(1)*
Koz-3	0.78 *(26)*	0.98 *(25)*	0.97 *(20)*	0.93 *(24)*	1.40 *(22)*	1.10 *(22)*	0.84 *(9)*	0.97 *(18)*

3.3.2 Confirmation activity

Four lines diverging for resistance to *R. solani* AG8 in the screening activity were chosen for inclusion in a confirmation activity: lines Adi-10, Bd 30-1, BdTR 13a and Koz-3. In the earlier screen, using *Method A*, lines Koz-3 and Bd 30-1 had the greatest *R. solani*/control root length ratio ($\sqrt{RLR_A}$), while BdTR 13a had the lowest ratio. All measurements for Bd 30-1, except total root length in *R. solani*, were the lowest of all the lines tested. Line Adi-10 was chosen for its vigorous growth in the control treatment, like BdTR 13a, but with root length in *R. solani* treatment similar to Koz-3.

The confirmation activity was designed to test whether resistance rankings would hold in a different soil mix, a different growth cabinet and a slightly longer growth period (26 days). All plants emerged at 3 – 4 days after planting. Days to emergence did not have a significant impact on phenotype measurements and was thus not included as a term in the linear mixed model analysis. Total root length and leaf number measurements are shown in Figure 3.11.

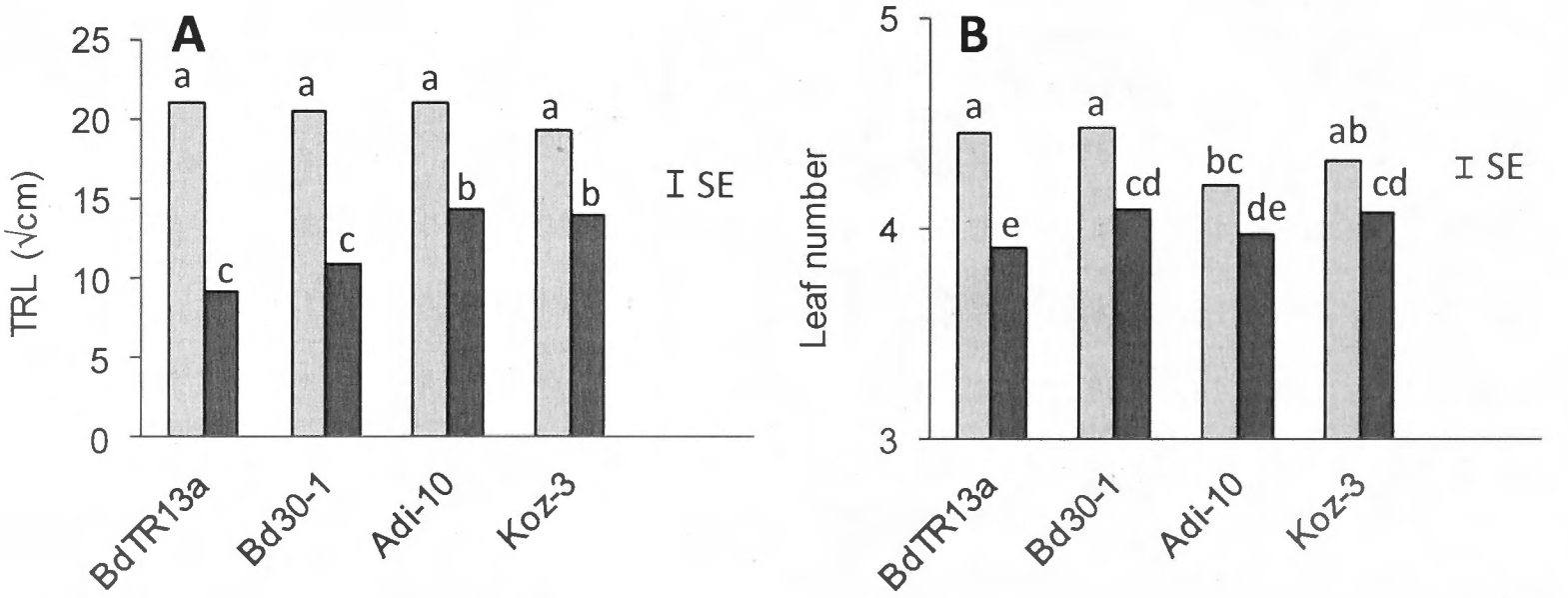

Figure 3.11 Confirmation activity phenotype measurements. Predicted means for a) total root length (TRL), and b) leaf number, for 4 accessions grown in control (light grey bars) and *R. solani*-inoculated soil (dark grey bars); A, n≤12; B, control, n≤16; B, *R. solani* n≤28. Values are given in Table 3.6.

Table 3.6 Predicted means of total root length and leaf number for *B. distachyon* in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values.

Line	Total root length (√cm)			Leaf number		
	Rs	C	Ratio	Rs	C	Ratio
BdTR 13a	9.2	21	0.44	3.9	4.5	0.88
Bd 30-1	11	20	0.53	4.1	4.5	0.91
Adi-10	14	21	0.68	4.0	4.2	0.95
Koz-3	14	19	0.72	4.1	4.3	0.94

Lines Adi-10, BdTR 13a and Koz-3 maintained similar $\sqrt{RLR_A}$ ratios and the same ranking order as seen in the earlier screen. Ratios were 0.64 and 0.68 for Adi-10 (earlier screen and this activity, respectively), 0.48 and 0.44 for BdTR 13a, and 0.78 and 0.72 for Koz-3 (Tables 3.5 and 3.6).

Line Bd 30-1 exhibited different growth and resistance behaviour from the screening activity under confirmation activity conditions. The line dropped in the resistance ranking, with its $\sqrt{RLR_A}$ falling from 0.77 to 0.53, largely due to an increase in root growth under control conditions. Line Bd 30-1 also differed in having the maximum mean leaf number in these experiments and the minimum mean leaf number in the earlier screen.

3.3.2.1 Alternative resistance phenotypes and allometry

The capacity of alternative phenotypes to measure *R. solani* disease resistance and the relationship of biomass allocation for different lines in infested and control treatments was explored by measuring root and shoot fresh weights, leaf area and by comparing root/shoot biomass ratios (Figure 3.12).

Rankings for the *R. solani*/control ratios of root length ($\sqrt{RLR_A}$), leaf area, root fresh weight, shoot FW, TRL/leaf area ratio and root/shoot FW ratio were all in the same order, from lowest to highest: BdTR 13a, Bd 30-1, Adi-10 and Koz-3 (Tables 3.6 and 3.7). The only exception was leaf number.

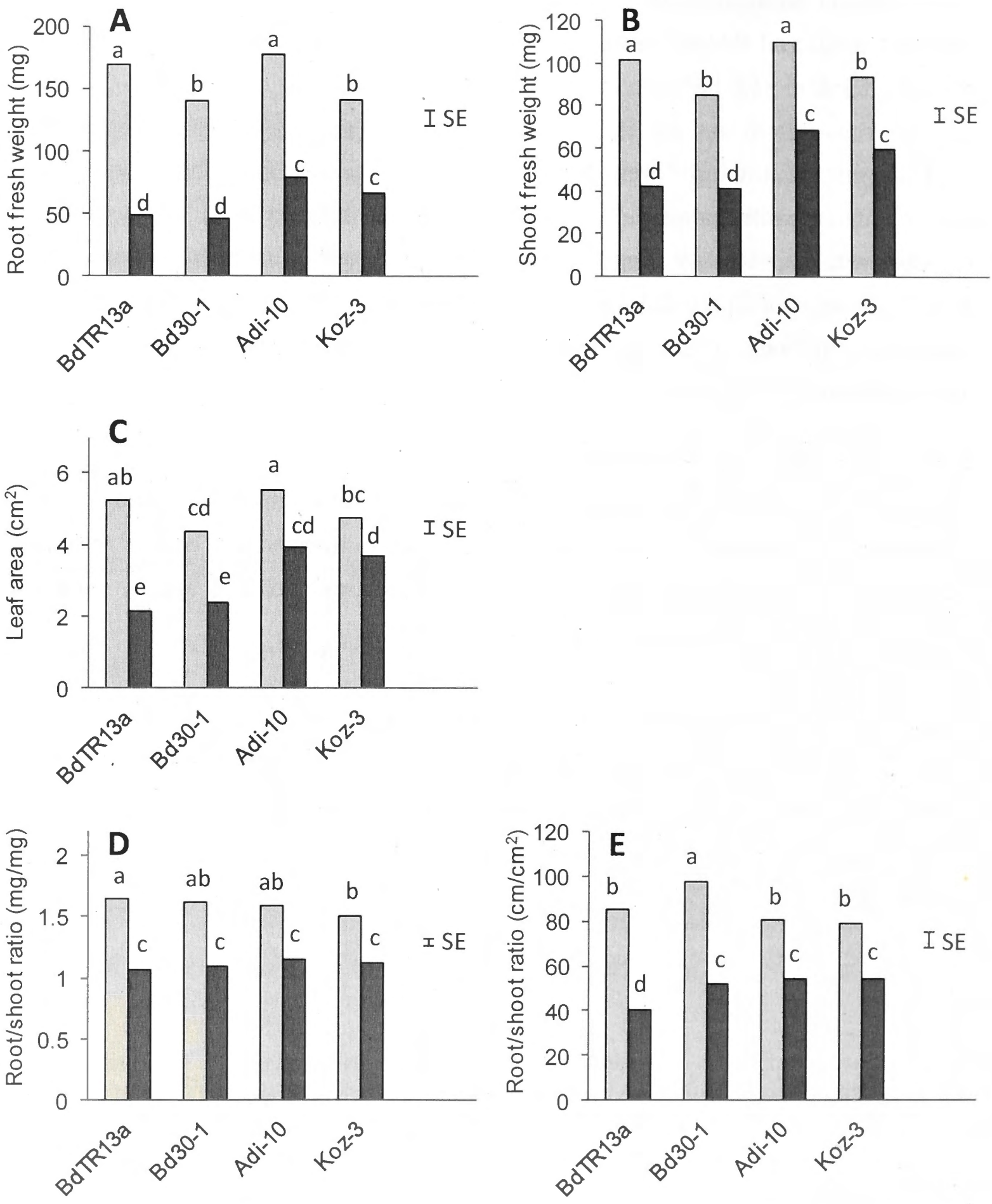

Figure 3.12 Confirmation activity phenotype measurements. Predicted means for a) root fresh weight, b) shoot fresh weight, c) leaf area, d) root/shoot fresh weight ratio, and e) root length/leaf area ratio, for four accessions grown in control (light grey bars) and *R. solani*-inoculated soil (dark grey bars); A, B, D, control, n≤16; A, B, D, *R. solani* n≤28; C, E n≤12. Values are given in Table 3.7.

Table 3.7 Predicted means of total root length, leaf number, leaf area, root fresh weight, shoot fresh weight, root/shoot ratio for root length/leaf area and root/shoot ratio for fresh weight for *B. distachyon* in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values.

Line	Root fresh weight (mg)			Shoot fresh weight (mg)		
	Rs	C	Ratio	Rs	C	Ratio
BdTR 13a	48	169	0.29	42	102	0.41
Bd 30-1	46	140	0.33	42	86	0.48
Adi-10	79	177	0.44	69	111	0.62
Koz-3	67	141	0.47	60	94	0.64

Line	Leaf area (cm^2)		
	Rs	C	Ratio
BdTR 13a	2.2	5.2	0.41
Bd 30-1	2.4	4.4	0.55
Adi-10	3.9	5.5	0.71
Koz-3	3.7	4.7	0.78

Line	Root/shoot FW ratio (mg/mg)			Root/shoot ratio (cm/cm^2)		
	Rs	C	Ratio	Rs	C	Ratio
BdTR 13a	1.07	1.65	0.65	40	85	0.47
Bd 30-1	1.10	1.63	0.68	52	98	0.53
Adi-10	1.15	1.59	0.72	54	81	0.67
Koz-3	1.13	1.50	0.75	54	79	0.69

The significance of plant-pathogen interactions between pairs of lines for all measurements is shown in Table 3.8, using only the data set common to all measurements. Lines Koz-3 and BdTR 13a differed in their response to *R. solani* in every case.

Total root length was the best single phenotypic indicator of the plant-pathogen interaction, revealing significant interactions between three pairs of lines, with other measurements showing a significant interaction between two pairs.

Only the Koz-3/BdTR 13a response difference was picked up for root and shoot fresh weights using the total data set of (control $n \leq 16$, *R. solani* $n \leq 28$), although the smaller data set used for root length and leaf area, i.e. excluding plants harvested for qPCR

analysis, (n≤12) picked up an additional significant difference for each fresh weight phenotype.

Two root/shoot ratios were tested: TRL/leaf area and root/shoot fresh weight. The TRL/leaf area ratio was more informative, with p-values splitting the lines into two distinct groups: less resistant BdTR 13a and Bd 30-1, and more resistant Adi-10 and Koz-3.

Table 3.8 Ability of different phenotypic measurements to reveal significant plant-pathogen interactions between four lines in the confirmation activity, using p-values for pair-wise *Host*Inoculum* interactions; green, p<0.05; n = 10 to 12.
*Interactions that were not significant (p>0.05) when additional fresh weight and leaf number data was included, i.e. the measurements used for Figure 3.12.

√TRL	BdTR 13a	Bd 30-1	Adi-10
Bd 30-1	0.198		
Adi-10	0.004	0.066	
Koz-3	<0.001	0.010	0.305

Leaf number	BdTR 13a	Bd 30-1	Adi-10
Bd 30-1	0.224		
Adi-10	0.005	0.115	
Koz-3	0.015	0.196	0.981

Root FW	BdTR 13a	Bd 30-1	Adi-10
Bd 30-1	0.196		
Adi-10	0.067	0.549	
Koz-3	<0.001	0.016*	0.052

Shoot FW	BdTR 13a	Bd 30-1	Adi-10
Bd 30-1	0.163		
Adi-10	0.021*	0.288	
Koz-3	0.005	0.118	0.589

Leaf area	BdTR 13a	Bd 30-1	Adi-10
Bd 30-1	0.084		
Adi-10	0.020	0.524	
Koz-3	0.001	0.114	0.297

Root/shoot FW ratio	BdTR 13a	Bd 30-1	Adi-10
Bd 30-1	0.933		
Adi-10	0.211	0.121	
Koz-3	0.008	0.002*	0.123

TRL/leaf area ratio	BdTR 13a	Bd 30-1	Adi-10
Bd 30-1	0.880		
Adi-10	0.007	<0.001	
Koz-3	0.004	<0.001	0.766

Even though root length and leaf area were better than fresh weight measurements for finding differences in *R. solani* disease response between lines, correlations between root FW and TRL, and shoot FW and leaf area were high ($p<0.001$) in both control and *R. solani*-inoculated treatments (Figure 3.13).

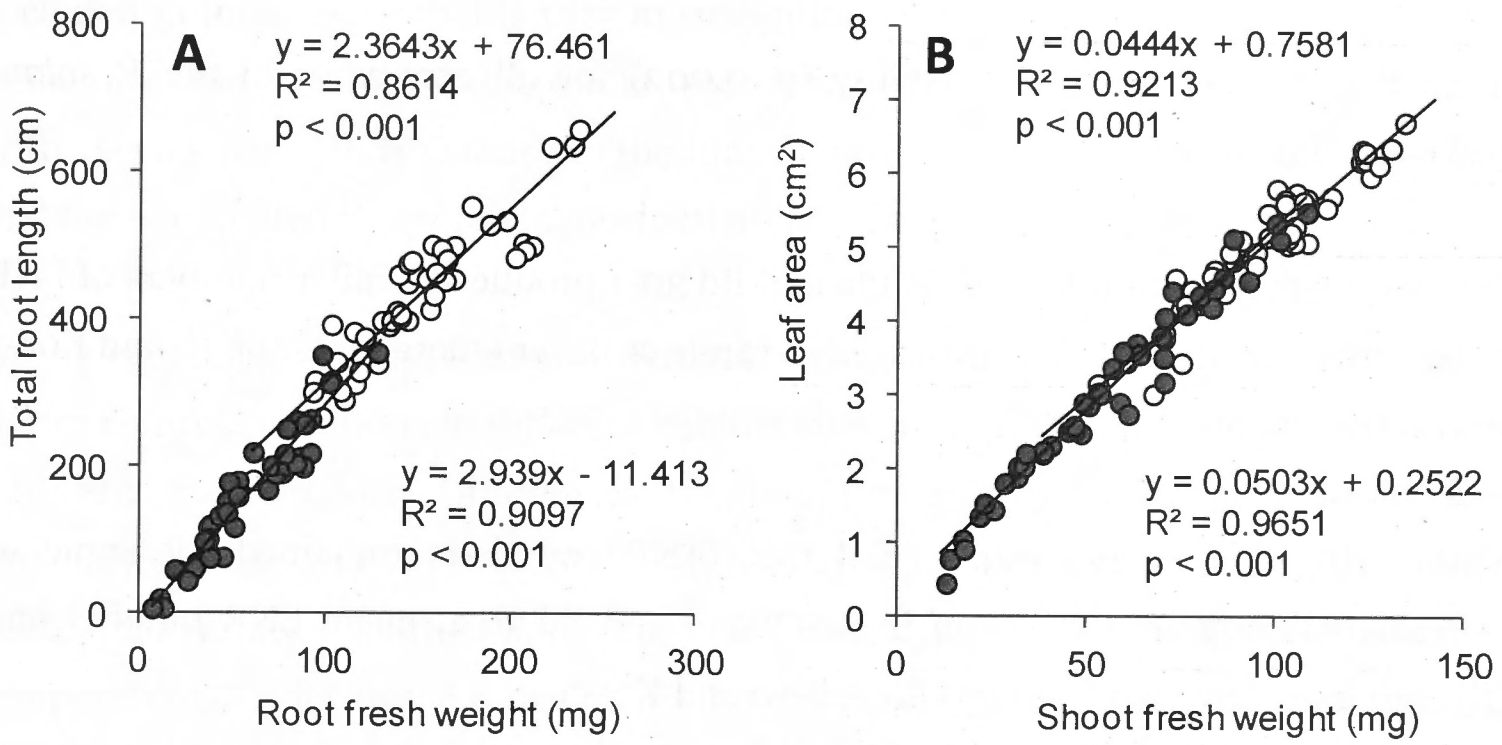

Figure 3.13 Correlation between a) root fresh weight and root length, and b) shoot fresh weight and leaf area, for individual plants of four accessions grown in control (white) and *R. solani* (grey) treatments.

3.3.2.2 Nodal root emergence

An advantage of the soil and growth conditions used in the confirmation experiments was that a substantial number of nodal roots had emerged by harvest and could be classified as coleoptile nodal roots (CNR) or leaf nodal roots (LNR). All distinguishable nodal roots were counted, even if they had been severely truncated.

Production of CNR increased slightly ($p=0.002$) for all genotypes in the *R. solani* treatment (Figure 3.14).

The two least resistant lines, BdTR 13a and Bd 30-1 produced similar numbers of LNR in the control treatment. In contrast, LNR rarely or never emerged in Adi-10 and Koz-3 control treatments.

While LNR number and total nodal root (TNR) numbers remained the same or decreased slightly with *R. solani* in BdTR 13a and Bd 30-1, mean LNR number and TNR number increased ($p<0.05$) for Adi-10 and Koz-3.

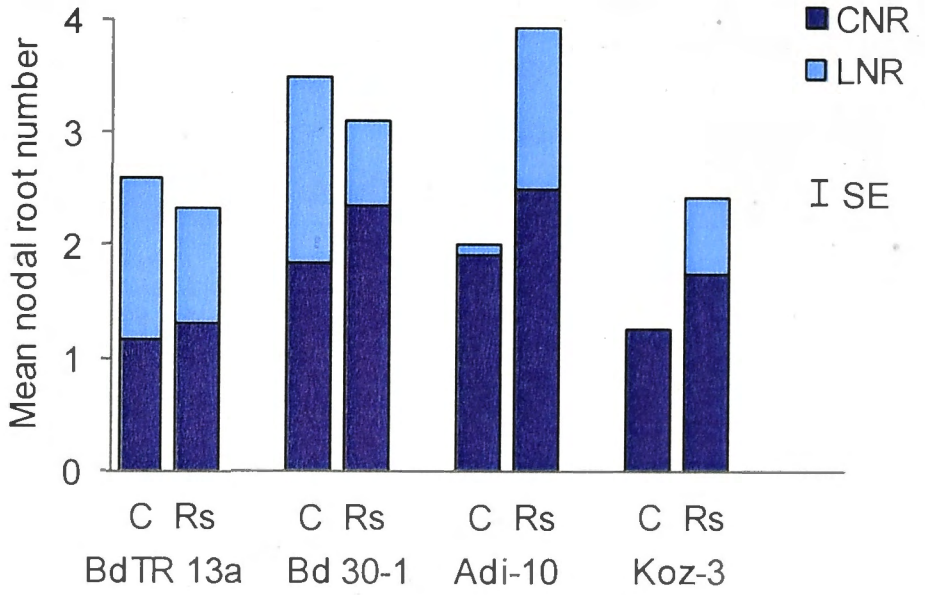

Figure 3.14 Emergence of leaf and coleoptile nodal roots in control (C) and *R. solani* infected (Rs) accessions of *B. distachyon*. CNR, coleoptile nodal root; LNR, leaf nodal root; n≤12; average SE

3.4 Discussion

The purpose of experiments in this chapter was to screen a diverse collection of *B. distachyon* natural accessions for variation in resistance to *R. solani* AG8. Twenty-six inbred lines of *B. distachyon* were grown in replicated experiments in uninoculated soil or soil infested with 0.09 ppg *R. solani* AG8 prior to sowing. Several of the lines included in these experiments vary in susceptibility to other plant pathogens. Disease resistance was assessed through total root length and leaf measurements. Four lines with varying levels of resistance to the fungus were identified in the screening activity and further studied in a confirmation activity.

In the screening activity a large difference was seen in root lengths of plants grown under control conditions in different cabinets, with a 245% increase between the first and final experiments. Differences between the growth cabinet conditions were probably due to variation in light quality and quantity as a result of aging fluorescent tubes, and variation in airflow, cooling and humidity. Spatiotemporal variation in soil temperature of pots, in the range of around 1°C for daytime temperatures and 0.5°C at night, was measured over the course of these experiments; however measurements were not taken consistently. Variation across experiments was greater for root measurements than shoot measurements. In control treatments root length showed a greater response to environmental conditions than shoot measurements. This may be partly due to the nature of the measurements. The shoot measurements taken in the screening activity do not estimate total shoot biomass, whereas total root length does.

Variation between batches of potting mix may also have contributed to some of the growth differences. Contamination of the soil with an unidentified sporulating fungus was thought to be behind the variation seen in experiments 2 and 3. The fungus was isolated from many of the pots during the toothpick bait re-isolation check and appears to be linked with greater *R. solani*/control ratios for root length and shoot measurements in these experiments. Control root length did not appear to increase in these experiments. Rather, the fungus may have inhibited the pathogenicity of *R. solani*. This could be due to a mycoparasitism of *R. solani* by a fungus such as *Trichoderma* or *Gliocladium* spp. (Raaijmakers *et al.*, 2009). As the unidentified fungus grew from toothpicks onto agar, it is not likely to be a mycorrhizal symbiont such as *Glomus mosseae,* which induces systemic resistance to *R. solani* in maize (Song *et al.*, 2011). Non-pathogenic strains of *Rhizoctonia* and other fungi can compete with virulent strains, induce resistance or even transmit mycoviruses to reduce disease (Sneh, 1998).

3.4.1.1 Variation in resistance across accessions in the screening activity

After correcting for spatiotemporal variation and days to emergence, genotypic variation in growth under control conditions and in response to *R. solani* AG8 was measured across the 26 accessions. Both root and shoot measurements were affected by *R. solani*, with the predominant effect being on total root length.

The maintenance of total root length in the inoculated treatment was earlier established to be the best indicator of plant resistance to *R. solani* with this experimental method. Using square-root transformed *R. solani*/control total root length ratio ($\sqrt{RLR_A}$) as a measure of relative disease resistance, Koz-3 was rated as the line most resistant to *R. solani* AG8 in the screening activity, with line BdTR 13a the least resistant. The predicted $\sqrt{RLR_A}$ for Koz-3 was 0.78, with a ratio of 0.48 for BdTR 13a.

In Chapter 2 experiments there was not often a significant reduction in *B. distachyon* leaf 1 and leaf 2 length, but in the screening activity half of all accessions had significantly lower leaf 1 lengths and 81% of accessions had significantly reduced leaf 2 length. This difference may be due to disease becoming established earlier in these experiments or the higher level of replication allowing these small variations to become significant.

3.4.1.2 An alternative resistance ranking method using only *R. solani* treatment

Attempting to reduce the number of plants required for screening per line, two methods for ranking relative resistance of each accession were compared. The first method, *Method A*, ranks greater resistance as greater square-root transformed *R. solani*/control total root length ratio ($\sqrt{RLR_A}$) and untransformed *R. solani*/control shoot ratios. This is the ideal method, as it includes both endogenous and disease-affected growth data. A second ranking method, *Method B*, was based solely on measurements taken in *R. solani*-infested conditions, using ratios of genotype/reference line (Bd 21-3) values (RLR_B). Having the ability to rank *R. solani* resistance based only on an infested treatment, would allow double the number of genotypes to be grown in each experiment. The correlation between ranking *Method A* and *Method B* for total root length was improved by including average seed mass in the *Method B* model, thereby accounting for some of the influence of endogenous plant vigour on resistance rankings.

For both methods, rankings for the rate of leaf appearance, measured as leaf number at harvest, correlated strongly ($p<0.001$) with root length ratio rankings, suggesting that

the plant development rate is dependent on root growth. Leaf 1 and 2 length ranks also correlated well ($p<0.001$) with each other, indicating a consistent response to the pathogen over the first two weeks of growth. The lack of correlation between root length and leaf number rankings with leaf length rankings and, indeed, a strong negative correlation ($p<0.001$) using *Method B*, is intriguing.

Leaf length is determined by the leaf expansion rate and duration of elongation. Granier and Tardieu (2009) state that leaf elongation rate is the more plastic trait and thus has the greater influence on final length. In monocots leaf appearance rate (or its inverse, phyllochron) is independently controlled from leaf expansion rate and final leaf length, e.g. Tesarová and Nátr (1990); Bultynck *et al.* (2004); Sugiyama and Gotoh (2010). This appears also to be the case in *B. distachyon*, taking leaf 2 length as an indicator of leaf expansion rate and leaf number as an indicator leaf appearance rate. In plants grown in control treatment of the screening activity, the genotype-leaf 2 length interaction was significant for leaf number ($p<0.001$), indicating that the relationship between these two traits varied between different *B. distachyon* lines.

The negative *Method B* shoot ratio correlations are probably due to the strong influence of average seed mass on these ranks. This was not investigated further, as root length ratio was the primary resistance rank and correlated well with the *Method A* root length rank.

3.4.2 Confirmation activity

Experiments for the confirmation activity were carried out in a different growth cabinet from the screening activity and with a different soil mix that improved emergence, root growth and quality of root scans while maintaining the same level of *R. solani* disease severity.

Lines Adi-10, BdTR 13a and Koz-3 were ranked in the same order of resistance in the confirmation activity as in the screening activity. The predicted $\sqrt{RLR_A}$ of 0.72 for Koz-3 was significantly higher than the ratio of 0.44 for BdTR 13a. Koz-3 and BdTR 13a also differed significantly for leaf number at harvest, leaf area, root fresh weight, shoot fresh weight and ratios of TRL/leaf area and root/shoot fresh weight.

Line Bd 30-1 growth and resistance rankings varied between the screening and confirmation activity. Root and shoot growth in control conditions of the confirmation activity was much greater than in the screening activity. The reason for this discrepancy is unknown. It may be that Bd 30-1 grows poorly in the potting mix used

for the screening experiments, perhaps due to an abiotic physical or chemical stress, or by succumbing to an unknown pathogen in this soil. The low leaf number resistance rank for Bd 30-1 in the screening activity (Table 3.5, *Method A*) appears to have been a better indicator of the line's poor performance in the confirmation activity than its root length resistance rank.

3.4.2.1 Alternative resistance phenotypes and allometry

Although there was a strong correlation between root fresh weight and total root length, and between shoot fresh weight and leaf area, fresh weight measurements gave weaker plant-pathogen interactions than measurements based on length or area. These results align with the view of Okubara *et al.* (2009) that total root length and disease severity ratings are better indicators of tolerance to *Rhizoctonia* spp. than fresh weight measurements.

Root/shoot fresh weight ratios were significantly lower in *R. solani* treatments compared with control treatment for all lines. The more resistant lines maintained root/shoot ratios closer to the control ratios in infested treatments, even though shoot measurements were also greater in these lines. As discussed in §2.4.2.1, the reason behind infected plants having proportionately greater leaf biomass is not understood.

3.4.2.2 Nodal root emergence

Coleoptile and leaf nodal roots in cereals are also known as adventitious roots or crown roots. Cereal nodal roots emerge in response to abiotic conditions such as availability of soil water in the nodal root emergence zone, and timing of nodal root emergence may be related with plant vigour (Rostamza *et al.*, 2013). The role of nodal roots in disease resistance has not been established; however in an experiment with *R. solani* AG8 inoculated barley, Schroeder and Paulitz (2008) noted an increase in crown root number once a particular threshold inoculum level was reached. Nodal root initiation allows barley seedlings to compensate for loss of primary root length (Crossett *et al.*, 1975).

Not much is known about the molecular processes leading to nodal root emergence, although auxin does appear to be involved in some stages of the development process (Osmont *et al.*, 2007; Pacheco-Villalobos *et al.*, 2013). Auxin is known to be involved in many root processes, including lateral root branching and symbiotic interactions with soil bacteria (Mathesius, 2010). Auxin signalling also appears to have contrasting roles in pathogen defence, having a positive effect in resistance to necrotrophs while increasing susceptibility to biotrophs (Kazan and Manners, 2009). The complex role of

auxin in the interplay between root development and plant defence against pathogens is recently starting to be demonstrated (Nibau *et al.*, 2008).

The production of leaf nodal roots (LNR) in response to *R. solani* appeared to be correlated with increased disease resistance in the confirmation activity. Under control conditions lines BdTR 13a and Bd 30-1 produced leaf nodal roots by 26 days after planting, while the more resistant lines, Adi-10 and Koz-3, did so rarely or never, even though total root length was not significantly different between the lines. Lines Adi-10 and Koz-3 produced significantly more LNR in response to *R. solani* treatment, resulting in a negative correlation (p=0.019) between control LNR/total nodal root ratio and the $\sqrt{RLR_A}$ resistance ranking (Figure 3.15).

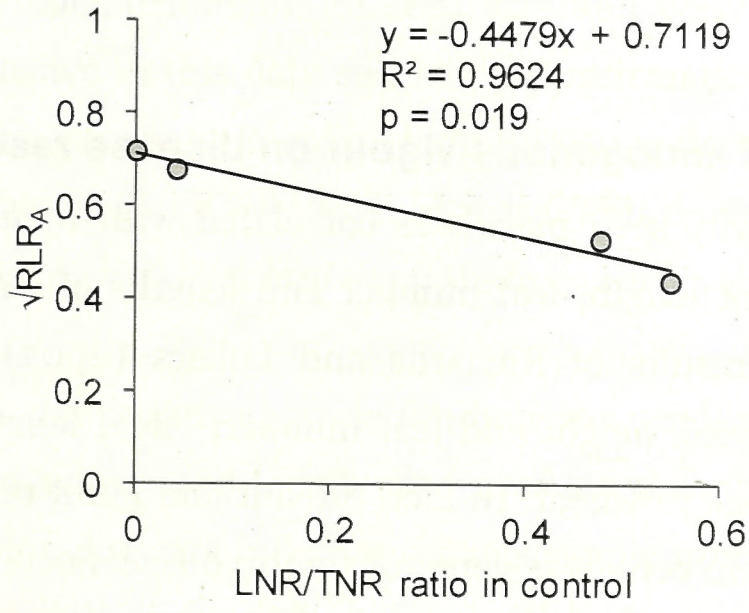

Figure 3.15 Relationship between the ratio of total nodal roots emerging from leaf nodes in control treatment with the *R. solani*/control total root length ratio resistance rank ($\sqrt{RLR_A}$) in the confirmation activity at 26 days after planting.

These results suggest that the early endogenous production of leaf nodal roots was disadvantageous for *R. solani* resistance. In a wheat field experiment Meagher *et al.* (1978) found that *R. solani* lesions covered a greater percentage of nodal roots than seminal roots nine weeks after sowing. On the other hand, Harris and Moen (1985) found that *R. solani* AG2 was less able to infect mature root tissue or nodal roots of wheat, but allowed secondary infections to continue to cause damage. *Rhizoctonia solani* AG8 causes wheat stunting when infected at the seedling stage, but plants tend not to be affected to the same extent when infected at later stages of development (MacNish and Neate, 1996).

Certainly it appears that *R. solani* is more active near the soil surface. In the course of this project is has been noted that *R. solani* hyphae could be seen to proliferate between the soil surface and toothpicks during the re-isolation check, and that *R. solani* grew aerial mycelium on plates when placed agar-down, but not when placed

agar-up, suggesting the fungus may be negatively gravitropic. Blair (1942) found that wheat isolates of *R. solani* grew faster horizontally than downwards. There are some suggestions that the activity of *R. solani* of different anastomosis groups can be inhibited by increasing concentrations of carbon dioxide (Blair, 1943; Durbin, 1959), which may restrict the fungus' growth and phytopathogenic activity to the surface layer of soil. Thus, it may be that emerging leaf nodal roots extend into the zone of highest danger for *R. solani* infestation.

These results suggest it is worthwhile for *B. distachyon* to delay endogenous LNR emergence, but to initiate LNR emergence in response to *R. solani* infection. While it is interesting to speculate on the role of nodal root development in plant disease resistance, the correlations observed in this small set of genotypes need to be investigated further.

3.4.3 The effect of endogenous vigour on disease resistance

In the screening activity seed mass was correlated with *B. distachyon* early vigour, measured as total root length, leaf number and lengths of leaf 1 and leaf 2. This is consistent with the results of Richards and Lukacs (2002), who found a strong correlation between seed weight and leaf number, leaf 1 length and leaf 2 length at about the 4.5 leaf stage in wheat. In fact, the authors point out that the determining factor of early vigour in cereals is generally embryo size. Seed weight was linked with embryo size in the wheat study and probably is in *B. distachyon* too, as these two traits are also linked in other cereals (López-Castañeda *et al.*, 1996). The number of days to emergence was also correlated with phenotypic indicators of early seedling vigour. Rapid germination is a characteristic of seedling vigour (Maguire, 1962).

The results of the screening data suggest that early vigour in *B. distachyon* is correlated with decreased resistance to *R. solani*. Greater root growth in control treatment was not compensated with increased root growth in *R. solani*, resulting in a negative relationship between root growth and disease resistance. The correlation between seed mass and $\sqrt{RLR_A}$ was also negative, but less strong. This is consistent with the results of Okubara and Jones (2011), who found that in a collection of wheat-*Thinopyrum* addition lines there was a tendency for smaller root systems to have greater resistance to *R. solani* AG8. On the other hand, a wheat mutant with increased tolerance to *R. solani* AG8 had greater endogenous root weight and length than the wild type (Okubara *et al.*, 2009).

The negative correlation between vigour and disease resistance points to a possible 'fitness trade-off' between plant growth and defence. This is a well-studied phenomenon, particularly in plant-herbivore interactions and more recently in plant-pathogen interactions with fungi (Todesco *et al.*, 2010; García-Guzmán and Heil, 2013). Herms and Mattson (1992) showed that under favourable growth conditions, when photosynthetic assimilation reaches a maximum, resources are allocated either to secondary metabolism or growth, thereby resulting in a trade-off between growth and defence.

Are *B. distachyon* resistance rankings entirely dependent on seed size and seedling vigour? Do all lines reach a similar *R. solani*/control root length ratio when they reach the same control root length? The results of the screening activity suggest that there is variation in resistance not attributable to seed size and early vigour; however the evidence was not conclusive in this data set. In the confirmation activity, line Adi-10 was significantly more resistance to *R. solani* than BdTR 13a despite reaching the same control root length and having 11% greater seed mass. This demonstrates that there is genetic variation in resistance to *R. solani* separate from early vigour.

It should be noted that the negative correlation between seedling vigour and disease resistance only occured when root length was used as the phenotypic measure of resistance. The correlation does not hold if resistance is measured as *R. solani*/control ratios of shoot measurements. A possible reason for this observation is that unlike leaf length and leaf number, root length increases exponentially, facilitated by root branching. This difference is seen in the need for transformation of root, but not shoot, values prior to statistical analysis. The negative correlation may be an artefact emanating from an imperfect transformation equation. Alternatively, it could be that as shoot effects follow on from primary effects of root disease, the correlation may not be noticeable in shoot data until a later stage of development. In the end, as roots are the primary site of *R. solani* attack, root length is expected to be a more informative phenotypic measure of disease severity than shoot observations.

It may be useful to weigh individual seeds before sowing, although this is a difficult and time-consuming exercise with the seed surface disinfection and germination technique used in these experiments. As emergence rate is not affected by *R. solani* disease under these conditions, this measurement could be a useful indicator of endogenous vigour of seedlings grown in infested treatments.

3.4.4 Variation in resistance to *R. solani* in the *B. distachyon* natural accessions

Previous studies that measured differences in resistance based on ratios of total root length found large variability in differences between more and less resistant genotypes (Table 1.6), partly due to variations in growth conditions (Okubara and Jones, 2011).

Calculation of the values given in Table 3 of the study by Okubara *et al.* (2009) shows that, at 14 days after planting, a more tolerant mutant of 'Scarlet' wheat, 'Scarlet-Rz1', had inoculated/control total root length ratios (RLR_A) of 0.75, 0.92 and 0.79, while wild type 'Scarlet' had ratios of 0.81, 0.53 and 0.73 in soil infested with *R. solani* AG8 at 20, 100 and 400 ppg, respectively. The change in RLR_A from wild type in the mutant was -6%, 39% and 6%, respectively.

The same calculation can be used for the values given in Table 2 of the publication by Okubara and Jones (2011) for two experiments with Chinese Spring wheat – *Thinopyrum* addition lines grown for 14 days in soil infested with *R. solani* AG8 at 200 - 300 ppg. The average change in RLR_A from Chinese Spring (CS) wheat for *Thinopyrum elongatum,* CS-*Th. elongatum* chromosome 4E addition line, and CS-*Th. bessarabicum* chromosome 4J addition line were 16%, 19% and 30%, respectively.

In experiments for this thesis, too, it is difficult to put a numerical value on the difference between the most and least resistant genotype. Root lengths in these experiments were square-root transformed prior to analysis, so they cannot be directly compared with previous root length studies. Back-transforming predicted total root lengths of the least resistant line, BdTR 13a, and most resistant line, Koz-3, in the screening activity gives inoculated/control total root length ratios (RLR_A) of 0.23 and 0.61, respectively. This represents a 38% greater RLR_A for Koz-3 than BdTR 13a. Again, back-transformation of predicted root lengths of BdTR 13a and Koz-3 in the confirmation activity gives RLR_A of 0.19 and 0.53, respectively, representing a 33% greater RLR_A for Koz-3 than BdTR 13a.

The difference in resistance between the most and least resistant natural accessions of *B. distachyon* tested here is comparable to resistance previously found in close relatives or mutants of wheat. Even with a substantial improvement in root growth between lines, there were no clear observable differences in root system morphology, such as branching or root diameter. Emergence of leaf nodal roots was an exception, but even so leaf nodal roots were often truncated in both more and less resistant lines.

3.5 Conclusion

Variation in resistance to *R. solani* AG8 was found in 26 natural accessions of *B. distachyon*. The level of resistance between the most and least resistant lines (Koz-3 and BdTR 13a, respectively) in these experiments was consistent and similar to levels measured in previous studies with wild relatives of wheat and wheat mutants.

These results provide a good basis for further work to discover genes involved in resistance to *R. solani* AG8 in *B. distachyon*. Strategies for gene discovery based on observed phenotypic variation are described in the General Discussion.

Chapter 4

Screening T-DNA lines of *Brachypodium distachyon* for resistance to *Rhizoctonia solani* AG8

Summary

This chapter uses the phenotyping method developed in Chapter 2 and used for natural accession screening experiments in Chapter 3 to look for variation in *Brachypodium distachyon* resistance to *Rhizoctonia solani* AG8 attributable to genes targeted in a T-DNA mutant collection. Preliminary evidence is presented of repeatable increased resistance to the fungus associated with disruption of a putative β-1,3-galactosyltransferase gene. This chapter describes:

- Selection of T-DNA mutant lines for screening based on a literature review and the likelihood that targeted genes could be involved in disease resistance or root development;

- Screening of 25 lines representing 19 T-DNA insertion events in three experiments;

- Confirmation that line 5088-4, with a disruption in a putative β-1,3-galactosyltransferase gene, *Bradi3g14370*, had 20% more root length in *R. solani* infested treatment compared with control treatment, compared with infested and control treatment root lengths for the reference line Bd 21-3. Further lines of the same T-DNA insertion event, 5088-2 and 5088-5, however did not have the same significant increase in root length ratios;

- Confirmation that, in uninfested controls, event 2426 and line 9212-15 were associated with greater root growth than the reference line Bd 21-3. This increased endogenous vigour may be partly due to greater seed mass; and

- Further investigation of potential resistance mechanisms, showing that the relationship between reduced vigour and resistance to *R. solani* was weaker in the T-DNA lines than in the natural accessions. An increase in coleoptile nodal root emergence in response to disease was consistent with natural accession results, but the link between leaf nodal root emergence and resistance was not repeated in lines assayed for this chapter.

4.1 Introduction

The aims of this chapter were to test the effect of genes silenced by transfer DNA (T-DNA) insertions in *B. distachyon* line Bd 21-3 on its resistance to *R. solani* AG8.

The T-DNA lines used in this study were developed in John Vogel's group by Jennifer Bragg, at the USDA, Albany. The development of T-DNA mutant collections for *B. distachyon* provides an excellent resource to study genes involved in diseases that affect both *B. distachyon* and wheat, such as *R. solani* AG8. While no plant-pathogen studies have been published to date using *B. distachyon* T-DNA mutants, mutants from Arabidopsis collections have previously been used to study *R. solani* and other necrotrophic diseases, e.g. Oñate-Sánchez *et al.* (2007); Zheng *et al.* (2006). *Brachypodium distachyon* T-DNA mutants have been used to study genes affecting stem elongation and root growth (Vain *et al.*, 2011; Pacheco-Villalobos *et al.*, 2013). If a resistance or susceptibility phenotype is found in the T-DNA collection lines the benefits are substantial, as verifying the tagged gene would be far easier than identifying a resistance gene in the natural accession collection.

A T-DNA insertion site in or near a gene is known as an event, with multiple lines available for some events. Lines for inclusion in experiments for this chapter were chosen based on a literature review of tagged gene descriptions received from J. Bragg (pers. comm.) and then on availability. Even with a limited collection, many of the mutants in the available transformed lines carried insertions in genes that have been linked to disease resistance, stress tolerance or root development (Table 4.1).

By selecting candidate lines most likely to respond differently to *R. solani*, the chances of finding variation in resistance are increased. Nevertheless, the chance of discovering a gene involved in resistance to *R. solani* AG8 in the chosen lines is still small. This is partly due to the small number of T-DNA mutant lines available at the time, but also to the expectation that resistance to necrotrophic diseases like *R. solani* is likely to require multiple quantitative gene interactions (Poland *et al.*, 2009). Furthermore, single gene knock-out mutants often do not have an observable phenotype due to redundancy in genetic pathways (Weigel *et al.*, 2000).

Lines were primarily chosen if there was evidence of the tagged gene being differentially expressed in response to pathogens. These are presented with their sources in the literature in Table 4.1. Reactive oxygen species (ROS) are produced by necrotrophic pathogens, but also by plants as a signal to initiate a basal defence response. In *R. solani* infection of tomato, this leads to the production of antioxidant

enzymes, as well as cell-wall strengthening and detoxifying compounds, such as phenolic acids (Nikraftar *et al.*, 2013). Genes involved in the transport or modification of antioxidants, including ascorbate, polyols and flavonoids, may thus be involved in pathogen defence or stress tolerance (Hoekstra *et al.*, 2001; Mittler, 2002). Genes that are known to be upregulated in response to chitin, including transcription factors and ubiquitin-ligase, may also be useful in recognition and defence against *R. solani*, as can genes encoding chitinases (Libault *et al.*, 2007). As *R. solani* destroys cell walls with a multitude of enzymes (Bora *et al.*, 2005), several genes that are involved in cell wall processes were chosen for the screen. Genes expressed during root development were also included, as root architecture and formation of lateral roots can be influenced by pathogens (Nibau *et al.*, 2008) and plant morphological traits can be potentially linked with quantitative disease resistance (Poland *et al.*, 2009). Ten of the nineteen tagged lines studied in this chapter align with expressed sequence tags (ESTs) in wheat, suggesting that orthologues of these genes are expressed in wheat. Potential markers for orthologous genes in wheat are available, with thirteen of the nineteen tagged genes aligning with one or more bread wheat single-nucleotide polymorphisms (Gramene, 2013a).

The T-DNA lines were phenotyped using the methods developed in Chapter 2. Maintenance of total root length in the infested treatment was used as the primary indicator of resistance to *R. solani*. The rate of leaf appearance, leaf lengths and appearance of nodal roots was also measured in some experiments.

Two methods for ranking resistance to *R. solani*, developed in Chapter 3 (§3.2.9.1), were used to analyse experimental results in this chapter. The first, *Method A*, compares root or shoot phenotypic measurements in *R. solani*-inoculated treatments with those in untreated controls. Higher *R. solani*/control ratios indicate greater resistance. Using *Method A*, root length measurements were transformed prior to analysis, to improve comparisons between the large range of values. A second resistance ranking method, *Method B*, was evaluated and used in some screening experiments in this chapter. This method requires lines to only be grown in *R. solani*-infested treatments, thus potentially allowing double the number of lines to be screened, compared with *Method A*. Using *Method B*, root and shoot measurements of lines in *R. solani* treatment are compared with those of the reference line, Bd 21-3, in *R. solani* treatment. Average seed mass was included in the analysis, as it appears to correct for some endogenous variation in plant vigour (Figure 3.10).

Table 4.1 Information on genes tagged in the T-DNA lines included in screening experiments in this chapter. UniPROT entry numbers are given; accessed 19 June 2013 unless otherwise specified (The UniProt Consortium, 2012). Wheat expressed sequence tag (EST) and single nucleotide polymorphism (SNP) information was acquired through the Gramene database (Gramene, 2013b).

T-DNA event	Construct[a]	Tagged gene; T-DNA location	Wheat ESTs and SNPs?[b]	NCBI Protein-BLAST sequence homology, *E*-value	Notes on predicted gene family homologues
77	pOL001	*Bradi3g36010*; in gene	No	Predicted: nucleobase-ascorbate transporter 6-like [*B. distachyon*], 0.0	NAT6 expressed in Arabidopsis seedling roots and lateral root primordia (UniPROT Q27GI3) (Maurino *et al.*, 2006). Ascorbate involved in *R. solani*-tomato pathogenesis (Nikraftar *et al.*, 2013).
654	pOL001	*Bradi1g23450*; in gene	ESTs SNP: B	Predicted: polyol transporter 5-like [*B. distachyon*], 0.0	Sugar-proton symporter; highly expressed in Arabidopsis roots, induced by wounding and insect feeding (UniPROT Q8VZ80). Polyols have antioxidant activity (Williamson *et al.*, 2002).
705	pOL001	*Bradi2g48120*; in gene	ESTs SNP: AD,B	Predicted: ras-related protein Rab7-like [*B. distachyon*], 3e-150	Small GTP-binding proteins; wheat Rab7 involved in response to stripe rust and abiotic stress (Liu *et al.*, 2012).
2426	pJJ2LBA activation tagging	*Bradi3g29780*; ~ 250 bases downstream	ESTs SNP: AD	Predicted: probable aquaporin TIP3-1-like [*B. distachyon*], 0.0	Aligns with *OsTIP3;1*, expressed in leaves and at lower levels in roots; 100% coverage, 87% identity, 2e -150, (UniPROT Q9FWV6, 16 August 2013) (Sakurai *et al.*, 2005). Aligns with seed-specific *AtTIP3;1*, 99% coverage, 64% identity, 4e-113, UniPROT P26587 (Höfte *et al.*, 1992). An Arabidopsis TIP3;1 is expressed in seeds and during germination and early seedling development (Gattolin *et al.*, 2011).
2596	pJJ2LB	*Bradi3g04080*; ~240 bases upstream	No	Predicted: endoglucanase 5-like [*B. distachyon*], 0.0	Glycosyl hydrolase 9 family; secreted protein hydrolyses linkages in cellulose, lichenin and cereal beta-D-glucans (UniPROT Q9M995).

Table 4.1 continued Information on genes tagged in the T-DNA lines included in screening experiments in this chapter.

T-DNA event	Construct[a]	Tagged gene; T-DNA location	Wheat ESTs and SNPs?[b]	NCBI Protein-BLAST sequence homology, *E*-value	Notes on predicted gene family homologues
2771	pJJ2LB	*Bradi3g54950*; in gene	SNP: AD	Predicted: dihydroflavonol-4-reductase-like [*B. distachyon*], 0.0	Involved in flavonoid and pigment biosynthesis (UniPROT P51102); DFR involved in response to fungal and bacterial pathogens in rice (Hayashi *et al.*, 2005).
2892	pJJ2LB	*Bradi3g33510*; in gene	ESTs SNP: AD,B	Predicted: protein ARABIDILLO 1-like [*B. distachyon*], 0.0	Promoter of lateral root development in Arabidopsis (Coates *et al.*, 2006).
3175	pJJ2LB	*Bradi3g39050*; in gene	No	Predicted: DNA (cytosine-5)-methyltransferase 3-like [*B. distachyon*], 0.0	May be involved in DNA methylation and gene silencing (UniPROT Q8LPU5).
3400	pJJ2LB	*Bradi2g47210*; intergenic ~4.2 kb downstream	ESTs SNP: AD	Predicted: basic endochitinase A-like isoform 1 [*B. distachyon*], 0.0	Glycosyl hydrolase 19 family; hydrolysis of linkages in chitin and chitodextrins (UniPROT P29022).
3794	pJJ2LB	*Bradi1g54290*; ~ 790 bases upstream	ESTs	Predicted: uncharacterized protein LOC100842709 [*B. distachyon*], 2e-175 Late embryogenesis abundant hydroxyproline-rich glycoprotein [*Arabidopsis thaliana*], 2e-28	LEAs are involved in desiccation survival, abiotic stress tolerance (Hundertmark and Hincha, 2008), root architecture and necrotrophic disease response (Salleh *et al.*, 2012).
4243	pJJ2LB	*Bradi5g27660*; ~ 590 bases upstream	No	Predicted: U-box domain-containing protein 25-like [*Fragaria vesca* subsp. *vesca*], 5e-54 Predicted: U-box domain-containing protein 25-like [*B. distachyon*], 1e-37	Ubiquitin ligase (UniPROT Q9LT79). Involved in defence response; response to chitin (Libault *et al.*, 2007).
4774	pJJ2LB	*Bradi3g39700*; ~ 350 bases upstream	SNP: AD	Predicted: wall-associated receptor kinase 5-like [*B. distachyon*], 0.0	Serine/threonine protein kinase; induced by salicylic acid (UniPROT Q9LMN7).

Table 4.1 continued Information on genes tagged in the T-DNA lines included in screening experiments in this chapter.

T-DNA event	Construct[a]	Tagged gene; T-DNA location	Wheat ESTs and SNPs?[b]	NCBI Protein-BLAST sequence homology, *E*-value	Notes on predicted gene family homologues
5088	pJJ2LBP2 gene trap	*Bradi3g14370*; in gene	ESTs SNP: AD,B	Predicted protein [*Hordeum vulgare* subsp. *vulgare*], 0.0 Predicted: probable beta-1,3-galactosyltransferase 20-like [*B. distachyon*], 0.0	Glycosyltransferase family 31, galectin domain; protein modification and glycosylation; expressed mainly in stems (UniPROT A7XDQ9) (Strasser *et al.*, 2007). Galectins are involved in mammalian defence response (Sato *et al.*, 2009).
7557	pJJ2LBP2 gene trap	*Bradi3g16430*; in gene	SNP: AD,B	Predicted: probable LRR receptor-like serine/threonine-protein kinase At3g47570-like [*B. distachyon*], 0.0	LRRs often involved in plant defence response (UniPROT C0LGP4).
8634	pJJ2LBA activation tagging	*Bradi2g50980*; in gene	SNP: AD,B	Predicted: uncharacterized protein LOC100836340 [*B. distachyon*], 0.0 Glycosyl hydrolase, family 43 protein [*Zea mays*], 0.0	Synonym = glycoside hydrolase family 43 (www.cazy.org/GH43.html, 19 June 2013).
8913	pJJ2LBA activation tagging	*Bradi1g69610*; ~ 580 bases upstream	ESTs SNP: AD	Predicted: glucan endo-1,3-beta-glucosidase 3-like [*B. distachyon*], 0.0	Glycosyl hydrolase family 17; involved in wheat abiotic and biotic defence response (UniPROT P52409).
9212	pJJ2LBA activation tagging	*Bradi1g62970*; in gene	No	RING-H2 finger protein ATL5 [*Triticum urartu*], 5e-27 Predicted: RING-H2 finger protein ATL2-like [*B. distachyon*], 1e-14 Predicted: E3 ubiquitin-protein ligase EL5-like [*B. distachyon*], 4e-13	Aligns with a capsicum RING-H2 E3 ubiquitin ligase protein involved in biotroph defence response, 90% query coverage, 32% amino acid identity, 4e-17 (UniPROT G0T3B3) (Lee *et al.*, 2011). Aligns with an E3 ubiquitin-protein ligase EL5 involved in rice root growth, 25% query coverage, 62% identity, 3e-17, (UniPROT Q9LRB7) (Koiwai *et al.*, 2007).

Table 4.1 continued Information on genes tagged in the T-DNA lines included in screening experiments in this chapter.

T-DNA event	Construct[a]	Tagged gene; T-DNA location	Wheat ESTs and SNPs?[b]	NCBI Protein-BLAST sequence homology, *E*-value	Notes on predicted gene family homologues
9278	pJJ2LBA activation tagging	*Bradi1g68540*; in gene	ESTs SNP: AD,B	Predicted: translocase of chloroplast 34, chloroplastic-like [*B. distachyon*], 0.0	GTPase, imports protein precursors into chloroplast, light-induced, expressed mostly in roots and flowers (UniPROT Q38906).
		Bradi1g68530; ~ 3.1 Mb upstream	No	Predicted: U-box domain-containing protein 21-like [*B. distachyon*]; 0.0, 100%	Ubiquitin ligase, response to chitin (UniPROT Q38906, 28 July 2013) (Libault *et al.*, 2007).
9840	pJJ2LBA activation tagging	*Bradi1g23640*; in gene	ESTs SNP: AD	Predicted: glucan endo-1,3-beta-glucosidase 4-like [*B. distachyon*], 0.0	Glycosyl hydrolase family 17; inferred carbohydrate metabolism and defence response (UniPROT Q94CD8).

[a]Detailed construct information is available in Bragg *et al.* (2012). All constructs have the potential to knock out genes. In lines with pJJ2LBA expression of nearby genes may be increased by the insertion of the 'activation tagging' construct. Lines with the 'gene trap' construct, pJJ2LBP2, may express GUS and/or GFP instead of the original gene product if the construct lands downstream of a promoter region.

[b]ESTs, *Triticum aestivum* 'Expressed Sequence Tags' (ESTs) are available from dbEST (www.ncbi.nlm.nih.gov/dbEST/, 20 August 2013); SNP: AD, SNPs that differ between the A and D genomes (where the B genome is unknown); SNP: B, SNPs that are the same between the A and D genomes, but differ in B (www.gramene.org/Brachypodium_distachyon/Info/Annotation/, 20 August 2013).

4.2 Materials and methods

4.2.1 Seed preparation

Brachypodium distachyon T-DNA lines were donated by Dr John Vogel from the Western Regional Research Center Brachypodium T-DNA insertional mutant collection. Mutant lines were generated in Bd 21-3 background. Four T-DNA constructs were used in the transformation of lines used in this chapter (Table 4.1). All constructs have the ability to disrupt gene function if they are incorporated into coding or regulatory regions. Construct pJJ2LBA contains four copies of the CaMV 35S enhancer sequence, which can increase transcription of nearby genes. Construct pJJ2LBP2 contains GUS and GFP genes that may be expressed if the insertion occurs adjacent to a promoter. Inverse PCR was used to generate flanking sequence tags (FSTs), to identify genes with T-DNA insertions and to generate gene-specific PCR primers. Homozygous mutant plants were identified with gene specific PCR, prior to distribution of T2 seeds (Bragg *et al.*, 2012).

The first shipment of seeds arrived at CSIRO Black Mountain in May 2011. The quantity of seeds varied from around 10 to over 100 per line, with seed size and germination also variable. Lines required a round of seed increase to produce sufficient germplasm for replicated experiments. Seed increase for experiments was carried out at CSIRO Plant Industry, Black Mountain, by Drs Richard Poiré and Vincent Chochois.

Lines were chosen for inclusion in experiments based on the predicted function of genes hit by the T-DNA (Table 4.1) and on availability. At commencement of the first screening experiment 50 T-DNA lines with sufficient numbers of seeds were available, with the number increasing over time.

Seeds were surface disinfected in ethanol and sodium hypochlorite according to the method of Alves *et al.* (2009), then germinated overnight on agar. A detailed method is given in §2.2.1.

4.2.2 *Rhizoctonia solani* inoculum

The *R. solani* AG8 inoculum used for experiments in this chapter was grown on millet and stored at -20°C. The full method is described in §2.2.2.

4.2.3 Soil

The soil mixes used for experiments in this chapter were prepared, sieved to remove coarse materials and steam-sterilized by the CSIRO Plant Industry potting shed.

In the first and second screening experiments the soil mix used was 'Barley Mix', described in §2.2.3, a blend of recycled soil, leaf mulch, river loam, peat moss, perlite, vermiculite, river sand, straw and fertilizers.

The soil used for subsequent experiments was a blend of 50% river sand and 50% 'Special' potting mix, described in §3.2.3. 'Special' potting mix was a composted mixture of recycled soil, straw and fertilizers.

4.2.4 Cone preparation and sowing

Cones were prepared and sown according to the method described in §2.2.4. *Rhizoctonia solani* inoculum was incorporated into soil at 0.09 propagules per gram on the day of sowing. Control treatments were prepared in the same manner at *R. solani* treatments, but with no addition of inoculum to the soil. The rate of plant emergence from the soil surface was very high, with no difference in emergence or days to emergence between the *R. solani*-infested or control lines.

4.2.5 Growth conditions

The experiments described in this chapter were conducted during the same period of time as those for Chapter 3. Growth conditions were the same as those described in §2.2.5, except plants were grown in a PC2 facility in Adaptis A1000 growth cabinets (Conviron, Winnipeg, Canada). Cones were watered every two to three days with either 5 mL or 10 mL. All cones received the same volume of water.

4.2.6 Re-isolation of *Rhizoctonia* from soil

The toothpick bait method, initially described in §2.2.6 was used to re-isolate *Rhizoctonia* from soil at 8 days after planting (DAP) to ensure a consistent level of the fungus in *R. solani* treatments and the absence of cross-contamination in control treatments. Only control treatments with toothpick scores of zero and *R. solani*-infested treatments with toothpick scores of 3 were included in analysis. The toothpick inoculum check showed that inoculum levels were consistently high, with no contamination of control treatments.

4.2.7 Phenotype measurements

At harvest plants were removed from pots and roots rinsed gently. Plants were stored in ethanol (50% v/v) in plastic sauce containers.

The major phenotypic indicator of resistance to *R. solani* is maintenance of total root length in infested treatments. Roots were scanned at 400 dpi on an Epson Perfection V700 Photo flatbed scanner (Epson, Australia) to measure total root length using the WinRHIZO™ system (Regent, Quebec). Roots were stained with toluidine blue prior to scanning in the first and second screening experiments.

Emergence of nodal roots was measured, as this trait appeared to be linked with resistance in the natural accession confirmation experiment (§3.3.2.2). Coleoptile and leaf nodal root numbers were measured by counting emerged roots in scans. Roots were counted if they had clearly emerged, even if severely truncated.

Leaf number and leaf length measurements, when taken, were recorded at harvest. These shoot measurements are secondary indicators of *R. solani* disease severity.

Further details of phenotype measurement methods are given in §2.2.7.

4.2.8 General observations for characterisation of infection patterns and responses in roots

Seeds were surface disinfected, germinated overnight at room temperature on agar plates and incubated in the dark at 16°C. Six days after disinfection a square of agar from the growing edge of a *R. solani* colony was placed adjacent to *B. distachyon* roots. After 48 h vibratome sections (~100 μm) of the tip regions of colonized roots were observed with a Leica DMLB microscope (Leica Microsystems) under bright field and with fluorescence filters.

Root diameters were measured on six roots per line from seedling growing on uninoculated agar plates for 12 days. Root images were captured with a Leica M205 C microscope (Leica Microsystems). ImageJ 1.43u software (National Institutes of Health, USA) was used to measure diameters 1 mm from the root tip and 1 mm from the base of the seed. Average diameters and least significant differences were calculated using GenStat.

For GUS (β-glucuronidase) staining, seedlings were briefly vacuum infiltrated and then incubated overnight at 37°C in the histochemical stain, containing 0.1 mM X-Gluc

(5-bromo-4-chloro-3-indolyl glucanoride; X-Gluc DIRECT, UK), 5 mM EDTA and a mixture of 0.5 mM each of ferri- and ferro-cyanate, after Larkin *et al.* (1996).

4.2.9 Experiments

Four experiments are described in this chapter: three screening experiments and a confirmation experiment.

4.2.9.1 Screening experiments

The first screening experiment was of the same design as the experiments for the screening activity described in §3.2.8.1. Three replicates of a treatment were sown in adjacent pots in a randomised location within a flow tray and harvested at 22 DAP. Each *Line*Inoculum* treatment was repeated once per flow tray.

Design of the second screening experiment took a different approach. In an attempt to increase the number of lines that could be screened only one control treatment was included, the reference line Bd 21-3. Each line was harvested at three time-points: 10, 18 and 26 DAP. Every *Line*Days to harvest* treatment was sown in three randomised locations within a flow tray.

The third screening experiment was of the same design as for the second experiment, but treatments were only harvested at a single time-point, at 26 DAP. Control treatments were only included for four of the eleven lines, including line Bd 21-3.

4.2.9.2 Confirmation experiment

The confirmation experiment included both control and *R. solani*-infested *Line*Inoculum* treatments for every line. Six replicates per treatment were sown in randomised locations within each flow tray and harvested at 26 DAP. Lines carrying four T-DNA insertion events were included in this experiment, with additional lines included for events 5088 and 2596.

4.2.10 Statistical analysis

Experiments were set up to test the *Line* (genotype) x *Inoculum* interaction in randomized block designs across two flow trays, with each flow tray considered to be one block. The trays were placed on upper and lower shelves within the same growth cabinet.

Statistical analyses were carried out in GenStat (VSN International, UK) using a linear mixed model (REML), which is suitable for unbalanced data sets. Measurements were removed from analysis if the plant failed the toothpick re-isolation check (§4.2.6) or did not emerge within 6 DAP.

4.2.10.1 Analysis methods

Two analysis methods were used to rank resistance in this chapter. Abbreviations used to describe these different methods are summarised in Table 4.2.

Relative resistance of lines was measured by comparing the ratio of the predicted means of square-root transformed *R. solani*-infested/control total root length using *Method A* ($\sqrt{RLR_A}$), described in §3.2.9.1. In the first screening experiment predicted means were calculated using a linear mixed model (REML) in GenStat written as

Fixed Model: *Days to emergence + Line*Inoculum*

Random Model: *Experiment/Tray*

Total root length measurements were square-root transformed prior to analysis.

The pair-wise *Line*Inoculum* interaction for individual T-DNA lines and the reference line Bd 21-3 was calculated by including only the data for those lines in the analysis. This model was also used for the confirmation experiment, omitting the term *Experiment*.

A second analysis strategy, *Method B*, was developed using data in the Chapter 3 screening method to compare lines using only *R. solani*-inoculated treatment values (RLR_B, §3.2.9.1). The untransformed total root length values were averaged and divided by the Bd 21-3 reference line value within the same tray using Excel (Microsoft). Average seed mass was included as this was found to improve correlation with *Method A* resistance rankings, particularly for root length, by accounting for some of the endogenous plant vigour. To test *Method B* with data from the first screening experiment, predicted means were calculated using a REML analysis written as

Fixed Model: *Average days to emergence + Average seed mass+ Line*

Random Model: *Experiment/Tray*

A greater ratio of line/Bd 21-3 was used as an indicator of increased resistance to *R. solani*. This model was also used for the third screening experiment, but without the term *Experiment*.

The *Method B* model for the second screening experiment was written as

Fixed Model: *Average days to emergence + Average seed mass+ Days to Harvest*Line*

Random Model: *Tray*

In this experiment the variance component for *Tray* was negative (-0.00048), so the term was bound to zero. This may partly be due to one line, 8634-6, having a strong growth response in the second tray at all time-points, while the other lines did not. Daytime soil temperatures in Tray 2 were on average 1°C lower than in Tray 1. During the first eleven days of the experiment Tray 2 had 1 h shorter day length than Tray 1, due to an undetected cabinet fault.

The effect of moss growth on plant-pathogen interactions was investigated in the confirmation experiment with the model written as

Fixed Model: *Average days to emergence + Line*Inoculum*Moss growth*

Random Model: *Tray*

Table 4.2 Abbreviations used to describe root length and root length ratios. These ratios are the basis for comparing resistance to *R. solani* in *B. distachyon* lines.

Abbreviation	Measurement	Notes
TRL	Total root length	
$\sqrt{RLR_A}$	Square-root transformed root length ratio (*Method A*)	Ratio of the predicted means for *R. solani*-infested/control square-root transformed TRL using *Method A*. This is the primary method used to rank *B. distachyon* lines for increasing resistance to *R. solani*.
RLR_A	Root length ratio (*Method A*)	Ratio of means of *R. solani*-infested/control untransformed TRL using *Method A*. This ratio is used to compare results in this chapter with observations from the literature.
RLR_B	Root length ratio (*Method B*)	Ratio of the line/Bd 21-3 predicted means for untransformed TRL using *Method B*. This is an alternative method tested with the aim to reduce the number of plants per line required for screening experiments.

4.3 Results

Results of the screening and confirmation experiments describe variation in plant growth due to growth cabinet conditions, followed by variation in resistance to *R. solani* AG8 attributable to genetic differences in T-DNA mutant lines.

4.3.1 Screening experiments

4.3.1.1 Spatiotemporal variation in plant growth

In the previous chapter (§3.3.1.1) it was seen that plant growth varied across experiments due to variations in growth cabinet conditions. Some spatiotemporal variation in growth was also present in experiments for this chapter, as seen in the variation of root length and leaf number at harvest for the three individual experiments within the first screening experiment (Figure 4.1). Variation of growth due to cabinet conditions between screening experiments in this chapter was lower than in the natural accession experiments. This is likely due to smaller growth cabinets with better temperature control and the location of these growth cabinets inside a climate-controlled room.

Differences in mean root length and leaf number across experiments were accounted for by including *Experiment* as a random factor term in statistical analysis.

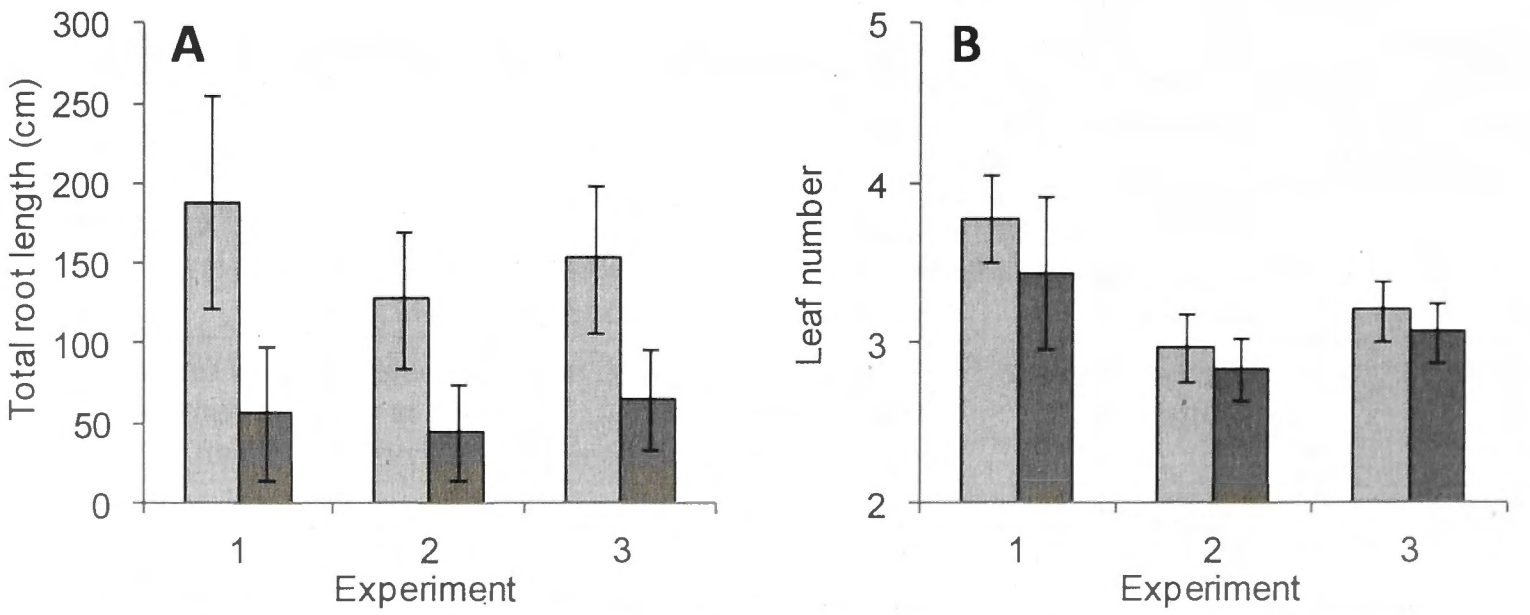

Figure 4.1 Overall variation due to growth cabinet conditions between individual experiments within the first screening experiment for a) total root length, and b) leaf number. Each bar represents the combined mean of the means of all 11 lines in an experiment with control (light grey) and *R. solani*-infested (dark grey) treatments. Every line mean was of up to 3 plants, SD.

4.3.1.2 Variation across T-DNA lines

Three screening experiments revealed some variation in levels of resistance to *R. solani* AG8, as well as variation in endogenous growth of different T-DNA lines under control conditions. Twenty-five lines representing 19 T-DNA insertion events in the Bd 21-3 wild type background were included in the screening experiments, with the reference line Bd 21-3 included in every experiment.

4.3.1.2.1 First screening experiment

The plant-pathogen response varied significantly across the twelve lines included in the first screening experiment for square-root transformed total root length (√TRL, $p=0.002$) and leaf number ($p=0.025$), but not for leaf lengths (Figures 4.2 and 4.3, Tables 4.3 and 4.4).

The lines most resistant to *R. solani* AG8 were 77-5, 3794-5 and 5088-4, with greater ($p<0.05$) *R. solani*/control root length ratios ($\sqrt{RLR_A}$) than the reference line Bd 21-3 (Table 4.4). These lines also maintained leaf number in *R. solani* treatment not significantly different from the control. Leaf 2 lengths for lines 3794-5 and 5088-4 in *R. solani* were also not significantly different from the control, indicating that the impact of the root disease on leaf measurements was lower in these lines. Leaf 1 length was not affected by *R. solani* in any line.

The increased resistance ratio of 5088-4 appeared to be partly due to significantly lower root length growth than the reference line in the control treatment ($p<0.05$). Lines 2426-11 and 4774-6 also had significantly ($p<0.05$) greater and lower root growth than Bd 21-3 in control, respectively.

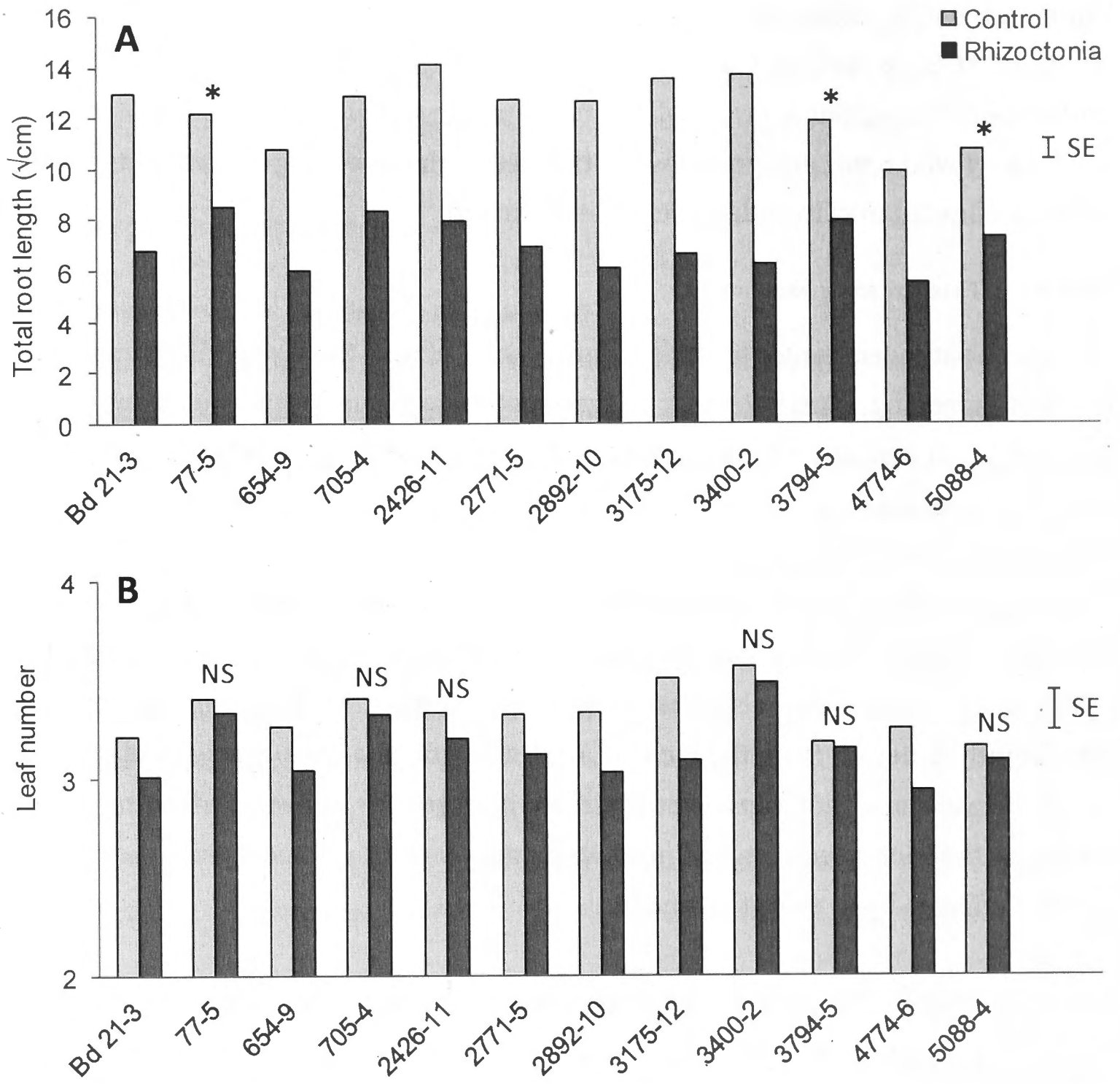

Figure 4.2 First screening activity phenotype measurements. Predicted means for a) total root length and b) leaf number, for 12 lines grown in control (light grey) and *R. solani* (dark grey) inoculated soil. Each line-pathogen treatment was sown in 3 experiments, except 3400-2 (one experiment) and 654-9 (2 experiments); n≤18, except 654-9 (n≤11) and 3400 (n≤6); average SE. Values are given in Table 4.3.
*Pair-wise line response to *R. solani* is significantly different ($p<0.05$) from the reference Bd 21-3 for total root length.
NS, no significant difference between *R. solani* and control treatments at 5% LSD.

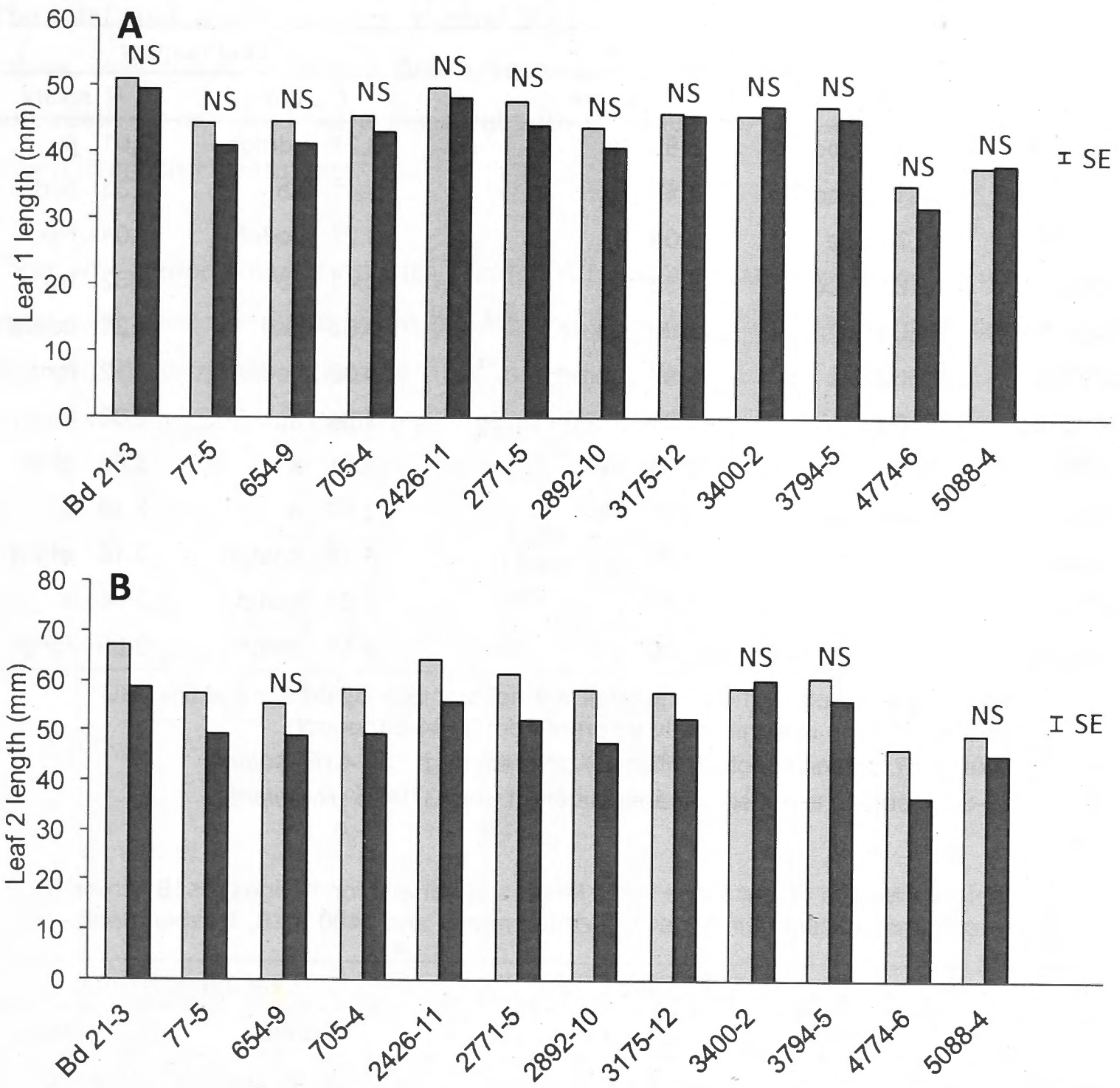

Figure 4.3 First screening activity phenotype measurements. Predicted means for a) leaf 1 length length and b) leaf 2 length, for 12 lines grown in control (light grey) and *R. solani* (dark grey) inoculated soil. Each line-pathogen treatment was sown in 3 experiments, except 3400-2 (one experiment) and 654-9 (2 experiments); n≤18, except 654-9 (n≤11) and 3400 (n≤6); average SE. Values are given in Table 4.4.
NS, no significant difference between *R. solani* and control treatments at 5% LSD.

Table 4.3 Means of total root length and leaf number at harvest for 12 lines; n≤18 across 3 experiments, except 654-9 (n≤11, 2 experiments) and 3400 (n≤6, 1 experiment).

Line	Total root length (√cm)[1]				Leaf number[1]			
	Control		*R. solani*		Control		*R. solani*	
Bd 21-3	12.98	abc	6.81	hij	3.21	cdefgh	3.01	jk
77-5	12.17	bcd	8.52	fg	3.41	ab	3.33	bcd[4]
654-9	10.76	de	6.04	ij	3.27	bcdef	3.04	hijk
705-4	12.85	abc	8.33	fg	3.41	ab	3.32	bcde
2426-11	14.09	a	7.98	gh	3.33	bcd	3.21	cdefgh
2771-5	12.68	bc	6.97	ghij	3.32	bcd	3.12	fghij
2892-10	12.66	abc	6.06	ij	3.34	bc	3.03	ijk
3175-12	13.46	ab	6.64	hij	3.51	a	3.09	ghijk
3400-2	13.69	ab[2]	6.28	hij[3]	3.57	a	3.49	ab
3794-5	11.83	cd	7.96	gh	3.18	cdefghi	3.16	efghij
4774-6	9.89	ef	5.53	j	3.25	bcdefg	2.94	k
5088-4	10.76	de	7.30	ghi	3.16	defghij	3.09	fghijk

[1]Numbers followed by the same letter are not significantly different at 5% LSD
[2]3400-2 (control) is significantly different from 3794-5 (control).
[3]3400-2 (*R. solani*) is not significantly different from 705-4 (*R. solani*).
[4]77-5 (*R. solani*) is not significantly different from 3794-5 (*R. solani*).

Table 4.4 Means of leaf 1 and leaf 2 lengths at harvest for 12 lines; n≤18 across 3 experiments, except 654-9 (n≤11, 2 experiments) and 3400 (n≤6, 1 experiment).

Line	Leaf 1 length (mm)[1]				Leaf 2 length (mm)[1]			
	Control		*R. solani*		Control		*R. solani*	
Bd 21-3	50.9	a	49.5	abc	67.2	a	58.6	cd
77-5	44.4	defghijk	41.1	ijk[2]	57.4	cde	49.4	fghi
654-9	44.6	defghijk	41.3	hijk[3]	55.3	def	48.8	fghi
705-4	45.4	defgh	42.9	ghijk	58.1	cd	49.5	fghi
2426-11	49.9	ab	48.1	abcd	64.5	ab	56.0	cde
2771-5	47.7	abcde	44.1	efghijk	61.4	bc	52.3	efgh[4]
2892-10	43.6	fghijk	40.6	jk[2]	58.4	cd	47.7	ghi
3175-12	45.9	cdefg	45.6	defgh	58.0	cd	52.5	efg[5]
3400-2	45.4	bcdefgh	46.9	abcdefg	58.5	bcd	60.1	abcd
3794-5	47.0	bcdef	45.1	defghi	60.5	bcd	56.4	cde
4774-6	35.3	lm	31.9	m	46.5	hi	37.0	j
5088-4	38.0	kl	38.3	kl	49.2	fghi	45.2	i

[1]Numbers followed by the same letter are not significantly different at 5% LSD
[2]3400-2 (control) is not significantly different from 77-5 (*R. solani*) and 2892-10 (*R. solani*).
[3]654-9 (*R. solani*) is not significantly different from 3400-2 (*R. solani*).
[4]3400-2 (control) and (*R.* solani) are not significantly different from 2771-5 (*R. solani*).
[5]3400-2 (control) and (*R.* solani) are not significantly different from 3175-12 (*R. solani*).

4.3.1.2.2 An alternative resistance ranking method (Method B) using only *R. solani* treatment

The total root length ranking *Method B* (§4.2.10.1), using only *R. solani* treatment values, was tested with the first screening experiment data set. More lines can potentially be screened per experiment with this method, as plants do not need to be grown in control treatments.

There was a good correlation with *Method A* resistance rankings based on root length when seed mass was included in the *Method B* model (p=0.005), as seen earlier with the natural accession screening data in §3.4.1.2 (Figure 4.4). Seed mass essentially becomes a substitute for endogenous plant vigour.

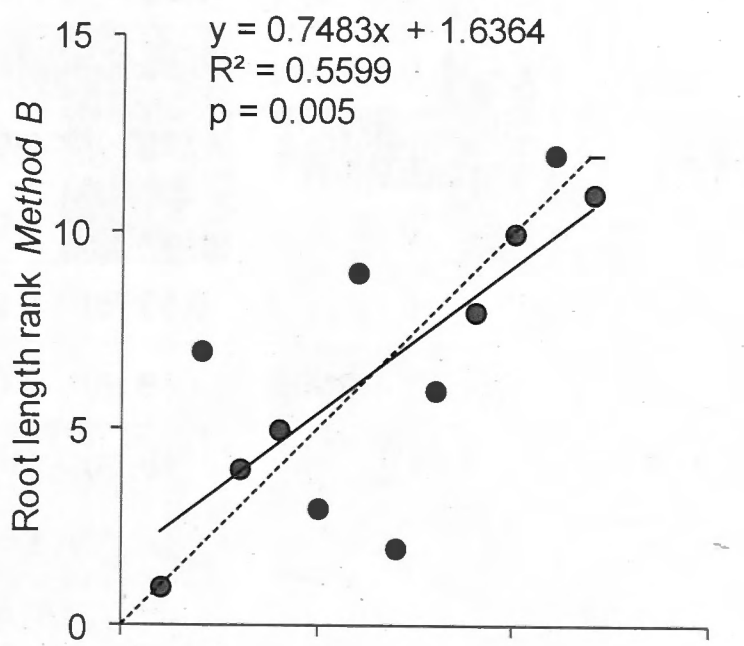

Figure 4.4 Correlation between resistance rankings based on total root length using *Method A* ($\sqrt{RLR_A}$) and *Method B* (RLR_B). Rankings are from 1, least resistant, to 12, most resistant; dotted line, y=x.

Using total root length ratio *Method B* (RLR_B), the two most resistant lines were 5088-4 and 77-5, with ratios of 1.86 and 1.82, respectively (Table 4.5). These were also the two most resistant lines using $\sqrt{RLR_A}$. Refer to Table 4.2 in §4.2.10.1 for explanation of abbreviations.

While ratios of leaf 1 length and leaf 2 length were correlated ($p<0.05$) within each method, leaf length ratios did not correlate with rankings based on root length or leaf number ratios.

Table 4.5 Summary of rankings using different methods for measuring resistance to *R. solani*. Rankings 1 to 12 (in parentheses) are from least to most resistant; green, more resistant; orange, less resistant. $\sqrt{RLR_A}$, *Method A* square-root transformed root length ratio; RLR_B, *Method B* root length ratio; L#, leaf number ratio; L1, leaf 1 length ratio; L2, leaf 2 length ratio.

Line	*Method A* - ratios of *R. solani*/control treatment from linear mixed model predicted means; (*rank*)				*Method B* – ratios of *R. solani* treatment (line mean)/(Bd 21-3 mean) from linear mixed model predicted means; (*rank*)			
	$\sqrt{RLR_A}$	L#	L1	L2	RLR_B	L#	L1	L2
3400-2	0.46 *(1)*	0.98 *(11)*	1.03 *(12)*	1.03 *(12)*	0.42 *(1)*	1.08 *(11)*	1.16 *(10)*	1.24 *(12)*
2892-10	0.48 *(2)*	0.91 *(3)*	0.93 *(5)*	0.82 *(2)*	0.98 *(7)*	0.98 *(6)*	0.95 *(4)*	0.94 *(4)*
3175-12	0.49 *(3)*	0.88 *(1)*	0.99 *(10)*	0.90 *(9)*	0.92 *(4)*	0.97 *(4)*	1.08 *(9)*	1.06 *(7)*
Bd 21-3	**0.52 *(4)***	**0.94 *(5)***	**0.97 *(9)***	**0.87 *(7)***	**0.93 *(5)***	**0.95 *(1)***	**1.17 *(11)***	**1.18 *(10)***
2771-5	0.55 *(5)*	0.94 *(6)*	0.92 *(2)*	0.85 *(3)*	0.90 *(3)*	0.96 *(3)*	1.07 *(7)*	1.08 *(8)*
4774-6	0.56 *(6)*	0.90 *(2)*	0.91 *(1)*	0.79 *(1)*	1.15 *(9)*	0.98 *(7)*	0.72 *(1)*	0.71 *(1)*
654-9	0.56 *(7)*	0.93 *(4)*	0.93 *(4)*	0.88 *(8)*	0.87 *(2)*	0.97 *(5)*	0.94 *(3)*	0.90 *(3)*
2426-11	0.57 *(8)*	0.96 *(7)*	0.96 *(8)*	0.87 *(6)*	0.93 *(6)*	0.95 *(2)*	1.17 *(12)*	1.18 *(11)*
705-4	0.65 *(9)*	0.97 *(8)*	0.95 *(6)*	0.85 *(4)*	1.08 *(8)*	1.01 *(8)*	1.07 *(8)*	1.05 *(6)*
3794-5	0.67 *(10)*	0.99 *(12)*	0.96 *(7)*	0.93 *(11)*	1.72 *(10)*	1.03 *(9)*	1.04 *(6)*	1.09 *(9)*
5088-4	0.68 *(11)*	0.98 *(10)*	1.01 *(11)*	0.92 *(10)*	1.86 *(12)*	1.07 *(10)*	0.83 *(2)*	0.83 *(2)*
77-5	0.70 *(12)*	0.98 *(9)*	0.93 *(3)*	0.86 *(5)*	1.82 *(11)*	1.08 *(12)*	0.97 *(5)*	1.01 *(5)*

4.3.1.2.3 Second screening experiment

Several changes were made to the screening protocol between the first screening experiment and the subsequent screening experiments, as *Method B* analysis appeared to be a useful initial measure of resistance rankings of different lines. The reference line Bd 21-3 was included in the control treatment, with all other lines sown only in the *R. solani* inoculated treatment.

In the second screening experiment lines were sown on the same date and harvested at 10, 18 and 26 days after planting. During method development in Chapter 2, an infection time-course experiment (§2.3.2.1) showed that root length was reduced significantly by *R. solani* from 10 DAP.

Only one line had a significantly different line-pathogen interaction from the reference line, and only at one harvest time-point. Line 9212-15 had a significantly ($p<0.05$)

larger RLR_B than Bd 21-3 at 26 DAP (Figure 4.5). Resistance rankings varied considerably, with little correlation between harvest dates.

Reference line Bd 21-3 was included in a control treatment. It was noted that the transformed *R. solani*/control root length ratio ($\sqrt{RLR_A}$) for Bd 21-3 was higher than in previous experiments, at 0.74 and 0.70 for the 18 and 26 DAP harvest, respectively. The first screening experiment ratio of 0.52 at 22 DAP was similar to the average ratio of 0.50 at 22 DAP for Bd 21-3 in Chapter 3 screening experiments 1, 4, 5, 6, 7 and 8. The higher root length ratios of 0.79 seen in Chapter 3 screening experiments 2 and 3 coincided with the growth of an unidentified fungus. There was, however, no evidence of contamination in this second screening experiment.

The time-course experiment was replaced with greater replication at a single time-point in subsequent experiments, to increase statistical power.

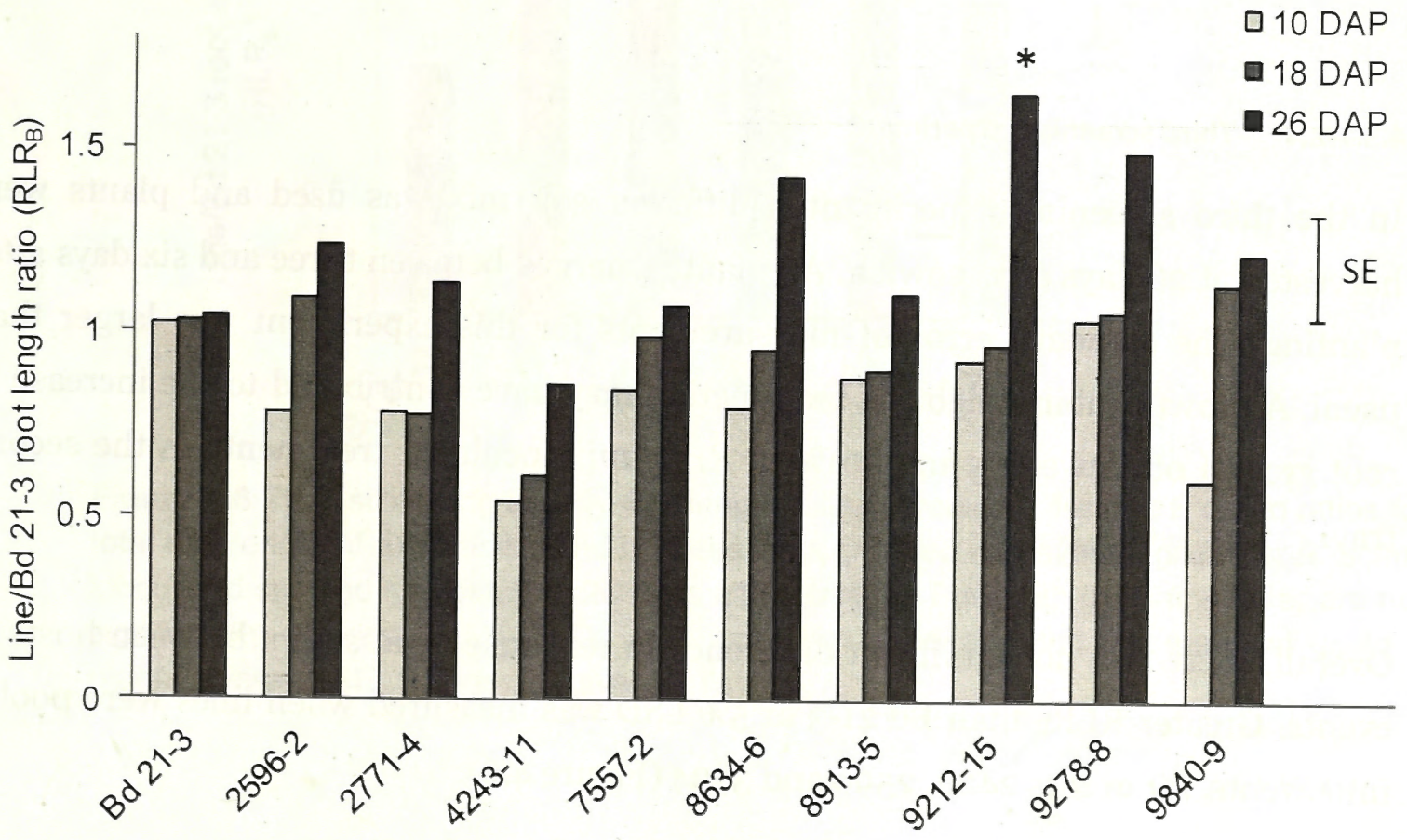

Figure 4.5 Second screening activity root length ratios. Predicted *Method B* mean ratios for total root length of 10 accessions grown in *R. solani* inoculated soil and harvested at 10, 18 and 26 days after planting; n=2, with every n being the mean of up to 3 plants; average SE. Values are given in Table 4.6.
*Line is different from the reference line Bd 21-3 at the same time-point at 5% LSD.

Table 4.6 Summary of *Method B* line/Bd 21-3 total root length ratios (RLR_B) for harvest time-points 10, 18 and 26 days after planting. Rankings increase from least to most resistant.

Line	RLR_B; (*rank*)		
	10 DAP	18 DAP	26 DAP
Bd 21-3	1.02 *(9)*	1.03 *(7)*	1.04 *(2)*
2596-2	0.78 *(3)*	1.09 *(9)*	1.23 *(7)*
2771-4	0.78 *(4)*	0.78 *(2)*	1.13 *(5)*
4243-11	0.54 *(1)*	0.61 *(1)*	0.85 *(1)*
7557-2	0.84 *(6)*	0.99 *(6)*	1.07 *(3)*
8634-6	0.79 *(5)*	0.96 *(4)*	1.42 *(8)*
8913-5	0.88 *(7)*	0.90 *(3)*	1.10 *(4)*
9212-15	0.92 *(8)*	0.97 *(5)*	1.65 *(10)*
9278-8	1.03 *(10)*	1.06 *(8)*	1.49 *(9)*
9840-9	0.60 *(2)*	1.13 *(10)*	1.21 *(6)*

4.3.1.2.4 Third screening experiment

In the third screening experiment a different soil mix was used and plants were harvested at 26 days after sowing. All plants emerged between three and six days after planting. The standard error of measurements for this experiment was larger than usual. A growth cabinet lighting malfunction may have contributed to the increase in root growth of between 5 and 201% in *R. solani* inoculated treatments in the second tray.

Overall, there was no significant difference in response to *R. solani* between hosts or events. Greater RLR_B than Bd 21-3 at 5% LSD was measured when lines were pooled into events, for events 2426, 2596 and 3794 (Figure 4.6).

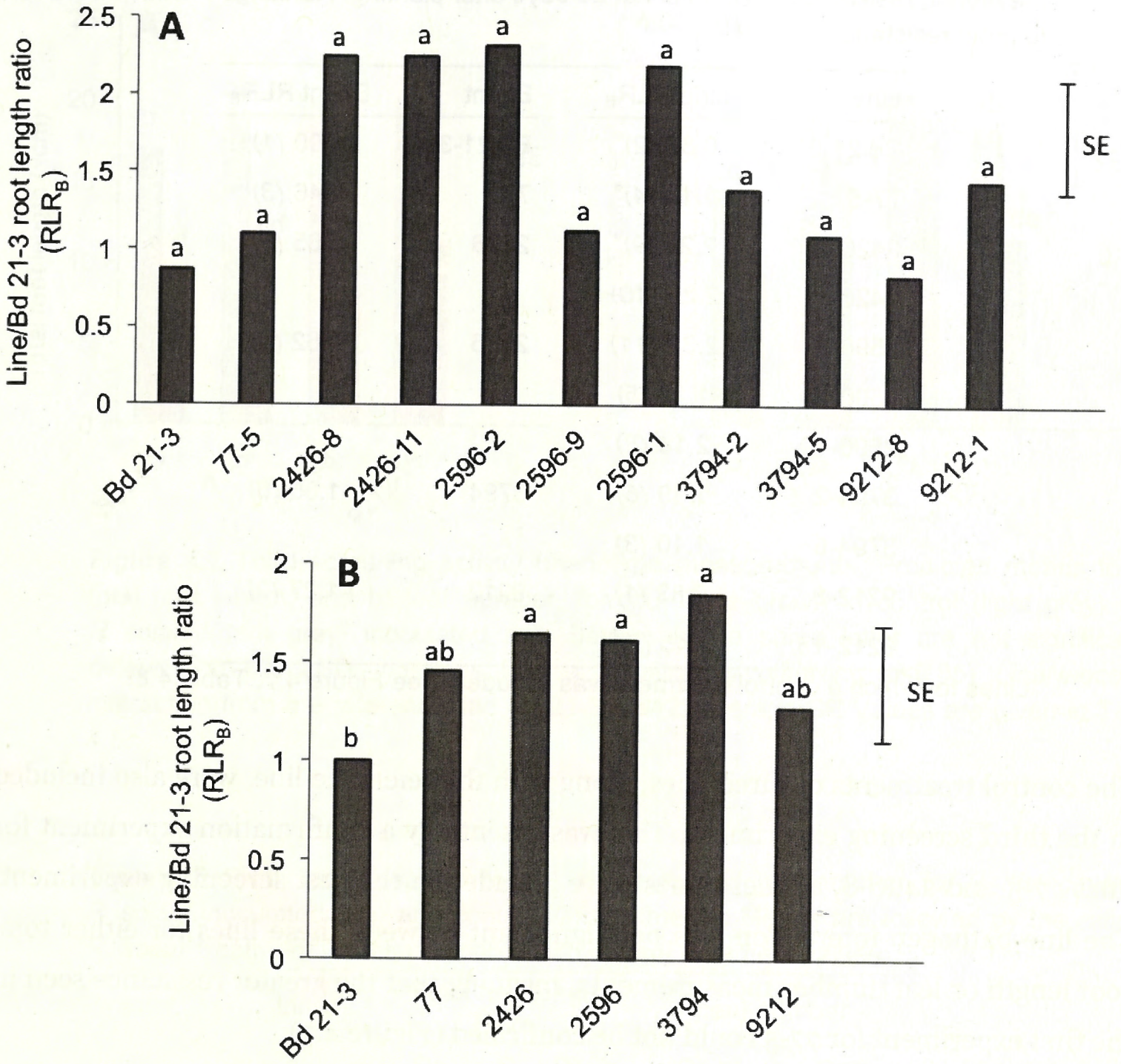

Figure 4.6 Third screening activity root length ratios. Predicted *Method B* mean ratios for total root length of a) 11 lines, and b) these lines grouped into events, grown in *R. solani* inoculated soil and harvested at 26 days after planting. Bars with the same letter are not significantly different at 5% LSD; in a) n=2, with every n being the mean of up to 6 plants; average SE. Values are given in Table 4.7.

Table 4.7 Summary of *Method B* (line/Bd 21-3) total root length ratios (RLR_B) for measuring resistance to *R. solani* at 26 days after planting. Rankings increase from least to most resistant.

Line	Line RLR_B	Event	Event RLR_B
Bd 21-3	0.87 *(2)*[a]	Bd 21-3	1.00 *(1)*
77-5	1.10 *(4)*[a]	77	1.46 *(3)*
2426-8	2.25 *(9)*[a]	2426	1.65 *(5)*
2426-11	2.25 *(10)*		
2596-2	2.32 *(11)*	2596	1.62 *(4)*
2596-9	1.12 *(5)*		
2596-10	2.18 *(8)*		
3794-2	1.39 *(6)*	3794	1.85 *(6)*
3794-5	1.10 *(3)*		
9212-8	0.83 *(1)*	9212	1.27 *(2)*
9212-15	1.45 *(7)*[a]		

[a]Lines for which a control treatment was included (see Figure 4.7, Table 4.8)

The control treatments of three lines, along with the reference line, were also included in the third screening experiment. This was essentially a confirmation experiment for lines 77-5 and 2426-8, as these lines were included in the first screening experiment. The line-pathogen interaction was not significant between these lines for either total root length or leaf number measurements, meaning that the greater resistance seen in the first experiment for 77-5 could not be confirmed (Figure 4.7).

Line 9212-15 had a significantly different resistance response from the reference line at the 26 DAP time-point in the second screening experiment. In the third screening experiment this line was not more resistant than the reference line, but did display greater endogenous root growth than Bd 21-3 ($p<0.05$). Comparison of untransformed predicted means for root length showed that 9212-15 produced 37% more root length than Bd 21-3 in the control treatment.

Likewise, although line 2426-8 was not more resistant to *R. solani* in the third screening experiment, it mirrored the increased endogenous root length of line 2426-11 in the first screening experiment ($p<0.05$). Comparing untransformed predicted means, line 2426-11 had 20% greater TRL than Bd 21-3 in the first screening experiment, while line 2426-8 had 32% greater TRL in the third screening experiment.

Leaf number at harvest was significantly greater in the control treatment for the three transformed lines, compared with Bd 21-3 ($p<0.05$).

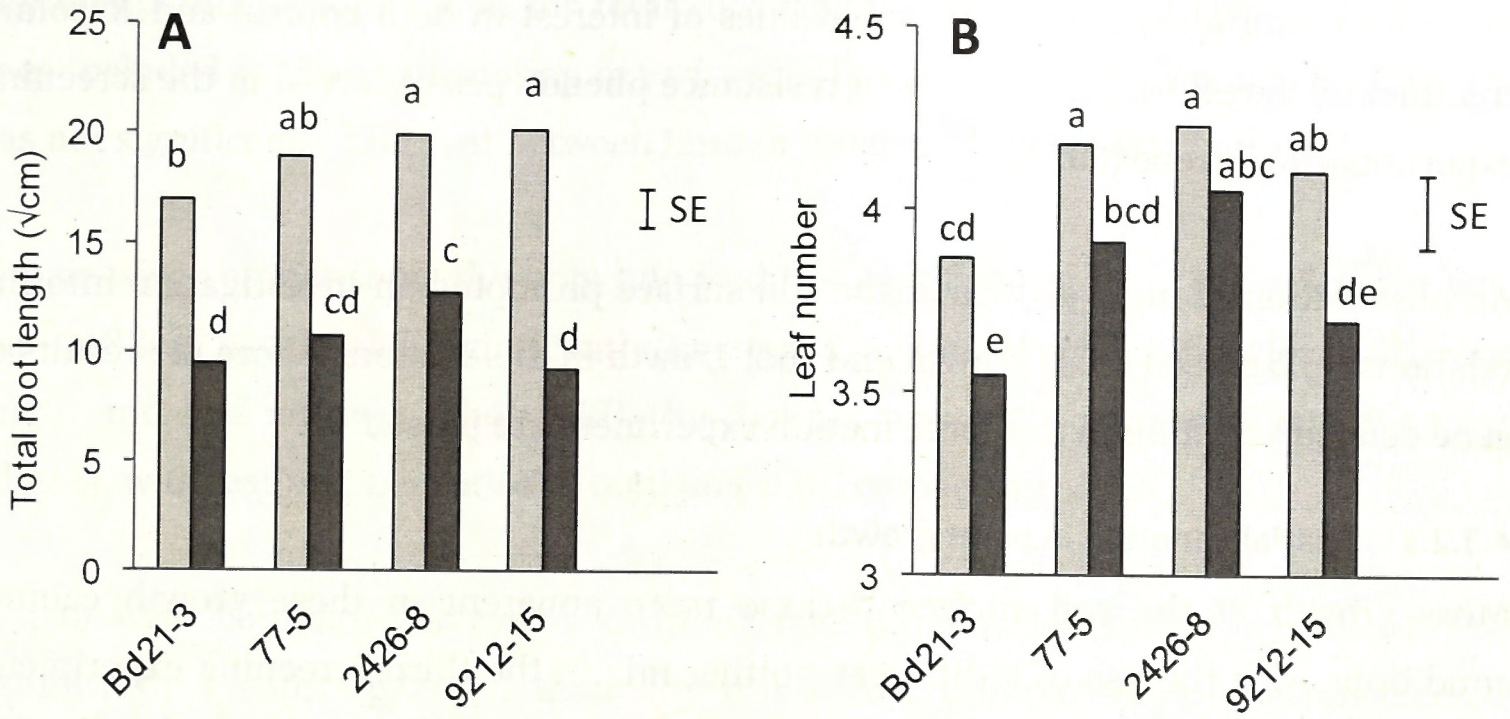

Figure 4.7 Third screening activity phenotype measurements. Predicted means for a) total root length, and b) leaf number, for four lines grown in control (light grey) and *R. solani* (dark grey) inoculated soil. Bars with the same letter are not significantly different at 5% LSD; no lines had a significantly different ($p<0.05$) *Line*Inoculum* interaction from the reference line Bd 21-3; n≤12; average SE Values are given in Table 4.8.

Table 4.8 Predicted means of total root length and leaf number for *B. distachyon* in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values.

Line	Total root length (√cm)			Leaf number		
	Rs	C	Ratio	Rs	C	Ratio
9212-15	9.2	20.2	0.45	3.7	4.1	0.90
Bd 21-3	9.5	16.9	0.56	3.5	3.9	0.92
77-5	10.8	19.0	0.57	3.9	4.2	0.93
2426-8	12.7	19.9	0.64	4.1	4.2	0.96

4.3.2 Confirmation experiment

The confirmation experiment included lines of interest in both control and *R. solani* treatments, to test whether increased resistance phenotypes observed in the screening experiment were repeatable.

An observation of moss growth at the soil surface prompted an investigation into the relationship between moss growth and root growth in this section, before the results of gene-related variation in the confirmation experiment are presented.

4.3.2.1 Spatial variation in plant growth

Moss growth at the soil surface became more apparent in these growth cabinet conditions with the use of a different potting mix in the third screening experiment. During the confirmation experiment there was an obvious difference in the localization of moss growth. A visual score for moss growth was taken a 20 DAP, with a range of 0 (no moss growth) to 3 (maximum growth).

A contributing factor to moss growth was likely to be the higher light intensity in Tray 2 than Tray 1 (averaging around 785 and 655 μEinstein, respectively), with light intensity increasing toward the centre of each tray. Factors that may influence moss growth and also likely to be affected by light intensity, are local temperature and humidity. Local temperature and humidity measurements were not taken.

Correlations were calculated between moss growth scores at 20 DAP and phenotypic measurements taken at the conclusion of the experiment (26 DAP). Moss growth was significantly correlated with increased root length ($p=0.007$), greater leaf number at harvest ($p<0.001$) and more leaf nodal roots ($p<0.001$).

The significant *Inoculum*Moss* interaction for total root length points to greater root growth in control plants when moss was present, while there was no difference in root growth between mossy and bare pots for *R. solani* infected plants.

Importantly however, moss growth did not appear to affect the *Line*Inoculum* interaction as *Line*Inoculum*Moss* never became significant. This suggests spatial randomisation of lines and treatments was effective.

It should be noted that these results cannot be used to suggest that greater plant growth was caused by the presence of moss or vice versa. An environmental factor may have affected both measurements.

4.3.2.2 Variation across lines

The line-pathogen interaction for total root length was significant ($p<0.001$) for the lines included in the confirmation experiment. The leaf number response to *R. solani* was not significantly different between lines in the overall analysis (Figure 4.8).

In pair-wise comparisons, the only line to differ significantly from the reference line was 5088-4, which had significantly greater *R. solani*/control ratios for both root length and leaf number. The $\sqrt{RLR_A}$ for 5088-4 was 0.70 compared with 0.53 for Bd 21-3, with leaf number ratios of 0.98 and 0.91, respectively.

In pair-wise comparisons root growth of line 9212-15 in control treatment was significantly greater ($p<0.05$) than any other line. Comparison of untransformed predicted means for root length shows that TRL for 9212-15 was 19% greater than for Bd 21-3 in control treatment. Leaf number was also greater ($p<0.05$) than the reference line for 9212-15 control treatment.

Root lengths in 5088-2 and 5088-4 were 20% and 30% lower than the reference line in the control treatment, respectively, according to linear mixed model analysis of untransformed values ($p<0.05$). In the first screening experiment, line 5088-4 had 26% less total root length than Bd 21-3 ($p<0.05$). There was no significant difference in control leaf number compared with Bd 21-3 for lines of event 5088.

The line-pathogen interaction for total root length remained significant ($p<0.001$) when lines were pooled into T-DNA insertion events, with the interaction also becoming significant ($p=0.024$) for leaf number (Figure 4.9). Again, only event 5088 was significantly more resistant that the reference line based on root length and leaf number measurements.

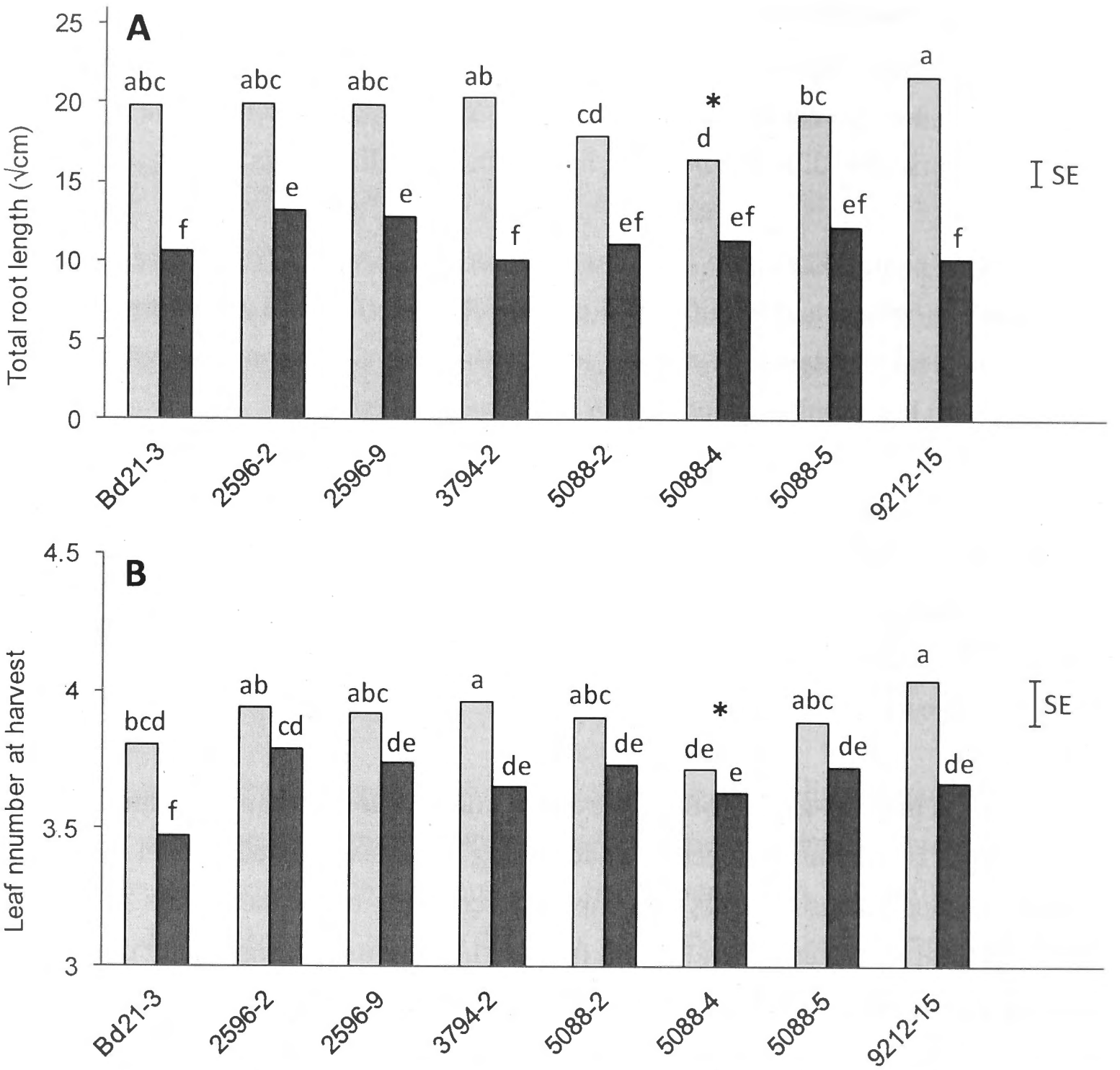

Figure 4.8 Confirmation activity phenotype measurements. Predicted means for a) total root length, and b) leaf number, for eight lines grown in control (light grey) and *R. solani* (dark grey) inoculated soil (n≤12). Bars with the same letter are not significantly different at 5% LSD; lines with an asterisk had a significantly different ($p<0.05$) *Line*Inoculum* interaction to the reference line Bd 21-3; average SE. Values are given in Table 4.9.

Table 4.9 Predicted means of total root length and leaf number for *B. distachyon* in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values.

Line	Total root length (√cm)			Leaf number		
	Rs	C	Ratio	Rs	C	Ratio
Bd 21-3	11	20	0.53	3.5	3.8	0.91
2596-2	13	20	0.66	3.8	3.9	0.96
2596-9	13	20	0.64	3.7	3.9	0.96
3794-2	10	20	0.49	3.7	4.0	0.92
5088-2	11	18	0.62	3.7	3.9	0.96
5088-4	11	16	0.70	3.6	3.7	0.98
5088-5	12	19	0.63	3.7	3.9	0.96
9212-15	10	22	0.47	3.7	4.0	0.91

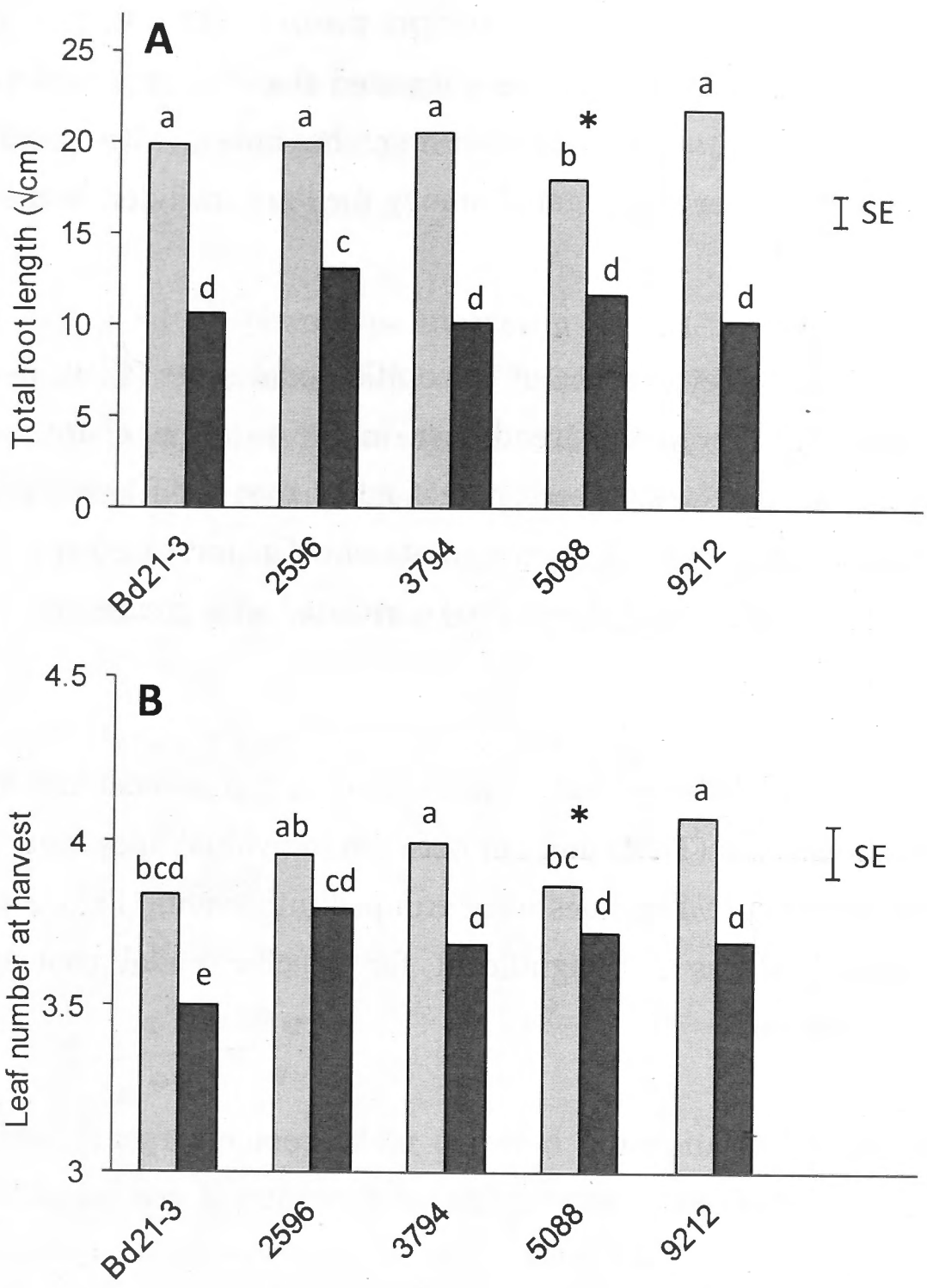

Figure 4.9 Confirmation activity phenotype measurements for lines pooled into insertion events. Predicted means for a) total root length, and b) leaf number, for four T-DNA insertion events grown in control (light grey) and *R. solani* (dark grey) inoculated soil. Bars with the same letter are not significantly different at 5% LSD; lines with an asterisk had a significantly different ($p<0.05$) *Line*Inoculum* interaction to the wild type Bd 21-3; average SE. Values given in Table 4.10.

Table 4.10 Predicted means of total root length and leaf number for *B. distachyon* in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values.

Line	Total root length (√cm)			Leaf number		
	Rs	C	Ratio	Rs	C	Ratio
Bd 21-3	11	20	0.53	3.5	3.8	0.91
2596	13	20	0.65	3.8	4.0	0.96
3794	10	21	0.49	3.7	4.0	0.92
5088	12	18	0.65	3.7	3.9	0.96
9212	10	22	0.47	3.7	4.1	0.91

4.3.2.3 Nodal root emergence

Results of the natural accession screen suggested that the emergence of leaf nodal roots in response to *R. solani* infection may be linked with greater resistance (§3.3.2.2). This was further investigated among the lines included in the confirmation activity for this chapter.

Here, an increase in the emergence of coleoptile nodal roots (CNR, $p<0.001$) in the *R. solani* treatment followed the trend seen in the previous chapter (§3.3.2.2). In contrast to the natural accession screen, leaf nodal root (LNR) emergence increased ($p=0.013$) across all lines in the *R. solani* treatment. The increased appearance of LNR was also found to be significantly ($p<0.001$) correlated with greater moss growth at the cone surface (§4.3.2.1).

In this confirmation experiment a significant ($p=0.022$) line-inoculum interaction was found for total nodal root (TNR) number between individual lines, but this interaction was no longer significant when lines were grouped into events (Figure 4.10). The line-inoculum interaction was not significant for specific nodal root types between individual lines or events.

Overall, the inferred relationship between nodal root emergence and resistance to *R. solani* in the confirmation experiment of the natural accession screen was not observed in this group of T-DNA lines.

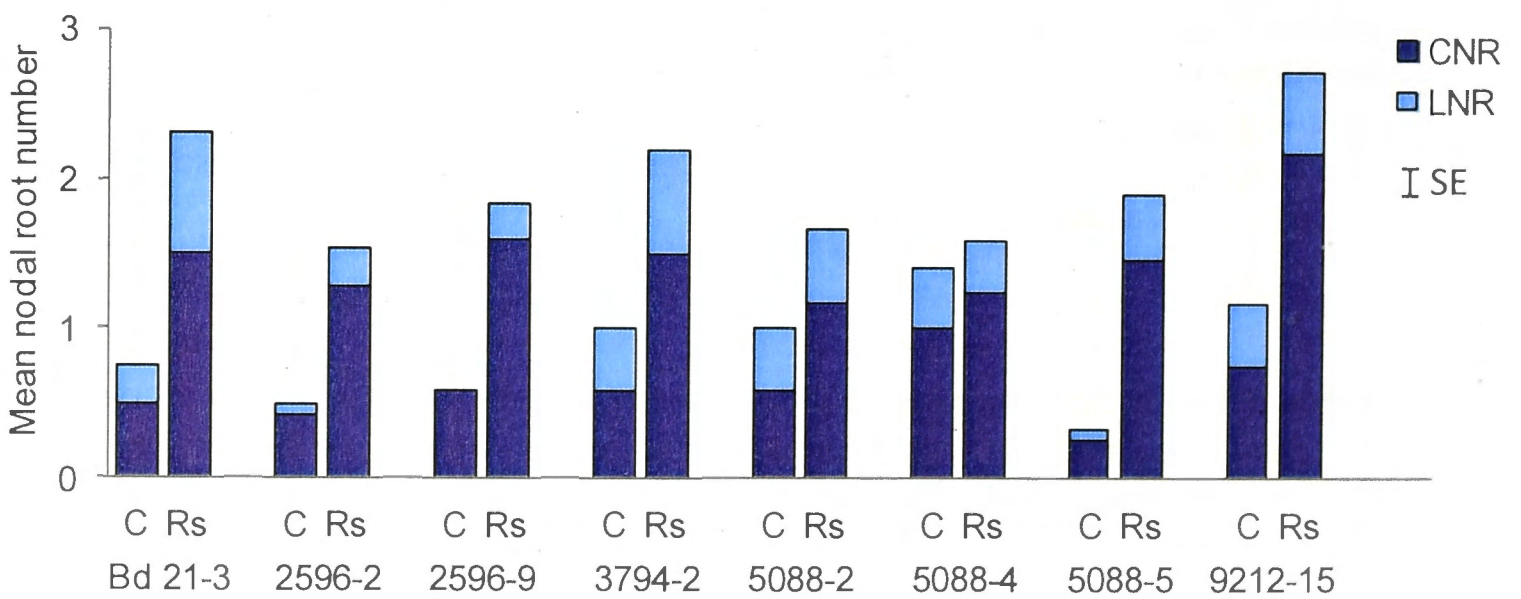

Figure 4.10 Emergence of leaf and coleoptile nodal roots in control (C) and *R. solani* infected (Rs) treatments for seven T-DNA insertion lines and the reference line Bd 21-3. CNR, coleoptile nodal root; LNR, leaf nodal root; n≤12; average SE

4.3.3 The effect of endogenous vigour on disease resistance

In the Chapter 3 natural accession screen a correlation was noted between endogenous seedling vigour, measured as seed mass or root length in the control treatment, and reduced resistance to *R. solani* (§3.3.1.3).

The effect of seed mass on phenotype measurements in the first T-DNA screening experiment mirrored the relationship with seedling vigour seen in the natural accession screen. Using a simple linear regression analysis, seed mass was again found to be correlated with increasing total root length and leaf number in control treatments, but not in *R. solani* treatments (Figure 4.11). Average seed mass also correlated with leaf length measurements in both treatments.

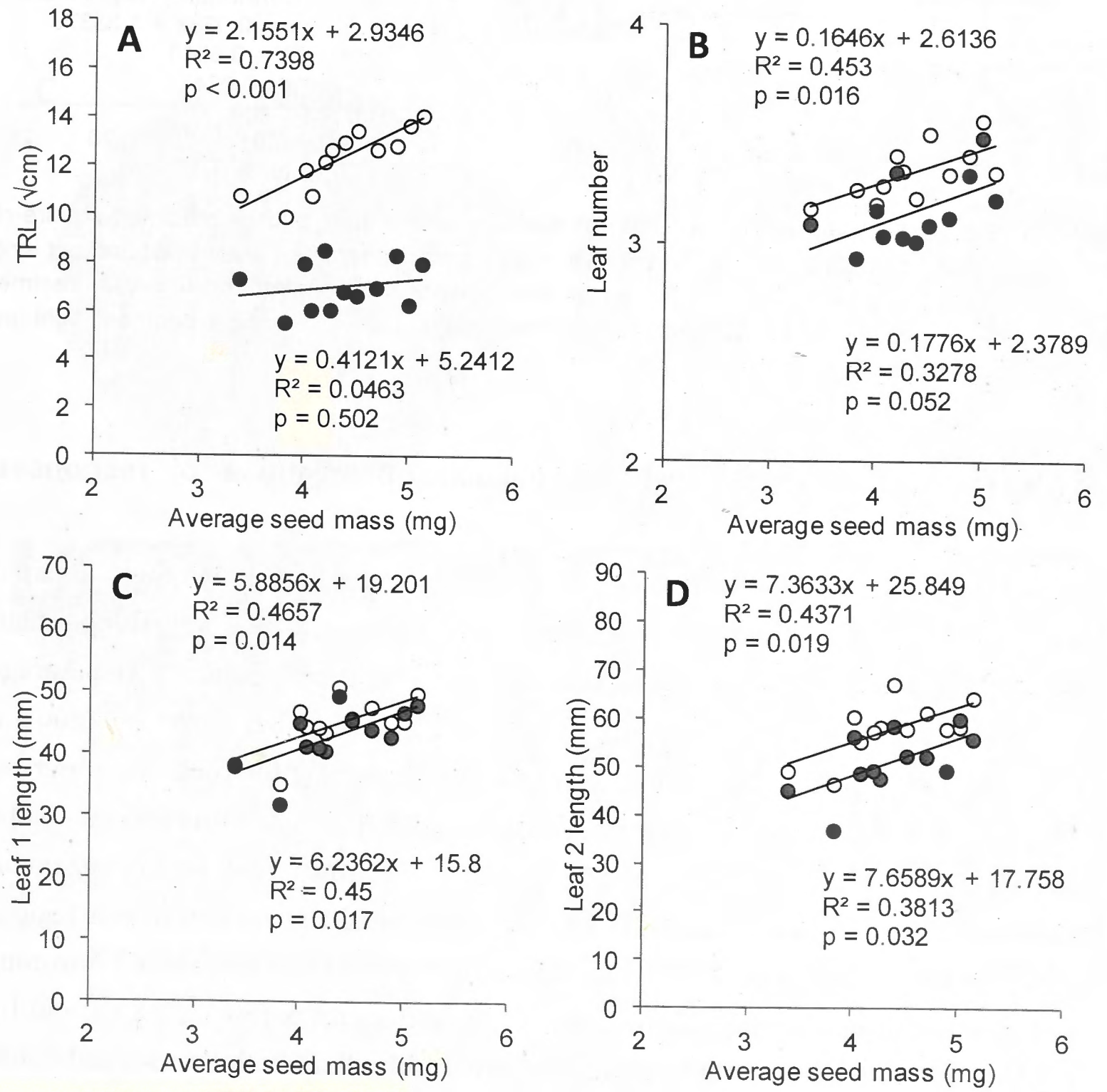

Figure 4.11 Correlations of root and shoot measurements with seed mass. Predicted means for a) total root length, b) leaf number, c) leaf 1 length, and d) leaf 2 length, plotted against average seed mass for 26 accessions grown in control (white circles) and *R. solani*-inoculated soil (grey circles).

In the natural accession screening activity $\sqrt{RLR_A}$ was negatively correlated with seed mass ($p=0.046$) and root length in the control ($p<0.001$) (§3.3.1.3). A similar trend was seen across the T-DNA experiments, but only control root length in the confirmation experiment correlated significantly with $\sqrt{RLR_A}$ (Figure 4.12).

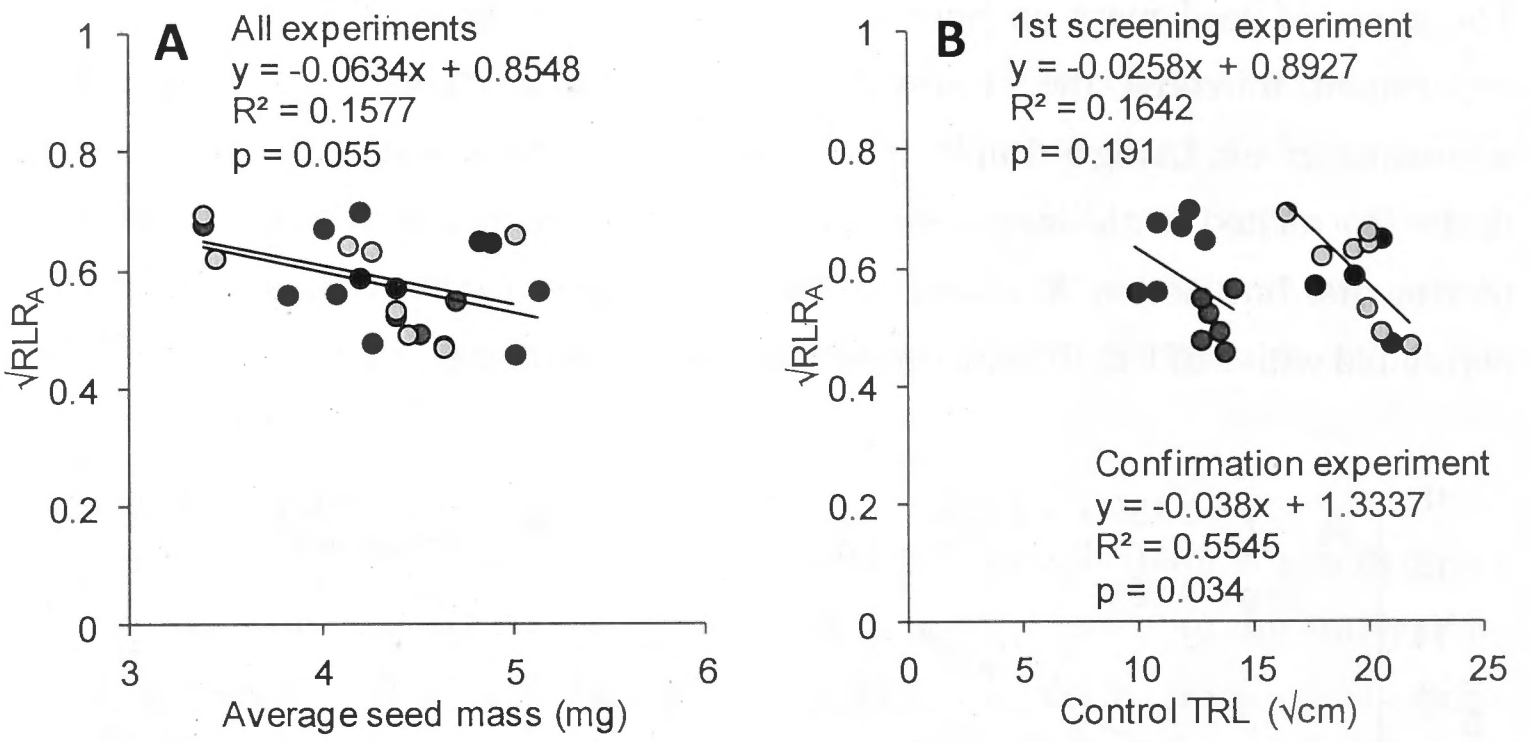

Figure 4.12 Correlation between a) average seed mass, and b) predicted square-root transformed total root length in control, with resistance rankings based on total root length ratios using *Method A* ($\sqrt{RLR_A}$) for all lines sown in both control and *R. solani* treatment. Dark grey circles, first screening experiment; black, third screening experiment; light grey, confirmation experiment.

4.3.4 General morphological and cellular observations of responses within infected roots

Growth on agar plates and response to *R. solani* 48 h after inoculation was compared between line 5088-4 and the reference line Bd 21-3. Roots of 5088-4 were thinner than those of Bd 21-3, even though root lengths were not significantly different. The average diameter of uninoculated roots of 5088-4 was 155 µm, while Bd 21-3 averaged 209 µm ($p=0.001$, LSD 30 µm). Light browning over larger sections of the roots was observed on uninoculated plates when roots grew aerially, indicating that this is also an abiotic stress response. Shorter (~5 mm) sections of dark brown root in the region of inoculation were observed mainly in *R. solani* inoculated roots. Dark brown regions appeared more often in Bd 21-3 than 5088-4. In cross-sections generalized browning was observed in epidermal cells and cells of the outer cortex (Figure 4.13 a and b). Hyphae of *R. solani* were seen to proliferate in cortical cells of both Bd 21-3 and 5088-4 without apparent cell deformation. Protoxylem appeared to mature earlier and fluoresce more strongly in Bd 21-3 than in 5088-4 (Figure 4.13 d).

As observed in Chapter 2, vacuolar inclusions of root border cells fluoresced strongly when excited at 420 – 490 nm (Figure 4.13 d and e) in both Bd 21-3 and 5088-4.

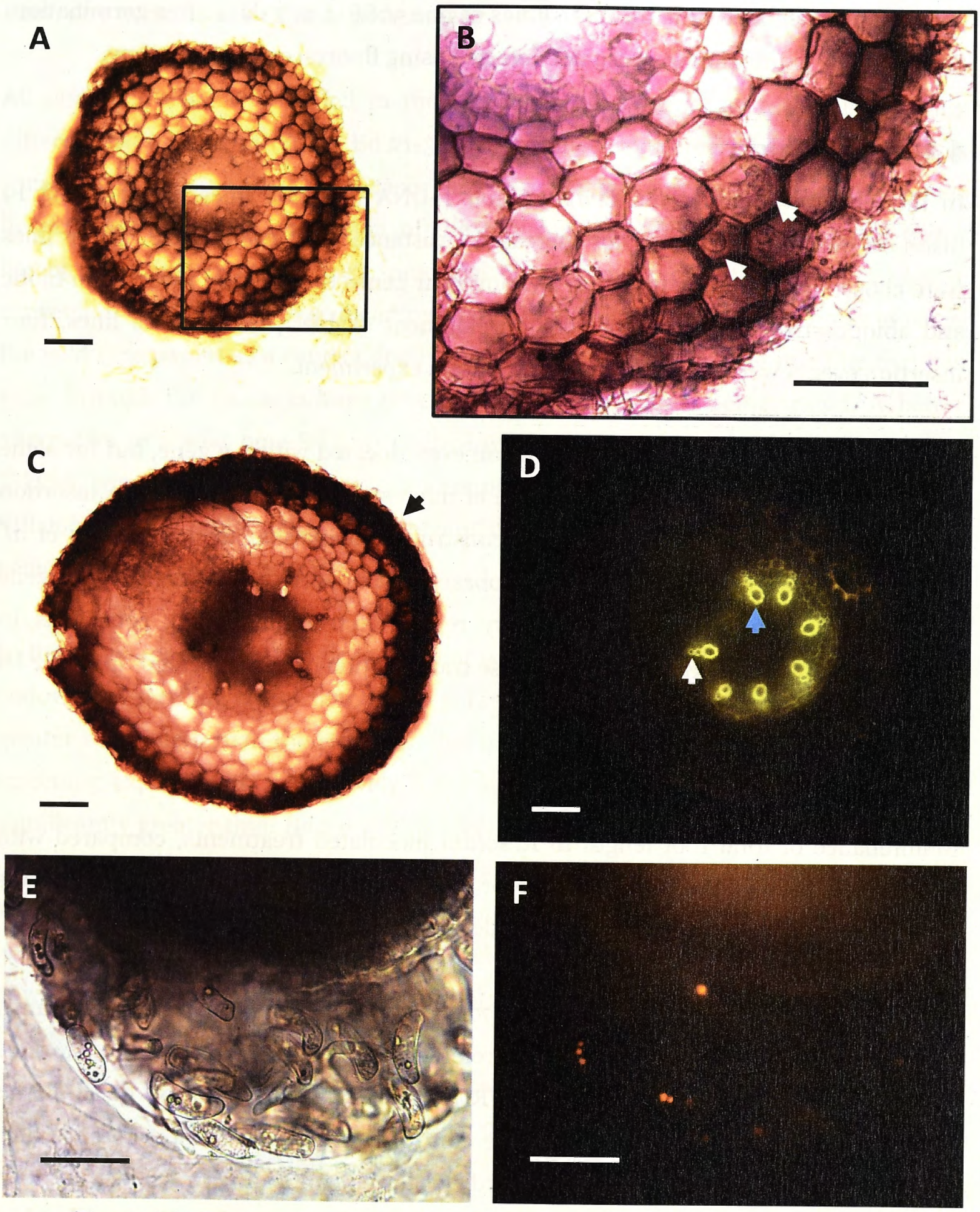

Figure 4.13 Cross-sections through browned regions of *B. distachyon* roots, close to the root tip, 48 h after inoculation with *R. solani* AG8 on agar plates; a) 5088-4 with the inset (b) showing *R. solani* hyphae extending through the cortex (arrows); c) and d) Bd 21-3 lateral root emerging under bright field and UV fluorescence with Leica H3 filter (black arrow, general darkening of root periphery; blue arrow, peripheral metaxylem; white arrow, protoxylem); e) and f) Bd 21-3 root border cells in mucilage surrounding the root tip under bright field and Leica H3 fluorescence filter (Ex 420 – 490 nm, Em-LP 515 nm). Scale bars, 100 µm.

The T-DNA insertion construct for event 5088 may result in GFP or GUS expression depending on its placement in the gene. Less than 10% of lines with this construct express GUS in vegetative tissue (Bragg *et al.*, 2012). GUS expression could not be detected in inoculated or control seedlings of line 5088-4 at 7 days after germination. Seedling GFP expression was also not detected using fluorescence microscopy.

4.4 Discussion

In this chapter twenty-seven *B. distachyon* T-DNA insertion lines, representing 19 insertion events, were tested for increased resistance to *R. solani* AG8. These lines were chosen based on the insertion location near genes that may be involved in biotic and abiotic stress resistance or root development (Table 4.1). Of these lines, four insertion events were included in a confirmation experiment.

Most chosen lines had the T-DNA insertion event located within a gene, but for some the event was intergenic or near the gene of interest. The effect of a T-DNA insertion on genes not directly disrupted by the construct is somewhat unclear. Bragg *et al.* (2012) state that insertions within one kilobase of an adjacent gene may affect the gene if the T-DNA lands within a regulatory region. Activation tagging constructs in Arabidopsis have been reported to increase transcription of genes located up to 8.2 kb from the enhancer (Ichikawa *et al.*, 2003).

4.4.1 Screening experiments

Maintenance of total root length in *R. solani* inoculated treatments, compared with control, was used as the primary indicator of disease resistance. Shoot measurements were also taken to study changes in plant development.

In the first screening experiment, lines 77-5, 3794-5 and 5088-4 were significantly more resistant to *R. solani* AG8 than the reference line Bd 21-3, based on transformed *R. solani*/control root length ratios ($\sqrt{RLR_A}$). The higher ratio of 5088-4 was associated with lower root growth in the control compared with the reference line. Lines 3794-5 and 5088-4 also notably did not have significantly lower leaf number, leaf 1 length nor leaf 2 length in *R. solani* treatment than control, indicating that leaf growth was less affected by *R. solani* by 22 DAP in these lines than in the reference line Bd 21-3.

Although analysis *Method B* looked promising based on the natural accession screen and first T-DNA screening experiment data, only one line, 9212-15, was picked up with potentially increased disease resistance in the second and third screening experiments

using this method. Events 2426, 2596 and 3794 were only significantly different from the control in the third experiment when lines of the same event were pooled. This lack of identifiable variation may be due to a dearth of resistance variation in the selected lines, due to reduced statistical power using this method or a combination of both.

All lines and events identified in the screening experiments as being significantly different from the control line Bd 21-3 were instances of increased resistance. This is somewhat surprising, as in most cases the construct type and insertion location would be expected to reduce expression of the targeted gene. With T-DNA tagged genes having been selected for their potential to improved disease resistance, lower expression should result in reduced resistance. This may be due to the choice of using the wild type parent as a control line in T-DNA experiments, instead of a line that has gone through the tissue culture process alongside the lines being tested. A better alternative to a wild type Bd 21-3 control would be a transformed line in which the T-DNA construct is located in a non-annotated region of the genome (Pacheco-Villalobos *et al.*, 2013) or a nil segregant that has lost the T-DNA insert in the T_2 generation (Vain *et al.*, 2011).

In the screening activity two lines of event 2426 were linked with significantly greater endogenous seedling vigour than Bd 21-3. Lines 2426-11 and 2426-8 had 20% and 32% greater root length than the reference line in control treatments of the first and third screening experiments, respectively. Leaf appearance rates for both lines were also significantly greater than Bd 21-3. This increased vigour is expected to be due to seed masses 17% and 10% greater than Bd 21-3 for lines 2426-11 and 2426-8, respectively. While this result is not of interest in the question of pathogen resistance, it may be worth investigating the role in seed development of the nearby T-DNA insertion downstream of an aquaporin TIP3;1 (tonoplast intrinsic protein) gene, *Bradi3g29780*. The gene has 87% amino acid identity (E-value 6e-152) with a rice aquaporin protein (GenBank AAG13544.1) expressed in leaves and roots (Sakurai *et al.*, 2005). Other TIP3;1 genes are seed-specific and may be expressed during initial stages of seedling germination and growth (Höfte *et al.*, 1992; Gattolin *et al.*, 2011).

4.4.2 Confirmation experiment

Seven T-DNA lines representing four insertion events, that had previously been included in screening experiments, were included in a confirmation assay to test whether these events were involved in *B. distachyon* resistance to *R. solani* AG8.

4.4.2.1 A candidate gene for increased resistance to *R. solani*

Only one line in the confirmation experiment, 5088-4, was more resistant to *R. solani* than the reference line Bd 21-3, based *R. solani*/control root length ratio. This increased resistance was also found in the first screening experiment.

The effect of *R. solani* on shoot development of line 5088-4 was also found to be minimal at 22 and 26 days after planting, compared with the reference line. In the first screening and confirmation experiment, leaf number at harvest for line 5088-4 did not differ significantly between the control and inoculated treatments; whereas leaf number was significantly lower in inoculated treatments of Bd 21-3 in both experiments.

4.4.2.1.1 T-DNA event 5088 is an insertion in a putative *β*-1,3-galactosyltransferase gene

The T-DNA insertion location for event 5088 is located within an intron of gene *Bradi3g14370* (Figure 4.15). The insertion is a bidirectional gene trap vector (pJJ2LBP2) containing promoterless GUS and GFP genes (Bragg *et al.*, 2012). In practice only a low percentage of tagged lines express GFP (2.3%) or GUS (4.8-7.2%) in any tissue (Ryu *et al.*, 2004; Bragg *et al.*, 2012), thus it not surprising that expression of GUS or GFP could not be detected in seedlings of line 5088-4.

The construct can nevertheless disrupt a tagged gene. In Arabidopsis research it is usually found that T-DNA insertions within introns have an effect on transcription and knock out or reduce protein expression (Wang, 2008). It is unclear how disruption of *Bradi3g14370*, a gene encoding a protein with β-1,3-galactosyltransferase (GT31) and galectin (galactose-binding lectin) domains, would increase resistance to *R. solani*.

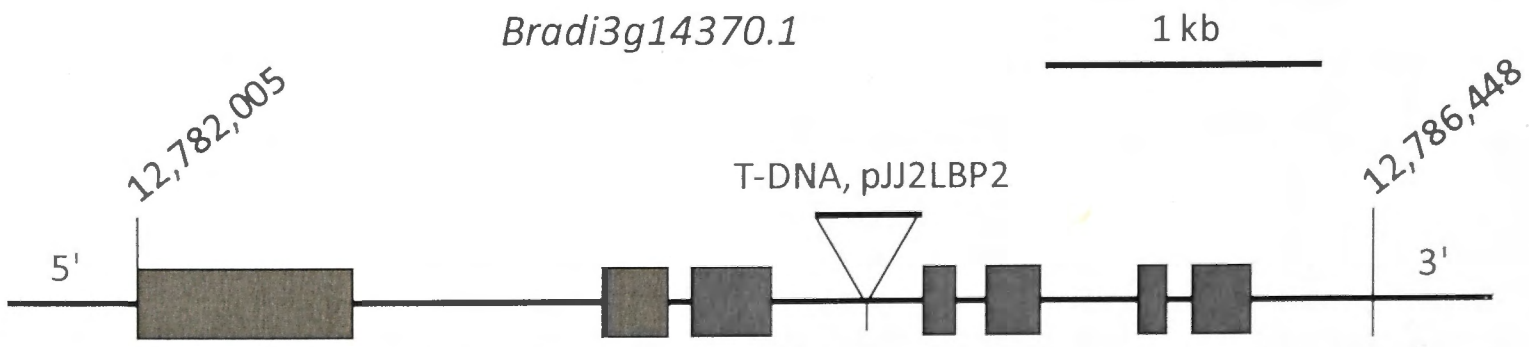

Figure 4.15 Location of the T-DNA insertion event 5088 in an intron of gene *Bradi3g14370.1*.

Family GT31 is one of the most abundant families of glycosyltransferase proteins in wheat, rice and Arabidopsis, members of which have been identified as wheat cell wall biosynthesis enzymes (Sado *et al.*, 2009). Griffitts *et al.* (2001) described a GT31-encoding gene, *bre-5*, in *Caenorhabditis elegans* that is required for susceptibility to *Bacillus thuringiensis* toxin (GenBank accession AY038065). The β-1,3-galactosyltransferase is involved in binding *Bt* toxin at the gut surface, thus allowing it to be internalized.

While β-1,3-galactosyltransferases are common in cereals, galectins are rarely described in plants (Strasser *et al.*, 2007; Qu *et al.*, 2008), but are known to be involved in the animal immune response as pattern recognition receptors of pathogen-associated molecular patterns (PAMPs) (Sato *et al.*, 2009). The galectin domain of *Bradi3g14370* does not align strongly with animal galectins, the closest match being with galectin-8 of *Condylura cristata* (E-value 0.023, 33% protein sequence identity).

Dunaeva and Adamska (2001) described a putative β-1,3-galactosyltransferase 19 gene in Arabidopsis, *Lsr5*, the protein of which also contains the galectin motif and aligns with the protein encoded by *Bradi3g14370.1* (Figure 4.16, GenBank protein accession BAA97209.1, UniProt Q9LV16, E-value 0.0, 57% protein sequence identity). Both proteins are predicted to be localised to Golgi bodies, which are involved in preparing compounds for secretion. *Lsr5* was induced by cold stress, wounding, light and desiccation stress. It has been shown that necrotrophic pathogens can exploit the plant immune response to biotrophic pathogens (Mengiste, 2012). The interplay between abiotic stress tolerance and resistance to necrotrophic pathogens appears to vary. For example, in Arabidopsis *BOS1* increased resistance to necrotrophic *Alternaria brassicola* and was also required for tolerance to water deficit, salinity and oxidative stress (Mengiste *et al.*, 2003), whereas an *exla2* mutant with increased resistance to this pathogen was more sensitive to salt and cold stress (Abuqamar *et al.*, 2013). Further work may show that this *B. distachyon* gene is involved in both biotic and abiotic stress tolerance.

Score	Expect	Method	Identities	Positives	Gaps
701 bits(1808)	0.0	Compositional matrix adjust.	327/571(57%)	418/571(73%)	13/571(2%)

```
Query  83   RYDRVSLPDIAARNRSALDRMADDAWSLGLTAWED--AAAFAGDPWELAAAGSAASSTDK  140
            R+D  +    +       L + A  AW +G   WE+  +          +        T+
Sbjct  111  RFDPETFNPSSKDGSVELHKSAKVAWEVGRKIWEELESGKTLKALEKEKKKKIKEHGTNS  170

Query  141  CPSAVSVRA-----RGRVVFLPCGLAAGSSVTVVGTPRPAHKEYVPQLARMRQGDGTVLV  195
            C  +VS+        RG ++ LPCGL  GS +TVVG PR AH E  P+++ +++GD  V V
Sbjct  171  CSLSVSLTGSDLLKRGNIMELPCGLTLGSHITVVGKPRAAHSEKDPKISMLKEGDEAVKV  230

Query  196  SQFMVELQGLRAADGEDPPRILHLNPRLRGDWSQRPIIEHNTCYRMQMGGAQRCDGLPPE  255
            SQF +ELQGL+A +GE+PPRILHLNPRL+GDWS +P+IE NTCYRMQMG AQRC+G
Sbjct  231  SQFKLELQGLKAVEGEEPPRILHLNPRLKGDWSGKPVIEQNTCYRMQMGSAQRCEGWRSR  290

Query  256  DNEDKVDGFTKCEKWIRDDIVDTKESKTTS----WLKRFIGRAKKPAMTWPFPFVEDRLF  311
            D+E+ VDG  KCEKW RDD + +KE +++      WL R IGR+KK  + WPFPF  D+LF
Sbjct  291  DDEETVDGQVKCEKWARDDSITSKEEESSKAASWWLSRLIGRSKKVTVEWPFPFTVDKLF  350

Query  312  VLTIQAGVEGFHIYVGGRHVTSFPYRPGFTLEEATGLYVKGDVNVHSVYATALPMSHPSF  371
            VLT+ AG+EG+H+ V G+HVTSFPYR GFTLE+ATGL + GD++VHSV+A +LP SHPSF
Sbjct  351  VLTLSAGLEGYHVSVDGKHVTSFPYRTGFTLEDATGLTINGDIDVHSVFAGSLPTSHPSF  410

Query  372  SLQQVLEMSEKWRSQPLPKDPVYLFIGILSASNHFAERMAVRKTWMQTSEIRSSKVVARF  431
            S Q+ LE+S  W++  LP + V +FIGILSA NHFAERMAVR++WMQ   ++SSKVVARF
Sbjct  411  SPQRHLELSSNWQAPSLPDEQVDMFIGILSAGNHFAERMAVRRSWMQHKLVKSSKVVARF  470

Query  432  FVALNSRKEVNVMLKKEAEYFGDIVILPFIDRYELVVLKTIAICEYGVQNLTATHIMKCD  491
            FVAL+SRKEVNV LKKEAE+FGDIVI+P++D Y+LVVLKT+AICEYGV  L A  IMKCD
Sbjct  471  FVALHSRKEVNVELKKEAEFFGDIVIVPYMDSYDLVVLKTVAICEYGVNQLAAKFIMKCD  530

Query  492  DDTFVRVDVVLRHIRAYSFGKPLYMGNLNLLHRPLRTGKWAVTEEEWPEDIYPPYANGPG  551
            DDTFV+VD VL   +     + LY+GN+N  H+PLR GKW+VT EEWPE+ YPPYANGPG
Sbjct  531  DDTFVQVDAVLSEAKKTPTDRSLYIGNINYYHKPLRQGKWSVTYEEWPEEDYPPYANGPG  590

Query  552  YVISGGIAKFVVSQHANQSLRLFKMEDVSMGLWVEKYNS-TTPVRYSHSWKFCQYGCLKN  610
            Y++S  I++F+V +     LR+FKMEDVS+G+WVE++N+ T PV Y HS +FCQ+GC+KN
Sbjct  591  YILSNDISRFIVKEFEKHKLRMFKMEDVSVGMWVEQFNNGTKPVDYIHSLRFCQFGCIKN  650

Query  611  YYTAHYQSPRQMLCLWDKLV-RGRPSCCNYR  640
            Y TAHYQSPRQM+CLWDKLV  G+P CCN R
Sbjct  651  YLTAHYQSPRQMICLWDKLVLTGKPQCCNMR  681
```

Figure 4.16 Protein BLAST alignment between *Bradi3g14370.1* and Arabidopsis *Lsr5* (gi|8809658|dbj|BAA97209.1) (NCBI, 2 August 2013).

4.4.2.1.2 Additional lines of event 5088 were less responsive than 5088-4

Two additional lines, 5088-2 and 5088-5, were included in the confirmation experiment in order to test the validity of T-DNA insertion event 5088 and exclude the possibility that increased resistance seen with line 5088-4 was the result of an unintended effect of the transformation process. Although root length and leaf number ratios for these two lines were higher than those of the reference line, the differences were not significant. Further work is therefore needed to ascertain whether gene *Bradi3g14370.1* is involved in *B. distachyon* resistance to *R. solani*.

There was a significant decrease in root growth in control treatments for lines 5088-2 and 5088-4, producing 20% and 30% less root length in the confirmation experiment than Bd 21-3, respectively. Likewise, in the screening experiment control treatment 5088-4 had 26% less root length than the reference line. This decrease in endogenous root growth combined with improved growth in *R. solani* treatments is reminiscent of a trade-off between plant 'fitness' and disease resistance, discussed briefly in §3.4.3.

Disruption of an orthologous GT31 gene (protein E-value 0.0) in Arabidopsis did not result in a noticeably altered endogenous phenotype (Strasser *et al.*, 2007) and indeed, this reduction in endogenous root growth may well be due to average seed masses of 5088-2 and 5088-4 being over 20% lower than Bd 21-3.

Some of the variation seen between the three lines of event 5088 may be due to variation in seed quality. Line 5088-4 had the greatest quantity and best quality of increased seed, with an average seed mass of 3.4 mg. Line 5088-2 had the second best seed quality and quantity followed by 5088-5, with average seed masses of 3.4 mg and 4.3 mg, respectively. It may be that somaclonal variation during tissue culture has led to differences in both seed quality and resistance to *R. solani* in the three lines of event 5088 (Barrell and Conner, 2011). Further work is needed to confirm the increased resistance phenotype observed for line 5088-4 and the reason behind its difference from the lines 5088-2 and 5088-5. This can be achieved through screening additional T-DNA lines with insertions in gene *Bradi3g14370.1* or through complementation of line 5088 with the wild type gene (Chochois *et al.*, 2012).

4.4.2.2 Further observations on variation in T-DNA mutant resistance and growth

Overall there was little variation seen between most lines in the confirmation experiment for resistance to *R. solani* AG8. Line 3794-5 was more resistant to the fungus than the reference line in the screening experiment, but in the confirmation experiment a line carrying the same T-DNA insertion event, 3794-2, did not maintain the same level of resistance. This may be due to differences in growth conditions, namely the different soil mix, or differences between the two lines.

Event 9212 appeared to have greater endogenous root vigour than the reference line. Root length of line 9212-15 in the control treatment was 19% greater than the reference line in the confirmation experiment. This significant trend was first observed in the third screening experiment, in which line 9212-15 had 37% greater root length than Bd 21-3 and may be explained partially by a 6% greater average seed mass. The T-DNA insertion event 9212 is located in the exon of *Bradi1g62970*, a gene encoding a protein with a RING-finger domain (Figure 4.14). Disruption of this gene is not expected to increase root growth as, for example, in rice a weak orthologue E3 ubiquitin-protein ligase (E-value 3e-17) is required for maintenance of the root meristem and normal root growth (Koiwai *et al.*, 2007). Another weak orthologue RING-H2 protein (E-value 4e-17) in capsicum is involved in salicylic acid-mediated defence against biotrophic and hemibiotrophic oomycete and bacterial pathogens (Lee *et al.*, 2011). Disruption of *Bradi1g62970* did not have a measurable effect on resistance to necrotrophic *R. solani*.

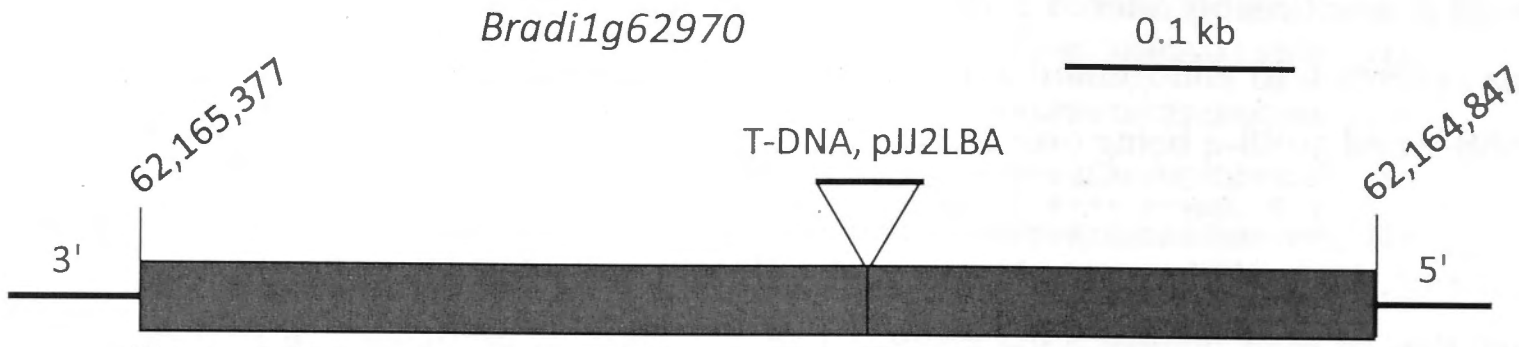

Figure 4.14 Location of the T-DNA insertion event 9212 in the exon of gene *Bradi1g62970.*

4.4.3 Phenotypes previously associated with resistance

In experiments with *B. distachyon* natural accessions described in Chapter 3, resistance to *R. solani* was linked with variation in nodal root emergence and endogenous seedling vigour.

4.4.3.1 Nodal root emergence

An increase in emergence of coleoptile nodal roots in *R. solani* treatment was consistent across the confirmation experiments for natural accessions and T-DNA lines. Emergence of coleoptile nodal roots may be the result of lateral root initiation, mediated by auxin signalling, in response to primary root truncation (Pacheco-Villalobos *et al.*, 2013).

The increase in leaf nodal root emergence in *R. solani* treatment reflects the observations of Schroeder and Paulitz (2008) in barley, in which crown roots were formed under greater *R. solani* AG8 disease severity. The apparent relationship between leaf nodal root emergence and resistance observed in the natural accession confirmation experiment was not seen in the T-DNA data. Genes targeted by T-DNA insertions in this screen are not known to be involved in leaf nodal root initiation, so this trait would not be expected to vary between these lines or in response to *R. solani.*

4.4.3.2 The effect of endogenous vigour on disease resistance

A relationship very similar to that seen in the natural accession screening activity (§3.3.1.3) was found between average seed mass and phenotype measurements for the first T-DNA screening experiment (§4.3.3). Greater seed mass correlated significantly with increased total root length, leaf number and leaf lengths in control and inoculated treatments, with the exception of total root length and leaf number in the *R. solani* treatment.

The negative correlation seen in the natural accession screen between endogenous seedling vigour and resistance rankings based on *R. solani*/control root length ratios

(Figure 3.9) was only repeated with root length, but not seed mass, in the T-DNA confirmation experiment.

The negative trend between endogenous plant vigour and resistance to *R. solani* may become significant with more replication or testing of further T-DNA events; however it is also possible, as seen in the natural accession confirmation activity (§3.4.3), that vigour is not the only determinant of resistance.

4.4.4 Variation in resistance to *R. solani* in *B. distachyon* T-DNA mutants

As discussed in §3.4.4, there are difficulties with putting a numerical value on the difference in resistance between lines. Back-transformation of predicted square-root transformed root lengths allows comparison with resistance measured in other studies and genotypes. Back-transformed total root lengths of the reference line Bd 21-3 and 5088-4 in the confirmation experiment gives untransformed *R. solani*/control ratios (RLR_A) of 0.30 and 0.50, respectively, representing a 20% increase in RLR_A for 5088-4. The same calculations for the first screening experiment give ratios for Bd 21-3 and 5088-4 of 0.28 and 0.44, respectively, or a 17% increase in RLR_A for the transformed line.

Less variation in resistance to *R. solani* was detected between lines in the T-DNA screen than in the natural accession screen. Eight of 25 natural accessions had significantly greater root length ratios than Bd 21-3 in the Chapter 3 screening activity, compared with four of 25 lines in the T-DNA screening experiments. The most resistant line in the natural accession screen had 33 – 38% greater RLR_A than the least resistant line, while the T-DNA mutant line 5088-4 had 17 – 20% greater RLR_A than the reference line Bd 21-3. A greater variation in resistance across the natural accessions is not surprising as *R. solani* is necrotrophic pathogen with a broad host range and thus, resistance is expected to require multiple quantitative genes (Mengiste, 2012).

4.5 Conclusions

Less variation in resistance to *R. solani* AG8 was found in *B. distachyon* T-DNA lines than in the natural accessions, as would be expected in quantitative resistance to a necrotrophic pathogen.

Line 5088-4, with a T-DNA insertion in gene *Bradi3g14370*, displayed a consistent quantitative level of increased resistance to *R. solani*. The failure of two additional lines of T-DNA insertion event 5088 to replicate this increased resistance puts the result into question. Resistance to *R. solani* could not be separated from its general negative correlation with endogenous seedling vigour for event 5088, with two lines having reduced root growth in control treatment.

Repeatable increase in endogenous vigour was measured for two lines of event 2426 and line 9212-15, with tagged genes *Bradi3g29780* and *Bradi1g62970*, respectively. These lines had slightly increased endogenous root growth over the reference line and other T-DNA mutant lines, with no difference in resistance to *R. solani*. Differences in early root growth may be partly due to differences in seed mass.

Further work is needed to validate these findings. These results indicate that, at this stage, the T-DNA collection is a less useful resource than the natural accession collection for discovering genes involved in quantitative disease resistance to *R. solani* and other root diseases of wheat.

Chapter 5

General discussion

Summary

This thesis describes the first investigation into disease resistance of *Brachypodium distachyon* to the wheat root rot pathogen *Rhizoctonia solani* AG8. Both wheat and *B. distachyon* were affected to a similar degree by the pathogen, with reduced root length, leaf number and lengths of the first and second leaves. Screening of a diverse collection of *B. distachyon* natural accessions revealed variation in resistance to *R. solani* AG8, providing a basis for future research to discover resistance markers. Screening of a collection of T-DNA insertional mutants revealed a gene that may be involved in quantitative resistance to the disease.

This chapter summarises the major findings of the thesis and discusses applications to related research questions, the use of *B. distachyon* as a research model for root diseases and future directions in development of *R. solani* resistant wheat.

The root pathogen *Rhizoctonia solani* AG8 is an important disease of wheat in Australia and around the world. The impact of *R. solani* AG8 is often felt most by farmers cultivating marginal land, where continuous wheat rotations are common. With no varieties of Rhizoctonia-resistant wheat available, farmers must use management techniques such as tilling to reduce disease levels, thereby increasing the risk of soil erosion (Paulitz *et al.*, 2002).

There is currently little opportunity to breed resistance into commercial wheat cultivars due to limited variation in resistance in wheat germplasm. A mutant line of wheat with increased tolerance to *R. solani* AG8 is being developed (Okubara *et al.*, 2009) and greater levels of resistance to *R. solani* found in a wild grass species were able to be introgressed into wheat (Okubara and Jones, 2011), however resistance mechanisms in wheat are still largely unknown.

The study of resistance to root diseases in wheat is firstly hampered by a large hexaploid genome, currently sequenced but not annotated, which makes finding genes or markers for quantitative traits difficult. Secondly, the large vigorous root system of wheat quickly reaches the base of pots, restricting the ability to study root traits to the very early stages of growth (Watt *et al.*, 2009).

To this end, the aim of this thesis was to develop *Brachypodium distachyon* as a model for the wheat-*R. solani* AG8 pathosystem. *Brachypodium distachyon* is a close relative of wheat developed as a model cereal since the turn of the century and has already proven useful in the study of foliar fungal diseases and root architecture.

5.1 Summary of main results

The development of a robust screening method for *B. distachyon* response to *R. solani* AG8 was a key component of this project. Researchers have previously written about the difficulty of achieving consistent inoculum pathogenicity, as well as useful phenotypic indicators of disease (Smith *et al.*, 2003a; Okubara and Jones, 2011).

Consistent disease levels were maintained through the use of a millet inoculum based on the method of McDonald and Rovira (1985), incorporated into the soil on the day of sowing. Rhizoctonia re-isolation from pots using toothpick baits, based on the method of Paulitz and Schroeder (2005), was a useful check of inoculation distribution. Consistent germination and emergence of *B. distachyon* was achieved through dehusking seeds prior to surface disinfection. Roots of plants grown to the four-leaf stage in small volume containers under these conditions were easily separated and scanned for root length measurements.

Maintenance of total root length in the inoculated treatment was the primary phenotypic measure of resistance to Rhizoctonia root rot. Shoot measurements including leaf number at harvest, lengths of first and second leaves and leaf area were also affected by the disease. The use of qPCR as a measure of root colonisation was tested without success.

A significant impact of *R. solani* was seen on root growth from 10 days after planting and on leaf number and leaf area from 18 DAP. The symptoms and severity of *R. solani* disease were very similar in *B. distachyon* and wheat, with rotting of the root cortex leading to truncated root 'spear tips'.

Total root length was found to be the most sensitive single measure of the disease phenotype. Root fresh weight measurements correlated well with root length, but could only pick up some of the differences in resistance found using root length. The lack of distinct root architecture differences between more and less resistant lines, apart from leaf nodal root emergence, may warrant in future the use of quicker fresh weight measurements in initial *R. solani* disease screens, followed by confirmation with total root length measurements for lines of most interest. An experienced operator can separate and scan a 26-day-old uninfested root system of *B. distachyon* on average every twelve minutes. Measuring root fresh weight and counting nodal roots could considerably reduce the time taken to conduct initial screening experiments. Scanning root length is recommended to confirm variation in resistance between lines.

Variation in resistance to *R. solani* AG8 was found across 26 *B. distachyon* natural accessions that were chosen on genotypic diversity and included in a replicated screen. After 26 days growth in infested soil the most resistant line, Koz-3, maintained a 33% greater total root length ratio than the least resistant line, BdTR 13a. Across the diverse natural accessions screened in this project, endogenous seedling root vigour and, to a lesser extent, seed mass were negatively correlated with resistance to *R. solani*, but this was not the only determinant of resistance.

Variation in resistance to *R. solani* AG8 was also seen in experiments with T-DNA insertion lines, however this was generally not able to be confirmed in subsequent experiments. Line 5088-4, an insertion in gene *Bradi3g14370*, was consistently found to be more resistant to *R. solani* than the reference line Bd 21-3, maintaining a 20% greater total root length ratio. This result requires further validation, as two additional lines of the same T-DNA insertion event did not display the same level of increased resistance.

An increase in coleoptile nodal root appearance in response to *R. solani* was seen in both the natural accession and T-DNA screen. In the natural accession screen, lines that produced leaf nodal roots in response to *R. solani* inoculation were more resistant than those that produced early endogenous leaf nodal roots. The lack of variation in leaf nodal root emergence between T-DNA lines derived from the same Bd 21-3 background is consistent with this being a genetically controlled trait, with the tagged genes not affecting leaf nodal root emergence.

Comparing results of the natural accession and T-DNA resistance screens, greater variation in resistance was found in the more genetically diverse natural accessions. Plant resistance to broad host-range nectrotrophic pathogens such as *R. solani* is generally quantitative, requiring multiple genes for effective levels of resistance (Mengiste, 2012), so a greater range of resistance was expected across the natural accessions than the mutant lines.

These results demonstrate that *B. distachyon* can be a useful model for the study of wheat root disease. The variation in resistance to *R. solani* AG8 found in the *B. distachyon* germplasm provides a basis for further work to determine genes and mechanisms involved in reducing the severity of this disease. Hopefully this knowledge will eventually be applied to develop wheat varieties with greater resistance to Rhizoctonia root rot.

5.2 Genetic variation in resistance to *R. solani* AG8

Experiments for this thesis demonstrated genetic variation in *B. distachyon* resistance to *R. solani* AG8. Phenotypic observations provide clues to genes that may be involved in resistance mechanisms.

Nodal root emergence and hormone signalling

The emergence of nodal roots in response to *R. solani* infestation, seen here in the *B. distachyon* natural accessions and earlier noted by Schroeder and Paulitz (2008) in barley, points to an auxin signalling response mediated by ethylene and/or cytokinin (Kitomi *et al.*, 2011; Pacheco-Villalobos *et al.*, 2013). It is speculated, e.g. Kazan and Manners (2009), that altered auxin signalling patterns may direct external and internal root architectural changes that affect plant-pathogen dynamics. Comparison of auxin signalling between more and less resistant *B. distachyon* lines may reveal interesting differences in expression patterns to explain the potential link between nodal root emergence and disease resistance.

As a cautionary note, an intriguing recent result from Foley *et al.* (2013) suggests that the defence-related hormones auxin, ethylene, jasmonic acid, abscisic acid and salicylic acid are not involved in Arabidopsis host resistance to *R. solani* AG8, even though the group had previously shown that ethylene signalling was involved in the defence response in soybean and barrel medic (Anderson *et al.*, 2010). Instead, oxidative stress, cell wall associated protein, transcription factor and heat shock protein gene families were most affected by *R. solani* pathogenesis in Arabidopsis. In light of the observations of Pacheco-Villalobos *et al.* (2013), that the modulation of auxin by ethylene differs between Arabidopsis and *B. distachyon*, it would be worthwhile to investigate expression changes seen in the Arabidopsis-*R. solani* pathosystem in *B. distachyon*.

Seed size and endogenous root vigour

Seed mass is controlled by a combination of genetic, environmental and seed position factors (Martínez-Andújar *et al.*, 2012). In cereals seed size, embryo size and seedling vigour are generally correlated (Richards and Lukacs, 2002). A significant increase in endogenous root vigour in lines of two *B. distachyon* T-DNA insertion events appeared to be partially related to increased seed mass.

Considerable variation was seen in the natural accession collection for seed size and root vigour. Across all lines these traits were negatively correlated with disease resistance, a trend also reported by Okubara and Jones (2011).

During the course of experiments attempts were made to remove the effect of endogenous vigour from resistance rankings by using different analysis strategies and by including *Seed size* and/or *Days to emergence* as factors. These factors are both correlated with endogenous vigour (Maguire, 1962; Richards and Lukacs, 2002). By using a ratio of inoculated to control root length, the possibility always remains that a correlation between endogenous vigour and *R. solani* disease resistance is an artefact of the analysis and not biologically relevant. This is a limitation of the current and previous studies, which could be further explored. Perhaps shoot ratios or seed set would be a better indicator of overall plant fitness. Scoring root disease severity is problematic, especially with the single primary root of *B. distachyon*. Quantification of *R. solani* DNA present in roots may be a better way to compare disease severity between lines, especially this measurement is not affected by seedling vigour.

In any case, reduced early seedling vigour was not the exclusive determinant of *R. solani* disease resistance in the *B. distachyon* collections, as demonstrated in the natural accession confirmation assay. A wheat mutant with increased tolerance to *R. solani* developed by Okubara *et al.* (2009) also bucked the trend, having greater inherent vigour. It is important that disease resistance can be separated from seedling vigour, as increased early vigour is a key strategy in breeding wheat for dry environments (Richards *et al.*, 2010).

Based on observations in this thesis, the *B. distachyon* germplasm collection may be a useful resource for further detailed work into the relationship between seed size, seedling vigour and *R. solani* disease resistance.

A candidate T-DNA tagged gene

Screening activities for the T-DNA collection were possibly confounded by an inconsistent approach between experiments, such as harvesting at different days after planting and using an alternative method to calculate resistance in the second and third screening experiments. Thus some lines may have failed to be correctly identified as significantly different in resistance to *R. solani* compared with the control line Bd 21-3.

The most promising line identified in the T-DNA screen for *R. solani* resistance, had an insertion in gene *Bradi3g14370*. This tagged gene, encoding a protein with β-1,3-galactosyltransferase and galactose-binding lectin (galectin) domains, has few described orthologues in plants (Dunaeva and Adamska, 2001). Of particular interest is the galectin domain, rarely described in plant genes, which is involved in animal

innate immunity (Sato *et al.*, 2009). The gene sequence aligns with wheat expressed sequence tags (ESTs), meaning that if the increased resistance trait is confirmed in *B. distachyon*, there is a good chance that an orthologous gene is present and expressed, and may thus be manipulated, in wheat. Two additional mutants of this insertion event were not more resistant to *R. solani* than the reference line. Further work that would be required to confirm *Bradi3g14370* as a quantitative defence gene for *R. solani* is described in the following section.

Further genes for investigation

Only a small number of candidate genes were able to be included in the T-DNA screen. Literature and database searches reveal many more *B. distachyon* genes that could be targeted in future investigations into quantitative resistance to *R. solani*. A selection of these genes is listed in Table 5.1. Genes for quantitative resistance cover a broad range of functions, including plant architecture, effector perception for basal disease resistance and production of detoxification enzymes or phytoalexins (Poland *et al.*, 2009). Genes that have not previously been associated with defence responses are also still being discovered as mediators of quantitative resistance.

Table 5.1 Candidate genes for potential future investigations into the *Brachypodium distachyon-Rhizoctonia solani* AG8 pathosystem. UniProtKB/SwissProt descriptions were retrieved from http://www.uniprot.org on 27 Feb 2013.

BLAST gene prediction for *B. distachyon*	*B. distachyon* genes	Wheat ESTs and SNPs?[b]	Predicted gene function
snakin-1-like	*Bradi1g07890* *Bradi1g07900*	No AD	Involved in resistance to *R. solani* in potato (Almasia *et al.*, 2008)
zeamatin-like	*Bradi1g13060* *Bradi1g13070*[a]	EST; AD EST	UniProt: (Maize) Has antifungal activity. Inhibits *Candida albicans* and *Trichoderma reesei*; marginal inhibition observed against *Alternaria solani* and *Neurospora crassa*.
UDP-glycosyltransferase 85A2-like	*Bradi1g27270* *Bradi1g27290*	AD No	Two of several orthologues of genes within a putative *Rsn1* locus, involved in rice sensitivity to *R. solani*. (Costanzo *et al.*, 2011)
basic endochitinase C-like	*Bradi1g29880*	No	UniProt: (Rye) Defence against chitin containing fungal pathogens. Binds the hyphal tips of fungi and degrades nascent chitin.
basic endochitinase A-like	*Bradi1g29887* *Bradi2g47210*	No EST; AD	UniProt: (Rye) Defence against chitin containing fungal pathogens. Binds the hyphal tips, lateral walls and septa of fungi and degrades mature chitin.
protein synthesis inhibitor I-like	*Bradi1g63700*	No	UniProt: (Barley) Inhibits the elongation phase of protein synthesis. Inactivates fungal ribosomes even more effectively than mammalian ribosomes and is thought to function as a constitutive antifungal agent in plants.
germin-like protein 8-4-like	*Bradi2g21010*	EST; AD,B	UniProt: (Rice) Germin-like protein 8-4 and 8-9 play a role in broad-spectrum disease resistance.
germin-like protein 8-9-like	*Bradi3g15190* *Bradi3g15200* *Bradi3g15220* *Bradi3g15230* *Bradi3g15240* *Bradi3g17330*	AD No EST No EST AD	
chitinase 2-like	*Bradi2g26000*	No	UniProt: (Rice) Hydrolyzes chitin and plays a role in defense against fungal pathogens containing chitin. Its overexpression confers enhanced resistance to sheath blight pathogen (*R.solani*) [Sequence caution].
polygalacturonase inhibitor-like	*Bradi2g35490*[a]	B	UniProt: (Grape) Inhibitor of fungal polygalacturonase. It is an important factor for plant resistance to phytopathogenic fungi.

Table 5.1 continued Candidate genes for potential future investigations into the *Brachypodium distachyon-Rhizoctonia solani* AG8 pathosystem.

BLAST gene prediction for *B. distachyon*	*B. distachyon* genes	Wheat ESTs and SNPs?[b]	Predicted gene function
glucan endo-1,3-beta-glucosidase GII-like	*Bradi2g60490*[a]	EST; AD,B	UniProt: (Barley) May provide a degree of protection against microbial invasion of germinated barley grain through its ability to degrade fungal cell wall polysaccharides.
thaumatin-like pathogenesis-related protein 3-like	*Bradi3g07960*	No	UniProt: (Oat) Associated with resistance against stem rust fungi.
ethylene-responsive transcription factor ERF098-like	*Bradi3g12565*	No	UniProt: (Arabidopsis) Probably acts as a transcriptional activator. Binds to the GCC-box pathogenesis-related promoter element. May be involved in the regulation of gene expression by stress factors and by components of stress signal transduction pathways
14-3-3-like protein GF14-E-like	*Bradi3g46960*[a]	EST; AD	UniProt: (Rice) Induction by wounding, drought and salt stresses, benzothiadiazole (BTH), ethephon, methyl jasmonate (MeJa) [GF14-E only], hydrogen peroxide, abscisic acid (ABA) and incompatible and compatible races of rice blast fungus (*M. grisea*) and rice bacterial blight (*X. oryzae*).
14-3-3-like protein GF14-B-like	*Bradi5g12510*[a]	EST; AD	
barwin-like (chitinase activity)	*Bradi4g14920*	AD	Sequence similarity to *T. aestivum* wPR4a gene (GenBank: AJ006098.1) encoding a pathogenesis-related protein.
uncharacterised	*Bradi1g63910.1*	EST; AD,B	WRKY transcription factors are involved in plant response to biotic and abiotic stresses (Pandey and Somssich, 2009).
uncharacterised	*Bradi2g02430*	EST; AD	Sequence similarity to *O. sativa OsWAK1* (GenBank: AK065470.1). *OsWAK1* is involved in resistance to rice blast (Li *et al.*, 2009).
RbohD (GenBank: AF055357.1) orthologue	*Bradi4g17020.1*[a]	EST; AD,B	Best orthologues of Arabidopsis NADPH oxidase genes *atrbohf* and *atrbohd*, a double mutant of which is less resistant to *R. solani* AG8 (Foley *et al.*, 2013).
Atrboh F (GenBank: AB008111.1) orthologue	*Bradi2g49040.1*[a]	AD, B	

[a] tagged in JJ T-DNA collection (Feb 2013).

[b]ESTs, *Triticum aestivum* 'Expressed Sequence Tags' (ESTs) are available from dbEST (www.ncbi.nlm.nih.gov/dbEST/); SNP: AD, SNPs that differ between the A and D genomes (where the B genome is unknown); SNP: B, SNPs that are the same between the A and D genomes, but differ in B. (www.gramene.org/Brachypodium_distachyon/Info/Annotation/)

5.3 Applications to related research questions

Resistance versus tolerance

The question remains whether the differences in root length ratio found between natural accessions and in a T-DNA line are due to 'resistance' or 'tolerance', as discussed in §1.3.4. By combining phenotypic resistance measurements with qPCR data on root colonisation, the plant-pathogen interaction could be classified as resistant, moderately resistant, susceptible or tolerant, as demonstrated by Dan *et al.* (2001). During this project quantification of the relative colonisation of root tissue in the most and least susceptible natural accessions was attempted using qPCR of fungal and plant DNA. Although unsuccessful, some further adjustments to the qPCR protocol and the use of appropriate *B. distachyon* primers may allow the host-pathogen interaction in this system to be better understood.

Which genes are involved in B. distachyon *resistance to* R. solani *AG8?*

Quantitative PCR is more often used to measure gene expression. Transcriptomic techniques may reveal differences in the level or timing of gene expression between the most and least resistant lines (Schenk *et al.*, 2012). In T-DNA lines, changes in the expression level of the targeted gene would show that the targeted gene had been disrupted, while expression changes of other genes could reveal downstream signalling effects. As discussed in the previous section, auxin signalling could be investigated as a link between altered nodal root appearance and disease resistance.

Chochois *et al.* (2012) discuss the elucidation of genes responsible for differences in traits of interest in a natural accession screen. To find a *B. distachyon* gene via the sequencing/EcoTILLING pathway (Chochois *et al.*, 2012, Figure 6B) at least 200 to 300 lines, preferably a couple of thousand lines, need to be phenotyped and sequenced (Vincent Chochois, pers. comm.). The second option of finding quantitative trait loci (QTL) by crossing more and less resistant lines is discussed in the following section.

Adult plant resistance

Plant resistance to diseases can vary depending on the stage of development. For example, resistance to leaf rust mediated by *Lr34* does not normally occur in seedlings, but can be induced by cold treatment (Risk *et al.*, 2012). Onset of resistance to *R. solani* during the vegetative growth phase has been noted in common bean and cotton (Develey-Rivière and Galiana, 2007). In *R. solani* AG8 bare patch disease of wheat, patches often have distinct boundaries, suggesting that wheat may also become resistant to the fungus at a defined stage of development.

The short stature of *B. distachyon* allows plants to easily be grown to later stages of development in pot assays than crop cereals (Watt *et al.*, 2009). Thus, onset of cereal adult plant resistance to *R. solani* AG8, if found to be present, could be studied using the *B. distachyon* - *R. solani* pathosystem.

Rhizoctonia decline and plant-microbe interactions

A well-established phenomenon in *R. solani* AG8 research is 'Rhizoctonia decline' in wheat, in which successive rotations of wheat favour disease-suppressive micro-organisms that reduce the development of root rot (Barnett *et al.*, 2006). Current rapid developments in the ability to sequence the soil microbiome provide an opportunity to revisit 'Rhizoctonia decline' using lines of *B. distachyon*, to study the interaction between plants, *R. solani* and beneficial soil micro-organisms (Berendsen *et al.*, 2012). By comparing 16*S* ribosomal DNA in *R. solani* conducive and suppressive soils, Mendes *et al.* (2011) identified the Pseudomonadaceae as key members of the consortia of *R. solani*-suppressive bacteria, and showed that protection was linked with production of a putative chlorinated lipopeptide by these bacteria. Mavrodi *et al.* (2012) indentified strains of *Pseudomonas* spp. that suppressed Rhizoctonia root rot and Pythium root rot in pot experiments with wheat, but could not attribute disease suppression to a particular bacterial metabolite. Plants also have some control over the activity of resident rhizosphere bacteria, with Jousset *et al.* (2011) showing that plants infected with the necrotrophic root pathogen *Pythium ultimum* could systemically increase the production of a *Pythium* spp. inhibiting compound by *Pseudomonas fluorescens* in non-infected roots.

The more and less *R. solani* resistant lines of *B. distachyon* could be investigated for variation in root exudate production. If variation is present, the effect of different root exudate compounds on numbers and activity of rhizosphere bacteria may reveal biocontrol mechanisms for disease suppression.

Root exudation and rhizosphere interactions

The abundant yellow vacuolar inclusions in root and root border cells of *B. distachyon* (Figure 2.21) may contain phenolic compounds or other metabolites that could be exuded in defence against soil pathogens (Jousset *et al.*, 2011) or in allelopathy (Wu *et al.*, 2002). Border cells secrete antimicrobial compounds, proteins and extracellular DNA into 'slime' surrounding the root cap (Driouich *et al.*, 2013). Although no difference in abundance of these inclusions was observed between infected and uninfected roots, their nature could be investigated further to see if they are involved

in the defence response of *B. distachyon* to *R. solani* AG8 or in other rhizosphere interactions.

5.4 Future directions

Brachypodium distachyon *as a model plant for non-specific quantitative resistance to multiple pathogens*

The variation in resistance to *R. solani* AG8 seen in the *B. distachyon* collection is not necessarily specific to this fungus. In wheat a single locus confers quantitative resistance to the foliar fungal pathogens leaf rust, stripe rust, stem rust, powdery mildew and spot blotch in wheat, barley leaf rust and barley powdery mildew, as well as *Barley yellow dwarf virus* (Risk *et al.*, 2013). Manosalva *et al.* (2009) found that germin-like proteins were involved in quantitative resistance to both *Magnaporthe oryzae* and *R. solani* in rice. Again, quantitative resistance suggestive of a basal defence response in tobacco was attributed to a calmodulin gene that helped protect against necrotrophic pathogens of three different kingdoms: *Ralstonia solanacearum, R. solani* and *Pythium aphanidermatum* (Takabatake *et al.*, 2007). Thus, the lines of *B. distachyon* most and least resistant to *R. solani* AG8 may upon further investigation be found to also have differing levels of resistance to other pathogens. Although they offer only intermediate levels of disease resistance, breeders are becoming more interested in quantitative resistance genes as they provide a more durable resistance trait (Boyd *et al.*, 2013).

Discovery of QTL markers in B. distachyon *natural accessions*

A first step in learning about inheritance of resistance and which genes are behind the variation in resistance seen in the natural accession collection is to cross the most and least resistant lines, Koz-3 and BdTR 13a. Barbieri *et al.* (2012) describe the elucidation of quantitative trait loci (QTL) using two *B. distachyon* natural accessions with variable levels of resistance to *Puccinia brachypodii*. Transgressive segregation in a cross between Bd 1-1 and Bd 3-1 resulted in F2 and F3 offspring with a broader range of resistance than the parents. Transgressive segregants have previously been found by Zhao *et al.* (2005) for *R. solani* resistance in crosses of susceptible soybean lines. Thus, it is possible that a larger range of resistance to *R. solani* could be seen in the offspring of a cross between Koz-3 and BdTR 13a. Barbieri *et al.* (2012) developed and used AFLP and SNP markers, along with SSR markers developed by Vogel *et al.* (2009), to create a linkage map for resistance in seedlings and adults of the F2 and F3 population, finding three QTL for false brome rust resistance that together explained 39-54% of variation in resistance in seedlings and 22% in adults. Identifying intermediate

resistance phenotypes for *R. solani* with the root length assay would be more difficult than finding intermediate resistance phenotypes for rust resistance, as endogenous root growth could also vary in the F2 and F3 population, whereas rust severity scores are independent of control scores.

Confirmation of tagged genes in B. distachyon *T-DNA mutant lines*

To confirm that a targeted T-DNA insertion gene is responsible for a phenotype, an independent mutant of the same gene should be shown to produce the same phenotype or complementation with the wild-type gene demonstrated to restore the wild-type phenotype (Krysan *et al.*, 1999). A similar gene silencing approach can also be used to verify genes that are found through natural accession screening. If an orthologue of the known *B. distachyon* gene is present in wheat, then reverse genetics techniques can be employed to see if it has the same function in both species (Chochois *et al.*, 2012; Fitzgerald *et al.*, 2012b).

It is important that gene expression of T-DNA tagged genes is measured, as gene expression is not necessarily completely disrupted by the insert. Expression may be down- or even up-regulated depending on insertion type and location (Bragg *et al.*, 2012). For example, Pacheco-Villalobos *et al.* (2013) measured mRNA levels of gene *BdTAR2L* in roots of two independent T-DNA tagged lines. Expression in line *Bdtar2l*^{hypo} was 5% of wild type, while expression in line *Bdtar2l*^{qnull} was 1-2% of wild type. The difference in expression corresponded with differences in severity of phenotypes.

Comparing R. solani *AG8 mode of infection in* B. distachyon *and wheat*

Further histological observations should be made to demonstrate that the *R. solani* AG8 infection process in *B. distachyon* is comparable with that in wheat, and to compare infection between the more and less resistant lines of *B. distachyon* identified in this thesis. Microscopic comparisons between lines can help to deduce and substantiate putative disease resistance mechanisms, e.g. Nikraftar *et al.* (2013).

Developing wheat with resistance to R. solani *AG8*

The ultimate aim of this project was to work towards discovering genetic resistance that can be used to develop *R. solani* AG8 resistance in wheat. Chochois *et al.* (2012) outline the steps needed to move from observation of a phenotypic trait of interest in *B. distachyon* natural accessions or T-DNA lines to discovery of a gene of interest in wheat. Validation is then required to ensure this gene is also associated with an increased resistance phenotype in wheat. Wasson *et al.* (2012) describe the subsequent

inclusion of a desirable phenotype in breeding programmes to develop a commercial wheat cultivar with the trait of interest.

At present, the reality is that quantitative traits are unlikely to be adopted into commercial breeding programs, especially if the trait is not already in an elite background. Breeders prefer to have genetic traits associated with single-nucleotide polymorphism (SNP) markers at less than one centiMorgan distance (Steve Jefferies, pers. comm., 21 May 2013). A strong selection marker is particularly important for root disease resistance traits, as the phenotype is difficult to select in the field and may not occur every year. Discovery of SNPs in wheat has advanced along with the bread wheat genome sequencing project, with good alignment of wheat SNPs to the *B. distachyon* genome (Allen *et al.*, 2011; Brenchley *et al.*, 2012). This will help with finding markers in wheat for genes characterised in *B. distachyon.*

Building on cell wall research in plant-pathogen interactions

Research shows that cell wall associated genes are important in different plant-pathogen interactions (Foley *et al.*, 2013) and that altering the structure of cell wall polysaccharides can affect resistance to fungal pathogens (Pogorelko *et al.*, 2013). This ties in well with research into the use of *B. distachyon* as a model for biofuel crops, aimed at improving the efficiency of energy production from plant biomass. For example, Bouvier d'Yvoire *et al.* (2013) found a *B. distachyon* gene that altered lignification to improve the saccharification process, while other studies have looked at the role of caffeic acid *o*-methyltransferase in lignification (Dalmais *et al.*, 2013; Wu *et al.*, 2013b). Do these genes affect the increased plant cell wall lignification response that occurs after inoculation with *R. solani* (Taheri and Tarighi, 2010) or the effectiveness of lignin-degrading laccases produced by *R. solani* AG8 (Bora *et al.*, 2005)?

5.5 Conclusion

The aim of this thesis was to develop a method to screen the model grass *B. distachyon* for resistance to the wheat root rot pathogen *R. solani* AG8. A rigorous repeatable method was developed and used to screen a diverse population of *B. distachyon* natural accessions and T-DNA insertion mutant lines for resistance to the fungus.

Variation in resistance to *R. solani* AG8 of the magnitude found in wild relatives and mutant lines of wheat was found in the *B. distachyon* natural accession and T-DNA collections. The level of resistance was higher across natural accessions than in the T-DNA lines, as would be expected of a quantitative resistance trait.

The variation in resistance to *R. solani* AG8 seen in the *B. distachyon* collections adds to previous studies showing variation in resistance to fungal diseases of the shoots and crown. This supports the continued use of *B. distachyon* as a model plant for cereal root diseases, with the aim of accelerating the development of disease resistant crops.

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Appendix A

A.1 Media recipes

Potato dextrose agar (PDA)

Potato dextrose agar	*Difco Laboratories, Sparks, US*	39	g
Milli-Q water	make up to	1	L

Quarter-strength Potato Dextrose Agar (¼ PDA)

After Kirkegaard *et al.* (1996).

Potato dextrose agar	*Difco Laboratories, Sparks, US*	10	g
Agar-agar	*Merck, Darmstadt, DE*	12	g
Milli-Q water	make up to	1	L

Water agar 3%

Agar-agar	*Merck, Darmstadt, DE*	30	g
Milli-Q water	make up to	1	L

Rhizoctonia-selective medium (KHF)

After Ko and Hora (1971), modified after Gill *et al.* (2000).

$NaNO_2$	*Ajax Chemicals, Sydney, AU*	200	mg
K_2HPO_4	*Fronine Pty Ltd, Riverstone, AU*	1	g
$MgSO_4.3H_2O$	*Ajax Chemicals, Sydney, AU*	354	mg
KCl	*BDH, Poole, GB*	500	mg
$FeSO_4.7H_2O$	*Ajax Chemicals, Sydney, AU*	10	mg
Agar-agar	*Merck, Darmstadt, DE*	20	g
Milli-Q water	make up to	1	L

Autoclave at 121°C for 20 minutes.

Cool to 50°C. Add gallic acid and antibiotics:

gallic acid	*Sigma, St Louis, US*	400	mg
Fongarid® (250 g/kg furalaxyl)	*Amgrow Garden King, Staplyton, AU*	400	mg
chloramphenicol	*Sigma, St Louis, US*	50	mg
streptomycin sulfate	*Sigma, St Louis, US*	50	mg

Potato dextrose broth

Difco™ Potato dextrose broth	*BD, Sparks, US*	24	g
Milli-Q water	make up to	1	L

Pythium semi-selective medium (VP3)

From a Pythium Training Manual prepared by Rosemary Warren (2010), modified from the recipes of Ali-Shtayeh *et al.* (1986) and Pankhurst *et al.* (1995).

100x micronutrient stock solution:

$MgSO_4.7H_2O$	*ChemSupply, Gillman, AU*		500	mg
$ZnCl_2$	*Ajax Chemicals, Sydney, AU*		50	mg
$CuSO_4.5H_2O$	*BDH, Poole, GB*		1	mg
MoO_3	*BDH, Poole, GB*		1	mg
$MnCl_2.4H_2O$	*BDH, Poole, GB*		1.3	mg
$FeSO_4.7H_2O$	*Ajax Chemicals, Sydney, AU*		1	mg
Thiamine HCl	*Sigma, St Louis, US*		5	mg
$CaCl_2.2H_2O$	*Ajax Chemicals, Auburn, AU*		786	mg
Milli-Q water		make up to	500	mL

Autoclave at 121°C for 20 minutes. Store at 4°C.

Media preparation:

Sucrose	*ChemSupply, Gillman, AU*		20	g
BBL™ Corn meal agar	*BD, Sparks, US*		17	g
Bacto™ agar	*BD, Sparks, US*		23	g
Milli-Q water		make up to	980	mL

Autoclave at 121°C for 20 minutes. Cool to 50°C and add 10 mL sterile micronutrient solution and antibiotics.

Antibiotics:

Vancomycin HCl, (75 mg/mL stock in MQ-water)	*Sigma, St Louis, US (included here, but may be omitted as very expensive)*	1	mL
Penicillin G sodium salt, (50 mg/mL stock in MQ-water)	*Fluka/Sigma, St Louis, US*	1	mL
Pimaricin, ~2.5% aqueous suspension	*Sigma, St Louis, US*	200	µL
Pentachloronitrobenzene, 95%	*Sigma-Aldrich, St Louis, US*	100	mg
Rifampicin crystalline, (10 mg/mL stock in methanol)	*Sigma, St Louis, US*	1	mL

Mix thoroughly and pour immediately. Store media at 4°C.

Water agar 0.1% for isolation of Pythium from soil

Bacto™ agar	*BD, Sparks, US*		100	mg
Milli-Q water		make up to	100	mL

Prepare in 100 mL Schott bottles.

A.2 Root staining with toluidine blue

After Watt *et al.* (2005).

Preparation of toluidine blue solution

Sodium benzoate	*Sigma-Aldrich, St Louis, US*		290	mg
Benzoic acid	*Sigma, St Louis, US*		250	mg
Toluidine blue	*Sigma, St Louis, US*		100	mg
Deionised water		make up to	200	mL

Add sodium benzoate and benzoic acid to water in a Schott bottle. Place on a stirring plate and stir on medium speed until dissolved. This may take several hours. Add toluidine blue powder and mix until toluidine blue is evenly dispersed.

Root staining

Remove plants from ethanol (50% v/v), cut off roots and place in water. Briefly dry the root system on paper towel, then place into a container with 0.05% toluidine blue. Stain for 3 min, then place roots into a beaker of fresh water. Rinse for a minute. Continue rinsing in fresh water until the rinsate remains clear. Separate roots using plastic forceps in water in a glass tray placed on a flatbed scanner.

Reuse the 0.05 % toluidine blue solution until it loses effectiveness. Dispose of the toluidine blue solution and the first rinsate into a waste container with a charcoal tea-bag. Light blue solutions of stain can be flushed down the sink with plenty of water. If roots are to be viewed using UV fluorescence under the microscope, do not stain with toluidine blue, as this interferes with cell autofluorescence.

A.3 Primers for PCR

Primer	Sequence 5'-3'; primer length	Source	Primer target; product size
Rs-F	AGAGTTGGTTGTAGCTGGTCC; 21 bp	Dr Jonathan Anderson, CSIRO Plant Industry, Perth, WA, Australia	*R. solani* ribosomal internal transcribed spacer region; 200 bp
Rs-R	CCGTTGTTGAAACTTAGTATTAGA; 24 bp		
Ta-F	ATAGGATTCCGGTCCTATTGTGT; 23 bp		Wheat 18s rDNA (also amplifies *B. distachyon* DNA); 201 bp
Ta-R	TAGGACGGTATCTGATCGTCTTC; 23 bp		
Bd-F	GTTCGCCATCCATTGCTT; 18 bp	Dr Jennifer Bragg, USDA-ARS, Albany, CA, USA	*B. distachyon* T-DNA JJ3794 flanking sequence; ~1000 bp
Bd-R	TGGTGCCCACGGATAAAT; 18 bp		

A.4 Average seed mass

Mean seed mass for natural accession and T-DNA lines used in experiments (n=16).

Natural accession line	Average mass (mg)
Abr 2	3.3
Adi-10	4.6
Adi-12	4.2
Arn 1	3.4
Bd 1-1	3.6
Bd 18-1	3.5
BD 21	4.2
BD 21-3	4.4
BD 2-3	5.2
Bd 30-1	2.9
BD 3-1	4.8
BdTR 10c	4.3
BdTR 10o	4.8
BdTR 11i	4.9
BdTR 12c	4.5
BdTR 13a	4.1
BdTR 13c	4.1
BdTR 1i	4.7
BdTR 2g	4.2
BdTR 3c	4.4
BdTR 5i	4.4
BdTR 9k	4.9
Cas 2	3.2
Koz-1	5.0
Koz-3	3.9
Tek-4	2.8

T-DNA line	Average mass (mg)
2426-11	5.1
2426-8	4.8
2596-10	4.9
2596-2	5.0
2596-9	4.1
2771-4	4.7
2771-5	4.7
2892-10	4.3
3175-12	4.5
3400-2	5.0
3794-2	4.4
3794-5	4.0
4243-11	4.8
4774-6	3.8
5088-2	3.4
5088-4	3.4
5088-5	4.3
654-9	4.1
705-4	4.9
7557-2	4.0
77-5	4.2
8634-6	4.9
8913-5	5.2
9212-15	4.6
9212-8	4.1
9278-8	4.9
9840-9	4.9

Appendix B
Preliminary experiments

B.1 Exploratory experiment with *Pythium* and *Rhizoctonia*

An exploratory experiment was carried out, prior to the experiments conducted for this thesis, to test the effect of five isolates of *Pythium* spp. and two isolates of *Rhizoctonia solani* AG8 on root and shoot growth of wheat and *B. distachyon*. The aim was to test pathogenicity of these isolates in a pot growth experiment in order to choose an isolate to continue with in future experiments. The experimental procedure for this experiment differs markedly from that described in the thesis.

B.1.1 Materials and Methods

B.1.1.1 Seed source and preparation

Brachypodium distachyon accession Bd 21-3 and *Triticum aestivum* cv. Janz were sourced as described in §2.2.1. Seeds were surface-sterilized for 2 min in sodium hypochlorite (1.3% v/v) and rinsed thoroughly in Milli-Q water before planting.

B.1.1.2 Inoculum preparation

Isolates of *P. irregulare* P15, *P. irregulare* BH40 and *R. solani* Rs21 were donated by Dr Paul Harvey, CSIRO Ecosystems Sciences, Urrbrae, SA, Australia. *Pythium paroecandrum* (KS01) was isolated from the field soil used in this experiment (described in next section) and identified by PCR. The origin of the isolate of *R. solani* AG8 ZG1-1 is described in §2.2.2.

Inoculum was prepared in large glass jars by seeding a twice-autoclaved mixture of maize meal (15 g, Nature First Organic, Cheltenham, VIC, Australia), coarse sand (1500 g) and distilled water (360 mL) with a square cm surface area of mycelium from an agar plate. Inoculum was incubated at room temperature for three weeks, with gentle mixing every 4 days. The method is similar to one used by Harvey *et al.* (2001).

Inoculum levels were enumerated using the method for isolation of *Pythium* from soil. Briefly, 1 g of inoculum was added to 0.1% water agar (100 mL) and shaken for 1 h. A 1:9 dilution was made into water agar. Aliquots (1 mL) of the original and diluted agar were spread onto *Pythium*- or *Rhizoctonia*-selective media. After two days, water agar was washed off the plate and colonies counted. This method was not ideal for enumeration of *R. solani*.

B.1.1.3 Soil

Soil was taken from a field trial near Galong, NSW, known to have a history of Rhizoctonia and Pythium disease. The trial had been sown to wheat and triticale in recent years. Soil was collected in mid-April 2010, while the site was being prepared for sowing, from a buffer area that had been sown to triticale in 2008 and fallow in 2009. The sandy loam was considered to be suitable for pot experiments, with pH (1:5 $CaCl_2$) 5.0, pH (1:5 H_2O) 6.3 and EC 27.7 μScm^{-1}. Soil was passed through a 3 mm sieve to remove large pieces or organic matter and break up large clods. Sieved air-dry

soil (4 kg) was mixed with water (200 mL) in a small autoclave bag, placed at room temperature overnight and then pasteurised at 60°C for 1 h.

Maize meal/sand mix inoculum was mixed thoroughly with pasteurised field soil to give final inoculum levels of 500 ppg (Higginbotham *et al.*, 2004) and 80 ppg (Okubara *et al.*, 2009) at a ratio of 1 part maize meal/sand mix to 3 parts soil. Control treatments were prepared in the same ratio, with uninoculated maize meal/sand mix.

B.1.1.4 Cone preparation and sowing

Cones were half-filled with inoculated soil (90 g), placed into medium flow trays and incubated at 22°C overnight in a Conviron CMP 2023 growth chamber (Winnipeg, Manitoba, Canada). One flow tray was used per inoculum. The following day loose uninoculated soil medium mix (approximately 70 g) was added to cones, before being watered to saturation with 40 mL deionised water. Cones were incubated overnight at 7°C.

One seed was placed onto the soil surface in each cone and covered with approximately 40 g of 'Barley Special' potting mix: river sand (1:1, w/w) (see §2.2.3 for details of 'Barley Mix'). Cones were incubated in darkness at 7°C for five days for seed stratification. Following stratification, growth cabinet conditions were set at 14 h days with cool white fluorescent light (approximately 270 $\mu E.m^{-2}.s^{-1}$) and a constant temperature of 16°C (Harvey *et al.*, 2001; Higginbotham *et al.*, 2004). Logged daytime temperature of the air and at the pot surface was 20 – 21°C, varying within approximately one degree around the growth cabinet and due to the heating and cooling cycle of the cabinet. Night temperature was 16°C. Pots were watered with approximately 5 mL deionised water every three days. Wheat and *B. distachyon* were harvested at 33 and 39 days after planting, respectively.

B.1.1.5 Phenotype measurements

At harvest, roots were washed out in water and stored in 50% ethanol in plastic sauce containers. Length of the long root was measured using a ruler. Shoots were cut from the root system, dried in paper envelopes at 70°C for 3 d and weighed.

B.1.1.6 Statistical analysis

Emergence was low for wheat and *B. distachyon* across all treatments, with average rates of 56, 65 and 60% at 0, 80 and 500 ppg, respectively. The 80 and 500 ppg treatments were thus combined into a general 'inoculated' treatment for analysis. Plants that had not emerged by 7 days after planting were excluded from analysis. Statistical analysis was carried out using REML linear regression in Genstat (VSN International, UK). Predicted means and p-values were calculated for control-inoculated pair-wise comparisons for all combinations of host and pathogen.

B.1.2 Results and discussion

Several problems with the experimental procedure were identified and corrected before proceeding with experiments for the thesis.

The pasteurisation technique used did not appear to effectively reduce pathogen load in the field soil. A low level of emergence in control treatment of *B. distachyon* and wheat at 30 – 70% and 40 – 70%, respectively, may have been due to damping off. For

example, steam sterilization kills *Pythium* hyphae in soil, but oospores may germinate after a month or so (R. Simpson, pers. comm).

Shoot dry weight in control treatment differed markedly between flow trays. Few significant differences were found between control and inoculated treatments for shoot growth (Figure B1.1). In wheat there was an increase in shoot growth with *P. paroecandrum* inoculum and a decrease with *R. solani* ZG1-1 inoculum.

Root growth in *Pythium* treatments did not differ between inoculated and control treatments. In *R. solani* inoculated treatments, root growth was restricted to the upper half of the pot, with roots not entering into the lower inoculated half of the pot. This is seen by longest roots of wheat and *B. distachyon* reaching the bottom of the 21 cm deep cones, with roots in inoculated treatments only reaching around 10 cm (Figure B1.2).

Isolate *R. solani* AG8 ZG1-1 was chosen for experiments in this thesis.

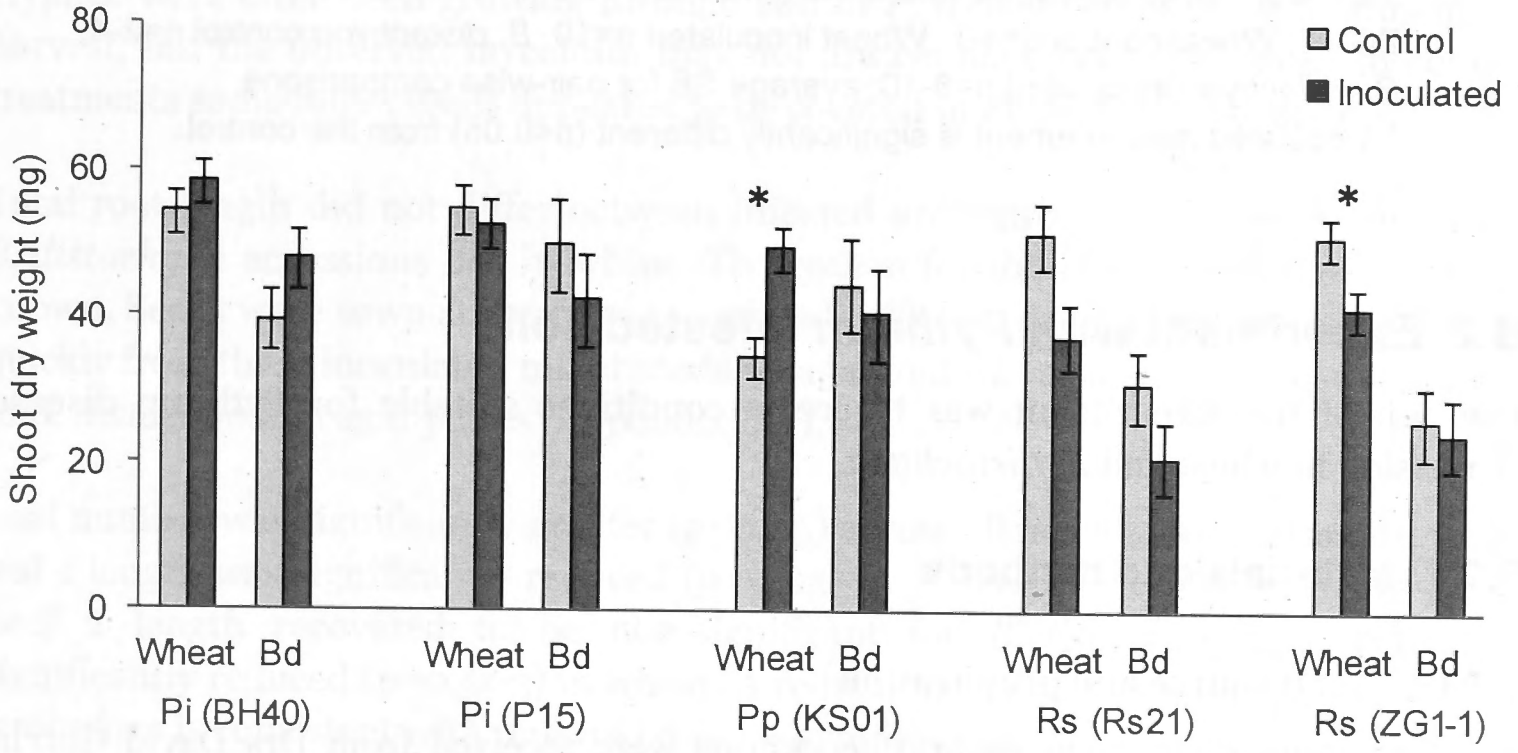

Figure B1.1 Average shoot dry weight for wheat and *B. distachyon* (Bd) grown in control or inoculated soil and harvested at 39 DAP. Inoculum was stratified into the lower half of cones. Inoculum: Pi (BH40), *P. irregulare* BH40; Pi (P15), *P. irregulare* P15; Pp (KS01), *P. paroecandrum* KS01; Rs (Rs21), *R. solani* Rs21; Rs (ZG1-1), *R. solani* AG8 ZG1-1; Wheat control n=3-7, Wheat inoculated n=10-13, *B. distachyon* control n=3-6, *B. distachyon* inoculated n=9-15; average SE for pair-wise comparisons.
* Inoculated measurement is significantly different ($p<0.05$) from the control.

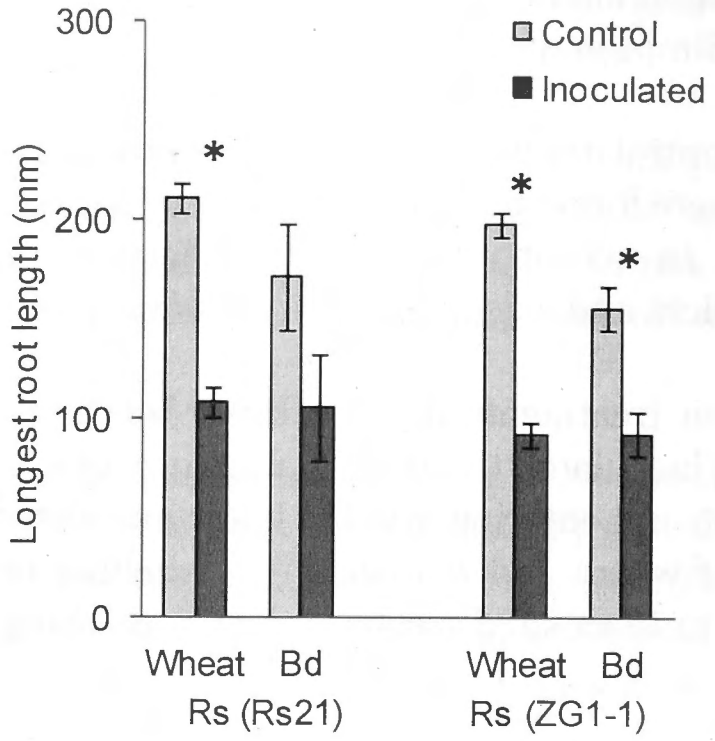

Figure B1.2 Average longest root length for wheat and *B. distachyon* (Bd) grown in control or *R. solani* inoculated soil and harvested at 39 DAP. Inoculum was stratified into the lower half of cones. Inoculum: Rs (Rs21), *R. solani* Rs21; Rs (ZG1-1), *R. solani* AG8 ZG1-1; Wheat control n=4-7, Wheat inoculated n=10, *B. distachyon* control n=2-4, *B. distachyon* inoculated n=9-10; average SE for pair-wise comparisons.
* Inoculated measurement is significantly different ($p<0.05$) from the control.

B.2 Experiment with *Pythium* infested soil

The aim of this experiment was to create conditions suitable for Pythium disease expression in wheat and *B. distachyon*.

B.2.1 Materials and methods

B.2.1.1 Seed source and preparation

Brachypodium distachyon natural accessions were received from Drs David Garvin and John Vogel, as described in §2.2.1. Seeds were surface-sterilized using the method described in §2.2.1, except that seeds were not de-husked, and then incubated on 3% water agar at room temperature for 3 – 5 days until germinated.

B.2.1.2 Inoculum preparation

The isolate of *Pythium irregulare* BH40 was donated by Dr Paul Harvey (CSIRO Ecosystems Sciences, Urrbrae, SA, Australia). Inoculum was prepared with white millet seed, described in §2.2.2. Moist autoclaved millet was inoculated with cubes of agar from the growing edge of a *P. irregulare* colony and incubated for 10 d. In contrast to *R. solani*-inoculated millet, *P. irregulare*-inoculated millet does not remain viable after storage at -20°C.

B.2.1.3 Cone preparation and sowing

'Barley Mix' potting mix, described in §2.2.3, was used for this experiment. Millet seed inoculum was mixed into soil at a rate of approximately 0.5 propagules (individual seeds) per gram. Cones were loosely filled with soil and watered with tap water (30 mL). A pre-germinated *B. distachyon* seed was placed alongside an inoculated millet

seed in the centre of the pot, covered with uninoculated soil and watered with an extra 5 mL. The control treatment was prepared as for the inoculated treatment, except millet was not inoculated with the oomycete. Cones were incubated at 16°C in 12 h day length and harvested 18 days after planting.

B.2.1.4 Phenotype measurements

Shoot measurements taken at harvest were leaf number, leaf 1 length and leaf 2 length. Total root length was measured using WinRhizo. Details of phenotypic measurements are given in §2.2.7.

B.2.1.5 Statistical analysis

Plants that had not emerged by 7 days after planting were excluded from analysis. Statistical analysis was carried out using a linear regression model (REML) in GenStat with the fixed model written as '*Days to emergence* + *Host*Inoculum*'. Wheat was analysed separately from the *B. distachyon* lines.

B.2.2 Results and discussion

Hyphae were often seen growing through soil in *P. irregulare*-infested treatments at harvest, but the observed mycelium may not always have been *Pythium*. In control treatments some millet seeds appeared to be covered in *Penicillium*-like spores.

Total root length did not differ between infested or control treatments in any of the *B. distachyon* accessions nor in wheat. The reason for the lack of root disease is not known. Seeds were sown adjacent to inoculated millet seeds. *Pythium irregulare* grew quickly from these inoculated millet seeds on agar and was also shown to cause disease in *B. distachyon* on agar plates (Appendix B.3).

Leaf number was significantly greater ($p<0.05$) across all inoculated treatments, while leaf 1 length was significantly reduced ($p<0.05$) by *P. irregulare* BH40 (Figure B2.1). Leaf 2 length recovered to be non-significant for *B. distachyon*, but remained significantly reduced ($p=0.003$) in wheat. A reduction in leaf 1 length in response to *P. irregulare* is consistent with reported disease symptoms (Ingram and Cook, 1990).

It is curious that leaf number generally increased in response to *P. irregulare* inoculum, as abiotic stress reduces the rate of leaf appearance. Leaf appearance rate in cereals increases with thermal time and decreases with soil hardness (Masle and Passioura, 1987; Masle *et al.*, 1989). The impact of soil pathogens on phyllochron has not been studied. It may simply be that during pot filling uninoculated soil was inadvertently packed harder than inoculated soil.

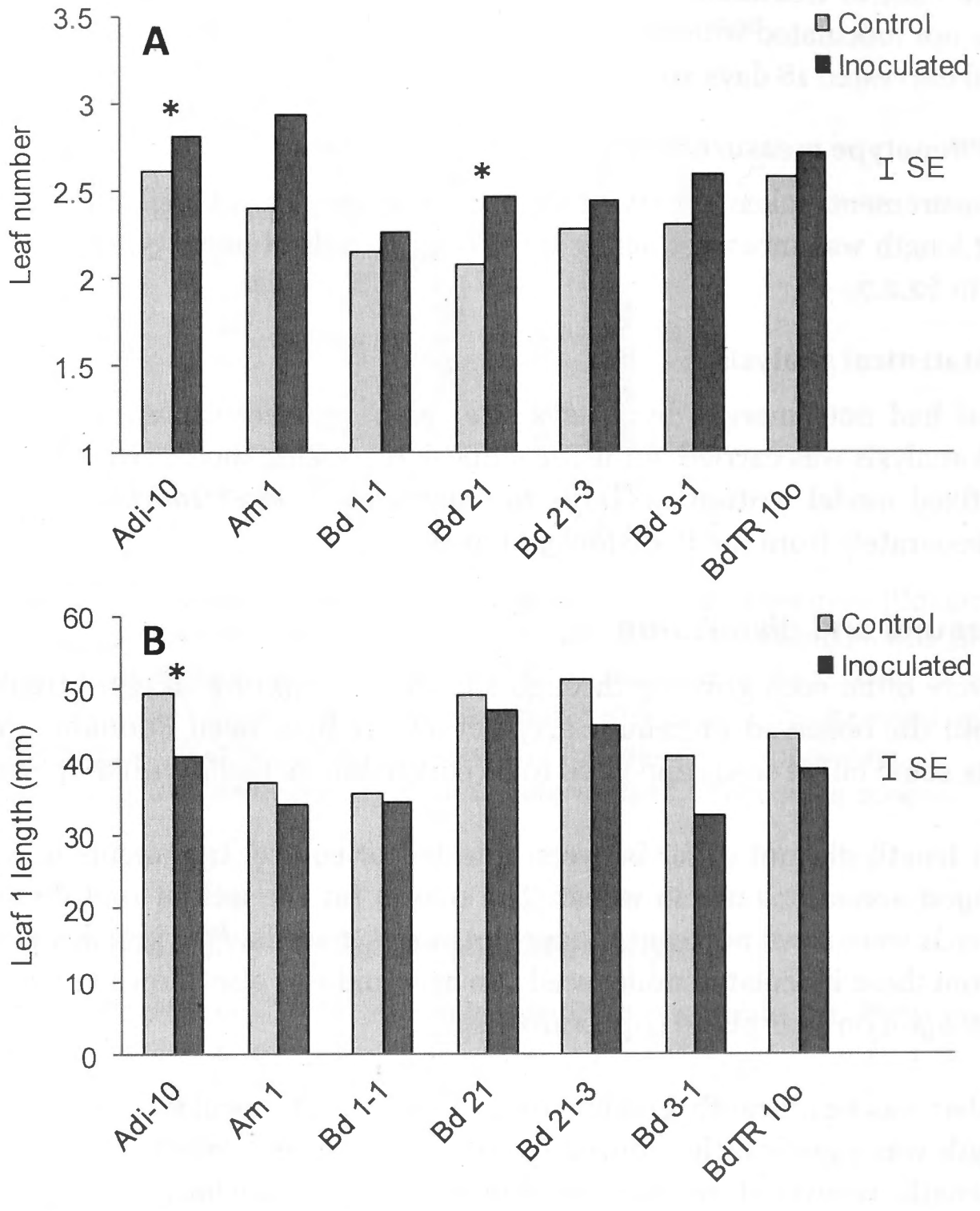

Figure B2.1 Average a) leaf number, and b) leaf 1 length *B. distachyon* accessions grown in control or *P. irregulare* BH40 inoculated soil and harvested at 18 DAP; control n=3-14; inoculated n=5-13; average SE.
* Inoculated measurement is significantly different ($p<0.05$) from the control in a pair-wise analysis.

B.3 Experiment with *Pythium* inoculated plates

As Pythium disease could not be achieved in pot assays, the pathogenicity of an isolate of *P. irregulare* was tested on inoculated agar plates.

B.3.1 Materials and methods

B.3.1.1 Seed source and preparation

Seeds of 134 *B. distachyon* natural accessions were used in this experiment. The germplasm source is described in §2.2.1. Seeds were neither dehusked nor surface sterilized before sowing.

B.3.1.2 Plate preparation

Water agar plates (1.2% w/v) were inoculated centrally with a 5 mm^2 surface area cube of agar from the growing edge of a *P. irregulare* BH40 colony grown on potato dextrose agar. Isolate origin is given in §B.1.1.2. Plates were incubated at 25°C for 3 d. Eight seeds were placed onto each plate, with embryos oriented toward the centre. Plates were incubated at 12°C in darkness. Control plates were prepared in the same way, but not inoculated.

B.3.1.3 Phenotype measurements

Germination was scored visually. Root lengths were measured by scanning plates on a flatbed scanner, then measuring root lengths using ImageJ 1.43u software (NIH, USA). Mean seed mass of 16 seeds across all lines was 4.2 ± 0.8 mg, ranging from 2.3 ± 0.4 mg (BdTR 2r) to 5.4 ± 0.7 mg (BdTR10 h).

B.3.1.4 Statistical analysis

Statistical analysis was carried out using the REML linear regression analysis in GenStat. Root length analysis included only the subset of seeds that had germinated. Simple linear regression was used to calculate the correlation between seed mass and root length.

B.3.2 Results and discussion

Significant differences ($p<0.001$) were observed between lines, between control and inoculated treatments, and for the line-inoculum interaction for both germination (Figure B3.1) and root length at 7 days after sowing (Figure B3.2). Root length of seeds germinated on the *P. irregulare* lawn were reduced to between 3 – 79% of control root length. At 7 DAP some plants growing on inoculum had germinated and produced a shoot, but no root. Roots that were able to penetrate into agar appeared to somewhat escape infection and tended to be longer than those growing at the plate surface.

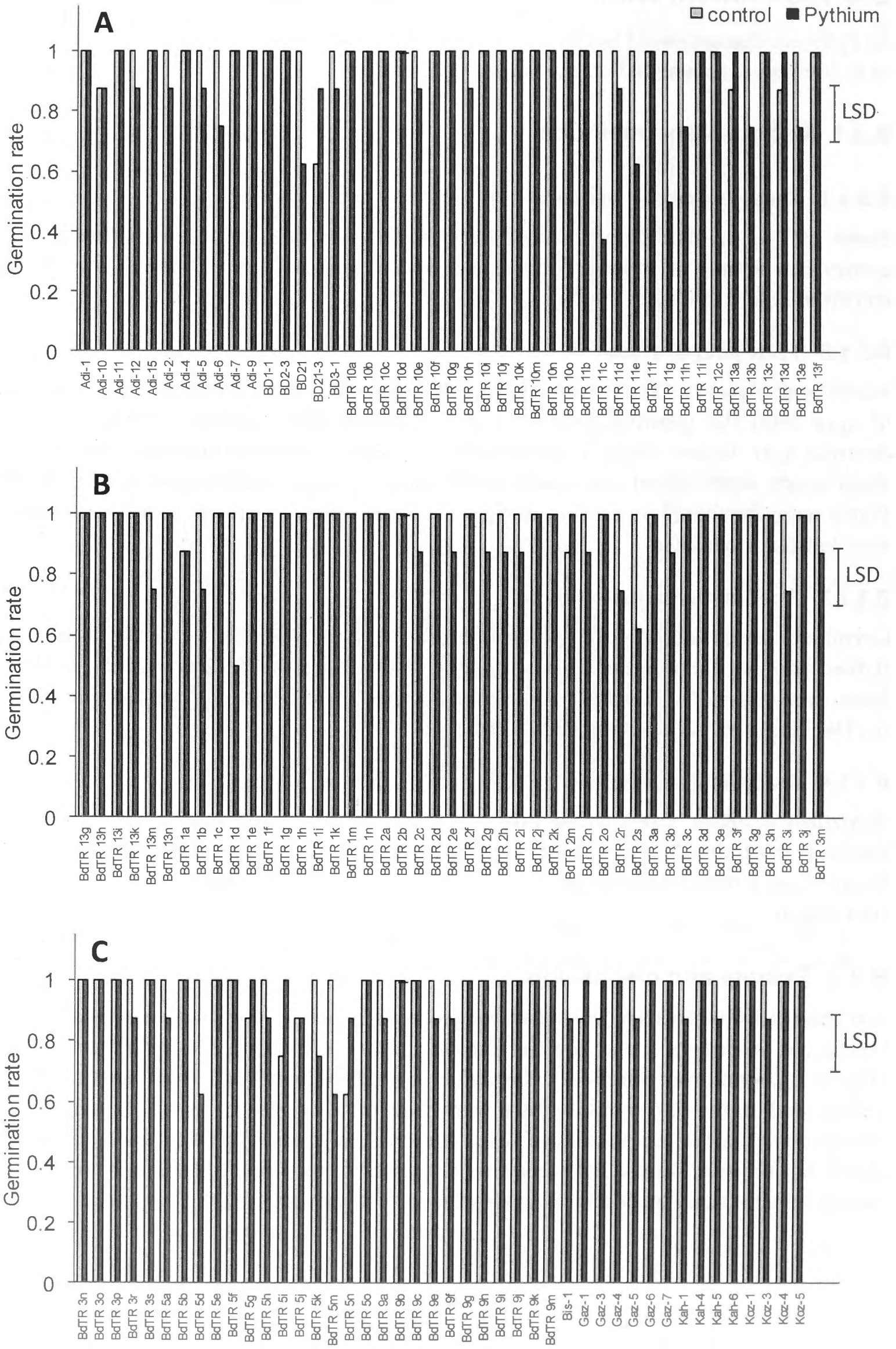

Figure B3.1 Germination rate of 134 *B. distachyon* natural accessions on *P. irregulare* BH40 inoculated and control plates; n=8 seeds per treatment; LSD, 5% least significant difference.

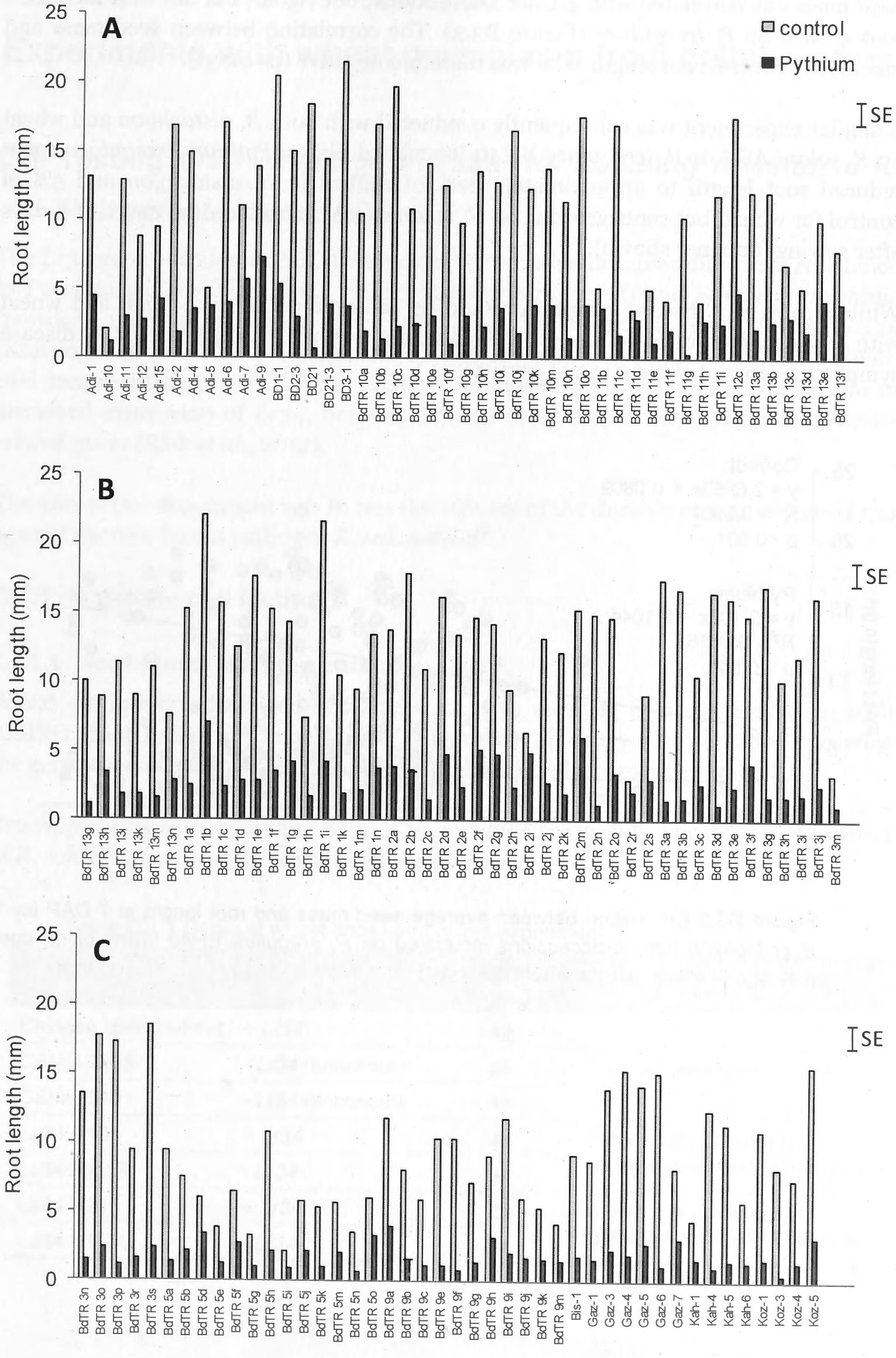

Figure B3.2 Average root length of 134 *B. distachyon* natural accessions grown on *P. irregulare* BH40 inoculated and control plates for 7 days; n=8 seeds per treatment; SE, average standard error.

Seed mass was correlated with greater endogenous root vigour, but not with increased root growth on *P. irregulare* (Figure B3.3). The correlation between seed mass and inoculated/control root length ratio was therefore negative (p=0.031).

A similar experiment was subsequently conducted with both *B. distachyon* and wheat on *R. solani* AG8 or *P. irregulare* BH40 inoculated plates. *Pythium irregulare* again reduced root length to approximately 22% of control in *B. distachyon* and 5% of control for wheat, but roots growing on *R. solani* were not reduced in length at 8 days after sowing (data not shown).

While strong root disease symptoms could be achieved on *B. distachyon* and wheat with *P. irregulare* in plate assays, this pathogen was not studied further as disease symptoms were not evident in pot assays.

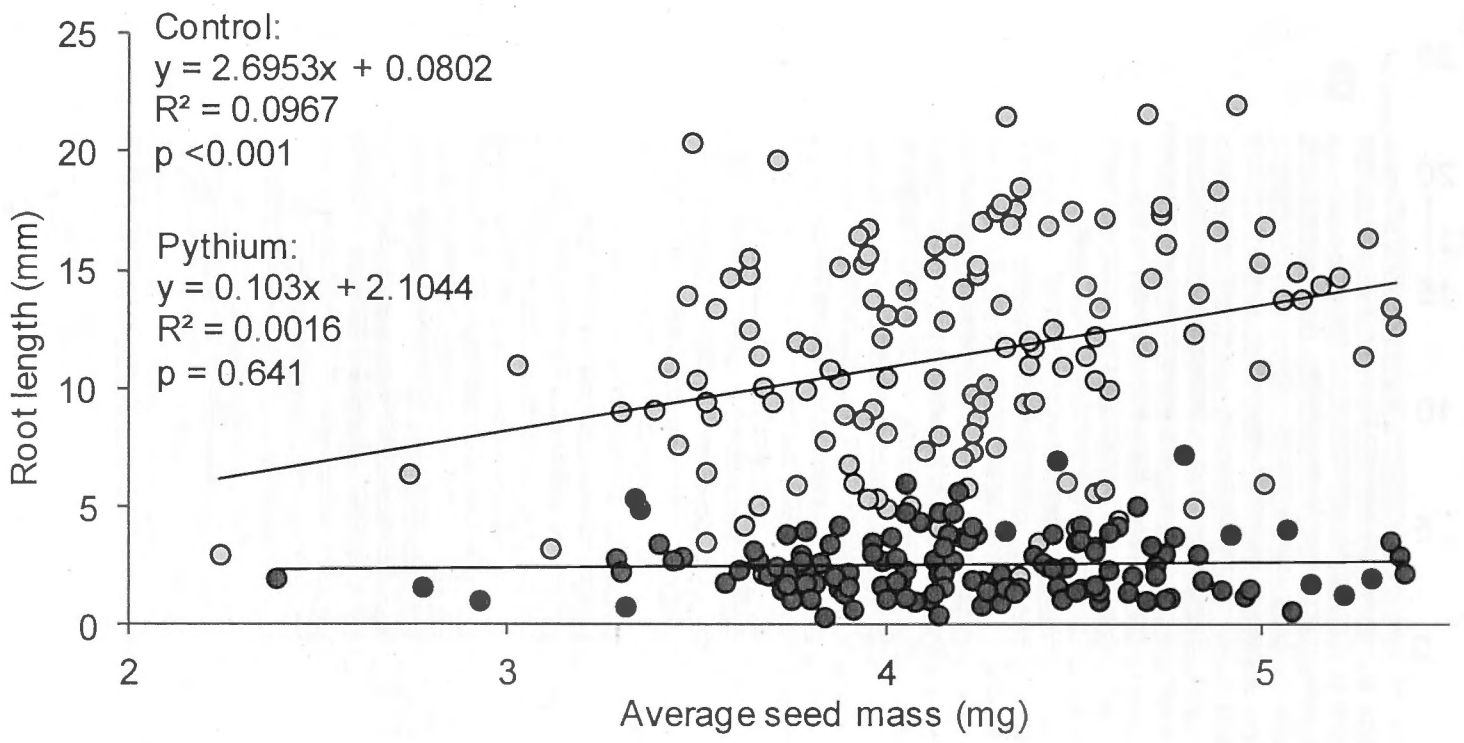

Figure B3.3 Correlation between average seed mass and root length at 7 DAP for 134 *B. distachyon* natural accessions incubated on *P. irregulare* BH40 lawns or on control plates; n≤8 seeds per treatment.

Appendix C

Experiments with wheat germplasm from collaborators

C.1 Testing *Lr34* wheat germplasm for seedling resistance to *Rhizoctonia solani* AG8

The *Lr34* gene encodes an ABC-transporter that confers durable adult plant resistance to *Puccinia triticina* (leaf rust), *Pu. striiformis* (stripe rust) and *Blumeria graminis* (powdery mildew) in wheat. The *Lr34* gene has been lost from *B. distachyon* (Krattinger *et al.*, 2011). Seedling resistance of lines carrying *Lr34* can be induced with cold treatment (4 to 10°C). This form of resistance does not appear to be due to increased expression of *Lr34*, but may be due to an interaction with pathogenesis-related genes (Risk *et al.*, 2012).

The aim of this experiment was to test the efficacy of the durable resistance gene *Lr34* against the root fungal pathogen *R. solani* AG8.

C.1.1 Materials and Methods

C.1.1.1 Seed source and preparation

Wheat germplasm, described in Table C.1.1, was received from Dr Evans Lagudah (CSIRO Plant Industry, Canberra ACT, Australia). Spielmeyer *et al.* (2008) describe the generation of mutants. Seeds were surface-sterilized as described in §2.2.1.

Brachypodium distachyon line Bd 21-3 was included alongside wheat to test the effect of *R. solani* infection at the lower temperature.

Table C.1.1 Origin of wheat lines used in this experiment

Genotype	*Lr34* expression	Average seed mass (mg)	Notes
Chinese spring wheat	+ *Lr34*	42	
CSMU 2215	- *Lr34* (knockout)	24	Chinese spring wheat EMS mutants
CSMU 5101	- *Lr34* (knockout)	18	
La34 R19	+ *Lr34*	43	Indian line (Lalbahadur), gamma-irradiated M2 sib pairs
La34 MU19	- *Lr34*	34	
La34 R21	+ *Lr34*	28	Indian line (Lalbahadur), gamma-irradiated M2 sib pairs
La34 MU21	- *Lr34*	34	

C.1.1.2 Experimental conditions

Inoculum was prepared as described in §2.2.2. Plants were sown into 'Barley Mix' potting mix (§2.2.3) using the method described in §2.2.4.

Trays were placed in Adaptis A1000 growth cabinets (Conviron, Winnipeg, Canada) programmed with 12 h days at a constant temperature of 10°C. A lower temperature is required for seedling expression of *Lr34* (Rubiales and Niks, 1995; Risk *et al.*, 2012). Soil temperature at seed level was 9°C overnight and ~10.2°C during the day (Figure C.1.1). Irradiance was measured to be around 200 µEinsteins. Plants were harvested at 16 and 26 days after planting.

When plants were harvested at 16 DAP, the potting mix was quite wet. Therefore the remaining plants were only watered once more before the Day 26 harvest.

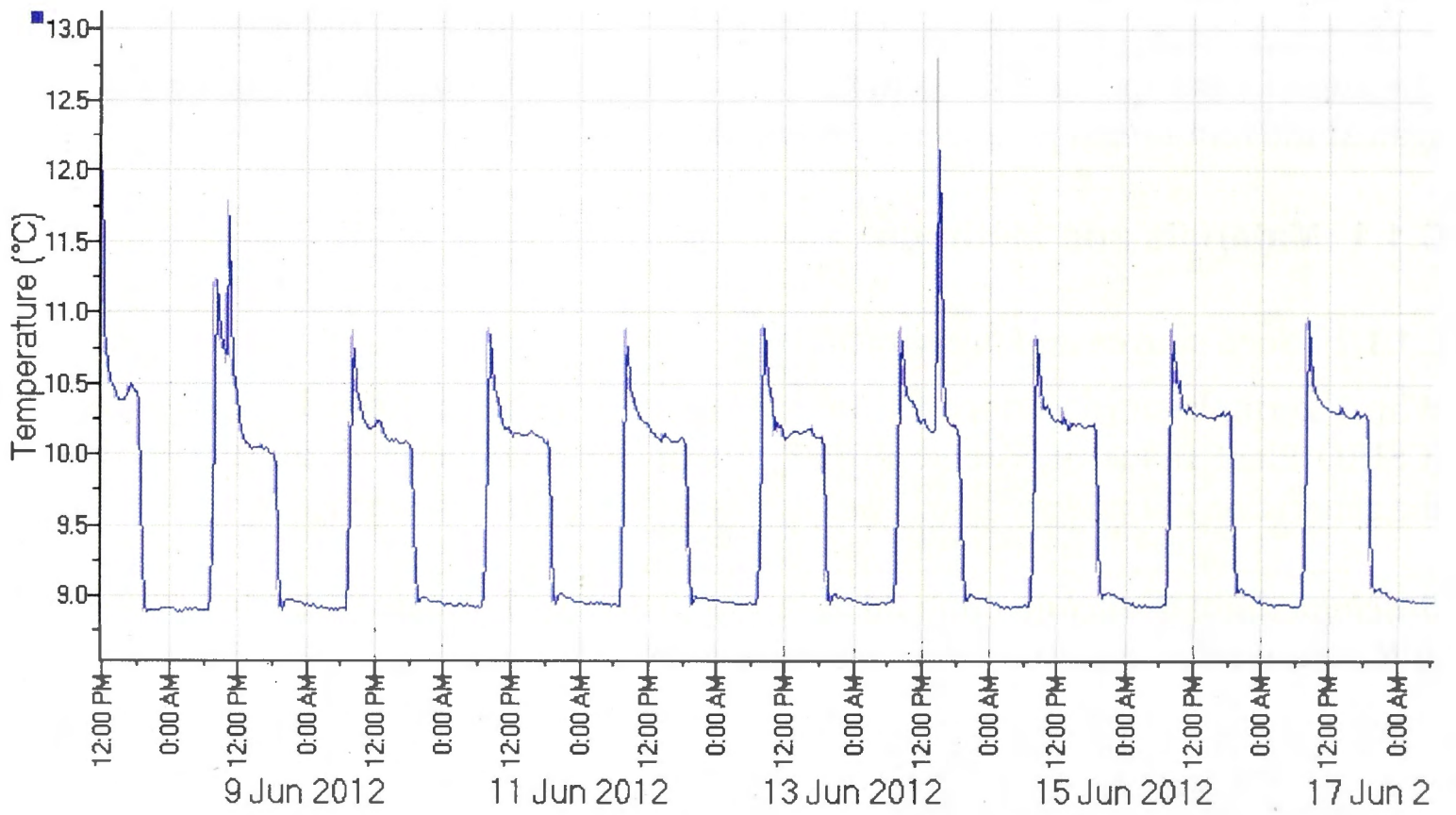

Figure C.1.1 Soil temperature recorded at seed level in a Tray 1 cone over nine days during the course of the experiment. Temperature spikes on the first and sixth full day were due to watering.

C.1.1.3 Re-isolation of Rhizoctonia from cones

Rhizoctonia was reisolated from cones (§2.2.6) at 15 days after planting. The toothpick analysis was delayed to 15 DAP because many plants hadn't emerged by 8 DAP, when the toothpick assay is usually carried out.

C.1.1.4 Phenotypic measurements

Total root length and leaf number at harvest were measured according to the protocol described in §2.2.7

C.1.1.5 Statistical analysis

With temperature set at 10°C and humidity at 50%, plants grew more slowly and there was far less evaporation than in previous experiments at 16°C. Wheat took on average 7 days to germinate, and Bd 21-3 took 8 days. Plants were excluded from analysis if they had not germinated by 10 DAP. Predicted means were calculated using the REML linear regression model in GenStat, with the fixed model '*Days to emergence* + *Host*Inoculum*' and '*Tray*' included as the random model. Root length was square-root transformed prior to analysis.

C.1.2 Results and discussion

C.1.2.1 Growth under cooler temperature

All experiments carried out for the main body of this thesis were conducted at 16°C. At 10°C *B. distachyon* and wheat took longer to emerge from soil and plant growth decreased.

Plants used water at a lower rate than in experiments run at 16°C. This affected the *Rhizoctonia* re-isolation toothpick assay, which could not clearly determine the presence or absence of *Rhizoctonia* in control and *R. solani* treatments. Root scans clearly showed, however, that there was no *Rhizoctonia* damage in control treatments of wheat and that all *R. solani* treatments had truncated roots. Previous experiments with *R. solani* have shown that cross contamination between cones rarely occurs in this system.

Factors that could have resulted in few toothpicks having strong growth of *Rhizoctonia* at 15 DAP with 10°C temperature, may include:

- Rhizoctonia needing longer than 24 h to colonise toothpicks, due to reduced growth rate at cooler temperature. Refshauge (2007) showed that the growth rate of *R. solani* AG8 on agar decreases from 5.5 mm d^{-1} at 16°C to 3.4 mm d^{-1} at 10°C.
- Inhibition of *Rhizoctonia* due to reduced evaporation at cool temperatures resulting in wetter soil. Evidence of disease on all inoculated roots indicates this was not the case (Figure C.1.2).
- Growth of other soil fungi and changing population dynamics at this lower temperature and delayed time-point. Non-*Rhizoctonia* colonies with dense hyphae emerged from many toothpicks and were tentatively identified as *Fusarium* spp. Rhizoctonia quickly invades and damages roots, allowing secondary pathogens to enter and exacerbate root rot as a 'disease complex' evolves (Moen and Harris, 1985).

While control wheat roots looked quite healthy at 26 DAP, some *B. distachyon* control roots were distorted and may have been affected by another soil pathogen.

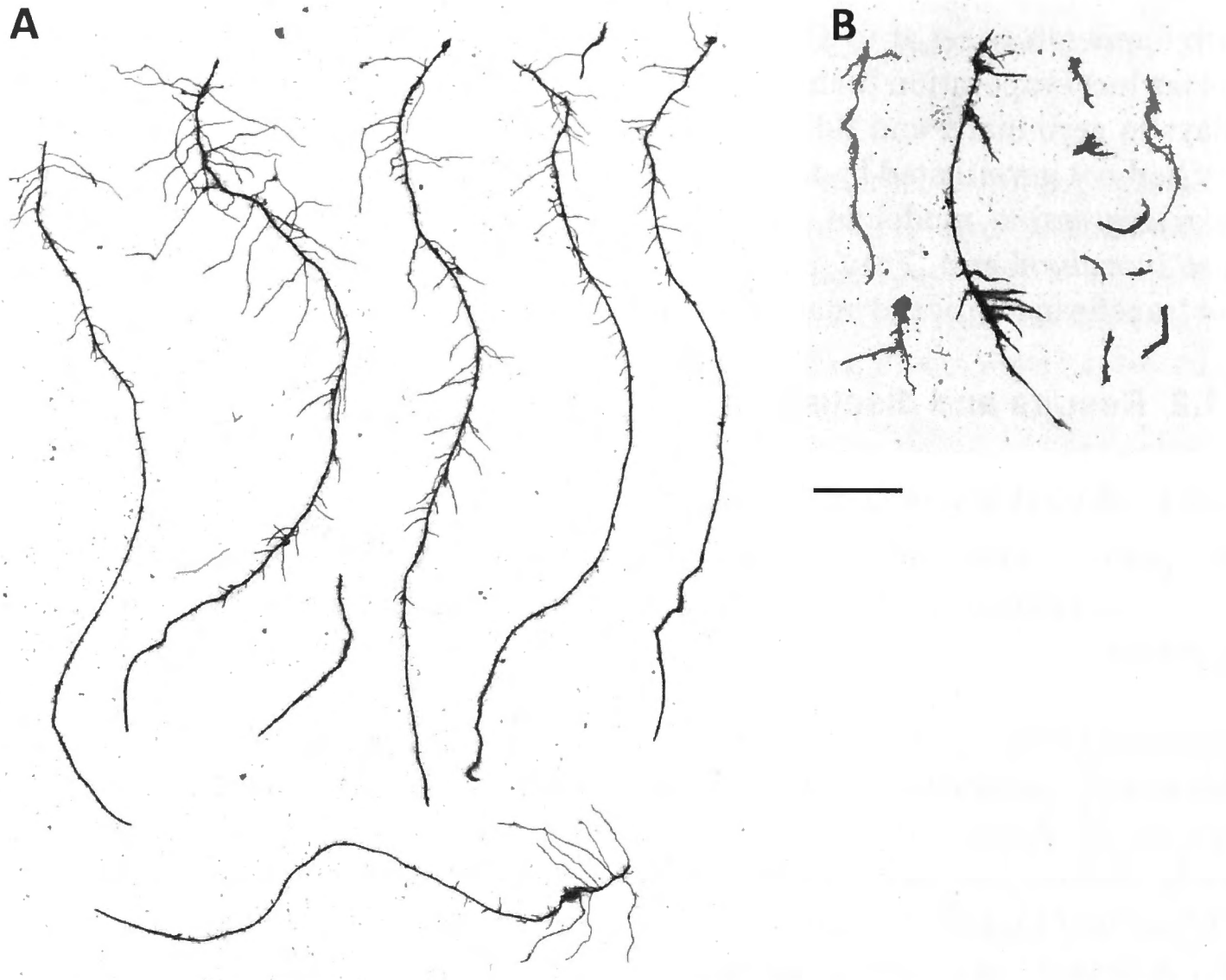

Figure C.1.2 An example of root scans of Chinese Spring wheat in a) uninfested, and b) *R. solani* infested soil, at 26 DAP harvest; scale bar, 2 cm.

C.1.2.2 Root and shoot growth

The plant-pathogen interaction for square-root transformed total root length was only significant (p=0.015) at the 26 DAP harvest (Figure C.1.3). This was due to the Chinese Spring wheat mutant CSMU 5101 (-*Lr34*) having a greater control/*R. solani* square-root transformed total root length ratio (RLR_A, see Table 4.2) than Chinese Spring wheat (+*Lr34*).

A significant plant-pathogen interaction (p=0.037) was also seen at the 16 DAP harvest for leaf number (Figure C.1.4), the result of a greater control/*R. solani* leaf number ratio for CSMU 2215 (-*Lr34*) than Chinese Spring wheat (+*Lr34*).

Brachypodium distachyon line Bd 21-3 root length and leaf number was calculated separately from wheat (Table C.1.4). Growth was strongly reduced at 10°C, compared with 16°C (compare with Figure 2.15).

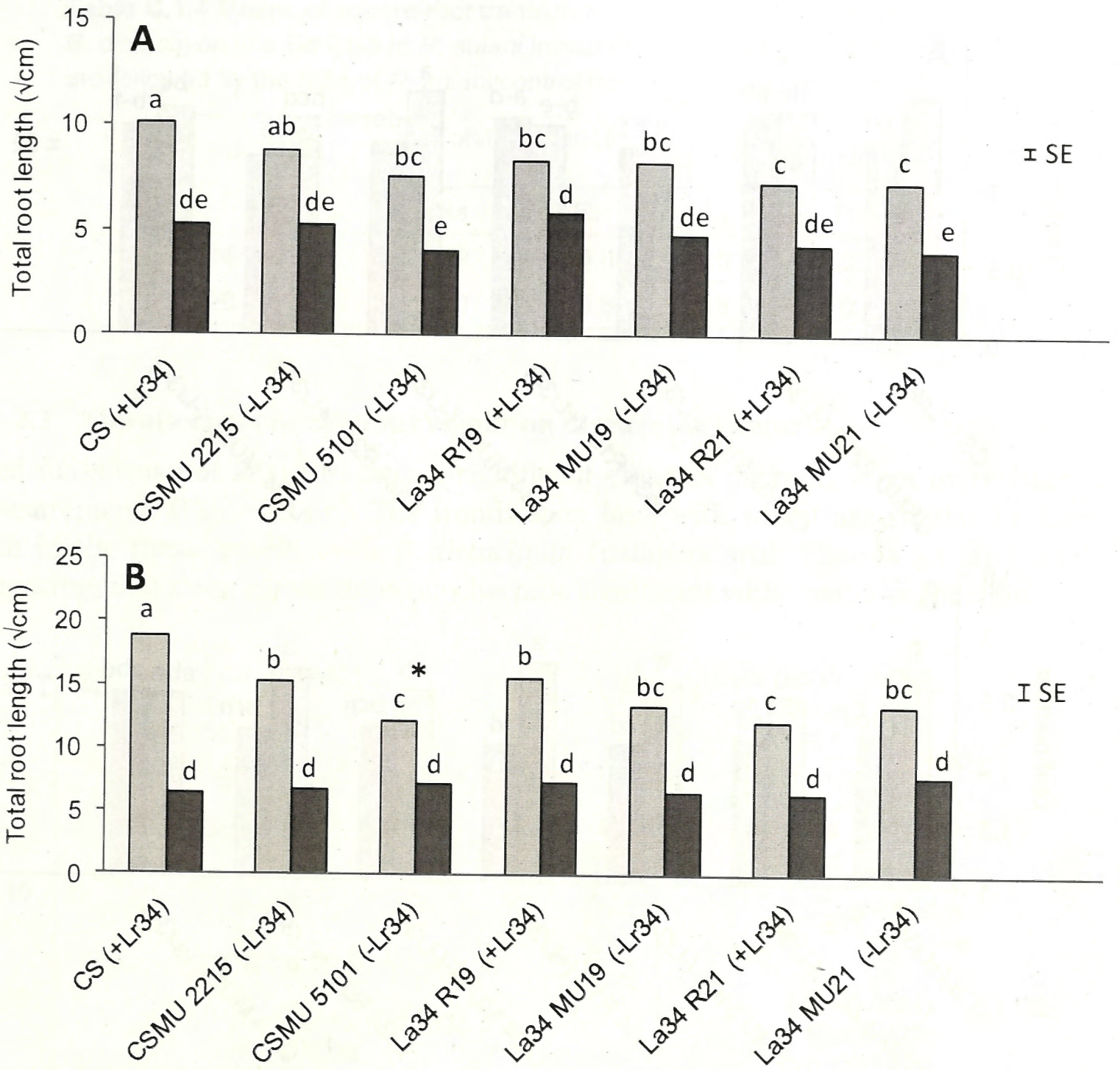

Figure C.1.3 Predicted means for total root length, for 7 lines grown in control (light grey) and *R. solani* (dark grey) inoculated soil and harvested at a) 16 DAP, and b) 26 DAP; n=3 to 6; average SE. Values are given in Table C.1.2.
*Pair-wise line response to *R. solani* is significantly different ($p<0.05$) from CS for CSMU 5101 for root length at 16 DAP.

Table C.1.2 Predicted means of square-root transformed total root length (√cm) for wheat in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values; n=3 to 6.

Genotype	*Lr34* expression	16 DAP			26 DAP		
		Rs	C	Ratio	Rs	C	Ratio
Chinese spring wheat	+ *Lr34*	5.3	10	0.52	6.5	19	0.35
CSMU 2215	- *Lr34* (knockout)	5.3	8.8	0.60	6.7	15	0.44
CSMU 5101	- *Lr34* (knockout)	4.1	7.6	0.53	7.2	12	0.60
La34 R19	+ *Lr34*	5.8	8.3	0.69	7.4	16	0.48
La34 MU19	- *Lr34*	4.8	8.3	0.58	6.6	13	0.50
La34 R21	+ *Lr34*	4.3	7.3	0.59	6.5	12	0.54
La34 MU21	- *Lr34*	4.1	7.3	0.55	7.9	13	0.59

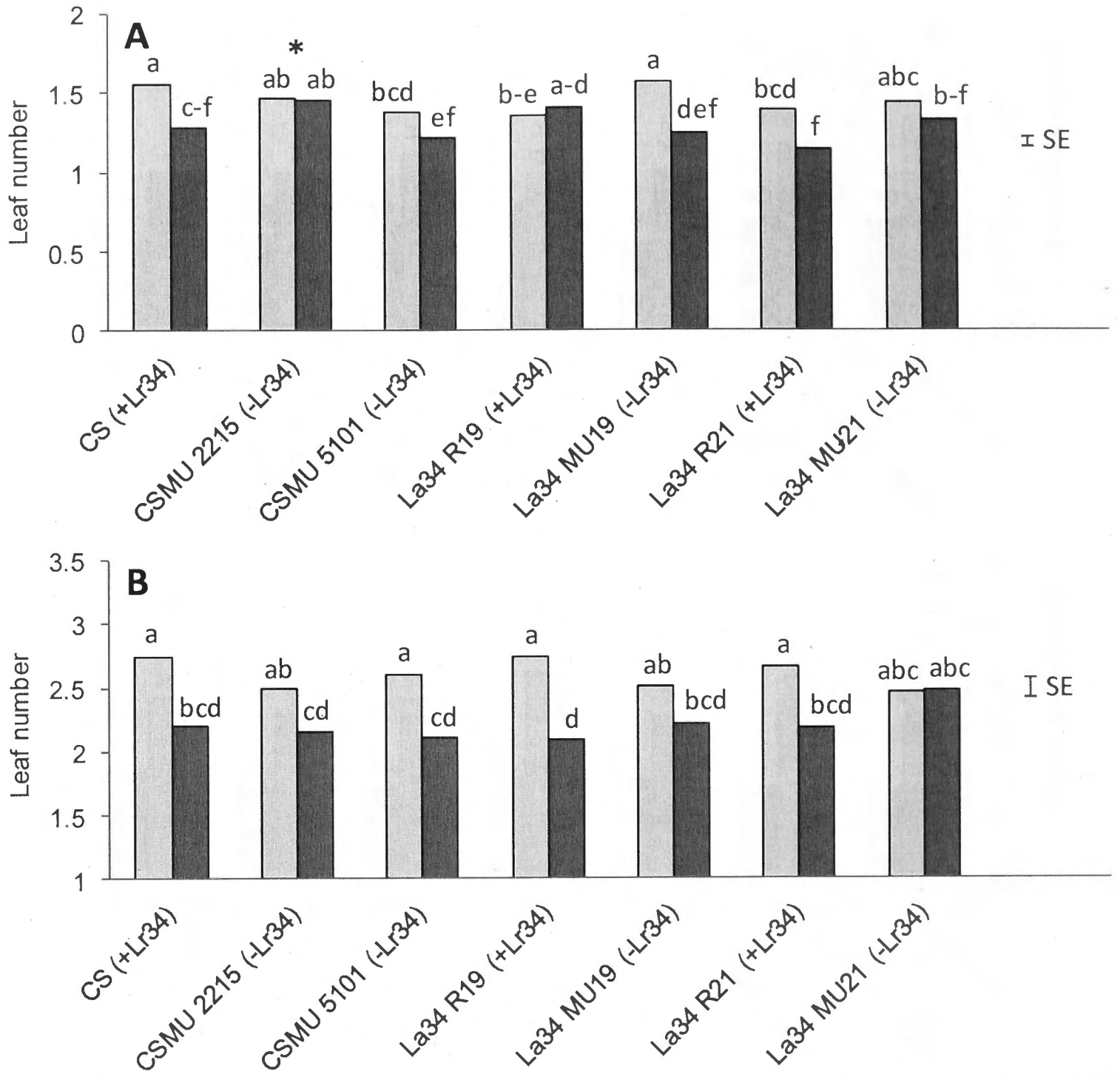

Figure C.1.4 Predicted means for leaf number, for 7 lines grown in control (light grey) and *R. solani* (dark grey) inoculated soil and harvested at a) 16 DAP, and b) 26 DAP; n=3 to 6; average SE. Values are given in Table C.1.3.
*Pair-wise line response to *R. solani* is significantly different ($p<0.05$) from CS for CSMU 2215 for leaf number at 16 DAP.

Table C.1.3 Predicted means of leaf number for wheat in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani* treatment values compared with control treatment values; n=3 to 6.

Genotype	*Lr34* expression	16 DAP			26 DAP		
		Rs	C	Ratio	Rs	C	Ratio
Chinese spring wheat	+ *Lr34*	1.3	1.6	0.82	2.2	2.7	0.80
CSMU 2215	- *Lr34* (knockout)	1.5	1.5	1.00	2.1	2.5	0.86
CSMU 5101	- *Lr34* (knockout)	1.2	1.4	0.88	2.1	2.6	0.81
La34 R19	+ *Lr34*	1.4	1.4	1.04	2.1	2.7	0.76
La34 MU19	- *Lr34*	1.2	1.6	0.80	2.2	2.5	0.89
La34 R21	+ *Lr34*	1.1	1.4	0.82	2.2	2.7	0.82
La34 MU21	- *Lr34*	1.3	1.5	0.92	2.5	2.5	1.01

Table C.1.4 Means of square-root transformed total root length (√cm) and leaf number for *B. distachyon* line Bd 21-3 in *R. solani* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of *R. solani*/control treatment values; n=3 to 5.

Bd 21-3	Total root length (√cm)			Leaf number		
	Rs	C	Ratio	Rs	C	Ratio
16 DAP	1.9	2.9	0.64	1.0	1.3	0.63
26 DAP	3.0	4.7	0.63	1.8	2.0	0.91

C.1.2.3 The effect of endogenous vigour on disease resistance

Seed mass was not found to have a significant effect of on root, shoot or resistance measurements (Figure C.1.5). The trends seen here with wheat are similar to those seen in the thesis results with *B. distachyon* (compare with Figures 3.8 and 4.11), suggesting that these correlations may become significant with greater replication.

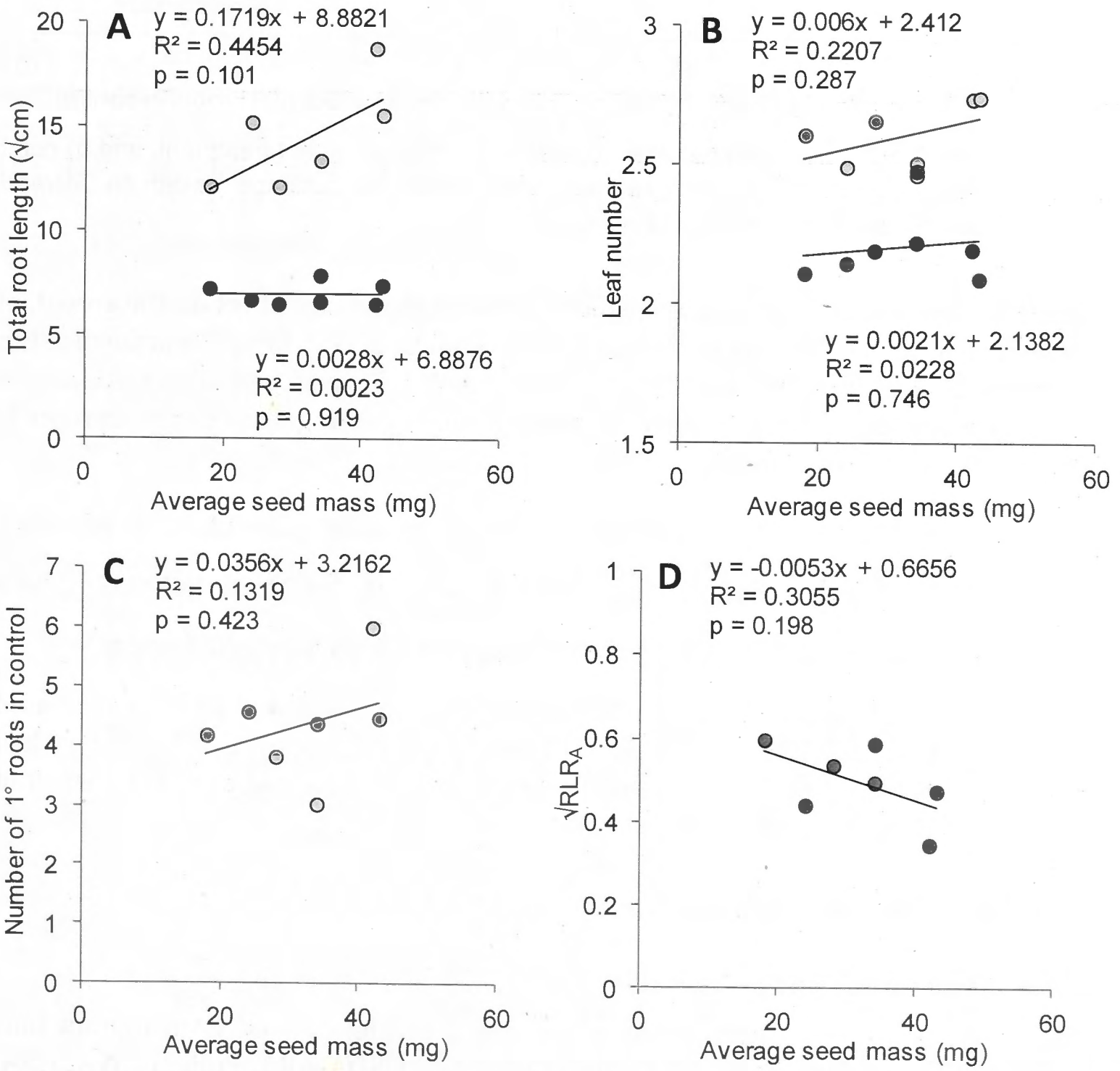

Figure C.1.5 Predicted means for a) total root length, b) leaf number, c) number of seminal roots in control, and d) the ratio of *R. solani*/control root length, plotted against average seed mass for 7 accessions grown in control (light grey circles) and *R. solani*-inoculated soil (dark grey circles) and harvested at 26 DAP.

Total root length in control treatment was positively correlated with the number of primary roots in control treatment (p=0.024). As also observed in two thesis experiments with *B. distachyon*, endogenous root vigour was significantly correlated with reduced resistance to *R. solani*, determined by *R. solani*/control total root length ratios determined using *Method A* described in §3.2.9.1 (Figure C.1.6).

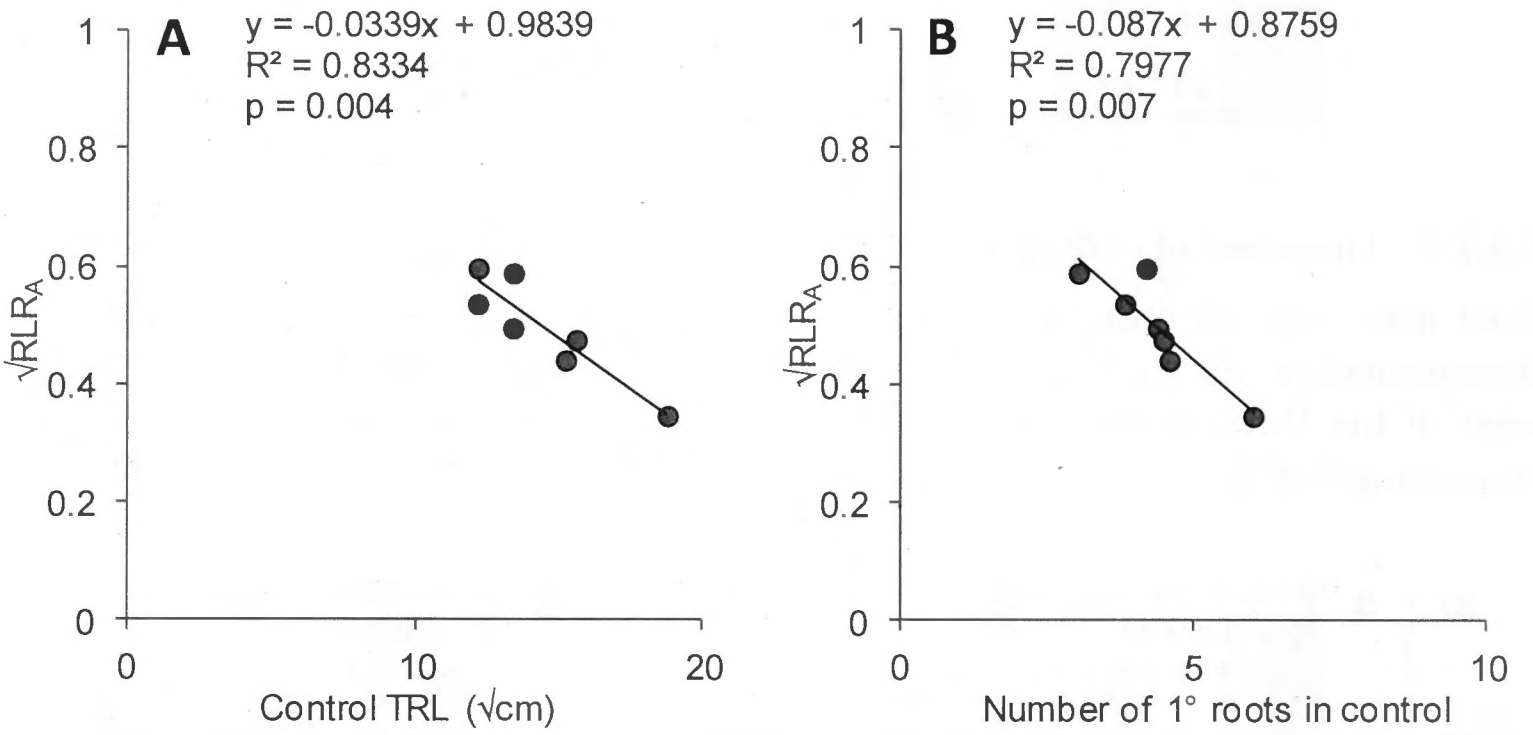

Figure C.1.6 Correlation between a) total root length in control treatment, and b) number of primary roots in control treatment, with resistance rankings based on *Method A R. solani*/control total root length ratios.

Overall, the presence of the *Lr34* gene did not have a significant effect on the growth of *R. solani* AG8 infested wheat in this assay. This indicates that *Lr34* is not involved in resistance to *R. solani*, but could also be the result of experimental conditions not allowing expression of *Lr34*, or required complementary endogenous genes, in roots at this early stage of development.

C.2 Effect of *Rhizoctonia solani* AG8 on Scarlet wheat lines with reported differences in tolerance to Rhizoctonia

A mutant line of wheat cultivar Scarlet, Scarlet-Rz1, was reported to have increased tolerance to *R. solani* AG8 than its parent (Okubara *et al.*, 2009). Our laboratory had access to seeds from this line, so a small experiment was designed to test the effect of our isolate of *R. solani* AG8 on these lines.

C.2.1 Materials and methods

C.2.1.1 Seed source and preparation

Triticum aestivum cv. Scarlet spring wheat and a Scarlet-derived EMS mutant line, 'Scarlet-Rz1', were donated by Dr Patricia Okubara (USDA-ARS, Pullman WA, USA) (Okubara *et al.*, 2009). In this experiment seeds were stratified to try to synchronise germination of *B. distachyon* (results not reported). Seeds were imbibed overnight in tap water at 4°C, surface-sterilized in sodium hypochlorite (1.3% v/v) and rinsed

thoroughly in water before being placed onto 3% agar plates. Plates were sealed and incubated at 4°C for 5 days, then at 12°C for 3 days.

C.2.1.2 Cone preparation and sowing

Rhizoctonia solani AG8 ZG1-1 inoculum was prepared according to the protocol in §2.2.2. Inoculum was mixed through 'Barley Mix' potting mix (§2.2.3) at 0.1 propagules per gram of soil, as described in §2.2.4.

C.2.1.3 Growth conditions

Growth cabinets were set at 16°C and 12 h day length, as described in §2.2.5. The toothpick re-isolation check (§2.2.6) was performed at 8 DAP. Plants were harvested at 12 days after planting.

C.2.1.4 Phenotype measurements

Total root length, leaf number, leaf 1 length and leaf 2 length were measured according to procedures described in §2.2.7.

C.2.1.5 Statistical analysis

This was a small experiment with the values of only 4 – 5 plants per treatment included after removing those that did not emerge or failed the toothpick re-isolation check. Statistical analysis was carried out with GenStat linear regression analysis (REML) using a fixed model '*Host*Inoculum*'.

C.2.2 Results and discussion

Total root length was significantly decreased by *R. solani* ($p<0.001$) in both lines. Leaf 1 length was not significantly affected by the pathogen, but mean leaf 1 length of the combined control and Rhizoctonia treatments for Scarlet-Rz1 was greater than Scarlet ($p=0.005$, 147 mm and 127 mm respectively). Okubara *et al.* (2009) also noted the more vigorous growth of Scarlet-Rz1 compared with Scarlet.

Ratios of root and shoot measurements between *R. solani* and control treatments in the two lines are presented in Table C.2.1. The genotype-pathogen interaction was not significantly different for any measurement. There was also no significant difference in leaf 2 length or leaf number between either genotype or inoculum treatment.

Table C.2.1 Predicted means of total root length, leaf 1 length, leaf 2 length and leaf number for Scarlet and Scarlet Rz-1 wheat in *Rhizoctonia* inoculated (Rs) and control (C) treatments. Means are followed by the ratio of Rhizoctonia treatment values compared with control treatment values.

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
Scarlet	179	367	0.49	2.0	2.2	0.90	127	127	1.00	190	207	0.92
Scarlet-Rz1	161	380	0.42	2.2	2.2	1.00	147	140	1.05	197	202	0.97

No significant difference was seen between the two Scarlet lines in the resistance response to *R. solani* AG8. The small but significantly greater leaf 1 length of Scarlet-Rz1 may be the result of higher early vigour in this line (Richards and Lukacs, 2002).

While the sample size for this experiment was very low, the trend in total root length ratio did not follow the results of Okubara *et al.* (2009), perhaps due to differences between the *R. solani* isolates used in the two studies. Thus, no further experiments were conducted to compare the Scarlet lines.