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The role of strigolactones and ethylene in disease caused by *Pythium irregulare*

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SUMMARY

Plant hormones play key roles in defence against pathogen attack. Recent work has begun to extend this role to encompass not just the traditional disease/stress hormones, such as ethylene, but also growth-promoting hormones. Strigolactones (SLs) are the most recently defined group of plant hormones with important roles in plant–microbe interactions, as well as aspects of plant growth and development, although the knowledge of their role in plant–pathogen interactions is extremely limited. The oomycete *Pythium irregulare* is a poorly controlled pathogen of many crops. Previous work has indicated an important role for ethylene in defence against this oomycete. We examined the role of ethylene and SLs in response to this pathogen in pea (*Pisum sativum* L.) at the molecular and whole-plant levels using a set of well-characterized hormone mutants, including an ethylene-insensitive *ein2* mutant and SL-deficient and insensitive mutants. We identified a key role for ethylene signalling in specific cell types that reduces pathogen invasion, extending the work carried out in other species. However, we found no evidence that SL biosynthesis or response influences the interaction of pea with *P. irregulare* or that synthetic SL influences the growth or hyphal branching of the oomycete *in vitro*. Future work should seek to extend our understanding of the role of SLs in other plant interactions, including with other fungal, bacterial and viral pathogens, nematodes and insect pests.

Keywords: ethylene, pea, *Pythium irregulare*, root pathogen, strigolactones.

INTRODUCTION

Plant hormones play a central role in the response to and defence against plant pathogens. In particular, the classic defence hormones ethylene, salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) play central roles, with a general split between responses to necrotrophic (ethylene and JA) and hemibiotrophic and biotrophic (SA) pathogens (Derksen *et al*., 2013; Robert-Seilaniantz *et al*., 2011). Cross-talk between these disease pathways is also crucial in plant defence. However, the involvement of a hormone in the development of a specific disease is dependent on a number of factors, including not only the lifestyle of the pathogen (biotroph vs. necrotroph) and the plant species involved, but also on variables within a pathosystem, such as the tissue type, inoculation method and the particular disease parameters measured (Denancé *et al*., 2013; Van der Ent and Pieterse, 2012).

The soil-borne oomycete *Pythium irregulare* has a wide host range, causing seedling damping off and root rot in many species. It is a particularly aggressive pathogen of several cereal and legume crops (Harvey *et al*., 2000), including field pea (*Pisum sativum* L.) (Hwang *et al*., 2000; Kraft *et al*., 1998), and continues to be difficult to control with chemical or cultural approaches because of little host resistance. A role for ethylene in the defence against this necrotrophic pathogen has been proposed in Arabidopsis and tobacco through the use of ethylene-insensitive lines (Adie *et al*., 2007; Geraats *et al*., 2002). Increased susceptibility to *P. irregulare* was observed in Arabidopsis mutants defective in essential ethylene signal transduction elements [ETHYLENE RECEPTOR1 (ETR1) and ETHYLENE INSENSITIVE2 (EIN2)] and also transgenic tobacco expressing a mutant version of the AtETR1 protein. However, a closer examination of the stages of infection in which ethylene signalling is crucial, including early molecular events and the specific tissues affected, as well as a broader understanding of ethylene's role in major crops affected by this disease, is required.

Considerably less attention has been paid to the role played by the 'growth-promoting' hormones auxin, gibberellin, brassinosteroids and cytokinin in defence (Derksen *et al*., 2013; Robert-Seilaniantz *et al*., 2011). This lack of information is particularly acute for the most recently defined hormone group, the strigolactones (SLs) (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). SLs are carotenoid derivatives with regulatory roles in shoot branching, secondary growth, root development and phosphate stress responses (Foo and Reid, 2013; Smith and Li, 2014). Indeed, even before their definition as plant hormones, SLs were known to play important roles in the rhizosphere in interactions with parasitic weeds and in the formation of symbioses with beneficial arbuscular mycorrhizal (AM) fungi (Akiyama *et al*., 2005; Cook *et al*., 1966). This role in plant–microbe interactions has most recently been

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extended to include SL's promotion of the symbiosis of leguminous plants with nitrogen-fixing bacteria (Foo and Davies, 2011; Foo *et al*., 2013; Liu *et al*., 2013; Soto *et al*., 2010). Given the widespread nature of SLs throughout the plant kingdom (Challis *et al*., 2013; Delaux *et al*., 2012), their presence in root exudate and their ability to interact with soil microbes, it is essential we understand how they may influence plant interactions with disease-causing pathogens. Indeed, given the current focus on the development of commercial products to suppress parasitic weed interactions by targeting SLs (e.g. Lachia *et al*., 2014, 2015), the impact on other plant–microbe interactions is essential information.

There are several conflicting reports that the synthetic SL, GR24, may or may not influence the growth, activity and/or hyphal branching of disease-causing fungi when grown in culture (Dor *et al*., 2011; Sabbagh, 2012; Steinkellner *et al*., 2007; Torres-Vera *et al*., 2013). At low GR24 concentrations, similar to those of SLs that would be present in the rhizosphere and that induce AM hyphal branching ($\leq 1 \times 10^{-6}$ M), there was little effect on radial growth or hyphal branching in a range of fungal species. However, at higher doses of GR24, some negative effects on radial growth and positive effects on hyphal branching were observed by Dor *et al*. (2011), although their method employed the heating of relatively unstable GR24 which may yield breakdown products with unknown effects. Importantly, a recent report has examined the role of endogenous SLs in the development of disease in tomato plants challenged with two foliar fungal pathogens: *Botrytis cinerea* and *Alternaria alternata* (Torres-Vera *et al*., 2013). Detached leaves of transgenic tomato lines with reduced SL synthesis (as a result of *RNAi* suppression of the SL biosynthesis gene *SlCCD8*) challenged with these pathogens developed larger lesions than wild-type (WT) plants, suggesting that SLs may play a positive role in plant defence. The authors suggested that SLs may act as an endogenous signal during plant defence, rather than on the fungi directly, as low doses of GR24 did not appear to directly influence the growth of either fungus in culture (Torres-Vera *et al*., 2013), although, as outlined above, there are reports of higher doses of GR24 affecting growth and/or hyphal branching of these fungal species (Dor *et al*., 2011). The authors also reported a somewhat decreased level of the defence hormones SA, JA and ABA in leaves of intact unchallenged *SlCCD8 RNAi* lines compared with WT. It is now essential to determine whether SLs play a more general role in plant defence by examining other plant species and a range of pathogens with different life strategies. Given that SLs are a component of root exudate, it is particularly important to examine their role in interactions with soil-borne pathogens.

In this article, we used pea as a model crop legume to examine the role of the plant hormones ethylene and SLs in disease caused by the soil-borne pathogen *Pythium irregulare*. Pea is an ideal system, given that it is an economically important crop that is impacted by *P. irregulare* infection and has well-defined mutants defective in ethylene signalling (*Psein2*; Weller *et al*., 2015) and SL biosynthesis and response (*Psccd7*, *Psccd8*, *Psf-box*; Foo and Reid, 2013). We found an important role for ethylene signalling in defence against *P. irregulare* and showed that ethylene signalling in specific cell types appears to reduce pathogen invasion. In contrast, we found no evidence that SL biosynthesis or response influences interactions with *P. irregulare*, either by SLs acting directly on oomycete spore germination or hyphal growth, or by influencing events after infection.

RESULTS

Ethylene plays a key role in defence against pea root rot

In pea, all ethylene signalling appears to be through the single copy gene *PsEIN2*, which encodes an N-RAMP metal-transporter-like protein, whose degradation on ethylene perception ultimately promotes the expression of ethylene-responsive genes (Lin *et al*., 2009; Merchante *et al*., 2013; Qiao *et al*., 2012; Weller *et al*., 2015). *Psein2* mutant plants are ethylene insensitive at both the seedling and adult stage and across tissue types (Weller *et al*., 2015). As described previously, *ein2* seedlings are almost indistinguishable from WT, with many of the effects of the mutant only apparent in mature plants (e.g. delayed petal abscission, Weller *et al*., 2015).

In contrast with the large healthy root systems of mock-inoculated plants, plants of both genotypes inoculated with *P. irregulare* developed classic symptoms of root rot, including a stunted root system caused by tap root tip necrosis, failure of many lateral roots to develop (aborted lateral roots) and discoloration of lateral roots that did develop (Martin and Loper, 1999; Fig. 1). However, when challenged with *P. irregulare*, *ein2* mutants developed more severe disease symptoms than WT plants (Fig. 1). This was particularly the case for shoot symptoms, with inoculated *ein2* shoots developing significantly smaller, less developed shoots that were severely wilted compared with those of inoculated WT plants (Fig. 1A–D). This severe wilting only happened in a proportion of WT plants (<30%), whereas the vast majority of *ein2* mutant shoots challenged with the pathogen collapsed completely (Fig. 1D). The lateral roots of the ethylene-insensitive mutant plants also developed significantly more discoloration than those of WT plants (Fig. 1I). However, it is interesting to note that other root disease symptoms, such as the proportion of discoloration on the tap root and the number of aborted lateral roots, were similar in WT and *ein2* mutant plants (Fig. 1E–H).

We explored the influence of ethylene signalling on the specific cell types colonized by *P. irregulare* in infected WT and *ein2* mutant roots (Fig. 2). To examine disease progression at a cellular level, longitudinal sections of tap roots were taken from WT plants and *ein2* mutants 9 days after infection and stained to observe the

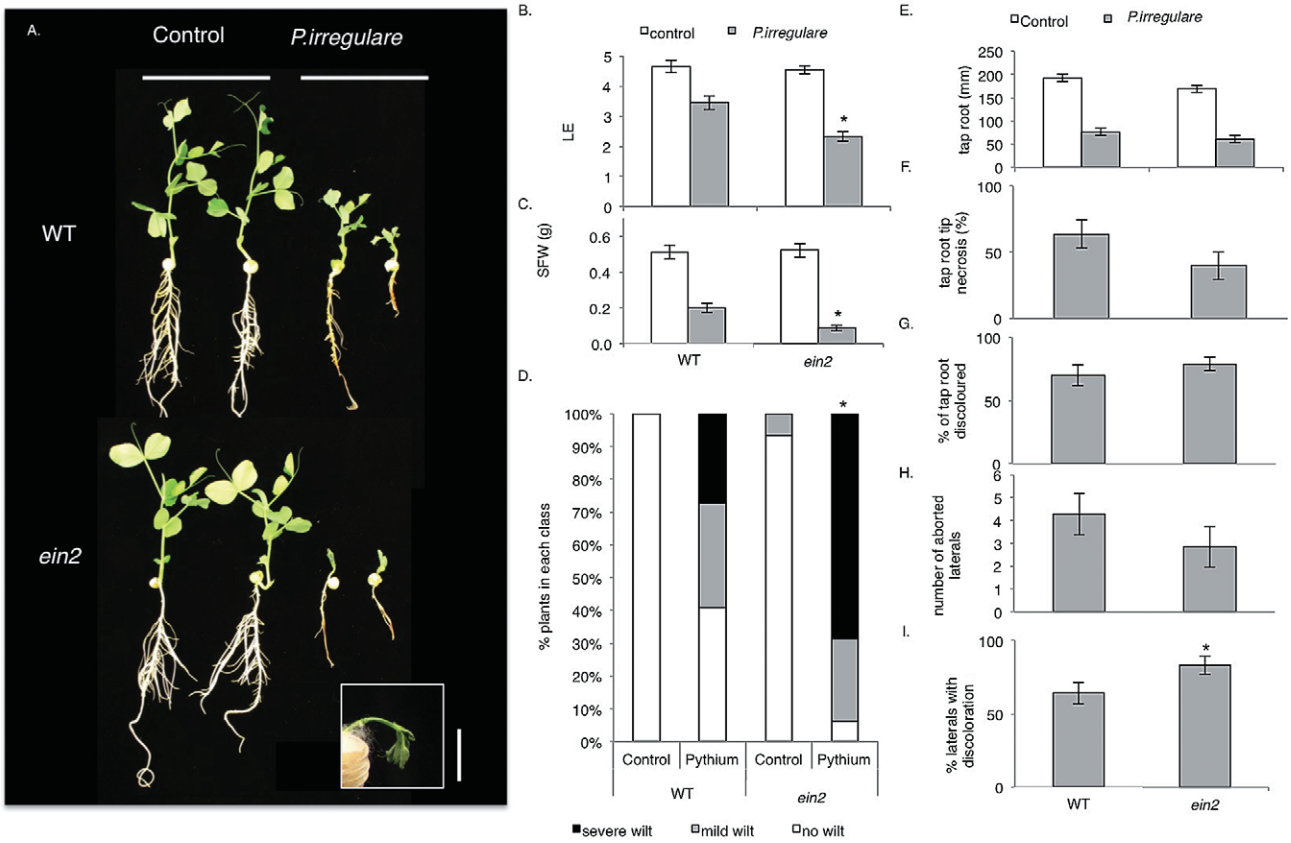

Fig. 1 Disease symptoms of wild-type (WT) and the ethylene-insensitive *ein2* mutant 9 days after inoculation. (A) Photographs of whole plants of control and *Pythium irregulare*-infected WT and *ein2* showing the range of phenotypes observed (scale bar, 5 cm); inset is a close up of the strongly wilted *ein2* shoot. (B) Leaves expanded (LE). (C) Shoot fresh weight (SFW). (D) Shoot wilt. (E) Tap root length. (F) Percentage of plants with tap root tip necrosis. (G) Percentage of tap root classed as discoloured. (H) Number of aborted lateral roots per plant. (I) Percentage of lateral roots with any discoloration. Control plants did not have any discoloration on their roots and so control values are not displayed for (F)–(I). (B–I) Values are means ± standard error (n = 20–22). (B–D) * indicates significant genotype × treatment interaction ($P < 0.05$). (F–I) * indicates significant difference between genotypes ($P < 0.05$).

pathogen. *Pythium irregulare* hyphae were found within the root of *ein2* mutant plants, from the outer cortex to deep within the inner cortical cell layers (Fig. 2A,B). In contrast, these root tissues were largely free of the oomycete in WT plants (Fig. 2A,B), despite the fact that large amounts of hyphae were observed on the surface of the root of both genotypes (data not shown). Interestingly, *P. irregulare* hyphae were found to a similar extent in aborted lateral root tips of WT and *ein2* mutant plants (Fig. 2C,D). Indeed, in both genotypes, advanced lateral root infection was characterized by the almost complete degradation of the lateral root meristems (Fig. 2D). This invasion of the pathogen into the cortex of the ethylene-insensitive roots, including the cells closest to the vascular sheath, may be the cause of the more dramatic shoot wilt and collapse observed in this mutant.

We also examined the expression of key defence markers in the root at the site of inoculation 24 h after challenge with *P. irregulare* to establish whether, in addition to later roles in the patterns of invasion, ethylene signalling was also essential for the earliest responses to the pathogen (Fig. 3). Two enzymes in the phenolic pathway were monitored: *Pal1* (*phenylalanine ammonia lyase*; Yamada *et al*., 1992) and the downstream enzyme *Hmm6* (*6-α-hydroxymaackiain methyltransferase*), which catalyses the last step in the synthesis of the phytoalexin pisatin (Preisig *et al*., 1989; Wu *et al*., 1997). The expression of the hydrolase *β-Gluc* (*β-1,3-glucanase*), which breaks down one of the major components of fungal and oomycete cell walls (Balasubramanian *et al*., 2012; Ferreira *et al*., 2007), was also monitored. Compared with mock-inoculated plants, there was a significant induction of expression of all three genes in inoculated WT and *ein2* plants (Fig. 3A). Two-way analyses of variance (ANOVAs) revealed a significant influence of inoculation ($P < 0.05$), but no genotype × treatment interactions. However, the expression of *β-Gluc* was significantly lower in *ein2* mutants (i.e. a genotype effect). Consistent with these data, which indicate no clear role for ethylene during the early stages of infection, we found no significant change in the expression of ethylene biosynthesis genes *ACS1* (*1-aminocyclopropane-1 carboxylic acid synthase*) and *ACOX* (*ACC oxidase*) in the first 24 h after pathogen challenge in either genotype, although *ACS1*

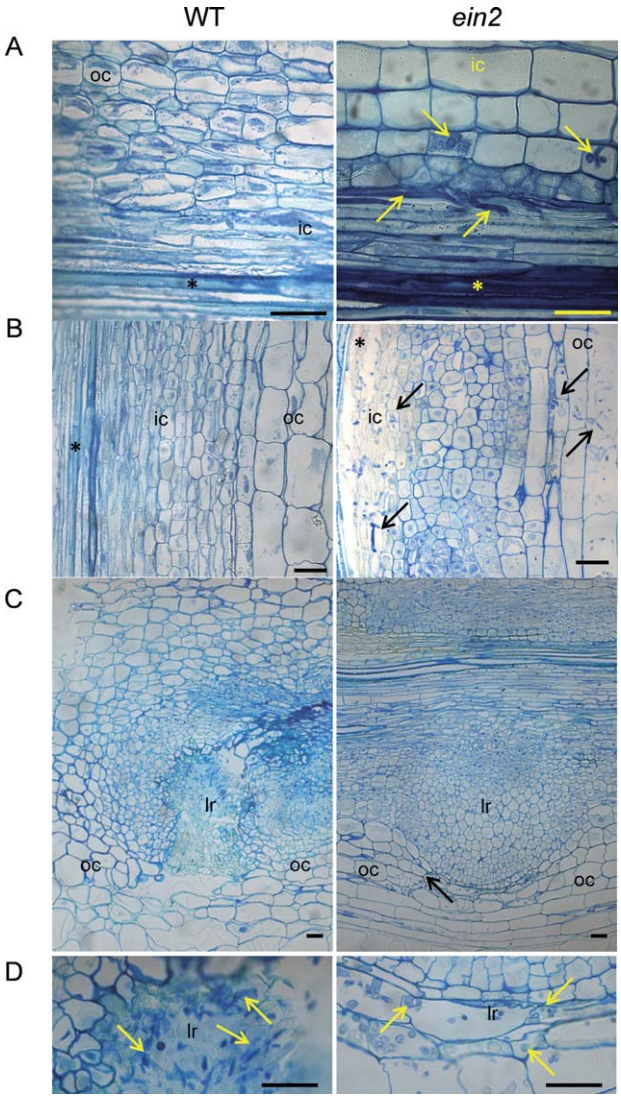

Fig. 2 *Pythium irregulare* is found in the root cortex of the ethylene-insensitive *ein2* mutant, but not in wild-type (WT) plants. Transverse sections of roots of *P. irregulare*-infected WT and *ein2* mutants stained with toluidine blue O. (A) Inner cell layers of tap root. (B) Outer cell layers of tap root. (C) Aborted lateral root tips. (D) Close up of advanced infection in aborted lateral root tips. Arrows indicate intracellular hyphae. ic, inner cortex; lr, lateral root; oc, outer cortex; asterisk, vascular cylinder. All scale bars are 50 μm.

expression was somewhat elevated under all conditions in *ein2* plants (Fig. 3B), presumably as a result of the feedback regulation seen for the synthesis of many plant hormones in insensitive mutants, including ethylene (Chan *et al*., 2013; Croker *et al*., 1990; Nomura *et al*., 1999; Weston *et al*., 2008).

SLs do not affect the growth, hyphal branching or oospore germination of *P. irregulare in vitro*

There is little information on the role of SLs during the invasion of plant tissue by pathogenic oomycetes. Given the recent sug-

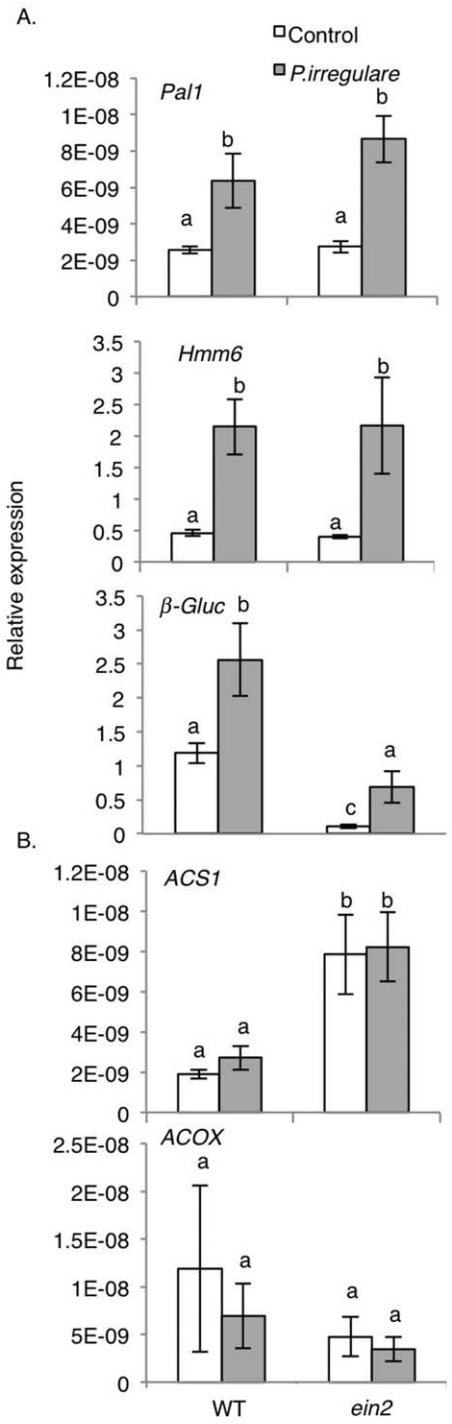

Fig. 3 Expression of defence and ethylene metabolism genes in wild-type (WT) plants and ethylene-insensitive *ein2* mutants 24 h after inoculation with *Pythium irregulare* in root tissue at the site of inoculation. (A) Defence genes *Pal1* (*phenylalanine ammonia lyase*), *Hmm6* (*6-α-hydroxymaackiain methyltransferase*) and *β-Gluc* (*β-1,3-glucanase*). (B) Ethylene metabolism genes *ACS1* (*1-aminocyclopropane-1 carboxylic acid synthase*) and *ACOX* (*ACC oxidase*). Values within a panel with different letters are significantly different at $P < 0.05$. Values are means ± standard error ($n = 5$).

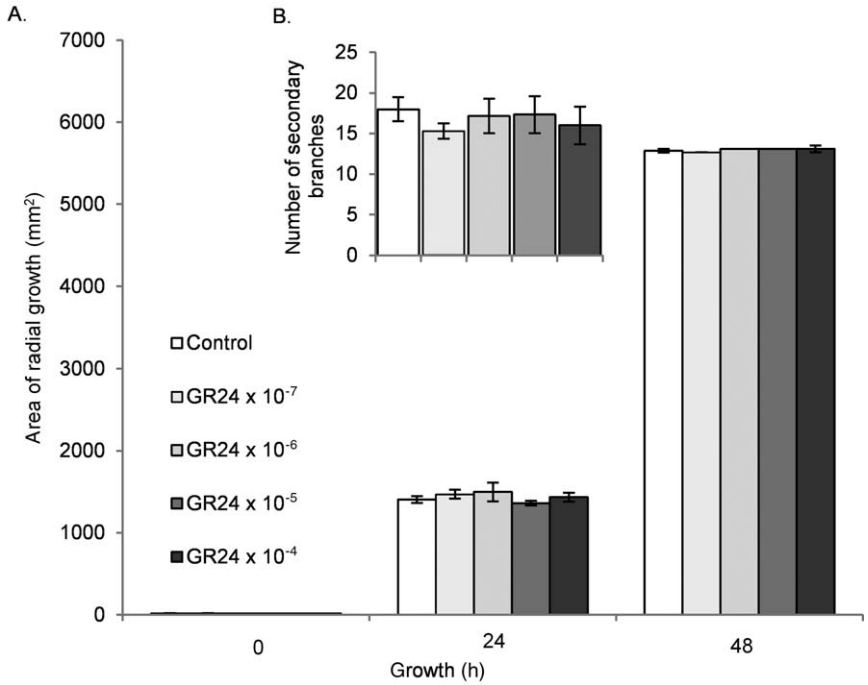

Fig. 4 Effect of the synthetic strigolactone (+)-GR24 (1×10^{-4}–10^{-7} M) or control on the growth of *Pythium irregulare* in culture. (A) Radial growth over time. (B) Hyphal branching after 24 h. Values are means ± standard error ($n = 10$).

gestion that SLs play a positive role in defence against necrotic foliar fungal pathogens (Torres-Vera *et al*., 2013), we examined whether SLs may be important in defence against the soil-borne pathogen *P. irregulare* in pea. SLs are components of the plant root exudate, with potent effects on symbiotic AM fungi, including the stimulation of spore germination and hyphal branching (Akiyama *et al*., 2005; Besserer *et al*., 2006, 2008). Therefore, it was important to first establish whether SLs may have a direct effect on *P. irregulare* before contact with the plant. It is important to note that different stereoisomers of SL analogues can have quite specific biological activities (e.g. Akiyama *et al*., 2010; Scaffidi *et al*., 2014). In contrast with previous studies that employed a racemic mix of GR24 containing both stereoisomers (Dor *et al*., 2011; Sabbagh, 2012; Steinkellner *et al*., 2007; Torres-Vera *et al*., 2013), we employed (+)-GR24 (also known as 5DS-GR24), which is known to mimic naturally occurring SL (Scaffidi *et al*., 2014). We found no influence of a range of concentrations of (+)-GR24 on either radial growth or hyphal branching of *P. irregulare* (Fig. 4). Although the inoculum used contained overwhelmingly hyphal material and few spores (data not shown), both oospores and mobile zoospores may be an important source of disease-causing inoculum in field situations. Mobile zoospore must normally first contact the plant host tissue and encyst before germination. We therefore examined the effect of (+)-GR24 on the germination of the normally overwintering oospores that can germinate *in vitro* (e.g. Harvey *et al*., 2000). As with hyphal growth and branching, we found no significant effect of (+)-GR24 on spore germination (Fig. S1, see Supporting Information).

SL mutants of pea develop similar disease symptoms to WT plants

The above *in vitro* studies suggest that SLs are not likely to act directly on *P. irregulare* in the soil to influence disease progression. However, SLs are also found inside the root and, like many other plant hormones, may act inside the plant as a signal during the response to pathogen attack. To explore this, we examined disease progression after infection with *P. irregulare* in a range of pea SL mutants. The *ccd7* and *ccd8* mutants are disrupted in carotenoid cleavage dioxygenase (CCD) enzymes that catalyse essential steps in the biosynthesis of SLs and are both severely deficient in SLs (Foo *et al*., 2013; Gomez-Roldan *et al*., 2008). The *f-box* mutant is disrupted in a key element of the SL perception pathway that has been proposed to complex with the SL receptor (Hamiaux *et al*., 2012; Johnson *et al*., 2006). At the seedling stage, all mutants have a similar phenotype to WT, except for the presence of small shoot branches on some plants and some small changes in root architecture (for details, see Urquhart *et al*., 2014).

Both SL-deficient and SL-insensitive mutants developed the same pattern and severity of symptoms as seen for WT plants (Fig. 5). This was the case for shoot stunting and wilting in SL-deficient *ccd8* mutants (Fig. 5B–D) and all root symptoms measured, including stunted growth and extent of discoloration of all SL-deficient and SL-insensitive mutants (Fig. 5E–I). The fact that we could not distinguish a difference in disease development in SL mutants when compared with WT plants is consistent with the disease progression observed at a cellular level. A similar pattern of *P. irregulare* hyphae in aborted lateral root tips, but not in the

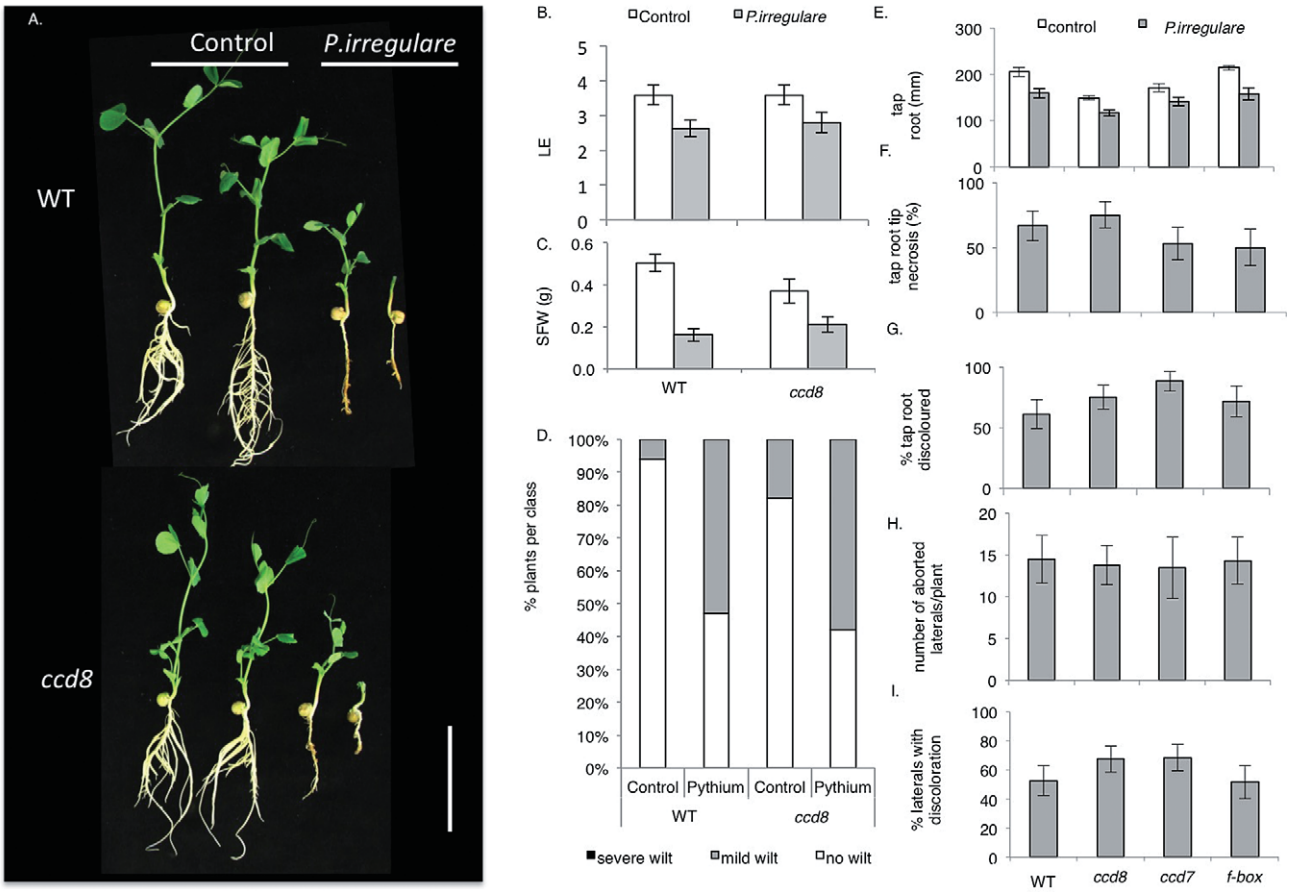

Fig. 5 Disease symptoms of wild-type (WT) and strigolactone (SL) mutants (*ccd7*, *ccd8* and *f-box*) 9 days after inoculation. (A) Photograph of whole-root systems of control and *Pythium irregulare*-infected WT and *ccd8* plants showing the range of phenotypes observed (scale bar, 5 cm). (B) Leaves expanded (LE). (C) Shoot fresh weight (SFW). (D) Shoot wilt. (E) Tap root length. (F) Percentage of plants with tap root tip necrosis. (G) Percentage of tap root classed as discoloured. (H) Number of aborted lateral roots per plant. (I) Percentage of lateral roots with any discoloration. Control plants did not have any discoloration on their roots and so control values are not displayed for (F–I). (B–I) Values are averages ± standard error (n = 10–20).

tap root cortex, was seen in WT plants and SL-deficient *ccd8* mutants (Fig. 6). These results were also consistent with no marked change in the induction of expression of disease markers in *ccd8* SL-deficient mutants compared with WT plants following pathogen challenge (Fig. 7). A two-way ANOVA revealed a significant influence of inoculation on the expression of *Pal1* and *β-Gluc* ($P < 0.05$), but no significant genotype or genotype × treatment interactions.

Given that recent studies have revealed that SL production may be influenced by a number of abiotic stresses, in particular nutrient deficiency and osmotic stress (e.g. Foo *et al*., 2013; Liu *et al*., 2014; Yoneyama *et al*., 2007), we examined whether SL production was influenced by pathogen attack. We examined the expression of the key SL biosynthesis genes *PsCCD7* and *PsCCD8* in WT plants in the root at the site of inoculation 24 h after *P. irregulare* challenge, and found a small but significant increase ($P < 0.05$) in the expression of *PsCCD7*, but not of *PsCCD8*, compared with mock-inoculated controls (Fig. 8A). It is difficult to assess whether this small up-regulation in *PsCCD7* expression may result in a small increase in SL production during the early stages of infection, as young pea seedlings (<10 days old) produce very low levels of SL (data not shown) and gene expression is not always a reliable indicator of endogenous hormone levels (Symons and Reid, 2008). However, there was no change in SL levels in root tissue of older pot-grown *P. irregulare*-infected WT plants compared with mock-inoculated controls (Fig. 8B).

DISCUSSION

Seedling damping off diseases, such as the root rot caused by the oomycete *P. irregulare*, cause significant damage to crops, including the field legumes pea and bean (Kamoun *et al*., 2015; Martin and Loper, 1999). In this article, we define an important role in pea for ethylene signalling in specific root cells that results in defence against *P. irregulare* invasion, extending the findings in other species (Adie *et al*., 2007; Geraats *et al*., 2002). In contrast,

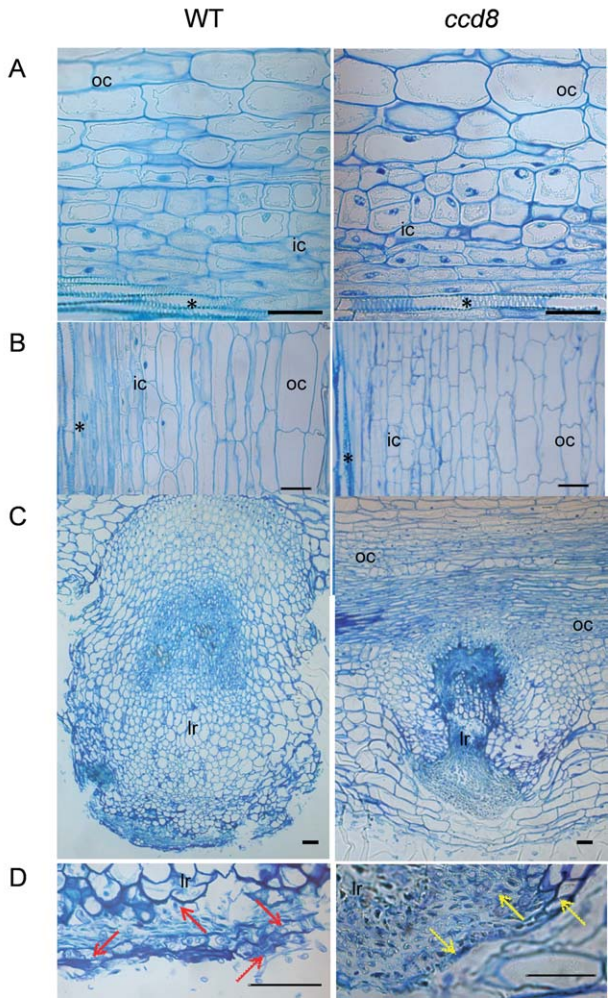

Fig. 6 *Pythium irregulare* is only found in the lateral root tips of *ccd8* and wild-type (WT) plants. Transverse sections of roots of *P. irregulare*-infected WT and *ccd8* mutants stained with toluidine blue O. (A) Inner cell layers of tap root. (B) Outer cell layers of tap root. (C) Aborted lateral root tips. (D) Close up of advanced infection in aborted lateral root tips. Arrows indicate intracellular hyphae. ic, inner cortex; lr, lateral root; oc, outer cortex; asterisk, vascular cylinder. All scale bars are 50 μm.

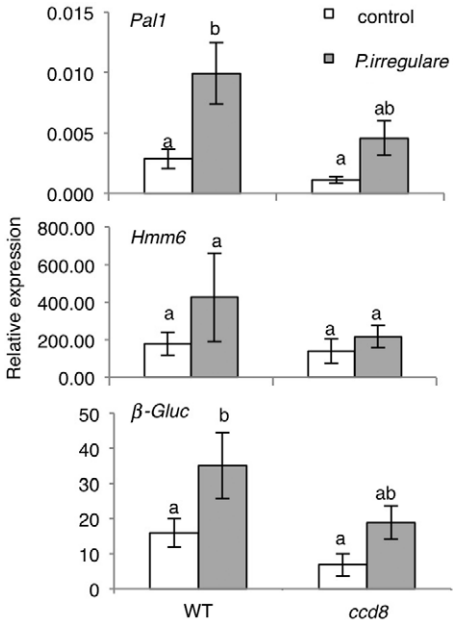

Fig. 7 Expression of defence genes *Pal1* (*phenylalanine ammonia lyase*), *Hmm6* (*6-α-hydroxymaackiain methyltransferase*) and *β-Gluc* (*β-1,3-glucanase*) in wild-type (WT) plants and strigolactone (SL)-deficient *ccd8* mutants 24 h after inoculation with *Pythium irregulare* in root tissue at the site of inoculation. Values within a panel with different letters are significantly different at $P < 0.05$. Values are means ± standard error (n = 3–5).

although SL has been proposed to play a positive role in defence against foliar necrotrophic fungi in tomato (Torres-Vera *et al*., 2013), we found no such protective effect of SLs in pea interactions with the soil-borne necrotroph *P. irregulare*. This highlights the fact that the specific role of a plant hormone in plant–pathogen interactions must be established empirically, because of the clear differences seen in specific plant species and plant tissues, as well as those caused by the pathogen itself. This is especially the case for the growth-promoting hormones, whose role in plant defence and disease response is poorly understood.

Ethylene signalling plays an important role in limiting the invasion of *P. irregulare* infection in pea roots. Intracellular invasion was observed in WT plants at tap and lateral root tips. In contrast, intracellular hyphae were also present in the epidermis, outer and inner cortex of the tap roots of ethylene-insensitive *ein2* mutants, suggesting a role for ethylene signalling in restricting invasion in the root. Indeed, the extensive invasion of the pathogen into the inner root tissue of *ein2* mutant seedlings was linked to more severe wilting than observed in WT plants, and premature death. This action of EIN2 in the cortical cell layers of pea offers a possible explanation of the severe disease reaction also observed in *P. irregulare* infection of orthologous ethylene-insensitive lines of Arabidopsis and tobacco, where it has been speculated that ethylene signalling may act to restrict the pathogen to outer cell layers (Adie *et al*., 2007; Geraats *et al*., 2002). Most plant species only develop mild infections when challenged with the non-host-specific *P. irregulare*, and this restriction of disease does not appear to involve the hypersensitive response (Kamoun *et al*., 1999). The restriction of disease in WT plants compared with ethylene-insensitive lines challenged with *P. irregulare* suggests an important role for ethylene in a partial ubiquitous resistance to this common oomycete across species.

SLs are a fascinating group of plant compounds, as they act not only *in planta* as a hormone, influencing shoot and root development, but also *ex planta* as a rhizosphere signal, where they influence interactions with parasitic weeds and symbiotic AM fungi (Foo and Reid, 2013; Smith and Li, 2014). This dual role of

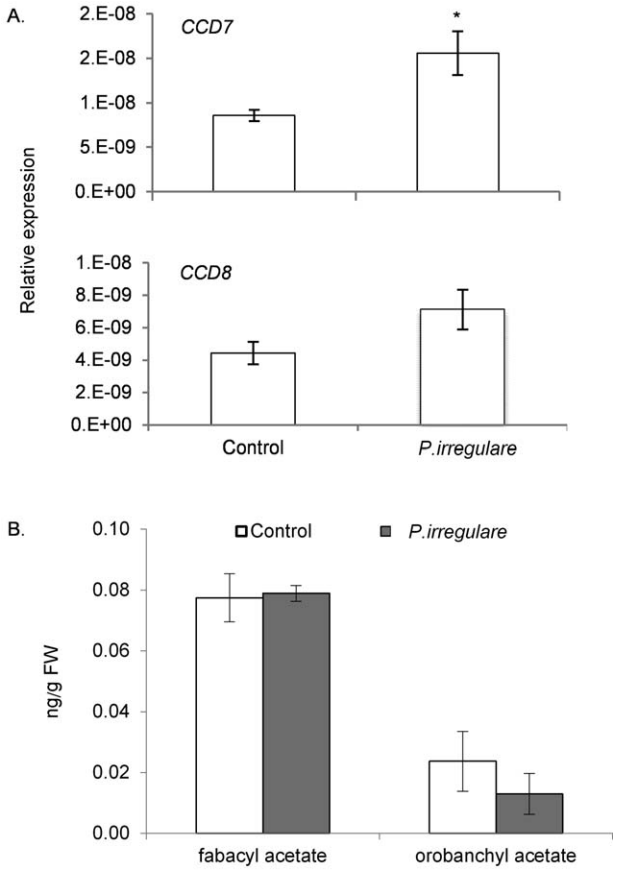

Fig. 8 Strigolactone levels and expression of strigolactone biosynthesis genes in wild-type (WT) (cv. Parvus) control and *Pythium irregulare*-inoculated plants. (A) Relative expression of strigolactone biosynthesis genes *CCD7* and *CCD8* in tube-grown plants 24 h after control or *P. irregulare* inoculation. (B) Strigolactone levels in root tissue of pot-grown plants 14 days after control or *P. irregulare* inoculation. FW, fresh weight. Values are means ± standard error (n = 3–5). * indicates significant difference between genotypes (P < 0.05).

SLs as internal and external signals makes them a potentially important signal in interactions with other organisms, including plant pathogens. Indeed, one report in tomato using an SL-depleted RNAi line suggested a protective role for SL in defence against two fungal necrotrophs that infect the shoot (Torres-Vera *et al*., 2013). We examined both potential roles of SL in disease development in pea using a combination of application and mutant studies. Several reports have indicated that the synthetic SL GR24 may have some impact on the growth or hyphal branching of some plant pathogens tested in culture, although it should be noted that not all responses were reproducible (Dor *et al*., 2011; Steinkellner *et al*., 2007; Torres-Vera *et al*., 2013). In the case of the pathogen investigated here, *P. irregulare*, we found no evidence that the synthetic SL, (+)-GR24, influenced the growth, hyphal branching or oospore germination in this oomycete across a range of concentrations. This indicates that SLs in root exudates are unlikely to directly influence the growth of this oomycete or oospore germination before infection.

If SLs are important regulators of the development of disease once the pathogen and plant have come into contact, mutants with reduced production or perception of SLs would be predicted to show altered disease progression. We did not find any significant difference in the development of disease symptoms in SL mutants compared with WT, at the whole plant, tissue or molecular level. All disease symptoms, in both the shoots and the roots, were the same in their nature and intensity in SL mutants and WT plants. This similarity between genotypes at the whole-plant level was reflected in the sectioning of the *ccd8* SL-deficient mutant, which showed no difference in the cell types infected or in the abundance of infection when compared with WT plants. Consistent with this, the *ccd8* SL-deficient mutant did not differ markedly from WT in the induction of early defence markers. This contrasts with the significant changes in interactions with symbiotic microbes (AM fungi and rhizobia) reported previously for these SL mutants (Foo and Davies, 2011; Foo *et al*., 2013; Gomez-Roldan *et al*., 2008). There was also no strong indication that a change in endogenous SL content is induced by infection. This is striking, as other abiotic and biotic signals have been shown to induce large changes in SL production and/or exudation, including nutrient stress, drought and AM colonization (e.g. Foo *et al*., 2013; Liu *et al*., 2014; López-Ráez *et al*., 2011; Yoneyama *et al*., 2007).

In conclusion, unlike the highly conserved role for SLs in interactions with AM fungi and their positive role in interactions between legumes and nitrogen-fixing rhizobial bacteria (Foo *et al*., 2014), our study suggests no general role for SLs in defence against necrotrophic plant pathogens. This is in contrast with the consistent protective role for ethylene signalling in interactions between the oomycete *P. irregulare* and several plant species, including the results presented here for pea. However, this only highlights the need for further studies examining the role of SLs in a range of pathosystems, including interactions with biotrophic pathogens, diseases caused by bacteria, viruses and nematodes, as well as interactions with insect pests. By employing the range of SL mutants/transgenics across a number of plant species (pea, Arabidopsis, petunia, rice, etc.), we will begin to establish the interactions that are influenced by SL and thus, ultimately, inform farming practices to protect plants from disease whilst enhancing plant architecture, symbiotic interactions and yield.

EXPERIMENTAL PROCEDURES

Plant and fungal material

The *Pisum sativum* L. lines used were WT cv. Torsdag and cv. Parvus, SL-deficient lines *ccd8-1* (*rms1-1* derived from WT Parvus; Beveridge *et al*., 1997) and *ccd8-2* (*rms1-2*T; crossed into the cv. Torsdag background as described in Foo *et al*., 2013), and the *ccd7* mutant (*rms5-3*T; crossed into

cv. Torsdag as described by Foo *et al*., 2013). The SL-insensitive line *Psf-box*, also known as *rms4-1*, was derived from cv. Torsdag (Beveridge *et al*., 1994, 1996), as was the ethylene-insensitive *Psein2* mutant (Weller *et al*., 2015).

Fungal cultures of *P. irregulare* were sourced from the University of Sydney and their molecular identity was confirmed by BLAST of the sequence obtained from the internal transcribed spacer (ITS) region (White *et al*., 1990, Schroeder *et al*., 2013, data not shown).

Fungal growth and SL application studies

Pythium irregulare was cultured on potato dextrose agar (PDA) plates at 25 °C in the dark. For GR24 *in vitro* studies, (+)-GR24 (also known as 5DS-GR24; Scaffidi *et al*., 2014) was dissolved in a minimal volume of acetone and then made up to the correct concentration with sterile water. One hundred microlitres of the appropriate solution were spread onto PDA plates and a small square of PDA-containing hyphae was placed in the middle of the plate. All concentrations of GR24 were adjusted to contain the same concentration of acetone and control plates received acetone in water only. Radial growth was measured every 24 h and hyphal branching (number of secondary branches per hypha in a field of view) was assessed under a dissecting microscope.

Plant growth, inoculation and disease assessment

For all studies, unless otherwise stated, plants were grown in growth chambers at 20 °C/15 °C day/night under an 18-h photoperiod with cool-white fluorescent tubes (100 μmol/m^2/s) at pot height. Seeds were surface sterilized with 70% ethanol, nicked, planted into sterile vermiculite and watered with milliQ water and grown for 4 days. At 4 days, seedlings were transferred to 50-mL tubes containing a slant of half-strength-modified LANs medium (1.85 mM KNO_3, 1.3 mM $Ca(NO_3)_2.4H_2O$, 2.5 mM NaH_2PO_4, 0.5 mM $MgSO_4.7H_2O$, 2.5 μM $MnSO_4.H_2O$, 0.25 μM $CuSO_4.5H_2O$, 0.3 μM $ZnSO_4.7H_2O$, 12 μM H_3BO_3, 21 μM NaCl, 0.02 μM $(NH_4)_6$ $Mo_7O_{24}.4H_2O$ and 14.5 μM $Na_2FeEDTA$), solidified with 5 g/L Phytagel (Sigma-Aldrich Pty Ltd., Castle Hill, NSW, Australia) and affixed with a sterile band aid, so that the tap root grew along the slant and the shoot emerged beside the sterile cotton wool plug. Roots were protected from light with a black cardboard sleeve and tubes were placed on a slight angle so that the roots grew vertically. On day 5, a small plug of agar from a 2-day-old culture of *P. irregulare* containing hyphae was placed on the surface of the tap root close to the root tip. Control (mock)-inoculated plants had a sterile plug of agar applied to the roots.

At 9 days after inoculation, seedlings were removed from the slant, and root and shoot parameters were measured, including shoot fresh weight, number of leaves expanded, degree of shoot wilting, number of lateral roots, number of lateral roots aborted, number of lateral roots with discoloration, tap root length, percentage of tap roots with discoloration and the number of tap roots with a necrotic root tip.

For sectioning of the roots, excised root segments, 5 mm in length, were fixed in 2.5% buffered glutaraldehyde under vacuum, dehydrated in acetone and embedded in Spurr's resin (Spurr, 1969). Semi-thick sections (4–5 μm) were stained with toluidine blue O (Feder and O'Brien, 1968).

Gene expression studies

Plants were inoculated as described above. Twenty-four hours after inoculation, approximately 2 cm of root tissue at the site of inoculation were harvested. Each replicate consisted of three to four plants. Tissue was snap frozen, ground and RNA extracted using an Isolate II RNA Plant Kit (Bioline, Australia). cDNA was synthesized with 1 μg of RNA using a Sensi Fast cDNA Synthesis Kit (Bioline, Alexandria, NSW, Australia) and real-time polymerase chain reaction (PCR) was carried out in duplicate using SensiMix SYBR Master Mix (Bioline, Alexandria, NSW, Australia) in a Rotor Gene 2000 (Corbett, Mortlake, NSW, Australia). Real-time PCR was performed using 100–200 pmol of *Ps18s* primers (O'Neill *et al*., 2010), *PsPAL1* primers (PsPAL1 F, 5'-CGGTGTTACTACCGGTTTCG-3'; R, 5'-TCCAGCATTCAAAAACCTGA-3'), *PsHmm6* primers (Fondevilla *et al*., 2011), *PsB-Gluc* primers (PsB-Gluc F, 5'-TCCCACAACAGATGCTCAAA-3'; R, 5'-ACCGAGAATGAGTTCGATGC-3'), *PsCCD8* primers (referred to as *RMS1* F and R; Foo *et al*., 2005), *PsCCD7* primers (*RMS5* F and R; Johnson *et al*., 2006), and *PsACS1* and *PsACO* primers (Foo *et al*., 2006). A standard curve for each gene was generated using serially diluted plasmids containing the cloned fragment of each amplicon, and the average concentration of duplicate samples was calculated. Relative gene expression of three to five biological replicates was achieved by comparing the concentration of the gene of interest with the 18s concentration for that sample.

SL measurements

For SL measurements, plants were grown in a pot system to obtain sufficient root tissue for analysis. A layer of macerated 10-day-old *P. irregulare* from PDA plates (described above) was placed half way down the 1-L pots filled with 50 : 50 gravel : vermiculite, topped with potting mix and sterilized seeds were planted into the potting mix. Control plants received sterile PDA. Plants were grown in cabinets as described above for 14 days. Comparable inoculated plants grown until 28 days old developed classic root rot symptoms, whereas control plants showed no signs of disease (data not shown). Whole roots were harvested, with three plants per replicate, into ethyl acetate and labelled SL standards were added (1 ng each of [6'-$^2H_1$]-orobanchol, [6'-$^2H_1$]-orobanchyl acetate and [6'-$^2H_1$]-fabacyl acetate). SLs were extracted and measured as described by Urquhart *et al*. (2014) by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS).

Statistical analysis

All data were analysed using the statistics package StatPlus (Softonic, Barcelona, Spain). For pairwise comparisons, *t*-tests were performed. For multi-factorial experiments, two-way ANOVAs were performed followed by least significant difference (LSD) post-test.

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SUPPORTING INFORMATION

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Fig. S1 Effect of the synthetic strigolactone (+)-GR24 on oospore germination rates of *Pythium irregulare*. Oospores were generated on modified SuperV8 plates (Chee *et al.*, 1976) for 7 days at 25 °C in the dark and separated by homogenization at high speed with a Physcotron (Microtech Co. Ltd., Funabashi-shi, Chiba, Japan). (+)-GR24 (1×10^{-4}–10^{-7} M) or solvent alone (0) was added to the resulting slurry and incubated on slides for 3 days at 25 °C in the dark. The germination rate is the percentage of oospores with clear germ tubes. A freshly homogenized control sample is also shown (control, time 0). Values are the means ± standard error of three to four replicates with an average number of 360 spores counted per treatment. There was no significant effect of GR24 on oospore germination compared with oospores exposed to control solution after 3 days of incubation.