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ORIGINAL ARTICLE

The role of strigolactones during plant interactions with the pathogenic fungus *Fusarium oxysporum*

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Abstract

Main conclusion **Strigolactones (SLs) do not influence spore germination or hyphal growth of *Fusarium oxysporum*. Mutant studies revealed no role for SLs but a role for ethylene signalling in defence against this pathogen in pea.**

Strigolactones (SLs) play important roles both inside the plant as a hormone and outside the plant as a rhizosphere signal in interactions with mycorrhizal fungi and parasitic weeds. What is less well understood is any potential role SLs may play in interactions with disease causing microbes such as pathogenic fungi. In this paper we investigate the influence of SLs on the hemibiotrophic pathogen *Fusarium oxysporum* f.sp. *pisi* both directly via their effects on fungal growth and inside the plant through the use of a mutant deficient in SL. Given that various stereoisomers of synthetic and naturally occuring SLs can display different biological activities, we used (+)-GR24, (−)-GR24 and the naturally occurring SL, (+)-strigol, as well as a racemic mixture of 5-deoxystrigol. As a positive control, we examined the influence of a plant mutant with altered ethylene signalling, *ein2*, on disease development. We found no evidence that SLs influence spore germination or hyphal growth of *Fusarium oxysporum* and that, while ethylene signalling influences pea susceptibility to this pathogen, SLs do not.

A contribution to the special issue on Strigolactones.

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Keywords Ethylene · *Fusarium* · Pea · Root pathogen · Strigolactones

Abbreviations

AM Arbuscular mycorrhizal
SL Strigolactone

Introduction

Strigolactones (SLs) are a fascinating group of ancient plant-derived signalling molecules. They have important roles both outside the plant as rhizosphere signals as well as internally where they regulate an expanding list of plant developmental processes (Foo and Reid 2013; Smith and Li 2014). This dual function makes them an unusual hormone group when considering the role of plant hormones in interactions with both beneficial and pathogenic soil microbes. SLs are well known for their positive effect on the beneficial arbuscular mycorrhizal (AM) symbiosis and act by promoting spore germination, growth and hyphal branching of AM fungi from the phylum Glomeromycota (Akiyama et al. 2005; Besserer et al. 2006, 2008). Parasitic weeds exploit SLs as an indicator of host presence to trigger their seed germination (Cook et al. 1966, 1972; Matusova et al. 2005). SLs also influence nodulation, the beneficial symbiosis between legumes and nitrogen-fixing soil bacteria, although this does not appear to be due to direct effects of the SLs on the bacteria themselves (Soto et al. 2010; Foo and Davies 2011).

Recent advances in our understanding of plant symbioses have revealed striking similarities between the genes and signals that mediate responses to symbiotic and

pathogenic microbes via a shared evolutionary history (e.g. Evangelisti et al. 2014; Gourion et al. 2015). There is now intense interest in defining the elements that allow plants to distinguish between, and cross-talk with, potential symbionts and pathogens, including prominent roles for signals exchanged in the rhizosphere (Antolin-Llovera et al. 2014; Hayachi and Parniske 2014). Thus, investigating the direct effects of SLs on the growth of pathogenic microbes as well as during the infection process in the plant is now timely.

There are a handful of reports that use mutants or transgenic plants with altered SL-biosynthesis or perception to examine the role of endogenous SLs in disease development. Studies using tomato RNAi lines with reduced SL production due to disruption of a key SL biosynthesis gene indicate SLs may have a positive role in defence against necrotic foliar fungal pathogens (Torres-Vera et al. 2014). These authors also provided some information on possible interactions between SLs and several defence hormones. An important element of the SL perception pathway, MAX2, has also been shown to influence resistance to the bacterial pathogens, *Pectobacterium carotovorum* and *Pseudomonas syringae* in Arabidopsis, possibly due to influences on stomatal conductance and subsequent pathogen entry (Piisila et al. 2015). However, as MAX2 appears to also act in a SL-independent perception pathway (see Smith and Li 2014) and SL-deficient mutants were not examined, it is not certain that the altered disease development in *max2* mutants is due to disruption of SL signalling. In contrast to these studies, a recent paper using pea mutants disrupted in either SL signalling or biosynthesis has shown that SLs do not appear to influence the susceptibility of the garden pea to infection by the necrotrophic soilborne oomycete *Pythium irregulare* (Blake et al. 2015).

There are also a limited number of studies examining the influence of SLs directly on the growth of oomycete and true fungal pathogens (Steinkellner et al. 2007; Dor et al. 2011; Sabbagh 2012; Torres-Vera et al. 2014; Blake et al. 2015). A range of responses to the synthetic SL GR24 have been reported for the same pathogen, including no change in radial growth or branching of hyphae (Steinkellner et al. 2007; Torres-Vera et al. 2014; Blake et al. 2015) to some promotion of branching but suppression of radial growth (Dor et al. 2011). It is also important to note that all of this work has been carried out using a mixture of stereoisomers of the synthetic SL, GR24. These mixtures contain up to four stereoisomers (Thuring et al. 1997) with potentially different biological activities (e.g. Scaffidi et al. 2014; Artuso et al. 2015) and the importance of this in interpreting experiments is discussed in detail by Flematti et al. (2015) in this special issue. With the recent ability to synthesise specific SL stereoisomers it is now possible to individually test the activity of these isomers in a range of processes. Given that the naturally occurring SLs synthesized by plants can also have different biological activities from GR24 (Scaffidi et al. 2014) it is also important to test a range of endogenous SLs found within and across species.

As observed for some other plant hormones, distinct responses by different pathosystems to SLs is not unexpected. A range of roles in defence for the growth promoting hormones auxin, gibberellin and brassinosteroids have been identified depending on the host and pathogen species, tissue type, inoculation method and the particular disease parameters measured (e.g. Van der Ent and Pieterse 2012; Denancé et al. 2013). Although there is a general split between positive roles for ethylene and jasmonic acid in defence against necrotrophic pathogens, and a role for salicylic acid in protection from biotrophic pathogens, there are important exceptions to this simple classification (Robert-Seilaniantz et al. 2011; Derksen et al. 2013). For example, instead of ethylene acting in defence, the ethylene signalling pathway is exploited by the necrotrophic fungus *Fusarium graminearum* to aid colonisation of Arabidopsis and barley plants (Chen et al. 2009). In addition, the role of individual plant hormones in response to the same pathogen species can differ markedly between plant hosts. For example, unlike the protective role for ethylene response factors in Arabidopsis against *Fusarium oxysporum* infection (Onate-Sanchez et al. 2007), *Medicago truncatula* lines overexpressing an ethylene response transcription factor did not display altered disease when challenged with *F. oxysporum* (Anderson and Singh 2011).

In this paper we used (+)-GR24, (−)-GR24, (*rac*)-GR24, the naturally occurring SL, (+)-strigol, and a racemic mixture of 5-deoxystrigol (Fig. 1) to examine the response of the hemi-biotrophic fungus *Fusarium oxysporum* to SLs. *F. oxysporum* is an ascomycete that is widely distributed and can persist in soil and contains many strains, some of which can be pathogenic to plants. While the species has a broad host range, different strains can be quite specific. We used a strain, *F. oxysporum* f.sp. *pisi,* that attacks the roots of pea and can invade the xylem of this species resulting in wilting and death of the shoot. We have examined hyphal branching, fungal growth and spore germination in response to these SL compounds. Further we have used pea as a model legume to examine the role of endogenous SLs in the disease caused by this soil-borne pathogen. Pea is a good system for this work given it is an important grain legume crop, is susceptible to infection by this pathogen (Castillejo et al. 2015) and has well-defined SL biosynthesis mutants (Foo and Reid 2013). We used the *ccd8* mutant that is disrupted in a carotenoid cleavage dioxygenase (CCD) enzyme that catalyses an essential step in the biosynthesis of SLs and is severely deficient in SLs

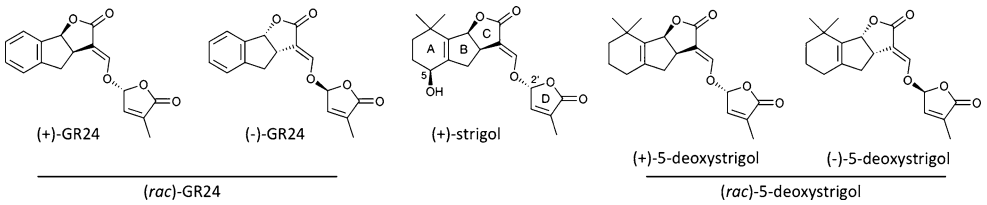

Fig. 1 Structures of synthetic and naturally occurring strigolactones used in this study

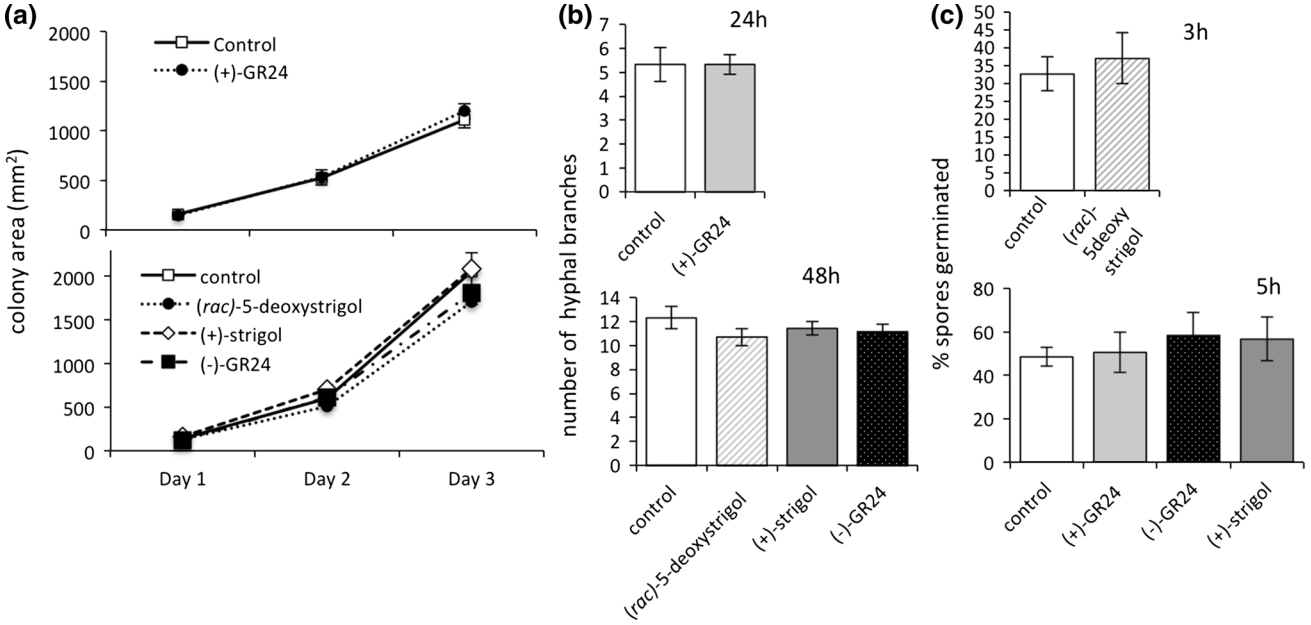

Fig. 2 Influence of the synthetic SL stereoisomers (+)-GR24 and (−)-GR24), the naturally occurring SL, (+)-strigol, and a racemic mix of 5-deoxystrigol (*rac*-5-deoxystrigol) on the growth and spore germination of *F. oxysporum* in culture. **a** Colony area over 3 days. **b** Hyphal branching after 24 or 48 h. **c** Germination of predominantly microconidial spores after 3 or 5 h (approximately 100 spores was examined for each treatment). Values shown are for the 1×10^{-6} M treatments, other treatment concentrations are shown in Tables 1 and 2. Values shown are mean ± SE ($n = 4$–6) for colony area and hyphal branch measurements and ($n = 12$) for spore germination. One-way ANOVAs were performed for **a**–**c** and no significant effect of any SL was found on the parameters measured

(Gomez-Roldan et al. 2008; Foo et al. 2013). At the seedling stage, this mutant has a similar phenotype to wild type, 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). We provide evidence about the role of SLs in this interaction between pea and *F. oxysporum.* Given that studies in non-legumes have highlighted an important role for ethylene in response to *F. oxysporum* (e.g. Geraats et al. 2003; van Loon et al. 2006) that was not found in the legume *M. truncatula* (Anderson and Singh 2011), we also examined disease caused by *F. oxysporum* in the recently characterised pea ethylene-insensitive *ein2* mutant (Weller et al. 2015). The EIN2 protein is a key component of ethylene signalling, an N-RAMP metal-transporter-like protein whose degradation upon ethylene perception ultimately promotes the expression of ethylene-responsive genes (Lin et al. 2009; Qiao et al. 2012; Merchante et al. 2013). In pea *EIN2* is a single copy gene and thus *ein2* mutant plants are ethylene-insensitive at both the seedling and adult stage and across tissue types (Weller et al. 2015). We provide evidence that ethylene signalling does contribute to defence against *F. oxysporum,* adding to our understanding of the regulation of this important disease in legumes.

Materials and methods

Plant and fungal material

The *Pisum sativum* L. lines used were the strigolactone-deficient line *ccd8* (*rms1-1*, Beveridge et al. 1997) derived from wild-type cv. Parvus, and the ethylene-insensitive *ein2* mutant (Weller et al. 2015) derived from wild-type cv. Torsdag. *ein2* was crossed to cv. Torsdag three times to ensure purity as described in Weller et al. (2015).

Table 1 Influence of a range of concentrations of the stereoisomers of the synthetic SL GR24 ((+)-GR24 and (−)-GR24) and (*rac*)-GR24, the natural SL, (+)-strigol, and a racemic mix of 5-deoxystrigol ((*rac*)-5-deoxystrigol) on colony area and hyphal branching of *F. oxysporum* in culture

Treatment	Concentration (M)	Colony area (mm^2)			Number of hyphal branches
		1 Day	2 Day	3 Day	
Control		159.7 ± 14.45	528.0 ± 75.69	1113.2 ± 87.04	5.3 ± 0.71
(+)-GR24	1×10^{-7}	140.9 ± 18.16	459.0 ± 173.84	1120.2 ± 73.92	5.3 ± 0.66
(+)-GR24	1×10^{-6}	147.0 ± 16.54	531.1 ± 20.42	1195.5 ± 80.37	5.3 ± 0.42
(+)-GR24	1×10^{-5}	140.5 ± 5.20	531.8 ± 50.03	1090.6 ± 69.64	7.5 ± 1.15
(+)-GR24	1×10^{-4}	143.5 ± 17.23	547.1 ± 46.76	1127.4 ± 66.79	5.5 ± 0.56
Control		141.0 ± 18.83	603.3 ± 51.77	2054.7 ± 219.08	12.3 ± 0.92
(rac)-5-deoxystrigol	1×10^{-7}	133.9 ± 17.69	551.7 ± 12.02	1759.8 ± 24.87	11.9 ± 0.87
(rac)-5-deoxystrigol	1×10^{-6}	127.2 ± 17.23	510.8 ± 11.56	1710.7 ± 32.40	10.7 ± 0.72
(rac)-5-deoxystrigol	1×10^{-5}	144.1 ± 15.81	544.7 ± 6.87	1798.4 ± 69.12	12.4 ± 0.75
(rac)-5-deoxystrigol	1×10^{-4}	132.9 ± 5.90	531.3 ± 20.22	1650.6 ± 47.65	11.2 ± 0.76
(+)-Strigol	1×10^{-7}	137.4 ± 7.92	594.5 ± 24.94	1954.4 ± 126.4	10.7 ± 0.31
(+)-Strigol	1×10^{-6}	162.2 ± 16.22	707.1 ± 19.24	2083.3 ± 33.03	11.4 ± 0.58
(+)-Strigol	1×10^{-5}	133.3 ± 11.79	573.1 ± 24.49	1637.9 ± 11.98	12.6 ± 1.11
(+)-Strigol	1×10^{-4}	107.5 ± 10.8	531.1 ± 11.8	1674.0 ± 12.11	12.1 ± 0.34
(−)-GR24	1×10^{-7}	101.5 ± 10.91	459.5 ± 27.72	1638.6 ± 48.17	11.1 ± 1.31
(−)-GR24	1×10^{-6}	117.1 ± 14.01	608.5 ± 7.26	1812.0 ± 93.70	11.2 ± 0.63
(−)-GR24	1×10^{-5}	98.1 ± 5.76	498.7 ± 34.53	1735.9 ± 56.77	10.5 ± 0.82
(−)-GR24	1×10^{-4}	110.6 ± 11.38	497.6 ± 13.09	1906.1 ± 172.28	11.2 ± 0.40
Control		147.2 ± 2.48	503.5 ± 6.33	1088.9 ± 13.01	na
(rac)-GR24	1×10^{-6}	137.4 ± 4.51	496.4 ± 8.38	1094.6 ± 15.02	na
(rac)-GR24	1×10^{-5}	137.5 ± 4.35	482.5 ± 7.61	1066.7 ± 4.60	na

Concentrations ranged from 1×10^{-7} to 1×10^{-4} M. As different SLs were tested across different experiments controls for each experiment are shown. Values are mean ± SE (n = 4–6). One-way ANOVAs were performed and no significant effect of any SL at any concentration was found on the parameters measured. na, not assessed

Comparisons were always made between the mutant line and its appropriate wild-type progenitor line. Fungal culture of *F. oxysporum* f.sp. *pisi* (RBG 6444) was sourced from The Royal Botanic Gardens and Domain Trust collection (Sydney, Australia) and the molecular identity of this culture was confirmed by BLAST of sequence obtained from the internal transcribed spacer (ITS) region (White et al. 1990; Schroeder et al. 2013; data not shown).

Fungal growth and strigolactone application studies

Fusarium oxysporum was cultured long-term on carnation leaf agar (CLA containing streptomycin, Leslie et al. 2006) plates at 25 °C in the dark. To generate hyphae for hyphal growth studies *F. oxysporum* was subcultured onto PDA containing streptomycin and grown for 5 days at 25 °C. For SL in vitro studies a range of SLs were tested. These were (+)-GR24 (also known as 5DS-GR24; Scaffadi et al. 2014), (−)-GR24 (also known as *ent*-5DS-GR24), (*rac*)-GR24, (*rac*)- deoxystrigol and (+)-strigol (Fig. 1). GR24 isomers were synthesized by Dr Chris McErlean (Bromhead et al. 2014), and (*rac*)-5-deoxystrigol (racemic mix of the natural (+) and non-natural (–) isomers) was synthesized by Steven Abel after Reizelman et al. (2000). The (+)-strigol was synthesized by BJ Fisher and JA Smith (unpublished results) using the Bromhead et al. (2014) asymmetric synthesis of (+)-GR24 as inspiration. The SLs were dissolved in a minimal volume of DMSO (a solvent in which GR24 has been found to have minimal degradation, Bromhead et al. 2015) then made up to the correct concentration with sterile water. Concentrations ranged from 1×10^{-7} to 1×10^{-4} M. All concentrations of SL were adjusted to contain the same concentration of DMSO and control plates received DMSO in water only (0.01 % v/v). 100 uL of the appropriate solution was spread onto standard circular 9 cm water agar plates. For hyphal growth studies a 1×1 cm^2 of PDA from the leading edge colonised with hyphae was aseptically transferred to the middle of the plate. Colony area was measured from 4 replicate plates every 24 h and hyphal branching (number of secondary branches per hyphae in a field of view) was assessed at the leading edge of fungal growth under a

Table 2 Influence of a range of concentrations of the stereoisomers of the synthetic SL GR24 ((+)-GR24 and (−)-GR24) and (*rac*)-GR24, the natural SL, (+)-strigol, and a racemic mix of 5-deoxystrigol ((*rac*)-5-deoxystrigol) on spore germination of *F. oxysporum* in culture. Concentrations ranged from 1×10^{-7} to 1×10^{-4} M

Treatment	Concentration (M)	Spore germination
Control		32.7 ± 4.72
(rac)-5-deoxystrigol	1×10^{-7}	31.0 ± 1.91
(rac)-5-deoxystrigol	1×10^{-6}	37.0 ± 7.1
(rac)-5-deoxystrigol	1×10^{-5}	38.7 ± 3.99
(rac)-5-deoxystrigol	1×10^{-4}	35.9 ± 4.33
Control		48.5 ± 4.35
(+)-GR24	1×10^{-7}	52.6 ± 7.08
(+)-GR24	1×10^{-6}	50.6 ± 9.24
(+)-GR24	1×10^{-5}	48.3 ± 7.61
(+)-GR24	1×10^{-4}	56.5 ± 7.15
(+)-Strigol	1×10^{-7}	47.2 ± 7.65
(+)-Strigol	1×10^{-6}	56.6 ± 10.04
(+)-Strigol	1×10^{-5}	54.8 ± 10.28
(+)-Strigol	1×10^{-4}	62.7 ± 13.08
(−)-GR24	1×10^{-7}	52.1 ± 8.21
(−)-GR24	1×10^{-6}	58.4 ± 10.66
(−)-GR24	1×10^{-5}	46.2 ± 6.69
(−)-GR24	1×10^{-4}	53.6 ± 9.69
Control		97.9 ± 2.08
(rac)-GR24	1×10^{-7}	90.9 ± 3.71
(rac)-GR24	1×10^{-6}	91.2 ± 3.06
(rac)-GR24	1×10^{-5}	77.7 ± 9.72
(rac)-GR24	1×10^{-4}	86.6 ± 7.08

As different SLs were tested across different experiments controls for each experiment are shown. Values are mean ± SE ($n = 12$). One-way ANOVAs were performed and found no significant effect of any SL at any concentration on germination

dissecting microscope across 4 replicate plates, with three fields of view per plate.

For spore production, a small square of CLA culture was placed into 100 mL of CDAZ (Czapek Dox plus micronutrients, Coddington et al. 1987) containing streptomycin and grown on a rotary shaker at 120 rpm in the dark at 25 °C for 5 days. Spores (predominantly microconidia) were collected by spinning cultures at 8000*g* for 15 min and quantified using a haemocytometer. Spore concentrations were adjusted with sterile water to 2.5×10^6 spores/mL and 100 µL of spore suspension was plated onto three replicates water agar plates with control or various SL solutions as outlined above for hyphal growth studies. Plates were incubated at 25 °C in the dark and spore germination was quantified 3–5 h later under a light microscope with approximately 100 spores observed per treatment across three fields of view per replicate plate.

Plant growth, inoculation and disease assessment

For all studies, plants were grown as described by Blake et al. (2015). Briefly, plants were grown under an 18 h photoperiod at 20 °C/15 °C day/night temperatures under cool-white fluorescent tubes (100 µmol m^{-2} s^{-1}). Seeds were germinated in sterile vermiculite. At 4 day seedlings were transferred to 50 mL tubes containing a slant of half-strength modified LANS media solidified with 5 g L^{-1} Phytagel (Sigma-Aldrich Pty Ltd). The roots were protected from the light with a cardboard sleeve. The tap-root grew along the slant and the shoot emerged beside the sterile cotton wool plug. On the following day a solution of *F. oxysporum* (1.8 mL of a 2.5 x 10^6 spores/mL solution) was sprayed onto the surface of the root. Control (mock) inoculated plants had sterile water applied. Small quantities of sterile modified LANS were added to the tubes as required.

At 21 day after inoculation seedlings were removed from the slant and six root and shoot parameters were measured, including shoot fresh weight, degree of shoot wilting, tap root length and the percentage of the root system that was discoloured. The tap-root was severed at the cotyledons and 2 and 4 cm down the root and inspected for discolouration of the vascular bundle.

Statistical analysis

All data were analysed using the statistics package StatPlus. For pair wise comparisons, *t*-tests were performed. For multi-treatment experiments either one-way or two-way ANOVAs were performed followed by Tukeys HSD post-tests where appropriate. For frequency data Chi squared tests were performed.

Results and discussion

Strigolactones do not affect hyphal growth, branching or spore germination of *F. oxysporum* in vitro

SLs are contained in plant root exudates and early work in pea revealed that root exudates could influence the germination of *F. oxysporum* spores (Buxton 1957a, b) and in tomato this is influenced by AM status (Scheffknecht et al. 2006). There are conflicting reports of the effect of the synthetic SL, GR24, on *F. oxysporum* hyphal branching, including reports of no effect (Steinkellner et al. 2007) or increased branching (Dor et al. 2011). GR24 is a relatively unstable molecule (Akiyama et al. 2010; Bromhead et al. 2015) and heating to 60 °C causes significant degradation (Enzer et al. 2013). Given this we followed the method of

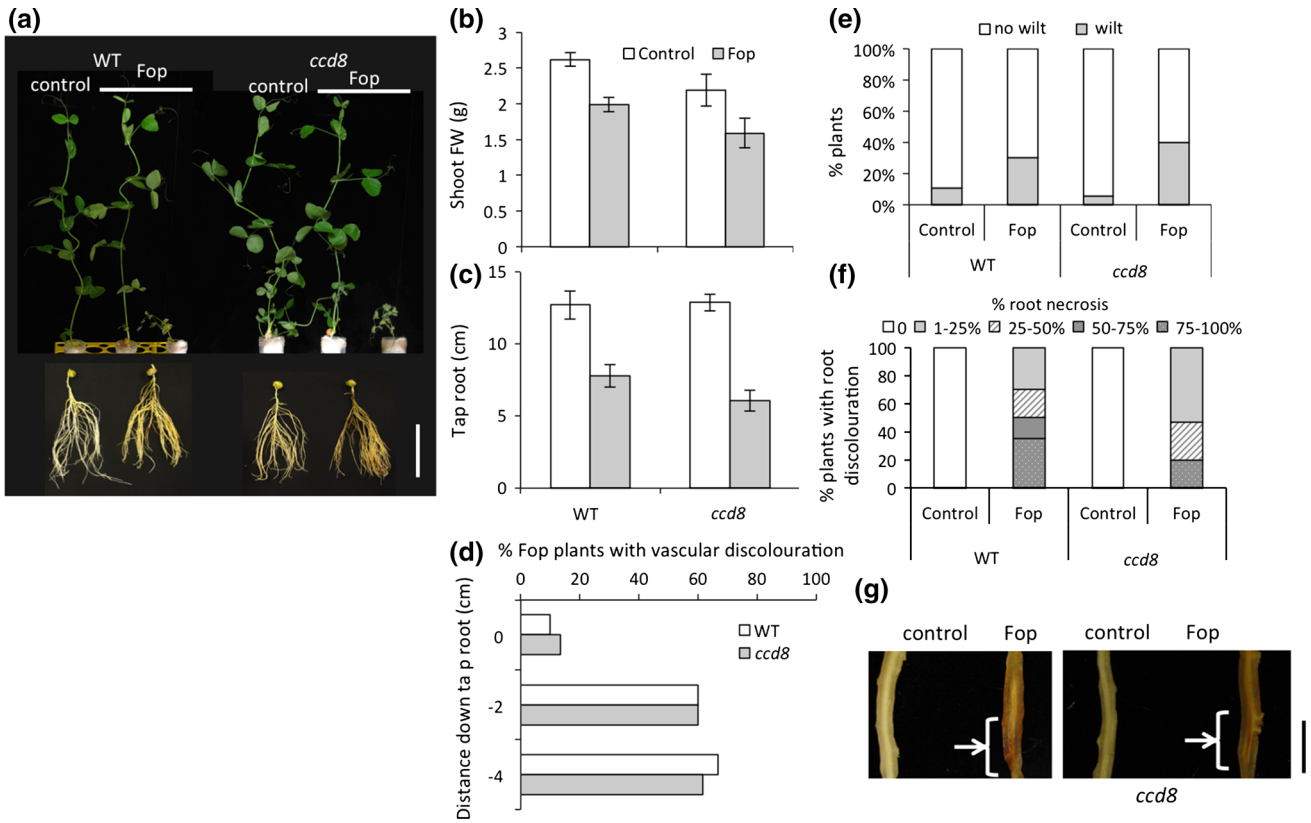

Fig. 3 Disease symptoms of wild-type (WT) and the SL-deficient *ccd8* mutant 21 d after inoculation. **a** Photo of whole plants of control and *F. oxysporum* (Fop) infected WT and *ccd8* plants showing the range of phenotypes observed. *Scale bar* 5 cm. **b** Shoot fresh weight. **c** Tap root length. **d** Percentage of plants with vascular discolouration at distances down the tap root. **e** Shoot wilt. **f** Percentage of plants in each class of root discolouration. **g** Cross section of the tap root at the shoot-root boundary. *White arrows* indicate the extent of red-brown discolouration of the vasculature. *Scale bar* 1 cm. **b**–**f** Values are mean ± SE (n = 15–20). Appropriate statistical tests were performed but no significant differences between WT and *ccd8* plants were observed

Steinkeller et al. (2007) by adding SLs onto the surface of plates. In addition, unlike the mixture of GR24 stereoisomers used in previous studies, we also employed (+)-GR24 (also known as 5DS-GR24), the stereoisomer that is known to mimic naturally occurring SLs (Scaffadi et al. 2014).

We found no influence of (+)-GR24 on either the area of radial growth of the hyphae or hyphal branching of *F. oxysporum* f.sp *pisi* (Fig. 2a, b) and this was consistent across a range of concentrations (Table 1). A similar method of SL application to the agar surface has been shown to influence hyphal branching of symbiotic fungi (e.g. Akiyama et al. 2005). Spores are an important source of disease-causing inoculum in field situations, so we also examined the effect of (+)-GR24 on the germination of microconidia and also found no significant effect (Fig. 2c). The stereoisomer (−)-GR24 and the racemic mixture of both stereoisomers also had no significant effect on spore germination, colony area or hyphal branching of *F. oxysporum* (Fig. 2a–c, Tables 1 and 2).

The naturally occurring SL, (+)-strigol, and the (*rac*)-5-deoxystrigol also did not significantly influence colony area, hyphal branching or spore germination of *F. oxysporum* (Fig. 2a–c). Similar results were obtained across a range of concentrations (Tables 1 and 2). These results are consistent with Steinkellner et al. (2007) and suggest that, unlike in interactions with AM fungi, root-exuded SLs in the rhizosphere are unlikely to be influencing spore germination or hyphal branching of *F. oxysporum* before infection of the plant takes place.

A strigolactone-deficient mutant of pea develops similar disease symptoms to wild-type plants

The above in vitro studies suggest that SLs are not likely to act directly on *F. oxysporum* in the soil to influence pathogen behaviour and subsequent infection. However, SLs are also found inside the root and, like many other plant hormones (e.g. Robert-Seilaniantz et al. 2011; Derksen et al. 2013), may act inside the plant as a signal during the response to pathogen attack. To explore this, we examined disease progression after infection with *F. oxysporum* in an SL-deficient pea mutant, *ccd8*. This mutant developed the same pattern and severity of symptoms as seen for wild-type plants in response to *F.*

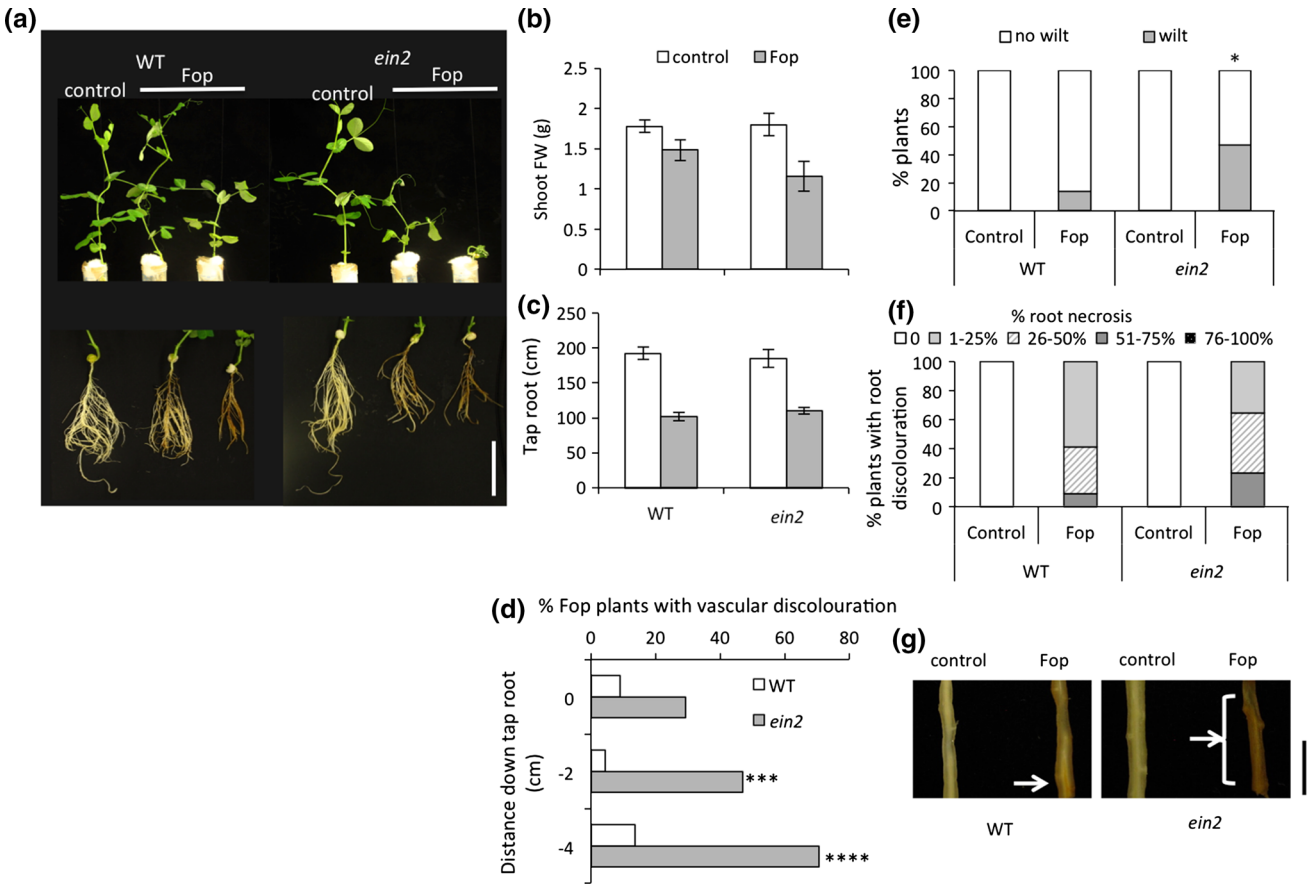

Fig. 4 Disease symptoms of wild-type (WT) and the ethylene insensitive *ein2* mutant 21 d after inoculation. **a** Photo of whole plants of control and *F. oxysporum* infected WT and *ein2* plants showing the range of phenotypes observed. *Scale bar* 5 cm. **b** Shoot fresh weight. **c** Tap root length. **d** Percentage of plants with vascular discolouration at distances down the tap root. **e** Shoot wilt. **f** Percentage of plants in each class of root discolouration. **g** Cross section of tap root at the shoot-root boundary, *white arrows* indicate the extent of red-brown discolouration of the vasculature. *Scale bar* 1 cm. **b–f** Values are mean ± SE ($n = 14$–23). For **d**, Chi squared test found significant difference between WT and *ein2*, $^{***}P < 0.001$ and $^{****}P < 0.0001$, and for **e** a two-way ANOVA showed a significant interaction between genotype and treatment, $^{*}P < 0.05$

oxysporum infection (Fig. 3). This was the case for shoot stunting, shoot wilting and all root symptoms measured, including the stunted growth of the tap-root and the extent of root necrosis (Fig. 3). Vascular discolouration inside the tap-root occurred to the same degree in both genotypes with the least discolouration near the root/shoot junction in both genotypes (Fig. 3 d, g). Therefore, unlike the clear roles for SLs in interactions between symbiotic microbes and plant hosts such as pea (e.g. Gomez-Roldan et al. 2008; Foo and Davies 2011), SLs do not appear to have a major influence on the interaction between pea and *F. oxysporum*, a fungus that undergoes a sequential biotrophic and then necrotrophic interaction with the plant host.

Ethylene plays a role in defense against *F. oxysporum* infection in pea

The role of ethylene signalling in response to the hemi-biotroph *F.oxysporum* has not been explored to any great extent in legumes. Indeed, in the only report using genetic tools to examine this question in legumes, studies in *M.truncatula* did not find an influence of ethylene response factors on the development of the disease following challenge with *F. oxysporum* (Anderson and Singh 2011). With the recent characterisation of the ethylene-insensitive *ein2* mutant of pea (Weller et al. 2015) we now have another tool with which to explore the role of ethylene in this important legume disease.

Under baseline ethylene conditions, such as mock-inoculation, the *ein2* mutation has little influence on seedling development and *ein2* seedlings are almost indistinguishable from wild type (Fig. 4) with many of the effects of the mutant only apparent in mature plants (e.g. delayed petal abscission, Weller et al. 2015). However, when challenged with *F. oxysporum*, *ein2* mutants developed somewhat more severe disease symptoms than wild-type plants (Fig. 4). This included shoot symptoms, with inoculated *ein2* shoots developing significantly more

wilted shoots compared with inoculated wild-type plants ($P < 0.05$, Fig. 4e). The most striking difference was seen in discolouration of the vascular system where the discolouration was significantly more extensive in *ein2* mutants than infected wild-type plants ($P < 0.001$; Fig. 4d, g). We note however that other root disease symptoms, such as the proportion of necrosis on the tap-root surface and the tap-root length were similar in wild-type plants and *ein2* mutants (Fig. 4c, f). The increased spread of the disease seen in the *ein2* vascular system would presumably accelerate the spread and impact of the disease and indicates that ethylene signalling is an important element in the defence of peas to *F.oxysporum* disease progression. These results with ethylene also provide a positive control for the results with strigolactones (Fig. 3) by showing that the pathosystem developed is capable of responding differentially to different plant genotypes.

In conclusion, we have defined a role for ethylene signalling, but not SLs, in the defence of pea plants against the disease caused by the hemibiotrophic fungus *F.oxysporum*. The minimal influence of SLs on root infection and disease by *F.oxysporum* in pea is consistent with reports of pea root disease caused by the necrotrophic oomycete *Pythium irregulare* (Blake et al. 2015) but is in contrast to reports of increased disease in tomato RNAi-SL depleted plants challenged with foliar necrotrophic fungi (Torres-Vera et al. 2014). This suggests that, unlike the conservation of the role of SLs in developmental processes such as shoot branching and interactions with AM fungi (in AM hosts), this does not appear to be the case for the role of SLs in the response of host plants to disease. However, given the ubiquitous nature of SLs in land plants (Delaux et al. 2012; Challis et al. 2013) and the development of new agrichemical tools to target the use of SLs for crop improvement (e.g., Lachia et al. 2014, 2015), future work focusing on the role of SLs in a wide variety of plant interactions with other organisms, including additional pathogenic and endophytic fungi, growth promoting as well as pathogenic bacteria, viruses and insects will provide essential baseline information. In addition to a protective role, plant hormones can also be exploited by pathogens to aid their colonisation of the plant, such as the roles for gibberellin in "foolish seedling disease" and auxin/cytokinin balance in *Agrobacterium tumefaciens* infection (e.g. Takahashi et al. 1955; Akiyoshi et al. 1983). Similar types of interactions may also occur for the SLs. For example it has recently been shown that SLs play a role in leafy-gall syndrome in Arabidopsis (caused by the bacterial actinomycete *Rhodoccus fascians*) by interacting with cytokinin to contribute to increased shoot branching in infected plants (Stes et al. 2015).

Author contribution statement EF and JBR planned the study. JAS and BNF synthesised many of the chemicals used in the study. EF and SN performed the experiments. EF analysed the data and EF wrote the manuscript with assistance from JBR. The authors declare no conflict of interest.

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