Different effects of phytohormones on Fusarium head blight and Fusarium root rot resistance in *Brachypodium distachyon*

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Phytohormones affect *B. distachyon* FHB and FRR differently

Highlight

The tissue-dependent effects of defence phytohormones and tissue-independent effects of development phytohormones on *F. graminearum*-induced Fusarium head blight and Fusarium root rot diseases in the model cereal *Brachypodium distachyon*.

Abstract

Fusarium graminearum is a devastating pathogen of small grain cereals causing both Fusarium head blight (FHB), Fusarium root rot (FRR). Phytohormones play important roles in hosts during Fusarium infection and studies have found that application of phytohormones can significantly impact FHB resistance. In contrast to FHB, FRR remains a poorly characterised disease and it is unknown whether phytohormones play similar roles in FHB and FRR. In this present study, *B. distachyon* floral tissues at mid anthesis and root tissues from seedlings were exogenously treated with a range of phytohormones before inoculation with *F. graminearum*. Application of salicylic acid (SA) significantly increased susceptibility to FRR but not to FHB. On the other hand, both jasmonic acid (JA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) increased resistance to FRR but increased susceptibility to FHB. The auxins indole-3-acetic acid (IAA) and 1-napthaleneacetic acid (NAA) greatly increased FHB and FRR resistance. The cytokinin trans-Zeatin and the defence-related compound 3-aminobutanoic acid (BABA) induced high levels of susceptibility in both FHB and FRR. In FRR the phytohormones SA, JA, and ACC appeared to exert their effects early in infection whereas auxin, cytokinins, and BABA altered the rate of colonisation rather than affecting initial infection.

Key Words

ACC, auxin, BABA, *Brachypodium*, cytokinin, *Fusarium*, head blight, root rot, jasmonic acid, salicylic acid.

Abbreviations

ACC, 1-aminocyclopropane-1-carboxylic acid; BABA, 3-aminobutanoic acid; dpi, days post inoculation; FHB, Fusarium head blight; FRR, Fusarium root rot; IAA indole-3-acetic acid; JA, jasmonic acid; NAA, 1-napthaleneacetic acid; PDA, potato dextrose agar; RNL, root necrosis length; SA, salicylic acid.

Introduction

Fusarium graminearum is one of the most important plant pathogens in the world (Dean *et al.*, 2012), affecting small grain cereals such as bread wheat (*T. aestivum*), barley (*H. vulgare*), and rye (*S. cereale*). *F. graminearum* can infect almost the entire plant (Miedaner, 1997) causing a range of diseases. The most well-known and economically important disease is fusarium head blight (FHB). Other diseases include fusarium crown rot, fusarium root rot (FRR), and seedling blight. Plant pathogens often adopt specific trophic lifestyles. Biotrophs obtain nutrients from living tissue, necrotrophs from dead tissue, and hemibiotrophic pathogens adopt an early biotrophic phase that is followed by a necrotrophic phase sometime later in infection (Glazebrook, 2005; Zeilinger *et al.*, 2016). *F. graminearum* is generally considered a facultative hemibiotroph in the developing cereal spike (Jansen *et al.*, 2005; Boddu *et al.*, 2006). Infection patterns of floral tissues show that *F. graminearum* grows as a biotroph at the advancing hyphal front with necrotrophic feeding behind this, where hyphae are ramifying through tissues already killed by the fungus (Brown *et al.*, 2010). Current FHB control methods rely on the effective use of fungicides and genetics resistance. However novel control mechanisms are required.

Fungal root diseases are widespread in wheat and barley fields and are becoming more prevalent with the increased use of cereal crop rotation and no tillage practices (Cook, 2001). FRR is caused by several Fusarium species including *F. graminearum*. Colonisation and sporulation can occur rapidly (Wang *et al.*, 2015) causing root browning and necrosis (Beccari *et al.*, 2011). FHB and Fusarium crown rot can also manifest from FRR infection due to systemic migration via the vascular system (Beccari *et al.*, 2011; Wang *et al.*, 2015). Ultimately this results in reduced root, shoot length, biomass and yield loss (Mergoum *et al.*, 1998; Beccari *et al.*, 2011; Wang *et al.*, 2015). Control of FRR is also difficult due to limited effectiveness of fungicides, lack of genetic resistance, and persistence in the soil for many years facilitated by the predominantly saprotrophic lifestyle of *F. graminearum* (Cook, 2001). Furthermore, FRR is not as well characterised as FHB due to the difficulty of research on root diseases.

Brachypodium distachyon (purple false brome) is a valuable model for small-grained

81 cereals as it is a small inbreeding annual with short generation times, minimal growth
82 requirements, and abundant genetic resources (Draper *et al.*, 2001; Vogel *et al.*, 2006; Vogel
83 *et al.*, 2010; Brkljacic *et al.*, 2011; Kellogg, 2015; Scholthof *et al.*, 2018). *B. distachyon* is a
84 good model for investigating shoot and root development due to high anatomical and
85 developmental similarities to wheat (Draper *et al.*, 2001; Watt *et al.*, 2009; Opanowicz *et al.*,
86 2011; Kellogg, 2015). Furthermore, hormone signalling pathways appear well conserved
87 (Goddard *et al.*, 2014; Kouzai *et al.*, 2016). Importantly, *B. distachyon* is an excellent model
88 for investigating FHB and FRR as both roots and florets are susceptible to *F. graminearum*
89 infection (Peraldi *et al.*, 2011; Pasquet *et al.*, 2014).

90 Phytohormones are critical components of plant defence signalling and their impact on *F.*
91 *graminearum* resistance response has been investigated in numerous studies on *A. thaliana*,
92 wheat, and barley. Exogenous application of salicylic acid (SA) or methylsalicylate has been
93 shown to have positive effects on FHB resistance in wheat (Makandar *et al.*, 2011; Qi *et al.*,
94 2012; Sorahinobar *et al.*, 2016) with direct effects on *in-vitro* growth (Qi *et al.*, 2012).
95 Application of methylsalicylate also showed positive effects on wheat leaf resistance to *F.*
96 *graminearum* (Ameye *et al.*, 2015). Jasmonic acid (JA) and methyl-jasmonic acid application
97 was also reported to have a positive effect on FHB resistance in wheat (Li and Yen, 2008; Qi
98 *et al.*, 2016; Sun *et al.*, 2016). In contrast Makandar and colleagues (2010) found that
99 methyl-jasmonic acid compromised resistance during early infection but had positive effects
100 during late infection in *A. thaliana* (Makandar *et al.*, 2010). Similar results were reported in
101 wheat where pre-infection application of methyl-jasmonic acid promoted susceptibility,
102 whereas post-infection application promoted resistance of wheat leaves to *F. graminearum*
103 (Ameye *et al.*, 2015). JA was also found to reduce *F. graminearum* growth in vitro (Qi *et al.*,
104 2016). The precise role of ethylene on resistance to Fusarium remains unclear. Compounds
105 that produce ethylene have been reported to have positive effects (Li and Yen, 2008; Foroud
106 *et al.*, 2018), negative effects (Chen *et al.*, 2009), and no significant effect (Sun *et al.*, 2016)
107 on Fusarium infection.

108 More recently other important hormones were shown to have roles in defence and
109 contribute significantly to the infection response. Exogenous application of gibberellic acid
110 reduced FHB spread in wheat heads (Buhrow *et al.*, 2016), whereas exogenous application
111 of abscisic acid negatively affected resistance to FHB (Buhrow *et al.*, 2016; Qi *et al.*, 2016).

112 Exogenous application of brassinosteroid had positive effects on resistance (Ali *et al.*, 2013)
113 while brassinosteroid receptor mutants in which brassinosteroid levels are believed to be
114 increased, exhibited enhanced resistance to *Fusarium culmorum* (Goddard *et al.*, 2014).
115 Finally, the auxin indole-3-acetic acid (IAA) had positive effects on barley FHB (Petti *et al.*,
116 2012), and was even found to reduce Fusarium growth *in vitro* (Luo *et al.*, 2016). There are
117 hormones that have not been investigated regarding exogenous application and response to
118 *F. graminearum* infection. These include cytokinins which have been implicated in plant
119 defence (Choi *et al.*, 2010; Choi *et al.*, 2011; Albrecht and Argueso, 2017) and the recently
120 classed non-protein amino acid and signalling molecule 3-aminobutryric acid (BABA) (Cohen,
121 2001; Jakab *et al.*, 2001; Cohen, 2002; Cohen *et al.*, 2016; Thevenet *et al.*, 2017).

122 The reported effects of phytohormones on resistance to *F. graminearum* is complex or in
123 some cases contradictory. Most studies have focused on FHB with a lack of research on *F.*
124 *graminearum*-induced FRR pathogenesis. The main aim of this research was to investigate
125 the effect of several phytohormones on resistance to FRR and compare these with the
126 effects of the same compounds on FHB. We then generated a model for how each
127 phytohormone influences resistance or susceptibility to the two diseases in *B. distachyon*.

Materials and Methods

Plant Material and Growth Conditions.

The *Brachypodium distachyon* line Bd3-1 was obtained from the John Innes Centre, Norwich, UK. To soften the floret, seeds were soaked in water for 10-30 min. Subsequently, the lemma and palea were peeled off the individual seeds. Seeds were placed between two layers of dampened filter paper (9 cm, Sartorius Grade 292) with 5 ml sterile water, and were stratified at 5 °C for five days in the dark, and incubated for one day at 22 °C (16h/8h light/dark photoperiod, variable humidity) in controlled environment growth cabinets (Peraldi *et al.*, 2011). For FHB assays, peeled Bd3-1 seeds were sown in 50% Peat/Sand and 50% John Innes mix 2 (two seeds per pot). Plants were kept for four weeks at 22°C (20h/4h light/dark photoperiod, 70% humidity) in controlled environment growth cabinets or rooms (Peraldi *et al.*, 2011). The *B. distachyon* line Bd21 was obtained from BASF SE Agricultural Centre Limburgerhof, Germany, For the FHB assay, approximately five Bd21 unpeeled seeds were grown for 6 weeks at 20h/4h (day/night) in a glasshouse (Autumn conditions).

Preparation and Maintenance of *Fusarium graminearum*

The *Fusarium graminearum* isolate PH1 was used for all experiments. In order to produce mycelial inoculum for FRR assays, *F. graminearum* was maintained on approximately 20 ml potato dextrose agar (PDA) in 9 cm diameter plastic Petri-dishes in a controlled growth cabinet at 22°C under 16h/8h light/dark photoperiod. Conidial *F. graminearum* inoculum was produced in Mung Bean broth as reported previously (Makandar *et al.*, 2006). After one week, conidia were harvested, washed and concentration adjusted to 1 x 10^6 conidia/ml. Inoculum was stored at 5 °C for up to two weeks before inoculation. For Bd21 FHB assay, *F. graminearum* isolate Li600 was grown alternating on malt agar and oatmeal agar for two weeks and conidia were harvested in 10 ml of water amended with 0.05 % Tween 20 and concentration adjusted as above.

Fusarium Root Rot Assays with Chemical Amendment.

157 Aspects of the FRR assay were derived from (Peraldi *et al.*, 2011; Goddard *et al.*, 2014)
158 and modified for chemical amendment experiments. A sterile 9 cm^2 filter paper square (cut
159 from Chromatography Paper 46 cm x 57 cm from Slaughter Ltd R & L) was placed on square
160 plastic square Petri-dishes (10 cm^2) containing 50 ml autoclaved 0.8% agar (Fischer Science).
161 Under sterile conditions, ten cold-stratified and germinated Bd3-1 seeds were placed on the
162 filter paper. A minimum of 30 seedlings was used for each treatment. All plates were placed,
163 angled at 70° from the horizontal to ensure uniform downward root growth, in covered
164 plant propagators containing wetted paper towel to maintain high humidity (Supp Mat. Fig.
165 x). Plant propagators with square Petri-dishes were incubated at 22 °C (16h/8h light/dark
166 photoperiod, variable humidity) in controlled environment growth cabinets. After three
167 days, the filter paper with seedlings attached was carefully transferred to different square
168 Petri-dishes containing 0.8% agar amended with phytohormone or control solvent alone
169 and returned to controlled environment growth cabinets. All compounds were ordered
170 from Merck/Sigma-Aldrich UK unless otherwise stated. The final concentration for SA, JA,
171 ACC, and 3-aminobutanoic acid (BABA) were derived from a dilution series experiment
172 including (Kakei *et al.*, 2015) and (Peraldi, unpublished). The concentration for IAA was
173 derived from (Kakei *et al.*, 2015) whereas trans-Zeatin was derived from (Großkinsky *et al.*,
174 2013). The concentrations for the 1-napthaleneacetic acid (NAA) and kinetin were derived
175 from the concentrations chosen from IAA and trans-Zeatin respectively. The compounds
176 ACC, IAA – sodium salt, kinetin, and BABA were dissolved in water. The compounds SA, JA
177 and NAA were dissolved in ethanol and trans-zeatin was dissolved in DMSO. All non-water
178 solvent concentrations in final treatment were at or below 0.1 %. The same concentration of
179 solvent was applied to respective control treatment 0.8 % agar plates.

180 Roots were inoculated six hours after transfer to hormone amended medium. Inoculum
181 was prepared by blending mycelium and PDA from one-week old cultures (adding 1 ml
182 water per PDA plate). Subsequently, approximately 0.1 ml to 0.2 ml of homogenized slurry
183 was transferred with a 10 ml syringe onto the root tip. This mycelial slurry induced more
184 severe and consistent symptoms than a conidial slurry (PDA slurry amended with a conidial
185 suspension) during early FRR (Unpublished data). Inoculated plants were then incubated at
186 22 °C (16h/8h light/dark photoperiod, variable humidity) in controlled growth cabinets. The
187 slurry was removed when necrosis was visible at the root tip, often at 1-day post inoculation

188 (dpi). Roots were photographed at intervals to monitor disease development. For repeated
189 independent experiments with different measurement dates, the days were combined and
190 denoted as 'Score Date' 1 to 3 with each measurement at 3-day intervals (Fig. 1B, Fig. 1C).

191 Fusarium Head Blight Assays with Chemical Pre-Treatment.
192

193 Numerous features of the FHB assay were derived from (Peraldi *et al.*, 2011). With
194 Bd3-1 FHB assays, once extruding anthers were visible around mid-anthesis, the entire Bd3-
195 1 plant was sprayed with 50 ml of phytohormone or solvent with 0.05% Tween 20 onto a
196 tray of 10 to 11 pots. All compounds were ordered from Merck/Sigma-Aldrich UK unless
197 otherwise stated. The concentrations used for BABA and trans-Zeatin were derived from
198 those used in FRR assays, the SA concentrations was obtained from previous publications
199 (Mandal *et al.*, 2009; Makandar *et al.*, 2011; Sorahinobar *et al.*, 2016) and JA and ACC were
200 experimentally determined. ACC and BABA were dissolved in water, trans-Zeatin in DMSO,
201 and SA and JA in ethanol. The final concentration of non-water solvents was kept at or
202 below 0.2 %. The same concentration of solvent was applied to respective control treatment
203 groups. Twenty-four hours later the soil was watered, and Bd3-1 spikes were sprayed to
204 run-off with conidia of *F. graminearum* (0.25-1 x 10^6) amended with wetting agent (0.05%
205 Tween 20). A control pot without inoculum was included for each treatment. Approximately
206 30 ml of inoculum was sprayed onto a total of 20 plants' spikelets per treatment. Spraying
207 was performed immediately before the dark period and pots were then collectively held in a
208 large plastic humidity chamber to maximise humidity for three days 22°C (20h/4h light/dark
209 photoperiod). Symptoms were scored every four days by counting the number of infected
210 florets per spike. For repeated independent experiments with different measurement dates,
211 the days were combined to 'Score Date' 1-3 with each measurement at 4-day intervals (Fig.
212 3B, Fig. 3D).

213 Some experiments were performed at BASF SE using Bd21 instead of Bd3-1. Bd21 and
214 Bd3-1 are both highly susceptible to FHB (Peraldi *et al.*, 2011). For the Bd21 auxin trial, the
215 entire plant was sprayed with respective compounds until run-off. The auxin concentration
216 was experimentally determined (Unpublished data). The final solvent concentration for each
217 auxin treatment with Bd21 including the control treatment was 5 % acetone. After 24h, a

working concentration of $5x10^5$ *F. graminearum* LI600 was prepared in 0.05 % tween 20 water, and 175 ml of inoculum was evenly sprayed above all plants. The base matting was watered until run-off and plants were encased in plastic covers for six days in an elevated humidity glasshouse room (Autumn conditions) before scoring.

Statistical Analysis.

All statistical tests were performed using the software package GENSTAT v.19.1.0.21390 (VSN international Ltd). A Generalised liner model with an accumulated analysis of variance (ANOVA) was used for all experiments using a normal distribution for FRR assays or log-linear modelling for FHB assay count data. Individual time points were analysed separately. All data was untransformed except for a square root transformation for Fig. 1A at 7 dpi, Fig. 1C at score date 1, Fig. 2A at 2 dpi, Fig. 2C at 4 dpi, Fig. 2D at 2dpi and 4dpi. A log10 transformation was used for Fig. 2C at 2dpi. A few ANOVA's for combined independent experiments showed a significant interaction between the independent experiment and the hormone treatment at specific time points: Fig. 1A at 3 dpi, Fig. 1B at Score Date 3, Fig. 1D at 2 dpi, Fig. 2A at 2 dpi, Fig. 2B at 2 dpi, Fig. 3C at 11 dpi, and Fig. 3E at 7 dpi and 11dpi ($p < .05$). However for all the remaining time points, no such interaction was observed ($p > .05$). For Bd21, a randomised complete block design was used from the R package 'agricolae' and plant pots were randomized before chemical application. Microsoft Office was used for writing, data collection, diagrams, and analysis. All graphs were prepared using Graphpad Prism (V5.04).

Results

The Differential Effect of Plant Hormones on Fusarium Root Rot Disease Progression.

The SA amendment significantly increased root necrosis length (RNL) at all time points (Fig. 1A), while amendment with JA (Fig. 1B), or the ethylene precursor (ACC) (Fig. 1C), significantly reduced RNL at all time points ($p < .01$). Interestingly, the difference in RNL for all three compounds remained similar with the regression lines remaining parallel rather than diverging over time. The compound BABA had the greatest effect in increasing RNL compared to other defence-related hormones (Fig. 1D). The differential RNL symptoms resulting from pre-application of BABA increased over time with application leading to near doubling of necrosis at 4 dpi and 6 dpi ($p < .001$) relative to the control. Overall exogenous application of defence-related hormones had a substantial effect on FRR disease severity but whether the effect on resistance was positive or negative was dependant on the particular phytohormone.

Exogenous application of IAA resulted in the most pronounced decrease in RNL compared to the control at all time points ($p < .001$) since FRR symptoms were reduced to approximately half those in control treatments (Fig 2A). Like IAA, the synthetic auxin NAA also reduced RNL at all time points ($p < .001$, Fig 2B) but auxin induced FRR resistance was most pronounced with IAA. With both auxins, the increased resistance was observed as early as 2 dpi yet the regression lines diverged over time with a greater reduction in RNL at later time points (Supp. Fig. S1). Overall, auxin treatment produced a very positive effect on resistance towards FRR.

The cytokinin trans-Zeatin increased RNL at all time points ($p < .001$, Fig. 2C) and the differential increased over time. The extent of necrosis was twice that in the control by 6 dpi (Supp. Fig. S2) leading to the most severe symptoms observed with any of the phytohormone treatments. The cytokinin kinetin exhibited no significant effect on symptoms at 2dpi ($p = .785$) but increased the rate of RNL so that the differential increased over time ((4 dpi and 6 dpi $p < .01$, $p < .001$ respectively) Fig. 2D). Strikingly the effect of cytokinin amendment increased dramatically over time, with the effect being even greater than that observed for the positive effect of auxins. Overall cytokinin amendment appeared

to have a very negative effect on FRR resistance which became more pronounced as the disease progressed.

The phytohormones abscisic acid, gibberellic acid, and the brassinosteroid epibrassinolide were also tested on FRR (Supp. Fig. S3A and S3B and Supp. Fig. S4). Gibberellic acid and epibrassinolide had no significant effect on FRR symptoms while abscisic acid induced extensive root discolouration that was indistinguishable from FRR symptoms and so prevented assessment (Supp. Fig. S4).

Most Plant Hormones Promoted Susceptibility to Fusarium Head Blight.

In order to compare the effects of hormones on FRR to those on FHB, the same hormones were applied to the entire *B. distachyon* plant and subsequently the spikes were inoculated with *F. graminearum* conidia. Unless otherwise stated, the effects described are from a single dose of phytohormone pre-treatment as opposed to continual exposure as during the FRR assays. As a result, often a higher concentration of phytohormone was applied to maximise the dose of phytohormone received by the plant.

Although pre-inoculation treatment with SA marginally increased FHB symptoms (Fig. 3A), the increase was not statically significant at any time point ($p > .05$). In additional experiments, SA was applied repeatedly (four applications) before, during, and after inoculation on Bd3-1 but again, no significant effect on resistance was observed (Supp. Fig. S5). Pre-inoculation treatment with JA resulted in an increase in the number of infected florets at all time points ($p < .05$, Fig. 3B). Similarly, pre-treatment of ACC (Fig. 3C) also significantly increased the number of infected florets at all time points ($p < .05$), but the effect on symptoms diminished over time and by 11 dpi, the effect was not significant ($p > .05$). Pre-inoculation application of BABA significantly increased the number of infected florets at all time points ($p < .001$) (Fig. 3D) and application of cytokinin trans-Zeatin caused the largest increase in infected florets relative to the control treatment of any of the compounds examined (Fig. 3E) ($p < .001$). With both BABA and trans-Zeatin, the differential in the number of infected florets relative to the control treatments increased after the first score date although the divergence from the controls was not as great as observed in the FRR assays where the phytohormone amendment was present throughout the course of the experiment. Overall pre-inoculation application of all the phytohormones except for SA

301 promoted FHB susceptibility leading to a significant increase in the number of florets
302 exhibiting symptoms.

303 Using Bd21 instead of Bd3-1, two concentrations of IAA and NAA were exogenously
304 applied to Bd21 plants before inoculation with the isolate *F. graminearum* LI6000 (Fig. 4). All
305 auxin pre-inoculation applications resulted in a reduction in the number of infected florets.
306 Pre-inoculation application of synthetic auxin NAA at 8 mM resulted in the most significant
307 decrease in the number of infected florets ($p < .001$) and even 4 mM NAA significantly
308 decreased the number of infected florets ($p < .01$). IAA appeared to be less effective than
309 NAA since 8 mM IAA showed a similar reduction in infected florets as 4 mM NAA ($p < .01$)
310 and at 4 mM IAA the reduction in the number of infected florets was not significant ($p =$
311 $.33$). Overall, these data indicate that auxins have a positive effect on FHB resistance when
312 applied at relatively high concentrations prior to inoculation.

313 To ascertain whether the effects of phytohormones on FRR reflected an altered plant
314 response or an effect on the fungus itself, hormones at the same concentrations as used in
315 the experiments were applied to *F. graminearum* growth medium to measure any changes
316 in growth over time. Compared to the respective control treatments there was no
317 significant effect on *F. graminearum* growth of any of the tested phytohormone (Supp. Fig.
318 S6). Due to the high concentrations of auxins tested for FHB, a different assay was
319 performed. Auxins or solvent controls were applied to filter disks at four equidistant points
320 from a mycelial plug of *F. graminearum* PH1 growing on PDA. There was no difference in
321 mycelial growth near any of the auxin-treated filter disks (Supp. Fig. S7).

Discussion

Given some of the proposed differences between *F. graminearum* infection of floral and root tissues, the effect of a range of phytohormones on FRR was assessed and compared to their effect on FHB. Research into the role of exogenous phytohormones on FHB resistance has been investigated in several plant species. SA was found to increase resistance to FHB in wheat and Arabidopsis (Makandar *et al.*, 2011; Qi *et al.*, 2012; Sorahinobar *et al.*, 2016). We were not able to replicate the positive FHB effect of SA (Fig. 3A) despite using similar concentrations to those used previously. We speculate that the different results are due to alternative SA application methods. In earlier studies, SA (200 µM) was applied as a soil drench (Makandar *et al.*, 2011) (Sorahinobar *et al.*, 2016). This application method may have more pronounced effects on resistance due to initial uptake in the roots as opposed to florets and because the phytohormone remained in contact with the roots throughout the experiment. Furthermore prolonged exposure to SA may influence JA/ethylene dependent Induced systemic resistance (ISR) (Pieterse *et al.*, 2014) which might impact the resistance response to FHB.

Our results on the effect of application of JA on FHB (Fig. 3B) support some studies (Makandar *et al.*, 2010), but contradict others (Li and Yen, 2008; Qi *et al.*, 2016; Sun *et al.*, 2016). The reasons for these differences are unclear. They may reflect the use of a higher concentration of JA in the present study than was used in some previous reports (Qi *et al.*, 2016; Sun *et al.*, 2016) or they may be due to differences in the timing of treatment (Makandar *et al.*, 2010). The difference may also be due to the application method. Qi and colleagues (2016) point-inoculated individual florets with *F. graminearum* rather than spraying the head (Qi *et al.*, 2016). Injecting high levels of inoculum into florets may have resulted in a by-passing or severe shortening of the biotrophic phase of infection/colonisation (Jansen *et al.*, 2005; Brown *et al.*, 2010). Similar to JA, our results with ACC (Fig. 3C) support the findings from some studies (Chen *et al.*, 2009), but not those of others (Li and Yen, 2008; Sun *et al.*, 2016; Foroud *et al.*, 2018). Ethylene can affect host resistance via synergism with classic defence hormones (Pieterse *et al.*, 2012) or via accelerated senescence (Abeles *et al.*, 2012; Häffner *et al.*, 2015). As a result, the outcome is very sensitive to several factors such as timing of exogenous application, tissue type, and

353 the type of invading pathogen (van Loon *et al.*, 2006). Differences in the experimental
354 procedures may also affect important factors for ethylene response. In some reports
355 detached head assays were performed (Chen *et al.*, 2009; Foroud *et al.*, 2018) which might
356 influence senescence processes as well as defence responses. Ethephon (releases ethylene
357 gas) was used instead of ACC as the ethylene treatment in some studies (Li and Yen, 2008;
358 Chen *et al.*, 2009; Sun *et al.*, 2016). Although Foroud and colleagues (2018) found that
359 ethephon and ACC had similar effects on FHB in most wheat varieties tested (Foroud *et al.*,
360 2018). The role of ethylene is further complicated given evidence that some isolates of *F.*
361 *graminearum* and *F. culmorum* can produce ethylene *in vitro* (Haidoulis et al. unpublished
362 data), however it is uncertain whether ethylene is produced *in planta*.

363 We found that auxin, (both IAA and NAA) greatly improved FHB resistance (Fig. 4). This
364 results supports evidence by Petti and colleagues (2012) who found that exogenous IAA
365 application reduced yield loss and general symptoms of *F. culmorum*-induced FHB in barley
366 (Petti *et al.*, 2012). However, the mechanism for the effects of auxin in FHB response are not
367 clear given that *F. graminearum* can also produce IAA (Luo *et al.*, 2016).

368 Overall, our results using *B. distachyon* as a model for investigating FHB responses were
369 broadly in line with those from studies on wheat, barley and Arabidopsis. Similar trends
370 were observed in the response to auxins, JA, and ethylene as reported in FHB of wheat and
371 barley. Wheat and *B. distachyon* have been show to share a common defence transcriptome
372 response following Fusarium crown rot infection caused by *F. pseudograminearum* (Powell
373 *et al.*, 2017). Furthermore *B. distachyon* and barley were found to share common
374 brassinosteroid-associated defence responses through the use of brassinosteroid receptor
375 (*bri1*) mutants in the two species (Goddard *et al.*, 2014). This suggests the observed effects
376 of phytohormones on FHB in *B. distachyon* can be effectively translated to wheat FHB
377 responses.

378 The pathology of *F. graminearum* in root tissues is poorly characterised, but evidence
379 suggests that the fungus rapidly penetrates roots and exhibits specific stages of infection
380 (Wang *et al.*, 2015). Investigation of exogenous phytohormones on FRR response revealed
381 that FRR was much more responsive than FHB in the response to infection. This is likely due
382 to the continual exposure during FRR assays as opposed to the single application used for
383 FHB assays. An example of this was observed JA (Fig. 3B) and ACC (Fig. 3C) that displayed

384 the most pronounced effect on FHB susceptibility at earlier time points suggesting a short-
385 lived response.

386 The classic defence hormones SA, JA, and ethylene (ACC) exhibited different effects on
387 FRR and FHB (Fig. 5). Despite the non-significant FHB response to SA, SA increased FRR
388 susceptibility as early as 1 dpi (Fig. 1A). This difference may reflect the differences in the
389 levels of phytohormones in different tissues. Endogenous free SA levels were found to be
390 substantially higher in rice (*O. sativa*) floral tissues compared to root tissues (Chen *et al.*,
391 1997). If this is also true for *B. distachyon*, exogenous SA application might be expected to
392 have more impact on the endogenous SA profile in roots than florets resulting in a more
393 pronounced change in resistance in FRR compared to FHB. The similar response between JA
394 and ethylene in FHB and FRR supports the evidence that JA and ethylene often function
395 similarly in defence (Pieterse *et al.*, 2012). However, both JA and ethylene had contrasting
396 effects on FRR and FHB, increasing resistance to the former and susceptibility to the latter
397 (Fig. 1B-C and Fig 4B-C). The SA pathway is generally considered to promote resistance to
398 biotrophic pathogens and susceptibility to necrotrophic pathogens whereas JA and ethylene
399 pathways generally promote resistance to necrotrophic pathogens and susceptibility to
400 biotrophic pathogens (Glazebrook, 2005; Bari and Jones, 2009; Pieterse *et al.*, 2012). In both
401 FHB and FRR, hormones were applied before inoculation and would most likely influence
402 events relating to the biotrophic phase of *F. graminearum* infection and colonisation. Given
403 the increase susceptibility to FHB in response to JA and ethylene, this supports the JA/
404 ethylene pathway antagonism to the biotrophic phase of FHB. However, since the opposite
405 effect of JA and ethylene was observed on FRR, we speculate that *F. graminearum* is
406 adopting a more necrotrophic lifestyle on roots. This difference in trophism between tissues
407 is not well documented. Differences in trophic state have been observed for other plant
408 pathogens. Marcel and colleagues (2010) found that *M. oryzae* behaves as a biotroph
409 during rice root infection despite adopting a hemibiotrophic lifestyle on leaves (Marcel *et*
410 *al.*, 2010). Hypothetical reasons for switching to necrotrophy may be due to a demand for a
411 change in the resource acquisition method, the time required to overcome defences, or a
412 change in plant defence response (Kabbage *et al.*, 2015; Zeilinger *et al.*, 2016). One or more
413 of these factors may be causing this difference in fungal lifestyle between roots and shoots
414 in the *B. distachyon* - *F. graminearum* pathosystem. Wang and colleagues (2015) found that

415 wheat Sumai3 roots were susceptible to FRR despite this variety being known for its potent
416 FHB resistance (Wang *et al.*, 2015). Furthermore, Lyons et al. (2015) identified differing
417 expression patterns between root and leaf infection by *Fusarium oxysporum*. These
418 examples suggest that a unique defence mechanism might be occurring in roots which
419 promotes a different trophic lifestyle in Fusarium.

420 Contrary to the classic defence hormones, the development-related phytohormones
421 induced similar effects on FHB and FRR (Fig. 5). Auxin application resulted in greatly
422 increased resistance in both FHB and FRR in *B. distachyon*. Like the effect of auxins, the
423 cytokinins trans-Zeatin and BABA also functioned in a tissue-independent manner, but they
424 greatly increased susceptibility to FHB and FRR (Fig. 5). Aside from roles in plant defence,
425 BABA can also affect plant development through reduction of growth and endogenous iron
426 content (Wu *et al.*, 2010; Koen *et al.*, 2014). Therefore, BABA could be considered to be
427 functioning similarly to the classic development-associated hormones auxin and cytokinin.
428 There was a substantially larger effect of these three phytohormones on resistance
429 compared to the classic defence phytohormones. We speculate that auxins, cytokinins, and
430 BABA are affecting resistance in an SA/JA independent manner. In support of this, (Qi *et al.*,
431 2016) found that exogenous application of IAA did not lead to an increase in endogenous
432 levels of SA or JA. Furthermore auxin signalling independent of JA and SA was found to be
433 important for *A. thaliana* resistance to two other necrotrophic pathogens (Llorente *et al.*,
434 2008). In another example, elevated expression of a the IAA-amido synthetase gene *GH3-8*
435 that regulates IAA levels promoted resistance to *X. oryzae* in rice independently of SA and JA
436 (Ding *et al.*, 2008). Cytokinins were also found to improve *N. tabacum* resistance to *P.*
437 *syringae* in an SA and JA independent manner (Großkinsky *et al.*, 2011). BABA functions
438 synergistically with the abscisic acid defence pathway (Ton and Mauch-Mani, 2004) and
439 there is evidence suggesting that abscisic acid promotes FHB susceptibility (Buhrow *et al.*,
440 2016; Qi *et al.*, 2016). The different effect of development hormones may be related to
441 either phytohormone homeostasis, antimicrobial production linked to metabolic pathways
442 of development hormones, or trade-offs with growth (Kazan and Manners, 2009; Huot *et*
443 *al.*, 2014; Albrecht and Argueso, 2017). Additionally, the opposite and marked response
444 observed between cytokinins and auxins may be due to an antagonism between the two
445 response networks (Naseem and Dandekar, 2012; Naseem *et al.*, 2012). Overall, the effect

446 of development-associated hormones on FHB and FRR may depend on their influence on
447 basal resistance or specific growth/defence trade-offs independent of the trophic lifestyle.
448 In contrast, the role of the classic defence hormones may be dependent on the trophic
449 lifestyle of the pathogen.

450 Since multiple time points for both FHB and FRR were recorded, we observed differences
451 in slope regression lines between phytohormones. We were able to separate the responses
452 to compounds into two broad groups. The first 'parallel' group are those compounds that
453 induce a significant difference in resistance at the first time point, and that the difference
454 remained similar at later time points. The phytohormones SA, JA, and ACC (Fig. 1A-C) had
455 this effect on FRR whereas no phytohormone displayed this on FHB. This trend implies that
456 these compounds have an immediate effect on initial *F. graminearum* infection, after which
457 the absence of deviation relative to the first time point suggests that the effect on FHB
458 colonisation was much reduced or absent. The second 'divergent' group are those that have
459 minor effects at the first time point, but where the differential in resistance increased at
460 later time points. The growth and development-associated phytohormones exhibited this in
461 FRR assays: BABA (Fig. 1D), both auxins (Fig. 2A and Fig. 2B), and both cytokinins (Fig. 2C and
462 Fig. 2D). This trend implies that these compounds have a minimal effect on initial infection
463 but affect the ability of the host to restrict *F. graminearum* colonisation. These trends are
464 summarised in Fig. 5. Together the evidence supports the idea of fundamental differences in
465 the functioning of growth/development associated phytohormones compared to the classic
466 defence phytohormones in resistance to FRR. Secondly there were differences between FHB
467 and FRR in response to the classic defence hormone. This supports our view that for FHB
468 there is a period of biotrophic growth before switching to necrotrophy whereas in FRR this
469 initial phase is much reduced or absent.

470 A model was produced to summarise the effects of the tested phytohormones on FHB
471 and FRR in *B. distachyon* (Fig. 5). Phytohormones altered FRR and FHB resistance in *B.*
472 *distachyon*. The results reflect the generally antagonistic nature of SA to JA/ ethylene in the
473 two diseases. However they also show that these defence hormones induce disease-specific
474 effects between FHB and FRR. Unlike the classical defence-related hormones, the
475 development-associated hormones auxin and cytokinin had large tissue-independent effects
476 on *B. distachyon* response to FHB and FRR, highlighting an important role of these

hormones in the *B. distachyon* - *F. graminearum* pathosystem. Furthermore our data suggest that each hormone functions at different phases with some affecting on initial *F. graminearum* infection and others playing a greater role during colonisation. Future experiments are required to examine the host and pathogen transcriptome response during both FHB and FRR to provide additional evidence on the mode of trophism within infected tissues and gene expression of both partners in the interaction.

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Conflicts of Interest

The authors declare no conflict of interest.

Supplementary Figures

Fig. S1: The growth (measured as radius from mycelial epicentre) of *F. graminearum* on hormone-amended PDA at 2 dpa.

Fig. S2: The change in *F. graminearum*-induced FRR necrosis after application of gibberellic acid (A) and epibrassinolide (B) on Bd3-1 seedling roots.

Fig. S3: The visual effect of 10 µM abscisic acid on 7-day old Bd3-1 roots in the absence of disease.

Fig. S4: The visual decrease in RNL after IAA application (B) compared to control (A) at 6 dpi.

Fig. S5: The visual increase in RNL after Zeatin application (B) compared to DMSO control (A) at 6 dpi.

Fig. S6: The change in number of *F. graminearum*-infected florets after four doses of salicylic acid 24 h before, 30 min before inoculation, 3 dpi, and 7 dpi on Bd3-1.

Fig. S.7: *F. graminearum* PH1 mycelial growth on PDA after 3 dpa with 8 mM IAA and NAA treated filter disks.

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Figure legends

Fig. 1: The change in *F. graminearum*-induced FRR necrosis after application of defence-related hormones 100 µM SA (**A**), 1 µM JA (**B**), 100 µM ACC (**C**), and 9 mM BABA (**D**) on Bd3-1 seedling roots. Each data point is the mean root necrosis length ± SE from two (**A**, **B**, **C**) or three (**D**) independent experiments, except score date 3 from **C** which was from one independent experiments. Score Date numbers (3-day intervals between each number) for **B** and **C** are the combined dpi from different experiments. Significance levels: ** $p < .01$, *** $p < .001$ compared to control.

Fig. 2: The change in *F. graminearum*-induced FRR necrosis after application of development-related auxins 10 µM IAA (**A**) and 5 µM IAA NAA (**B**) and cytokinins 10 µM trans-Zeatin (**C**) and 10 µM Kinetin (**D**) on Bd3-1 seedling roots. Each data point is the mean root necrosis length ± SE from three (**C**), two (**A** and **B**) or one (**D**) independent experiments. Significance level ** $p < .01$, *** $p < .001$ compared to control. Visual difference for **A** in Supp. Fig. 4 and **C** in Supp. Fig. 5.

Fig. 3: The change in number of *F. graminearum*-infected florets after pre-application of 200 µM SA (**A**), 50 µM JA (**B**), 1 mM ACC (**C**), 10 mM BABA, (**D**) and 10 µM trans-Zeatin (**E**) on Bd3-1. Each data point is the mean number of florets infected ± SE from one (**A**), two (**B**, **C**, **E**) or three (**D**) independent experiments. Score Date numbers (4-day intervals between each number) for **B** and **D** are the combined dpi from different experiments. Significance levels: * $p < .05$, ** $p < .01$, *** $p < .001$ compared to control.

Fig. 4: The number of *F. graminearum*-infected florets at 8 dpi after pre-application of IAA or NAA on Bd21. Each bar is the mean number of florets infected ± SE from one experimental replicate. Significance levels: ** $p < .01$, *** $p < .001$ compared to control.

Fig. 5: Model summarising all exogenous hormone application experiments (Fig. 1-5). Compares the effects between FHB and FRR in *B. distachyon* (on the left). This summary is a generalisation of the trend over time and includes different arrow thicknesses based on the potency of response caused by each phytohormone. Includes the predicted slope regression groups for each phytohormone presented as different arrow caps (excluding auxins and SA as these were not measured or not significantly different to control). Novel abbreviations: ET (ethylene), Aux (auxins), CK (cytokinins).

Figures

Fig. 1

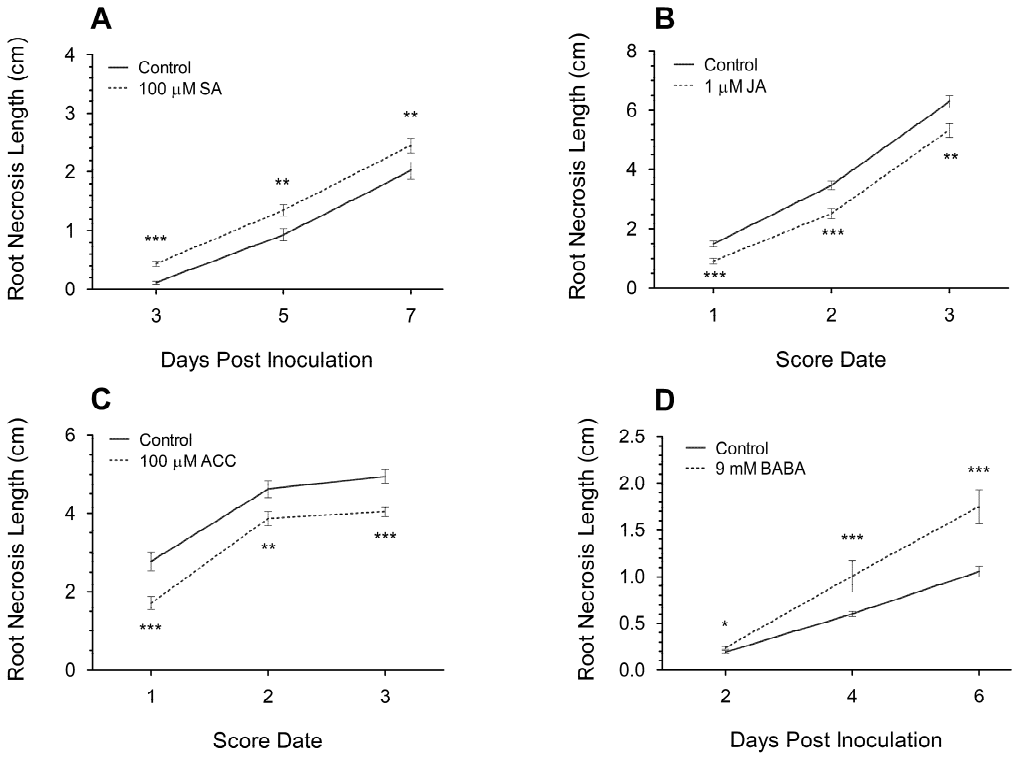


Fig. 2

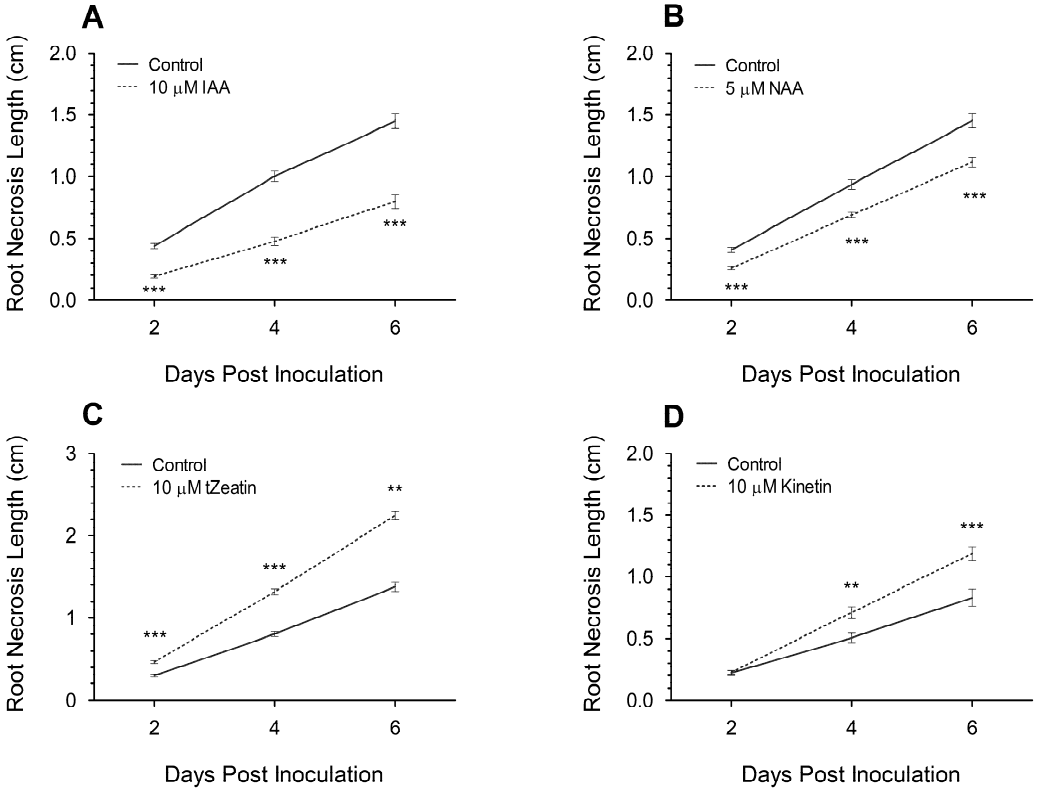

A
Root Necrosis Length (cm)
Control
10 μM IAA
Days Post Inoculation
B
Root Necrosis Length (cm)
Control
5 μM NAA
Days Post Inoculation
C
Root Necrosis Length (cm)
Control
10 μM tZeatin
Days Post Inoculation
D
Root Necrosis Length (cm)
Control
10 μM Kinetin
Days Post Inoculation

Fig. 3

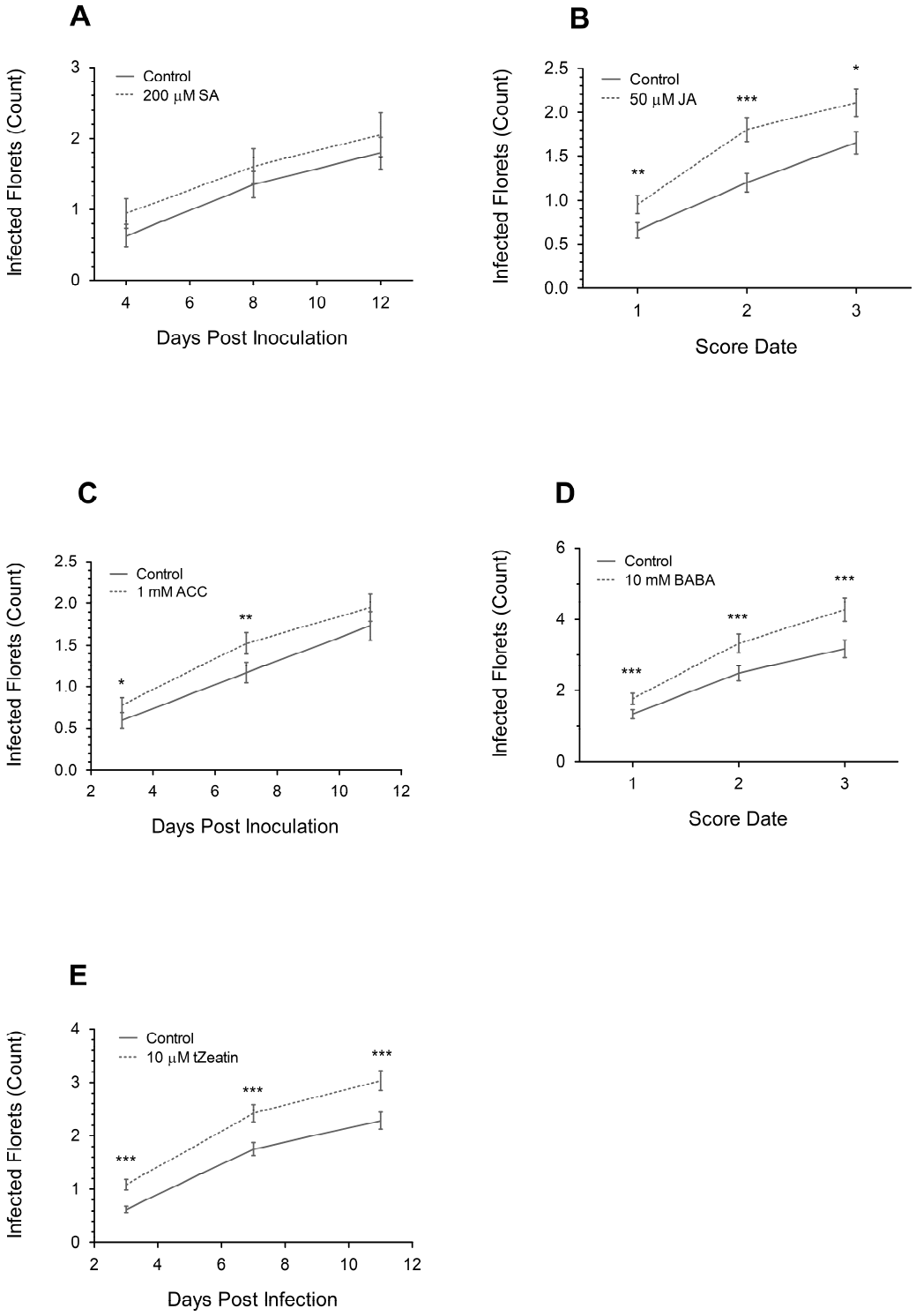

A
Control
200 μM SA
Infected Florets (Count)
Days Post Inoculation
B
Control
50 μM JA
Infected Florets (Count)
Score Date
C
Control
1 mM ACC
Infected Florets (Count)
Days Post Inoculation
D
Control
10 mM BABA
Infected Florets (Count)
Score Date
E
Control
10 μM tZeatin
Infected Florets (Count)
Days Post Infection

Fig. 4

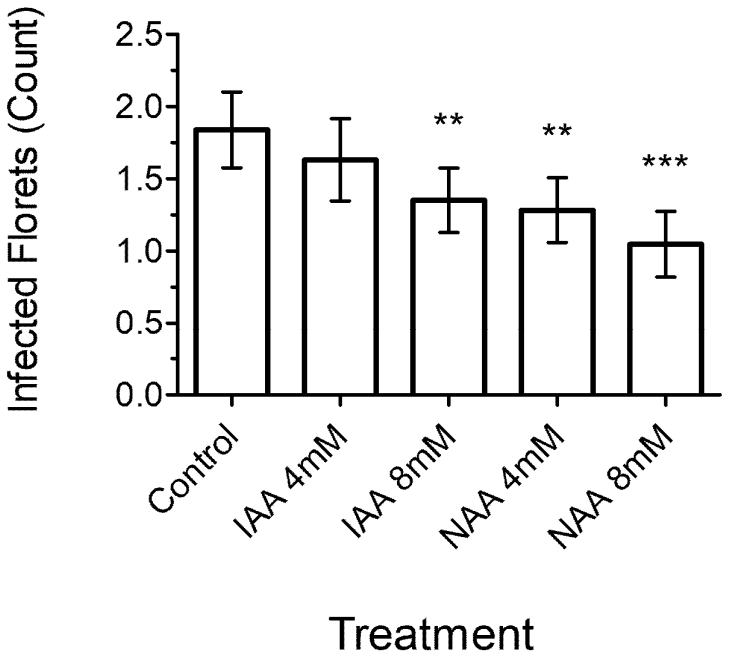
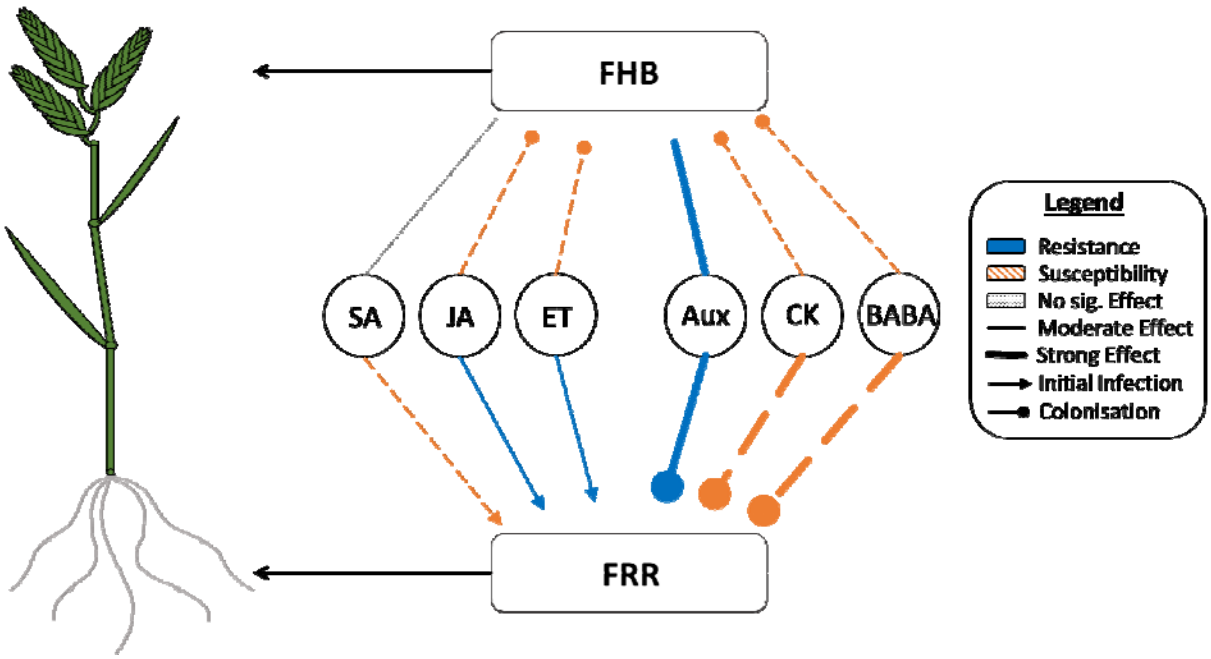

Infected Florets (Count)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
***
Control
IAA 4mM
IAA 8mM
NAA 4mM
NAA 8mM
Treatment

Fig. 5


FHB
SA
JA
ET
Aux
CK
BABA
FRR
Legend
Resistance
Susceptibility
No sig. Effect
Moderate Effect
Strong Effect
Initial Infection
Colonisation