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Use of the volatile trichodiene to reduce Fusarium head blight and trichothecene contamination in wheat

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Summary

Fusarium graminearum **is the primary cause of Fusarium head blight (FHB), one of the most economically important diseases of wheat worldwide. FHB reduces yield and contaminates grain with the trichothecene mycotoxin deoxynivalenol (DON), which poses a risk to plant, human and animal health. The first committed step in trichothecene biosynthesis is formation of trichodiene (TD). The volatile nature of TD suggests that it could be a useful intra or interspecies signalling molecule, but little is known about the potential signalling role of TD during** ***F. graminearum*****-wheat interactions. Previous work using a transgenic** ***Trichoderma harzianum*** **strain engineered to emit TD (*Th* + *TRI5*) indicated that TD can function as a signal that can modulate pathogen virulence and host plant resistance. Herein, we demonstrate that *Th* + *TRI5* has enhanced biocontrol activity against *F. graminearum* and reduced DON contamination by 66% and 70% in a moderately resistant and a susceptible cultivar, respectively. While *Th* + *TRI5* volatiles significantly influenced the expression of the *pathogenesis-related 1* (*PR1*) gene, the effect was dependent on cultivar. *Th* + *TRI5* volatiles strongly reduced DON production in *F. graminearum* plate cultures and downregulated the expression of *TRI* genes. Finally, we confirm that TD fumigation reduced DON accumulation in a detached wheat head assay.**

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Introduction

Fusarium graminearum is the primary cause of Fusarium head blight (FHB), a devastating fungal disease of wheat that causes billions of dollars in annual economic losses worldwide (Wilson *et al*., 2018). FHB reduces crop yield and contaminates grain with trichothecene mycotoxins that make the grain unsafe for use as food or feed. The trichothecene analog deoxynivalenol (DON) occurs in highest abundance in *F. graminearum*-infected wheat and is considered a virulence factor that enables the pathogen to overcome plant defences and spread from infected to uninfected tissues (Desjardins *et al*., 1996; Audenaert *et al*., 2014). This connection between DON and virulence indicates that the inhibition of trichothecene biosynthesis would be a practical control strategy of FHB.

The use of antagonistic microorganisms is gaining popularity as an effective, sustainable, and ecofriendly method of pathogen control. Competitive filamentous fungi belonging to the genus *Trichoderma* can provide effective biocontrol of FHB under at least some field conditions, and can outcompete *F. graminearum* by reducing growth and sporulation on crop debris (Sarrocco *et al*., 2019). One isolate of *Trichoderma harzianum* was reported to have reduced *F. graminearum* colonization of wheat straw and perithecia development by as much as 96% (Schoneberg *et al*., 2015). Additionally, the antifungal volatile 6-pentyl-2H-pyran-2-one (6PP) produced by some *Trichoderma* species was shown to reduce *F. graminearum* DON production on agar medium (Cooney *et al*., 2001).

The first committed step in trichothecene biosynthesis is cyclization of the primary metabolite farnesyl diphosphate (FPP) to form trichodiene (TD), a volatile sesquiterpene hydrocarbon. This reaction is catalysed by a sesquiterpene synthase enzyme, trichodiene synthase, encoded by the gene *TRI5* (McCormick *et al*., 2011). In

Fusarium, TD undergoes four oxygenations to form isotrichotriol, and these reactions are catalysed by a multifunctional oxygenase encoded by the *TRI4* gene. Isotrichotriol undergoes spontaneous cyclization to form isotrichodermol, which can then undergo multiple enzyme-catalysed oxygenations, acetylations, acylations, and/or deacetylations to form the diverse array of trichothecene analogs that have been described (McCormick *et al*., 2006; McCormick *et al*., 2011). In *Fusarium* species, most of the enzyme-encoding genes required for trichothecene biosynthesis (*TRI* genes) are located adjacent to one another in the 25 kb *TRI* cluster. Two transcription factor genes, *TRI6* and *TRI10*, that regulate transcription of *TRI* genes are also located in the cluster (Seong *et al*., 2009; Proctor *et al*., 2018). Despite the synchronized expression of *TRI* genes, a substantial amount of TD passes through the lipid membranes and escapes into the surrounding environment. TD can easily be detected in the headspace above trichothecene producing fungal cultures, *F. graminearum*-infected wheat, and contaminated grain (Jeleń *et al*., 1997; Eifler *et al*., 2011; Girotti et al., 2012). Given that the formation of trichothecenes almost certainly exacts a significant energy cost to *F. graminearum*, loss of TD from the trichothecene pathway into the surrounding air space suggests that TD has a signalling function in addition to its role as an essential intermediate in trichothecene biosynthesis.

Fungal volatiles have been shown to function as antimicrobial compounds, plant growth and/or defence response regulators, and intra or interspecies signals (Li *et al*., 2016). Previous studies in which exogenous TD was added to liquid cultures of *F. graminearum* reported numerous transcriptional responses, suggesting that TD may have an intraspecies signalling role (Seong *et al*., 2009). However, the expression levels of most *TRI* genes where not significantly affected, and the added TD was incorporated into DON (Seong *et al*., 2009). Nevertheless, the response of *F. graminearum* to TD in a liquid culture may differ from its response during growth on a solid substrate or in planta.

Trichoderma harzianum T34 (herein designated *Th*) has been widely used in basic biocontrol research (de la Cruz and Llobell, 1999), and produces a great variety of cell-wall degrading enzymes and secondary metabolites on different natural substrates (Delgado-Jarana *et al*., 2002; Vizcaíno *et al*., 2005). In previous research, *Th* was genetically modified to emit TD. This was done by silencing the ergosterol biosynthetic gene *erg1* and heterologously overexpressing *TRI5* from a trichothecene producing *Trichoderma* species (Malmierca *et al*., 2015b). The resulting TD-emitting (approximately 20 $\mu g\ h^{-1}$) strain (herein termed *Th* + *TRI5*) induced expression of *Botrytis cinerea* virulence genes and tomato plant defence-related genes (Malmierca *et al*., 2015a; Malmierca *et al*., 2015b). Among the plant defence-related genes, the expression of tomato pathogenesis-related (PR) gene *PR1*, which is regulated by the salicylic acid (SA) defence signalling pathway, was most strongly induced both by exposure to volatiles emitted from the *Th* + *TRI5* strain and purified TD.

These results suggest that TD can function as a signal that modulates pathogen virulence and host plant resistance. Therefore, we hypothesized that TD emitted by *Th* + *TRI5* could also function as an intra and interspecies signal that could regulate trichothecene biosynthesis in *F. graminearum* and stimulate wheat defences. Furthermore, we hypothesized that due to these signalling functions, *Th* + *TRI5* would exhibit enhanced biocontrol activity against FHB relative to *Th*. To test these hypotheses, we compared the antagonistic efficacy of *Th* and *Th* + *TRI5* against *F. graminearum* in intact plants of a susceptible and moderately resistant wheat cultivar. Because differences were observed, we also compared the defence responses in wheat seedlings exposed to *Th* and *Th* + *TRI5* volatiles. To assess the potential regulatory role of *Th* and *Th* + *TRI5* volatiles on trichothecene biosynthesis, the DON content and expression of *TRI* genes were analysed in *F. graminearum* cultures exposed to the volatiles emitted by *Th* and *Th* + *TRI5*. Finally, we evaluated the direct effect of TD fumigation on DON accumulation of three *F. graminearum* strains inoculated on detached wheat heads.

Results

Th + TRI5 *displayed enhanced biocontrol activity on mature wheat plants*

To determine if *Th* + *TRI5* had enhanced biocontrol activity against FHB, wheat heads of intact potted plants of FHB susceptible wheat cultivar Norm$^{(S)}$ and moderately resistant cultivar Alsen$^{(MR)}$ were pretreated with sterile water (control), *Th*, or *Th* + *TRI5* prior to inoculation with *F. graminearum* (strain Gz3639) or sterile 0.04% Tween20 solution (mock-inoculation). Volatiles were collected from mock-inoculated heads 1 day after inoculation and every 7 days thereafter for 3 weeks. TD emission was only detected from heads treated with *Th* + *TRI5,* and similar levels of TD were detected throughout the 21-days time course (Fig. 1). *Th* + *TRI5*-treated heads of both Alsen$^{(MR)}$ and Norm$^{(S)}$ exhibited significantly reduced FHB severity (Fig. 2A and B) and less head weight loss due to the disease when compared to control heads which did not have any pretreatment with a biocontrol strain (Fig. 2C and D). The amount of DON in *Th* + *TRI5*-pretreated heads of Alsen$^{(MR)}$ and Norm$^{(S)}$ inoculated with *F. graminearum* was 83% and 76% less, respectively compared to

control heads (Fig. 2E and F). *Th* pretreatment significantly reduced disease severity only in Alsen$^{(MR)}$. *Th* + *TRI5* pretreated Alsen$^{(MR)}$ heads had 62% less disease than *Th*-pretreated heads (Fig. 2A). On average *Th* pretreatment reduced DON contamination in comparison to controls, but this difference was not statistically significant. However, *Th* + *TRI5*-pretreated heads had 66% and 70% less DON contamination in Alsen$^{(MR)}$ and Norm$^{(S)}$, respectively, than *Th*-pretreated heads.

To determine whether the enhanced biocontrol activity of *Th* + *TRI5* was due to an increased colonization ability, we compared the ability of *Th* + *TRI5* and *Th* to colonize wheat heads. We also assessed the relationship between colonization by each *T. harzianum* strain and abundance of *F. graminearum*. Both colonization by the *T. harzianum* strains and spread of *F. graminearum* were estimated by measuring the biomass of the organisms, and biomass was assessed using quantitative polymerase chain reaction (qPCR) assays (Fig. 3). The qPCR data indicated that colonization by both *Trichoderma* strains differed between the two wheat cultivars, but within a cultivar colonization by *Th* + *TRI5* and *Th* was not statistically significantly different. However, in wheat heads of both cultivars inoculated with *F. graminearum*, colonization by *Th* + *TRI5* was significantly less than *Th* (Fig. 3A and B). Furthermore, heads treated with *Th* + *TRI5* had the lowest levels of *F. graminearum*. That is, the treatment with the lowest level of colonization by *T. harzianum* was the treatment with the lowest level of *F. graminearum*. The results were similar for both cultivars. These findings indicate that the enhanced biocontrol activity of *Th* + *TRI5* compared to *Th* did not result from increased colonization of wheat heads by *T. harzianum*.

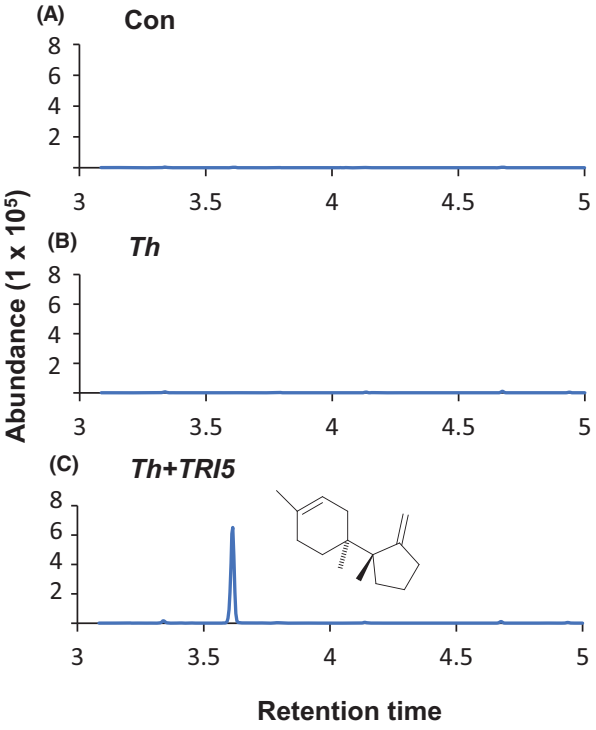

Fig. 1. Flowering wheat heads of both Alsen$^{(MR)}$ and Norm$^{(S)}$ cultivars were dipped in either sterile water (Con), or a 10^5 conidia ml^{-1} suspension of wild-type *T. harzianum* (*Th*), or *T. harzianum* overexpressing *TRI5* (*Th* + *TRI5*) for approximately 5 s. A subset of three heads from each treatment were excised on day 1, 7, 14 and 21 post-treatment and volatiles were collected for 24 h on a Porapak™ filter via the closed-loop stripping method. The samples were analysed using GCMS. Trichodiene was only detected from the *Th* + *TRI5* treated heads and was still detected 21 days after the treatment. Graphs display GC chromatographs of volatiles collected from Alsen$^{(MR)}$ heads at 21-days post-treatment with Con (A), *Th* (B) and *Th* + *TRI5* (C). Results were identical for Norm.

Impact of Th + TRI5 *on wheat seedling defences*

We also determined whether *Th* + *TRI5* volatiles were capable of influencing the expression of defence-related genes in wheat, as previously shown in tomato (Malmierca *et al*., 2015b). To do this, we used large assay plates to grow wheat seedlings of Alsen$^{(MR)}$ or Norm$^{(S)}$ in proximity to but not in contact with agar media inoculated with sterile water (control), *Th*, or *Th* + *TRI5* (Fig. 4A). After one week of growth and exposure to volatiles produced by the *T. harzianum* strains, the aboveground tissues of the seedlings were collected, and relative gene expression of the defence-related gene *PR1* was analysed via qPCR. Expression of the gene in response to the same treatment differed in the two cultivars.

Exposure of Alsen$^{(MR)}$ seedlings to *Th* + *TRI5* volatiles caused an 3.2-fold increase in *PR1* expression compared to the control (Fig. 4B). Although exposure of Alsen$^{(MR)}$ seedlings to *Th* volatiles caused an increase in *PR1* expression on average compared to the control, the difference was not statistically significant. Exposure of Alsen$^{(MR)}$ seedlings to *Th* + *TRI5* volatiles did cause a significant difference in *PR1* expression compared to exposure to *Th* volatiles. In contrast, exposure of Norm$^{(S)}$ seedling to *Th* volatiles significantly reduced *PR1* expression compared to the control, whereas exposure of Norm$^{(S)}$ seedlings to *Th* + *TRI5* volatiles did not cause a significant difference in *PR1* expression compared to the control (Fig. 4C).

We further evaluated the potential of *Th* + *TRI5* to influence expression of wheat defence-related genes when in direct contact with plant tissue. Seeds from Alsen$^{(MR)}$ and Norm$^{(S)}$ were soaked in a conidial suspension of *Th*, *Th* + *TRI5* or sterile water for 48 h and then planted in clay pellets and allowed to grow for 2 weeks. The treatments did not have any visual effect on plant growth or development. The stems and roots of each

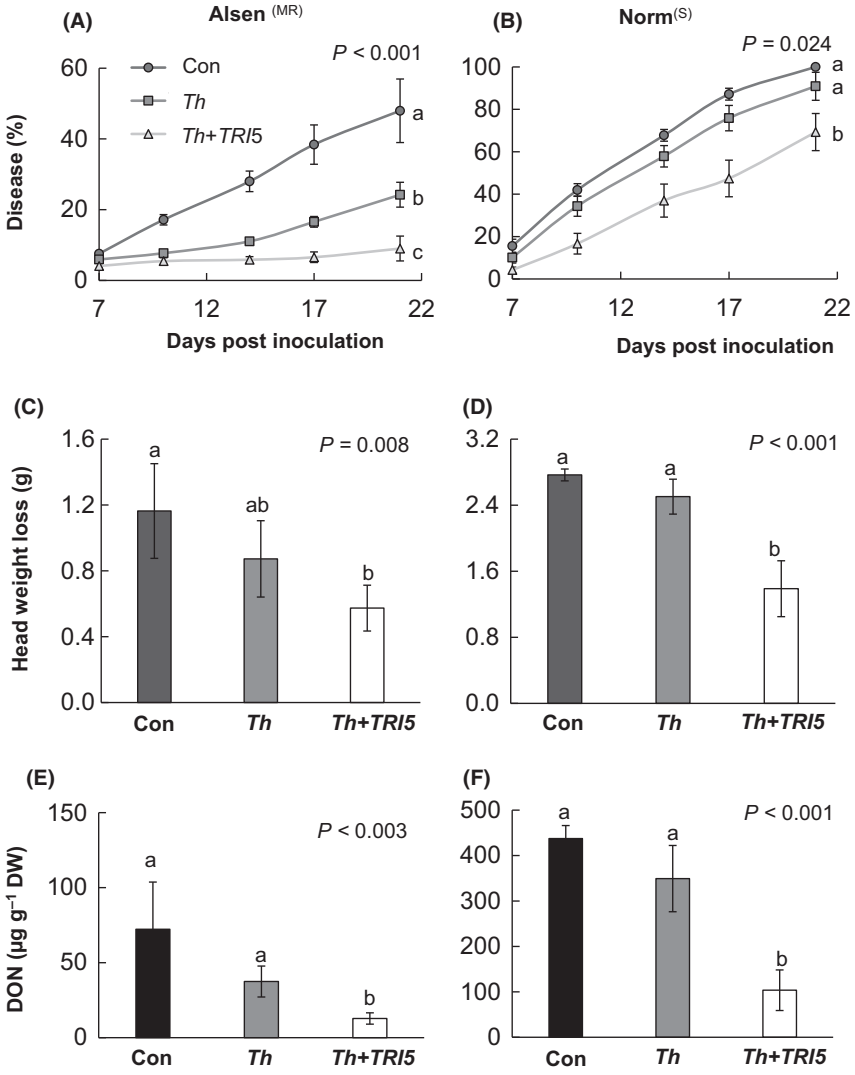

Fig. 2. The FHB moderately resistant and susceptible spring wheat cultivars Alsen$^{(MR)}$ and Norm$^{(S)}$, respectively, were grown to anthesis in environmentally controlled growth chambers and pretreated by dipping the heads in water (Con), or a 10^5 conidia ml^{-1} suspension of *T. harzianum* (*Th*), or *T. harzianum* overexpressing *TRI5* (*Th* + *TRI5*) for approximately 5 s. After 24 h, heads were point inoculated with 10 µL of 10^5 ml^{-1} conidial suspension of *F. graminearum* (*Fg*, Gz3639) or sterile medium as a mock-inoculation control. The per cent disease was estimated over a 21-day period (A, B). The average reduction in weight due to *Fg* was determined by subtracting the head weight of *Fg*-inoculated heads from the average head weight of mock-inoculated controls (C, D). Heads were then lyophilized, pulverized, and the average concentration of DON per g dry weight (DW) was estimated using GCMS (E, F). Significant differences are indicated by different letters (analysis of variance (ANOVA) followed by Tukey–Kramer honestly significant difference (HSD); $n = 15$ of individual heads for disease progression weighted by time and weight loss based on measurements at day 21; $n = 5$ [with each replicate representing three combined heads] for DON analyses)

plant were collected separately, and phytohormone and gene expression were evaluated independently for the different tissue types and cultivars. At the timepoint evaluated, the treatments did not result in any significant differences in plant phytohormone levels (data not shown). In contrast to the volatile exposure *PR1* expression in aboveground tissues of *Th* + *TRI5* treated seedlings was on average less than the control (Fig. S1A top panels). However, this difference was significant only in Alsen$^{(MR)}$. There were no other statistically significant differences in *PR1* expression among treatments for the shoots of either cultivar. On average, *PR1* expression was greater in roots from the *Th* + *TRI5* treatment compared to the control and *Th* treatments (Fig. S1A bottom panels). In Alsen$^{(MR)}$, higher levels of expression in the *Th* + *TRI5* treatment were statistically significant compared to both the control and *Th* treatments, whereas in Norm$^{(S)}$, the higher levels of *PR1* expression in the

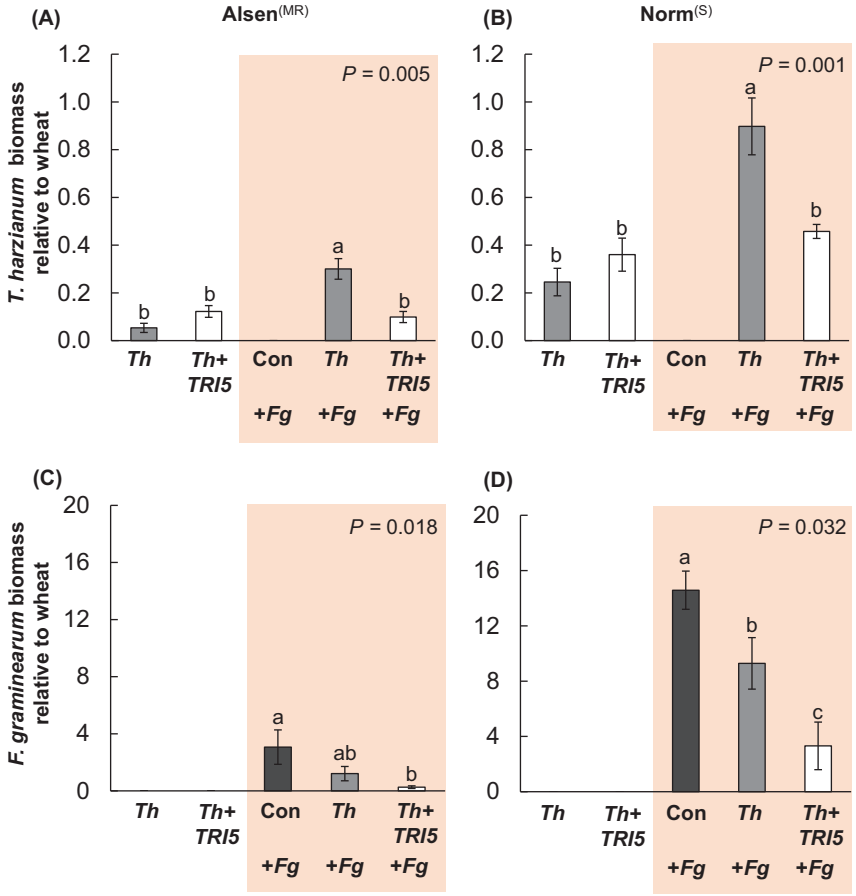

Fig. 3. Alsen(MR) and Norm(S) were pretreated by dipping the heads in water (Con), or a 10^5 conidia/mL suspension of *T. harzianum* (*Th*), or *T. harzianum* overexpressing *TRI5* (*Th* + *TRI5*) and then inoculated with 10 μL of 10^5 /mL conidial suspension of *F. graminearum* (*Fg*, Gz3639) or sterile medium as a mock-inoculation control. Disease was allowed to progress for 21 days and then the samples were collected. The relative amounts of *T. harzianum* (A, B) and *F. graminearum* (C, D) biomass in each sample were determined from the ratio of fungal DNA to wheat DNA via qPCR using species-specific primers (Table 1). Significant differences are indicated by different letters (ANOVA followed by Tukey–Kramer HSD; $n = 5$ [with each replicate representing three combined heads]).

Th + *TRI5* were significant relative to the *Th* treatment but not the control.

Expression of the phenylalanine ammonia-lyase gene (*PAL*), another defence-related gene, was also evaluated (Fig. S1B). Significant differences in *PAL* expression were detected only in Alsen(MR). In shoots, *PAL* expression was significantly less in the *Th* treatment compared to the control, and in the roots, expression was significantly less in the *Th* + *TRI5* treatment compared to the control. But no other statistically significant differences between treatments were observed.

Th + TRI5 *volatiles downregulate* F. graminearum *biosynthesis of DON*

To determine if *Th* + *TRI5* or *Th* volatiles had a direct influence on trichothecene biosynthesis, *F. graminearum* strain Gz3639 was grown on a solid trichothecene-inducing medium (agmatine medium) and exposed to the volatiles in the absence of the plant. Two Petri plates containing either *F. graminearum* cultures or uninoculated agmatine medium were placed in a larger assay plate along with two plates containing one of the following treatments: (i) *Th* cultures, (ii) *Th* + *TRI5* cultures, or (iii) or uninoculated growth medium (Fig. 5A). No significant differences in growth of *F. graminearum* were observed among the treatments ($P > 0.05$). However, levels of DON produced by *F. graminearum* were 40% less in the *Th* + *TRI5* treatment compared to the *Th* and control (no *T. harzianum*) treatments (Fig. 5B). No significant difference in DON production were observed in the *Th* treatment compared to the control.

In addition, the *Th* + *TRI5* and *Th* volatile treatments significantly affected *TRI* gene expression (Fig. 5C). The expression of *TRI6, TRI5*, and *TRI4* responded similarly to the different treatments. In comparison to the control,

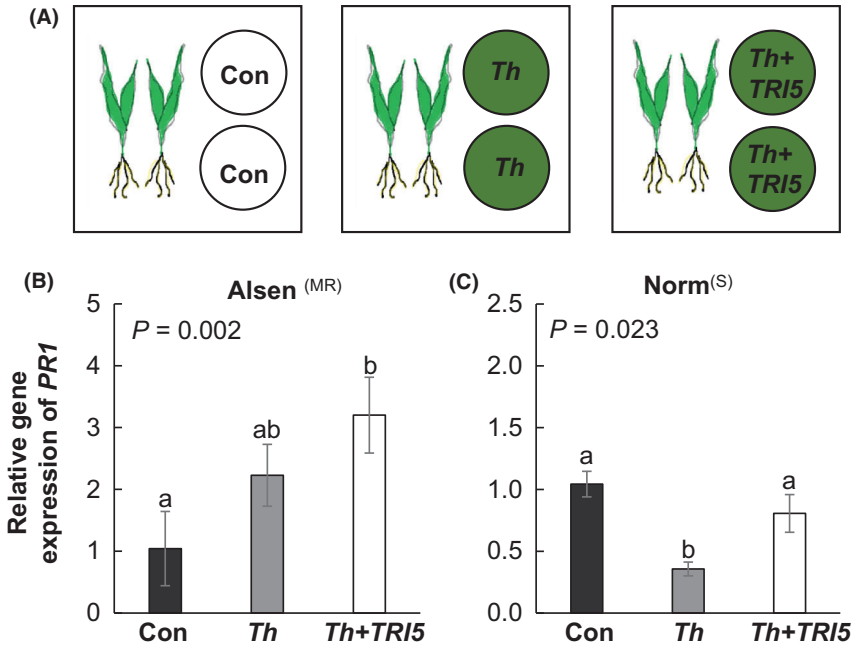

Fig. 4. Alsen$^{(MR)}$ or Norm$^{(S)}$ seeds were germinated and grown on water agar in a large 22 x 22 cm assay plate that contained two sterile V8 agar plates (Con), or two media plate inoculated with *Trichoderma harzianum* (*Th*), or *T. harzianum* overexpressing *TRI5* (*Th* + *TRI5*) (A). After one week of growth, the seedling leaves were collected, RNA was extracted, and relative gene expression of *pathogenesis-related 1* (*PR1*) was independently compared between the different treatments in Alsen$^{(MR)}$ (B) or Norm$^{(S)}$ (C). Different letters above bars indicate significant differences (ANOVA followed by Tukey–Kramer HSD; $n = 4$).

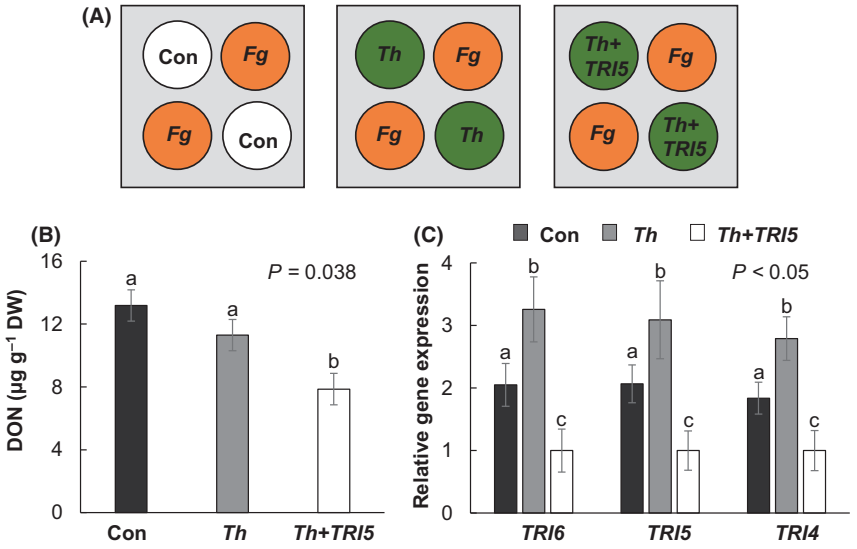

Fig. 5. *F. graminearum* (*Fg*, Gz3639) cultures grown on agmatine agar plates were placed into a larger 22 x 22 cm assay plate as shown in diagram (A) with sterile V8 agar plates (Con), or plates inoculated with *Trichoderma harzianum* (*Th*), or *Th* + *TRI5*. The fungi were exposed to each other's volatiles within the headspace of the assay plate, but do not physically come into contact with one another. After two weeks of growth, the amount of DON produced on the plates was determined using GCMS analyses and compared between treatments (B). Furthermore, the relative gene expression for *TRI6*, *TRI5* and *TRI4* under the different volatile treatments was estimated via qPCR and compared (C). Different letters above SEM bars indicate significant differences (ANOVA followed by Tukey–Kramer HSD; $n = 4$).

the levels of expression of the three *TRI* genes evaluated were 50% higher in the *Th* treatment and 50% lower in the *Th* + *TRI5* treatment (Fig. 5C). *TRI* gene expression was approximately 70% less in the *Th* + *TRI5* treatment compared to the *Th* treatment. Together, results of analyses of gene expression and

DON analyses suggest that TD downregulates *TRI* gene expression and trichothecene production.

TD fumigation reduced the accumulation of DON

In order to determine if TD was responsible for the reduction in DON production, detached wheat heads were inoculated with one of three *F. graminearum* strains (Gz3639, 06-219 or 06-255), placed in glass jars and fumigated with 1.25 μg cm^{-3} purified TD on the day of inoculation and again eight days later (Fig. 6A). Ten days after inoculation, the day on which the heads were collected for DON quantification, fungal hyphae were visible on all florets regardless of volatile treatment. Quantification of DON, via gas chromatography mass spectroscopy (GCMS), revealed that on average less DON accumulated in heads fumigated with TD compared to heads which were exposed to only the carrier solvent acetone (control). This difference in DON accumulation was statistically significant for Gz3639 (40% less DON) and 06-219 (32% less DON) (Fig. 6B, C), but not for 06-255 (Fig. 6D).

Discussion

In this study, we demonstrated that a genetically modified *T. harzianum* strain (*Th* + *TRI5*) emitting TD had enhanced biocontrol activity against *F. graminearum;* wheat plants pretreated with *Th* + *TRI5* had significantly less disease and DON contamination than plants treated with the wild-type progenitor *T. harzianum* strain T34 (*Th*). Assessments of biomass indicate that the enhanced biocontrol activity of *Th* + *TRI5* was not due to a difference in its ability to colonize wheat heads. *Th* + *TRI5* volatiles modestly stimulated wheat defences, as measured by *PR1* expression, but in a cultivar-specific manner. However, *Th* + *TRI5* volatiles strongly reduced DON production in *F. graminearum* cultures and downregulated the expression of *TRI* genes. Additionally, fumigation with purified TD confirmed that TD reduced DON accumulation. To the best of our knowledge, this study represents the first to demonstrate that TD, the volatile intermediate of trichothecene biosynthesis, can regulate DON production.

Biosynthetic enzymes are frequently controlled at the transcriptional level by transcription factors whose

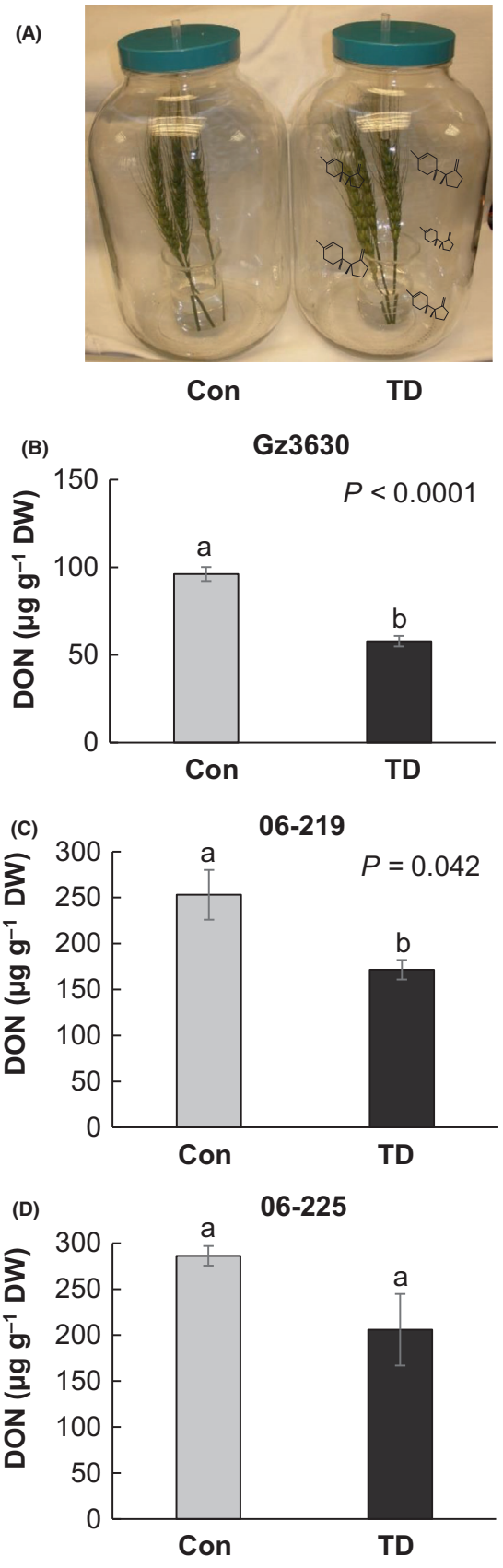

Fig. 6. Flowering wheat (cultivar Norm$^{(S)}$) heads were excised, point inoculated with 10 μL of 10^5 conidia/mL suspension of *F. graminearum* (Gz3639, 06-219, 06-255), placed in glass jars and fumigated with 1.25 μg cm^{-3} purified trichodiene (TD) or an acetone solvent control (Con) of equal volume (A). Ten-day postinoculation, the heads were lyophilized, pulverized, and analysed with GCMS for DON contamination (B–D). Different letters above standard error of the mean (SEM) bars indicate significant differences (*t*-test; $n = 3$).

presence and/or activity can be affected by pathway intermediates or end products (Chubukov *et al.*, 2012). Our findings suggest that TD can function as a negative regulatory signal of *TRI* gene transcription (Fig. 5C) and are consistent with the cost/benefit model for transcription of metabolic pathway genes to avoid loss of resources by dissipation into the atmosphere. However, unlike genes in many intermediate metabolite-regulated pathways (Chubukov *et al.*, 2012), TD negatively affected expression of genes both upstream and downstream of *TRI5* (Fig. 5C). The downstream gene, *TRI4*, was not upregulated to maximize conversion of TD into DON. Previous reports showed that the addition of TD to liquid cultures of *F. graminearum* did not affect *TRI* gene expression and that the added TD was incorporated into trichothecene biosynthesis (Seong *et al.*, 2009). However, the response of *F. graminearum* to TD added to a liquid culture is likely very different from the response to TD in the air space surrounding hypha growing on solid media or in planta. Additionally, since the added TD was incorporated into the trichothecene pathway in liquid culture, there was no loss of resources that would call for a negative regulatory function.

Our data further expand the pivotal role of *TRI5* in trichothecene biosynthesis to include gene regulation. Disruption of the *TRI5* gene not only eliminates the enzymatic cyclization of FPP to TD, the parent compound of all trichothecene analogs, but also disrupts the formation of toxisomes, the specialized subcellular structures made up of colocalized enzymes of the mevalonate- and trichothecene biosynthetic pathways on the endoplasmic reticulum and in which trichothecene biosynthesis occurs (Boenisch *et al.*, 2017; Boenisch *et al.*, 2019). However, these two functions can be uncoupled; replacement of wild-type *TRI5* with mutated *TRI5* that encodes an enzyme without trichodiene synthase activity restored toxisome formation without restoring trichothecene production (Flynn *et al.*, 2019). Thus, the Tri5 protein itself is required for the development of toxisomes, and the enzymatic product of Tri5 functions both as an essential intermediate in trichothecene biosynthesis and, as shown herein, a volatile regulatory signal.

Volatile signals are frequently concentration dependent (Lee *et al.*, 2016). For example, different concentrations of 6PP can either enhance or inhibit plant growth and health (Jeleń *et al.*, 2014). It is also possible that different individuals of the same species have variable levels of sensitivity to a signal and thus respond differently. This may explain why TD fumigation did not significantly affect DON production by *F. graminearum* strain 06-225 (Fig. 6). Nevertheless, further research is needed to determine concentration effects of TD and its mode of perception and action on trichothecene biosynthesis. Furthermore, our data do not address the specificity of the signal. It is possible that other sesquiterpene hydrocarbons can similarly regulate trichothecene biosynthesis.

Various chemicals that inhibit DON biosynthesis have previously been identified. For example, various succinate dehydrogenase inhibitors representing a fungicide class that inhibits fungal respiration decrease DON biosynthesis by inhibiting *TRI5* expression and toxisome formation (Xu *et al.*, 2019). Additionally, the fungicide validamycin exhibits dual efficacies in that it can control FHB by inhibiting DON biosynthesis in *F. graminearum* and inducing host resistance (Li *et al.*, 2019). In contrast to fungicides, TD is an essential component of trichothecene biosynthesis, and trichothecene production is essential for high levels of virulence of *F. graminearum* on wheat. Therefore, *F. graminearum* is less likely to overcome TD-based control strategies than fungicide-based strategies.

Although previous studies showed that TD and volatiles emitted by *Th* + *TRI5* strongly induced expression of tomato defence genes related to SA (Malmierca *et al.*, 2015a), our results indicate that *Th* + *TRI5* volatiles have a relatively modest effect on the wheat disease response, and this effect was not consistent between cultivars. *Th* + *TRI5* volatiles only significantly induced the expression of *PR1* in Alsen$^{(MR)}$ (Fig. 4), and this induction was a modest 3-fold induction compared to the 1000-fold induction observed in tomato (Malmierca *et al.*, 2015b). However, *PR1* expression was similarly induced 2 to 3-fold in response to validamycin treatment in wheat tissues, and this response was assumed sufficient to potentially enhance resistance of wheat to *F. graminearum* (Li *et al.*, 2019). *Th* volatiles suppressed *PR1* expression in Norm$^{(S)}$. This suggests that trichoacorenol, which was found to be produced by *Th* (Malmierca *et al.*, 2015b), or other unidentified *Th* volatiles may also influence wheat defences in a cultivar-specific manner, and in some cases this effect could impede rather than enhance the wheat defence response. *T. harzianum* strains with volatile blends that do not enhance *TRI* gene expression or produce other inhibitory volatiles may provide even greater control against FHB. Nevertheless, since the induction of defence-related gene expression was only observed in one cultivar and the enhancement of biocontrol activity was observed in both cultivars, it is unlikely that the enhancement was due to stimulated host defences alone.

PR1 expression declined in wheat seedling shoots treated with *Th* or *Th* + *TRI5* and was significantly less in Alsen$^{(MR)}$ treated with *Th* + *TRI5* (Fig. S1A). These changes in *PR1* expression could result from direct fungal exposure and/or in alterations in levels of nonvolatile metabolites. For examples, *Th* + *TRI5* produces lower

levels of ergosterol and higher levels of squalene than *Th* (Malmierca *et al*., 2015b), and both ergosterol and squalene can trigger transcription of plant defence-related genes in a concentration-dependent manner (Klemptner *et al*., 2014; Lindo-Yugueros *et al*., 2020). The ratio of ergosterol to squalene can also influence *T. harzianum* colonization of plants (Lindo-Yugueros *et al*., 2020). Therefore, differences in *PR1* expression that occurred in roots versus shoots in response to *Th* + *TRI5* may result from different metabolite concentrations, which in turn could result from differences in the extent of colonization of the roots and shoots by *T. harzianum*. Nevertheless, when applied directly to the wheat heads, *Th* and *Th* + *TRI5* did not exhibit significant difference in colonization as assessed by biomass (Fig. 3). The extent of colonization was not directly related to the efficacy of a strain as an FHB biocontrol agent. Although, *Th* + *TRI5* colonized *F. graminearum*-infected wheat heads less than *Th*, *Th* + *TRI5* was the more effective FHB biocontrol agent (Figs 2 and 3). This further supports the notion that metabolites produced by *Th* + *TRI5* enhance the efficacy of FHB control.

Genetically engineering microbes to enhance their beneficial uses in agriculture has been gaining acceptance. Numerous examples of genetically modified biocontrol agents with improved activity against phytopathogens have been reported (Farrar *et al*., 2014; Arora *et al*., 2020; Hanlon and Sewalt, 2020). For example, overexpression of *TRI5* in *T. brevicompactum* increased the antimicrobial activity of the strain via incorporation of TD into the antimicrobial compound trichodermin (Tijerino et al., 2011). The biocontrol activity of a *Pseudomonas fluorescens* strain against take-all disease in wheat was enhanced by introducing a seven-gene operon for synthesis of the antibiotic phenazine-1-carboxylic acid (Yang *et al*., 2017). Another strain of *P. fluorescens* was genetically modified to act as a biocontrol agent and biofertilizer with biological nitrogen fixation activity (Zhou *et al*., 2014; Jing *et al*., 2020). Genetically modified microbes (Pivot Bio PROVEN™) developed by California-based Pivot Bio are currently commercially available in the US and are being used to increase corn productivity and decrease fertilizer application. Thus, the use of genetically modified microorganisms in agriculture may be more easily accepted than transgenic crops.

Despite the extraordinary potential for the use of TD to enhance the FHB biocontrol activity of *T. harzianum*, it is essential to better understand the mechanism(s) by which TD enhances the biocontrol. Our data do not exclude the possibility that TD-emitting *T. harzianum* strains can stimulate trichothecene biosynthesis under certain conditions. Furthermore, environmental conditions can have a significant effect on emission and dispersal of volatiles (Misztal *et al*., 2018), and this could affect the efficacy of TD-enhanced biocontrol activity. Additionally, the intraspecies signalling potential of TD may result in non-target effects that could have negatives consequences on crop resistance to other pests or pathogens. Nevertheless, this study has identified a novel method to regulate trichothecene biosynthesis and our approach has potential as an effective control strategy for both FHB epidemics and DON contamination in wheat. *T. harzianum* can be applied as a foliar spray during anthesis as demonstrated herein, but could also be applied as a seed coating (Ferrigo *et al*., 2014; Rocha *et al*., 2019) as demonstrated in other studies.

Experimental procedures

Wheat head blight assays evaluating the biocontrol activity of Th + TRI5

FHB disease progression assays were performed using two hard red spring wheat cultivars, the moderately FHB resistant cultivar Alsen$^{(MR)}$ and the susceptible cultivar Norm$^{(S)}$. Both cultivars were grown in climate-controlled growth chambers programmed for 23°C day/20°C night, 500 μmol m^{-2}/s^{-2} photosynthetic photon flux density 14 h photoperiod and 50–60% relative humidity. Five wheat seeds were planted in each 20 x 15-cm pot containing 2.5 l of SunGrow Horticulture potting mix (Agawam, MA, USA). Plants were fertilized two-week postgermination and then every other week thereafter with a 500 ml solution containing 0.5 g l^{-1} of Peter's 20-20-20 (Grace-Sierra Horticultural Products, Milpitas, CA, USA) until inoculation.

The biocontrol activity of wild-type *T. harzianum* strain *Th* (T34) and the genetically modified strain *Th* + *TRI5* (E20-tri5.7), silenced in expression of *erg1* and heterologously expressing *TRI5* which emits approximately 20 μg h^{-1} TD (Malmierca *et al*., 2015a; Malmierca *et al*., 2015b), were compared. Detailed differences in metabolite profiles between the two strains have previously been described (Malmierca *et al*., 2015a; Malmierca *et al*., 2015b).

When the plants started flowering, they were pretreated by dipping heads for 5 seconds in either a 10^5 conidia/mL suspension of *Th*, or *Th* + *TRI5* or sterile water (control). After 24 hours, 15 wheat heads from each cultivar per treatment (*Th, Th* + *TRI5* or control) were point inoculated with 10 μl of a 10^5 conidia ml^{-1} suspension of *F. graminearum* (Gz3639) in 0.04% Tween 20 (Thermo Fisher Scientific, Waltham, MA, USA) or a sterile 0.04% Tween 20 solution (mock-inoculated). The pots were separated by treatment into different chambers to prevent any cross-contamination of volatiles with the other treatments. The experiment was repeated altering the chambers used for the designated treatments. Individual heads were bagged for 3 days to

Table 1. qPCR assays used for determination of fungal biomass within plant tissue. All probes were labelled with 5' 6-FAM and were double quenched, using internal ZEN and 3' Iowa Black fluorescent quenchers. All assays were designed in this work.

Organism	Target gene	Forward primer	Probe	Reverse primer
Fusarium graminearum	*translation elongation factor 1-α*	CAGTCACTAACCACCTGTCAAT	AACCCAGGCGTACTTGAAGGAACC	AATGGTGATACCACGCTCAC
Fusarium graminearum	*trichothecene 3-O-acetyltransferase (TRI101)*	GGACTCTGGGATTACGACTTTG	CGAGACTGTGAGACGGCCAATCTTT	ATCAGGCTTCTTGGGCATAAA
Fusarium graminearum	*reductase*	TGACAGCTTTGGTTGTGTTTG	CGGAAGACTGCTGAGTAACGCCAA	CTTGGCTGGAATGAGTCTGT
Trichoderma harzianum	*calmodulin*	TTCGGACGVTTTGAGTGCT	CGTACCATCACCGTCCTTGTCCTG	CGGYCGGMAGCAAGTCATTCG
Trichoderma harzianum	*endochitinase*	CGATCTCAGCTGGATGCTTAT	AATGGTGAGGAGGAAGTGGTAGCC	GCAGCTTGGAGTAGTTATCCTT
Trichoderma harzianum	*RNA polymerase II*	ATTGGATGGGAGGGATTGATTAG	CATGATCTGCATGACGCCAGAGGA	GCCTTCTGAAGACGATACAGC
Triticum aestivum	*actin*	CCAAGGCCAACAGAGAGAAA	TGCCCAGCAATGTATGTCGCAATC	GCTGGCATACAAGGACAGAA
Triticum aestivum	*phenylalanine ammonia-lyase (PAL)*	GTGTTCTGCGAGGTGATGAA	AAGCACCACCCTGGACAGATTGAA	GTATGAGCTTCCCTCCAAGATG

increase humidity and ensure disease establishment. The number of diseased florets was scored on day 7 and then every three days until 21-day postinoculation. The percentage of disease was determined as the number of florets displaying early whitening and necrosis divided by the total number of florets on the head. After 21 days, average yield loss was estimated by subtracting the excised head weight of the *F. graminearum* diseased heads from the average weight of the mock-inoculated controls. Three heads from each treatment were then combined as one sample (biological replicate), lyophilized, ground and analysed for estimation of fungal biomass and DON contamination. DON was extracted from approximately 1 g of tissue using 86:14 acetonitrile: water and quantified using GCMS following previously reported methods (Vaughan *et al*., 2020).

Additionally, a subset of 3 mock-inoculated heads were excised at 1, 7, 14, and 21 days post pretreatment with *Th*, or *Th* + *TRI5* and volatiles were collected on a Porapak^{TM} filter for 24 hours using a closed-loop stripping method (Tholl *et al*., 2006). The presence of TD was evaluated by GCMS analysis and confirmed using purified a standard of TD.

Estimation of fungal biomass

To determine the amount of fungal biomass in diseased wheat heads, genomic DNA was extracted from the lyophilized and pulverized tissue using the ZR Fungal/Bacterial DNA Miniprep Kit (Zymo Research, Boston, MD, USA). DNA quantity and quality were evaluated using a spectrophotometer (NanoDrop 2000; Thermofisher Scientific, Waltham, MA, USA). If the samples' 260/230 ratios were below 1.6, the DNA was further purified using the Genomic DNA Clean and Concentrate Kit (Zymo Research). The biomass of *F. graminearum*, *Th*, or *Th* + *TRI5* within the plant tissue was estimated as the relative quantity of fungal DNA to wheat DNA, using three sets of species-specific primers (Table 1).

Samples and assays were distributed across an integrated fluidic circuit and mixed in pairwise fashion using the Juno instrument (Fluidigm, San Francisco, CA, USA). Technical triplicates were run for each sample-assay pair. Template DNA concentration was standardized to 40 ng DNA μl^{-1}. The PCR components followed protocol PN 100-7222 C1 (Fluidigm), including the use of SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA). All qPCR assays (Table 1) were designed during this work, and all probes were labelled with 5' 6-FAM and were double quenched, using internal ZEN and 3' Iowa Black fluorescent quenchers (Integrated DNA Technologies). The specificity of the assays was validated by testing the primers and probes on different samples which included the individual species alone and in

combination with one another (Fig. S2). Thermocycling and fluorescence detection were performed with the Biomark HD (Fluidigm); thermocycling conditions were as defined by protocol PN 100-7222 C1 (Fluidigm).

The threshold cycle (Ct) values were used as calculated by the default parameters of the Fluidigm software. Per sample-assay pair, the mean Ct was calculated. The geometric mean was calculated across assays for each organism (i.e. 3 assays for *F. graminearum*, 3 assays for *T. harzianum*, 2 assays for *T. aestivum*). The biomass of each fungus within the plant tissue was estimated as a ratio of fungal DNA relative to wheat DNA, as: biomass = $2^{\Delta Ct}$, where Ct was the geometric mean across assays for each organism.

Effect of Th + TRI5 *volatiles on wheat seedlings*

To evaluate the potential of interspecies signalling, wheat seedlings of Alsen$^{(MR)}$ and Norm$^{(S)}$ were exposed to *Th*, or *Th* + *TRI5* volatiles. Two wheat seedlings were allowed to grow on water agar in square 22 cm x 22 cm assay plates in proximity but not in contact with two V8 juice agar plates inoculated with sterile water (control), *Th*, or *Th* + *TRI5* as shown in Fig. 4A. Seeds were surface sterilized and then germinated for 48 h in water prior to placement in the assay plates for volatile exposure. The seedlings were grown in the assay plates for one week during which they were continuously exposed to the volatiles released from the different treatments (control, *Th*, or *Th* + *TRI5*). For each treatment and cultivar, four large assay plates were set up and designated as independent biological replicates. On day 7, the seedling shoots from a single plate were collected together, lyophilized, pulverized and the combined tissue was used for expression analysis of the defence-related gene *PR1* via qPCR.

Effect of Th + TRI5 *application on wheat seedlings*

Seeds of Norm$^{(S)}$ and Alsen$^{(MR)}$ were germinated (48 h) in either sterile water (control), or a 1x10^5 conidia/mL suspension of *Th* or *Th* + *Tri5*. A single treated seedling was planted in each 'cone-tainer'™ (Stuewe and Sons Inc., Tangent, OR, USA) containing clay pellets which allow for easy separation of root tissue from the substrate (Vaughan *et al*., 2011; Vaughan *et al*., 2015). For each treatment and wheat cultivar, four biological replicates were prepared. The seedlings were placed in a climate-controlled growth chamber programmed to 23°C day/20°C night, 500 μmol m^{-2} s^{-2} photosynthetic photon flux density, 14 h photoperiod and 50–60% relative humidity. The clay pellets were watered daily. After 2 weeks of growth the shoots and roots of each plant were separated. The individual tissues were lyophilized, pulverized and separated for analysis of phytohormones and relative expression of plant defence genes.

Quantification of phytohormones

Phytohormones were analysed via chemical ionization gas chromatography/mass spectrometry (CI-GC/MS) profiling (Schmelz *et al*., 2004). Jasmonic acid (JA) and salicylic acid (SA) were extracted from approximately 100 mg lyophilized ground tissue with 1-propanol and methylene chloride, (Sigma-Aldrich, St. Louis, MO, USA), derivatized with trimethylsilyldiazomethane in hexanes (Sigma-Aldrich) for 30 min, and then collected by vapour-phase extraction (Schmelz *et al*., 2004). The CI-GC/MS analysis was performed on a 7890B GC coupled to a 5977A MS (Agilent Technologies, Germantown, MD, USA) run in CI mode with isobutane as the ionization gas. Compounds were separated on an Agilent Technologies DB-35MS column (30 m, 0.25 mm, 0.25 μm) held at 70°C for 1 min after injection, followed by a programmed temperature gradient of 15°C/min to 300°C where it was held for 7 min. Helium was used as the carrier gas with a 0.7 ml min^{-1} flow. Identification and quantification of JA and SA were based on a deuterated internal standard (CDN Isotopes, Pointe-Claire, Quebec, Canada) spiked into each sample prior to extraction. Quantity estimates of total JA (trans + cis) and SA were based on corresponding deuterated internal standards (Vaughan *et al*., 2014).

Fumigation of F. graminearum *cultures*

Two agmatine agar plates inoculated with *F. graminearum* (Gz3639) and two V8 juice agar plates inoculated with sterile water (control), *Th*, or *Th* + *TRI5* were placed into larger, square 22 x 22 cm assay plates (Fig. 5A). The fungi did not come into physical contact with one another but were exposed to the volatiles emitted into the headspace of the assay plate. For each of two experimental replicates performed, three assay plates (biological replicates) were set up per treatment. The assay plates were covered and kept in the dark at ambient laboratory conditions. Every other day throughout the first week, radial growth measurements were recorded from the centre of the inoculum plug to the extreme edge of the *F. graminearum* fungal mycelia development following two perpendicular lines on each plate. The average of the two radial measurements from each media plate within a single assay plate was used as a biological replicate resulting in a total of three biological replicates per treatment. Following 14 days of growth and volatile exposure, the Gz3639 mycelia were scraped from the surface of the agar plate and used for relative *TRI* gene expression analyses. DON was extracted from

the agar media using 86:14 acetonitrile: water and quantified using GCMS following previously reported methods (Vaughan *et al*., 2020).

Transcriptional analyses

RNA was isolated from approximately 60–80 mg lyophilized, ground tissue using a combination of the Trizol reagent (Life Technologies, Carlsbad, CA, USA) and the RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) extraction methods following standard manufacturer protocols with an additional DNase step using the RNase Free DNase Kit (Qiagen, Germantown, MD). First-strand cDNA was generated using a Superscript II Kit (Invitrogen, Carlsbad, CA, USA). The efficiency of each primer pair was estimated prior to experiments using a mixture of cDNA from the samples. qPCR was prepared in a total of 20 μl volume with 10 μl of Bio-Rad SybrGreen Supermix (Bio-Rad Laboratories), 300 nM of each primer and 1 μl of cDNA. All sample reactions were performed in triplicate. PCR was performed on a Bio-Rad CFX96 RealTime System (Bio-Rad Laboratories). The thermocycling programme consisted of an initial denaturation at 98°C for 2 min, 40 cycles each of 98°C denaturation for 15 s and 60°C annealing/elongation for 1 min and a final dissociation curve from 65 to 95°C.

Gene-specific oligonucleotides and their PCR efficiencies are listed in Table 2. The C_t values of *PR1* and *PAL* were normalized to the wheat housekeeping gene GAPDH and the Ct values of *TRI* genes were normalized to the *Fusarium* endogenous control β-tubulin (β-tub) encoding gene. The amount of each gene transcript was calculated relative to its corresponding average for control samples in each experiment. Transcript fold-changes were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008) using CFX Manager software, version 3.0 (Bio-Rad).

TD fumigation of detached wheat heads inoculated with F. graminearum

FHB susceptible hard red spring wheat cultivar Norm$^{(S)}$ was grown in a sunlit greenhouse supplemented with high-pressure sodium lights to maintain a 14 h day cycle. Greenhouse temperatures ranged from 25 to 28°C during the day and 17-20°C at night. At anthesis the inflorescences (heads) were excised with approximately 10 cm of stem. A single central floret was inoculated on each head by injecting 10 μl suspension of 10^5 *F. graminearum* conidia per ml. Three 15-acetyldeoxynivalenol (15-ADON) producing *F. graminearum* strains, Gz3639, 06-219 and 06-255 belonging to the North American 1 population were independently used as inoculum (Kelly and Ward, 2018). Three heads inoculated with the same strain were placed into a small beaker of water and then sealed in a 4 l (4000 cm^3) glass jar which contained a 5 mm diameter vent tube in the cap (Fig. 6A). This vent tube allowed for some gas exchange and dissipation of volatiles from the jar. Therefore, fumigation treatments were applied twice, on the day of inoculation (day 1) and again on day 7. Fumigation was performed by adding 50 μl of purified TD at a concentration of 100 μg μl^{-1} dissolved in acetone (100%). This resulted in a 1.25 μg cm^{-3} TD fumigation treatment that likely gradually declined with time. TD was isolated from yeast extract-peptone-dextrose liquid (YEPD) cultures of *F. sporotrichioides* Tri4- mutant strain F15 and purified as previously reported (Hohn *et al*., 1995). Three biological replicates represented by three individual jars each containing three inoculated heads were set up per volatile exposure treatment. Ten days following *F. graminearum* inoculations, the stems were removed, and the wheat heads were lyophilized, pulverized, and the tissue was separated for DON quantification as previously described (Vaughan *et al*., 2020).

Table 2. qPCR assays used to evaluate gene expression.

Organism	Target gene	Forward primer	Reverse primer	PCR efficiency
Triticum aestivum	*pathogenesis-related 1 (PR1)*	CGTCTTCATCACCTGCAACTA	CAAACATAAACACACGCACGTA	103%
Triticum aestivum	*phenylalanine ammonia-lyase (PAL)*	TTGATGAAGCCGAAGCAGGACC	ATGGGGGTGCCTTGGAAGTTGC	108%
Triticum aestivum	*GAPDH*	TTGCTCTGAACGACCATTTC	GACACCATCCACATTTATTCTTC	101%
Fusarium graminearum	*TRI6*	TAACCACATCGTCGGGACTG	GCCGACTTCTTGCAGGTCTT	101%
Fusarium graminearum	*TRI5*	TCTATGGCCCAAGGACCTGT	ACGCTCATCGTCGAATTCCT	105%
Fusarium graminearum	*TRI4*	CGCGAGCTTACGACATTGAG	GAACTTCGCCAAGGTGTTCC	95%
Fusarium graminearum	*β-tubulin (β-tub)*	TCCAGGGTTTCCAAATCACC	GGAACGACGGAGAAAGTTGC	95%

Statistical analyses

All analyses were conducted using statistical software JMP (15.0.0). Differences between means were determined using t-test and analysis of variance (ANOVA) followed by Tukey–Kramer honestly significant difference (HSD). Data presented as percentages were arc sign transformed and biomass ratios were square root transformed prior to analyses. Details of individual analyses and resulting *P* values are described and reported within the results and figure legends.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Alsen$^{(MR)}$ or Norm$^{(S)}$ seeds were soaked in water or a solution of *Trichoderma harzianum* (*Th*), or *T. harzianum* overexpressing *TRI5* (*Th* + *TRI5*) for 48 h and

then planted in clay pellets. The shoots and roots of the two-week-old seedlings were collected separately and the relative gene expression of *pathogenesis-related 1* (*PR1,* A) and *phenylalanine ammonia-lyase* (*PAL*, B) were compared between treatments. Statistical comparisons were conducted independently for the different tissue types and cultivars. Different letters above bars indicate significant differences (ANOVA followed by Tukey–Kramer HSD; $n = 4$)

Fig. S2. Heat map of Ct values generated by Fluidigm software depicting species specificity of qPCR assays listed in Table 1. The left-hand panel shows the three different species evaluated. Each assay was tested in triplicate. The top panel lists the different samples and which species DNA were predicted to be in each based on sample preparation. *T. aestivum* assays amplified samples from both Alsen and Norm varieties. *T. harzianum* assays similarly amplified both *Th* and *Th* + *TRI5* strains. The 12 samples on the right side of the map which contain only *T. harzianum* or *F. graminearum* DNA, depict a dilution serious of the DNA isolated from axenic culture of the fungal species. Colour key for Ct values is depicted in the right panel. Black designates (>35) no detectible amplification. No detectible amplification was shown for all assays which did not contain the intended species of the assay