THE INTERACTION OF FUSARIUM GRAMINEARUM AND FUSARIUM POAE INOCULATION IN BARLEY (HORDEUM VULGARE L.)

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ABSTRACT

INTERACTION OF FUSARIUM GRAMINEARUM AND FUSARIUM POAE IN BARLEY (HORDEUM VULGARE L.)

Fusarium head blight (FHB) is a horribly destructive fungal diseases of barley. FHB is caused by a species complex of *Fusaria*, of which *Fusarium graminearum* Schwabe is the main causal species of FHB epidemics in Canada. Field surveys show that two or more *Fusarium* species often co-exist within the same field or grain sample and *F. poae* is reported as another important species in barley in Ontario and Quebec. The aim of this study was to observe the pathogenicity of *F. graminearum*, *F. poae* and a co-inoculation of both species causing FHB in barley. Two susceptible barley cultivars were spray-inoculated at 10 to 14 days after heading. Phenotypic disease severity was rated on a scale of 0-9 at 4, 7, 14, 21, and 28 days after inoculation. There was a significant difference in FHB severity between *F. graminearum* and *F. poae*, where infection with *F. graminearum* produced more severe disease ratings. *F. poae* generated lower disease ratings and was not statistically different from the control. When heads were co-inoculated with both *Fusarium* species, the resulting FHB severity was unchanged 54relative to heads inoculated with *F. graminearum* only. The ratio of *F. graminearum* to *F. poae* genomic DNA was also no different than when heads were inoculated with *F. graminearum* alone, as quantified with ddPCR using markers specific to each species. Metabolomic analysis of sample extracts corroborated our other findings where *F. graminearum* appeared to outcompete *F. poae* in barley also at the mycotoxin level. Barley cultivar did not have a significant effect on visual FHB disease ratings and fungal DNA detection, however there were some chemical differences between cultivars in response to challenge by both *F. graminearum* and *F. poae*.

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LIST OF ABBREVIATIONS

FHB……...….Fusarium Head Blight

Fg…….……*Fusarium graminearum*

Fp……...……*Fusarium poae*

Fg+Fp……..*Fusarium graminearum* and *F. poae*

m/z…….mass-to-charge ratio

ORDC……...……..Ottawa Research and Development Centre

DAOMC……...…Department of Agriculture, Ottawa, Mycology

CCFC…….….Canadian Collection of Fungal Cultures

UPLC-HRMS....Ultra-Performance Liquid Chromatography-High Resolution Mass Spectrometry

DON……..deoxynivalenol

15A-DON……...……..15-acetyl-deoxynivalenol

INTRODUCTION

Fusarium Head Blight (FHB) is a devastating fungal disease that has been infecting cereal crops, like barley (*Hordeum vulgare* L.), for decades in Canada and worldwide. The main causal species of FHB is *Fusarium graminearum*. There is some variation among host species, but the disease is generally characterized by discoloured, shriveled, and/or aborted kernels. Of greater concern, however, is the accumulation of mycotoxins, like deoxynivalenol (DON), also known as vomitoxin, in infected grain and plant tissues (Clear & Abramson, 1986). When ingested in amounts above regulated toxicity levels, *Fusarium*-derived mycotoxins elicit many negative health effects. These effects include reduced weight gain and fertility in livestock, and vomiting in all consumers. With respect to malting barley, *Fusarium* infection reduces kernel plumpness and germination (up to 40% ungerminated), and degrades β-amylase, the enzyme responsible for cleaving maltose, the most important fermentable sugar in the beer brewing process (Fox, 2018; Schwarz et al., 2001; Yang et al., 2010). High concentrations of DON in grain are not marketable and require additional conditioning and processing for sale and safe consumption. FHB affects both grain quantity and quality, affecting growers' yields, profit and ultimately their livelihoods. Some of the most severe and recorded epidemics in North America were during the 1990's (Tekauz, 2018; Windels, 2000). Throughout this period, estimated economic losses to the agriculture and food industry attained nearly $2B USD, with yields reported at nearly 50% less than expected (Windels, 2000).

'The Disease Triangle' may be used to describe disease as the product of interactions between three key players: pathogen, host, and environment (Scholthof, 2007; Tekauz, 2018). To reduce the risk of infection, growers may implement strategies to limit each player's effect on producing disease. For instance, diversification of crop rotation interrupts FHB disease cycle and

reduces inoculum load in the soil (Dill-Macky & Jones, 2000). Sunflower residue, birdsfoot trefoil, and white clover do not support ascospore production and incorporating these species in the grower's crop rotation may help control FHB (Pereyra & Dill-Macky, 2008). Tillage impacts FHB by physically burying or exposing the inoculum: incorporation of infected crop residue reduces FHB but the increasing adoption of conservation tillage practices among growers contributes to the increased incidence of FHB (Dill-Macky & Jones, 2000; Windels, 2000). Growers may use fungicides to suppress fungal growth, and DON accumulation but these are not 100% effective at preventing and/or interrupting fungal growth entirely (Yoshida et al., 2008). Fungicide effectiveness is also dependent on the correct rate, placement, and most importantly, timing of the application. Growers can also choose to grow cultivars that have moderate resistance to FHB to avoid *Fusarium* damage and mycotoxin accumulation. No lone strategy is the silver bullet to FHB resistance, and an integrated management approach of combining all of the above is most effective.

Cereal crops like barley are most susceptible to infection when generous precipitation and periods of warm and humid weather coincide with anthesis to the soft dough stage of development, and especially so in the evening when spore release is highest (Schmale et al., 2006). In barley, flowering is a fairly inconspicuous event compared to other cereal crops like wheat and oats. There is some variation by cultivar but anthesis often occurs while the head is still enveloped by the flag leaf sheath, perhaps with the awns extruded, creating a clear shield from its external environment. This shield may also be concentrating disease by creating a warm and humid microclimate favourable to fungal growth. Inside the sheath is also where anthers emerge from the glume, physically opening the grain spike for *Fusarium* spores to enter. When

environmental conditions favourable to FHB infection coincide with anthesis, the host and *Fusarium* spp. may be in optimal position to produce disease.

FHB-causing species of *Fusarium* have a very broad range of hosts, mainly graminaceous plant species. Approximately 49% of world crop production area is susceptible to FHB infection; six of the top ten primary crop species are susceptible to this pathogen (Food and Agriculture Organization of the United Nations (FAO), 2018). One single species of *Fusarium* may infect multiple host species, and multiple *Fusarium* species may infect one host at once. For example, *F. graminearum* can cause crown rot in soybean, ear rot in corn, and FHB in wheat, barley, and other small grain cereals, and over 30 different species of *Fusarium* have been detected in symptomatic, *Fusarium*-damaged barley (Beccari et al., 2017, 2018; Bourdages et al., 2006; Castañares et al., 2016; Garmendia et al., 2018; Gonzalez Pereyra et al., 2011; Linkmeyer et al., 2016; Soledad Nogueira et al., 2018; A. Xue et al., 2013; A. G. Xue et al., 2019). Conversely, there are many species of *Fusarium* that are not pathogenic in certain hosts, like *F. verticillioides* in corn or *F. graminearum* in grassy weeds (Lofgren et al., 2018; Picot et al., 2012). The *Fusarium* genus itself is highly diverse and each species has nuanced behaviour in various hosts (Chandra et al., 2011).

Fusarium sp. spores are wind- and rain splash-dispersed from infected crop residue and the soil. In still conditions, spore gradients significantly decrease within 50m from the inoculum source, but spores may be carried far beyond this distance by stronger winds and microbursts, especially in cereal intensive production zones (Francl, 1997; Luna et al., 2002).

F. graminearum is well-documented as the dominant causal species of FHB epidemics in Canada. This may be attributed to several factors, including the array of virulence factors it produces to disrupt plant cell structure and metabolism, suppress host immune response, interfere

with pathogen recognition, and ultimately establish infection. However, in recent years, attention has been diverting to *Fusarium poae*, a generally less aggressive pathogen. Multiple surveys have detected *F. poae* in both *Fusarium*-damaged and asymptomatic grain (Beccari et al., 2017, 2018; A. G. Xue et al., 2019). This is of concern mainly because *F. poae* produces different mycotoxins, some of which are more toxic than those produced by *F. graminearum*, but also many of these are not yet regularly monitored in our feed and food systems (Gruber-Dorninger et al., 2017; A. G. Xue et al., 2019). Some current fungicide controls for *F. graminearum* are also less effective when *F. poae* is also present (Tan et al., 2021). A specific survey of putatively infected grain conducted by Xue et al. (2019) in Ontario reported that *F. graminearum* was most detected in wheat, *F. poae* was most detected in oats, but in barley, *F. graminearum* and *F. poae* were both detected equally as often. They also saw that in years where environmental conditions were favourable to disease (i.e. warm and humid), *F. graminearum* detection nearly doubled. This difference in species detection led to the question: do *F. graminearum* and *F. poae* interact in barley?

Most published studies of *F. graminearum* and *F. poae* in cereals are conducted in wheat. They observe *Fusarium* sp. infiltrate the wheat head and spread from spikelet to spikelet via vascular tissue in the rachis, thus choking out any spikelets above the point of infection. The pattern of infection in barley is different where hyphae extend and climb across the surface of the florets, and infiltrate by the paleal margins (Imboden et al., 2018). *Fusarium* spp. mycotoxin production also heavily influences fungal spread and FHB development, but their role varies with host species (Langevin et al., 2004). Many studies are also conducted in the field; these yield very complex results that are very challenging to analyze since there are many uncontrolled variables whose effects are not yet understood. While results from indoor controlled environments may not

be applicable or as easily transferrable in the field, they help increase our understanding of interspecific interactions. Therefore, our goal with this research was to compare visual FHB disease, *Fusarium* sp. biomass, and mycotoxin profiles of barley when infected with *F. graminearum*, *F. poae* or both simultaneously. We hypothesized that a competitive interaction exists, and that the presence of *F. poae* with *F. graminearum* may reduce FHB in barley.

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CHAPTER I:

Visual development of *Fusarium graminearum* and *Fusarium poae* causing Fusarium Head Blight in barley

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ABSTRACT

Fusarium head blight (FHB) is one of the most devastating diseases of barley. FHB is caused by a species complex of *Fusaria*, of which *Fusarium graminearum* Schwabe is the species responsible for most FHB epidemics in Canada. Field surveys show that two or more *Fusarium* species often co-exist within the same field or grain sample and *F. poae* is reported as another dominant species in barley in Ontario and Quebec. The aim of this study was to observe the pathogenicity of *F. graminearum*, *F. poae* and a co-inoculation of both species causing FHB in barley. Two susceptible barley genotypes were spray-inoculated at 10 to 14 days after heading. Phenotypic disease severity was rated on a scale of 0-9 at 4, 7, 14, 21, and 28 days after inoculation. There was a significant difference in FHB severity between *F. graminearum* and *F. poae*, where *F. graminearum* produced more severe disease ratings. *F. poae* was less pathogenic and not statistically different from the control treatment (inoculated with deionized water only). When heads were co-inoculated with both *Fusarium* species, the resulting FHB severity was unchanged relative to heads inoculated with *F. graminearum* only. This suggests that the presence of *F. poae* affects neither *F. graminearum* nor its pathogenicity in barley.

1.0 INTRODUCTION

Fusarium head blight (FHB) is a devastating fungal disease that causes massive losses in grain yield and quality in cereals and grasses (Windels, 2000). It is mostly distinguished by their shrivelled and/or discoloured 'tombstones'. Infected kernels are particularly dangerous to livestock and human health because of mycotoxins accumulated in the grain. When consumed beyond safe thresholds, these mycotoxins have adverse gastrointestinal and reproductive effects on consumer health. Since its first recording in Canada in 1919, FHB has become a chronic issue with economic losses estimated at 520M CAD lost in the 1990s in Canada alone (Tekauz, 2018; Xia et al., 2020).

FHB is caused by a complex of species, of which the predominant causal species in Canada is *Fusarium graminearum* Schwabe (Bourdages et al., 2006; Xue et al., 2006). Results from a 2001-2017 survey in Ontario, Canada showed that *F. graminearum* was most detected in grain samples in epidemic years (Xue et al., 2019). However, in non-epidemic years, a weaker pathogen, *F. poae*, was most detected, especially in barley. Furthermore, relative host species differences were observed where *F. graminearum* was most dominant in wheat, *F. poae* was most dominant in oat, but in barley *F. gramineaum* and *F. poae* were equally dominant. Seasonal and host differences prompted a question: what is the relationship between *F. graminearum* and *F. poae* in barley? The objective of this study was to observe the individual and interactive effect of *F. graminearum* and *F. poae* on visual Fusarium head blight symptoms in barley under controlled conditions and in field. To our knowledge, most published *Fusarium* species interaction studies are performed *in vitro*, in wheat, or in the field, and so this report below is the first observing *F. graminearum* and *F. poae* in barley under controlled conditions in a growth cabinet. From our observations, there appears to be no interaction between *F.*

graminearum and *F. poae* and the presence of *F. poae* with *F. graminearum* does not change disease symptoms in barley.

2.0 MATERIALS AND METHODS

2.1.1 Growth cabinet - Plant material

Two susceptible spring barley genotypes, Stander (six-row) (Rasmusson et al., 1993) and CDC Bold (two-row) (CFIA Variety Registration #4951), were tested in growth cabinets at Agriculture and Agri-Food Canada's Ottawa Research and Development Centre (ORDC) in 2021. Seeds were germinated on soaked Whatman paper, and then five seeds per 7.5" pot were transferred to a growth cabinet (Model PGC20, Conviron, Winnipeg, MB, Canada) at 20:17°C with a photoperiod of 16 h light:8 h dark and 70% relative humidity (RH). At two weeks after planting, plants were fertilized once a week with 20-20-20 (N:P:K) until harvest.

2.1.2 Growth cabinet – Inoculum preparation

One isolate, DAOMC 180378, from *F. graminearum* and one isolate, DAOMC 252242, from *F. poae* were used in the experiment. Both isolates were collected from the Ottawa Valley ecozone and procured from the Canadian Collection of Fungal Cultures (Ottawa, ON, Canada). DAOMC 180378 has the 15-ADON chemotype, is commonly used at the ORDC and is used in the FHB field nursery when screening FHB resistance in breeding programs (Kebede et al., 2016).

The liquid inoculum (spore suspension) was prepared in the Plant Pathology laboratory at ORDC, Ottawa, ON as described in Xue et al. (2004). To prepare the culture plates, 0.5 mL of concentrated conidial spore suspension was spread onto modified potato dextrose agar (dextrose, 10 g/L) amended with 20 ppm streptomycin sulfate in 90 mm Petri dishes. Petri dishes were

incubated at 22-25°C under mixed UV and fluorescent lighting for 48 h to stimulate sporulation. Each dish then received 10mL of sterile distilled water with 0.01% Tween 20 (polyoxyethylene sorbitan monolaurate) and was scraped gently with a sterile microscope slide to dislodge spores. The resulting suspension was filtered through two layers of cheesecloth, the spore concentration determined using a hemocytometer, and adjusted to 3.8 x 10^3 CFUs/mL. Separate suspensions were prepared for each isolate, and the final concentration of the co-inoculation treatment contained a 1:1 mixture of both species (Xue et al., 2004).

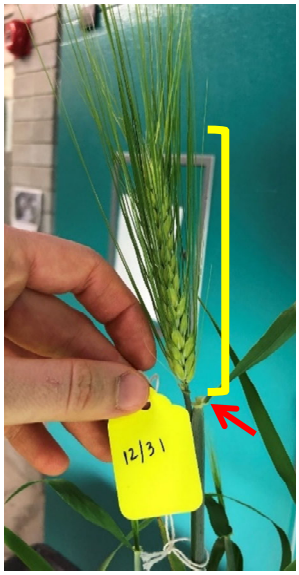


Figure 1.1: Visual explanation of a 'headed' spike. The base of the spike has cleared the flag leaf collar (red arrow)

2.1.3 Growth cabinet - Inoculation and visual disease rating

The two barley genotypes, Stander and CDC Bold, and four inoculation treatments (*F. graminearum* (*Fg*), *F. poae* (*Fp*), both species simultaneously (*Fg* + *Fp*), and a sterile water control were arranged in a randomized complete block design with four replications (n = 8 per treatment). Spikes were spray-inoculated at 10-14 days after heading (McCallum & Tekauz, 2001; Xue et al., 2006) (Figure 1.1). Spikes ready for inoculation had their awns trimmed, were sprayed with spore suspension (approximately 1x10^4 CFUs per spike) using a kitchen aerosol oil dispenser (Misto International LLC, Connecticut, USA), and each head was individually covered with its own plastic sample bag

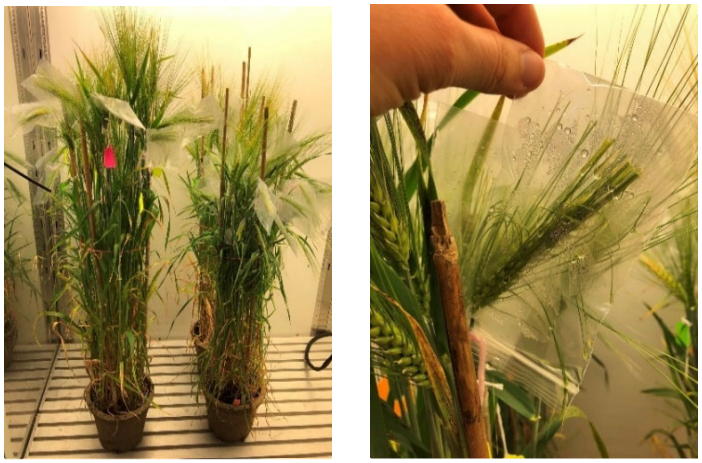


Figure 1.2: Photos from inside the growth cabinet showing the bagging technique over the barley heads

(Thermo Fisher Scientific, Waltham, MA, USA; Figure 1.2).

The inoculated pots were transferred to a new cabinet with 25:20°C, photoperiod of 16 h light : 8 h dark, and 90% RH. These small bags remained on the spikes to reduce cross-contamination between treatments, and at 4, 7, 14, 21, and 28 days post-inoculation, a visual disease rating was assigned to each spike on a scale of 0-9 (Xue et al., 2004). At 28 days post-inoculation, spikes were cut from the plant at the base of the head, individually wrapped in aluminum foil, flash-frozen in liquid nitrogen, and stored at -80°C (Thermo Fisher Scientific, Waltham, MA, USA) until ready for further molecular analysis.

2.2.1 Field experiment – Ottawa, ON

For one growing season in 2021 in two locations (Ottawa, ON and Harrington, PEI), the indoor growth cabinet study was applied outdoors. In Ottawa, the same two genotypes, Stander and CDC Bold, plus Chevron, a moderately resistant cultivar, were planted at 5 g of seed per row in the FHB nursery where one plot consisted of four 1.5 m rows with row spacing of 38 cm (Choo et al., 2015). At 10-14 days after heading spikes were spray-inoculated with approximately 1 L of spore suspension (5 x 10^4 spores/mL) using a hand-pumped 1-gallon lawn and garden sprayer with the filter removed (Chapin International, Batavia, NY, USA). Plots were irrigated for approximately 15 to 20 min once a day in the afternoon with irrigation sprinklers to create conditions favorable for infection by sprayed *Fusarium* inoculum. To minimize the cross-contamination between plots each treatment had its own sprayer, plants were sprayed when winds were non-existent or calm, later in the afternoon at least 1 hour after misting, and with the spray nozzle approximately 10cm away from the heads to limit drift between plots. The experiment was separated from other plots in the nursery by a spring-planted winter wheat border to create a physical barrier and medium height, FHB-susceptible buffer plots (cv. Island)

between test plots to estimate the level of inoculum already present in the environment (Figure 1.3). At 14 and 28 days post-inoculation, 10 heads at the centre of the plot were randomly selected and assigned a visual disease rating on the same scale as used in the growth cabinet experiments. At 28 days post inoculation (dpi), each entire plot was harvested (both test and buffer plots) and individually threshed. Harvested grain was stored in -20°C until further analysis, discussed later in Chapter 2 and 3.

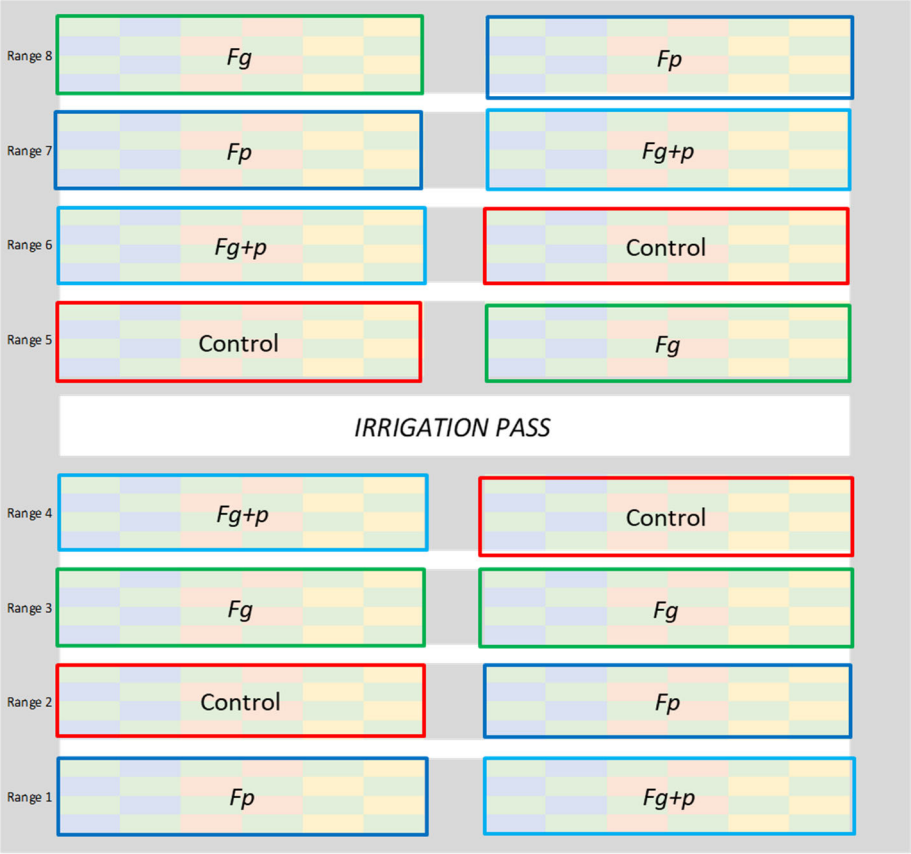


Figure 1.3: Schematic of field layout in FHB nursery at Ottawa Research and Development Centre. Light blue = Stander, light orange = CDC Bold, yellow = Chevron, green = Island (susceptible border/buffer), grey = winter wheat border; colour of bordering frames indicates which range of plots received which treatment. Only Stander, Bold and Chevron were spray-inoculated with a treatment, bordering Island plots were not treated.

2.2.2 Field experiment – Harrington, PEI, Canada

In Harrington, PEI, Canada, the trial was planted in a split-plot design with cultivar as the main-plots and inoculation source in sub sub-plots. Trial was planted May 25, 2021. Inoculum sources were *F. graminearum* (Isolate 20-35, collected in PEI; Johnstone et al. 2021), *F. poae* (Isolate 20-73, collected in PEI; Johnstone et al. 2021), both species, and non-inoculated control. Cultivars Chevron, CDC Bold and Stander were grown. Individual sub plots were a single 30cm row flanked on all sides by guard plots of AC Island. Rows were spaced at 15cm. Inoculum was applied as described in 2.2.1, but spore suspension was 5 x 10^4 spores/mL. Conidia inoculum was produced by inoculating liquid mung bean media (20 g/L boiled mung bean broth filtered with 15 g/L NaCl) with a plug from a 7-day old PDA culture of each species and incubated for an additional 7 days with gentle shaking at 50 rpm at room temperature. Irrigation is applied through a misting system that is activated for 1 min 24 times per day to generate constant leaf wetness. Inoculum was applied twice July 1, 2021, and July 19, 2021. FHB was rated at 0, 7 and 23 dpi for incidence and severity. Heads were harvested on August 25, 2021 and sent to Ottawa for further analysis. The data from the two locations were analyzed separately.

2.3 **In vitro** *germination of seeds to check for contaminants*

Seed for Stander was increased in the greenhouse in 2018 and seed for CDC Bold was increased in the field at the Morden Research and Development Centre in Manitoba. To check for any seed-borne pathogens and, more specifically, for *Fusarium* contaminants, we surface sterilized seed with bleach and germinated them on media *in vitro*.

Sixteen seeds of each genotype were placed in their own 15mL Falcon tube with 10 mL of sterilization solution (3 mL bleach, 7 mL sterile MQ H_2O bleach + a drop of 0.1% Tween). Falcon tubes were placed on the nutating mixer for 10 minutes. The sterilization solution was

decanted off, then seed was rinsed with 10mL of sterile MQH_2O and replaced on nutating mixer for another 5 minutes. The decant and rinse was repeated another 6 times for a total 7 rinses. Seeds were then placed on YM + C media (in 1 L of distilled MQH_2O, 10 g malt extract, 2 g yeast extract, 10 g glucose, 20 g agar, 18 g Instant Ocean, and 100 mg chloramphenicol), germinated at 20°C, and incubated for 5 days to culture any contaminants enclosed within the seed coat.

3.0 RESULTS

3.1 Effect of Fusarium sp. *on FHB symptoms in growth cabinet*

Two susceptible barley cultivars were inoculated with one of four possible *Fusarium* spp. spore suspensions. Two-way analysis of variance (ANOVA) was used to analyze effects of genotype and treatment on visual FHB rating (Table 1.1). At all timepoints, there was a significant treatment effect ($p < 0.0001$), while the genotype effect was significant ($p < 0.05$) in early stages of disease. In early disease progression, *Fp* alone was not significantly different from the control treatment, but the FHB severity was significant at 14 days post inoculation and beyond. FHB ratings from *Fp* alone were significantly lower than FHB ratings in *Fg* and co-inoculation (*Fg*+*Fp*) (Figure 1.4). The observed mean disease severity due to *Fg* appeared unchanged when co-inoculated with both species.

Table 1.1: Two-way ANOVA for genotype and treatment effects from growth cabinet once inoculated *with F. graminearum*, *F. poae*, or both (Note: *, **, ***: significant at $p < 0.05$, $p < 0.001$, $p < 0.0001$, respectively; df = degrees of freedom; MS = Mean Square)

		MS				
Source	**df[a]**	*4 dpi*	*7 dpi*	*14dpi*	*21dpi*	*28 dpi*
Genotype	1	2.65*	19.69**	4.43	3.45	1.09
Treatment	3	23.68***	96.92***	123.11***	116.82***	101.08***
Genotype x Treatment	3	1.02	6.46	0.97	1.14	2.97
Error	21	0.58	1.08**	2.25	2.47	2.78

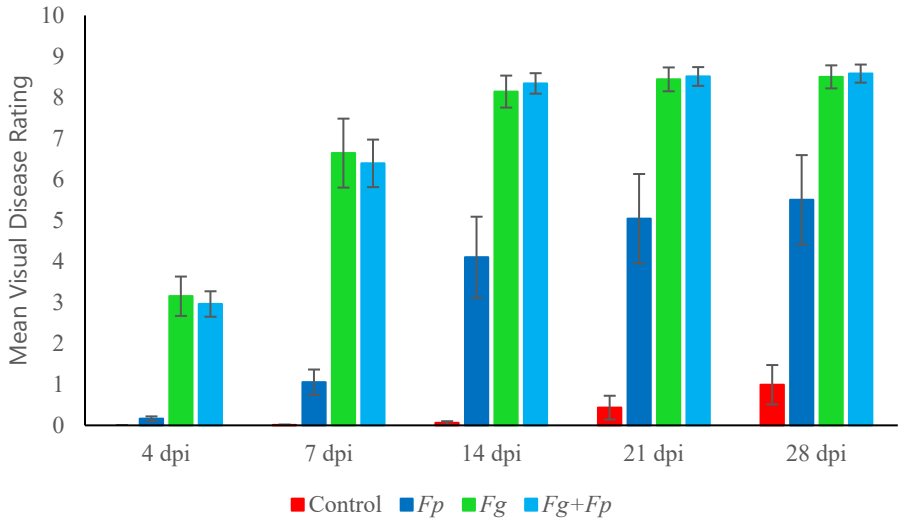


Figure 1.4: **Mean observed disease progression per treatment, using visual disease rating scale of 0-9 from Xue et al. 2004 (*n* = 8 per treatment)**. The scale was as follows: 0 = no symptoms, 9 = severely diseased, spike dead; 0, no visible symptoms; 1, one diseased spikelet; 2, two diseased spikelets; 3, three diseased spikelets; 4, >3 diseased spikelets but 1/4 spike area with symptoms (sas); 5, <1/3 sas; 6, <1/2 sas; 7, <2/3 sas, slight peduncle discolouration; 8, <3/4 sas, restricted peduncle discolouration; 9, >3/4 sas, extended peduncle discolouration, spike dead. Error bars represent the standard error.

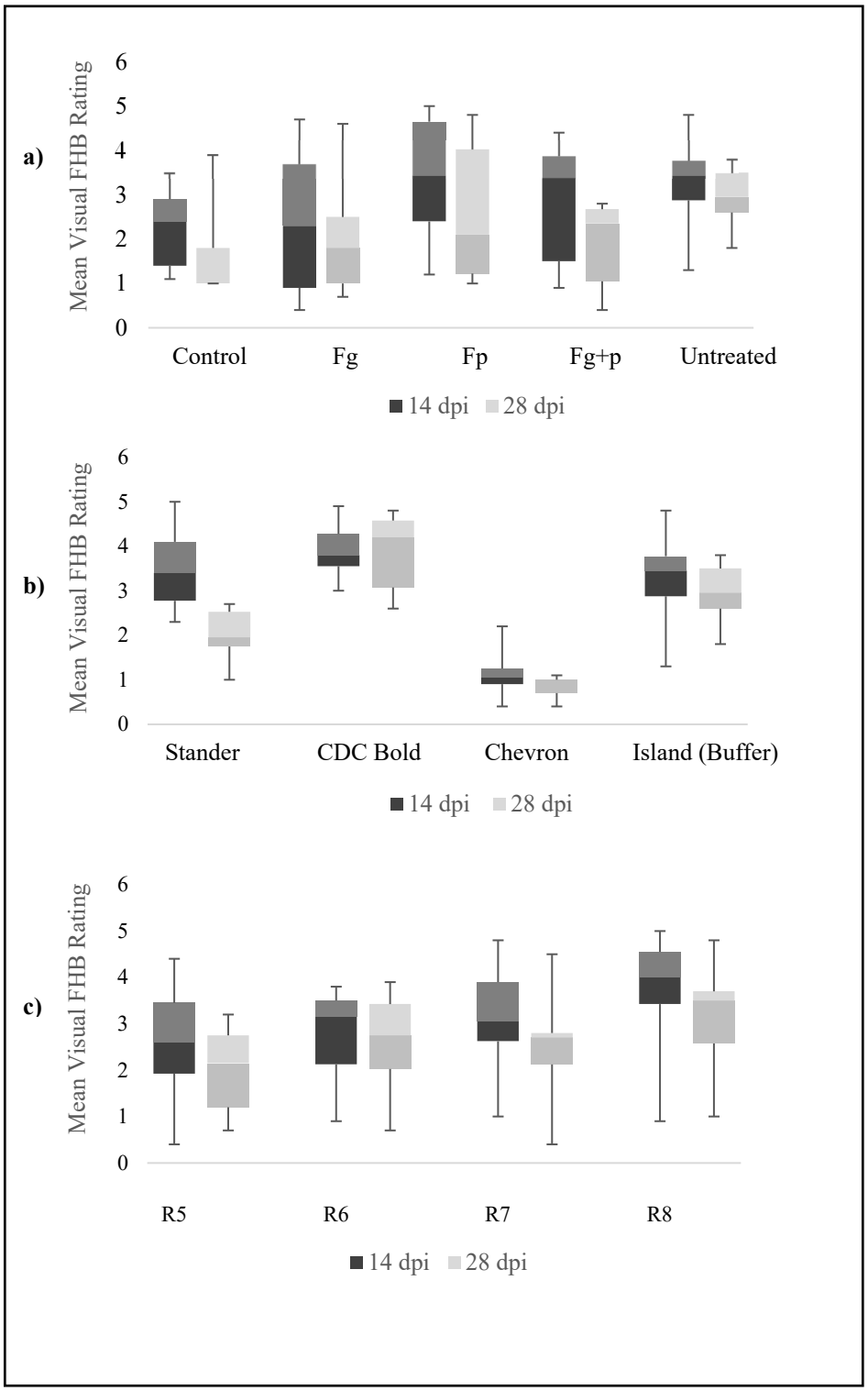
3.2 Effect of Fusarium sp. *on FHB symptoms in field*

In Ottawa, a multi-way ANOVA revealed significant genotype and range effect ($p < 0.05$) at both 14 and 28 days post inoculation and then significant treatment and interaction effect at 28dpi (Table 1.2). The significant genotype effect was likely driven by Chevron, the moderately resistant cultivar, who according to pairwise comparisons from Tukey's HSD test was the only significantly different cultivar at both 14 and 28 dpi.

Table 1.2: Multi-way ANOVA for genotype, treatment, and range effects from field experiments in Ottawa, ON

		p-value	
Source	***df***	***14 dpi***	***28 dpi***
Genotype	3	1.79×10^{-7}	4.14×10^{-4}
Treatment	3	0.2378	0.02667
Range	3	0.0409	0.00261
Genotype x Treatment	6	0.8435	0.01228

Figure 1.5: Mean visual FHB ratings by a) Treatment, b) Genotype, and c) Range from field experiment at Ottawa, ON. Error bars represent standard error.



Boxplots in Figures 1.5 illustrate these differences and demonstrate the level of variability in the data. Untreated buffer plots were not different from any treated plots, suggesting that the test plots were not well isolated from each other or from other environmental inoculum sources (i.e. soil borne and neighbouring FHB experiments in the nursery). These boxplots also show that there was a general decrease in mean FHB rating from 14 dpi to 28 dpi, which was quite unexpected given disease symptoms are assumed to worsen and not improve with time. As for the range effect, it was also significant according to ANOVA results, where Range 8 appeared to be driving most of this effect according to Tukey's test at 28 dpi. There was a gentle slope from range 5 towards range 8, and so differences in ranges may be partly due to this. It is important to note that ranges 1 to 4 were eliminated from the analysis because of the number of missing values due to uneven emergence.

In Harrington, PEI, the analysis of variance showed significant genotype and treatment effects on FHB incidence and severity at the beginning of the trial but became insignificant at 23 dpi (Table 1.3). Pairwise comparisons revealed significant differences between the resistant variety, Chevron, versus the susceptible others, for both FHB incidence and severity. Boxplots in Figure 1.6 show a general increase in disease with time; however, data is highly variable.

Table 1.3: Two-way ANOVA for genotype and treatment effects on FHB a) incidence and b) severity from field experiment in Harrington, PEI

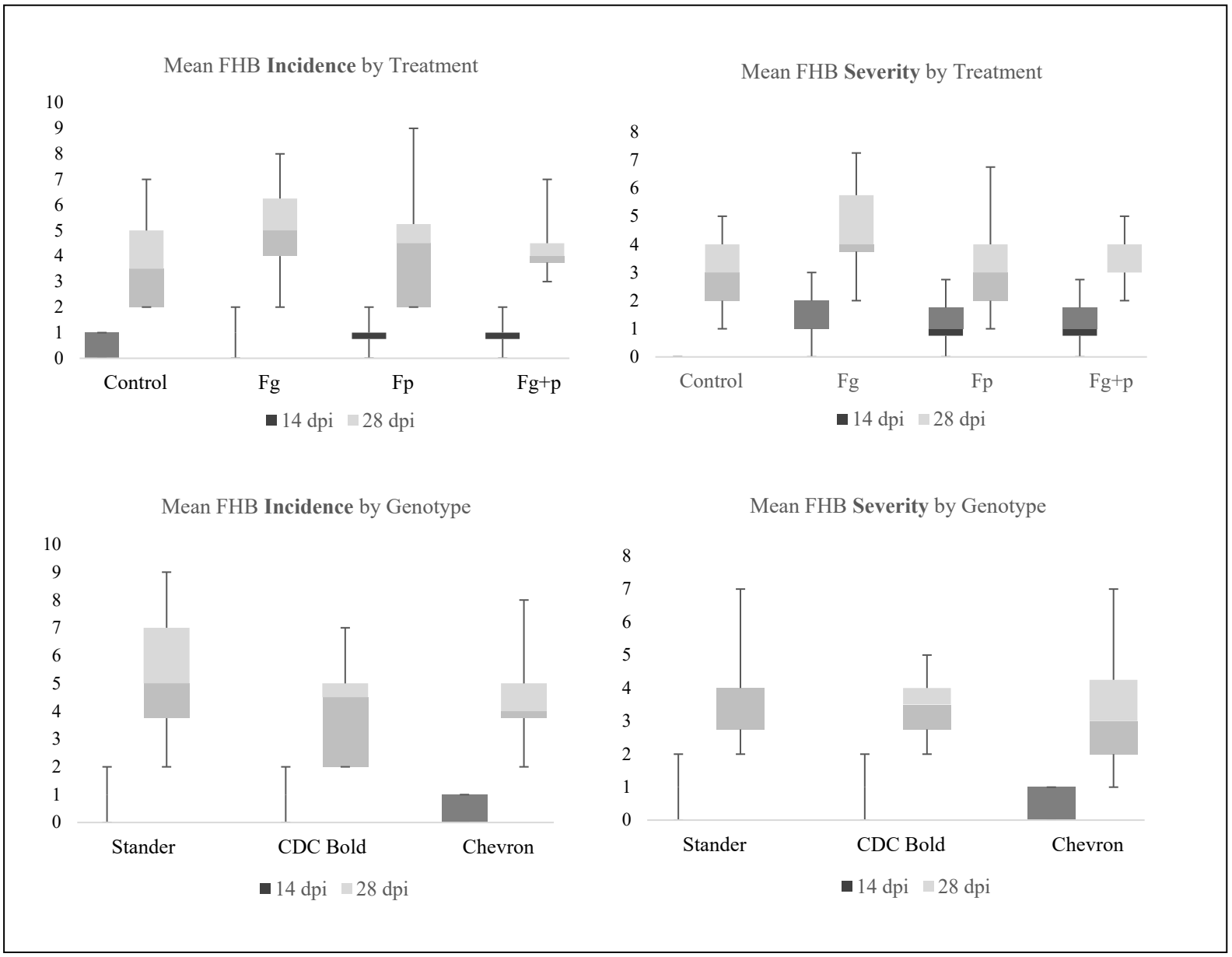
a)

		p-value		
Source	*df*	*7 dpi*	*14 dpi*	*28 dpi*
Genotype	2	0.0047	0.0047	0.2100
Treatment	3	0.0316	0.0316	0.3590

b)

		p-value		
Source	*df*	*7 dpi*	*14 dpi*	*28 dpi*
Genotype	2.0000	0.0062	0.0028	0.2100
Treatment	3.0000	0.0266	0.0289	0.3590

Figure 1.6: Mean visual FHB incidence and severity by Treatment and Genotype from field experiment at Harrington, PEI. Error bars represent standard error.



3.3 *Identification of seed contaminants*

Morphology observations and sequencing results identified mainly common cereal pathogens like *Bipolaris sorokiniana*, *Arthrinium* species and *Sarocladium* species. No species of *Fusarium* were identified in the seed stock for this assay (Figure 1.7 and 1.8). We later read a study by Xi et al. (2008) on vertical disease transmission showing that no head blight symptoms were a result of seed-borne *Fusarium* sp. in their growth cabinet study. By that rationale, had any *Fusarium* been identified, its effect would not have a large influence on visual disease ratings. However, the effect of the species of fungi identified here on visual FHB symptoms is unclear.

a)

b)

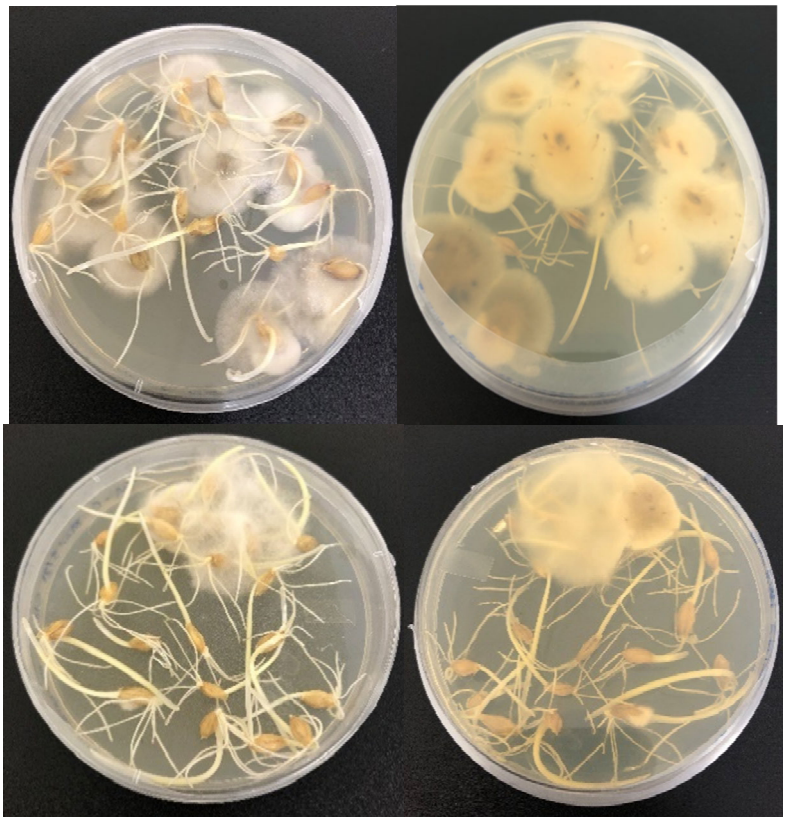


Figure 1.7: Top and bottom view photos of germinated surface-sterilized seed in 90 mm Petri dishes: a) CDC Bold, b) Stander

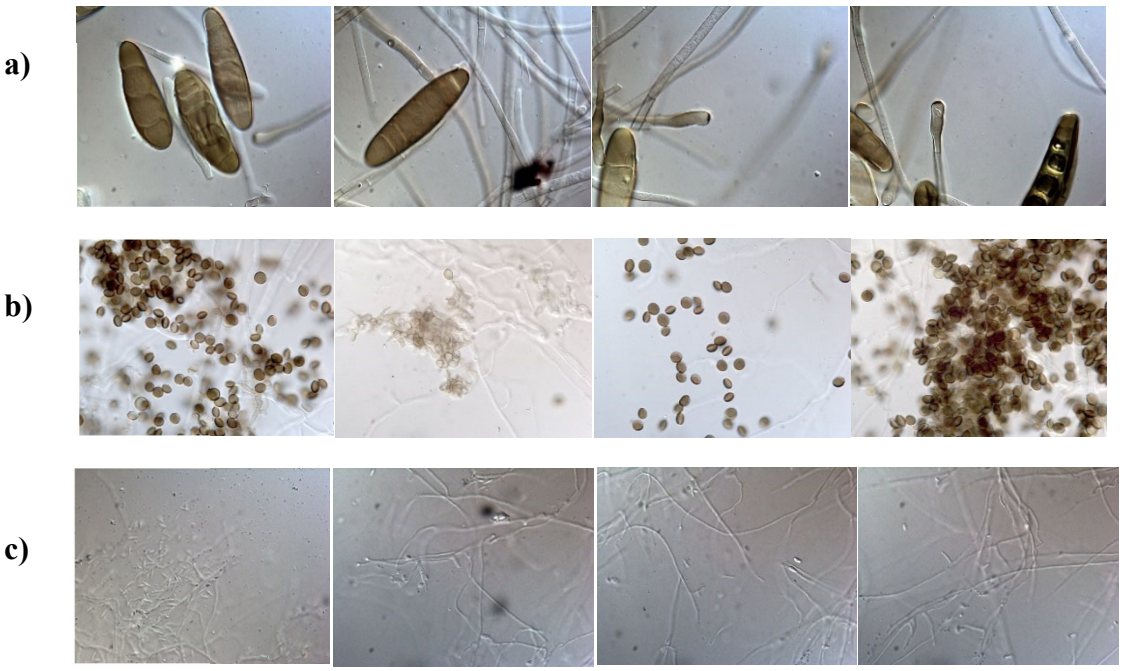


Figure 1.8: Photos of seed stock contaminants under microscope at 100x (Jonathan Mack)
a) *Bipolaris sorkiniana*, pale colony with grey and/or pink undersides on original plates, found in both Stander and CDC Bold; b) *Arthrinium* sp., found in Stander; c) *Sarocladium* sp., like *Acremonium* sp., slow white colony but conidia are in dry chain and not water droplet as typical of most species, found only in one seed of Stander

4.0 DISCUSSION

In most published pathogen interaction studies, 'competition is the rule and not the exception,' though from these phenotypic results, it seems like this competition was skewed (Xu et al., 2007). Although plants were grown at the optimal temperature for *Fp* (25°C), rather than at the optimal 28°C for *Fg*, *Fg* has been and remains to be the causal organism of FHB epidemics in cereal crops across Canada (Nazari et al., 2018). In fact, its range appears to be expanding as it is also becoming the dominant cause of epidemics in cooler regions of Europe (Osborne & Stein, 2007; Xue et al., 2019). There has been much intense investigation into what makes *Fg* such a force, but there is yet to be one straightforward reason. The trend among most searches is to examine the genetic regulation of *Fg*'s attack (and subsequent defence by the host,

to better develop resistant cultivars) as well as differences in mycotoxin production and accumulation – more on this is to come, especially in Chapter 3. For now, phenotypic effects of disease are the focus of Chapter 1.

There are many reports of *Fg*'s world domination over *Fp* and its culprit to epidemics beyond North America. It therefore came as no surprise when we saw *Fg*-infected heads had the highest visual FHB ratings and *Fp* had the lowest in the single-species inoculations (Starkey et al., 2007). Numerous disease surveys and pathogenicity tests in controlled environments and in the field reported similar results (Linkmeyer et al., 2016; Pereira et al., 2021; Salas et al., 1999; Soledad Nogueira et al., 2018). Brennan et al. cultured five species of *Fusarium in vitro* and conducted *in planta* pathogenicity tests in wheat coleoptiles. *F. graminearum* and *F. culmorum* were the most pathogenic, and damaged coleoptiles nearly twice as severely as *F. poae* (Brennan et al., 2003). Indoor experiments by Xue et al. (2006) examined the pathogenicity of eight species of *Fusarium* in barley under controlled conditions. Their group inoculated six genotypes with 48 isolates at 10-14 days after heading and also recorded *F. graminearum* among the highly pathogenic, and *F. poae* as weakly pathogenic. Additional work by Xue et al. (2004) in wheat reported similar findings: six wheat lines were inoculated with eight species of *Fusarium* (54 isolates) and *F. graminearum* not only produced the most severe disease symptoms, it produced the most severe symptoms the most rapidly.

When co-inoculated with multiple *Fusarium* sp. in one system, it was expected that again, 'competition is the rule and not the exception' and that we would observe interspecific competition. From our observations, *F. poae* does not appear to be in competition with *F. graminearum* when inoculated indoors and X. Xu et al. observed similar infections (Xu et al., 2007). Their group inoculated wheat heads with one of four different *Fusarium* species (*F.*

graminearum, *F. culmorum*, *F. avenaceum*, and *F. poae*), varying initial inoculation conditions and then inoculated up to 48h later with another two or three of the listed species. The authors reported that inoculation with additional species left wheat heads no more severely changed than single-species inoculation and did not significantly affect visual disease ratings. We observed similar behavior in our barley experiments where co-inoculation did not dramatically change visual disease ratings; disease severity observed in our trials appeared relatively unchanged when heads were co-inoculated with *Fg*+*Fp*, compared to *Fg* alone.

As for the field trials, the data collected in Ottawa was vastly different from that collected in the growth cabinet. Possible reasons for the extreme variability are the gentle slope from Range 5-8. Grassy weed pressure was also intense despite efforts to manually remove them, weakening barley and increasing competition for resources. Emergence of CDC Bold was poorer than Stander and Chevron and ranges 1-4 also had to be eliminated from the analysis due to the number of missing values. Ultimately, the data was likely confounded by existing inoculum in the soil and long-distance spore transport as well as multiple spore release events, beyond the two that were spray applied (de Luna et al., 2002). Additional physical barriers or greater separation between plots will need to be investigated since the winter wheat border may not have been sufficient isolation. Martínez et al. (2020) conducted a field experiment to study the interaction of *Fg* and *Fp* with five barley genotypes and a blend of four isolates for each *Fusarium* species. These authors had similar outcomes to our indoor study where they observed neither a ‘synergistic’ nor ‘antagonistic’ interaction between both species of *Fusarium*.

Data collected in Harrington, however, drew a few different conclusions. Similar to the indoor experiment, *Fg* produced the most severe and highest incidence of disease symptoms. Of the 117 isolates collected in a field survey of South American barley samples, 103 were

identified as *F. graminearum* (Castañares et al., 2016). A similar study conducted in Uruguay collected 154 barley samples (Garmendia et al., 2018), and another in Iran collected diseased wheat samples and identified 41 different isolates (Khaledi et al., 2017) – *F. graminearum* was the dominant cause of FHB in both cases. Meanwhile, from boxplots in Figure 1.6, it appears that the presence of *Fp* did indeed have some effect on disease symptoms: *Fg*-inoculated barley had the highest disease severity, *Fp* the lowest, and the co-inoculation appeared to be an intermediate severity of the two. This suggests that *Fp* may be competing with *Fg* for resources, however this effect was not significant. This may have been due to some degree of contamination from the soil or environment by other inoculum sources. A fluorescent protein tagging system to identify inoculated *Fusarium* versus pre-existing/endogenous *Fusarium* in the field could be useful in quantitative molecular studies especially when working in previously infected soil (Dufresne et al., 2007; Jansen et al., 2005). There would still however be no way to quickly determine which *Fusarium* spp. caused the diseased phenotype and necessitates further molecular investigation. Plus, the regulatory hurdles to spraying transgenic fungus outdoors in Canada are many.

Only one isolate per species was used in this study, and so results may only be isolate-specific rather than representative of the entire species. For instance, generally, 15-ADON chemotype *Fusaria* are less toxic and virulent in wheat than 3-ADON (Gilbert et al., 2010). While there have been reports of an increase in 3-ADON in Western Canada, whereas the 15-ADON chemotype has been more prevalent in Ontario and Quebec (Gilbert et al., 2014; Kelly et al., 2015; Tamburic-Ilincic et al., 2015). Using a blend of isolates with varied chemotypes may better capture the variation of the *Fg-Fp* interaction in Canada, bearing in mind that intraspecies interaction can reduce mycotoxin production and interfere with disease symptom development

(Walkowiak et al., 2015). Schöneberg et al. also saw optimal infection conditions of *Fg* in barley vary with the cultivar and so extending the panel of genotypes tested would also increase the confidence of our results (Schöneberg et al., 2018).

Indoors or outdoors, *Fp* was the less aggressive fungus and produced lower disease ratings than *Fg*. Looking back to work by Xue et al. (2019) where *Fp* was most detected from grain in years when there was not an FHB epidemic, this begs the question: is *Fusarium poae* even a pathogen? Could *Fp* be an opportunistic endophyte? Generally, an endophyte is an organism that establishes a symptomless infection in internal plant tissues, where tissues are apparently healthy on collection (Bacon & White, 2000; Petrini, 1991; Zakaria & Ning, 2013). Endophytic fungi may be vertically or horizontally transmitted and have likely co-evolved with their host (Lofgren et al., 2018). To that end, the product of the host-microorganism interaction may be host species dependent. Many *Fusarium* sp. have been isolated from asymptomatic grasses (Lofgren et al., 2018; Zakaria & Ning, 2013), *F. oxysporum* from bananas and tomatoes (Demers et al., 2015; Waweru et al., 2014), *F. verticillioides* from corn (Bacon & Hinton, 1996; Rodriguez Estrada et al., 2011), but when coupled with a different host elicits a diseased phenotype or a maladaptive host response. Again, harking back to Xue et al.'s (2019) multi-year survey of infected cereals, they observed that *F. graminearum* dominated wheat samples, *F. poae* in oat, but both in barley: could *Fusarium poae* then be endophytic in wheat and barley but pathogenic in oats? Although increased disease is not observed when *Fp* is added to the system, there are reports of greater mycotoxin accumulation and a more diverse range of toxins produced (Stenglein et al., 2012; Xu et al., 2007). Therefore, if *F. poae* is indeed an endophyte, that does not mean that it is any less dangerous and should be ignored. Considering *F. poae* can 1) produce other mycotoxins that are more toxic than those produced by *F. gramineaurm* and 2)

reduces the effectiveness of existing chemical and biological controls, there is the potential to collapse a large component of our only FHB management strategies, and still great reason to investigate the interaction of *F. gramineaurm* and *F. poae* (Tan et al., 2021). From the phenotypic data presented here, *F. poae* does not seem to be a serious cause of visual disease and *F. graminearum* remains to be the main player. Given visual disease ratings do not always correlate with mycotoxin accumulation, the following chapters however will investigate further at the molecular level of interaction between *F. poae* and *F. graminearum*.

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CHAPTER II

ddPCR Assay to Quantify Relative Species Dominance of *Fusarium graminearum* and *F. poae* in Barley

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ABSTRACT

Fusarium head blight (FHB) is one of the most devastating diseases of barley. FHB is caused by a species complex of *Fusaria*, of which *Fusarium graminearum* is the species responsible for most FHB epidemics in Canada. Field surveys show that two or more *Fusarium* species often co-exist within the same field or grain sample and *F. poae* is reported as another dominant species in barley in eastern Canada. The aim of this study was to quantify the relative levels of fungal genomic DNA of *Fusarium graminearum* and *Fusarium poae* in barley samples collected from greenhouse inoculations with these species. Two susceptible barley genotypes were spray-inoculated at 10 to 14 days after heading. Phenotypic disease development was tracked and at 28 days post-inoculation, heads were harvested, flash-frozen in liquid nitrogen and stored in -80°C. Genomic DNA was extracted from the harvested heads of each experimental unit, and the ratio of *F. graminearum* to *F. poae* DNA was quantified with ddPCR using markers for species-specific metabolites, GRA1 (gramillin, produced by *F. graminearum*) and APS1 (apicidin synthetase produced by *F. poae*). When barley was co-inoculated, the ratio of GRA1 to APS1 was no different than when heads were inoculated with *F. graminearum* alone.

11.0 INTRODUCTION

Fusarium head blight (FHB) remains to be a serious issue facing the agri-food sector as it threatens grain yield and quality worldwide (Windels, 2000). The main causal species of FHB is an aggressive pathogen, *Fusarium graminearum*, however, in recent years there has been increasing concern over *Fusarium poae* in Canada and beyond, as both *Fusarium graminearum* and *Fusarium poae* were most isolated from barley (Martínez et al., 2020; Xue et al., 2019). Given this history and that most research focus is placed on resistance to *F. graminearum*, it is important that we investigate the possibility for *F. poae* interacting with *F. graminearum*, and *Fp* interacting with *Fg* in barley.

A crux to studying the interaction of *Fg* and *Fp* in barley is the ability to measure it. Conventionally, FHB is measured by visual disease assessment and identified morphologically *in vitro* (Doohan et al., 1998). The adoption of molecular techniques have highlighted how subjective and misleading visual ratings can be. For instance, Stenglein et al. (2012) concluded that their visual estimation of *F. poae* infection in barley was significantly underestimating actual grain contamination relative to their PCR data. They went as far to say that "for each grain with visual symptoms two barley grains could actually contain the fungus" (Stenglein et al., 2012). To build on the observations in Chapter 1, our main objective for this section was to take a more objective approach to measuring the *Fg-Fp* interaction and quantify the relative amounts of *F. graminearum* and *F. poae* in barley when spray-inoculated with either alone or both simultaneously.

12.0 MATERIALS AND METHODS

12.1 Barley spike inoculation assay

Inoculation methods were listed in Chapter 1, but the following is a summary. Heads from two susceptible genotypes of barley were spray-inoculated with spore suspensions of one of 4 treatments at 10-14 days after heading (i.e. base of spike has emerged from the flag leaf sheath): *F. graminearum* (*Fg*), *F. poae* (*Fp*), both *Fg*+*Fp*, and ddH_2O (control). Each replicate contained 8 pots, and two of each were assigned to each treatment. Visual disease ratings were assessed at regular timepoints, whole heads were harvested 28 days post inoculation, flash frozen in liquid nitrogen and stored in -80°C until ready for molecular analysis.

12.2 Sample preparation

Seven heads (or less if not available) were pooled from each pot and hand-ground as finely as possible in liquid nitrogen using a mortar and pestle. Genomic DNA of each pooled sample was extracted using Macherey-Nagel's Nucleospin 96 Plant II Kit (Macherey-Nagel, Düren, Germany) with some modifications to the User Manual centrifuge processing protocol. At Step 2a, samples were incubated overnight for 18 hours. At Step 2b, each individual sample was vigorously mixed on a vortexer until all sample material was moistened by the solvent, and then spun for 1min at 1,500 x g. At Step 8, 80uL of the first DNA elution was placed back through the same column rather than washing the membrane with another 100µL of Buffer PE. Fungal DNA from *in vitro* positive controls was isolated using the E.Z.N.A Fungal DNA Mini Kit (Omega Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer's manual instructions. The concentration of each stock was measured using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Ottawa, ON, Canada).

12.3 Polymerase Chain Reaction (PCR) to confirm specificity

The list of species-specific primers used are listed in Table 2.1. Amplicons could not be longer than 150 bp in order to optimize the fluorescent signal: too small and fluorescence is too low, too long and fluorescence is too high. To remove secondary structure, 1500 ng of extracted genomic DNA was digested with EcoR1 at 37°C for 60 minutes. Polymerase chain reaction (PCR) amplification was performed on all digested samples in a 25 µL reaction volume. The final volumes of each reagent were as follows: 0.6 µL dNTPs, 0.35 µL 50X Advantage DNA Polymerase, 2.5 µL 10X Advantage Buffer, 0.6 µL each forward and reverse primer at 10 µM, 5 µL template DNA, and 15.35 µL water. Products were then visualized by gel electrophoresis on a 1% agarose gel stained with SYBR Safe (Figure 2.1 and 2.2).

Table 2.1: Primers used for PCR reactions

Target species	*Target*	*Forward primer*	*Reverse primer*	*Expected amplicon length (bp)*	*Reference*
F. poae	APS1, apicidin synthetase	GAAGCAGACA TTGGCAACCG	GAAGAGTCCG ATAGCAGGGC	150	Witte et al. 2021
F. graminearum	GRA1, gramillin, FGSG_15673	CTCGCATAGCA TACCTACATCAA	CGAGATGAGCG AGAAGAAGAAG	127	Bahadoor et al. 2018

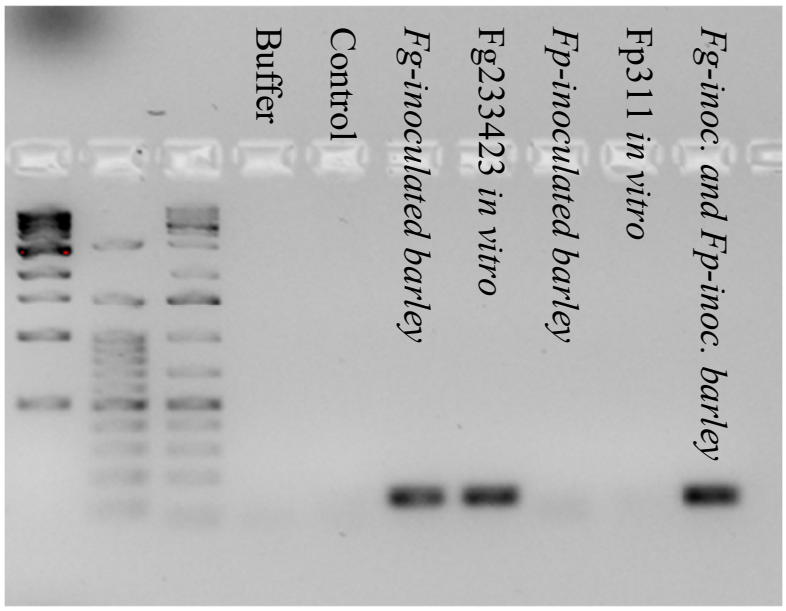


Figure 2.1: Verification of GRA1 primer specificity by qPCR and gel electrophoresis on 1% agarose gel and stained with SYBR Safe dye

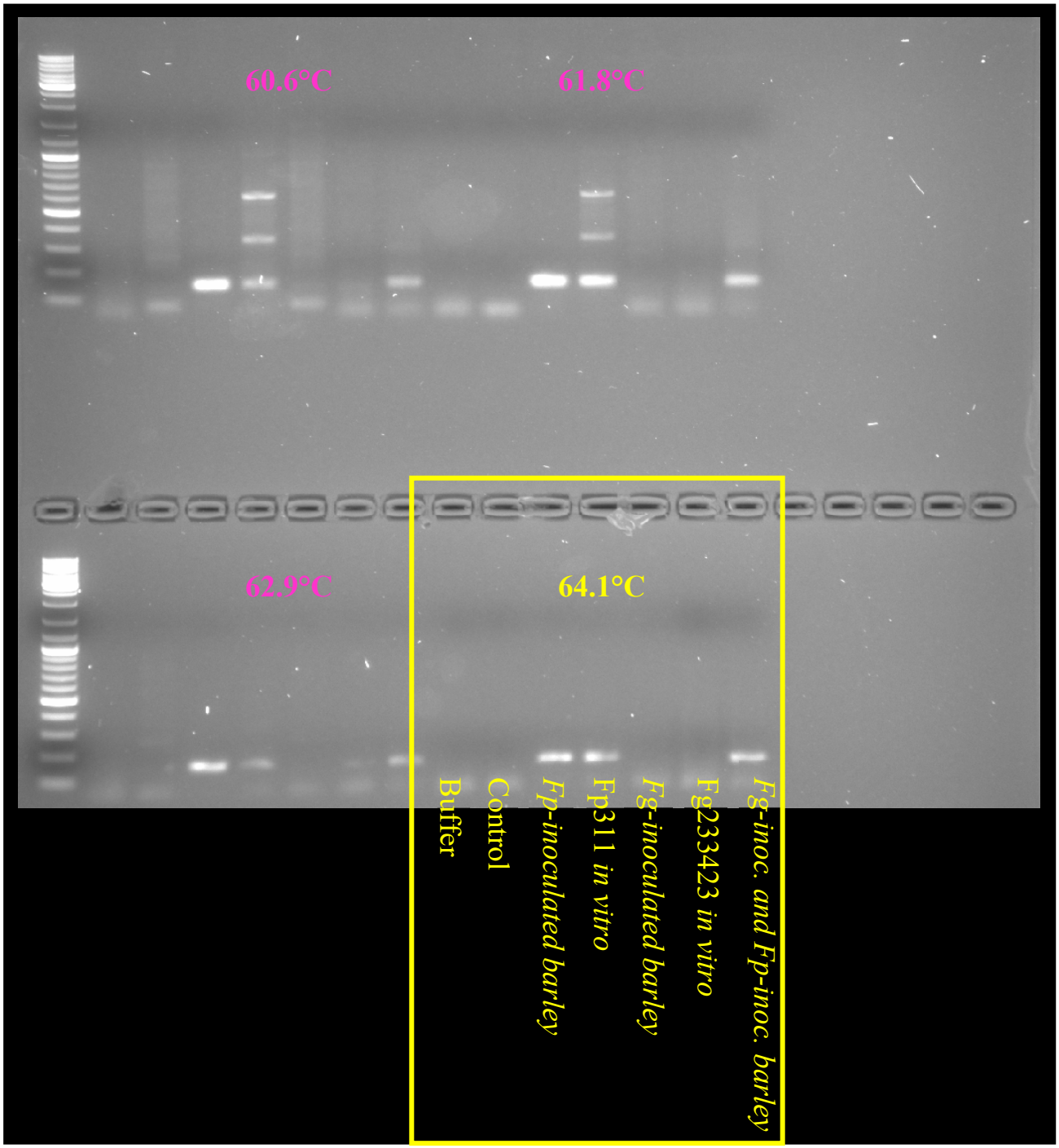


Figure 2.2: Verification of APS1 primer specificity by qPCR and gel electrophoresis on 1% agarose gel and stained with SYBR Safe dye

12.4 Sanger sequencing for amplicon confirmation

PCR products were directly transferred and amplified for Sanger sequencing in a reaction volume of 10 µL using the ABI BigDye Terminator 3.1 sequencing kit. The final volumes of each reagent were as follows: 8.5 µL BigDye Seq Mix diluted 1:8 with Seq buffer (Thermo Fisher Scientific, Ottawa, ON, Canada), 0.5 µL of reverse primer at 3.2 ng/µL, and 1 µL of PCR product from the initial amplification. PCRs were run on an Eppendorf MasterCycler (Thermo Fisher Scientific) with an initial denaturation of 95°C for 3 min, followed by 40 cycles of 15 s at 95°C, 15 s of annealing at 50°C, and 2.5 min of extension at 60°C, and then a final hold at 10°C. These PCR products were then submitted for in-house Sanger sequencing. The returned sequence was aligned in Geneious Prime version 2022.1.1 (Biomatters, Ltd, San Diego, CA, USA). The extracted consensus sequence was verified against reference sequences for *Hordeum vulgare*, *F. graminearum*, and *F. poae* using National Center for Biotechnology Information's (NCBI) blastn suite in their web Basic Local Alignment Search Tool (BLAST) (Sayers et al., 2021).

12.5 Droplet digital PCR (ddPCR) for fungal load quantification

Droplet Digital PCR (ddPCR) conditions were optimized for each reaction by running a series of concentration and temperature gradients with positive and negative controls, and blended test samples. This means when testing *Fg*-specific primers, for example, fungal DNA isolated from pure *in vitro* culture was the positive control, DNA from samples mock-inoculated with ddH_2O (assumed to be barley only) was the negative control, and a blend of DNA isolated from *Fg*-treated and co-inoculated samples were used. *Fg*-treated samples should contain *Fg* DNA, and co-inoculated samples should contain both *Fg* and *Fp* DNA to also test *Fg* primer

specificity in a background of *Fp* and barley DNA. The final volumes of each reagent were as follows: 12.5 µL of EvaGreen Supermix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), 1.6 µL each of forward and reverse primers at 10 µM, 4 µL of digested genomic DNA at 1.25 ng/µL, and 5.3 µL of sterile Milli-Q water, to produce a total reaction volume of 25 µL.

A separate ddPCR reaction was performed for each pathogen using species-specific primer with the QX200™ Droplet Digital™ PCR System (Bio-Rad Laboratories Ltd.), meaning the 64 samples were analyzed twice: once with *Fg*-specific primers, and then again with *Fp*-specific primers in a separate reaction. The droplets were prepared with the Bio-Rad Droplet Generator according to manufacturer's instructions. PCR amplification was completed on the Bio-Rad C1000 Touch Thermal Cycler with the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 62°C for 1 min, then hold at 4°C. Following amplification, the plate was transferred to the Bio-Rad Droplet Reader and droplets counted with the accompanying software, QuantaSoft (Bio-Rad Laboratories Ltd.). The positive-droplet threshold for GRA1 was set manually at 20K (Appendix A). One control sample (in well C1, barley DNA) was greatly contaminated with GRA1 and so it was removed from the remainder of the analysis. Background noise was detected in the APS1 samples and this was manually cleaned with thresholds above 22K and below 28K (Appendix B).

13.0 RESULTS

It is critical to refer to the ratio of GRA1 to APS1 when comparing response to the different inoculations; without normalizing to a barley-specific marker, direct comparisons between individual samples and/or treatments must be made with caution. Multiple ANOVA

was used to analyze effects of genotype, treatment, inoculation and replicate on ratio of mean GRA1 concentration to mean APS1 concentration; only treatment had a statistically significant effect ($p < 0.05$). Mean concentrations of GRA1 and APS1 per mL were plotted in Figure 2.3, and the ratio of GRA1 to APS1 between *Fg*-treated and co-inoculated samples did not differ significantly; the ratio in samples treated with *Fp* only, however, did.

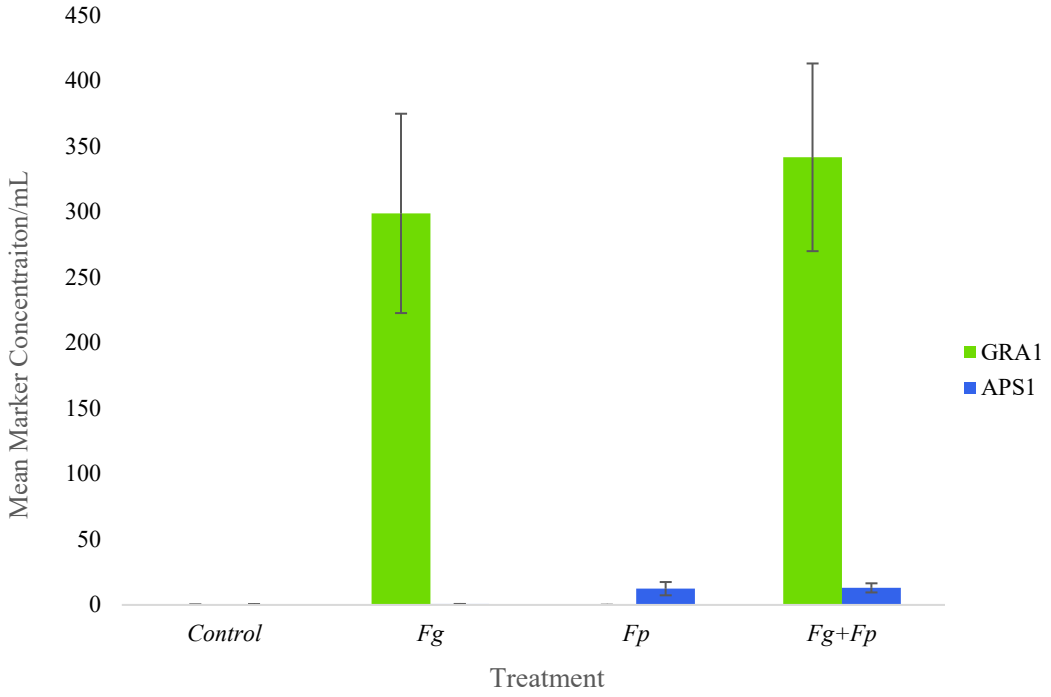


Figure 2.3: Markers detected by ddPCR on ground tissue inoculated with single-species or co-inoculation treatments (*n* = 8). Heads were harvested at 28 dpi, flash-frozen, and ground in liquid nitrogen. Error bars represent standard error.

14.0 DISCUSSION

To investigate fungal interactions, many groups have used mixed co-inoculations where spores from both species are in the same inoculum suspension as we did in our work, and/or have used sequential inoculations where spores from both species are each applied separated by

a period of time. After collecting levels of fungal DNA relative to notes on phenotypic disease changes, a number of possible explanations have been described (Table 2.3).

Table 2.3: A non-exhaustive list of relationships in mixed and sequential inoculations

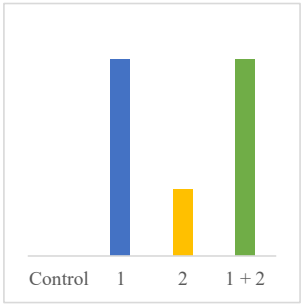
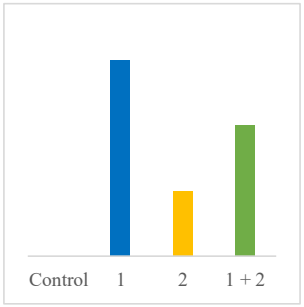
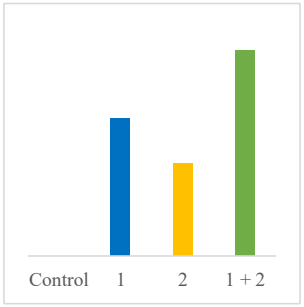
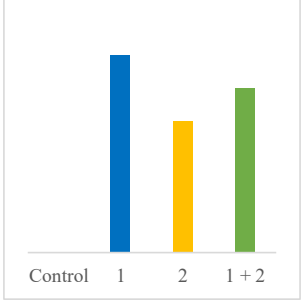
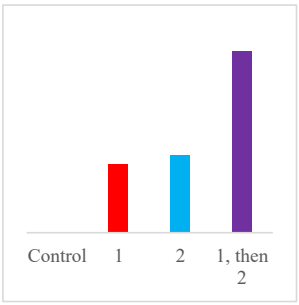
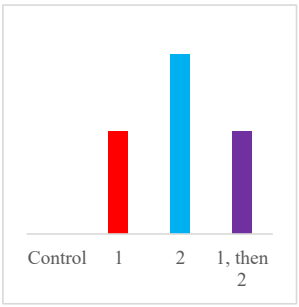
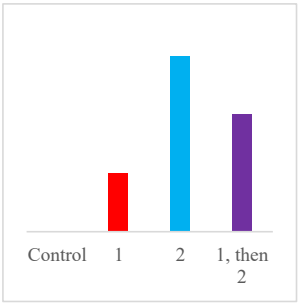
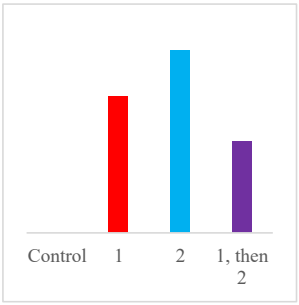
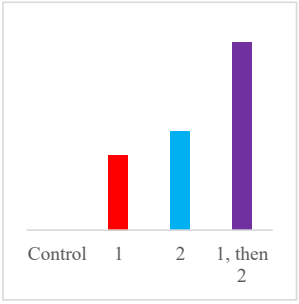
Relationship	***Expected phenotype***	***Literature***			
		Reference	*Potential explanation for observed results*	*DNA quantification of co-inoculation*	*Disease phenotype of co-inoculation*
Mixed inoculations					
Dominance		X.-M. Xu et al., 2007	*F. culmorum* is more aggressive than *F. poae*	*Fc* > *Fp*	Unchanged, compared to *Fc* alone
Antagonism		Schoneberg et al., 2015	*F. graminearum* is more aggressive, *Trichoderma* spp. antagonizes *Fg*	*Fg* > *Fp*	Decreased, compared to *Fg* alone
Synergism		Jakovljevic, 2020	*F. lateritium* LP7 and *Trichoderma viride* LP5 had enhanced biodegradation of environmental contaminants when cultured together (synergistic interaction)	LP7 = LP5	Increased, compared to either single species
Unrelated			Fungus 1 and Fungus 2 are not related	F1 ≠ F2	Unchanged, compared to either single species

Table 2.3 (cont.): A non-exhaustive list of relationships in mixed and sequential inoculations

Sequential inoculations					
Primary infection weakens host		Sturz and Johnston, 1983	*F. poae* weakens host defences and predisposes host to disease upon second challenge by *F. graminearum* and *F. culmorum*	*Fp* < *Fg* and/or *Fc*	Increased, compared to *Fg* or *Fc* alone
First pathogen established can better defend		Simpson et al., 2004	*M. nivale* var. *majus* was more established and was able to suppress *F. culmorum* in wheat seedlings	*Mn* > *Fc*	Unchanged, compared to *Mn* alone
Primary infection 'vaccinates' host and primes host's immune defenses		Tan et al. 2020	*F. poae* induces salicylic and jasmonic acid-related immune response, dampening subsequent *Fg* infection	*Fg* > *Fp*	Decreased, compared to *Fg* alone
Antagonism		Velluti et al., 2000	*F. graminearum* antagonizes *F. moniliforme* and *F. proliferatum* on irradiated maize grain	*Fm* and *Fp* < *Fg*	Decreased, compared to either single species
Synergism		Xing and Westphal, 2013; Nitzany et al., 1973	*Fusarium virguliforme* and *Heterodera glycines* have synergistic effect on Sudden Death Syndrome in soybean; *Fusarium* spp. and Cucumber Mosaic Virus have synergistic effect on disease symptoms	F1 = F2	Increased, compared to *Fusarium* spp. or CMV alone

Where there is a difference in aggressiveness in the pathogens, the more aggressive pathogen generally has more fungal DNA isolated (X.-M. Xu et al., 2007). Antagonistic action by the weaker pathogen can be observed in the disease phenotype, where the intensity of disease symptoms is dampened relative to the aggressive alone. In Chapter 1, visual disease ratings from inoculation with both *F. graminearum* and *F. poae* looked no different than barley spikes inoculated with *F. graminearum* alone, where at 28 days post-inoculation mean visual disease rating was 8.5 out of 9 when inoculated with *Fg* alone and 8.58 out of 9 when co-inoculated with both *Fg* and *Fp*. This suggests that *F. poae* does not antagonize *F. graminearum*. On the other hand, when comparing *F. poae* alone to in combination with *F. graminearum*, disease ratings climbed from 5.5 to 8.58 out of 9, respectively. Could *F. poae* be an opportunistic pathogen that requires the wake of destruction by *F. graminearum*? *F. graminearum* and *F. poae* disease phenotypes in the barley spike are essentially the same, and so it is impossible to identify the dominant species in co-inoculation without further analysis. Results from ddPCR reported the level of GRA1 relative to APS1 is unchanged when *Fg* is inoculated in combination with *Fp* versus when inoculated with *Fg* alone. It was expected that *Fg* was the dominant species as disease ratings appeared the same regardless of whether *Fp* was present in the inoculation. The dominant pathogen, *F. graminearum*, did not gain selective advantage when co-inoculated with the weaker *F. poae*. However, colonization by the weaker pathogen, *F. poae*, did not appear reduced by the more aggressive, *F. graminearum*. In fact, fungal DNA accumulation of both species did not even appear to be related (Figure 5). This is unusual relative to what has been reported in many other studies where *Fg* and/or *Fp* biomass are reduced in the presence of a competitor (Simpson et al., 2004; Velluti et al., 2000; X. Xu et al., 2007). Ultimately, *Fp* DNA accumulation does not appear affected by the presence of *Fg*.

In a co-inoculation of *F. avenaceum* and *F. poae*, the amount of *F. poae* DNA was less than when inoculated alone, suggesting that *F. avenaceum* suppressed or antagonized *F. poae* (X.-M. Xu et al., 2007). However, accumulated levels of nivalenol (NIV), a toxin only reported from *F. poae* and not *F. avenaceum*, were nearly 1500 times higher in co-inoculation than in *F. poae* alone. This suggested that the NIV productivity per unit of fungal biomass is dramatically increased in co-inoculation than single-species inoculation and that mycotoxin accumulation is another important parameter that should be investigated to understand the effect of *Fusarium* spp. interactions on grain quality, as it will be in Chapter 3. As it relates to the supposed disconnected levels of *Fg* and *Fp* DNA in our co-inoculated samples, there may be variations in the detection *Fg*- and *Fp*-specific metabolites.

Yang et al. (2010) observed the early stages of FHB infection in greenhouse-grown barley, and saw a sharp increase in *F. graminearum* DNA just 3 days after inoculation. This coincided with accumulation of β-amylase fragments, suggesting *F. graminearum*-induced β-amylase degradation. The presence of fungal DNA does not necessarily certify disease, so this additional confirmation of *Fg*-caused damage and examination of fungal gene expression and grain proteome is yet another avenue for confirmation of *F. graminearum* dominance in the *Fg*-*Fp* interaction.

The inclusion of sequential inoculations in experimental design allows the ability to make additional observations related to the order of establishment and to the host's response to inoculation with one versus multiple species. Picot et al. (2012) were interested in the order of colonization in presentation of disease. They compared single inoculations, mixed suspension

inoculations, and sequential inoculations of *F. graminearum* followed by *F. verticillioides* in corn in the field. They saw that *F. graminearum* DNA accumulation decreased or was unaffected by sequential inoculation with *F. verticillioides*. Inversely, *F. verticillioides* colonization, however, was favoured when pre-inoculated with *F. graminearum*. *Fusarium* spp., let alone *F. graminearum* only, do not behave the same in all host-species and tissue types and so it is unclear whether different responses would be observed in sequential *Fusarium* spp. inoculations in barley spikes (Harris et al., 2016). In field, *F. poae* and *F. avenaceum* have been isolated from barley before emergence from the boot, and they suppose that early colonization by *F. poae* is predisposing the host to infection by *F. graminearum* (Sturz & Johnston, 1983). This group views the end-of-season perspective to *Fusarium* spp. screening in grain samples as misleading and that the species order of establishment throughout the growing season may be critical to FHB disease development (Simpson et al., 2004; Sturz & Johnston, 1983).

From the results reported in Chapter 2, the *Fg*-*Fp* relationship appears most influenced by *F. graminearum* since levels did not appear impacted by the presence of *F. poae* in the co-inoculation treatment. Additional investigations into the mechanisms of *Fg* and *Fp* in barley are needed to better understand.

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CHAPTER III

Metabolomic analysis of *Fusarium graminearum*-*Fusarium poae* interaction in *in vitro* and *in planta* environments

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ABSTRACT

Fusarium head blight (FHB) is a major fungal disease of barley and other important agronomic crops in Canada and worldwide. The main causal species of FHB epidemics in Canadian cereal crops is *Fusarium graminearum*, but according to recent survey results, *Fusarium poae* isolated from diseased barley at consistently similar rates in Ontario. The aim of this study was to observe the effect on mycotoxin accumulation in grain when barley is co-inoculated with *F. graminearum* (*Fg*) and *F. poae* (*Fp*). Two susceptible barley cultivars were inoculated with one of four pathogen treatments in a growth cabinet 10-14 days after heading: *Fg*, *Fp*, both *Fg* and *Fp*, or a sterile water control. Heads were harvested 28 days post-inoculation and extracts from ground heads were analyzed by UPLC-HRMS and data analysis was performed using mzMine v2.53, ThermoXcalibur 2.2 SP1.48, and MetaboAnalyst 5.0, and annotated using R. Forty-six features were annotated, and visually represented in a heatmap and principal component analysis. The mycotoxin profile of the co-inoculated treatment appeared to be most influenced by *F. graminearum*-related metabolites and, though still present, less so by *F. poae*-related. There appeared to be some separation in barley genotypes in response to treatment with *F. graminearum*. From the features detected in this analysis, *Fg* appears to dominate the relationship of *Fg* and *Fp* in barley at the mycotoxin level.

21.0 INTRODUCTION

Fusarium head blight (FHB) is a devastating fungal disease that affects barley yield and, more importantly in this chapter, grain quality. From a malting perspective, fungal infection reduces germination and negatively affects the wort due to increased proteolysis by *Fusarium* spp. enzymes in infected grain (Martínez et al., 2020; Schwarz et al., 2001). *Fusarium* spp. also produce an array of secondary metabolites that accumulate in the grain, which if consumed in amounts above safe threshold tolerances, negative health effects will follow. Secondary metabolites are natural products not required for organismal growth or reproduction, but their production confers selective advantage to the pathogen. Mycotoxins, a group of fungal secondary metabolites, are associated with pathogenicity and are produced with the intent to infect as virulence or aggressiveness factors (Foroud & Eudes, 2009). Not all species, or even isolates of the same species, produce the same type and amount of mycotoxins. This also varies with environmental conditions, host species, and other abiotic and biotic stresses (Harris et al., 2016; Martínez et al., 2020). As a result, avoiding mycotoxin accumulation is not as straight forward as avoiding visual disease (Paul et al., 2005; Xian et al., 2022).

Due to the noxious effects from mycotoxin exposure, maximum tolerance levels have been legislated all around the world. These are mainly aflatoxins, fumonisins, ochratoxin A, zearalenone, and trichothecenes (Gruber-Dorninger et al., 2017). Trichothecenes are a related but structurally diverse class of mycotoxins produced by *Fusarium* spp., and can be further subdivided into four main groups, Type A, B, C, and D (Foroud & Eudes, 2009; Thrane et al., 2004). Type A and B can be distinguished structurally by differing features at C-8, and Type C and D are not associated with FHB (Foroud & Eudes, 2009). Of the known toxins produced by

Fusarium spp., deoxynivalenol (DON, a Type B trichothecene) and HT-2 toxin (a Type A trichothecene) are the only legislatively monitored mycotoxins in Canada (Government of Canada, 2015). The Canadian Food Inspection Agency, and other similar international regulatory counterparts, also has a group of 'emerging toxins' produced by *Fusarium* spp. that are increasingly becoming of concern (Gruber-Dorninger et al., 2017; Xue et al., 2019). These include but are not limited to mycotoxins like enniatins, zearalenone, diacetoxyscirpenol (DAS), culmorin, and beauvericin (Gruber-Dorninger et al., 2017). These are under review due to the high frequency and abundance of detection even in finished feed and grain products (Hao et al., 2022; Koletsi et al., 2021; Kovalsky et al., 2016).

Fusarium graminearum is the causal species of FHB epidemics in Canada and one of the main producers of DON among other mycotoxins. Recent multi-year survey results have isolated *Fusarium poae*, a weaker pathogen, equally as often as *F. graminearum* from *Fusarium*-damaged barley (Xue et al., 2019). What makes this result especially alarming is that *F. poae* is also reported to produce a number of the emerging toxins listed above. The effects of the co-occurrence of these mycotoxins on FHB or unlucky consumers are not yet clear (Xue et al., 2019).

In Chapter 1, co-inoculation with *F. graminearum* and *F. poae* produced neither more nor less severe disease symptoms than inoculation with *F. graminearum* alone, suggesting that *F. graminearum* dictates the relationship. Similarly in Chapter 2, the ratio of *F. graminearum* to *F. poae* DNA in the co-inoculation was neither more nor less than *F. graminearum* inoculated alone, again suggesting *F. graminearum* dominates the relationship in barley. Here in Chapter 3,

we seek to investigate at an even smaller scale at the molecular level. Given fungi use their arsenal of mycotoxins to invade their host, to respond to their environment, and to communicate with and ward off other microorganism stressors, we seek to observe differences in mycotoxin production when barley is inoculated with *F. graminearum*, *F. poae*, or both.

22.0 MATERIALS AND METHODS

22.1 In vitro *confrontation assay*

Two isolates of *Fusarium poae* (*Fp146* (DAOMC 252242) and *Fp122*) and one isolate of *Fusarium graminearum* (DAOMC 180378) from the Canadian Collection of Fungal Cultures (CCFC, Ottawa, ON) were cultured in confrontation or alone on yeast extract sucrose (YES) and Mannitol Murashige & Skoog Salts (MMK2) media in 90 mm Petri dishes. Four replicates of each plate combination were cultured on each media type, for a total of 8 plates per plate combination. Spore suspensions of each isolate were prepared using liquid CMC media and adjusted to 1.0-1.2 x 10^4 spores/mL using a hemocytometer. With a sterile microscope slide, a 'front' of *Fg*, *Fp122* and/or *Fp146* was transferred from the liquid spore suspension and stamped onto the plate (~500-600 spores). Plates were incubated at 25°C in the dark. At 4, 6, and 8 days after inoculation, 16 mini-plugs of agar and mycelium at the median line ('confrontation zone') were sampled with a sterile straw, placed in 2 mL scintillation vials, and stored at -80°C until ready for extraction (Figure 3.1). Photos representative of the visual variation in the treatment were also taken at day 8 for metadata (Figure 3.2).

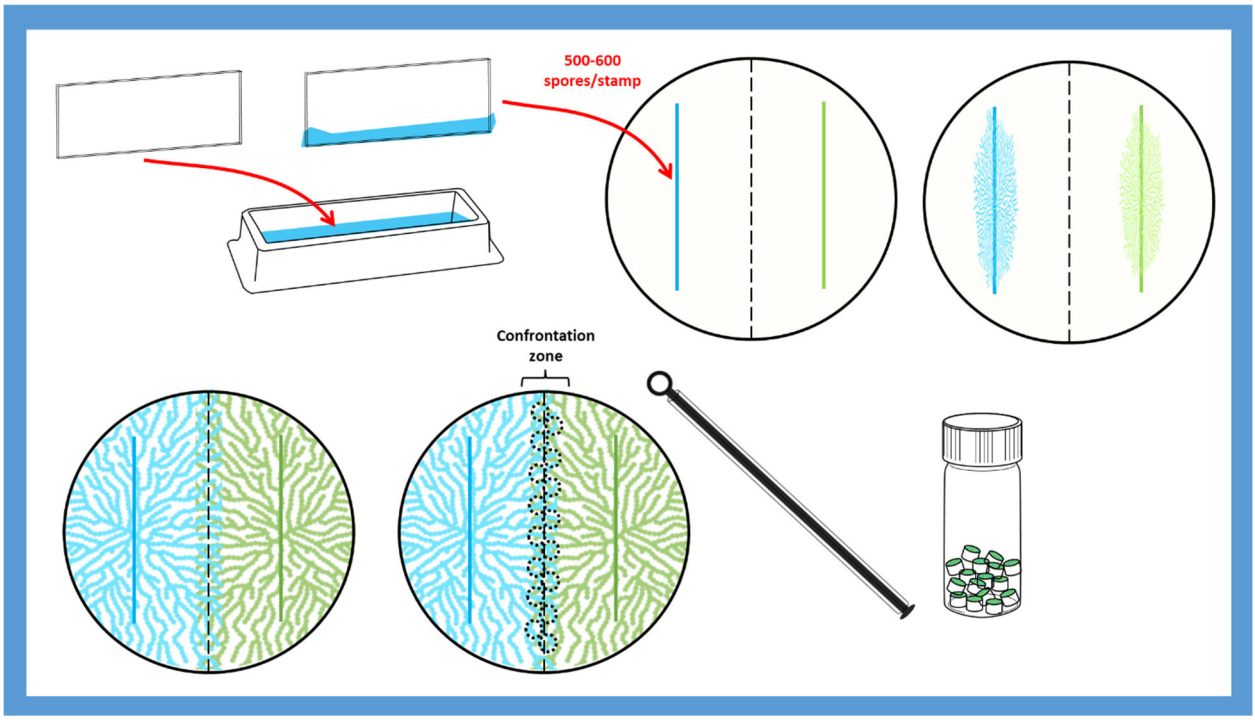


Figure 3.1: A schematic of the culturing and sampling method of the in vitro confrontation assay. The spore suspension of each *Fusarium* isolate was placed in the reservoir. To inoculate the plate, a sterile slide was dipped in the suspension and stamped the agar along the solid line. At 4, 6, and 8 days after inoculation, the agar and mycelium were sampled from along the dashed median ('confrontation zone') with a sterile straw. The plugs were frozen and stored at -80°C in a 2 mL scintillation vial until ready for extraction.

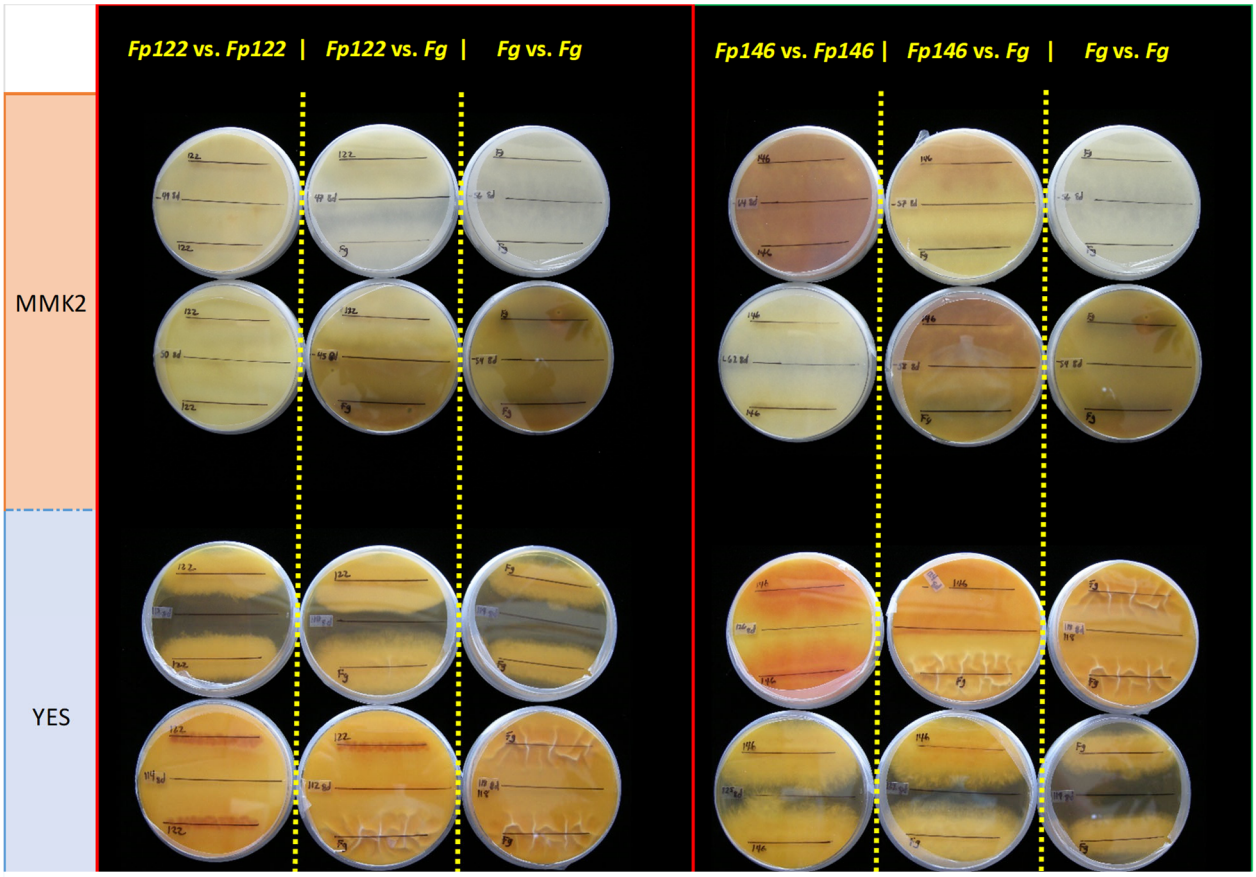


Figure 3.2: Photos from underside of in vitro confrontation assay at day 8.
Top row: on MMK2 media, bottom row: on YES media; left column*:* *Fp122* vs. *Fp122*, *Fp122* vs. *Fg*, *Fg* vs. *Fg*; right column: *Fp146* vs. *Fp146*, *Fp146* vs. *Fg*, *Fg* vs. *Fg*). Pictured here, each treatment contains 2 most representative of 4 total plates.

22.2 In vitro *sample extraction*

Two mL of ethyl acetate were added to each scintillation vial and placed on a rotary shaker at 100 rpm for 1 hour. The liquid was transferred to a new scintillation vial and these vials were dried under a pin dryer. These samples were re-suspended in 2 mL of acetonitrile (ACN) and transferred to HPLC-grade amber vials for submission to in-house UPLC-HRMS analysis.

22.3 In planta *sample extraction*

Seven heads (or less if not available) were pooled from each pot, flash frozen and hand-ground as finely as possible in liquid nitrogen using a mortar and pestle. A ball mill receptacle was filled halfway with pre-ground frozen tissue and milled for 5 minutes (Retsch MM2000, Newtown, PA, USA). About 500-600 mg of milled fresh tissue was placed in a 10 mL Falcon tube and 3 mL of extraction solvent was added; the solvent contained HPLC-grade 1:4 H_2O:ACN with 1% formic acid and 1 ppm of reserpine. Samples were placed in a sonicating bath (FS30D, Fisher Scientific, Waltham, MA, USA) for 5 minutes, placed on a nutator mixer for 60 minutes (Medmark Technologies LLC, Perkasie, PA, USA) and then spun for 10 minutes at 4740 x g (SORVALL RC-5B Plus, DuPont, Wilmington, DE, USA). A volume of 500 µL of the supernatant was placed into a 2 mL amber vial and submitted for in-house UPLC-HRMS analysis.

22.4 Ultra Performance Liquid Chromatography-High Resolution Mass Spectrometry (UPLC-HRMS) for both **in vitro** *and* **in planta** *samples*

The order of the extracts was randomized and loaded on the Thermo Ultimate 3000 for UPLC analysis. Chromatography was completed using a Phenomenex Kintex C18 column (50 mm x 2.1 mm, 1.7 µm, 100 Å) with flow rate of 0.35 mL/min and a gradient of two solvents: H_2O with 0.1% of formic acid (v/v) and acetonitrile (ACN) with 0.1% formic acid (v/v). Parameters for the cycle were as follows: start at 5% ACN and increase to 95% by 4.5 min, hold at 95% ACN until 8 min, return to 5% ACN by 9 min, and hold to equilibrate the column back to starting conditions by 10 min. HRMS detection was performed using a LTQ OrbitrapXL mass spectrometer (Thermo Fisher Scientific; Waltham, MA, USA). The machine was used with the following acquisition parameters (positive electrospray ionization (ESI+) mode, range of 100-2000 *m/z*, resolution of 30k (at 400 *m/z*), automatic gain control target of 5×10^5, and maximum injection time of 500 ms) and source parameters (ionization voltage of 4.0 kV, capillary voltage of 34V, tube lens voltage of 100 V, sheath gas of 40, auxiliary gas of 5, and sweep gas of 2).

22.5 **In vitro** *data curation*

Data preprocessing was performed using mzMine v2.51 (Pluskal et al., 2010). Mass features were detected using the profile mass detection algorithm with a noise level cut off of 1.0×10^4. Chromatograms were constructed using the ADAP chromatogram builder module, with a minimum groups size of 5 scans, a group intensity threshold of 1.0×10^5, a minimum highest intensity set to 8.0×10^6, and the *m/z* tolerance set to 5 ppm. Chromatogram deconvolution was performed using the local minimum module, with chromatographic threshold set to 35%, search minimum in RT range 0.05 minutes, minimum relative height at 15.0%, minimum absolute

height at 8.0 x 10^6, and minimum ratio of peak top/edge at 1.2, with a peak duration max of 2.00 minutes. Monotonic shape isotope grouping was performed using a *m/z* tolerance of 5.0 ppm, a retention time tolerance of 0.05 minutes, and a maximum charge of 1. Mass feature peak alignment was performed using the join aligner with the *m/z* tolerance set to 5.0 ppm with a weight value set to 2 and the retention time tolerance set to 0.05 min with a weight value set to 1. Finally, the multithreaded peakfinder gap-filling algorithm was applied to the dataset with an intensity tolerance of 15%, the *m/z* tolerance parameter specified as 5.0 ppm and the retention time tolerance set to 0.05 minutes. The data was then normalized to the total ion current prior to export. The selection of the utilized parameters was informed by inspection of the raw spectra.

A data matrix containing mass feature *m/z*, RT, and normalized peak areas was exported from mzMine and data curation, binary conversion and mass feature frequency phenotyping was performed in the R environment using in-house scripts according to Witte and Overy (2022). Mass features associated with media components and UPLC-HRMS system contaminants were removed. Statistical analysis was then performed in the 'R' environment using the freely available packages 'muma' (Gaude et al., 2013). For all multivariate analyses, data was scaled using the 'pareto' method of data scaling, wherein the square root of the standard deviation is used as the scaling factor for the purpose of retaining data structure while minimizing outliers, and then mean-centered. Univariate analysis was also performed using 'muma'. Variables were flagged as important to the confrontation treatments (*Fp122* vs *Fg* and *Fp146* vs *Fg*) if *p*-values from each comparison were < 0.05, and if the log2 fold changes were consistently either >1 or <1. Chromatograms of flagged variables were then manually inspected in mzMine for adduct annotation.

22.6 **In planta** *data curation*

Raw files from UPLC-HRMS were de-convoluted, aligned and gap-filled using mzMine v2.53 (Pluskal et al., 2010). Mass features were detected with a noise level threshold of 1.0×10^4 as this is generally the limit of detection on the UPLC-HRMS. Chromatograms were built using the ADAP chromatogram builder with a minimum group size of 7 scans, group intensity threshold of 1.0×10^5, minimum highest intensity of 1.0×10^6, and *m/z* tolerance of 0.001 *m/z* or 5.0 ppm. Peaks were smoothed with default settings then de-convoluted using the baseline cut-off algorithm where minimum peak height was set to 1×10^5, range duration at 0-2.5 minutes, and baseline level of 1×10^6. Chromatograms were aligned with the join aligner where *m/z* tolerance was 0.001 *m/z* or 5.0 ppm, weight of *m/z* of 20, retention time (RT) of 0.2 absolute min., and weight for RT of 10. Once gap-filled (*m/z* tolerance of 0.001 or 5.0 ppm), data was exported to Microsoft Excel for further curation.

To focus more specifically on fluctuations in fungal secondary metabolite production and unknown (fungal, or plant) metabolites associated with infection, a manual dataset curation was performed to only included mass features present in *in planta* pathogen-challenged plants and absent in mock infected controls. Conditional formatting in Excel was used to identify and eliminate background noise. This means features that were only present in the methanol blanks or whose peaks in test samples were approximately equivalent to the peaks in the methanol blanks, as these appeared to be carryover between samples or contamination in the blank, were removed, and features with clear and discernible mass feature pattern differences between mock-inoculated control plants and pathogen-challenged plants were kept. A subset of samples were

selected where, again, mass feature pattern differences were most representative of their treatment (n = 8 per treatment group, n = 7 for control, as one was observed to be contaminated by *F. graminearum* in Chapter 2). Using R, signals from these peaks were normalized to the total ion current (TIC) and a univariate analysis was completed using the Metabolomics Univariate and Multivariate Analysis (muma) package (Gaude et al., 2013). A manual inspection of each sample's raw chromatogram was performed in the QualBrowser package in ThermoXcalibur 2.2 SP1.48 to assign associated mass feature adduct and neutral loss states and selected mass features were assigned putative names based on accurate mass using an in-house database of *F. graminearum* and *F. poae* secondary metabolites to within 5.0 ppm of known *m/z*. To generate heat maps of mass feature vs samples and perform ANOVA of selected mass features, data from the selected mass features across the selected samples were inputted and auto-scaled in the one-factor module of MetaboAnalyst 5.0 (Xia et al., 2009).

23.0 RESULTS

23.1 In vitro *confrontation assay*

From photos taken at Day 8, there appears to be neither an antagonistic nor synergistic relationship between *F. graminearum* and *F. poae* in an *in vitro* environment (Figure 3.2). In co-culture plates, both species appeared to grow at similar rates, both compared to each other and to monoculture controls, and there does not appear to be a visible response when the two species converge at the median. There is also noticeable variation in colour and pigmentation of each isolate among biological plates cultured on MMK2. For example in Figure 3.2, when cultured on MMK2 the colour of *Fg* varied from light olive brownish to white, *Fp122* was fairly consistent white, and *Fp146* ranged from white to reddish-mauve. There did not appear to be any pattern in

changes of colour either in monoculture versus co-culture. Similar to observed variation in colour of the plates, it appears there was a great deal of variation in the chemical phenotype that was difficult to interpret as well (Figure 3.3). For example, Figure 3 illustrates this variation with 2 different patterns across 4 chromatograms: a group of peaks around RT of 4 minutes is present in two chromatograms (red box in Figure 3.3) and then the other two chromatograms have a distinct peak around RT of 5.67 minutes (blue arrow in Figure 3.3). No new mass features however appear to be consistently detected from co-culture samples that are not being detected from monoculture samples.

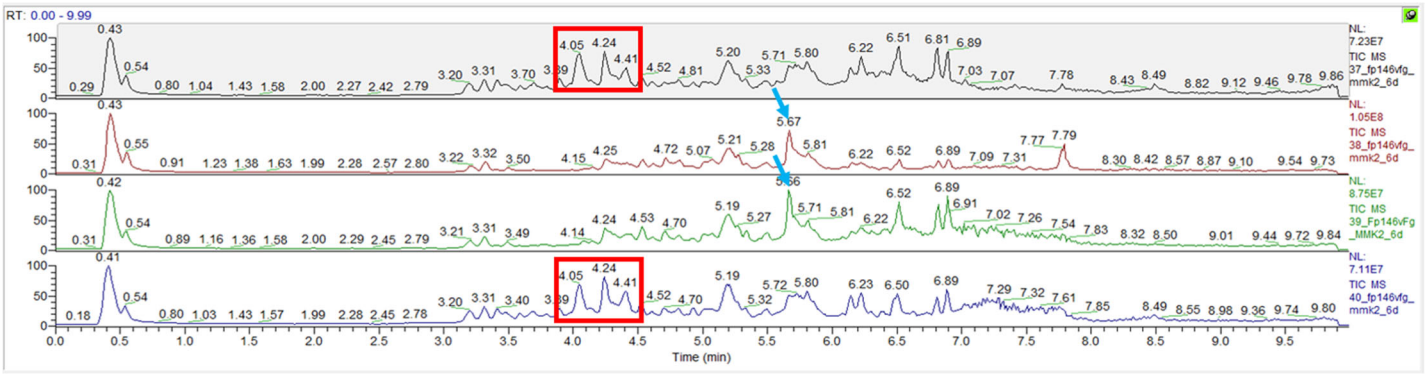


Figure 3.3: Screenshot of raw total ion current (TIC) chromatograms produced by QualBrowser in Xcalibur of extracts from confrontation zones between *Fg* and *Fp146* at 6 days post-inoculation on MMK2. This example screenshot shows the TIC chromatograms of four different samples, denoted by a different coloured line (black, red, green, and blue). The red boxes and blue arrows highlight two different patterns across the four samples.

23.2 In planta *growth cabinet study*

Initial pre-processing in mzMine pulled 1567 features. Given the complex nature of the dataset, a large number of these initial signals were background noise, as 41 detected metabolites had clear, discernible patterns and were assigned putative names. An important reminder, the *in planta* data set analyzed here represents a snapshot in the middle of many processes. The putatively detected molecules are not all final products of pathways, and as such many of the

annotated molecules are related to each other and were grouped together (i.e. trichothecenes). Of the selected list of 41 important features, five *m/z*'s did not correspond to within 5.0 ppm of any known molecule on the in-house list of mycotoxin annotations, and were therefore referred to as unknown. Tukey's HSD test was performed on all 41 metabolites across all 31 samples and only zearalenone was insignificant ($p > 0.05$, Figure 3.4a). Results of the separate Tukey tests for each cultivar (Figure 3.4b and c) follow below.

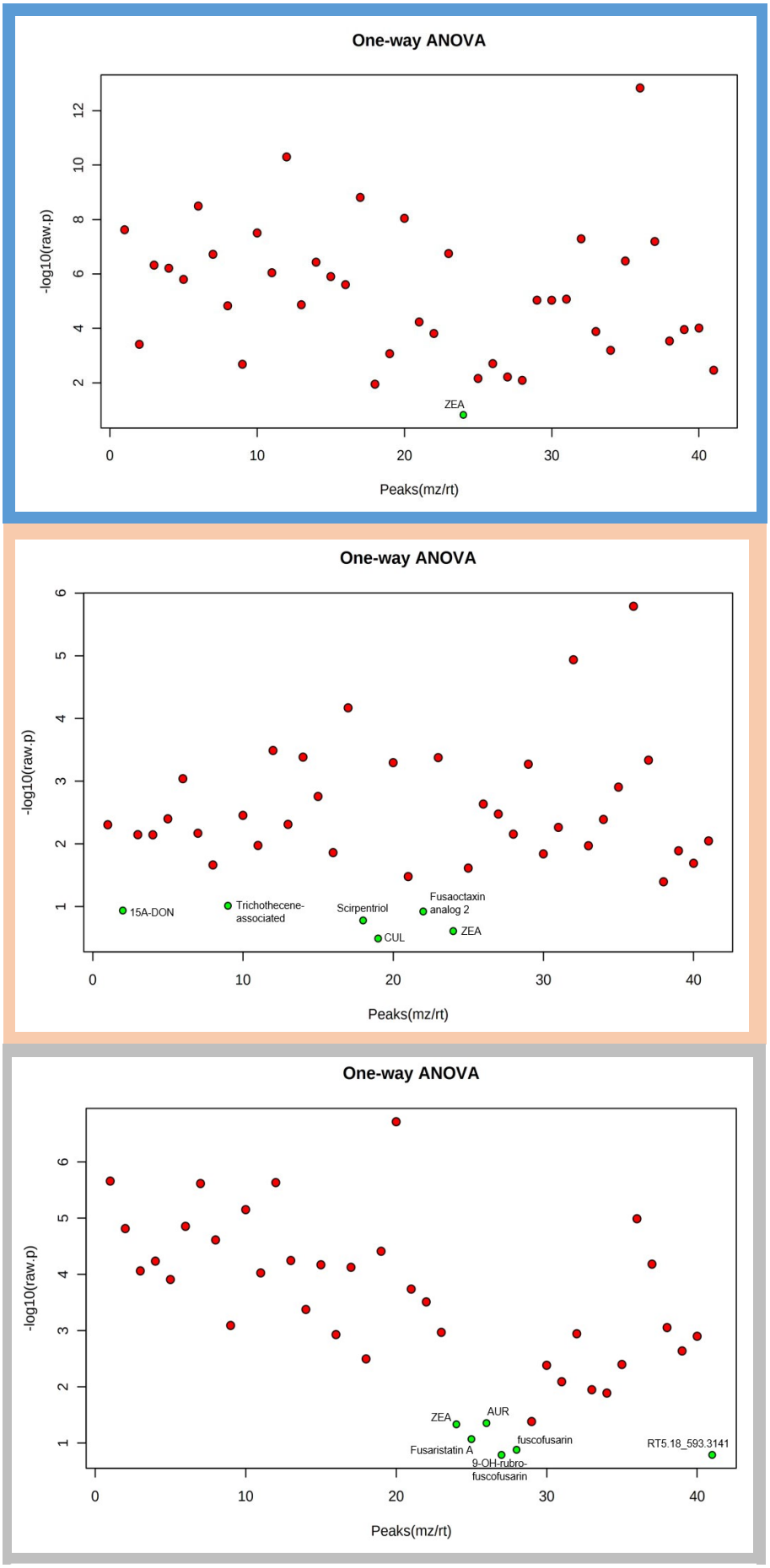
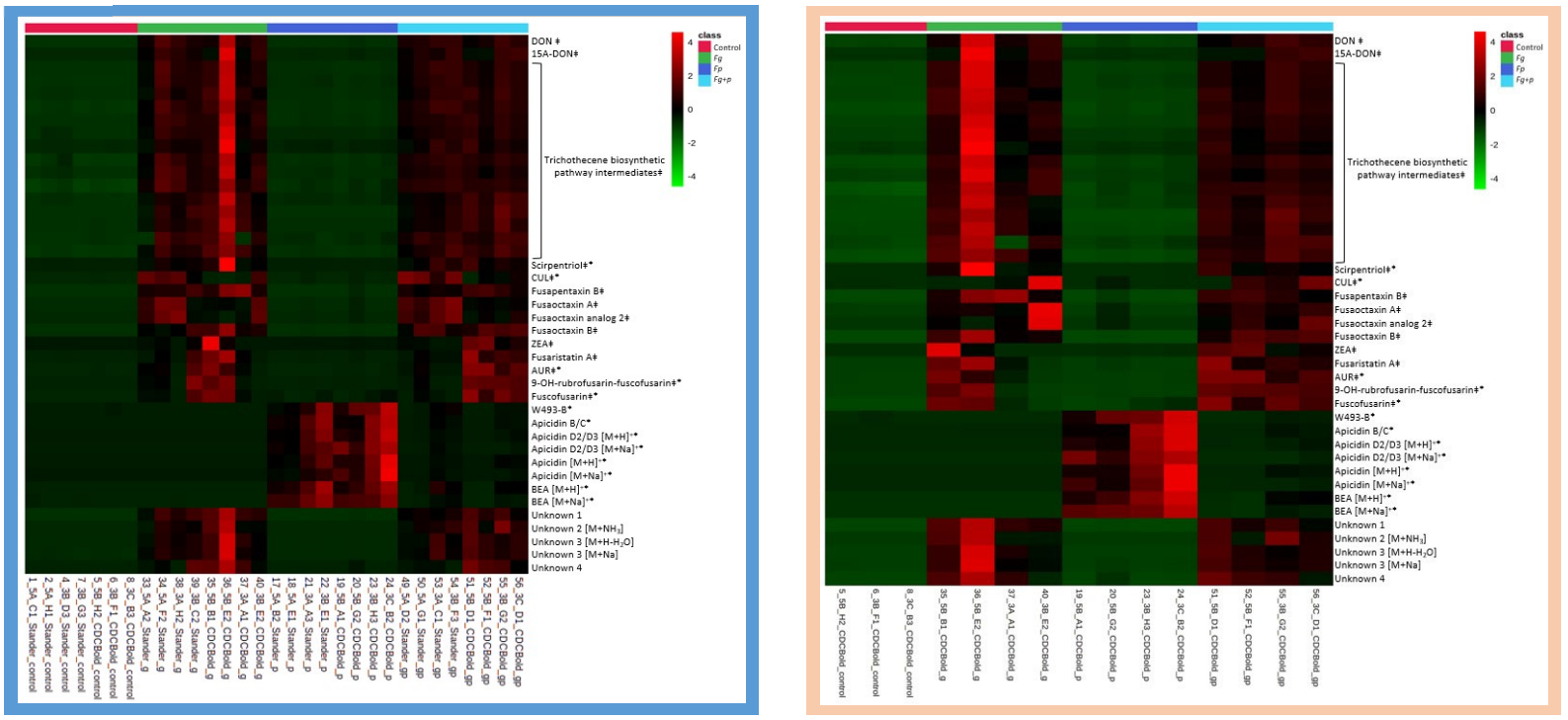


Figure 3.4: Graphical representation of ANOVA produced by MetaboAnalyst 5.0: a) all data, b) CDC Bold, c) Stander. Red dots represent significant and green dots represent insignificant raw.p-values ($p < 0.05$) according to Tukey's HSD (n = 7 control, n = 8 per infected treatment, total 31 samples; see table of significant p-values in Appendix F, G, and H).

b)

a)



c)

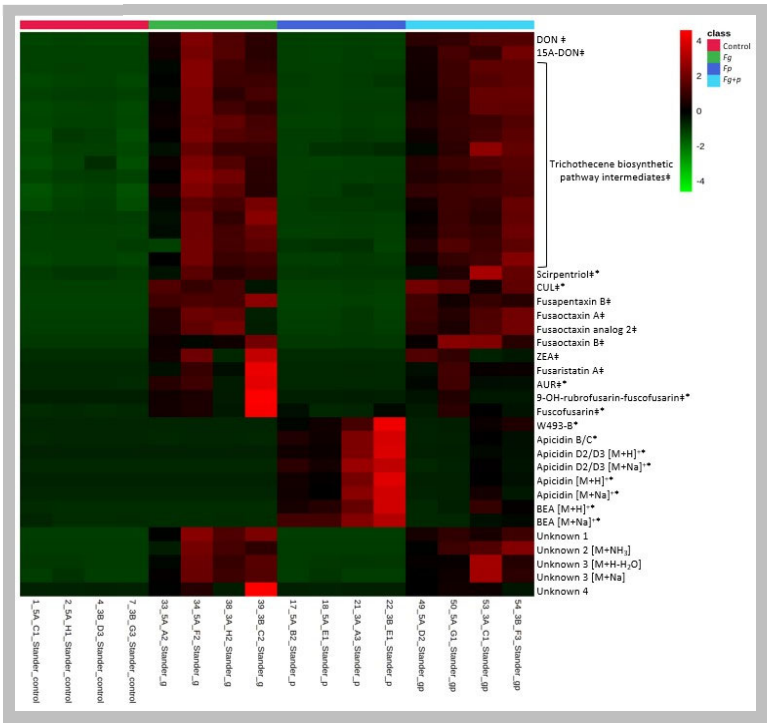


Figure 3.5: Non-binary heatmap of all 31 samples across 41 features produced by MetaboAnalyst 5.0 with some modifications: a) all samples, b) CDC Bold, c) Stander. Sample ID's are along the lower x-axis and feature names on the y-axis (‡ = Fg produces toxin, * = Fp produces toxin). The upper x-axis outlines the boundaries for each treatment group as represented in the legend.

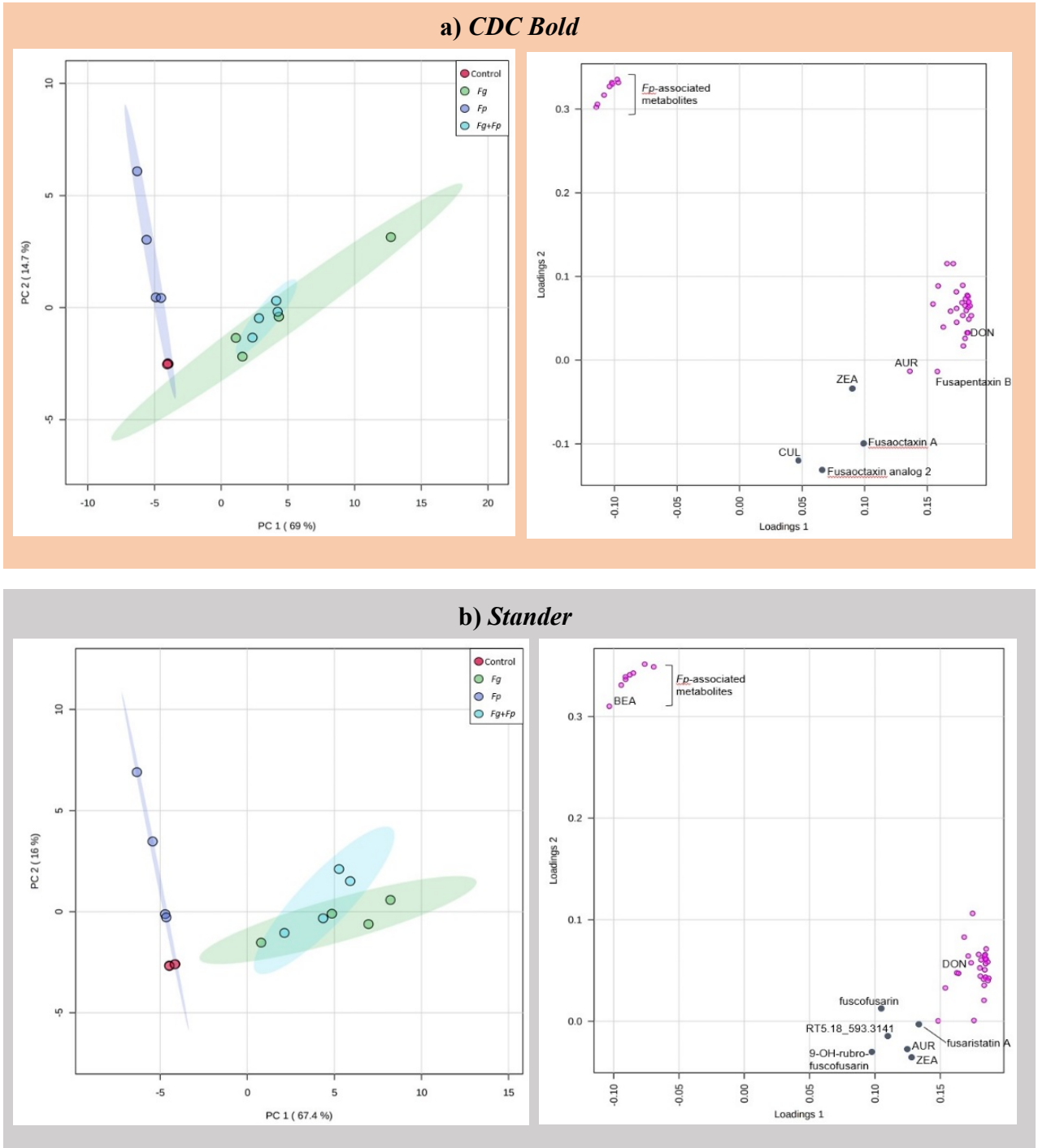


Figure 3.6: 2-D score plots (left) and loadings plots (right) from principal component analysis generated by MetaboAnalyst 5.0, separated by genotype: a) CDC Bold, b) Stander. Note: Black points in the loadings plot indicates the feature is not significant according to ANOVA.

According to the heatmap in Figure 3.5a and boxplots in Appendix C, detection of unknowns is strongest in *Fg*-treated samples. In fact, in the heatmap, most of all detected molecules appear to originate from *F. graminearum*-treated samples with 33 of 41 total features. The mycotoxin profile of the co-inoculation seems most influenced by *F. graminearum*, where signals appear up to the same intensity as recorded in the single *Fg* inoculation. For the most part, the median intensity of signals corresponding to *Fg*-type metabolites was the same from *Fg*-inoculated to co-inoculated (see boxplots in Appendix C). *F. poae* is capable of producing Type A and Type B trichothecenes and yet our assay does not detect any production of trichothecenes by *F. poae in planta*; in this case, they are produced exclusively by *F. graminearum*. *In planta*, apicidins, beauvericin, and cyclodepsipeptide W493-B are the predominant *F. poae*-associated metabolites. Furthermore, when *F. poae* is co-inoculated with *F. graminearum*, the detection of these *Fp*-type metabolites is dramatically lowered (Appendix C). While we detected greater mycotoxin diversity in the co-inoculation (as in *Fg*-treated produced A, *Fp*-treated produced B, and *Fg*+*p* produced A+B), accumulation intensity was not dramatically higher in the co-inoculation as compared to the single-species inoculations. Also, no new molecules were detected in co-inoculated samples versus *Fg*- or *Fp*-treated samples.

According to the heatmap in Figure 3.5a, signal intensity of metabolites varied with cultivar, particularly related to pigmentation metabolites (e.g. fusarins), culmorin, and fusaoctaxins. Separate ANOVA, heatmaps, and principal component analyses were therefore conducted in MetaboAnalyst for each cultivar. First, according to principal component analysis in Figure 3.6a, principal component 1 (PC1) explains 69% of variance on x-axis and principal component 2 (PC2) explains 14.7% of variance on y-axis. Metabolite patterns of co-inoculated

and *Fg*-treated samples trend along the x-axis, and *Fp*-treated in the y-axis. PC1 does account for some variance in *Fp*-treated samples, the majority of the variance in *Fg* and *Fg+Fp* samples. It appears that the variance of metabolite production explained by PC2 is mainly due to variance between replicates. It appears that metabolite patterns of CDC Bold is heavily influenced by *F. graminearum*, according to the nearly-overlapping confidence ellipses. Looking to the corresponding loadings plot in Figure 3.6a, *Fp*-associated metabolites, like beauvericin and apicidins, are clustered along the y-axis and are clear drivers in metabolite pattern variance related to *F. poae*. *Fg*-type metabolites like zearalenone, culmorin, fusaoctaxin A and fusaoctaxin analog 2 trail away from the main cluster towards the (0,0) origin. This suggests that the level of detection of these mass features are of a low abundance in particular sample replicates. Interestingly, according to Figure 4b, this is corroborated by the ANOVA where culmorin, zearalenone, fusaoctaxin A, and fusaoctaxin analog were not significant. Detection of 15A-DON, scirpentriol and a trichothecene biosynthetic pathway intermediate were also insignificant in CDC Bold-based ANOVA. Upon further inspection of the heatmap in Figure 3.5, the levels of detection of these metabolites are reduced in CDC Bold.

On the other hand, the principal component analysis from Stander samples was slightly different. In Figure 3.6b, PC1 explains 67.4% and PC2 explains 16% of variance. Similar to Figure 3.6a, PC1 explains most of the variance in metabolite patterns on the x-axis, and PC2 explains mainly variance between replicates. Here in Figure 3.6b, more of the variance in *Fg+Fp* metabolite pattern is explained by PC2. Looking to the confidence ellipses, *Fg* and *Fg+Fp* metabolism differs more in Stander than CDC Bold, but this appears due to variance between replicates. In the loadings plot, clustering similar to that observed in CDC Bold samples

is seen, however a different group of metabolites are trailing towards the origin: pigmentation-related molecules (i.e. aurofusarin, rubrofusarin, 9-OH-rubrofusarin-fuscofusarin). Different fungal metabolisms related to cultivar each are more clear when comparing Figure 3.5b and Figure 3.5c.

24.0 DISCUSSION

As discussed in previous chapters, *Fusarium graminearum* has long been accepted as the dominant cause of FHB worldwide. It appeared to be the main influencer in generating disease in Chapter 1 and outpaced *F. poae* in Chapter 2 where more *F. graminearum* DNA was isolated than *F. poae*; this theme continues here in Chapter 3 where features associated with *F. graminearum* treatment were more commonly detected by UPLC-HRMS.

Many other groups have seen that mycotoxin accumulation increases in co-inoculations when fungi are assumed to be competing for resources (X. Xu & Nicholson, 2009). Xu et al. (2007) observed mycotoxin accumulation up to 1000 times greater in wheat when *F. graminearum* was co-inoculated with *F. culmorum* and *F. poae* than in single-species inoculations. Simpson et al. (2004) investigated *in vitro* mycotoxin production of *F. culmorum* and *Microdochium majus* over time. They initially saw that pure *F. culmorum* and mixed cultures produced similar levels of DON, despite reduced *F. culmorum* fungal biomass from competition with *M. majus*, and suggested that DON productivity per unit of fungal biomass was higher in response to competition. By day 4, however, they saw that the amount of DON from the mixed culture decreased and that DON productivity was the same from pure and mixed cultures. Here in Chapter 3, no dramatic increases in DON production were observed when *F.*

graminearum was cultured with *F. poae* both *in vitro* or *in planta* in barley, recognizing that the study design may have missed critical timepoints to measure them.

When barley was co-inoculated with *Fg*+*Fp*, the observed mycotoxin profile closely resembled the single-*Fg* treatment. Given results collected in Chapter 1 and 2, this does not come as a surprise: disease was more intense in plants infected with *Fg*, simultaneously or not with *Fp*, and more *Fg* genomic DNA was isolated than *Fp*. *F. graminearum*'s apparent dominance here in Chapter 3 is an artifact of its fungal biomass advantage. However, signals of *Fg*-associated metabolites in co-inoculation were generally as high as that reported in single-*Fg* treatment (Appendix C). Moreover, levels of *Fp*-associated metabolites were significantly lower in the co-inoculation (Appendix C). It does not appear that there was any competition. As discussed in Chapter 1, the important takeaway may be that *F. poae* could indeed be an opportunistic pathogen. In Chapter 2, the ratio of *F. graminearum* to *F. poae* genomic DNA was unchanged in co-inoculation but here in Chapter 3, accumulation of *Fp*-associated metabolites decreases. Rather than *F. graminearum* actively suppressing *Fp*-associated metabolite production, perhaps *F. poae* downregulates its metabolite production in response to presence of *Fg*-associated metabolites. Again, trichothecene production here in Chapter 3 originates exclusively from *F. graminearum*, even when *F. poae* has the capability of producing them.

Toxin production is also highly dependent on the toxin-producing ability, and thereby relative competitiveness, of the isolate. For example, most North American isolates of *F. graminearum* produce either acetylated derivative of DON, 15-acetyl deoxynivalenol (15-ADON) or 3-acetyl deoxynivalenol (3-ADON) (Gilbert et al., 2010). Isolates can be

distinguished by their chemotype, or their ability to produce 15-ADON or 3-ADON. In recent years, there has been a shift in the Canadian *F. graminearum* population from the predominant 15-ADON chemotype to 3-ADON, raising concerns that it may be more aggressive (Gilbert et al., 2010, 2014; Ward et al., 2008). Wheat spray- and point-inoculated with a variety of isolates and chemotypes showed no significant differences in aggressiveness with respect to chemotype or origin; however, there was a significant increase in DON accumulation in 3-ADON-treated wheat (Gilbert et al., 2010). The isolate of *F. graminearum* used to infect our barley was of the 15-ADON chemotype, and so varying the isolates of *Fusarium* spp. used would likely affect results.

The use of *in vitro* cultures eliminates the plant matrix, and thereby the chemical communication that exists between the host and *Fusarium* spp.. For instance, *Fusarium graminearum* shows positive chemotropic growth of hyphae towards host-secreted peroxidases (Sridhar et al. 2020). From photos of MMK2 plates, the *Fg*-*Fp* relationship does not appear innately antagonistic, and suggests *Fg* and *Fp* are unaffected or do not communicate in an *in vitro* environment. The in-house database used for annotation is exclusively of *Fusarium* spp. metabolites and does not include plant defense metabolites. Important molecules that remain unknown after this analysis had the strongest signals when inoculated with *Fg*, suggesting they could be either generated by *F. graminearum* or produced by the host when infected with *F. graminearum*.

To study both pathogen and plant metabolites, Bollina et al. (2011) grew 6 six-row genotypes of barley with varying levels of resistance (5 resistant, 1 susceptible) under controlled

greenhouse conditions and inoculated with one isolate of *F. graminearum*. They categorized metabolites as resistance related (RR), metabolites whose signals were higher in resistant barley genotypes versus in susceptible, and resistance indicator metabolites, like DON and its partner DON-3-*O*-glucoside (D3G) once DON is detoxified by the plant host. They further filtered their list of plant resistance metabolites to those that held high levels of significance and higher fold changes, occurred in more than one genotype, and had history of plant immunity function as it relates to *Triticeae* fighting against *F. graminearum*. This produced a shortlist of 16 potential FHB-resistance related biomarker metabolites from phenylpropanoid, flavonoid, and fatty acid metabolic pathways. For each, *in vitro* studies showed *Fusarium* sp. biomass was inhibited by the plant metabolite, but their effect on *Fusarium* metabolites was not reported. To simplify the number of plant resistance mechanisms in barley, Chamarthi et al. (2014) performed similar work using doubled-haploid lines (4 resistant, 1 susceptible) with the rationale that these lines are homozygous and are therefore expected to have fewer traits at play from segregation. They employed similar strategies to Bollina et al. in identifying resistance related and resistance indicator metabolites, and filtered to a shortlist of potential FHB-resistance biomarkers, many of which overlapped with those identified by Bollina et al. in their work. Spiking samples with standards of suspected metabolites are also valuable to supporting molecule identification, as was done by Kumaraswamy et al. (2011). Spiking our *in planta* extracts with *in vitro* extracts and aligning their chromatograms to parse out which were plant-derived metabolites may be an avenue worth exploring, granted not all metabolites generated *in planta* will be produced when fungi may experience a different set of stressors cultured on media.

Thus far, six types of FHB resistance mechanisms have been described in cereal crops: resistance to initial infection (Type I), resistance to spread within infected spike tissue (Type II), resistance to kernel infection (Type III), tolerance to yield loss (Type IV), resistance to mycotoxin contamination (Type V), and resistance to alteration of grain components (Type VI) (Langevin et al., 2004; Martin et al., 2018; Mesterházy, 1995). Type V is also further subdivided into V-1, where the host may decompose or detoxify toxins produced, and V-2, where the host can prevent or inhibit toxin accumulation. Barley is inherently Type II resistant, however disease can spread between spikelets from the outside, instead of through the plant's vascular tissue like what is more often observed in wheat (Langevin et al., 2004). Six-row barley is generally more susceptible to disease than two-row, though this is unclear as to whether this is a product of plant architecture (i.e. lower spikelet density, prickly trichomes, more air circulation/less humid), or linkage of FHB-resistant QTLs to the *vrs1* locus, the gene conferring row-type (Langevin et al., 2004; Mesfin et al., 2003; Mesterházy, 1995; Stenglein & Rogers, 2010). Stander and CDC Bold are both FHB-susceptible varieties; however during literature review, CDC Bold was also referred to as an intermediate resistant variety (Geddes et al., 2008). Given the few differences in genotypes observed in our principal component analysis, could there also be variation in Type V resistance between Stander and CDC Bold? As mentioned above, Type V resistance is the resistance to mycotoxin accumulation, where the host decomposes or detoxifies toxins produced by pathogens (Type V-1), or where the host prevents or inhibits mycotoxin accumulation (Type V-2). For instance, acetylating trichothecenes has been reported as a detoxification strategy employed by various cereals (Kimura et al., 1998). Weber et al. (2018) examined culmorin, one of the same toxins putatively detected in our analysis, in wheat suspension cultures, and observed acetylation of culmorin. This same group also suggested the possibility of a synergistic

relationship between DON and culmorin accumulation in producing disease symptoms. Wipfler et al. (2019) examined the phytotoxic effects of culmorin alone and in combination with a number of trichothecenes, including DON and 15-ADON, on wheat root assays. Culmorin alone did not have any effect but when mixed with DON and 15-ADON, there was an even further reduction in wheat root elongation than when trichothecenes were applied alone. Interestingly, culmorin levels were generally lower in CDC Bold samples than in Stander. Could there be a barley genotype difference in culmorin activity modulation, thus conferring differences in Type V resistance?

While *in vitro* conditions may be 'less stressful' for fungi in that their substrate does not actively defend itself from colonisation, *in vitro* cultures are not always simple. In order to incubate plates all at the same level in the cabinet, the edges of some plates were stacked, potentially causing inconsistent humidity or gas levels inside plates. There also may have been some inconsistency in media formulation between plates, potentially limiting nutrient sources for some cultures.

In conjunction with genetic analyses, metabolomics approaches have been used to elucidate the major chemical mechanisms hosts use to defend and pathogens use to attack, and from there identify relevant biomarkers for breeders to develop more resistant germplasm. The reverse genetic approach proves to be especially difficult given resistance is the product of multiple small-effect genes working together, and is highly-influenced by the environmental growth conditions (Gauthier et al., 2015). Metabolomic analysis also extends as far as its annotation database, and while many of these are available, none are yet comprehensive.

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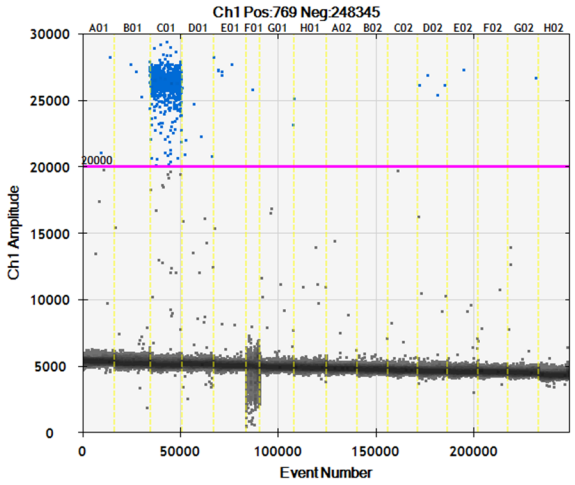
CONCLUSIONS AND FUTURE DIRECTIONS

In summary, challenging barley with *F. graminearum*, *F. poae*, or both in a controlled environment revealed a similar relationship across all three investigations: *F. graminearum* appears to outcompete *F. poae*. According to visual FHB ratings, disease was most severe when barley was inoculated with *F. graminearum* alone, and disease ratings neither worsened nor improved when *F. poae* was also present. These results suggested that *F. graminearum* outcompeted *F. poae* in the barley spike. When we zoom in further to quantify relative amounts of *F. graminearum* and *F. poae*, the ratio of *Fg* to *Fp* genomic DNA is unchanged comparing samples inoculated with both *F. graminearum* and *F. poae* versus either single species. These findings indicated that *F. poae* had no effect on *F. graminearum*'s ability to colonize barley, and that *F. graminearum* infection did not hinder *F. poae* colonization either. However, the amount of genomic DNA from *Fg* was greater than *Fp* indicating that *F. graminearum* was more successful in colonizing the barley spike. Previous literature has reported that *F. poae* detection is greater in non-epidemic conditions, and so further investigation is needed under other growth conditions that are less favourable to FHB epidemic to test whether individual species fitness is changed. The use of sequential inoculations, instead of mixed inoculations as it has been done in other fungal interaction studies with *Fusarium* spp., would also be valuable in observing competition between *F. graminearum* and *F. poae*. Previous literature has also shown that multiple species often co-exist in the same field, the same head and even grain. It is therefore also important that further investigations account for more species of *Fusarium* beyond *F. graminearum* and *F. poae* as these are not the only FHB-causing species relevant to Canadian farmers.

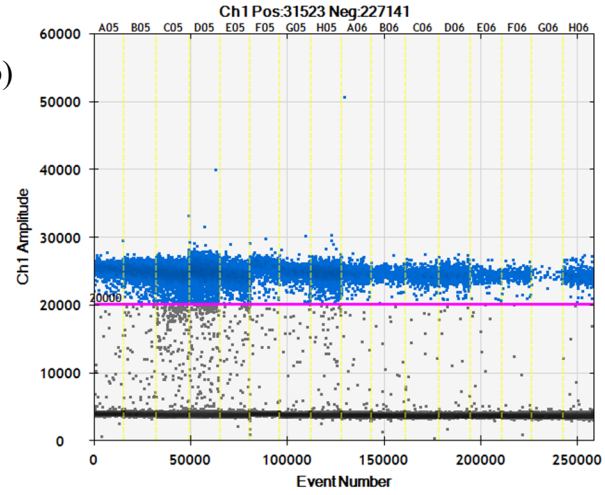
From metabolomic analysis, *F. graminearum*-associated metabolites dominate the mycotoxin profile of the co-inoculated samples. These findings indicate that treatment with *F. graminearum* largely influences the mycotoxins accumulated in grain when challenged with both species. Fungal metabolism did vary with barley cultivar. Future studies including more cultivars with varying levels of FHB resistance will also test whether all responses hold true against other genotypes. This is crucial for the practical application of our results to farmers. Most research is focused on protecting barley from *F. graminearum*. If resistant varieties behave similarly where they are relatively unaffected by *F. poae*, and by multiple strains of *F. graminearum* and *F. poae*, then growers can feel more confident in selecting a resistant cultivar to help protect their yield and grain quality from FHB. Even greater applicability would come with confirmation of these results in the field. Challenges lie in distinguishing barley response due to endemic *Fusarium* spp. in the soil and environment from artificially-applied inoculum.

APPENDIX A: QuantaSoft ddPCR outputs for GRA1 primers with DNA diluted to 1.25ng/uL and threshold of detection at 20K: (a) control, b) *Fg*-inoculated, c) *Fp*-inoculated, d) *Fg*+*Fp* co-inoculated, e) *Fg180378* and *Fp146* cultured *in vitro*

a)



b)



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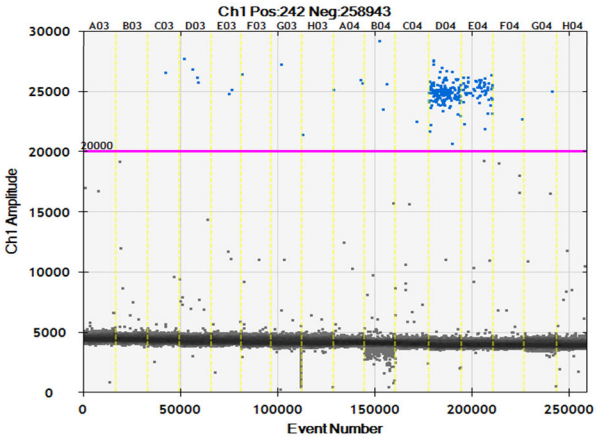
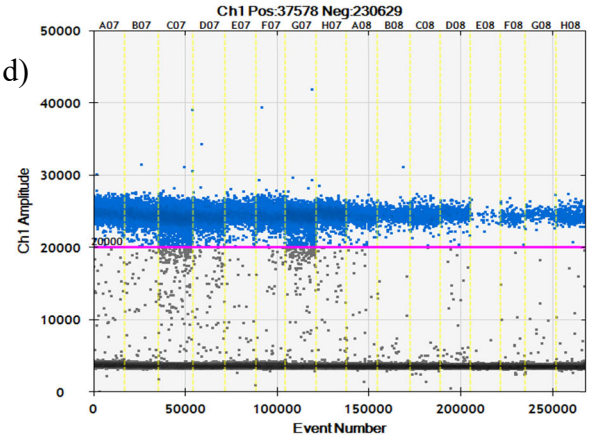
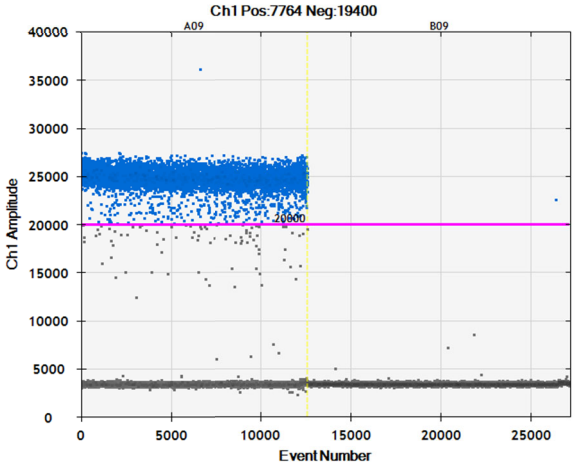


d)

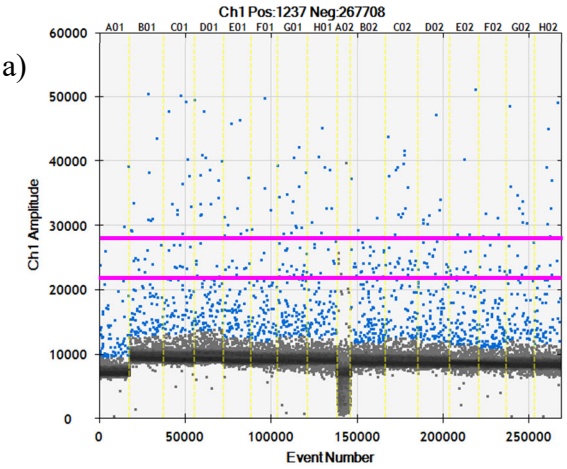


e)

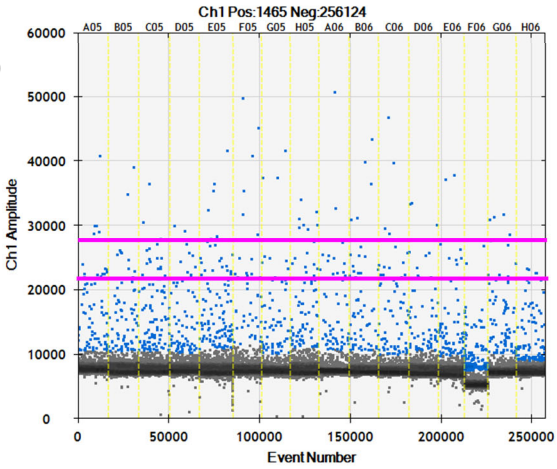


APPENDIX B: QuantaSoft outputs for APS1 primers with DNA diluted to 1.25ng/uL and thresholds of detection between 22 and 28K: (a) control, b) *Fg*-inoculated, c) *Fp*-inoculated, d) *Fg+Fp* co-inoculated, e) *Fg180378* and *Fp146* cultured *in vitro*

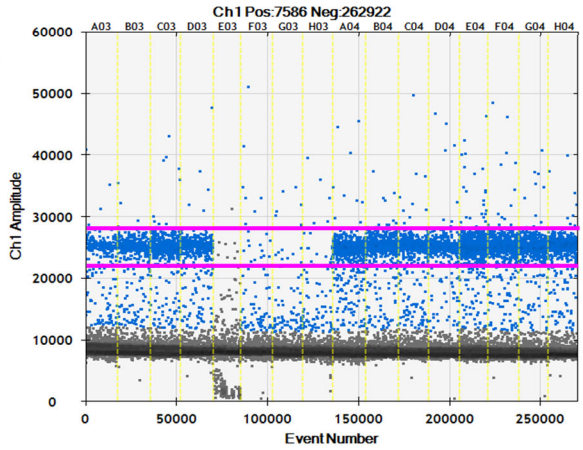
a)



b)



c)



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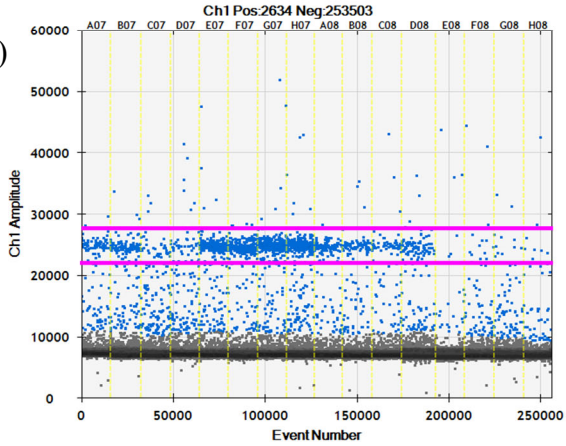

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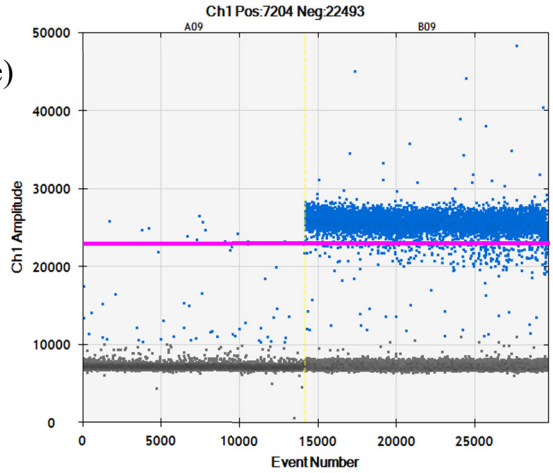
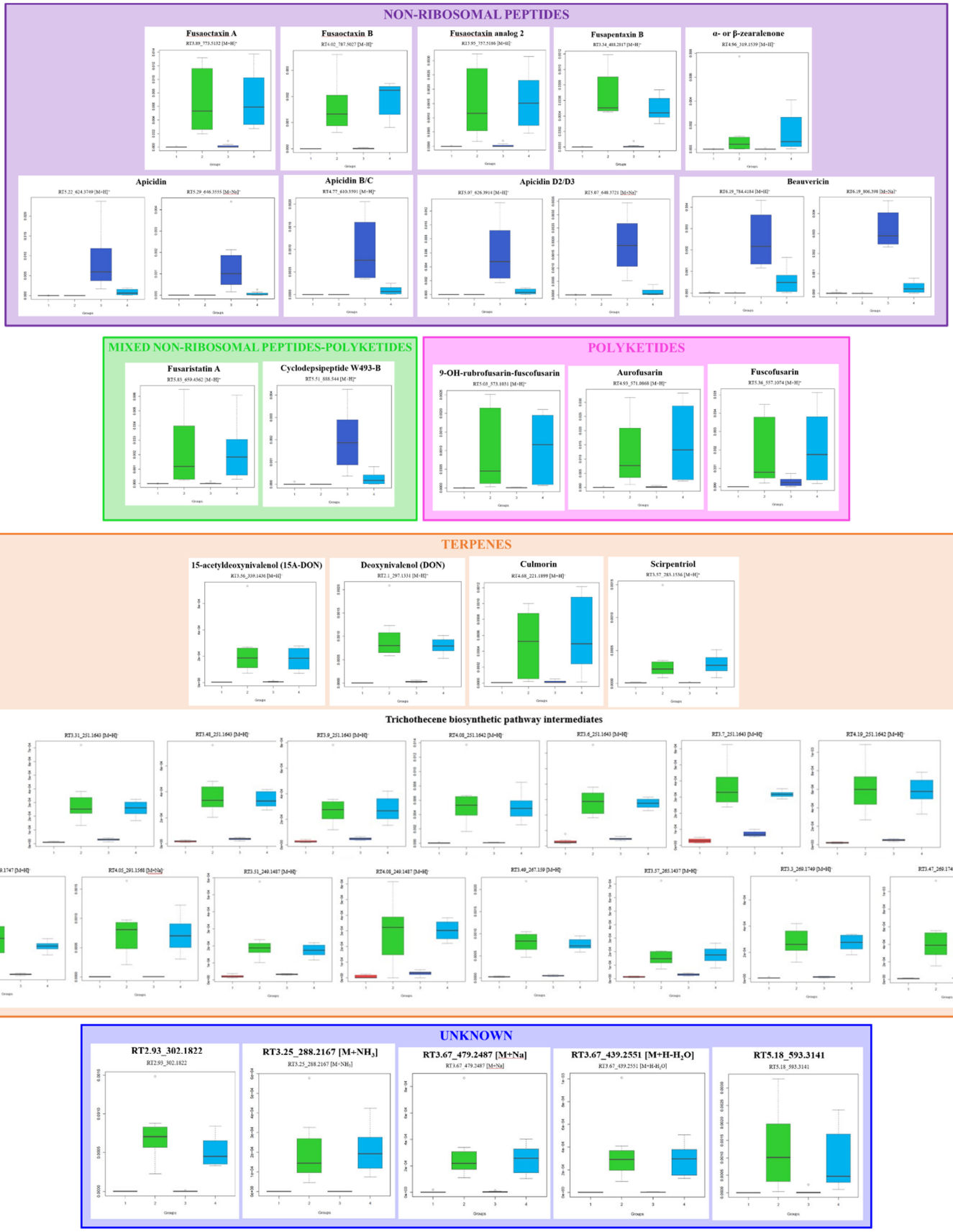


APPENDIX C: Collage of boxplots from univariate analysis generated by MUMA package in R, arranged by molecular family (purple = non-ribosomal peptide; light green = mixed non-ribosomal peptide-polyketide; pink = polyketide; orange = terpene; dark blue = unknown). Each y-axis represents intensity of signal on UPLC-HRMS normalized to the total ion current and on the x-axis, each group represents Treatment, where Group 1 = control, Group 2 = *Fg*, Group 3 = *Fp*, and Group 4 = *Fg*+*p*.



APPENDIX D: Feature details table from MetaboAnalyst 5.0 ANOVA using data from extracts of barley heads (cv. CDC Bold and Stander) infected with one of four *Fusarium* treatments (n = 31). Tissue was collected at 28 days post-inoculation.

Feature	*F-value*	*p-value*	*(-)LOG10(p)*	*FDR*	*Tukey's HSD*
RT6.19_806.398 Beauvericin [M+Na]+	80.106	1.47E-13	12.833	6.02E-12	3-1; 3-2; 4-3
RT3.7_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	48.711	5.02E-11	10.299	1.03E-09	2-1; 4-1; 3-2; 4-3
RT4.19_251.1642 Trichothecene biosynthetic pathway intermediate [M+H]+	35.688	1.55E-09	8.811	2.11E-08	2-1; 4-1; 3-2; 4-3
RT3.48_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	33.309	3.21E-09	8.4931	3.29E-08	2-1; 4-1; 3-2; 4-3
RT3.34_488.2817 Fusapentaxin B [M+H]+	30.156	9.05E-09	8.0435	7.42E-08	2-1; 4-1; 3-2; 4-3
RT2.1_297.1331 Deoxynivalenol [M+H]+	27.416	2.38E-08	7.6226	1.63E-07	2-1; 4-1; 3-2; 4-3
RT3.6_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	26.688	3.12E-08	7.5054	1.83E-07	2-1; 4-1; 3-2; 4-3
RT5.07_648.3721 Apicidin D2/D3 [M+Na]+	25.379	5.14E-08	7.289	2.63E-07	3-1; 3-2; 4-3
RT2.93_302.1822	24.809	6.43E-08	7.1921	2.93E-07	2-1; 4-1; 3-2; 4-3
RT4.02_787.5027 Fusaoctaxin B [M+H]+	22.319	1.78E-07	6.7494	7.09E-07	2-1; 4-1; 3-2; 4-3
RT3.49_267.159 Trichothecene biosynthetic pathway intermediate [M+H]+	22.165	1.90E-07	6.7209	7.09E-07	2-1; 4-1; 3-2; 4-3
RT6.19_784.4184 Beauvericin [M+H]+	20.868	3.35E-07	6.4752	1.14E-06	3-1; 3-2; 4-3
RT4.05_291.1568 Trichothecene biosynthetic pathway intermediate [M+H]+	20.643	3.70E-07	6.4314	1.17E-06	2-1; 4-1; 3-2; 4-3
RT3.3_269.1749 Trichothecene biosynthetic pathway intermediate [M+H]+	20.091	4.76E-07	6.3228	1.39E-06	2-1; 4-1; 3-2; 4-3
RT3.31_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	19.527	6.17E-07	6.2098	1.69E-06	2-1; 4-1; 3-2; 4-3
RT3.61_269.1747 Trichothecene biosynthetic pathway intermediate [M+H]+	18.718	9.04E-07	6.0438	2.32E-06	2-1; 4-1; 3-2; 4-3
RT4.08_251.1642 Trichothecene biosynthetic pathway intermediate [M+H]+	18.045	1.25E-06	5.9021	3.02E-06	2-1; 4-1; 3-2; 4-3
RT3.47_269.1749 Trichothecene biosynthetic pathway intermediate [M+H]+	17.567	1.59E-06	5.7994	3.62E-06	2-1; 4-1; 3-2; 4-3
RT4.08_249.1487 Trichothecene biosynthetic pathway intermediate [M+H]+	16.692	2.48E-06	5.6063	5.34E-06	2-1; 4-1; 3-2; 4-3
RT5.07_626.3914 Apicidin D2/D3 [M+H]+	14.417	8.45E-06	5.0733	1.73E-05	3-1; 3-2; 4-3
RT5.51_888.544 W493-B [M+H]+	14.261	9.23E-06	5.0348	1.73E-05	3-1; 3-2; 4-3
RT4.77_610.3591 Apicidin B/C [M+H]+	14.251	9.28E-06	5.0324	1.73E-05	3-1; 3-2; 4-3
RT3.9_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	13.591	1.36E-05	4.8671	2.42E-05	2-1; 4-1; 3-2; 4-3
RT3.51_249.1487 Trichothecene biosynthetic pathway intermediate [M+H]+	13.44	1.48E-05	4.8287	2.53E-05	2-1; 4-1; 3-2; 4-3
RT3.89_773.5132 Fusaoctaxin A [M+H]+	11.227	5.83E-05	4.2345	9.56E-05	2-1; 4-1; 3-2; 4-3
RT3.67_479.2487 [M+Na]	10.444	9.79E-05	4.0094	0.000154	2-1; 4-1; 3-2; 4-3
RT3.67_439.2551 [M+H-H2O]	10.259	0.00011089	3.9551	0.000168	2-1; 4-1; 3-2; 4-3
RT5.22_624.3749 Apicidin [M+H]+	10.018	0.00013083	3.8833	0.000192	3-1; 3-2; 4-3
RT3.95_757.5186 Fusaoctaxin analog 2	9.7781	0.00015448	3.8111	0.000218	2-1; 4-1; 3-2; 4-3
RT3.25_288.2167 [M+NH3]	8.8849	0.00029218	3.5344	0.000399	2-1; 4-1; 3-2; 4-3
RT3.56_339.1436 15A-DON/3A-DON [M+H]+	8.5046	0.00038677	3.4125	0.000512	2-1; 4-1; 3-2; 4-3
RT5.29_646.3555 Apicidin [M+Na]+	7.834	0.00064321	3.1916	0.000824	3-1; 3-2; 4-3
RT4.68_221.1899 Culmorin [M+H]+	7.4725	0.00085277	3.0692	0.00106	2-1; 4-1; 3-2; 4-3
RT4.93_571.0868 Aurofusarin [M+H]+	6.4359	0.0019778	2.7038	0.002385	4-1; 4-3
RT3.57_265.1437 Trichothecene biosynthetic pathway intermediate [M+H]+	6.3722	0.0020861	2.6807	0.002444	2-1; 4-1; 3-2
RT5.18_593.3141	5.7804	0.0034576	2.4612	0.003938	2-1; 3-2
RT5.03_573.1031 9-OH-rubrofusarin-fuscofusarin[M+H]+	5.1343	0.0061281	2.2127	0.006791	4-1; 4-3
RT5.83_659.4362 Fusaristatin A [M+H]+	4.9964	0.0069446	2.1584	0.007493	3-2; 4-3
RT5.36_557.1074 Fuscofusarin [M+H]+	4.8241	0.0081313	2.0898	0.008548	4-1; 4-3
RT3.57_283.1536 Scirpentriol [M+H]+	4.4727	0.011276	1.9479	0.011558	2-1; 3-2

APPENDIX E: Feature details table from MetaboAnalyst 5.0 ANOVA using data from extracts of barley heads (cv. CDC Bold) infected with one of four *Fusarium* treatments (n = 15). Tissue was collected at 28 days post-inoculation.

Feature	*F-value*	*p-value*	*(-)LOG10(p)*	*FDR*	*Tukey's HSD*
RT6.19_806.398 Beauvericin [M+Na]+	46.03	1.62E-06	5.7898	6.65E-05	3-1; 3-2; 4-3
RT5.07_648.3721 Apicidin D2/D3 [M+Na]+	31.017	1.16E-05	4.937	0.000237	3-1; 3-2; 4-3
RT4.19_251.1642 Trichothecene biosynthetic pathway intermediat	21.401	6.76E-05	4.1698	0.000924	2-1; 4-1; 3-2; 4-3
RT3.7_251.1643 Trichothecene biosynthetic pathway intermediate	15.107	0.00032379	3.4897	0.002442	2-1; 4-1; 3-2; 4-3
RT4.05_291.1568 Trichothecene biosynthetic pathway intermediat	14.277	0.00041335	3.3837	0.002442	2-1; 4-1; 3-2; 4-3
RT4.02_787.5027 Fusaoctaxin B [M+H]+	14.217	0.00042079	3.3759	0.002442	2-1; 4-1; 3-2; 4-3
RT2.93_302.1822	13.911	0.00046188	3.3355	0.002442	2-1; 4-1; 3-2; 4-3
RT3.34_488.2817 Fusapentaxin B [M+H]+	13.615	0.00050613	3.2957	0.002442	2-1; 4-1; 3-2; 4-3
RT5.51_888.544 W493-B [M+H]+	13.432	0.00053603	3.2708	0.002442	3-1; 3-2; 4-3
RT3.48_251.1643 Trichothecene biosynthetic pathway intermediat	11.822	0.00091266	3.0397	0.003742	2-1; 4-1; 3-2; 4-3
RT6.19_784.4184 Beauvericin [M+H]+	10.955	0.0012439	2.9052	0.004636	3-1; 3-2; 4-3
RT4.08_251.1642 Trichothecene biosynthetic pathway intermediat	10.051	0.001751	2.7567	0.005983	2-1; 4-1; 3-2; 4-3
RT4.93_571.0868 Aurofusarin [M+H]+	9.353	0.0023149	2.6355	0.007301	4-1; 4-3
RT5.03_573.1031 9-OH-rubrofusarin-fuscofusarin [M+H]+	8.4879	0.0033408	2.4762	0.009588	4-1; 4-3
RT3.6_251.1643 Trichothecene biosynthetic pathway intermediate	8.377	0.0035079	2.455	0.009588	2-1; 4-1; 3-2
RT3.47_269.1749 Trichothecene biosynthetic pathway intermediat	8.0937	0.0039817	2.3999	0.009825	2-1; 3-2
RT5.29_646.3555 Apicidin [M+Na]+	8.0433	0.0040737	2.39	0.009825	3-1; 3-2; 4-3
RT3.9_251.1643 Trichothecene biosynthetic pathway intermediate	7.6509	0.0048828	2.3113	0.010682	2-1; 4-1; 3-2; 4-3
RT2.1_297.1331 Deoxynivalenol [M+H]+	7.6217	0.0049501	2.3054	0.010682	2-1; 3-2
RT5.07_626.3914 Apicidin D2/D3 [M+H]+	7.4161	0.0054575	2.263	0.011188	3-1; 3-2; 4-3
RT3.49_267.159 Trichothecene biosynthetic pathway intermediate	6.9758	0.006765	2.1697	0.012238	2-1; 3-2
RT5.36_557.1074 Fuscofusarin [M+H]+	6.9084	0.0069965	2.1551	0.012238	4-1; 4-3
RT3.3_269.1749 Trichothecene biosynthetic pathway intermediate	6.871	0.0071289	2.147	0.012238	2-1; 3-2
RT3.31_251.1643 Trichothecene biosynthetic pathway intermediat	6.8613	0.0071637	2.1449	0.012238	2-1; 3-2
RT5.18_593.3141	6.4223	0.0089712	2.0471	0.014713	2-1; 3-2
RT3.61_269.1747 Trichothecene biosynthetic pathway intermediat	6.1087	0.010596	1.9749	0.016257	2-1; 3-2
RT5.22_624.3749 Apicidin [M+H]+	6.0895	0.010706	1.9704	0.016257	3-1; 3-2; 4-3
RT3.67_439.2551[M+H-H2O]	5.7509	0.012892	1.8897	0.018877	2-1; 3-2
RT4.08_249.1487 Trichothecene biosynthetic pathway intermediat	5.6318	0.013782	1.8607	0.019486	
RT4.77_610.3591 Apicidin B/C [M+H]+	5.5503	0.014434	1.8406	0.019726	3-1; 3-2; 4-3
RT3.67_479.2487 [M+Na]	4.9622	0.020378	1.6908	0.026951	3-2
RT3.51_249.1487 Trichothecene biosynthetic pathway intermediat	4.8605	0.021677	1.664	0.027773	2-1; 3-2
RT5.83_659.4362 Fusaristatin A [M+H]+	4.6735	0.02433	1.6139	0.030228	
RT3.89_773.5132 Fusaoctaxin A [M+H]+	4.1885	0.033195	1.4789	0.040029	
RT3.25_288.2167 [M+NH3]	3.9018	0.040213	1.3956	0.047106	

APPENDIX F: Feature details table from MetaboAnalyst 5.0 ANOVA using data from extracts of barley heads (cv. Stander) infected with one of four *Fusarium* treatments (n = 16). Tissue was collected at 28 days post-inoculation.

Feature	***F-value***	***p-value***	***(-)LOG10(p)***	***FDR***	***Tukey's HSD***
RT3.34_488.2817 Fusapentaxin B [M+H]+	58.648	1.93E-07	6.7141	7.92E-06	2-1; 4-1; 3-2; 4-3
RT2.1_297.1331 Deoxynivalenol [M+H]+	37.681	2.19E-06	5.6587	2.49E-05	2-1; 4-1; 3-2; 4-3
RT3.7_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	37.25	2.33E-06	5.6318	2.49E-05	2-1; 4-1; 3-2; 4-3
RT3.49_267.159 Trichothecene biosynthetic pathway intermediate [M+H]+	36.986	2.43E-06	5.6152	2.49E-05	2-1; 4-1; 3-2; 4-3
RT3.6_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	30.244	7.07E-06	5.1508	5.79E-05	2-1; 4-1; 3-2; 4-3
RT6.19_806.398 Beauvericin [M+Na]+	28.16	1.03E-05	4.9888	7.01E-05	3-1; 3-2; 4-3
RT3.48_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	26.525	1.40E-05	4.8542	7.86E-05	2-1; 4-1; 3-2; 4-3
RT3.56_339.1436 15A-DON/3A-DON [M+H]+	26.056	1.53E-05	4.8143	7.86E-05	2-1; 4-1; 3-2; 4-3
RT3.51_249.1487 Trichothecene biosynthetic pathway intermediate [M+H]+	23.789	2.44E-05	4.6123	0.000111	2-1; 4-1; 3-2; 4-3
RT4.68_221.1899 Culmorin [M+H]+	21.697	3.88E-05	4.4108	0.000159	2-1; 4-1; 3-2; 4-3
RT3.9_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	20.104	5.67E-05	4.2463	0.000198	2-1; 4-1; 3-2; 4-3
RT3.31_251.1643 Trichothecene biosynthetic pathway intermediate [M+H]+	20.002	5.82E-05	4.2354	0.000198	2-1; 4-1; 3-2; 4-3
RT2.93_302.1822	19.509	6.58E-05	4.1821	0.000198	2-1; 4-1; 3-2; 4-3
RT4.08_251.1642 Trichothecene biosynthetic pathway intermediate [M+H]+	19.406	6.75E-05	4.1707	0.000198	2-1; 4-1; 3-2; 4-3
RT4.19_251.1642 Trichothecene biosynthetic pathway intermediate [M+H]+	19	7.49E-05	4.1258	0.000205	2-1; 4-1; 3-2; 4-3
RT3.3_269.1749 Trichothecene biosynthetic pathway intermediate [M+H]+	18.435	8.67E-05	4.062	0.000222	2-1; 4-1; 3-2; 4-3
RT3.61_269.1747 Trichothecene biosynthetic pathway intermediate [M+H]+	18.122	9.42E-05	4.026	0.000227	2-1; 4-1; 3-2; 4-3
RT3.47_269.1749 Trichothecene biosynthetic pathway intermediate [M+H]+	17.134	0.000123	3.9088	0.000281	2-1; 4-1; 3-2; 4-3
RT3.89_773.5132 Fusaoctaxin A [M+H]+	15.775	0.000183	3.7384	0.000394	2-1; 4-1; 3-2; 4-3
RT3.95_757.5186 Fusaoctaxin analog 2 [M+H]+	14.089	0.000309	3.5106	0.000633	2-1; 4-1; 3-2; 4-3
RT4.05_291.1568 Trichothecene biosynthetic pathway intermediate [M+Na]+	13.162	0.00042	3.3763	0.000821	2-1; 4-1; 3-2; 4-3
RT3.57_265.1437 Trichothecene biosynthetic pathway intermediate [M+H]+	11.349	0.000809	3.0918	0.001509	2-1; 4-1; 3-2; 4-3
RT3.25_288.2167 [M+NH3]	11.124	0.000883	3.0543	0.001573	2-1; 4-1; 3-2; 4-3
RT4.02_787.5027 Fusaoctaxin B [M+H]+	10.626	0.001073	2.9692	0.001834	2-1; 4-1; 3-2; 4-3
RT5.07_648.3721 Apicidin D2/D3 [M+Na]+	10.482	0.001137	2.9441	0.001855	3-1; 3-2; 4-3
RT4.08_249.1487 Trichothecene biosynthetic pathway intermediate [M+H]+	10.399	0.001176	2.9295	0.001855	2-1; 4-1; 3-2; 4-3
RT3.67_479.2487 [M+Na]	10.23	0.00126	2.8997	0.001913	2-1; 4-1; 3-2; 4-3
RT3.67_439.2551 [M+H-H2O]	8.8364	0.002298	2.6387	0.003365	2-1; 4-1; 3-2; 4-3
RT3.57_283.1536 Scirpentriol [M+H]+	8.1357	0.003184	2.497	0.004501	4-1; 4-3
RT6.19_784.4184 Beauvericin [M+H]+	7.6674	0.004	2.398	0.005466	3-1; 3-2
RT4.77_610.3591 Apicidin B/C [M+H]+	7.6006	0.004135	2.3835	0.005469	3-1; 3-2; 4-3
RT5.07_626.3914 Apicidin D2/D3 [M+H]+	6.3251	0.008098	2.0916	0.010375	3-1; 3-2; 4-3
RT5.22_624.3749 Apicidin [M+H]+	5.7504	0.011248	1.9489	0.013974	3-1; 3-2; 4-3
RT5.29_646.3555 Apicidin [M+Na]+	5.5206	0.012893	1.8897	0.015547	3-1; 3-2
RT5.51_888.544 W493-B [M+H]+	3.7495	0.041347	1.3836	0.048435	