THE ROLE OF PLANT HORMONES IN FUSARIUM HEAD BLIGHT OF WHEAT

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Abstract

Fusarium Head Blight (FHB) is a devastating disease of cereals caused by a group of trichothecene-producing fungi belonging to the *Fusarium* genus. In plant-pathogen interactions, hormones (salicylic acid, jasmonic acid and ethylene) modulate a series of defence responses, including expression of pathogenesis-related (*PR*) genes, which limit pathogen spread or obliterates it entirely. The gene expression data from three wheat genotypes presented in this thesis suggests that *PR* gene regulation by plant hormones may be genotype-dependent in wheat. Exogenous application of ethylene activators/inhibitors in six wheat genotypes with different level of resistance to FHB showed that ET plays a positive role in the resistance response. A similar response was observed in *Fusarium* seedling blight (FSB) disease assays, where exogenous chemical treatments showed that ethylene is involved in a resistance response. In addition, SA was shown to play a positive role in resistance to FSB, while MeJA increased susceptibility.

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Chapter One: Literature Review

1.1 Introduction

Fusarium Head Blight (FHB) is a devastating disease of cereals caused by a group of fungi belonging to the Fusarium genus. The fungus, *F. graminearum* and related spp., infects wheat heads and produces toxic metabolites, such as deoxynivalenol (DON) and other trichothecene mycotoxins. Accumulation of trichothecenes in grains can reduce food quality and seed germination (Desjardins and Hohn, 1997). Cultivation of FHB resistant wheat varieties is recognized as the best way to control the disease and eliminate mycotoxins from the food chain (Foroud and Eudes, 2009). Several Types of FHB resistance have been described, where Type I (resistance to initial infection) and Type II (resistance to disease spread) are the best characterized (Schroeder and Christensen, 1963). For Type II resistance measurement, individual spikelets are inoculated with a quantifiable amount of inoculum. Resistance is measured as the number of diseased spikelets below the inoculation point (Rudd et al., 2001). Measurement of Type I resistance is less straightforward. The heads should be exposed to inoculum and percentage of diseased spikes per plant after exposure to pathogen (disease incidence) and percentage of infected spikelets on diseased spikes (severity) are measured as Type I resistance (Dill-Macky, 2003). However, this method does not accurately measure Type I resistance because diseased spikelets after exposure of the heads to inoculum may not be related to initial infection and can result from disease spread within the head (Rudd et al., 2001). One of the problems in Type I resistance assessment is a lack of uniform exposure of heads within a plant or a plot to FHB inoculum that affects the reliability and reproducibility of results in experiments, thus not allowing for an appropriate evaluation of the level of Type I

resistance. To address this issue a detached head assay was developed in this study to provide a better assessment for Type I measurement and also facilitate chemical application in other experiments.

Signalling pathways modulate a series of defence responses that limit the pathogen spread or obliterate it entirely. This signalling often involves plant hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). The expression of pathogenesis-related (*PR*) genes is one of the inducible defence responses that are activated by plant hormones. In the case of FHB, there are some contradictory results in the literature regarding which signalling pathways are involved in mediating resistance responses in cereals. This study was conducted with an objective to further investigate the role of plant hormones and cross-talk among them in different wheat genotype responses to FHB.

1.2 A brief history of FHB in North America

W. G. Smith first described FHB as "wheat scab" in 1884 in England (Arthur, 1891). Since W.G. Smith's first description of FHB, the disease has been reported in North America and in many areas worldwide. FHB was the main disease of wheat and barley during the early years of twentieth century (Stack, 2000).

Fusarium is a large genus of ascomycetes with teleomorphs in *Gibberella*, *Albonectria*, and *Haematonectria*, but in some species such as *F. sporotrichioides* the teleomorph has not been identified yet (Manka et al., 1989). *F. graminearum*, *F. avenaceum* and *F. culmorum* are the most common causal agents of FHB in North America and many other parts of the world (Goswami and Kistler, 2004). In Canada, *F. graminearum* was first reported on corn stubble in Manitoba in 1921 (Bisby and Bailey, 1923). During the period between 1920-1940, *F. graminearum* was described as a rare isolate from grain samples in western Canada (Sutton, 1982). In the next forty years and in 1980s there were outbreaks

of FHB in Ontario, Québec, Manitoba and the Peace River Region of Alberta. According to rainfall data from weather stations in these regions average rainfall was much higher than normal in the epidemic years. In 1984, *F. graminearum* heavily infected durum and spring wheat samples from the red river valley of southern Manitoba. In western Canada, *F. graminearum* was found in the black soil zone where the highest rainfall occurs and highly FHB-susceptible wheat varieties have been cultivated (Clear and Patrick, 2000; McMullen et al., 1997; Wang et al., 2003).

In the early part of 1989, the Canadian Grain Commission (CGC), responsible for official grading standards, reported that a few wheat kernels in southern Alberta were infected by *F. graminearum* demonstrating that conditions such as precipitation and moisture content for FHB spread in this region was favourable. Due to low precipitation and moisture at heading time for most of the Alberta regions, *F. graminearum* infection was not successful in Alberta (Tekauz et al., 2000). From 1991 to 1996, FHB outbreaks have been prevalent across eastern United States and localized epidemics also occurred in southern Manitoba, where the rain fall was above average during those years and led to high humidity conditions favourable for FHB development (Gilbert et al., 1995). In the 1990s, FHB caused severe losses for the Canadian grain industry, about $300 million in Manitoba alone (Windels, 2000). In addition to favourable weather conditions, other major factors contributing to FHB epidemics in 1990s include conservation tillage, cultivation of highly FHB susceptible cultivars, and short rotations between susceptible host plants (McMullen et al., 1997). *F. graminearum* spores can survive on the residue of infected crops such as wheat, barley and corn, therefore, conservation tillage can result in inoculum build up. By the year 2000, drier conditions in northern Alberta and cool weather in central and southern Alberta were not favorable for FHB (the Canadian Grain Commission, 2009).

In 2008, approximately 50% of the wheat grown in Manitoba was down degraded due to FDK. According to the CGC description, kernels having visible mycelium in the dorsal crease and orange-pink sporodochia or erumpent black spots (perithecia of *Gibberella* zeae) on their surface are defined as Fusarium-damaged kernels (FDK) (http://www.grainscanada.gc.ca/guides-guides/identification/fusarium). The following year, humid conditions at the heading stage resulted in substantial levels of FHB infection in wheat crops in southern Alberta. Unfortunately, from 2009 to 2016, the percentage of FDK detection and severity of Fusarium damage in western Canada has increased. In 2016, wheat samples collected from five out of nine regions in Saskatchewan had 100% FDK; meanwhile in Alberta, 100% FDK was observed in one out of five regions. Fusarium distribution data and maps from the CGC website have demonstrated a significant increase in severity of FHB in western Canada from 2011 to 2016. The highest level of FDK was reported for samples collected from Saskatchewan in 2014, while in other regions the disease severity only increased during the period from 2014 to 2016. In Alberta, in 2016, the frequency of FDK increased from an average of 0.68% to 3.5% (https://www.grainscanada.gc.ca/str-rst/fusarium/fhbmc-feccg-en.htm). The average daily mean temperature and average level of precipitation in the late spring and early summer in the western prairies from 2011 to 2016, precipitation and temperature increased (http://climate.weather.gc.ca/climatedata/dailydatae.html). Since warm temperature and high precipitation favors FHB (Al Masri et al., 2017; Del Ponte et al., 2009), this can explain the high incidence and severity of FHB in western Canada, and specifically southern Alberta, observed in those years.

The kernels that develop from FHB-diseased spikes are often contaminated with trichothecenes, such as 4-deoxynivalenol (DON) (Ward et al., 2008). Studies showed that

percentage of FDK can be used to reliably estimate DON content (Mesterházy, 2002; Miedaner et al., 2000). Historically, 15-O-acetyl DON (15-ADON) chemotype was known as the most common chemotype in the North America. Over the last twenty years, however, a shift in the trichothecene genotype and/or chemotypes of the *F. graminearum* species complex has taken place, resulting in evolution of chemotypes with increased 3-O-acetyl DON (3-ADON) in the North America (Ward et al., 2008). It has been shown that the 3-ADON chemotypes are more virulent than the 15-ADON chemotypes (Ward et al., 2008). The frequency of Fusarium genotypes of DON derivatives in 2016 showed that most of the samples were infected by 3-ADON genotypes in Alberta while most wheat samples harvested from Saskatchewan and Manitoba showed an equal frequency of 3-ADON and 15-ADON (50%-50%) (http://www.grainscanada.gc.ca/guides-guides/identification/fusarium).

1.3 The impact of FHB

Trichothecene accumulation in FHB-infected grains contributes directly to yield losses. Significant losses occur when wheat spikes become infected during anthesis to the early stage of kernel development resulting in FDK (Steffenson, 2003). FHB-infected spikes give rise to seeds that are contaminated with DON, which is the most prevalent trichothecene. Often, diseased grains can be easily distinguished as FDK; however, the kernels may not always show visible damage, and still be loaded with toxins. Consumption of trichothecene-containing grains can cause gastroenteric inflammation in mammals resulting in feed refusal, and also can lead to a mortality known as alimentary toxic aleukia (ATA) (Agag, 2005; Pierron et al., 2016). Symptoms of ATA include skin blistering, gastrointestinal irritation, vomiting, and abdominal cramps (Adhikari et al., 2017). During the 1920s, farmers reported that infected barley, which was grown mainly as a feed crop in

the Midwest, led to animal feeding problems including vomiting and food rejection. Pigs and horses were reported to be the most affected (Dickson et al., 1930; Mains and Curtis, 1929). Mundkur and Cochran (1930) reported that *Fusarium* species were the most recovered fungi from infected barley causing animal feeding issues. Shands (1937) clarified that it was a fungal product, rather than the fungus itself that led to feed refusal and digestion issues. After the second World War, in Russia, due to shortage in the food supply people consumed moldy overwintered grains which led to the deaths of over 100,000 people (Joffe, 1986). Joffe (1986) later identified as the trichothecene toxins, T-2 toxin and HT-2 toxin, produced by *Fusarium sporotrichioids* or *Fusarium poae*, from old grain samples collected at that time.

1.4 The causative agents of FHB

1.4.1 *Fusarium graminearum* morphology and disease cycle

During the 19th century, in most regions of North America affected by FHB, *Gibberella zeae* was found as the predominant pathogen (McMullen et al., 1997). However, during that time in Europe, the principal FHB causative agents were *F. avenaceum*, *F. culmorum* and *F. nivale* (Atanasov, 1920; Bennett, 1928). From 1937 to 1942, in Canada, *F. graminearum* and *F.culmorum* were rarely identified in cereal grains (Gordon, 1954). In eastern Canada in 1982, *F. poae* predominated in wheat heads (Sturz and Johnston, 1985). In recent years, among the many *Fusarium* species that cause FHB, *F. graminearum* and *F. culmorum* were the primary pathogens responsible for this disease in Canada (https://www.grainscanada.gc.ca/str-rst/fusarium/hfhb-hf-eng.htm).

1.4.2 Infection and disease cycle

Fusarium species produce asexual, fusiform spores (macroconidia) with elongated 5-6 septa and are pale orange that possess a foot-shaped basal cell and a tapered apical cell

(Figure 1.1A). The number and the size of the septa are used to differentiate between species (Leslie and Summerell, 2008). Ascospores (sexual spores) are produced during of some *Fusarium* species, and the sexual stage, are curved-shaped with 1-3 septa which are released from a dark purple to black perithecium (fruiting body).

F. graminearum overwinters on infected crop residues such as corn stalks, wheat straw or other host plants, and in the soil as saprophytic mycelia (Khonga and Sutton, 1988). On infested residues, the fungus produces macroconidia (Leslie and Summerell, 2008). Macroconidia are released from the sporodochia and transported in the air by rain-splash or the wind. Under favorable conditions (warm, humid, and wet), the *F. graminearum* moves into its sexual stage (*Gibberella zeae*) and perithecial fruiting bodies develop on the infected plant residues (Minnaar-Ontong, 2011). Infection occurs when the ascospores or macroconidia land on a susceptible host. After inoculation of the wheat spikes, stems are colonized systemically and mycelia move down the vascular bundles and radially colonize the stem tissue (Guenther and Trail, 2005). Florets that are infected produce diseased kernels that are shriveled and discolored. Kernels that are colonized by the pathogen during late kernel development may not appear to be affected, but are contaminated with mycotoxin produced by the fungus (Schmale and Bergstrom, 2003).

It has been reported that the flowering stage is the most susceptible stage to *F. graminearum* infection (Atanasov, 1920; Pugh et al., 1933; Selby and Manns, 1909). In 2009, Del Ponte et al. carried out a more detailed study on the influence of growth stage on disease outcomes, and found that the highest DON accumulation and reduction in grain weight occurred in wheat plants inoculated at flowering to late-milk stages. In the high yielding wheat cultivar, ‘Plump’, late infection led to significant levels of DON although there was no significant change for grain weight between non-inoculated and inoculated

grains. These results showed that time of infection has an important effect on grain weight reduction and mycotoxin accumulation thus suggesting that late infection and DON production should be considered in a grading system where only presence of visible FDK is employed for estimating DON content of the wheat.

Warm and humid conditions favor perithecial development and maturation on plant residues (Trail et al., 2002). Ascospores and macroconidia do not require dormancy and can germinate within 3 to 6 hours in water without exogenous nutrients (Cappellini and Peterson, 1971). Anderson (1948) demonstrated the importance of specific environmental parameters on disease development. He studied the relationship among temperature, humidity duration and flowering stage on *F. graminearum* infection. He indicated that FHB development after infection has been established depends on the developmental stage of the host plant, the duration of exposure to humidity, and particularly temperature. Among these factors temperature and incubation period under humidity after inoculation were shown to be critical for infection and disease development.

Pugh et al. (1933) studied the effect of temperature on FHB infection, and they found that infection had a reverse relationship with the duration of incubation at 28ºC, where the highest infection level in their experiment was achieved with the shortest incubation period (Pugh et al., 1933). Rossi et al. (2011) studied the influence of temperature and humidity on the infection of wheat spikes by some causative agents of FHB including *F. avenaceum*, *F. graminearum* and *F. culmorum*. They showed that infection rate was optimal at 28-29ºC for *F. avenaceum* and *F. graminearum*, and at 26.5ºC for *F. culmorum*. To study the effect of humidity, they inoculated the spikes of wheat with *F. graminearum* and *F. avenaceum* and incubated the infected tissues at 28-29ºC under different regimes of relative humidity (65-100%). They concluded that a reduction in humidity during the initial infection process

resulted in an unfavorable condition for *F. avenaceum* and *F. graminearum* to proliferate and cause disease (Rossi et al., 2001). These results support earlier work by McMullen et al. (1997) where early stages of disease development appeared to be most dependent on moisture.

1.4.3 Disease symptoms in wheat and barley infected with FHB

In wheat and barley, the most FHB-susceptible organ is the head (spike) and the highest infection level occurs during anthesis. The anatomy of the wheat spike is shown in (Figure 1.2). In wheat, the hyphae of the *Fusarium* species develop on the surface of the floret, allowing the fungus to grow toward susceptible sites such as stomata, leading to direct penetration through the epidermis (Bushnell et al., 2003). Other paths for direct penetration are fully exposed anthers, openings between the lemma and palea of the spikelet and the base of the wheat glumes, a bract leaf-like structure, where the epidermal cells are thin-walled (Bushnell et al., 2003). FHB disease symptoms first emerge as small reddish brown lesions on the base of the glumes below a spikelet around the middle of the head (Bushnell et al., 2003). As the disease progresses, lesions grow to form large necrotic areas, spread vertically through the vascular bundles in the rachis and rachilla of the spike (Ribichich et al., 2000) resulting in the appearance of bluish-black perithecia on the surface of FHB-infected wheat heads (Saharan et al., 2004). Infected spikelets are shriveled and chalkywhite compared to normal healthy green spikelets, and are covered with fine white filaments (hyphae) called mycelia (http://www.grainscanada.gc.ca/guides-guides/identification/fusarium). When infection spreads toward the bottom of the wheat head, the rachis may turn dark brown (Celetti et al., 1998).

1.5 Mycotoxins produced by FHB

1.5.1 Trichothecenes

Fusarium species produce mycotoxins, including trichothecenes, during the infection process, which accumulate in the kernels of infected spikelets. Mycotoxins affect grain quality and make the grain unsuitable for human or livestock consumption. Trichothecenes are toxic secondary metabolites composed of a tricyclic core and an epoxide function that has been shown to be essential for toxicity (Foroud and Eudes, 2009). Trichothecenes are protein translation inhibitors (McLaughlin et al., 1977) that bind to the A-site of the peptidyl transferase center in the 60S ribosomal subunit (de Loubresse et al., 2014). Additional cytotoxic effects of trichothecene include inhibition of DNA synthesis, RNA synthesis, negative impact on cell division, membrane integrity and mitochondrial function (Bin-Umer et al., 2011).

Four types of trichothecenes have been identified from trichothecene-producing fungi: type A, B, C and D. The type A trichothecenes T-2 toxin and diacetoxyscirpenol (DAS) both have an isovalerate ester at C-8 while all type B trichothecenes (including nivalenol (NIV), DON and their acetylated derivatives) have a keto group at C-8 (Mirocha and Xie Filho, 2003). Type C and D trichothecenes are not associated with FHB (Sudakin, 2003). Type A trichothecenes are extremely toxic in mammalian systems; T-2 toxin has been reported to be ten times more toxic in mammals than DON (Ueno, 1983). However, DON is more phytotoxic than T-2 toxin, and is the most common toxin associated with FHB and belongs to type B trichothecenes (Draeger et al., 2007; Eudes et al., 2001; Mudge et al., 2006; Wu et al., 2013). *Fusarium* species also produce other mycotoxins, such as fumonisins, zearalenone (ZON), moniliformin and butenolid (Desjardins et al., 2007).

Since consumption of diseased-grains which contain a high level of DON accumulation is harmful, Canadian Food Inspection Agency and Health Canada perform grain testing for DON to prevent contaminated grains from entering the food chain. DON-testing is conducted on the end-products by Canadian Food Inspection Agency and Health Canada to ensure the maximum allowable of DON in food products is not exceeded. Currently, the maximum allowable DON content in food is 2 ppm in uncleaned Canadian soft wheat for use in non-staple foods and 1 ppm in uncleaned soft wheat for use in baby foods (www.hc-sc.gc.ca/fn-an/securit/chem-chim/contaminants-guidelines-directives-eng.php).

1.5.2 Trichothecenes as aggressiveness factors in FHB

Different isolates of a given *Fusarium* species exhibit different levels of aggressiveness and pathogenicity. There is a relationship between FHB severity and DON accumulation in infected grain (Snijders and Krechting, 1992; Wong et al., 1995). It has been reported that within a barley field, a mixture of *F. graminearum* strains or other *Fusarium* species, often coexist within the same head (McCallum and Tekauz, 2002). The cumulative impact of multiple trichothecenes produced by different *Fusarium* isolates may increase disease severity (Mesterházy et al., 1999; Touati-Hattab et al., 2016). It was shown that an inoculum containing a mixture of *Fusarium* species (*F. culmorum*, *F. avenaceum* and *F. graminearum*) were able to produce a more severe FHB reaction than inocula containing individual species in winter triticale, wheat and rye; thus it can be concluded that a diversity of trichothecenes may contribute to severity of FHB (Arseniuk et al., 1999).

TRI5 is one of the virulence genes of *F. graminearum* that encodes the first committed enzyme (trichodiene synthase) in the trichothecene synthase pathway (Proctor et al., 1995). A trichothecene non-producing strain of *F. graminearum* was generated by disruption of the locus encoding trichodiene synthase (Proctor et al., 1995). In order to investigate the

role of trichothecenes in FHB aggressiveness, this mutant was compared with wild type parents. Results showed that the mutant produced lesser incidence and severity of infection which confirmed a role for trichothecenes in the disease (Bai et al., 2001; Eudes et al., 2001; Proctor et al., 1995). Similar results were observed in a Fusarium seedling blight assay in wheat, oats, and winter rye, where a reduced virulence was observed for a trichothecene non-producing strain of *F. graminearum* (Bai et al., 2001).

1.6 Fusarium head blight management strategies

FHB epidemics occur when the inoculum source is high and humidity levels are elevated at anthesis and, susceptible hosts are present (Parry et al., 1995). FHB management practices that reduce inoculum build-up, or intervene with dispersion of spores can effectively reduce the severity of FHB epidemics (Parry et al., 1995). *F. graminearum* survives on the debris of wheat, barley, oats, maize and rice (Bai et al., 2001; Leplat et al., 2013; Sutton, 1982). In the areas where small grains (cereals) and maize are grown, it is likely that maize is the more important source of inoculum, since the debris lasts longer than small grain. *Fusarium* survival is also increased by nutrient rich residues such as maize (Leplat et al., 2013). In the regions where small grain are grown more than maize, high quantity of wheat and barley debris are a major inoculum source (Leplat et al., 2013; McMullen et al., 1997). Since the inoculum source is often present in the form of ascospores in the soil or residues, crop rotation is an effective disease management practice that reduces the frequently suitable host for the pathogen and therefore reduces the amount of infected residue for pathogen overwintering (Vanova et al., 2008). Studies have shown that corn-wheat rotation increases inoculum level since both wheat and corn are susceptible hosts to FHB (Dill-Macky and Jones, 2000; McMullen et al., 2008; Pirgozliev et al., 2003),

but wheat-clover (nonhost crop for FHB) rotation decreases inoculum buildup (Dill-Macky and Jones, 2000; McMullen et al., 2008; Vanova et al., 2008).

Other management practices that can effectively reduce inoculum build-up in the soil include tillage. Inversion tillage buries the fungal spores that survive on top of the soil and on crop residue (David Miller et al., 1998; Dill-Macky and Jones, 2000). The effect of crop rotation (soybean-wheat and corn-wheat rotations) and tillage (Moldboard plow, chisel plow, and no-till treatments) on FHB of wheat was investigated. The corn-wheat rotation showed the highest FHB severity and incidence but severity and incidence were lower in moldboard plowed plots compared with either chisel plowed or no-tillage (Dill-Macky and Jones, 2000). It has been reported that when zero-tillage was used up to 9.10^3 colony-forming units of *F. graminearum* and of *F. culmorum* per g of soil were found whereas this level decreased 10 times when inversion tillage was applied (Leplat et al., 2013).

Chemical control is also practiced to control FHB in the field although this control shows limited success (Blandino et al., 2006; Yuen and Schoneweis, 2007). In addition to the cost of fungicides for producers, the available fungicides for FHB, such as tebuconazole, have not shown an effective impact on control of the disease (Yuen and Schoneweis, 2007). A fungicide called Prospero, which is a combination of prothicanozole and tebucanozole, has shown more promising results for FHB control (Paul et al., 2005). Yuen and Schoneweis (2007) studied strategies for managing FHB and showed that fungicide application slowed residue decomposition rate by 23%. Therefore, while fungicides may prevent the growth of fungi in crop residues, they can have a negative influence on decomposing microbes. The timing of fungicide application is an effective disease control strategy, but unfortunately, in the case of FHB there is a narrow window for successful application (Lechoczki-Krsjak et al., 2008). It has been documented that the

optimum time for fungicide application to prevent FHB in wheat is the beginning of anthesis (Wiersma and Motterberg, 2005) which corresponds to Zadok's growth stage GS60 (Wiersma and Motteberg, 2005; Zadoks et al., 1974). Generally, environmental effects of fungicides, their costs for producers and their restricted ability to successfully control FHB are limiting factors.

1.7 Fusarium head blight resistance

1.7.1 Mechanisms of FHB resistance

FHB resistance is a quantitative trait and a gene-for-gene resistance interaction is not available for this disease (Mesterhazy, 1995). While there are no reports of immunity, there is genetic variability for resistance to FHB in cereals. Although, resistance can be affected by environmental condition and aggressiveness of *Fusarium* species, it has been shown that highly resistant cultivars manifest a stable resistance (Miedaner, 1997). Many forms of resistance have been identified (Table 1.1). The resistance mechanisms can cooperate with each other to improve the entire resistance in the host. Resistance to initial infection (Type I) and resistance to disease spread through the rachis (Type II) first were described in the wheat plants by Schroeder and Christensen (1963). Other forms of resistance were later described (Mesterhazy, 1995): Type III (resistance to kernel infection), Type IV (tolerance against FHB and trichothecenes), and Type V (resistance to trichothecene accumulation by (a) chemical modification of trichothecenes or (b) inhibition of trichothecene synthesis (Boutigny et al., 2008)).

Type I resistance is typically reported as the FHB index, which is a combined measure of incidence (percentage of diseased heads) and severity (percentage of diseased spikelets within diseased heads). Plants are sprayed with (macroconidial or ascosporic) inoculum and then inoculated heads are kept under high humidity for a several days. Grain spawn

inoculation can also be used to evaluate FHB index in the field. In this method, *Fusarium* infested grain is dispersed in the field, and *Fusarium* ascospores are produced under mist-irrigation (Bai and Shaner, 1994; Imathiu et al., 2014). Many factors influence the reliability and reproducibility of results of Type I resistance evaluation: 1) a lack of uniform exposure of heads to inoculum within a plant or a plot; 2) the quantity of the inoculum that reaches the rachis; and 3) environmental conditions which are not controlled especially in field experiment (Eudes et al., 2004; Foroud, 2011). Moreover, 4) FHB index, as a combination of incidence and severity, does not give an accurate measurement of resistance to initial infection as these parameters are influenced by other forms of resistances, including disease spread (Mesterházy et al., 2008; Rudd et al., 2001).

Resistance measurement for Type II can be determined by injecting a quantifiable amount of spores into the spikelets at anthesis (point inoculation). The inoculated plants are kept at high humidity for several days. Since FHB disease spreads down the spike *via* the rachis, disease spread is measured as the number of infected spikelets below the inoculation point (Rudd et al., 2001). Type III, IV, and V resistances cannot be directly quantified. Type III resistance can be measured indirectly by FDK evaluation and Type IV resistance can be assessed indirectly by comparing FDK values to the DON level. Type V resistance (resistance to toxin accumulation) can be measured indirectly by DON quantification of FHB-infected spikes (Wang and Miller, 1988) and Mesterhazy (1995) also proposed that Type V resistance can be assessed indirectly by threshing infected spikes and observing damage to the kernels (FDK).

1.7.2 Different genetic sources of resistance to FHB in wheat

The increase in the rate of FHB disease within Canada and throughout the word results in more emphasis on the development of resistant cultivars (Bai et al., 2001; Mesterhazy,

1995). After the first description of resistance to FHB by Schroeder and Christensen (1963), improvement in field inoculation methods and application of those methods at accurate time resulted in a more exact approximation of FHB resistance (Mesterhazy, 1995).

Development of FHB resistance cultivars is a priority of breeding programs in China, Japan, several European countries, and in North America (Bai et al., 1999; Bai et al., 2001; Gilbert et al., 1997; Rudd et al., 2001). In Canadian wheat breeding programs, selection to produce high yielding varieties, early maturity and high protein content with improved resistance to diseases especially FHB is a priority (Gilbert and Tekauz, 2000; Khanizadeh et al., 2016; Randhawa et al., 2013). Durum wheat (*Triticum turgidum* subsp. *durum*) is a tetraploid (AABB) species and is more susceptible to FHB than hexaploid (AABBDD) bread wheat (Gilbert and Tekauz, 2000). FHB resistance in wheat is attributed to over 100 quantitative trait loci (QTL) from FHB-resistant wheat sources (Buerstmayr et al., 2009). Chinese hexaploid cultivar, ʻSumai3ʼ (spring wheat) is one of the best characterized sources of wheat FHB resistance (Rudd et al., 2001). ʻSumai3ʼ-derived resistance is associated to the *Fhb1* locus on chromosome 3BS (the main QTL granting Type II resistance), *Fhb2* on chromosome 6BS conferring Type II resistance, and *Qfhs.ifa-5A* on chromosome 5A, associated with Type I resistance (Häberle et al., 2009; Schweiger et al., 2013). Other QTL has been found on chromosome 4B which was named *Fhb4* (Xue et al., 2010). The aim of the breeding program in Canada is to have *Fhb1*, *2*, and *4* in their future lines (Randhawa et al., 2013).

ʻWaskadaʼ, a Canadian spring wheat may have *Fhb2* (Fox et al., 2009). A Canadian spring wheat cultivar that may carry *Fhb1* QTL is ʻCarberryʼ that was released in 2011 (DePauw et al., 2011). Fox et al. (2013) combined *Fhb1* and *Fhb-5AS* in released Canadian spring wheat cultivar ʻCardaleʼ. Generally, ʻWaskadaʼ, ʻCarberryʼ and ʻCardaleʼ all show

intermediate resistance to FHB. The expression and level of additivity of FHB QTL in elite Canadian spring wheat germplasm have been studied by McCartney et al. (2007). Their results showed that there is a negative relationship between grain protein content and the ʿSumai 3ʾ 5AS resistant allele.

Other sources of resistance are ʿFrontanaʾ (Brazilian spring wheat cultivar) and ʿArinaʾ (Swiss winter wheat cultivar) (Buerstmayr et al., 2009; Draeger et al., 2007). ʿFrontanaʾ shows moderate FHB resistance and QTLs position related to FHB resistance are 3A, 5A, 2B, 3AL, and 7AS (Mardi et al., 2006). Canadian cultivar ʿNeepawaʾ demonstrates intermediate resistance to FHB due to the presence of Frontana in its pedigree (Gilbert and Tekauz, 2000). ʿArinaʾ also shows moderate FHB resistance and the most significant QTL for FHB resistance is on chromosome 4DS and co-localised with the Rht–D1 locus for height (Draeger et al., 2007).

Using germplasm containing these alleles in breeding programs can enhance FHB resistance. The application of doubled haploid (DH) technology has effectively helped cultivar development, especially in winter wheat. Eudes et al (2008) described an *in vitro* selection process, for the development of a resistant plants to FHB, providing a tool for the production of DH transgenic wheat lines. In the current project, to study the role of plant hormones in mediating FHB responses in susceptible and resistant wheat genotypes three genotypes were used: GS-1-EM0040 (DH1), GS-1-EM0168 (DH2) and ʿSuperbʾ. DH1 (ʿCIMMYT 11ʾ/ʿSuperbʾ*2; Type I resistant) and DH2 (ʿCM82036ʾ/ʿSuperBʾ*2; Type II resistant) are resistant DH developed by *in vitro* selection of microspore-derived embryos using a trichothecene toxin screen (0.23 mg L-1 deoxynivalenol, 0.23 mg L-1 15-O-acety-4-deoxynivalenol, 0.47 mg L-1 nivalenol, and 0.7 mg L-1 T2 toxin) (Eudes et al., 2008) and ʿSuperbʾ is a moderately susceptible Canadian cultivar.

1.8 Plant hormones and host defences

The plant defence network is complex and includes different passive and active defence mechanisms that can be effective against a range of pathogens. Physical structure and chemical barriers such as secondary metabolites that prevent pathogen entry and infection are referred to as passive defence (Ding et al., 2011). Plants also employ inducible defence mechanisms that are activated upon pathogen recognition. Inducible or active defence mechanisms include biochemical, molecular and even morphological changes such as expression of pathogenesis-related proteins (PR proteins), oxidative burst, deposition of cell wall reinforcing materials, and/or programmed cell death (PCD) (Ding et al., 2011; Glazebrook, 2005; Van Loon et al., 2006b). These pathways are regulated by hormone signalling molecules in many pathogen-host systems (Arivalagan and Somasundaram, 2016; Nazareno and Hernandez, 2017; Pieterse et al., 2009; Tamaoki et al., 2013). For example, SA is generally involved in the activation of defence responses to biotrophic pathogens where SA signalling activates a hypersensitive responses in which localized PCD inhibits the biotrophic pathogens from feeding on the living plant cells (Glazebrook, 2005). By contrast, JA and ET are commonly associated with defence responses to necrotrophic pathogens and wounding (Glazebrook, 2005).

1.8.1 Plant hormone biosynthesis and signalling pathways

SA biosynthesis can occur either through the phenylpropanoid or shikimic acid pathways (Figure 3.1). In the former, phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine to *trans*-cinnamic acid, which then undergoes beta-oxidation by 3-ketoacyl thiolase 1 (KAT1) yielding a benzoic acid derivative (Widhalm and Dudareva, 2015) which then is hydroxylated to yield SA (Dempsey et al., 2011). SA biosynthesis *via* the shikimic acid pathway occurs in the chloroplast (Dempsey et al., 2011),

where isochorismate synthase (ICS) converts chorismate to isochorismate. Finally isochorismate is converted to salicylic acid (Dempsey et al., 2011; Seyfferth and Tsuda, 2014). In wheat, it is not clear which pathway is dominant, although in rice, which is a monocot, and therefore more closely related to wheat than *Arabidopsis*, it has been shown that SA is mainly formed from benzoic acid (phenylpropanoid pathway) (An and Mou, 2011). SA accumulation in the cytosol reduces the disulfide bonds in oligomeric non-expressor of pathogenesis-related 1 (NPR1) protein subunit, resulting in the release of NPR1 monomers that can enter the nucleus and up-regulates the expression of *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*) *via* TGA transcription factors (TFs) (Malamy et al., 1996).

SA signalling transduction has been shown to be necessary for wheat resistance to *Puccinia graminis*, a biotrophic pathogen responsible for stem rust (Crampton et al., 2009). SA accumulation leads to the activation of a hypersensitive response and localized programmed cell death that hinder biotrophic pathogens from feeding on living plant cells (Glazebrook, 2005). By contrast, necrotrophic pathogens favour host cell death and SA signalling does not typically provide suitable resistance to these pathogens. On the other hand, JA and ET signalling are generally found to offer effective defence responses against necrotrophs (Glazebrook, 2005).

JA biosynthesis takes place in the chloroplasts, where linolenic acid is converted to 12-oxophytodienoic acid by activities of lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) (Figure 5.1). 12-oxophytodienoic acid reductase (OPR) reduces 12-oxophytodienoic acid in peroxisomes, followed by β-oxidation steps to produce jasmonic acid (Bosch et al., 2014). *S*-adenosyl-l-methionine jasmonic acid carboxyl methyltransferase (JMT) catalyze the methylation of jasmonic acid to MeJA (Figure 1.6)

(Turner et al., 2002). Coronatine insensitive 1 (COI1), jasmonate resistant 1 (JAR1) and jasmonate insensitive 1/MYC2 (JIN1/MYC2) are three major JA-signalling components (Wasternack and Hause, 2013). *COI1* encodes an F-box protein that plays role in the SCF-mediated protein degradation by the 26S proteasome and is necessary for most JA-mediated responses (Chini et al., 2009). *JAR1* encodes an amino acid synthase that catalyzes conjugation of isoleucine to JA (JA-Ile), which is the active signal that can trigger defence responses in plants (Kombrink, 2012). JIN1/MYC2 is a transcription factor that regulates JA responsive gene expression (Kazan and Manners, 2008). JA accumulation in the plant cell activates the COI1 receptor involved in targeted protein ubiquitination leading to proteasome-mediated degradation (Devoto et al., 2002). This ultimately leads to the up-regulation of JA-inducible genes such as *PROTEINASE INHIBITORS* and *DEFENSIN* (Chini et al., 2009; Takahashi et al., 2004). In addition to offering defence against various pathogens, JA signalling plays a central role in activating plant defence responses against herbivores such as leafhoppers and spider mites (Howe and Jander, 2008) and also JA positively regulates the ET biosynthesis pathway (Pangesti et al., 2016; Shoresh et al., 2005).

ET biosynthesis is initiated by the conversion of S-adenosylmethionine (SAM) to 1 aminocyclopropane-1-carboxylic acid (ACC) *via* SAM synthase activity (Pech et al., 2010) (Figure 5.1). ACC oxidase then converts ACC to ET (Figure 1.8) (Pech et al., 2010). In *Arabidopsis,* five ET receptors have been identified; ET receptor 1 (ETR1) and ET response sensor 1 (ERS1) (subfamily I), ETR2, ERS2, and ET-insensitive 4 (EIN4) (subfamily II) (Hua et al., 1998). In rice, also five ET receptors have also been identified: ERS1 and ERS2 from subfamily I, and ETR2-like (ERL1), ETR4 and ETR5 from subfamily II (Wuriyanghan et al., 2009). In wheat, one ET receptor has been identified, namely the

ETR1 homologue (Ma and Wang, 2003). ET signalling is negatively regulated, in the absence of ET, receptors constitutively activate the mitogen-activated protein kinase kinase kinase, constitutive triple response 1 (CTR1), which is also a negative regulator of ET signalling pathway. In the presence of ET, CTR1 is deactivated, and in turn EIN2(a positive regulator of the ET signalling) is activated and interacts with TFs (such as ethylene response factors (ERRFs) resulting in up-regulation of ET-responsive genes such as *CHITINASE*, *PDF1.2* and *HEL* (Conrath et al., 2006; De Vos et al., 2005). ET promotes leaf senescence and fruit ripening, which in some pathogen interactions may result in an increase in disease susceptibility (Panter and Jones, 2002; van Loon et al., 2006a).

It is generally said that SA and JA/ET signal transduction pathways act antagonistically, but there is evidence that they can act synergistically as well (Spoel and Dong, 2008). While the host-resistance response depends on the nature of the pathogen (Glazebrook, 2005), many pathogens are not always clearly classified as biotrophic or necrotrophic. For example, *F. graminearum* has a bi-phasic lifestyle that includes both biotrophic and necrotrophic phase. Therefore, the timing and cross-talk among the different hormone signalling pathways may be critical in determining the appropriate response as the pathogen moves through the different phases of its lifecycle.

Antagonistic interactions between SA and JA/ET have been reported (Koornneef and Pieterse, 2008; Mur et al., 2006; Pieterse et al., 2009). The interaction of NPR1 with TGA transcription factors leads to induction of SA-responsive *PR* genes. *Arabidopsis* mutant *npr1* cannot transduce SA-mediated suppression of JA-responsive gene expression demonstrating the role of *NPR1* in SA-JA interaction (Spoel and Dong, 2008). Kempema et al. (2007) investigated the defence responses of *Arabidopsis* against silver leaf whitefly *Bemisia tabaci* and indicated that cross-talk between JA and SA resulted in SA activation

and JA suppression. Interestingly, it has been documented that ET is also involved in the SA-JA signalling interaction in a NPR1-dependent fashion (Leon-Reyes et al., 2009). An *Arabidopsis* double mutant, *npr1-1/ein2-1*, was used to confirm the effect of ET on NPR1 function in JA-SA cross-talk. Thus, if biotic or abiotic induction of ET results in high levels of cellular ET accumulation, SA cannot antagonize JA-dependent defense in an NPR1-dependent manner (Leon-Reyes et al., 2009). SA-regulated WRKY transcription factors also mediate cross-talk between SA and JA (Chen et al., 2012). Overexpression of *WRKY70* resulted in constitutive expression of SA-responsive *PR* genes such as *PR-1* and improved resistance to the biotrophic pathogen, but suppressed the expression of JA-responsive *PR* genes such as *PR-9* resulting in plants susceptible to the necrotrophic pathogens (Li et al., 2004). According to these results it can be concluded that WRKY70 is a positive regulator of SA-dependent and a negative regulator of JA-dependent defence responses. Verberne et al. (2003) showed that ET plays an important role in SAR induction. Ethylene-insensitive (Tetr) tobacco plants were grafted onto wild type plants and tobacco mosaic virus infection of rootstocks resulted in accumulation of PR-1 mRNA in the scion leaves, but grafting of wild type plants onto Tetr plants did not result in PR-1 mRNA accumulation in the scion. Their result showed that ET perception is necessary for production, release or transport of the SA mobile signal to other tissues.

Many studies have shown that JA and ET signalling act synergistically to initiate the expression of defence-related genes in response to pathogen attack (For example Gottwald et al., 2012; He et al., 2017; Kim et al., 2015; Pieterse et al., 2009; Sels et al., 2008; Zhu and Lee, 2015). It has been reported that there is a significant overlap in many of the defence-related genes induced by exogenous treatment with JA and ET (Schenk et al. 2000). Impairing JA/ET signalling perception in mutants such as the jasmonate insensitive

mutant (*coi1*) and ethylene insensitive mutant (*ein2*) led to inhibition of *PDF1.2* induction, which is a marker gene for JA-signalling (Devadas et al., 2002; Thomma et al., 2001; Zimmerli et al., 2004). The *Arabidopsis* transcription factors, ethylene response factor 1 (ERF1) and MYC2, are positive regulators of both JA and ET signalling (Lorenzo and Solano, 2005).

1.8.2 *PATHOGENESIS-RELATED* (*PR*) genes

Differential gene expression studies showed that many genes are up or down regulated in both positive and negative hormone cross-talk in response to stresses (Grobkinsky et al., 2016). Among the genes that are induced in response to biotic stress, many are PR-coding genes (Przymusiński et al., 2004; Van Loon et al., 2006b). PR proteins have been classified into different groups based on their amino acid sequences and enzymatic or biological activity (Van Loon et al., 1994). PR proteins currently include 17 families and their activities include inhibiting pathogen activity and growth (Van Loon, 1999). Within the PR protein family, the following subfamilies target the fungal cell wall: PR-2 (1, 3-endoglucanases), PR-3 (chitinase 1), PR-4 (chitinase 2), PR-8 (chitinase 3) and PR-11 (chitinase) (Van Loon et al., 2006b). In addition, PR-12 (defensin), PR-13 (thionin), PR-14 (lipid transfer proteins) have antifungal activities (Sels et al., 2008). Members of the PR-7 subfamily are proteinases and PR-9 subfamily are peroxidases that are involved in cell wall reinforcement by catalyzing lignification (Epple et al., 1995). The PR-10 subfamily has ribonuclease activity (Epple et al., 1995; Thomma et al., 2001; Van Loon, 1999). It has been shown that PR-15 and 16 are typical of monocot plants and are germin-like oxalate oxidases and oxalate oxidase-like proteins with superoxide dismutase activity (Van Loon et al., 2006b). Members of PR-17 family have antifungal activity and have been found in both monocots and dicots (Christensen et al., 2002).

1.8.3 The role of plant hormones in regulation of the expression of *PR* genes

PR gene expression is regulated, at least in part, by plant defence hormones. For example, SA-mediated activation of SAR induces *PR-1* expression (Van Loon, 2000). Increase in levels of SA, JA and ET leads to expression of SA-inducible PR genes such as *PR-1, PR-2*, and *PR-5* and ET and JA-inducible PR genes such as *PR-3, PR-4* and *PR-12*. Lawton et al. (1994) showed that exogenous application of ET decreased *PR-1* gene expression, indicative of the antagonistic effect of ET on SA signalling transduction. Interestingly, the same response was also observed with exogenous application of MeJA (Niki et al., 1998), supporting synergistic interaction between JA and ET and antagonistic interaction between SA and ET/JA. The effects of the cross-talk among these three defense hormones on the induction of *PR* genes is highly dependent on the timing and abundance of hormone accumulation in conjuction with the pathogen's life cycle.

A good portion of the available information on hormone signalling is derived from *Arabidopsis* and other dicots. Gene expression data collected from studies show the diversity and similarity of hormone-regulated gene expression in dicots and monocots (Kakei et al., 2015; Lu et al., 2006; Yu et al., 2001). Hormone-induced gene expression in *Brachypodium distachyon*, a new model plant for monocots, showed that transcriptional response is highly conserved between *Brachypodium* and rice, but there is an ACC gene synthase in rice that has no orthologues in *Brachypodium*. In *Brachypodium*, expression of ET reponsive genes was weaker than the expression of SA- and JA-responsive genes, a finding similar to that reported for *Arabidopsis* (Kakei et al., 2015).

As mentioned before, acquired resistance is an inducible defence mechanism by which plants protect themselves against a broad range of pathogens. In addition to pathogens and pathogen-derived elicitors, acquired resistance can be induced by chemicals such as SA,

benzothiadiazole (BTH) (an analogue of SA), or isonicotinic acid (INA) (analogues of SA) (Wang et al., 2016). It has been reported that application of BTH in dicot plants, such as tobacco and *Arabidopsis*, induces expression of SA-responsive genes whereas BTH application in wheat failed to induce the same set of genes (Schaffrath et al., 1997).

Plants employ complex signalling mechanisms to cope with different pathogens, but how plants prioritize one regulatory mechanism over others is not yet known. Exogenous treatment with different hormones can influence the host's defence-related gene-expression, which leads to regulation of metabolism and defence responses. Many components in charge of cross-talk among SA, JA, and ET signalling pathways have been characterized. However, the underlying molecular mechanisms involved in fine-tuning the balance in defence signalling network is not well understood. Investigating molecular mechanisms underlying defence regulating systems will expand our understanding of hormone-mediated defence responses in plants.

1.8.4 The role of plant hormones in FHB-wheat interaction

Studies from *Arabidopsis*, wheat and maize suggest that SA signalling is induced in parallel with JA signalling after *F. graminearum* infection (Ding et al., 2011; Makandar et al., 2010; Mika et al., 2010), although the timing of the activation of SA and JA signalling is important to distinguish between resistant and susceptible genotypes. In wheat in response to *F. graminearum* infection, it was reported that SA-induced enhanced expression of *PR-1* gene led to FHB resistance (Makandar et al., 2012). In another study, it was shown that putative SA silencing increased susceptibility to FHB in an F1 plants with a Type II resistant backbround, but no effect was observed in F1 plants with susceptible parents (Foroud, 2011). It was documented that *F. graminearum* mycelial growth and conidia germination are inhibited by SA, but this inhibitory effect was only

observed when SA-treated susceptible wheat cultivar ‘Roblin’ was co-inoculated with *F. graminearum* conidia together with SA; whereas, inoculation with SA-treated heads, where spores were not in direct contact with SA, showed no difference from control reactions without SA-treatment (Qi et al., 2012). These results suggest a direct effect for SA on *F. graminearum* through a reduction in efficiency of germination and growth. These results showed that SA-induced resistance contributed little to reduction of the FHB infection. Recently, it was reported that infection of the susceptible cultivar ‘Roblin’ with *F. graminearum* significantly increased accumulation of SA and JA, although the increase in JA was stronger than that for SA (Qi et al., 2016). Based on these results, it can be concluded that SA either positively contributes to FHB resistance or has no effect; it is further hypothesized that SA does not regulate FHB resistance in a negative way. The role of SA may be better defined in interaction with other plant hormones.

It was proposed that exogenous MeJA treatment limited SA-induced resistance during the early phase of infection, but increased resistance during the later stages (Makandar et al., 2010). Other studies showed that JA signalling is more important than SA in FHB resistance in dicot and monocot plants (Chen et al., 2006; Qi et al., 2016; Walter et al., 2010; Xiao et al., 2013). It was documented that exogenous MeJA treatment led to reduction in *F. graminearum* spore germination and FHB symptoms in wheat heads and infection of wheat head with *F. graminearum* increased JA level significantly higher than SA level (Qi et al., 2016). Phytohormone analyses of wheat heads infected with *F. graminearum* established that JA accumulates twice as much in a Type II resistance genotype compared with Type I resistance or susceptible cultivar ‘Superb’. Based on these results it was concluded that JA may play a role in activation of a local resistance mechanism in Type II resistance (Foroud, 2011). It was also shown that cellular JA content

increases in FHB infected spikes in a resistant wheat genotype, 'Whangshuibai', within 6 h of *F. graminearum* treatment followed by a reduction back to endogenous levels by 12 h; whereas, the susceptible variety NAUH117, which showed similar JA levels prior to exposure to the fungus, did not show an increase in JA accumulation (Sun et al., 2016). By contrast, Buhrow et al. (2016) observed a higher endogenous level of JA in 'Sumai3' compared with the susceptible cultivar 'Fielder', and a *F. graminearum*-induced increase in JA was only observed in 'Fielder', but this observation was made 14 days after treatment. According to these studies it can be concluded that MeJA plays a role in resistance to FHB but its role seems to be time-dependent and may also be genotype-dependent.

According to a microarray study by Li and Yen (2008), it was suggested that ET is also associated with FHB resistance in wheat. Gottwald et al. (2012, in a gene expression study, observed the expression of several JA- and ET-responsive genes including *LIPID-TRANSFERASE, THIONIN*, *DFENSIN* and *GDSL-LIKE LIPASE* in FHB-resistant wheat genotype (Gottwald et al., 2012). The positive effect of ET signalling on FHB resistance was further validated in a virus-induced gene silencing experiment carried out by Gillespie et al. (2012, where suppression of ET signalling resulted in FHB susceptibility in wheat. Gene expression data of ET responsive genes and cellular ET content in FHB-challenged spikes of wheat showed that ET signalling role was not significant in FHB resistance (Sun et al., 2016). Chen et al. (2009a showed that ET facilitated the colonization of *F. graminearum* in *Arabidopsis* and susceptible wheat cultivar, 'Hobbit' while experiment with another susceptible wheat line, landrace Y1193-6 had the opposite (Li and Yen, 2008). Interestingly, when three wheat genotypes ('Superb', GS-1-EM0040 (DH1, Type I resistance) and GS-1-EM0168 (DH2, Type II resistance)) were crossed with *EIN2*-silenced 'Bobwhite' cultivar (moderately susceptible), three different disease outcomes were

observed. ET silencing led to increase in resistance in 'Bobwhite'*ein2**DH2 cross, suggesting that ET had an impact in disease severity in Type II resistant background. However, in 'Bobwhite'*ein2**DH1 cross, ET silencing showed no effect on disease progress and in 'Bobwhite'*ein2**superb, ET silencing led to increase in susceptibility. In other words, similar trend was not observed in three wheat genotypes backgrounds silenced in the ET signalling pathway, and it was proposed that specific mechanisms of resistance or susceptibility are highly genotype-dependent (Foroud, 2011).

In general, these inconsistencies in results demonstrate the need for additional experiments to fully understand the role of ET and perhaps other plant hormones in the FHB response of multiple wheat genotypes. Mechanisms of mediating resistance responses to FHB may be genotype-dependent and it can explain the conflict among the results regarding the role of plant hormones in FHB-wheat interaction. In addition, cross-talk between plant hormones signalling may, in part, explain inconsistencies in the results from different studies describing the mechanism of FHB resistance. The work presented here is a more detailed characterization of the role of plant hormones and the relationship between plant hormones and *F. graminearum* infection in different susceptible/resistant wheat genotypes, with a particular focus on ET signalling.

1.9 Conclusion and research objectives

Significant progress has been made recently towards understanding the role of plant hormones in regulating defence responses. Knowledge of how different signalling pathways interact with each other and how plants prioritize one pathway over another in response to different pathogens can help us to better understand disease resistance mechanisms. Plant defence hormones act synergistically or antagonistically to mount the defence responses to various pathogens with different lifestyles. It is not clear which

signalling pathways are involved in FHB resistance in wheat, in part because the available evidence points in different directions, as described earlier. Based on contrasting results described in the literature, I hypothesized that up-regulation of specific pathways involved in mediating resistance responses to FHB is genotype-dependent.

In order to better understand the role of three defence hormones (SA, JA, and ET) in response to FHB in different wheat genotypes, three separate experiments were conducted. To address the original hypothesis of the study that the up-regulation of specific pathways involved in FHB resistance is genotype dependent, one of the objectives of the thesis was to investigate the genotype-dependent expression of defence-related *PR* genes. In order to validate marker genes for each of three hormone pathways (SA, JA and ET) in three wheat genotypes, a qRT-PCR experiment was carried out to determine significant differences in *PR* genes (*PR1.1*, *PR1.2*, *GLUCANASE*, *CHI1*, *CHI3*, *CHI4*, *PEROXIDASE* and *THIONIN*) expression in response to different concentrations of SA, MeJA and ET in three wheat genotypes (Chapter 2).

In this work, a detached head assay for FHB evaluation was adapted in order to facilitate exogenous hormone treatments to study their effects on FHB. Detached heads were treated with ET activators/inhibitors prior to *F. graminearum* infection with an objective to investigate the physiological changes in the response of different wheat genotypes to FHB; a disease assay on the detached wheat heads was carried out (Chapter 3).

In addition to the regulation of *PR* gene expression, and the putative role of ET signalling in different wheat genotypes, the role of plant hormones (JA, SA and ET) and cross-talk among them in the defence response of wheat seedling to FSB was also studied (Chapter 4).

Figure 1.1: The *F. graminearum* macroconidia (strain GZ3639). The image was produced in the image/cell biology unit with an Olympus FV1000 laser scanning confocal microscope using a 100x oil lens.

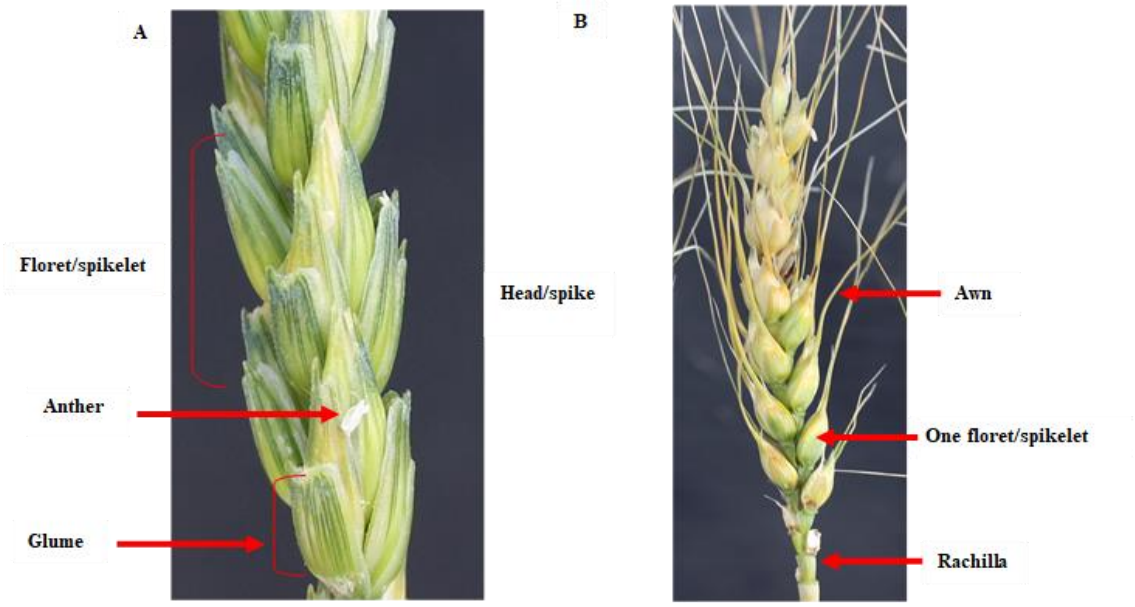

Figure 1.2: Anatomy of a wheat spike. A. Floret of wheat (‘Roblin‘cultivar) from a closer view. B. Anatomy of wheat (Superb cultivar) spike and phenotypic symptoms associated with Fusarium Head Blight of wheat from point inoculation with *F. graminearum*.

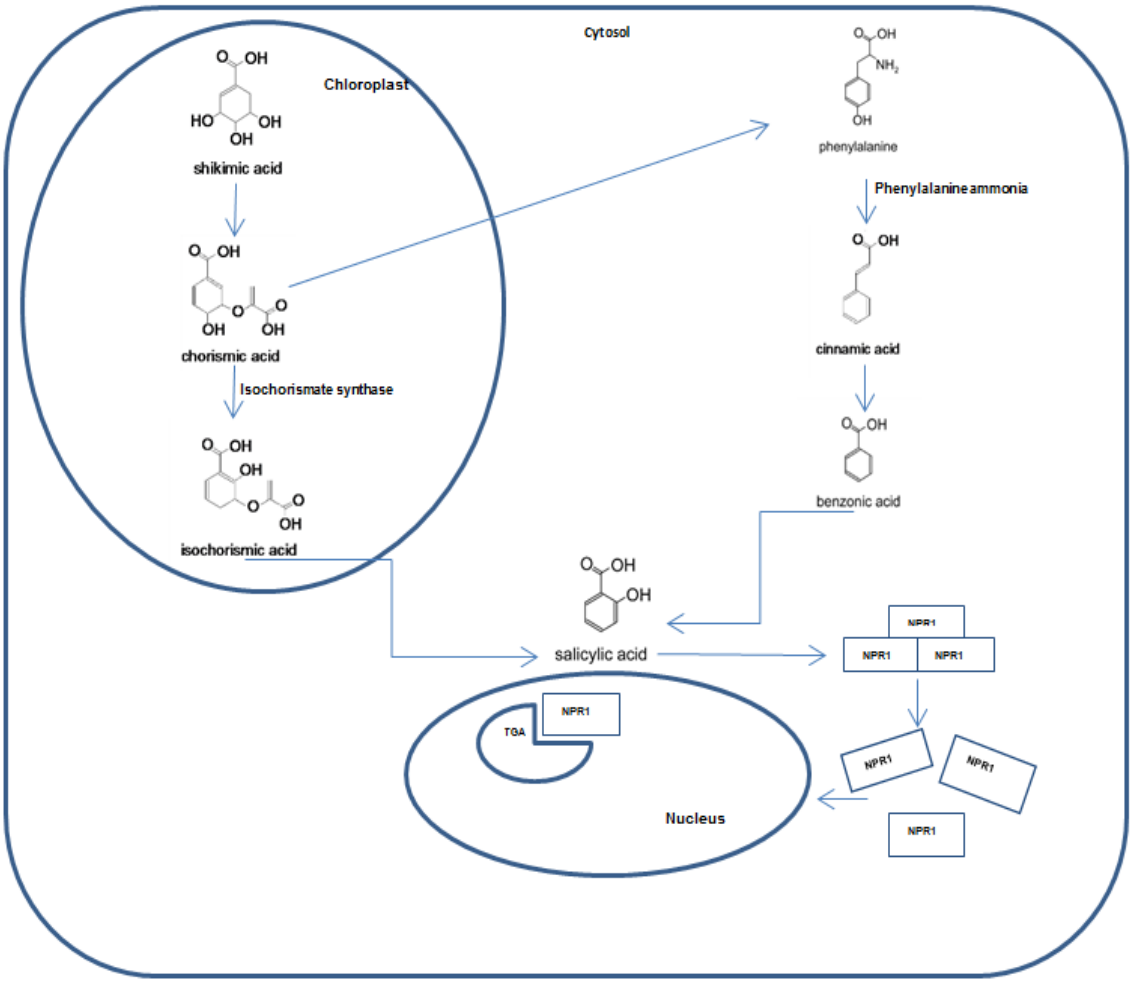

Figure 1.3: SA biosynthesis and signalling pathways.

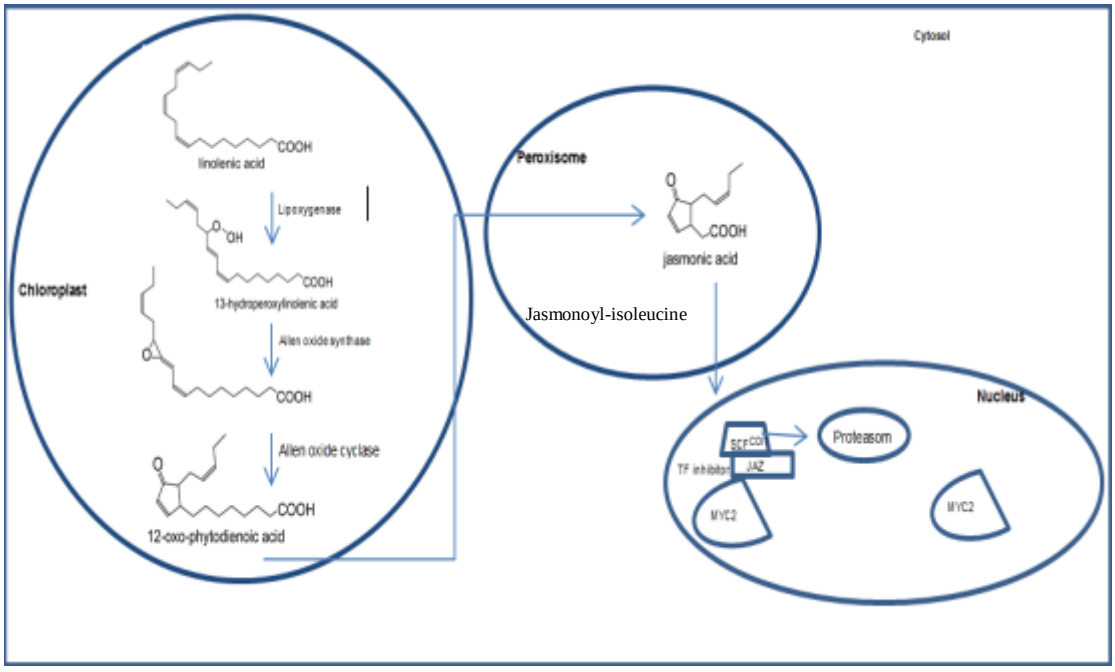

Figure 1.4: JA biosynthesis and signalling pathways

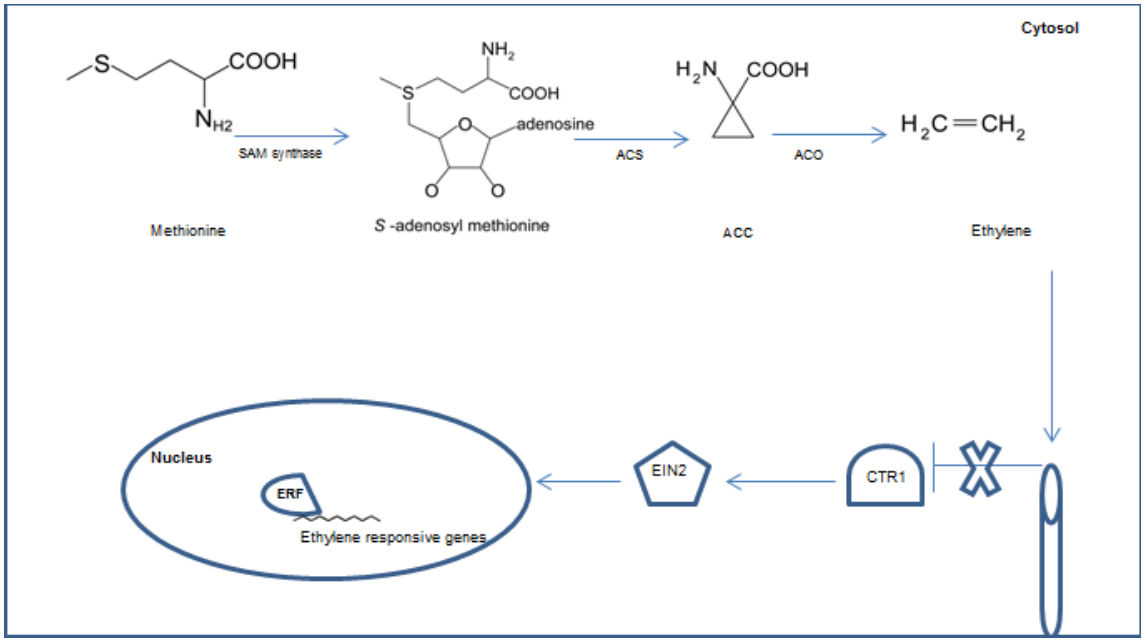

Figure 1.5: ET biosynthesis and signalling pathways

Chapter Two: Hormone-Regulated Expression of Pathogenesis-Related Genes in Three Wheat Genotypes

2.1 Introduction

Many plant-disease interactions are controlled through a series of hormone-regulated pathways (Bent, 1996; Veronese et al., 2003). The molecular mechanisms underlying plant defence responses are complex and are initiated with recognition of the pathogen by the plant. Plants employ pattern-recognition receptors (PRRs) to recognize microbes. Plant PRRs are cell surface-localized immune receptors that recognize pathogen-associated molecular patterns (PAMPs) such as chitin or flagellin leading to PAMP-triggered immunity (PTI) (Dodds and Rathjen, 2010). The perception of PAMPs by PRRs results in the induction of multiple defence signaling pathways. Also, there are NB-LRR (nucleotide-binding leucine rich repeat) proteins in the cytoplasm, which are encoded by plant resistance (*R*) genes. These proteins recognize pathogen-derived avirulence (Avr) proteins. The recognition of Avr proteins by plant R proteins leads to effector-triggered immunity (ETI), which is manifested in a hypersensitive response (HR) (Cui et al., 2015; Mauch-Mani et al., 2017; Zipfel, 2014). PTI and ETI alleviate microbe attacks by induction of downstream responses that lead to a local and systemic induced resistance. A number of defence responses are the activated in plants, including induction of intracellular signaling events such as ion fluxes, kinase cascades, and intercellular signalling events such as reactive oxygen species (ROS), nitric oxide (NO), SA, JA and ET. These signaling events lead to reinforcement of the plant cell wall, accumulation of secondary antimicrobial compounds and the production of pathogenesis-related (PR) proteins (Glazebrook, 2005; Greenberg and Yao, 2004; Shah, 2009). Exogenous application of plant hormones, such as

ET, MeJA and SA can mimic the effect of pathogen attack and results in accumulation of PR proteins (Renault et al., 2015; Tian et al., 2014; Zhang et al., 2015).

PR proteins were first identified in tobacco plants (*Nicotiana tabacum*) infected with tobacco mosaic virus (TMV) (Van Loon and Van Kammen, 1970). It was later shown that different PR proteins are produced in plant species in response to different biotic stresses, such as exposure to pathogenic fungi, bacteria, viruses, oomycetes, viroid, nematode and herbivorous insects (Datta and Muthukrishnan, 1999; García-Olmedo et al., 1995; Niderman et al., 1995; Van Loon et al., 2006b). They are also expressed in response to abiotic stresses, such as cold or drought (Goyal et al., 2016; Janská et al., 2010; Rustagi et al., 2015). PR proteins may be present in plants grown under normal conditions, but they are in low abundance in non-colonized organs and may not be readily detected (Van Loon and Van Strien, 1999). There are in excess of 17 families of PR proteins based on similarities among their amino acid sequences and structure (Table 2.1) (Christensen et al., 2002; Sinha et al., 2014). These proteins are low-molecular weight proteins in the range of 5 to 43 kDa, stable at low pH (< 3) and are mostly resistant to proteases which help them to survive in harsh conditions such as the vacuolar compartment (van Loon, 1985; Van Loon and Van Strien, 1999). PR proteins are found in all plant organs including roots, flowers, stems and leaves, although maximum abundance of these proteins is found in the leaves (Buchel and Linthorst, 1999; Gamir et al., 2017). PR proteins exhibit multiple functions in the plant. Most PR proteins have antifungal functions, although antibacterial, insecticidal, nematicidal, and antiviral functions of some of the PR proteins have also been reported (Caruso et al., 1996; Edreva, 2005; Liu et al., 2016; Xu et al., 2007). The known function of the different PR protein families is presented in Table 2.1.

Plant hormones up-regulate defence-related gene expression such as *PR* genes during pathogen attack and these responses may have transient or long-term effects (Casassola et al., 2015; Larrieu et al., 2015; Ren and Gray, 2015; Stegmann et al., 2017). Longer observation times after exogenous hormone application can reveal additional information about the hormone-mediated defence responses that occur in a long-term plant-stress interaction (Agrawal et al., 2001; Li et al., 2017; Martínez-Medina et al., 2016). Studies show that expression of *PR* genes is tightly regulated in a dose- and time-dependent manner (Reviewed in Agrawal et al., 2001; Eraslan et al., 2007; Leon-Reyes et al., 2010b; Lu et al., 2006; Miyamoto et al., 2012). Treatment of wheat leaves with 100 µM MeJA resulted in up-regulation of *PR-1.1*, *PR-1.2*, *CHI1*, *CHI3*, *CHI4*, *GU1* and *GLU3*, while treatment of rice leaves with 500 µM MeJA led to up-regulation of class III *CHITINASES* (Lu et al., 2006; Miyamoto et al., 2012). Interestingly, exogenous application of 0.1 mM MeJA in *Arabidopsis* led to up-regulation of *THIONIN* which has not been reported in monocots (Epple et al., 1997; Van Loon and Van Strien, 1999). These results indicate that JA regulates different *PR* genes in monocots and dicots. As observed for JA, it has been reported that SA also regulates different *PR* genes in monocots and dicots. *PR-1* and *PR-2* are up-regulated in *Arabidopsis* and other dicots following SA application (Banday and Nandi, 2017; Kuai et al., 2015; Lu et al., 2006; Wildermuth et al., 2006). In contrast, in some monocot plants, such as wheat and Brachypodium, *PR-1* and *PR-2* genes show responses to JA instead of SA (Garvin et al., 2008). Sequence data of *PR-1* families in *Brachypodium*, rice and *Arabidopsis* illustrates that *PR-1* family genes in *Brachypodium* and rice are different from *Arabidopsis* (Kouzai et al., 2016). Surprisingly, a study demonstrated that in rice a same set of genes is positively regulated by both SA and JA (Tamaoki et al., 2013). According to the study more than 313 genes were up-regulated by

benzothiadiazide (BTH), a functional analogue of SA, and were also up-regulated by JA (Tamaoki et al., 2013). In *Arabidopsis*, SA and JA act antagonistically (Kunkel and Brooks, 2002); for example, treatment with 1 mM SA suppresses JA-inducible *PR* genes such as *PDF1.2*, in *Arabidopsis* (Leon-Reyes et al., 2010). JA and SA do not regulate the same set of genes in *Arabidopsis*.

ET is another key player that regulates *PR* gene expression. ET signaling leads to the induction of *PR-4* (*CHITINASES*), *PR-5* (*THAUMATIN-LIKE*), *PR-2* (*GLUCANASE*), and *PR-1.2* genes, in both dicots and monocots (Botha et al., 1998; Thomma et al., 1999; Yu et al., 2001). Application of 7 mM ethephon (Etp; a chemical releaser of ethylene) induced expression of *PR-1* genes at 8, 12, 24 and 48 h after treatment in *Nicotiana tabacum* with a peak at 12 h and expression of the same set of genes was induced at 1 h after treatment with gaseous ethylene and reached the highest level 6 h later (Eyal et al., 1992). The delayed response after ETp treatment may be related to the additional time that takes for ETp to convert into ethylene. In rice, application of 100 µM and 1.0 mM ET resulted in up-regulation of *PR-1*, *PR-5* and *P-10* (Agrawal et al., 2001). These data suggests that additional gene expression studies on monocots such as wheat may reveal useful information about the role of SA, JA and ET in regulation of *PR* gene expression.

In the current chapter, three wheat genotypes, one FHB-susceptible cultivar ('Superb') and two FHB-resistant genotypes (GS-1-EM0040 and GS-1-EM0168) were used to study the responsiveness of defence-associated genes to exogenous application of plant defence hormones/hormone releaser, SA, MeJA and ETp in order to address the hypothesis that plant hormones induce *PR* genes expression differently in each wheat genotypes. In other words, I anticipate that genotype-dependent differences will be observed the pattern of PR gene expression. This hypothesis is part of the original hypothesis of the whole study which

is the role of plant hormones in FHB-wheat interaction is genotype dependent. To address the hypothesis of the current study, a time-dependent experiment was conducted where samples were harvested at 3, 6, 8, 12 and 24 h after treatment with different concentrations of SA, MeJA and ETp. Differences in the expression of *PR* genes were measured in three wheat genotypes.

2.2 Materials and methods

2.2.1 *Plant material*

Three wheat genotypes were used in this experiment: ʻSuperbʼ (Canadian cultivar), and derived doubled haploid lines: GS-1-EM0040 (ʻCIMMYT 11ʼ/ʻSuperbʼ*2) and GS-1-EM0168 (ʻCM82036ʼ/ʻSuperbʼ*2).

Seeds were planted into root-trainers (Spencer–Lemaire Industries, Edmonton, Alta.) containing a Cornell peat-lite mix (Boodley and Sheldrake, 1977). Wheat plants were grown in a greenhouse at the Lethbridge Research and Development Center, Agriculture and Agri-Food Canada, at 21^{o}C (day) and 18^{o}C (night) for 16 h photoperiod. Plants were watered as needed and fertilized biweekly with 20-20-20 (N-P-K). At the 5-7 leaf stage, they were treated with Tilt (2.5 mL L^{-1} propiconazole, Syngenta Crop Protection Canada, Guelph, ON) and Intercept (0.004 g L^{-1} of soil, Imidacloprid, Bayer Crop Science Canada, Toronto, ON) as preventative measures against powdery mildew and aphids.

2.2.2 *Chemical treatment*

Chemical treatments were applied to seedlings at 3-weeks post-emergence in the experiment, which corresponded to Zadoks growth stage 24 (Zadoks et al, 1974). SA (Aldrich Canada Ltd, Oakville, Ont., Canada, catalogue No. S-3007), MeJA (Sigma-Aldrich Chemical Company, Inc., Milwaukee, WI, USA, catalogue No. 392707) and ETp (Sigma-Aldrich Chemical Company, Inc., Milwaukee, WI, USA, catalogue No.

144491667287011) were each dissolved in distilled water to generate concentrations of 50, 100 and 200µM. For each replication fresh stock solutions were prepared on the same day of the experiment. Tween-20 (Fisher Scientific, Nepean, Ont., Canada, catalogue number, BP 337) was added (0.01%) as a surfactant. For each hormone concentration, seedlings were sprayed to run-off at 3-weeks post-emergence (Matsumoto et al., 1980). Control plants were sprayed with water supplemented with a 0.01% Tween-20. Plants were covered with a clear plastic lid immediately after treatments and incubated at room temperature. Three experimental repetitions were performed, each with five biological replicates, where experimental repetitions were differentiated by seeding dates. Each biological replicate consisted of five seedlings (one pot) for a given condition; biological replicates for each condition were harvested together. Leaf tissues were harvested at 3, 6, 8, 12 and 24 hours after treatment and immediately frozen in liquid nitrogen, and stored at -80^{o}C prior to RNA extractions.

2.2.3 *RNA isolation*

RNA was extracted from samples of each wheat genotype, treated with different concentrations of hormones and harvested at a different time points as described in section 2.2.2. Frozen leaf tissue was ground to a fine powder in liquid nitrogen with mortar and pestle and 100 mg of ground tissue was used for total RNA extraction using the Qiagen RNeasy Mini kit (QIAGEN Inc. - USA, Cat No/ID: 74106). The RNase-free DNase (QIAGEN Inc. - USA, Cat No/ID: 79254) was used for on-column digestion of DNA during RNA purification. RNA concentration and quality were determined at optical densities at 260 and 280 nm using a NanoDrop8000 (Thermo Scientific NanoDrop 8000 UV-Vis Spectrophotometers, NanoDrop products, USA). A 260/280 ratio between 1.8 and 2.0 and 260/230 ratio greater than 2.0 were denoted for all RNA samples used in this

experiment. RNA quality was further assessed with a Bioanalyzer RNA kit with a patented RNA integrity number (RIN) which is based on separating and visualizing total RNAs in agarose gels (Agilent RNA 6000 Nano Kit, Agilent technologies, USA). PCR reactions were carried out with RNAs using a house keeping gene, wheat *ELONGATION FACTOR1* (*TEF1*) to confirm the absence of contamination with DNA (HotStarTaq plus Master Mix Kit. QIAGEN Inc. - USA, Cat No/ID: 203645). The working concentration of all primer sets that were used in this experiment was 100 µM. PCR was carried out on each RNA sample in 10 µl reactions. For each reaction, 1 µl of RNA sample, 5 µl of Master Mix, 0.2 µl of each 100 µM forward and reverse TEF1 primer and 3.6 µl optima water were mixed. The PCR conditions for Taq were set according to the manufacturers' instructions (HotstartTaq plus Master Mix Kit. QIAGEN Inc. USA, Cat No/ID: 203645). The reactions were performed in an Eppendorf thermal cycler (Eppendorf Inc. Germany, Cat No: 6336000023) with an initial denaturation for 5 min at 95°C followed by 30 cycles of denaturation at 95ºC (60 s), annealing at 50ºC (60 s), and an elongation step at 72ºC (60 s), and a final extension at 72 ºC (10 min).

2.2.4 *cDNA synthesis*

To ensure no residual DNA was present, prior to cDNA synthesis another DNA digest was performed on 2 µg of each RNA sample. DNase I enzyme in RNase-free DNase set was used according to manufacturers' protocol (Invitrogen Inc. USA. DNase I kit, cat No: 18068-015). Reaction tubes containing, 2 µg of each RNA sample, 2 µL 10X DNase I Reaction Buffer, 2 µL DNase I, (Amp Grade, 1 U mL^{-1}) and DEPC-treated water to 10 µL were incubated for 15 min at room temperature. The DNase I was inactivated by the addition of 2 µL of 25 mM EDTA solution to the reaction mixture and a heat treatment at 65°C for 10 min. The reaction was used as a template for reverse transcription, such that 2

µg of RNA in 12 µL was combined with oligo $(dT)_{18}$ primers and transcribed with SuperScript III Reverse Transcriptase (First-Strand cDNA Synthesis, Invitrogen Canada, Inc., Catalogue No. 18064-014), according to the manufacturer's instruction. The reaction proceeded at 50ºC for 50 minutes and 85ºC for 5 min. The synthesized cDNAs were diluted 16.5 times in water to a final volume of 70 µL, and 4.4 µL of the diluted cDNAs were used as the template for each real-time PCR reaction, indicating that a template originating from 26.6 ng of total RNA was used in each reaction.

2.2.5 *Quantitative real-time PCR (qRT-PCR)*

Differential gene expression of PR proteins was assessed at 3, 6, 8, 12 and 24 h after hormone treatment to differentiate between short-term and long-term responses of exogenously applied plant hormones. Sequence data for wheat *PR-1.1*, *PR-1.2*, *b*-(*1*,*3*;*1*,*4*)-*GLUCANASE-2* (*GLU2*), *CHITINASE-1* (*CHI1*), *CHITINASE-3* (*CHI3*), *CHITINASE-4* (*CHI4*), *PEROXIDASE* (*POX*) and *THIONIN* (*THI*) were found in the GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA). The gene specific primers, the melting temperature (T_m) and amplicon size are presented in Table 2.2. The detection of all amplicons from each cDNA samples was performed by using a QuantiTect SYBR Green PCR Kit (QIAGEN, catalogue No. 204143) (Larionov et al., 2005). Threshold (C_t) values of real-time PCR results were collected from three biological replications with three technical replications for each sample. The relative expression for each defence-related gene was analysed with the REST software (Pfaffl et al., 2002). A $\geq$ 2.0-fold change threshold and p-value of < 0.05 were used for selection of gene candidates with altered expression. Values shown represent fold up-regulation in hormone-sprayed leaves compared with water-sprayed plants, after normalization to *TEF1* and *CONTIG 5* (Table 2.2).

2.3 Results

Since among the different concentrations and sampling times a more clear trend was observed at 24 hrs after treatment with 100 µM of SA, MeJA and ETp, only the results related to this concentration and sampling time are presented below. The complete data set, in the form of tables, along with RIN values of the RNA template used, are presented in the appendix.

2.3.1 *Effects of exogenous applications of SA, MeJA and ETp on expression of PR gene families in GS-1-EM0040*

Following SA, MeJA and ETp application, *PR-1.1* was up-regulated in GS-1-EM0040 (2.72 and 2.51 and 5.00 FC, respectively). Another member of the *PR-1* family, *PR-1.2*, was up-regulated also after SA and ETp treatment (3.21 and 6.52 FC, respectively). ETp application induced up-regulation of all PR genes evaluated, with the exception of *POX*. MeJA and SA application both induced up-regulation of *POX* in GS-1-EM0040 (4.81 and 1.80 FC, respectively). SA application also induced up-regulation of *CHI1* and *CHI3* (2.51 and 2.82 FC, respectively), but not *CHI4*.

2.3.2 *Effects of exogenous applications of SA, MeJA and ETp on expression of PR gene families in GS-1-EM0168*

Fewer genes were differentially regulated in GS-1-EM0168 compared with GS-1-EM0040. Following MeJA application, *CHI1* and *POX* were both up-regulated in GS-1-EM0168 (2.42 and 2.91 FC, respectively). Interestingly, SA application did not induce any changes, except for the down-regulation of *CHI3* (-2.91 FC). Application of ETp did not significantly induce up- or down-regulation of PR genes in GS-1-EM0168.

2.3.3 *Effects of exogenous applications of SA, MeJA and ETp on expression of PR gene families in 'Superb'*

Application of MeJA induced up-regulation of *PR-1.1* in ʻSuperbʼ (3.2 FC) while *PR-1.2* responded to exogenous application of ETp (3.21 FC). ETp also induced up-regulation of *CHI4* (2.22 FC). SA application induced down-regulation of *GLU2* in ʻSuperbʼ. *CHI1* and *CHI4* responded in a similar way to MeJA application and both were up-regulated (3.08 and 4.12 FC, respectively). As observed in the other two genotypes, application of MeJA induced up-regulation of *POX* in ʻSuperbʼ (4.11 FC), and in the case of ʻSuperbʼ SA application also lead to up-regulation of *POX*.

2.4 Discussion

In this study, the focus was on the regulation of *PR* gene expression in response to exogenous application of SA, MeJA and ETp in different wheat genotypes using qRT-PCR. It has been reported that transcription levels of *PR* genes, *PR-1.1*, *PR-1.2*, *CHI1*, *CHI3*, *CHI4* and *GLU2*, in response to exogenous hormone treatment increase with seedling development from one to three weeks, where the greatest effect is observed at the 3 week stage (Lu et al., 2006). A possible explanation for this increase in the level of *PR* gene expression with seedling growth is physiological changes which occur during developmental stages in both monocots and dicots (Lotan et al., 1989; Poethig, 1990; Vega et al., 2002).

In the present work, the differential expression of *PR* genes showed that application of 100 µM of SA induced up-regulation of *PR-1*, *PR-2*, *PR-3* (*CHI1*) and *PR-8* (*CHI3*) at 24 h in GS-1-EM0040 wheat genotype (Figures 2.1A). Different tested concentrations of MeJA up-regulated *PR-9* (*POX*) genes mostly at 24 h in all three wheat genotypes. Results for effect of different concentrations of the MeJA are presented in appendix (Table 6.7). In

general, ETp-induced up-regulation of *PR* genes was more pronounced in GS-1-EM0040 compared with other two wheat genotypes. For ʻSuperbʼ application of different tested concentrations of ETp resulted in up-regulation of *PR-2* and *CHI4* at 24 h and *THI* at 3 and 6 h (Appendix, Table 6.8). Different expression patterns were observed for some hormone-regulated *PR* genes between dicots and monocots (Balmer et al., 2012; Lawton et al., 1996; Molina et al., 1999). There are two different types of *PR-1* homologue genes in wheat, *PR-1.1* and *PR-1.2*. Molina et al. (1999) observed that expression of these two wheat genes are induced by pathogens, but not by SA or other SAR inducers; whereas, Makandar et al. (2006) found that the SA analogue BTH can induce up-regulation of a *PR-1* gene, but in a genotype and time-dependent manner. Meanwhile, in dicot plants, *PR-1.1* and *PR-1.2* responded to inducers of SAR (Uknes et al., 1993). In the current study, *PR-1.1* was up-regulated at 24 h after application of 100 µM SA in GS-1-EM0040. In addition, application of 50 µM SA resulted in up-regulation of *PR-1.2* at 3 h in ʻSuperbʼ (Appendix, Table 6.2).

PR-2 (*β-1,3-GLUCANASE*), which corresponds to the *GLU2* gene was up-regulated only after ETp treatment (50 and 100 µM, at 24 h) in GS-1-EM0040 (Appendix, Table 6.3). Simmons et al. (1992) reported that treatment of rice seedlings with hormones including ETp and SA resulted in up-regulation of rice *β-GLUCANASE* genes (*β-1, 3-; β-1, 4-GLUCANASE*). In another study application of 20 mM SA specifically induced *β*-(*1,3*;*1,4*)-*GLUCANASE-2* (*GLU2*) at 24 h in wheat seedlings (Lu et al., 2006).

In the current study, *CHI4* was up-regulated after treatment with 100 µM of MeJA and ETp at 24 h in ʻSuperbʼ, and *CHI1* was also up-regulated in response to MeJA in ʻSuperbʼ. Meanwhile, ETp induced up-regulation of both *PR-3* genes assessed here, namely *CHI1* and *CHI4*, in GS-1-EM0040 wheat genotype. These results are consistent with results of Lu et al. (2006) where *CHI4* was responsive to MeJA and generally unresponsive to SA.

Ding et al. (2002) showed that SA induced the expression of *PR-3* (*CHI1* and *CHI4*), but did not affect *PR-2* which was consistent with the results of my study. According to transcriptome analyses it has been shown that exogenous application of MeJA induced up-regulation of *CHI3* in rice at different time points where 24 h showed the highest fold changes (Miyamoto et al., 2012). MeJA treatment can induce expression of *PR-1.1*, *PR-1.2*, *CHI1*, *CHI3*, *CHI4*, *GLU1* and *LIPASE* at 24 h in wheat seedling (Lu et al., 2006). In rice MeJA application induced the expression of *PR-1*, *PR-2* (*GLU2*), *PR-3* (*CHITINASE*), *PR-5* and *PR-9* (*POX*) (Mei et al., 2006). In the present study, different concentrations of MeJA induced up-regulation of *CHI1*, some up-regulation was observed at 6 h, but mostly at 24 h in GS-1-EM0168 and ʻSuperbʼ wheat genotypes (Appendix, Table 6.4). *POX* encodes proteins involved in cell wall lignification, cell wall formation and ROS production (Passardi et al., 2004). In the current study, application of MeJA induced the highest up-regulation of *POX* at 24 h in all three wheat genotypes evaluated.

There is a link between *PR-1* and *PR-5* gene expression and SAR induction in dicot plants where genes that encode acidic PR-proteins are induced by SA and PR-proteins that accumulate in intercellular spaces (Ding et al., 2002). On the other hand, JA and ET induce expression of the *PR* genes that encode basic PR proteins such as basic chitinase (*CHI-B*) and PR-proteins accumulate in vacuoles (Niki et al., 1998). While *PR-5* was not investigated in this study, according to my results, *PR-1* gene expression in wheat does not follow those findings in dicot plants. In my study, the *PR-1* family responded to SA, MeJA and ETp application. These contradictory results show the complexity of the regulation of *PR* genes and are in line with the differences reported among other studies in monocots (Molina et al., 1999; Schweizer et al., 1997), including wheat (Lu et al., 2006; Makandar et al., 2006; Yu and Muehlbauer, 2001).

There may be many reasons for the observed differences in the hormone-induced expression patterns of *PR* genes in the present study and aforementioned publications. One reason can be the aforementioned effect of plant developmental stage. Lu et al. (2006) showed that seedling development stage at which MeJA and SA were applied had a great impact on the expression of defence-related genes. They reported that *PR-1.1, PR-1.2* and *CHI1* transcript levels increased with the increasing seedling age, from one week to three weeks post germination, in response to SA and MeJA application. However, transcript levels of *CHI3*, *GLU1* and *GLU2* increased with seedling age only in response to MeJA treatment and *GLU3* transcript level decreased with seedling age in response to both MeJA and SA treatments (Lu et al., 2006). *GLU3* is a seed-related gene in graminaceous plants (Laroche et al., 2015) that shows highest expression levels during and shortly after germination and its expression decreases during seedling growth and development. On the other hand, it has been shown that expression levels of *PR-1.1, PR-1.2, CHI1, CHI2, CHI4, GLU1, GLU2* and *LIPASE* increased with seedling age in response to SA and MeJA application from one week to three weeks (Lu et al., 2006). The factors regulating the responsiveness of *PR* genes to MeJA and SA with increasing seedling growth and development are not clear, but this phenomenon has also been observed in other plant species (Poethig, 1990; Ryals et al., 1996)

Another reason might be related to tissue-specificity or organ-specificity of gene expression. Hong et al. (2007) showed that *PR* genes may function in specific organs. They studied the role of a novel pathogen-activated protein (C3-H-C4 type RING finger protein), which physically interacts with the basic PR-1 protein, and concluded that the expression of this gene is organ-specific. Another possible explanation could simply be related to the difference among plant species. Indeed, interpreting results from one plant species to

another may not be meaningful (Bücking et al., 2004), and as a consequence, results from hormone-regulated gene expression experiments that have been done on dicots may not support the results obtained from a monocot plant.

Interpretation of the data on *PR* gene expression from hormonal applications to the exposure to actual pathogens may be even more problematic (Lu et al., 2006; Schweizer et al., 1997). Schweizer et al. (1997) compared gene expression in response to SAR inducer (BTH) and pathogen infection (*Pseudomonas syringae*) in rice. They found the expression pattern in response to BTH to be different from *P. syringae*, suggesting that hormones may not necessarily mimic pathogen attack. Kramell et al. (2000) studied the gene expression of octadecanoid-derived metabolite in stressed barley leaves and showed that there are two sets of JA-responsive genes; one set is induced in response to either exogenous MeJA treatment or increase in cellular MeJA, whereas another set only responds to exogenous application of MeJA. These results suggest that plant cells may respond differently to an exogenous source of hormones compared with a cellular source (Kramell et al., 2000; Parthier, 1990).

The method that is used to apply chemicals in different studies may also explain the differences between my results and published results. It has been reported that soil-drench application of SA increases the SA concentration in wheat spikes, induces *PR1* gene expression in wheat spikes and enhanced FHB resistance (Makandar et al. 2012), whereas spray application of SA/BTH may not be able to induce the same physiological effect (Li and Yen, 2008; Yu and Muehlbauer, 2001). Makandar et al. (2012) argued that it is likely that spray application is not able to build up high enough concentration of these chemicals to induce *PR1* gene expression or spray application may not deliver the chemicals to the location where SA-responsive genes are expressed.

In summary, in the current study it was demonstrated that exogenous application of 100 µM MeJA resulted in up-regulation of *PR-1.1* in ʻSuperbʼ, the *PR-3* gene *CHI1* in GS-1-EM0168 and ʻSuperbʼ and *PR-9 POX* at 24 h in all three wheat genotypes (Figure 2.2) confirming that JA signalling up-regulates *PR-1*, *PR-3* (*CHTINASE*) and *PR-9 POX* expression in wheat. It was noted that up-regulation of *PR-9 POX*, an indicator of MeJA signalling, was more pronounced. Up-regulation of *PR* genes of interest in this study by ETp was more pronounced in GS-1-EM0040 compared to the cultivar ʻSuperbʼ and GS-1-EM0168, suggesting that the response to ET in the form of *PR* gene induction may be genotype-specific. Among the different time points of *PR* gene induction, most of the *PR* genes of interest in the study were up-regulated at 24 h in all three wheat genotypes, demonstrating that at this time point the induction of a defence response is not causally related to a resistant phenotype, but is a part of a general defence response to a number of inducers including chemical treatments. Since different *PR* gene expressions were observed in three different wheat genotypes following exogenous hormone application, the results of this experiment support the original hypothesis of the thesis that the role of plant hormones in mediating defence responses is genotype-dependent.

Table 2.1: Main properties of classified families of PR proteins. Table was modified from (Sels et al., 2008)

Family	Typical size (kDa)	Function	Original reference
PR-1	15	Antifungal, involved in plant cell wall thickening	(Antoniw et al., 1980)
PR-2	30	β-1-3-Glucanase	(Antoniw et al., 1980)
PR-3	25–30	Chitinase (class I, II, IV, V, VI), hydrolyze the β-glycosidic bond in chitin	(Van Loon, 1982)
PR-4	15–20	Hevein-like proteins (class I), Acidic chitinase (classII)	(Van Loon, 1982)
PR-5	25	Thaumatin-like, Alternation of fungal membrane permeability	(Van Loon, 1982)
PR-6	8	Proteinase-inhibitor	(Green and Ryan, 1972)
PR-7	75	Endoproteinase	(Vera and Conejero, 1988)
PR-8	28	Chitinase class III	(Metraux et al., 1988)
PR-9	35	Peroxidase	(Lagrimini et al., 1987)
PR-10	17	‘Ribonuclease-like’	(Somssich et al., 1986)
PR-11	40	Chitinase	(Melchers et al., 1994)
PR-12	5	Defensin	(Terras et al., 1995)
PR-13	5	Thionin	(Epple et al., 1995)
PR-14	9	Lipid-transfer proteins (LTPs)	(García-Olmedo et al., 1995)
PR-15	20	Germin-like oxalate oxidase	(Zhang et al., 1995)
PR-16	20	Germin-like proteins without ‘Oxalate-oxidase function	(Wei et al., 1998)
PR-17	Tobacco	Peptidase	(Okushima et al., 2000)

Table 2.2: DNA primers used to assay the gene expression by qRT-PCR. [a] Tm values represent melting temperatures for primers.

Gene	Forward Primers (5'>3')	Reverse Primer (5'>3')	Size (bp)	[a]Tm (°C)	Family	Reference
PR 1.1	ACTACGACTACGGGTCCAACA	TCGTAGTTGCAGGTGATGAAG	155	57;56	PR-1	(Lu et al., 2006)
PR 1.2	CGTCTTCATCACCTGCAACTA	CAAACATAAACAACACGCACGT	156	54;54	PR-1	(Lu et al., 2006)
GLU2	AGCAGAACTGGGGACTCTTCT	CACATACGTACCGCATACACG	154	57;55	PR-2	(Lu et al., 2006)
CHI1	GGGCTACTTCAAGGAAGA	ACACTAGGTCTGGGTTGCTCA	156	56;58	PR-3	(Lu et al., 2006)
CHI4	TTCTGGTTCTGGATGACCAAC	ACTGCTTGCAGTACTCCGTGT	140	54;55	PR-3	(Lu et al., 2006)
CHI3	GTTTAAGACGGCGTTGTGGTT	ACCGTTGATGTTGGTGAT	151	54;56	PR-8	(Lu et al., 2006)
POX	CCTGCCAGGCTTTACATCTAG	TCGTAAGGAGGCCCTTGTTTCTG	150	57;58	PR-9	NCBI
THI	CGACCAAATCCATCAACCAAG	AACACACTATCACACCCTTGAAGC	150	57;58	PR-13	NCBI
TEF1	GGTGATGCTGGCATAGTGAA	GATGACACCAACAGCCACAG	126	55;64	Translation elongation factor 1 α-subunit	(Wang et al., 2014)
CONTIG 5	CTGCAGTGCGTGCATATTTT	AACAAGAACGATGCCGAGTT	143	54;55	Carbohydrate Transmembrane transporter allene	(Wang et al., 2014)

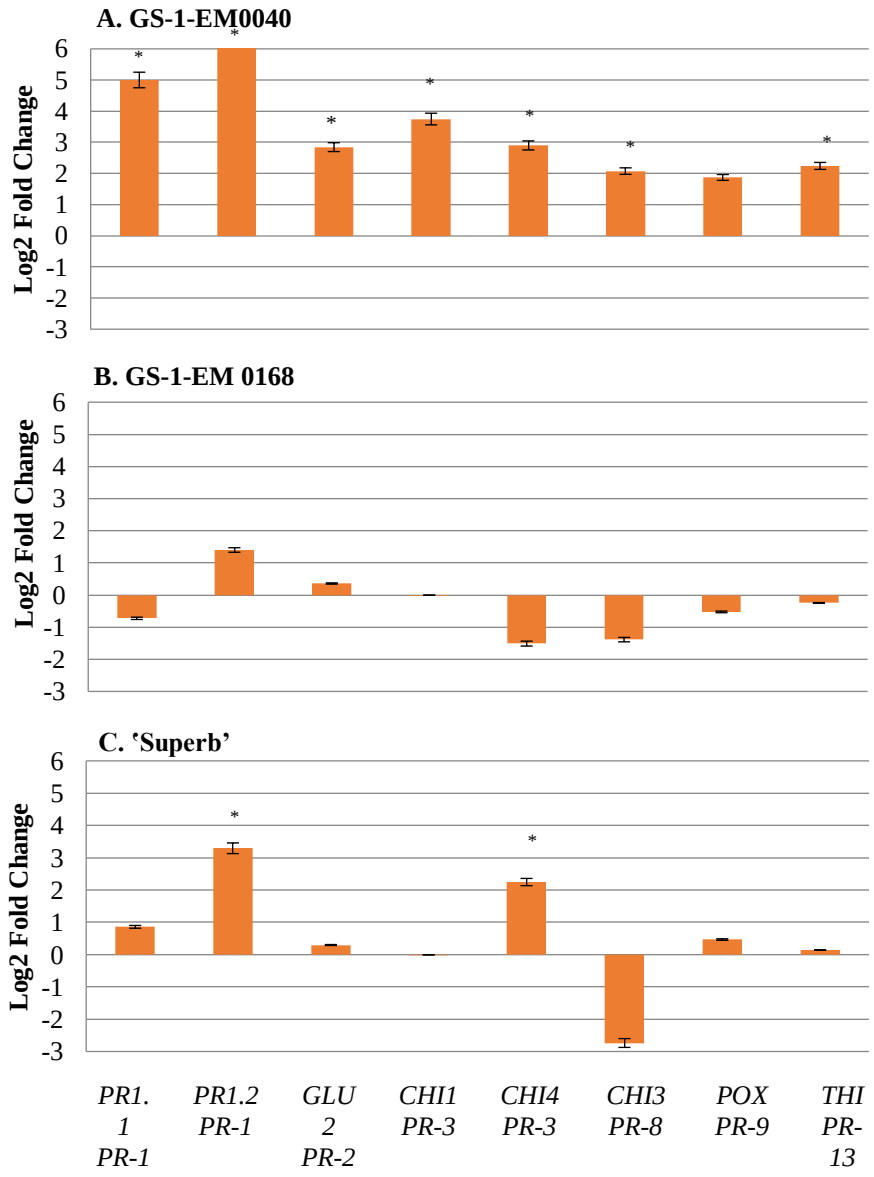

Figure 2.1 Effect of 100 µM of ETp on expression of the PR genes in wheat genotypes: A. GS-1-EM0040, B. GS-1-EM0168 and C. 'Superb'. Data is presented as $\log_2$ fold change. Error bars represent standard deviations and treatments with asterisk are significantly different ($P < 0.05$).

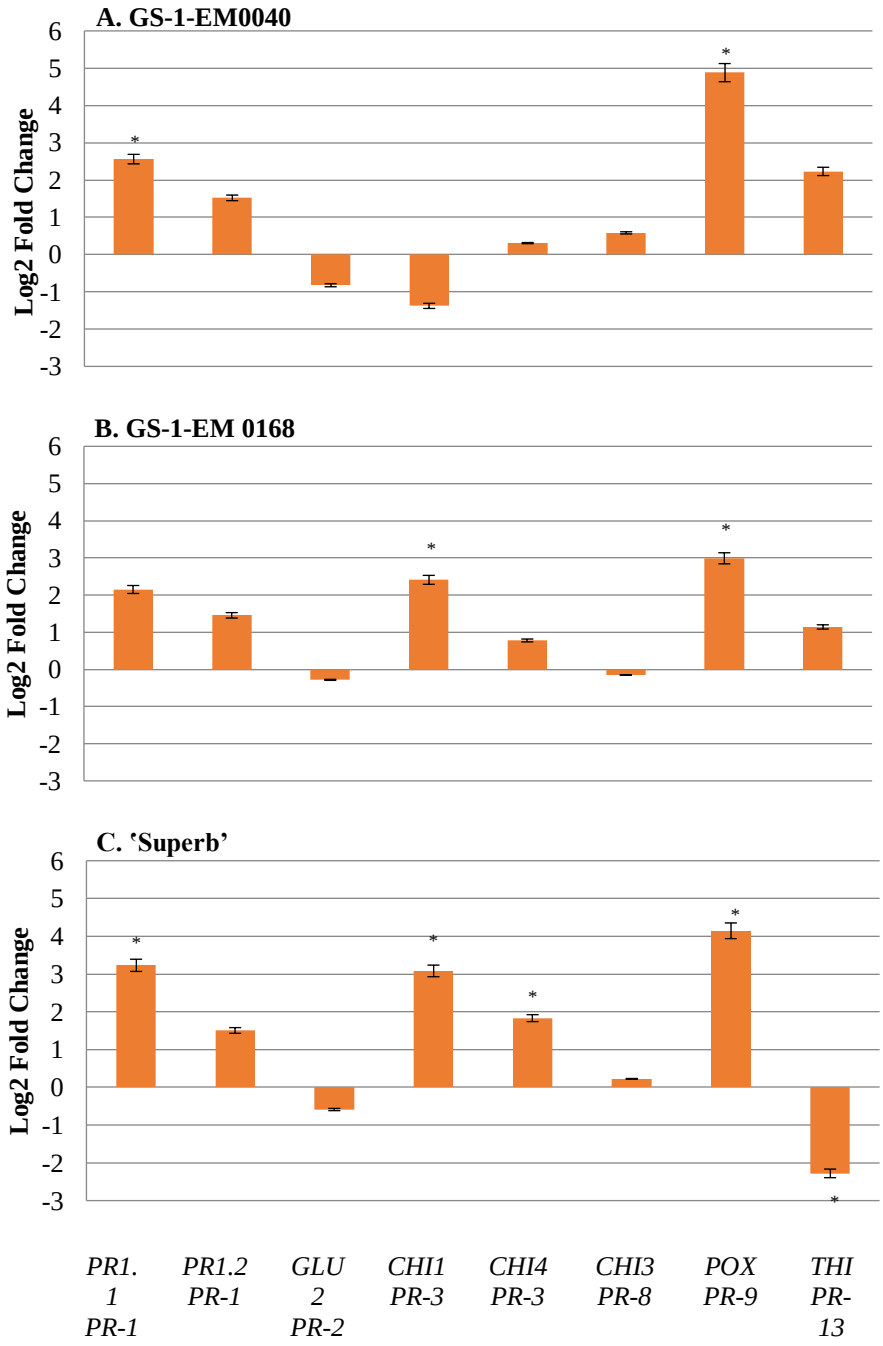

Figure 2.2 Effect of 100 µM of MeJA on expression of the PR genes in wheat genotypes; A. GS-1-EM0040, B. GS-1-EM0168 and C. 'Superb'. Data is presented as log_2 fold change. Error bars represent standard deviations and treatments with asterisk are significantly different ($P < 0.05$).

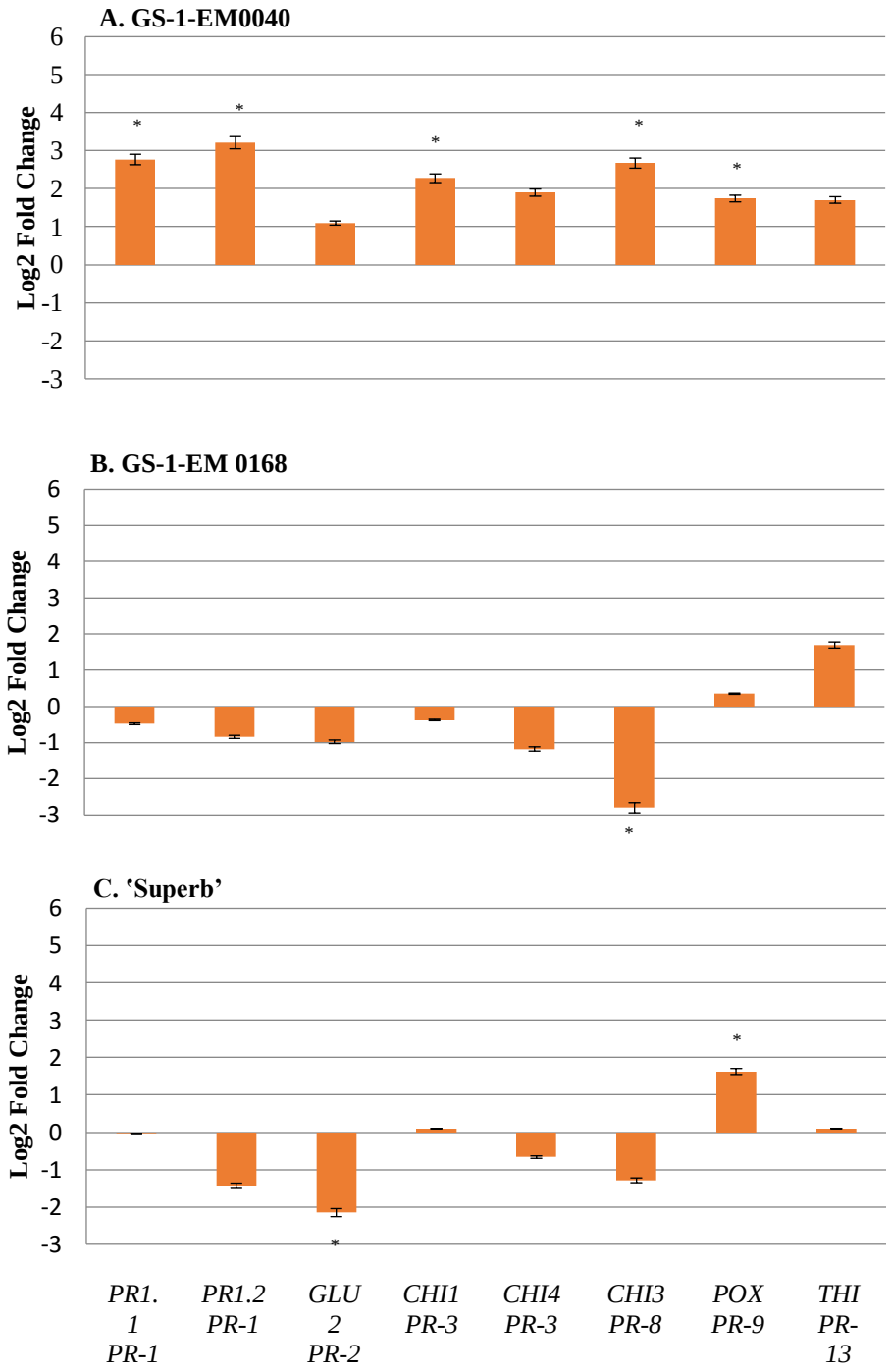

Figure 2.3 Effect of 100 µM of SA on expression of the PR genes in wheat genotypes; A. GS-1-EM0040, B. GS-1-EM0168 and C. 'Superb'. Data is presented as $\log_2$ fold change. Error bars represent standard deviations and treatments with asterisk are significantly different ($P < 0.05$).

Chapter Three: Investigating the Role of Ethylene Signalling in Fusarium Head Blight Disease Response in Wheat

3.1 Introduction

ET is a plant hormone that promotes leaf senescence and fruit ripening. It has been reported that ET plays a dichotomous role in plant-pathogen interactions and disease resistance (Yang et al., 2015). Since ET accelerates senescence and ripening, it is generally expected that exogenous treatments with ET or ET activators could result in enhanced disease development (Das et al., 2015). However, the underlying mechanisms of resistance to necrotrophic and hemi-biotrophic pathogens are complicated (Ding et al., 2011). The role of ET signalling in mediating susceptible response to *F. graminearum* has been documented in both wheat and *Arabidopsis* (Chen et al., 2009b). To further study the role of ET signalling impact in FHB-wheat interaction, ET signalling pathway in the FHB-susceptible wheat cv. 'Bobwhite' was successfully silenced by employing RNAi to target the *EIN2* transcripts (Travella et al., 2006). The ET silenced lines were further screened for changes in their response to FHB, and a significant decrease in susceptibility was reported (Chen et al., 2009b). These results are supported by exogenous treatment of ETp or silver thiosulphate (ET inhibitor) suggest that ET signalling increases FHB-susceptibility in wheat and barley, a result that may be associated with ET-mediated plant senescence (Chen et al., 2009b). In contrast, silencing of ET-biosynthesis and signaling genes in a resistant wheat genotype led to FHB resistant plants becoming susceptible (Gillespie et al., 2012). To confirm their results they applied exogenous chemical to inhibit ET signalling and observed that inhibition of ET signalling caused resistant wheat genotypes to become susceptible and significantly increased disease in susceptible wheat genotypes (Gillespie et al., 2012). Analysis of *F. graminearum*-induced differentially regulated genes in resistant

and susceptible wheat genotypes suggests that JA and ET signalling pathways are associated with a resistance response. These analyses were carried out on FHB-resistant Sumai3, FHB-susceptible wheat landrace Y1193-6 and also on FHB-resistant 'Wangshuibai' (Ding et al., 2011; Li and Yen, 2008). These results were further confirmed by Gillespie et al. (2012). They showed that suppression of the ET signaling pathway led to FHB susceptibility in FHB-resistant wheat lines. In another study, three wheat genotypes (FHB-susceptible cultivar 'Superb', FHB-resistant GS-1-EM0040 and another FHB-resistant GS-1-EM0168) were crossed with *EIN2*-silenced FHB-susceptible, 'Bobwhite'. It was shown that ET silencing led to an increase in resistance in the 'Bobwhite'-Δ*ein2**GS-1-EM0168 cross. However, in the 'Bobwhite'-Δ*ein2**GS-1-EM0040 cross, ET silencing showed no effect on disease spread and in 'Bobwhite'-Δ*ein2**'Superb', ET silencing led to an increase in susceptibility (Foroud, 2011). Since in one experimental design, where three different wheat genotypes were used, three different responses were observed it was proposed that specific mechanisms of resistance or susceptibility may be genotype-dependent. Considering all of the reported experiments, the role of the ET signalling pathway in the resistance mechanism to FHB is unclear. Conflicting data indicates that there is still much to be studied on the role of ET in mediating FHB-resistance or susceptible responses.

According to the results from Foroud (2011), where ET silencing in three wheat genotypes resulted in different disease outcomes, I hypothesized that the role of ET signalling in FHB-wheat interactions is varied among different wheat genotypes. To address this hypothesis, the objective of the current study was to further investigate the role of ET in the FHB-wheat interactions.

In order to modify the ET signalling pathway in wheat heads using exogenous chemical treatments, a detached head assay was adapted and used here (section 3.2.3). Three wheat genotypes with different FHB indices and different level of resistance to FHB disease spread were used for detached head assay optimization, ʿSuperbʾ; a FHB susceptible Canadian cultivar, GS-1-EM0040; Type I resistant, and GS-1-EM0168; Type II resistant (Foroud et al., 2012). The wheat genotypes were the same as genotypes that were used in the first experiment (Chapter 2). Disease incidence (percentage of diseased heads) can be equated to some extent with Type I resistance, but a lack of uniform exposure of heads within a plant or a plot affects the reliability and reproducibility of results. The detached head assay is a suitable method to assess Type I resistance or resistance to initial infection. The disease assay for dip inoculation method of detached heads will enable assessment of resistance to initial infection. Wheat heads were cut at anthesis and placed in a culture medium containing one of four chemicals that affect the ET signalling pathway: two inhibitors of ET (1-methylcyclopropene (MCP), cyclopropane-1,1-dicarboxylic acid (CDA)) and two activators of ET (1-aminocyclopropane-1-carboxylic acid (ACC), and ETp (ET) (a chemical releaser of ET)). Heads were inoculated with *F. graminearum* spores and disease evaluation was carried out at 3, 6 and 9 days after inoculation (DAI). According to the results of this study, ET plays a positive role in resistance response to FHB and its role is genotype-dependent.

3.2 Materials and methods

3.2.1 Plant material and growth condition

Six wheat genotypes namely ʿSuperbʾ, a moderately FHB-susceptible Canadian cultivar; ʿRoblinʾ, a FHB-susceptible Canadian cultivar (developed from the four-way

cross RL4302/RL4356//RL4359/RL4553) (Campbell and Czarnecki, 1987), ʻAwesome', a moderately FHB-susceptible (93FHB37/2*Andrew//SWS366 (L06015)) (Harpinder Randhawa, Agriculture and Agri-Food Canada, personal communication), a Type I FHB-resistant line, GS-1-EM0040 (ʻCIMMYT 11'/ʻSuperb'*2) (Foroud et al., 2012), a Type II FHB-resistant line, GS-1-EM0168 (ʻCM82036'/ʻSuperb'*2) (Foroud et al., 2012), and ʻTenacious', a FHB-resistance cultivar (HY665/BW346) (Brown et al., 2015), were used for investigation of the role of ET in FHB response of wheat in this experiment. Three wheat genotypes; ʻSuperb', GS-1-EM0040 and GS-1-EM0168 were used for the detached head assay optimization.

Seeds were planted into 1 gallon sized nursery pots (greenhouse megastore, Danville, USA) containing a Cornell peat-lite mix (Boodley and Sheldrake, 1977). Wheat plants were grown in a greenhouse at the Lethbridge Research and Development Center, Agriculture and Agri-Food Canada, at 21^{o}C (day) and 18^{o}C (night) for 16/h photoperiod. Plants were watered as needed and fertilized biweekly with 20-20-20 (N-P-K). At the 5-7 leaf stage, they were treated with Tilt (2.5 mL L^{-1} propiconazole, Syngenta Crop Protection Canada, Guelph, ON) and Intercept (0.004 g $L^{-1}$of soil, Imidacloprid, Bayer Crop Science Canada, Toronto, ON) as preventative measures against powdery mildew and aphids.

3.2.2 Preparation of inoculum

For the *F. graminearum* (GZ3639 wild-type strain) inoculum, one mycelial plugs (1 cm^2 each) from a culture grown on potato dextrose agar plates (Desjardins and Hohn, 1997) were used to inoculate 100 mL CMC broth (1.5% carboxymethylcellulose (SIGMA C1011), 0.1% NH4NO3, 0.1% KH2PO4, 0.05% MgSO4 · 7H2O, 0.1% yeast extract) (Cappellini and Peterson, 1965). The culture was incubated at 27ºC with gentle agitation

(180 rpm) for 5 days and then filtered through cheese cloth. The spores were collected by centrifugation (Eppendorf^{TM} 5810R, Eppendorf Canada) at 4000 rpm, for 10 min in a swinging bucker rotor (Rotor number A-4-81,). The supernatant was poured and washed three times. The washed-spores were subsequently diluted with sterile ddH_2O to a working concentration of 100,000 macroconidia mL^{-1}.

3.2.3 Disease assay

In order to modify the ET signalling pathway in wheat heads using exogenous chemical treatments, a detached head assay was adapted based on the protocol described in (Chen et al., 2009b) and used here to facilitate chemical treatments during infection with *F. graminearum.* The detached head assay (described in 3.2.3.2) was compared with the traditional disease assessment method (described in 3.2.3.1). The detached head assay was subsequently used to modify the ET signalling pathway to study the role ET signalling in FHB-wheat interaction (described in section 1.8.1).

3.2.3.1 Traditional point inoculation

Spikes of three wheat genotypes (‘Superb’, GS-1-EM0040 and GS-1-EM0168) were point inoculated at mid-anthesis by pipetting a 10 µL macroconidia suspension (*F. graminearum* strain GZ3639). A single spikelet near the center of the spike was point-inoculated with 10 µL inoculum (Figure 3.1.A), and incubated in a mist-irrigated greenhouse (25^{o}C, 95% humidity, 16 h photoperiod) for 3 days after point inoculation and plants were returned to a normal humidity greenhouse and grown under normal humidity. Disease was evaluated as the number of infected spikelets below the inoculation point at 3, 5, 7, 9, 15 and 18 DAI. Three biological replicates were completed for each line and inoculation.

3.2.3.2 Optimization of detached head assay: point and dip inoculation

Spikes of three wheat genotypes (‘Superb’, GS-1-EM0040 and GS-1-EM0168) were cut from plants at mid-anthesis and the stems were sterilized by spraying with 70% ethanol and subsequently wiped off. The detached heads were point or dip inoculated. Point inoculation was carried out as described for whole plant assays. Dip-inoculation (Figure 3.2.B) was carried out by submersing spikes to the base of the spike, and held for 1 s in a spore suspension with 5×10^4 macroconidia mL^{-1}, then removed them from the macroconidia suspension. The stem of the detached heads were placed in 15 mL tubes with culture medium consisting of 50 g L^{-1} sucrose and 0.4 g L^{-1} L-glutamine, buffered with 0.5 g L^{-1} morpholinoethanesulfonic acid. The pH of the medium was adjusted to 6.2 (Ganeshan et al., 2010). Since the tubes containing culture medium were covered with parafilm a small hole was made with scissors (cleaned with 70% ethanol) to facilitate passing the stems of detached heads. All the 15 mL tubes were covered with parafilm. The tube racks with the heads were then incubated in a closed container with a plastic lid in a greenhouse. Moisture content inside the lidded container was sufficient for disease spread. Disease was evaluated at 3, 5, 7 and 9 DAI. Three biological replicates were completed for each line and treatment and also a different batch of inoculum was used in each replicate.

3.2.3.3 Detached head assay to assess the effect of chemical treatments

The effect of four chemical treatments on the FHB response was assessed using the detached head assay method described above (section 3.2.3.2). Chemical treatments include two ET activators (ETp and ACC) and two inhibitors (CDA and MCP). ETp (Sigma-Aldrich Chemical Company, Inc., Milwaukee, WI, USA, catalogue No. 144491667287011) is a chemical regulator that breaks down into ET, hydrochloric acid and phosphonic acid in the plant. ACC (Sigma-Aldrich Chemical Company, Inc.,

Milwaukee, WI, USA, catalogue No MFCD00009944) is a precursor in the ET biosynthesis pathway. CDA (Sigma-Aldrich Chemical Company, Inc., Milwaukee, WI, USA, and catalogue No 343412) is an inhibitor of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO). MCP (SmartFresh^{TM}, AgroFresh Inc., Spring House, PA, USA) blocks the ET receptors and inhibits ET signalling.

The following modifications to the detached head assay described above (section 3.2.3.2) were employed to investigate the role of ET signalling in FHB-wheat interaction. The culture medium was supplemented with 0.01% Tween-20 and 100 µM ETp, ACC, CDA or MCP; for control reactions the medium was only supplement with 0.01% Tween-20. Each of the four chemicals affecting the ET pathway was added to the medium within 20 min of incubating with the detached heads. The tube racks with the heads were then incubated in a container with a closed lid in the greenhouse. Detached heads were inoculated by point or dip inoculation 2 h later, as described in section 3.2.3.2. To avoid cross-talk among chemical treatments, each five biological replicates per experiment, which were in one container, were kept in different regions of the greenhouse, and the ACC and ETp treatments which could lead to volatile ET release were kept in a separate greenhouse under the same growing conditions. Three experimental repetitions were performed; each with five biological replicates, where experimental repetitions were differentiated by seeding dates and a different batch of inoculum was used. Each biological replicate consisted of one spike from one plant for a given condition.

Disease spread was evaluated at 3, 6 and 9 DAI. For point inoculation, the number of diseased spikelets was evaluated by observing infection symptoms below the inoculation point (note that the disease typically spreads down the spike through the rachis). For dip inoculation, a measurement of severity (percent infected spikelet per spike) was used.

3.2.4 Statistical analysis

ANOVA was carried out with SAS program, version 9.3 software (SAS, USA). For each of the data collected under a specific condition. Comparisons were made (a) between the traditional inoculation method (section 3.2.3.1) and with the detached head assay (section 3.2.3.2); (b) between each chemical in different wheat genotypes at each rating date using Fisher's LSD.

For (a) mean comparison test were made among three wheat genotypes. The measured variable was disease spread among three wheat genotypes. Data considered significantly different ($p < 0.05$) within each group of plant lines at each rating date were marked with letters.

For (b) the analysis was done for each data set of each wheat genotype treated with different chemicals at rating dates. When significant interactions of factors were observed, comparison tests were performed within each plant line, at each rating date (3, 6 and 9 DAI). Data considered significantly different ($p < 0.05$) between chemical treatments for each wheat genotype at each rating date are marked with letters.

3.3 Results

3.3.1 Results for the detached head assay optimization

Results for the detached head assay compared reasonably well with the whole plant assay for point inoculation (Figure 3.3.B). There were differences ($p < 0.05$) in disease between the resistant and susceptible wheat genotypes. According to the results of this experiment the detached head assay (Figure 3.3.B and C) is suitable to assess resistance to disease spread by point inoculation, and can distinguish between Type II resistant and susceptible wheat within 9 DAI.

3.3.2 Investigating the role of ethylene signalling in FHB disease response in wheat using the optimized detached head assay

The point inoculation method assesses Type II resistance or resistance to spread of infection within the head (Schroeder and Christensen, 1963). At 6 and 9 DAI, application of ET inhibitors/activators resulted in different levels of disease spread between treatments.

For the susceptible cultivar ʻSuperbʼ, at 9 DAI, MCP and CDA treatment did not affect disease spread compared to control, 5.1 and 5.4 diseased spikelets in MCP and CDA versus 5.0 in control (Figure 3.4.A). However, both ETp and ACC application reduced disease spread as compared to the control, showing only 1.5 and 1.2 diseased spikelets following ETp and ACC treatments, respectively.

The moderately susceptible cultivar ʻAwesomeʼ showed the highest level of disease spread in response to the MCP at 6 DAI, where disease was higher compared with the control ($p < 0.05$), but was not statistically different from the CDA treatment. Furthermore, no differences were observed between CDA and the control treatments (Figure 3.4.B). ACC treatment resulted in the lowest disease spread at 6 DAI, 1.4 diseased spikelets compared to 2.5 in control. By 9 DAI, ACC or ETp treatments resulted in the lowest number of diseased spikelets, 2.3 and 2.7 for ACC and ETp, respectively, compared to 6.8 in the control.

For the susceptible cultivar ʻRoblinʼ, the highest number of diseased spikelets was observed in response to MCP, 3.3 compared to 2.3 in the control at 6 DAI. No significant difference was observed between the control and CDA treatments at 6 DAI (2.5 and 2.4 for control and CDA, respectively) (Figure 3.4.C). At both 6 and 9 DAI, there were no significant difference for disease spread between the ACC and ETp treatments, which both showed less disease spread compared with the control and other treatments (Figure 3.4.C).

At 6 DAI, no significant difference was observed between ETp and ACC for the GS-1-EM0040 (1.1 and 1.0 diseased spikelets for ETp and ACC, respectively) (Figure 3.5.D). On the other hand, MCP and CDA treatments led to a significant increase in disease spread, where MCP showed the highest number of diseased spikelets 3.6, as compared to 1.7 in the control at 6 DAI. The same trend was observed for MCP and CDA at 9 DAI compared with the control. At 9 DAI, ACC and ETp did not show any difference in the number of disease spikelets compared with the control (Figure 3.5.D).

By 6 DAI, MCP and CDA treatments significantly increased the number of diseased spikelets compared to control for the GS-1-EM0168 (2.4 and 1.7 for MCP and CDA, 1.5 for control, respectively; no significant effects were observed in response to ETp or ACC (Figure 3.5.B). At 9 DAI, CDA and MCP resulted in the highest number of diseased spikelets, 3.6 and 3.4 for CDA and MCP, respectively as compared to 1.6 in the control.

At 6 DAI, for the 'Tenacious', MCP and CDA treatments resulted in a significant increase in disease spread compared to control (2.1 and 2 for MCP and CDA, respectively) (Figure 2.C). There was no significant difference among the effects of ETp or ACC and control treatments at 6 DAI, 2.1, 1.8 and 2.1, for ETp, ACC and controls, respectively. These differences were evident at 6 DAI for disease spread and remained throughout.

Dip inoculation assesses a combination of Type I (resistance to initial infection) and Type II (resistance to spread within the head) resistance. Generally in the current study, unlike point inoculation, disease incidence in dip inoculated spikes differed at 3 DAI among most of the wheat genotypes. At 6 and 9 DAI, disease assay data showed that disease spread in MCP and CDA-treated wheat genotypes (resistant and susceptible) was significantly higher than the control, although some exceptions were observed in the susceptible lines (Figure 3.6). Application of ETp or ACC affected disease progression differently in

resistant and susceptible genotypes. With the exception of ʻRoblin', the susceptible genotypes showed reduced disease spread compared with the control ($p < 0.05$) in response to ETp and ACC treatments, but ETp and ACC treatment in the resistant genotypes showed little to no difference from the control. In general, the same trend observed for point inoculation was also found in the dip inoculation experiment.

At 3 DAI, ETp and ACC treatment decreased the number of infected spikelets (8.5% and 8% for ETp and ACC, respectively) significantly from the control (20%) in ʻSuperb' (Figure 3.6.A). At 6 and 9 DAI, MCP treatment led to the highest number of infected spikelets (34.85% and 69.7% for 6 and 9DAI, as compared to 30% at 6 and 58% at 9 DAI in the control respectively) and ACC treatments resulted in the lowest disease spread (10.4% and 16.6% infected spikelets for 6 and 9 DAI, respectively, as compared to 30% at 6 and 58% at 9 DAI in the control) (Figure 3.6.A).

In ʻAwesome', a significant difference was observed between CDA and control treatments at 3 DAI where CDA showed the highest number of diseased spikelets (15%) compared to the control, 12% (Figure 3.6.B). By 6 DAI, no significant difference in disease spread was observed between CDA, 27%, compared to the control, 28%. ETp and ACC treatments led to the lowest number of diseased spikelets at 3 DAI (5% for both ETp and ACC) as compared to the control, 13%, and the same trend was observed at 6 and 9 DAI.

No treatment effect was observed in 'Roblin' at 3 DAI. However, at 6 DAI, CDA and MCP treatments resulted in a higher number of diseased spikelets compared to ACC and ETp treatments (Figure 3.6.C). Meanwhile, no statistical difference was observed between the CDA or MCP treatments with the control, and the same is true for the ACC or ETp treatments compared with the control.

MCP and CDA treatments led to the highest number of infected spikelets at 3 DAI in GS-1-EM0040 (14.03%, 14.8% for CDA and MCP, respectively, as compared to 5% for the control) (Figure 3.7.D). The same trend for MCP and CDA was observed at 6 and 9 DAI. ACC and ETp treatments resulted in a similar level of disease as the control treatment across all three evaluation dates, with the exception of 6 DAI, where the ETo differed from the control ($p < 0.05$).

Except for MCP, all other treatments showed no significant differences with the control at 3 DAI in GS-1-EM0168 (6% for CDA, ACC and ETp and 13.3% for MCP as compared to 6% in the control). At 6 DAI, MCP continued to show the highest level of disease, but a difference was observed between the CDA and all other treatments, where the ACC, ETp and control treatments showed less disease ($p < 0.05$). By 9 DAI, the same high level of disease was observed for MCP and CDA (Figure 3.7.E).

By 6 DAI, MCP and CDA treatments showed the highest number of diseased spikelets compared to the control in 'Tenacious' (15.8% and 14.5% for MCP and CDA and 6.5% for the control, respectively) (Figure 3.7.F). At 6 and 9 DAI, while ACC and ETp treaments resulted in a lower percentage of disease spikelets, the control reaction showed the least amount of disease.

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3.4 Discussion

The role of ET in plant resistance responses appears to differ, depending on the pathogen and level of host susceptibility to the pathogen (Dıaz et al., 2002; Foroud, 2011). In this study, different wheat genotypes with different levels of susceptibility/resistance to FHB were used to examine the role of ET in resistance toward *F. graminearum*. A general trend of increased in disease in wheat heads impaired in ET signalling or biosynthesis,

particularly in the resistant wheat lines, implicated ET in a FHB-resistance responses. Findings from the work presented show that, in both point and dip inoculation methods, the application of ACC or ETp in FHB-resistant genotypes (GS-1-EM0040, GS-1-EM0168 and ʻTenaciousʼ) resulted in little to no difference in disease response from the control treatment; however, treatment with MCP or CDA increased disease spread significantly compared with the control ($p < 0.05$). In FHB-susceptible genotypes (ʻSuperbʼ, ʻAwesomeʼ and ʻRoblinʼ), in the point inoculation method, inhibition of ET signalling with MCP application resulted in a significant increase in disease spread compared with the control at 6 DAI ($p < 0.05$) (Figure 3.4). By contrast, with one exception (ʻSuperbʼ) CDA treatments showed no difference from the control reaction. Meanwhile, application of ACC and ETp (with one exception for ETp in ʻAwesomeʼ at 6 DAI) led to a significant reduction in disease spread compared to the control at 6 and 9 DAI ($p < 0.05$).

It was previously demonstrated that ET silencing by RNA interference (ETi) in a resistant background (ʻBobwhiteʼΔ*ein2**GS-1-EM0168), resulted in an increase in resistance to FHB. The same results were observed by Chen et al. (2009) where ET silencing in FHB-susceptible ʻBobwhiteʼ led to a decrease in susceptibility. The positive interaction between ETi and FHB resistance stand in opposite to the results of the current study where ET inhibition in GS-1-EM0168 (FHB resistance) resulted in higher FHB spread. Foroud (2011) also observed that in the susceptible background (ʻBobwhiteʼΔ*ein2**ʻSuperbʼ), ETi resulted in an increase in host susceptibility. The same results were observed in the current study where ET inhibition increased FHB susceptibility in susceptible genotypes. Cross-talk between ET and other plant hormone signalling molecules may, in part, explain the contrasting results.

Previously, it has been shown that ET signalling may down-regulate chlorophyll a/b–binding protein, a major light-harvesting complex of photosystem II (LHCII) (Schenk et al., 2000). In another study, it was shown that following FHB infection in ʻSumai3', a FHB-resistant cultivar, genes that encode chlorophyll a/b–binding protein were down-regulated (Li and Yen, 2008), suggesting the induction of ET signaling after FHB infection in the resistant genotype ʻSumai3'. A transcriptome profiling study of a FHB-resistant wheat cultivar, 'Wangshuibai', and its FHB-susceptible mutant, Meh0106, inoculated with *F. graminearum* indicated that the transcript level of *PR3*, a marker gene for ET-mediated defence response, and the wheat *ERF1* homolog only increased in the resistant cultivar, where the transcription level of *ERF1* and *PR3* increased significantly at 3 and 6 h after infection, respectively (Ding et al., 2011). However, expression levels of both genes showed no changes in the susceptible mutant within 36 h after infection. These results clearly suggest that ET signalling is involved in resistance responses to FHB (Ding et al., 2011; Li and Yen, 2008).

Chen et al. (2009) used genetic study to suggest that the necrotrophic phase of *F. graminearum* infection may be most relevant to symptom development and that it is during this phase that ET signalling is important. It is likely that ET regulates the transition phase between SA and JA, as it has previously been shown (Leon-Reyes et al., 2010a) in *Arabidopsis*, where ET signalling inhibits SA antagonistic interactions with JA signalling. Based on gene expression data observed in FHB-resistant 'Wangshuibai', up-regulation of ACO reached the highest level at 12 h after *F. graminearum* inoculation (Ding et al., 2011), but in the FHB-susceptible ʻBobwhite', *F. graminearum* appears to exploit the ET signalling pathway and leads to an increase in susceptibility (Chen et al., 2009b). These results show that ET plays different roles in mediating the response to FHB and its role

seems to be genotype-dependent. Gene expression study of JA- and ET-associated genes following *F. graminearum* inoculation in two wheat lines, FHB-resistant 'Wangshuibai' and the FHB-susceptible mutant NAUH117, showed that *F. graminearum* infection induced ET-associated genes in both genotypes (Sun et al., 2016). It has also been observed that the change in ET hormone content of a susceptible mutant was similar to the resistant line. According to these observations, it can be concluded that ET signalling plays a role in basal resistance in the wheat response to FHB (Geraats et al., 2003; Sun et al., 2016). Comparing expression of the genes associated with JA and ET in the FHB-diseased spikes showed that JA signalling is a key pathway in FHB resistance (Foroud et al., 2012; Sun et al., 2016). Because of the synergistic effect of JA and ET signalling, it is possible that ET also takes part in wheat FHB resistance.

Allen et al. (2016) studied the role of ET in PAMP-triggered immunity in Fusarium crown rot (FCR) disease resistance in two wheat cultivars and reported that FCR-susceptible wheat cultivar 'Kennedy' treated with ACC resulted in lower disease development, but inhibition of ET signalling with MCP in the FCR-resistant cultivar 'Bobwhite' resulted in higher infection. This supports the results of the current study where treatment with ETp/ACC prior to *F. graminearum* inoculation did lead to reduced disease spread in FHB-susceptible genotypes and inhibition of ET signalling/biosynthesis increased disease spread in FHB-resistant genotypes, although FHB was the disease of interest in this experiment not FCR. Further, RNA-sequencing data of the ACC treated FCR-susceptible cultivar 'Kennedy' during enhanced resistance has shown that 7,466 genes were up-regulated, where many of these genes were associated with disease-response category, including peroxidase, and ethylene response factors (Allen, 2016).

Altogether, the disease assay results from this study show that inhibition of ET led to a significantly higher number of diseased spikelets compared to controls in both FHB-susceptible or resistant genotypes ($p < 0.05$). However, activation of ET decreased disease spread significantly from the control in susceptible genotypes, but not in resistant genotypes ($p < 0.05$). It can be concluded that ET plays a positive role in FHB-wheat interaction and mediate resistance responses although the role of ET in the resistance response in FHB-resistant genotypes is more pronounced than in FHB-susceptible genotypes. Since in both resistance and susceptible genotypes ET plays role in resistance responses the hypothesis of the study was not supported that the role of ET in FHB disease outcomes is genotype dependant.

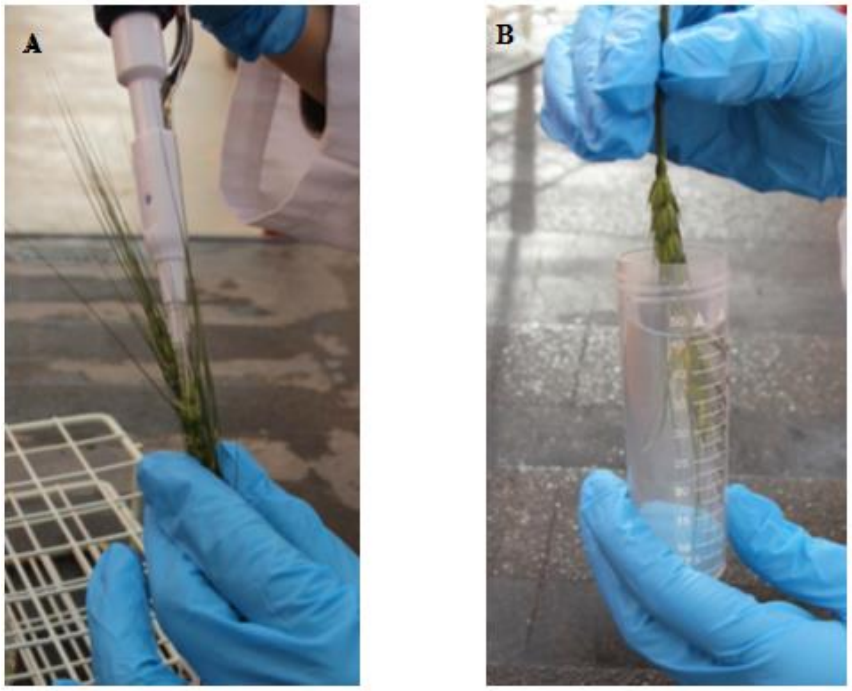

Figure 3.1: Point (**A**) and dip (**B**) inoculation of detached heads

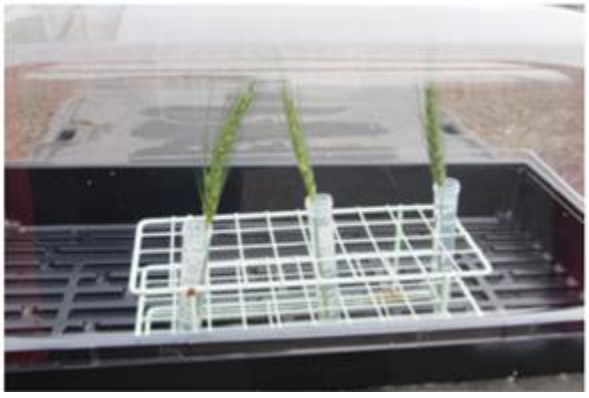

Figure 3.2: Incubation of inoculated heads in the lidded container

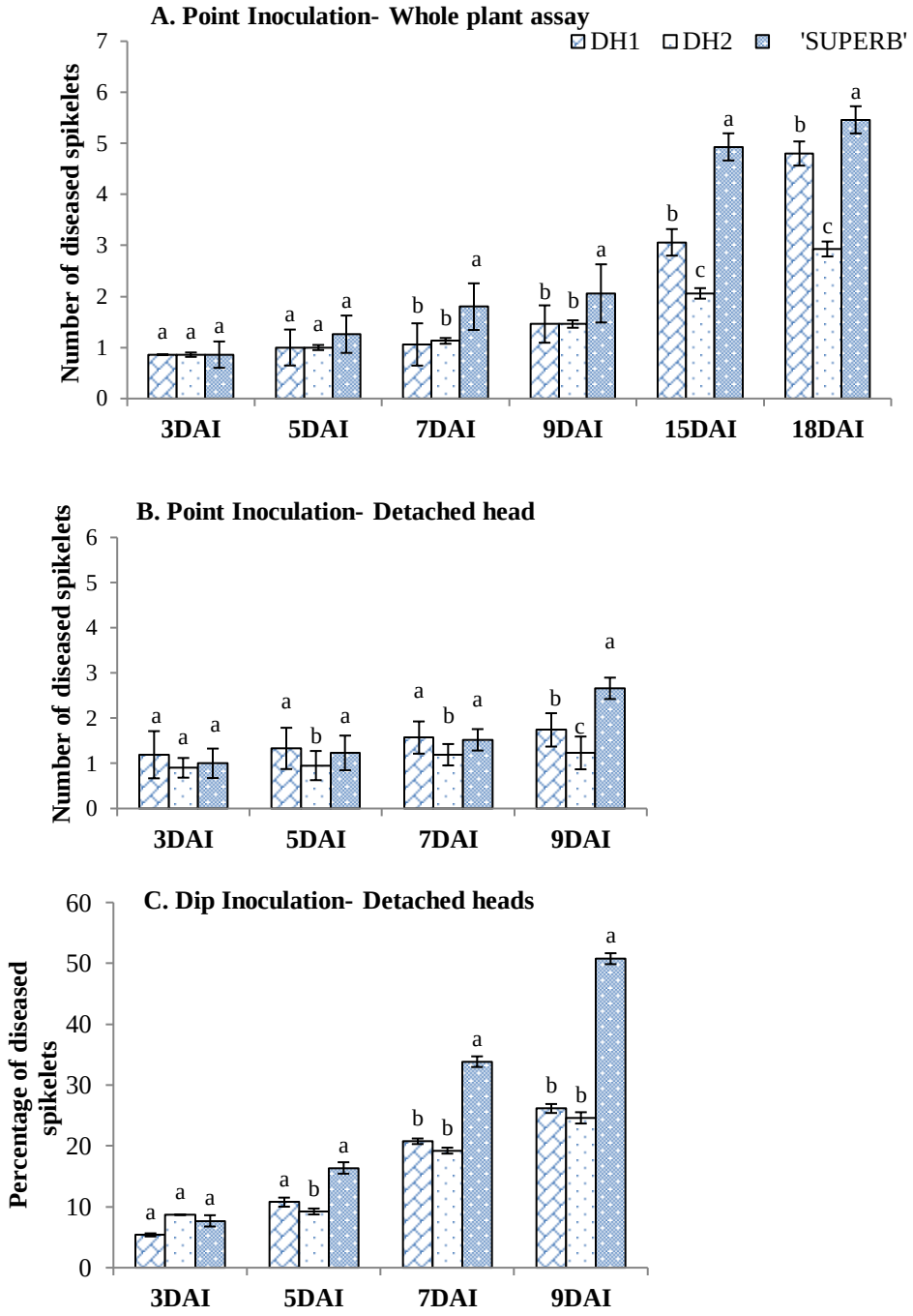

Figure 3.3: A. Whole plant assay, point inoculation method. B. Detached head assay, point inoculation method. C. Detached head assay, dip inoculation method. Error bars represent standard deviations and data considered significantly different ($p < 0.05$) between plant lines if they are not connected with the same letter above the bars.

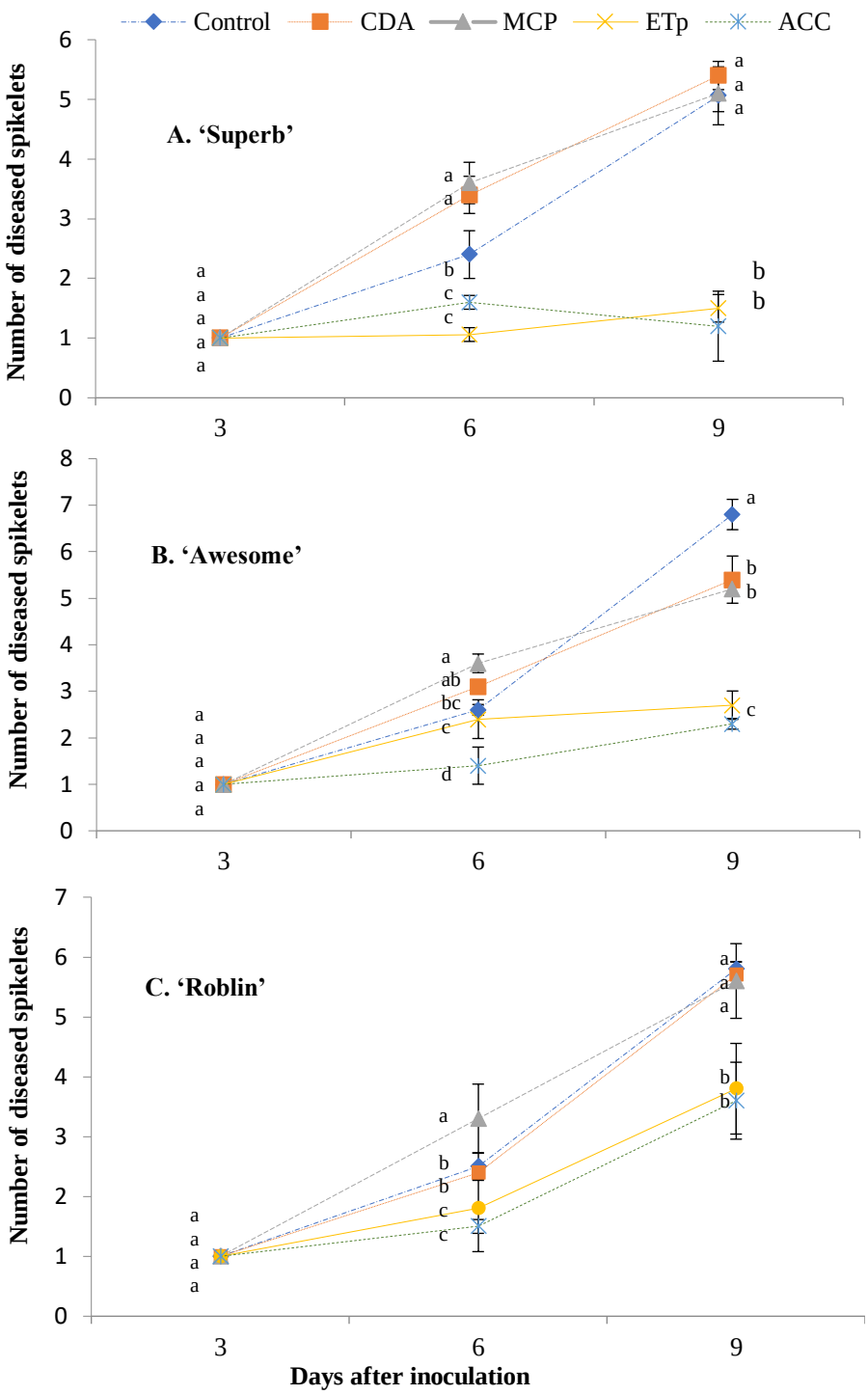

Figure 3.4: Disease assay following chemical treatment and point inoculation method in FHB-susceptible wheat genotypes. A. 'Superb' B. 'Awesome' C. 'Roblin'. Error bars represent standard deviations and treatments connected by the same letter are not significantly different at each rating date ($P < 0.05$).

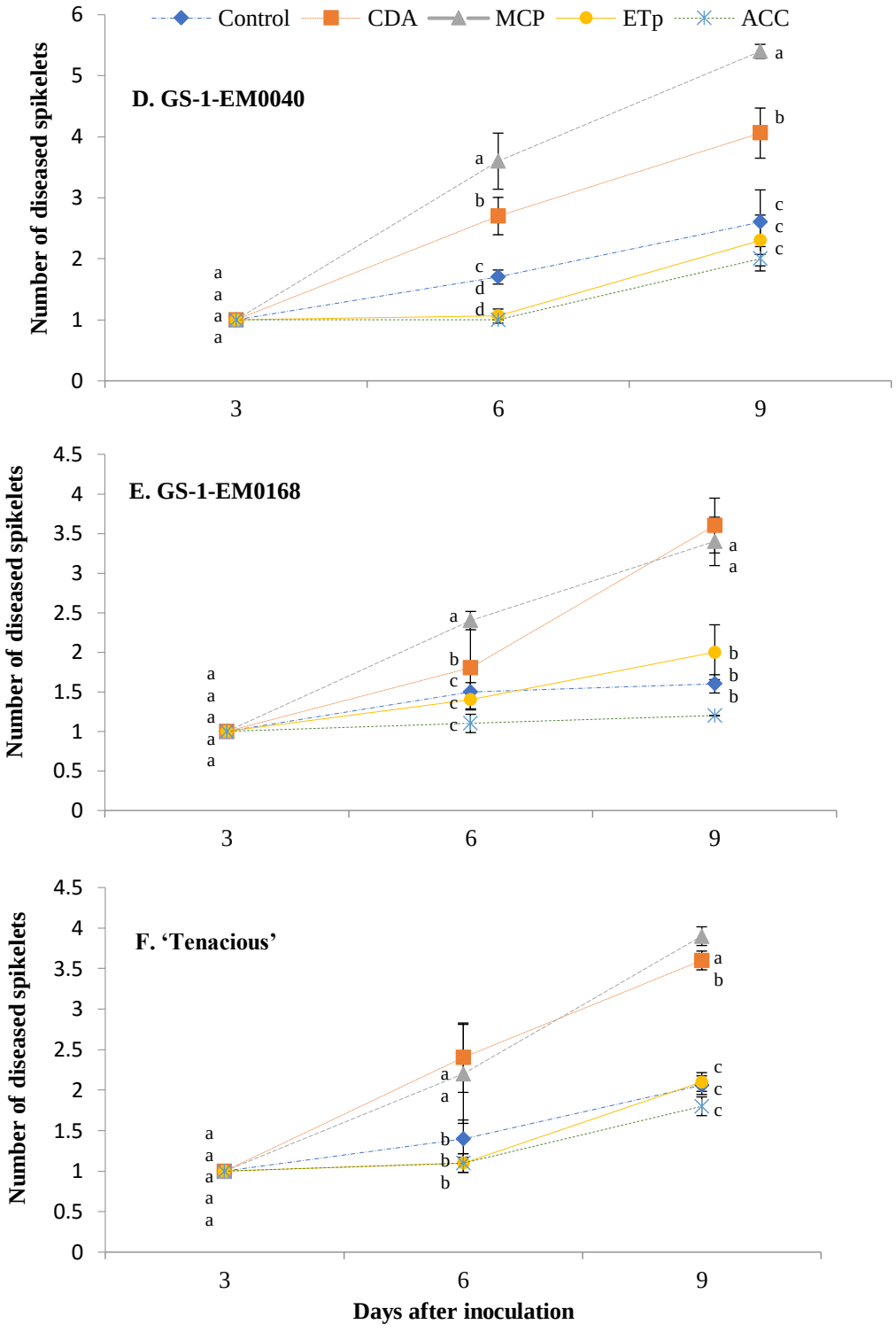

Figure 3.5: Disease assay of point inoculated heads following chemical treatments in FHB-resistant wheat genotypes. D. GS-1-EM0040 E. GS-1-EM0168 F. 'Tenacious'. Error bars represent standard deviations and treatments connected by the same letter are not significantly different at each rating date ($P < 0.05$).

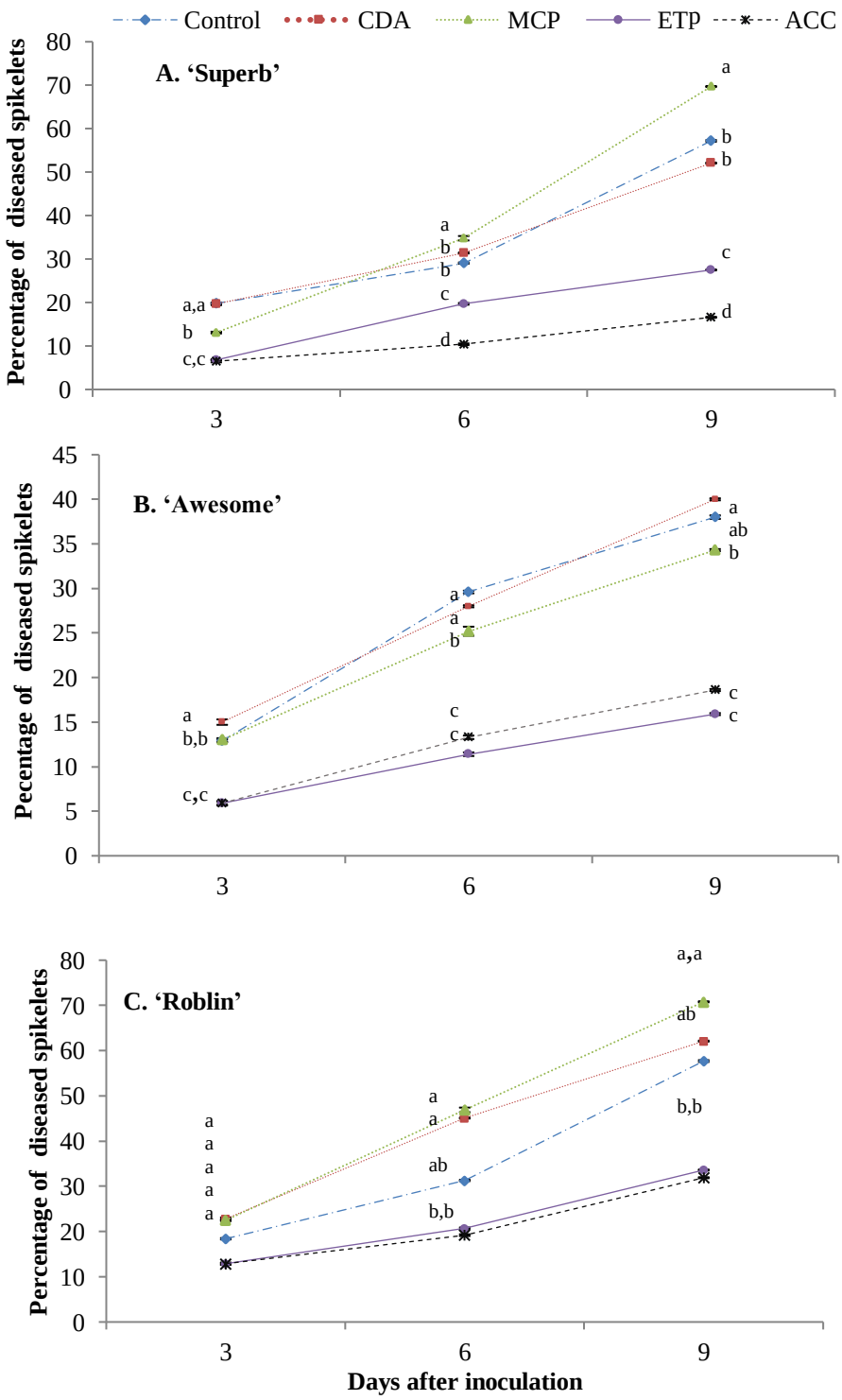

Figure 3.6: Disease assay following chemical treatment and dip inoculation method. A. 'Superb' B. 'Awesome' C. Roblin. Error bars represent standard deviations and treatments connected by the same letter are not significantly different at each rating date ($P < 0.05$).

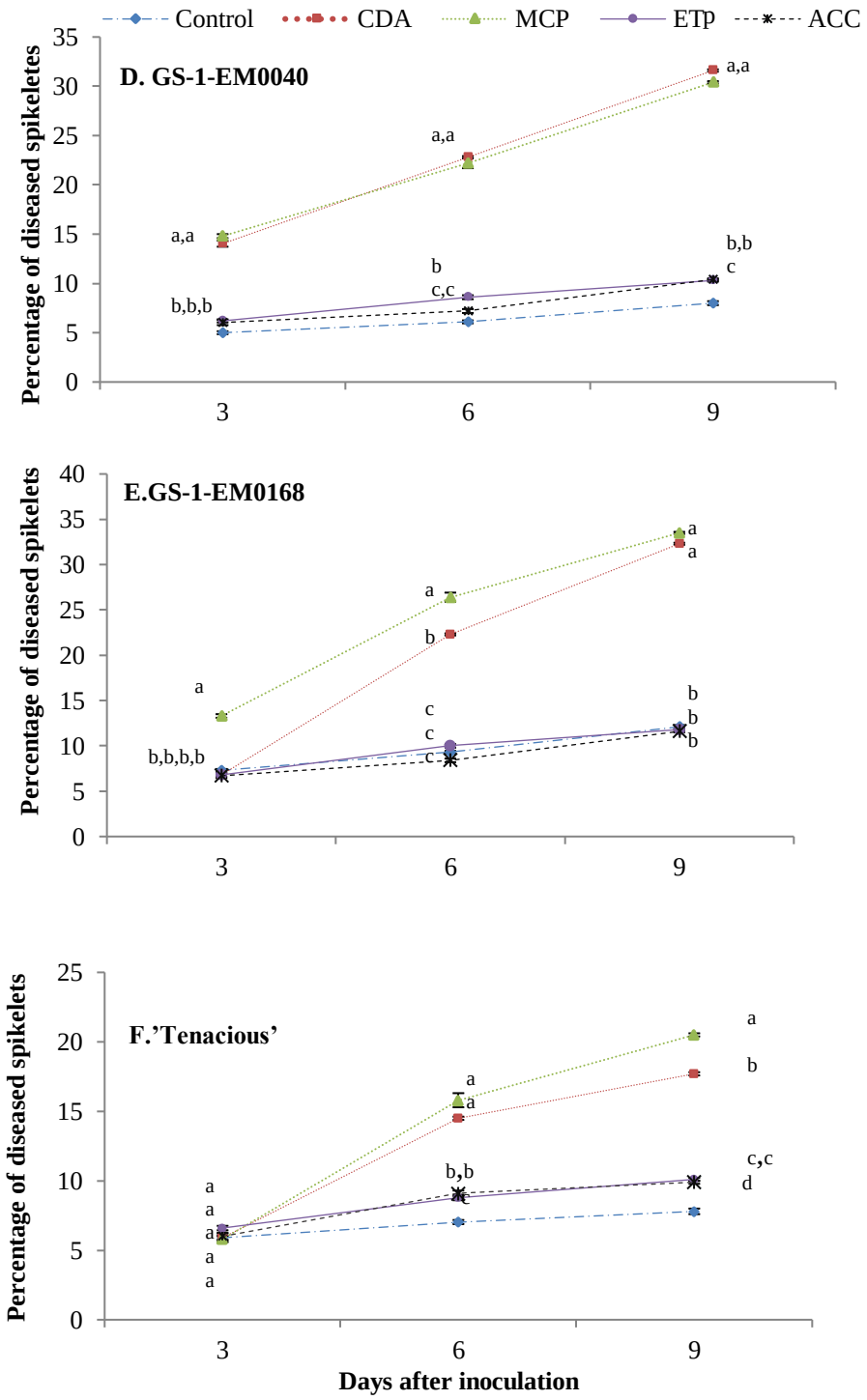

Figure 3.7: Disease assay following chemical treatment and dip inoculation method. D. GS-1-EM0040 E. GS-1-EM0168 F. 'Tenacious'. Error bars represent standard deviations and treatments connected by the same letter are not significantly different at each rating date ($P < 0.05$).

Chapter Four: Investigating the Role of Plant Hormone Signalling in Fusarium Seedling Blight Disease Response in Two Wheat Genotypes

4.1 Introduction

Among the numerous *Fusarium* species which are associated with diseases of cereals, the most prevalent species are *F. graminearum* and *F. culmorum.* It has been reported that these ascomycetous fungi are common causative agents of (FHB), Fusarium Seedling Blight (FSB), Fusarium Crown Rot (FCR), Fusarium Root Rot (FRR) and Fusarium Foot Rot (FFR) (Parry et al., 1995; Walter et al., 2010). FSB in wheat occurs at germination and throughout the seedling development, and it provides a pathogen source for subsequent FHB infections. FSB can be soil-borne through infection with mycelia or infected debris or spores in the soil (Rawlinson and Colhoun, 1969). FSB can damage growing seedlings extensively resulting in reduction of plant establishment, number of heads per square meter, and grain yield (Humphreys et al., 1998; Wong et al., 1992). However, because of the high risk of mycotoxin contamination in grains in FHB, disease management for FHB received more attention than FSB or foot rot in *Fusarium* disease complex. Pesticide application and biological the control shows some promising results in controlling FSB although these approaches can only partially reduce disease loss (Bhaskara Reddy et al., 1999). The best means to prevent losses due to FSB is the use of cereal cultivars with high resistance to Fusarium diseases (Dal Bello et al., 2002), however FSB-resistant germplasm is inadequate in nature and it is a challenge to develop resistant wheat varieties with suitable agronomic traits.

In general, the resistance level is related with the timing of induction of defence response and its strength. In plant-pathogen interactions, the regulation of plant defence responses are mediated by hormone signalling (Pandey et al., 2016). The role of plant

hormones in host resistance responses appears to differ depending on the pathogen lifestyle and also host susceptibility, but generally SA is involved in the activation of SAR following plant infection by biotrophic pathogens (Beckers and Spoel, 2006) while ET and JA offer defence against necrotrophic pathogens and herbivores (Bari and Jones, 2009; Glazebrook, 2005). Mei et al. (2006) reported that resistance to a hemibiotrophic pathogen, *Magnaporthe oryzae,* was higher in *OsAOS2*-overexpression rice plants. *M. oryzae* is a hemibiotrophic pathogen, but overexpression of *AOS2,* which is one of the genes involved in JA biosynthesis, increased overall resistance to this pathogen indicating that in monocots JA can contribute to resistance to hemibiotrophic pathogens. Moreover, in another study it has been observed that exogenous application of MeJA enhanced resistance to *Xanthomonas oryzae* pv *oryzae* (*Xoo*), which is a biotrophic pathogen in rice (Tamaoki et al., 2013). These results show that the JA signalling pathway can play a role in defence responses against biotrophic or hemibiotrophic pathogens in monocots (Deng et al., 2012; Kanno et al., 2012). In the case of FHB, Makandar et al. (2010) reported that JA plays a dichotomous role in the plant-FHB interactions. Over a time-course experiment they showed that MeJA application at the beginning (0 h) and 6 h after the *F. graminearum* infection led to disease enhanced disease severity, but exposure to MeJA at later stages of infection enhanced resistance (Makandar et al., 2010). These findings suggest that JA may mediate resistance response at the appropriate time point which in the case of *F. graminearum*, is probably when the fungus has entered the necrotrophic stage of infection. Interestingly, signalling pathways may be exploited by pathogens for spreading disease in the host plant. For example, Nalam et al. (2015) showed that knocking down *9-LOX*s genes, involved in JA biosynthesis pathway in both *Arabidopsis* and wheat resulted in a reduction of FHB disease severity. Moreover, up-regulation of these genes in wild-type plants

inoculated with *F. graminearum* confirmed that the pathogen may be exploiting the JA pathway to facilitate infection.

In most studies it has been shown that JA and ET signalling pathways act synergistically, but antagonistic effects have been observed between ET and SA signalling or JA and SA signalling (Koornneef and Pieterse, 2008; Niki et al., 1998). In *Arabidopsis*, the expression of JA-responsive genes such as *PDF1.2* is down-regulated by SA (Leon-Reyes et al., 2010b). The role of cross-talk among SA, JA and ET has not been investigated well in the monocot plant defence response. In a study it has been shown that SA and JA act antagonistically in mediating the defence response to *F. graminearum* infection. In case of FHB in monocots, it is not clear which signalling pathway is involved, in part because of contradictory data that has been described previously for example, according to microarray analysis, Li and Yen (2008) proposed that ET signalling plays a role in FHB resistance response in wheat, but Chen et al. (2009) documented that ET signaling facilitated *F. graminearum* colonization in wheat (Chen et al., 2009b; Li and Yen, 2008; Makandar et al., 2010; Zhou et al., 2005). These inconsistent results may be associated with the observation that *F. graminearum* is believed to be hemibiotrophic and displaying both biotrophic and necrotrophic phases (Van Loon et al., 2006b). Thus, it is possible that the particular signalling pathway that regulates resistance responses would dependent on the timing required for *F. graminearum* to switch from biotrophic to necrotrophic phase.

One of the techniques that are available to study the role of the hormone signalling metabolites in the resistance mechanism to FSB is modifying the signalling pathway by exogenous chemical (hormones, hormone activators and hormone inhibitors) treatments and observing the effect on FSB disease outcome. Modification of signalling pathways can be carried out by priming approach where chemicals are applied exogenously with the aim

of influencing the pathway of interest. Through this approach, defence responses can be activated and resistance to a variety of pathogens can be improved (Makandar et al., 2012; Qi et al., 2016). Prevalent priming agents include: methyl jasmonate (MeJA) (Farmer and Ryan, 1992); ETp, a chemical precursor of ethylene (Navet et al., 2003); ACC, precursor of ethylene (Yang and Hoffman, 1984); CDA, an inhibitor of 1-aminocyclopropane-1-carboxylic acid oxidase in ethylene biosynthesis pathway (Dourtoglou et al., 2000); MCP, an inhibitor of ethylene signalling pathway (Sisler and Serek, 1997); SA, plant hormone (Raskin, 1992); and benzothiadiazole (BTH), a synthetic plant defence inducer which is SA analogue (Wendehenne et al., 1998). Besides chemicals, priming with pathogens, or their elicitors, can also improve resistance, showing that priming is able to successfully activate the proper set of signalling pathways for an effective defence response to the pathogen of interest (Conrath, 2011; Foroud, 2011). In the priming (chemical or pathogen) approach, when the pathways of interest are involved in regulating a resistance or susceptible response to a specific plant disease, changes in disease response can be observed and assayed.

This project was conducted with a focus on the clarification of the early response of two wheat cultivars (FHB-resistant GS-1-EM0168 and FHB-susceptible ʻRoblinʼ) to FSB following priming with chemicals. The objective of the project was to further explore the putative roles of JA, SA and ET hormone signalling pathways and cross-talk among them in FSB-resistance of wheat genotypes infected with *F. graminearum*. A seedling assay protocol, which was modified in our lab (Goyal and Foroud, unpublished), was applied to screen hormones and hormone cross-talk effects on FSB-wheat interaction.

4.2 Material and methods

4.2.1 Plant material

Two wheat genotypes namely ‘Roblin’ (a susceptible Canadian cultivar) (Campbell and Czarnecki, 1987) and resistant ‘GS-1-EM0168’ (Foroud et al., 2012) were used for exogenous chemical priming in the current experiment. Seeds were planted into 24-well culture plates (VWR, 24 well tissue culture plates, Catalogue No. 10861-558) filled with vermiculite. Plates were sprayed with water and kept in containers with a lid for 5 days. The vents on the lids were left half opened and the plates sprayed with water daily. A basic germination test was carried out and a 90% germination rate was observed for both wheat genotypes. To account for the number of seeds that did not germinate, more seeds were planted.

4.2.2 Preparation of chemicals and chemical treatment

Chemical treatments were applied to seedlings at 5-days post-emergence, which corresponded to Zadok’s growth stage code 10 (Zadoks et al., 1974). SA (Aldrich Canada Ltd, Oakville, Ont., Canada, catalogue No. S-3007), MeJA (Sigma-Aldrich Chemical Company, Inc., Milwaukee, WI, USA, catalogue No. 392707) were first dissolved in distilled water to a stock concentration of 200 µM then, diluted in distilled water to a concentration of 100 µM. ACC (Sigma-Aldrich Chemical Company, Inc., Milwaukee, WI, USA, catalogue No MFCD00009944) and CDA (Sigma-Aldrich Chemical Company, Inc., Milwaukee, WI, USA, catalogue No 343412) were first dissolved in distilled water to a stock concentration of 0.1 M then, diluted in distilled water to a concentration of 100 µM. For co-treatment of hormones (SA+MeJA, ACC+MeJA, ACC+SA), first a stock concentration of 200 µM for each chemical was prepared and then, 100 µM concentration for each chemical was obtained by mixing equal amounts together. Tween-20 (Fisher

Scientific, Nepean, Ont., Canada, catalogue no. BP 337) was added (0.01%) as a surfactant to final solutions. Fresh stocks were prepared at the day of treatment application for each replication. The final solutions were sprayed onto seedlings until droplets formed (Matsumoto et al, 1980). Control plants were sprayed with water supplemented with 0.01% of Tween-20. Plants were immediately placed in containers with a lid on for 2 h prior to inoculation. To avoid crosstalk effects among treatment containers were kept in separate labs at the same temperature. Three experimental repetitions were performed; each with 70 biological replicates, where experimental repetitions were differentiated by seeding dates and a different batch of inoculum was used. Each biological replicate consisted of one seedling for a given condition.

4.2.3 Preparation of inoculum, seedling inoculation and incubation with the inoculum

F. graminearum (GZ3639 wild-type strain) inoculum was prepared as described in chapter 3 and the stock was diluted to 1 x 10^5 macroconidia mL^{-1}. After 2 h of chemical priming the seedlings were cut 2-3 mm from the top and inoculated by placing a droplet (3µL) of the *F. graminearum* macroconidia suspension onto the cut surface. The same procedure was followed for the control seedlings. Since the seedlings were wounded prior to inoculation, in order to separate the effect of wounding chemical treatments and/or from *F. graminearum* infection, mock inoculated control plants (with no chemical treatment) were also included (data not shown). Inoculated seedlings were then incubated in a closed container with a plastic lid for 3 days. Water was sprayed inside the containers to provide the necessary humidity for disease progression. After 3 days, infected seedlings were transferred into root-trainers (Spencer–Lemaire Industries, Edmonton, Alta.) containing Cornell peat-lite mix (Boodley and Sheldrake, 1977) and were placed in the greenhouse at

21°C (day) and 18°C (night) for 16 photoperiod. In each replication, disease was evaluated at 6, 9 and 12 days after inoculation (DAI) and the percentage of the seedlings that survived after inoculation with *F. graminearum* following treatment with chemicals was recorded.

4.2.4 Statistical analysis

ANOVA was carried out using SAS program, version 9.3 software (SAS, USA). The analysis was done for each data set of each wheat genotype treated with different chemical. When significant interactions of factors were observed, comparison tests were performed within each plant line, at each rating date (6, 9 and 12 DAI) with Fisher's LSD test to determine significant differences. Data considered significantly different ($p < 0.05$) between chemical treatments for each wheat genotype at each rating date are marked with letters.

4.3 Results

No changes were observed in mock inoculated plants compared with inoculated controls confirming that a wounding effect did not interfere with the results of the disease assay (data not shown).

4.3.1 Disease assay in inoculated seedlings following treatment with ET activators/inhibitors

For the cultivar 'Superb', CDA application resulted in the lowest percentage of the surviving seedlings (56%) and ACC treatment showed the highest seedling survival (93.9%) compared to 76% in the control at 6 DAI (Figure 4.1.A). At 9 DAI, ACC treatment resulted in a significant increase in the percentage of surviving seedling (88%) compared to the control (68%) ($P < 0.05$).

No significant difference was observed between the control and ACC treatments at 6 DAI in the genotype GS-1-EM0168 (96% and 94% for the control and ACC, respectively)

(Figure 4.1.B). CDA treatment showed the lowest percentage of surviving seedlings, 78.5%, compared to 75% in the control ($P < 0.05$) at 6 DAI. At 9 DAI, CDA treatment decreased the percentage of surviving seedlings (77%) significantly from the control (95%). At 12 DAI, no significant difference in percentage of surviving seedlings was observed between ACC treatment (90%) compared with the control (93%).

4.3.2 Disease assay in inoculated seedlings following treatment with SA/MeJA and effect of cross-talk between SA and MeJA on seedlings survival

In the cultivar 'Roblin', application of SA increased seedling survival; it was significantly higher than upon application of MeJA or in the control (92% for SA, 70% and 78% for MeJA and the control, respectively). In contrast, application of MeJA significantly decreased the percentage of surviving seedlings at 6 DAI, 70%, as compared to 78% in the control (Figure 4.2.A). No significant difference was observed between co-treatment of SA with MeJA and the control at 6 DAI (80% and 78% for SA+MeJA co-treatment and the control, respectively). By 9 DAI, SA treatment showed the highest percentage of surviving seedlings compared to the control (88% and 67% for SA and the control, respectively). No significant difference in the percentage of surviving seedlings was observed between MeJA and the control at 9 DAI (67% and 65% for MeJA and the control, respectively). Co-treatment of SA+MeJA increased percentage of surviving seedlings significantly from the control at 9 DAI (78% and 65% for SA-MeJA treatment and the control, respectively). At 12 DAI, the same observed-trend at 9 DAI was observed for different treatments.

No significant difference was observed between SA and the control treatments at 6 DAI in GS-1-EM0168, where both showed the highest percentage of surviving seedlings (95% and 94% for SA and the control treatments, respectively) ($P < 0.05$) (Figure 4.2.B). Application of MeJA resulted in the lowest percentage of surviving seedlings (85%)

compared with the control (94%) at 6 DAI. Co-application of SA and MeJA significantly decreased percentage of surviving seedlings, 87%, compare to 94% in the control at 6 DAI (Figure 4.2.B). By 9 DAI, MeJA treatment resulted in the lowest percentage of surviving seedlings (80%) compared to the control (85%). SA treatment showed no significant difference with the control and both led to the highest percentage of surviving seedling at 9 DAI (90% and 88% for SA and the control, respectively).

4.3.3 Disease assay in inoculated seedlings following ACC+MeJA co-treatment and ACC+SA co-treatment

At 6 DAI, co-treatment of ACC+SA showed no significant difference compared to the control and both resulted in the highest percentage of the surviving seedlings (76% and 73% for ACC+SA and the control, respectively) in 'Roblin' (Figure 4.3.A). ACC+MeJA treatment resulted in the lowest percentage of surviving seedlings (61%) compared with the control (73%) at 6 DAI. By 9 DAI, co-treatment of ACC+ SA showed the highest percentage of surviving seedlings (72%) compared to the control (66%). The same observed-trend at 9 DAI was observed at 12 DAI.

In the genotype GS-1-EM0168, co-treatment of ACC+MeJA resulted in the lowest percentage of surviving seedlings (83.9%) compared with the control (98%) at 6 DAI ($p < 0.05$) (Figure 4.3.B). Co-treatment of ACC+SA (88%) and co-treatment of ACC+MeJA (83.9%) decreased seedling survival significantly compared to the control (98%), but co-treatment of ACC+SA (88%) decreased seedling survival more profoundly (Figure 3.B). By 9 DAI, co-treatment of ACC+MeJA resulted in the lowest percentage of surviving seedlings (80%) compared to the control (98%). Co-treatment of ACC+SA significantly decreased percentage of the surviving seedlings (87%) compared to the control (98%) at 9 DAI ($p < 0.05$).

4.4 Discussion

The role of SA and JA signalling pathways in defence responses has been well studied in dicot and monocot species (Balmer et al., 2013; Glazebrook, 2005). The role of ET signalling pathway in defence response has been investigated in dicot plants more than monocots. Moreover, despite many studies that have investigated cross-talk among SA, JA/ET in dicots, there are not many related studies in monocots. In the current study, the putative role of SA, JA and ET signalling and cross-talk among these pathways was investigated in the FSB-wheat interaction. According to the results of the current study I propose that JA contributes to susceptibility, SA contributes in resistance response and ET plays a role in the defence response. Furthermore, negative cross-talk among SA and JA was observed where JA attenuated the effect of SA (Figure 4.3).

In this experiment, two wheat genotypes (FHB-resistant DH line GS-1-EM0168 and FHB-susceptible cultivar 'Roblin') were initially used to examine the role of ET signalling pathways in response to FSB. The findings showed that triggering ET biosynthesis increased resistance in the susceptible, but not resistant genotype, whereas inhibition of ET biosynthesis resulted in an increase in susceptibility in both genotypes.

Following analysis of the effect of ET signalling on FSB, the same two wheat lines were used to study SA and JA signalling, as well the effect of hormonal cross-talk, on FHB response. Treatment with SA prior to FSB infection resulted in resistance responses (with an average of 89% seedling survival compared to 67% in the control) in the cultivar, 'Roblin' (with an average of 89% seedlings survivals compared to 67% in the control). In the resistant genotype (GS-1-EM0168) no significant difference was observed between the control treatment (average of 87.1% surviving seedlings) and SA treatment (average of 86.8% surviving seedlings). SA has been reported in many studies as the main metabolite

which regulates SAR induction through NPR1 in monocots and dicots (Kogel and Langen, 2005; Makandar et al., 2006). As described in the introduction, SAR is activated systemically at sites distal to a localized infection (Chaturvedi and Shah, 2007). It has been reported that under normal conditions, a higher concentration of SA is maintained in rice compared with tobacco indicating that SA signalling is important in basal defence of monocots (Balmer et al., 2013; Enyedi et al., 1992; Silverman et al., 1995). In rice, it has been reported that the cellular concentration of SA is high and pathogen infection does not increase it beyond the cellular level (Silverman et al., 1995). However, SA-degrading enzyme salicylate hydroxylase (NahG) expression in transgenic rice increased susceptibility to *Magnaporthe grisea*, a hemibiotrophic fungus (Yang et al., 2004). Moreover, in NahG wheat plants, higher FHB severity was reported compared with a non-transgenic susceptible wild-type plants, namely 'Bobwhite' (Makandar et al., 2012). In wheat, induction of SAR has been reported in response to leaf and stem rust, powdery mildew, FHB and also with application of SAR inducers such as benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Barna et al., 1998; Görlach et al., 1996; Makandar et al., 2012; Schaffrath et al., 1997; Wang et al., 2010). In the current study, SA treated seedlings of the genotype GS-1-EM0168 hadthe same resistance response as the controls, which may show that SA levels in this genotype might be high under normal conditions and exogenous application does not alter that.

Treating wheat seedlings with MeJA resulted in a significant decrease in the percentage of seedling survival in the resistant genotype GS-1-EM0168 compared with the control treatments at 6, 9 and 12 DAI (Figure 4.2.B). However, in the FHB susceptible cultivar, 'Roblin', application of MeJA did not affect seedling survival compared to the control treatments at 9 and 12 DAI (Figure 4.2.A). JA signalling, unlike SA, regulates defence

mechanisms involved in responses against necrotrophic pathogens which obtain nutrients from dead host cells (Glazebrook, 2005). In addition, JA plays a role in the induction of induced systemic resistance (ISR), which is expressed in the leaves when roots are colonized by some rhizobacterial species (Pieterse et al., 2003). It has been documented that cellular concentrations of SA significantly decreased in response to exogenous application of MeJA in rice (Tamaoki et al., 2013). These results suggest that JA supresses SA signalling and may explain the susceptible response of GS-1-EM0168 to FSB after treatment with MeJA in the current experiment. It has previously been reported that, the level of JA in spikes of Type II FHB-resistant, GS-1-EM0168, is twofold higher than in FHB-susceptible ʻSuperbʼ or FHB-resistant GS-1-EM0040 (Foroud, 2011). These results may explain activation of a local resistance response in GS-1-EM0168 (Foroud, 2011). In a related study, Foroud (2011) reported that priming with FgTri5-, which is a trichothecene non-producing strain of *F. graminearum* (GZT3639 (FgTri5+); Proctor et al., 1995), induced up-regulation of JA-responsive genes in FHB-susceptible ʻSuperbʼ at 3 h after inoculation and also increased susceptibility to FgTri5+ infection. By contrast, inoculation with FgTri5- in FHB-resistant GS-1-EM0040 induced a delayed (18 DAI) up-regulation of JA signalling which resulted in resistance response. In this study, MeJA resulted in a susceptible response where treatment of seedlings with MeJA significantly decreased the percentage of seedling survival compared to the control at 6, 9 and 12 DAI in GS-1-EM0168 (Figure 4.2.B).

Generally, separate observations in ʻRoblinʼ and GS-1-EM0168 showed that co-treatment of MeJA+SA decreased percentage of seedling survival compared to SA treatment (Figure 4.2.A and 4.2.B). However, compared to MeJA application, co-treatment with MeJA+SA significantly increased the percentage of surviving seedlings (Figure 4.2.A

and 4.2.B). JA and SA biosynthesis/signalling are antagonistically regulated: when JA signalling is activated, cellular SA concentration is remarkably decreased, resulting in suppression of SA signalling (Navarro et al., 2008; Tamaoki et al., 2013). Cross-talk between JA and SA signalling modulates defence responses and both are required for basal resistance to *F. graminearum* (Makandar et al., 2010; Pieterse et al., 2009). It has been documented that JA plays a dichotomous role in *Arabidopsis* responses to *F. graminearum*, during the early phase of infection and improvement of the defence response to *F. graminearum* during later phases of infection (Makandar et al., 2010). Makandar et al (2012) reported that early application of MeJA, prior to *F. graminearum* inoculation or early stage of infection did not lead to a resistance response in the susceptible wheat cultivar, 'Bobwhite'. Similarly, in the current study application of MeJA showed no effect in the susceptible cultivar 'Roblin' compared with the control, but enhanced susceptibility in the genotype GS-1-EM0168. Also, in the genotype GS-1-EM0168, co-application of MeJA+SA attenuated the effect of SA, leading to a significant decrease in the percentage of surviving seedlings compared with the control ($p < 0.05$). It has been suggested that, during early stages of *F. graminearum* infection, when the pathogen is in the biotrophic stage (Goswami and Kistler, 2004), The induction of SA signalling decreases *F. graminearum* infection and application of MeJA suppresses SA/NPR1-induced defence signalling and SA-responsive gene expression such as *PR1* (Gaupels, 2015; Makandar et al., 2012). Thus, it is highly likely that pre-treatment with MeJA down-regulates *PR1* gene expression in response to *F. graminearum* infection (Makandar et al., 2012). In addition, Ding et al. (2011) showed that in a FHB-resistant wheat genotype 'Wangshuibai', expression of SA signalling associated genes, including *NPR1*, *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), *PHENYLALANINE AMMONIA LYASE* (*PAL*), which

contribute to biosynthesis of many secondary metabolites, were up-regulated faster compared with an FHB-susceptible mutant of 'Wangsuibai', Meh0106 (Ding et al., 2011). These data showed that SA signalling transduction is important at earlier stages, compared with JA signalling, to restrict *F. graminearum* infection. In this experiment it has been shown that application of SA prior to *F. graminearum* infection resulted in a resistance response in both genotypes, but MeJA treatment led to a susceptible response.

In the current study, co-treatment of ACC+SA significantly increased seedling survival in the FHB-susceptible genotype, 'Roblin' compared to the control (Figure 4.3.A). In contrast to the observed trend in 'Roblin', co-treatment of ACC+SA significantly decreased the percentage of surviving seedlings compared to the control in the FHB-resistant genotype, GS-1-EM0160 (Figure 4.3.B). Co-treatment of ACC+MeJA resulted in a decrease in the percentage of seedling survival in FHB-susceptible 'Roblin' compared to the control ($p < 0.05$) (Figure 4.3.A) and the same observed-trend in 'Roblin' was observed in FHB-resistant genotype, GS-1-EM0160 (Figure 4.3.B). It has been documented that there is a significant difference in the basal level of SA between FHB-susceptible and FHB-resistant wheat genotypes, where resistant genotypes have a higher concentration of SA compared with susceptible ones (Buhrow et al., 2016; Ding et al., 2011). The positive effect of ACC in the FHB-susceptible wheat cultivar response to *F. graminearum* infection in this experiment is in support of the observation in chapter 3. In the genotype GS-1-EM0168, the antagonistic effect of ET on SA is pronounced and co-treatment of SA+ACC likely resulted in suppression of SA signalling leading to a significant decrease in the percentage of surviving seedlings compared to the control. The cross-talk effect between SA and ACC in the genotype GS-1-EM0168 attenuated the SA effect, resulting in a significant decrease in seedlings survival compared to the control (Figure 4.3.B). Compared to co-treatment of

ACC+MeJA, application of ACC+SA significantly increased the percentage of seedling survival in the genotype GS-1-EM0168 ($p < 0.05$) (Figure 4.3.b). These results suggest that MeJA compromised SA effect and mediated susceptible responses to *F. graminearum* infection. Early stages in *F. graminearum* infection are biotrophic and SA plays an important role to limit the infection. It can explain the significant decrease in percentage of surviving seedlings treated with SA+MeJA compared to the control in the cultivar 'Roblin' and the genotype GS-1-EM0168. These results also indicate an antagonistic effect between SA and JA.

Taken together, the results from the mentioned studies and the results presented here propose that resistance to the hemibiotrophic fungus *F. graminearum* is related to fine tuning of defence responses by plant hormones where induction of a proper signalling pathway in biotrophic and necrotrophic phases of infection is important. In the current study according to the FSB disease assay data for 'Roblin' and GS-1-EM0168; it was shown that application of SA prior to infection with *F. graminearum* led to an increase in the percentage of surviving seedlings. In contrast, co-treatment of ACC +MeJA prior to FSB infection resulted in the lowest percentage of surviving seedlings compared to the control in the cultivar 'Roblin' and in the genotype GS-1-EM0168. Since similar observed-results for two wheat genotypes in the FHB response to ET activators/inhibitors (Chapter 3) were observed here for FSB, it can be concluded that similar pathways that were involved in mediating the response to FHB were induced in response to FSB as well. In the current study, the disease assay results along with the results from chapter 3 show that, in susceptible and resistant genotypes, it seems ET does not mediate susceptible responses. According to these results and observations from Allen et al. (2016) where similar results

were shown in response to FCR it can be concluded that the role of ET seems to be conserved in FHB, FSB and in FCR.

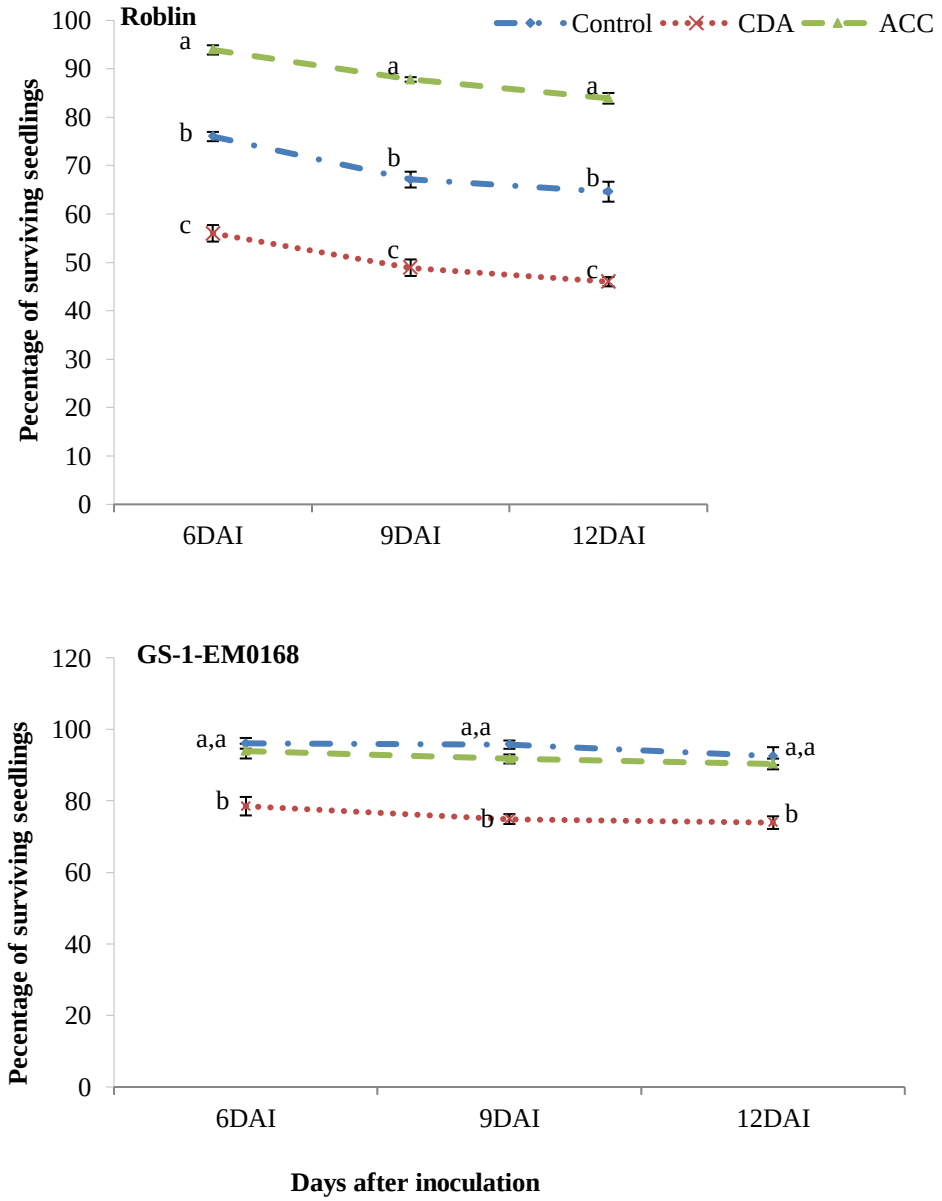

Figure 4.1: Effects of ET activator/inhibitor priming on the survival of **A**. 'Roblin' and **B**. GS-1-EM0168 wheat seedlings following inoculation with *F. graminearum*. Data were presented as percentage of surviving seedlings. Error bars represent standard deviations and treatments connected by the same letter are not significantly different ($P < 0.05$).

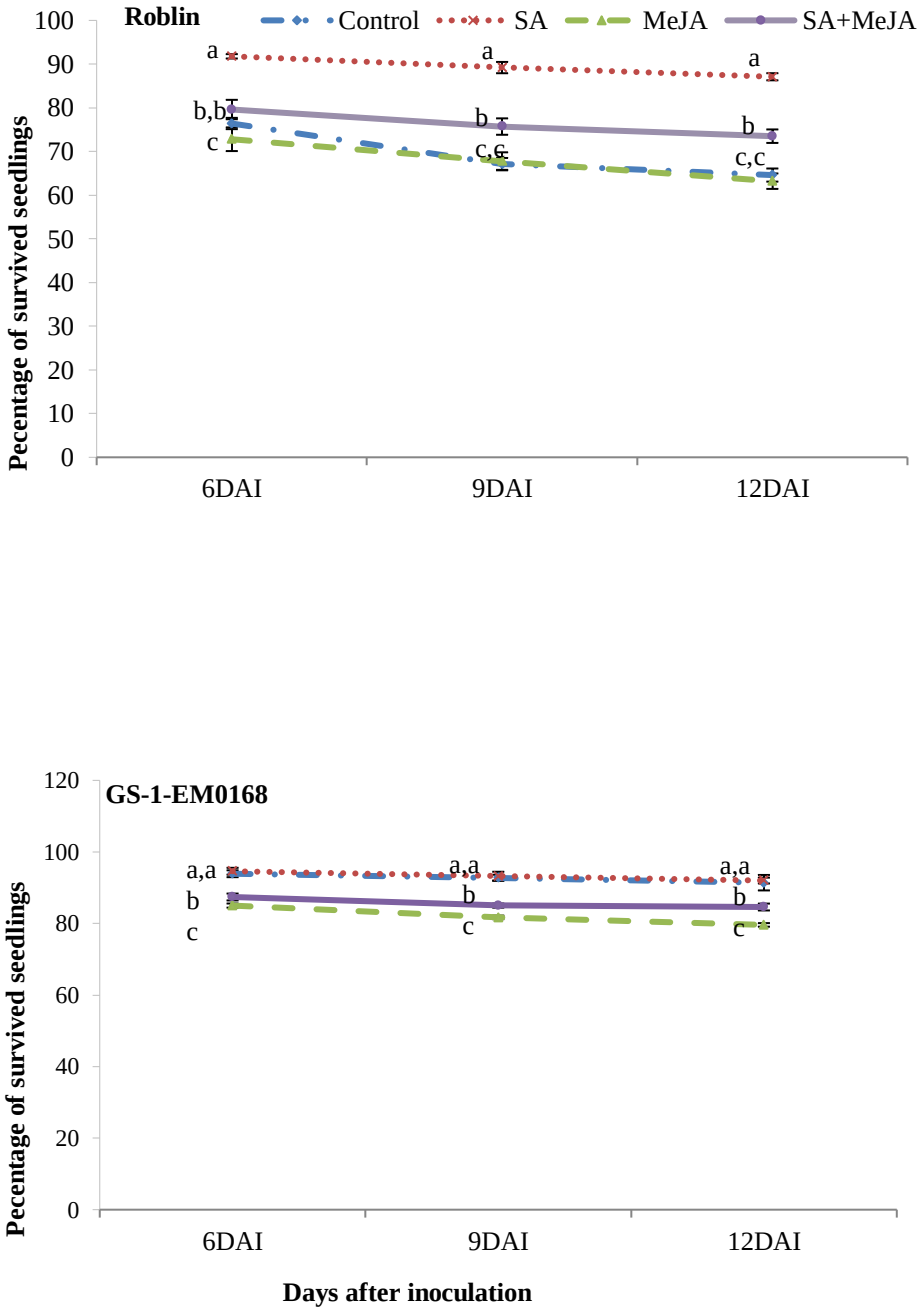

Figure 4.2: Priming effects of SA, MeJA and cross-talk between them on survival of **A**. 'Roblin' and **B**. GS-1-EM0168 wheat seedlings following inoculation with *F. graminearum*. Data were presented as percentage of surviving seedlings. Error bars represent standard deviations and treatments connected by the same letter are not significantly different ($P < 0.05$).

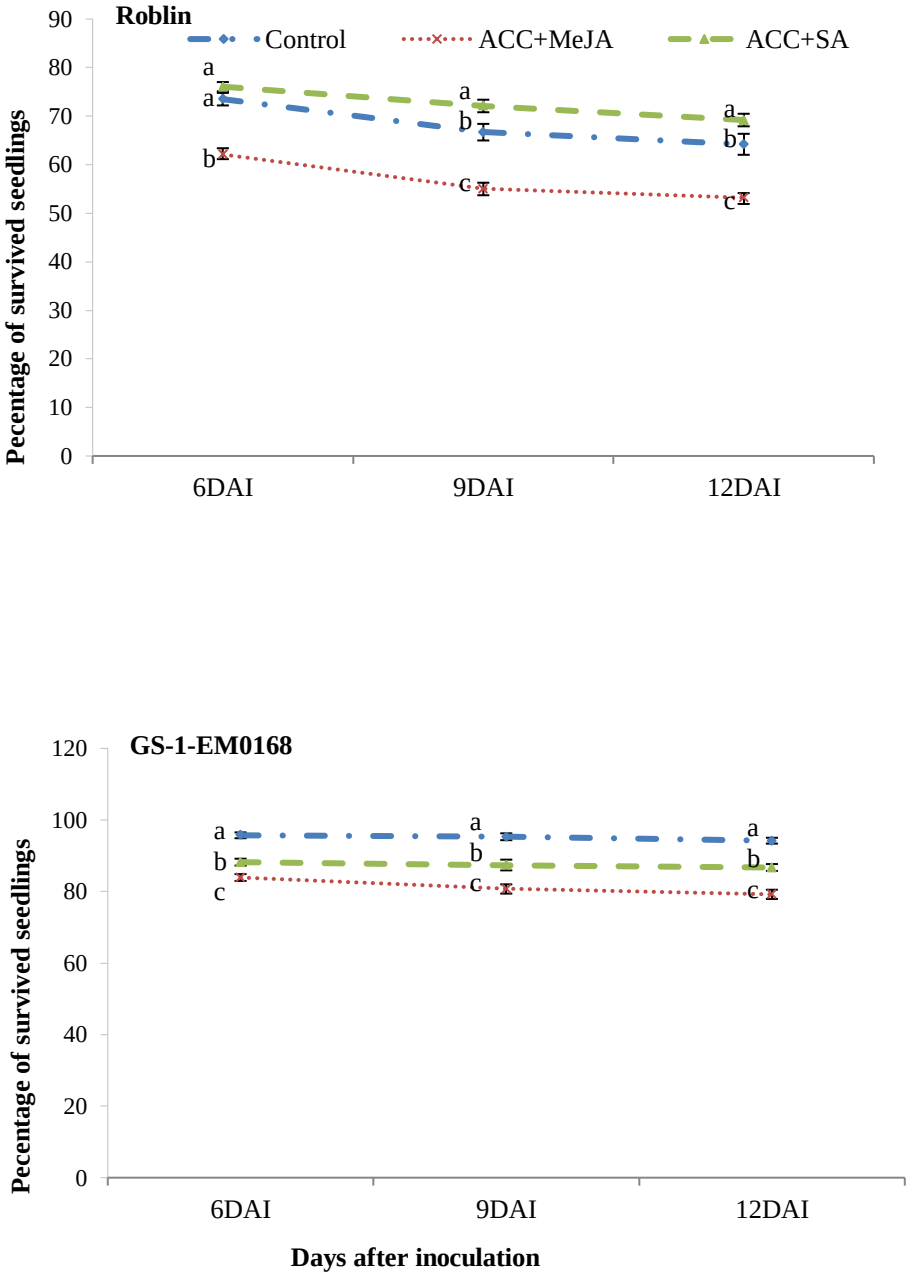

Figure 4.3:. Priming effects of cross-talk between ACC+MeJA and ACC+SA on survival of **A**. 'Roblin' and **B**. GS-1-EM0168 wheat seedlings following inoculation with *F. graminearum*. Data were presented as percentage of surviving seedlings. Error bars represent standard deviations and treatments connected by the same letter are not significantly different ($P < 0.05$).

Chapter Five: Final Conclusions and Future Directions

The regulation of plant disease resistance responses by hormone signalling pathways is a common process in many plant-pathogen interaction (Sun et al., 2016). The molecular mechanisms underlying the role of plant hormones in mediating resistance response to FHB is poorly understood, although, the physiological mechanisms are well defined. It was shown that SA signalling has a direct effect on *F. graminearum* spore germination while in wheat-FHB interaction activation of SA pathway either had positive effect or no effect on plant resistance (Foroud, 2011; Qi et al., 2016; Qi et al., 2012). It was reported that JA plays a positive role in FHB-wheat interaction (Li and Yen, 2008; Qi et al., 2016). Moreover, it was shown that the level of plant hormone MeJA in Type II resistance genotype is higher compared with other genotypes (Foroud, 2011). The high level of MeJA in Type II resistance can be related to the role of this plant hormone in lignification which plays an important role in cell wall thickening, an important factor in Type I and Type II resistance. The role of ET in FHB-wheat interaction is not clear in part because the available evidence points in opposite directions as described in Chapter 1 (Chen et al., 2009b; Foroud, 2011; Gottwald et al., 2012; Li and Yen, 2008; Sun et al., 2016).

These conflicting results regarding the role of plant hormones in mediating resistance responses in FHB-wheat interaction may be related to the observation that cross talk among plant hormones has an important impact in fine-tuning the defence response in plants. In addition, genotype dependent responses to FHB may in part explain the conflicting results. Foroud (2011) proposed that the role of ET in FHB-wheat interaction is genotype dependent. Based on these studies, I hypothesized that plant hormones regulate plant disease resistance response in a genotype dependent manner. To confirm this hypothesis,

first I tested whether the expression of various *PR* genes, which are essential components of hormone-induced disease response, is genotype dependent.

In Chapter 2, gene expression study of defence-related *PR* genes showed that up-regulation of different *PR* genes are induced strongly in Type I resistant genotype by different plant hormones while the same responses were not observed in Type II resistant genotype or susceptible cultivar. I further demonstrated that in three wheat genotypes with different resistant/susceptible background (FHB-susceptible 'Superb', Type I resistant GS-1-EM0040 and Type II resistant GS-1-EM0168) application of different plant hormones induced significant changes in *PR* genes (*PR-1*, *PR-2*, *PR-3*, *PR-8*, *PR-9* and *PR-13*) expression in each wheat genotype and a similar trend was not observed among three wheat genotypes. These results support the original hypothesis of the study that wheat genotypes with different types of resistance to FHB seemed to induce defence responses differently from each other and these responses were genotype-dependent. These results led to development of a new hypothesis that *PR* genes play more important role in response to FHB in Type I resistant genotype compared to Type II resistant.

One of the challenges regarding Type I resistance is the variability in the exposure of different spikelets or spikes to the inoculum and also the amount of inoculum that reaches the spikelets. To obtain a more controlled assay, I optimized a detached head assay with a novel method of inoculation that provides a uniform exposure to inoculum for wheat heads, making Type I assessment more accurate. Optimized detached heads showed promising results for point inoculation method when compared with whole plant assay. In Chapter 3, the detached head assay method was used for homogenized and highly controlled chemical application to evaluate the hypothesis that ET plays an essential role in FHB response of wheat in a genotype-dependent manner. The disease progress in six wheat genotypes with

different level of resistance/susceptibility to FHB was measured following chemical treatment. The data showed that ET signalling has a positive effect on mediating defence responses to FHB in both resistant and susceptible genotypes. Inhibition of ET signalling was shown to increase susceptibility in susceptible genotypes and enhanced disease progress in resistant genotypes. In addition, activation of ET signalling was shown to reduce susceptibility to FHB in susceptible genotypes, whereas no effect was observed on FHB resistance in resistant genotypes. Based on the data of this experiment I concluded that ET plays a positive role in mediating resistance responses to FHB in both resistant and susceptible genotypes and that these responses are more pronounced in resistant genotypes. These results do not support the original hypothesis of the project. However, the results led to the development of a new hypothesis that ET accumulates in higher quantities in the resistant genotypes.

Other hormone signalling pathways were also studied in the seedling assay experiment with one resistant and one susceptible wheat genotypes (Chapter 4). To confirm the results from Chapter 3, regarding the role of ET in FHB-wheat interaction in FSB-wheat interaction, seedlings were treated with ET activator/inhibitor. The same trend was observed - the inhibition of ET decreased seedlings survival in both resistant and susceptible wheat genotypes, whereas activation of ET only reduced susceptibility in the susceptible genotype. These results do not support the genotype dependent role of ET in FHB-wheat interaction and do not support the original hypothesis of the thesis. Regarding the role of other plant hormones, the data suggest that SA and MeJA signalling have a positive and a negative effect on FSB-wheat interaction, respectively, regardless of genotype. The results also show that SA and MeJA interact antagonistically in FSB-wheat interaction and it seems the positive effect of SA in FSB-wheat interaction is masked by

MeJA and lead to decreased seedlings survival in both genotypes. Cross talk between ET and MeJA was also antagonistic where co-treatment of ACC and MeJA decreased seedlings survival in both resistant and susceptible genotypes. Since positive and negative effects of SA and MeJA, respectively, are not genotype-independent, the original hypothesis is not supported.

Generally, the results from Chapter 3 and 4 do not support the original hypothesis of the study. Results from Chapter 3 suggest that ET plays a positive role in FHB resistance in three susceptible and three resistant wheat genotypes. Seedling assay results (Chapter 4) show that in an FHB-susceptible and a resistant genotype, ET also plays a positive role in resistance responses. Therefore, my results show that the positive role of ET signalling pathway in mediating defence response to FHB and FSB is conserved in the resistant or susceptible wheat genotypes thus, it is not genotype-dependent. These results make it clear that ET plays a role in FHB-wheat interaction, but it does not address the reason behind the conflicting on-going results about positive or negative role of ET signalling pathway in response to FHB.

Since *F. graminearum* is a hemibiotrophic pathogen and in each phase of the infection, induction of a proper signalling pathway is important, for future work I propose that the effect of different plant hormones in *F. graminearum* infection in wheat has to be studied through a time course experiments to compare the effect of early and late applications of plant hormones on disease outcome. Gene expression study of the diseased spikelets after hormone application to screen changes in hormone responsive genes during different stages of the infection can also reveal more information about the induction of each signalling pathway during *F. graminearum* infection. I also propose that the effect of each plant hormone has to be compared in single hormone treatment *versus* multiple hormone

treatments, to evaluate the potential effect of cross-talks. For example, the effect of SA has to be compared with the effect of ACC+SA. In the work present in Chapter 4 of this thesis, MeJA application decreased seedling survival, likely by increasing plant susceptibility to *Fusarium*. I propose that for future work the role of MeJA in FSB-wheat interaction has to be studied in a time course experiment, as MeJA's role in FHB-wheat interaction is likely time dependent and it may increase resistance in later phases of *F. graminearum* infection.

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Chapter Seven: Appendix: Supplementary Tables

Table 6.1: Time-course expression of wheat *PR-1.1* transcripts in three wheat genotypes exposed to SA, MeJA, ETp and water

	Treatment	Line	3 h	6 h	8 h	12 h	24 h
Salicylic Acid	50 µM	ʻSuperbʼ	-0.45	-0.82	1.60	-0.92	-1.68
		GS-1-EM0040	-3.49	-1.89	1.62	0.16	1.78
		GS-1-EM0168	*-2.30	-0.52	0.08	-5.11	1.90
	100 µM	ʻSuperbʼ	3.29	1.48	-0.34	-1.87	-0.03
		GS-1-EM0040	-2.37	*-4.35	1.76	*-2.95	*2.76
		GS-1-EM0168	-2.91	-0.55	-0.04	-0.84	-0.48
	200 µM	ʻSuperbʼ	-0.23	0.74	*-3.38	*-2.06	-0.44
		GS-1-EM0040	1.73	*-6.26	-0.55	-0.29	*2.02
		GS-1-EM0168	*-2.97	0.29	-0.43	*-3.79	-1.92
Methyl-Jasmonate	50 µM	ʻSuperbʼ	3.62	*2.95	-0.70	*-2.78	*2.76
		GS-1-EM0040	-1.08	*-6.38	0.62	-1.99	*4.10
		GS-1-EM0168	-1.00	-0.40	0.09	*-2.60	*2.99
	100 µM	ʻSuperbʼ	1.11	*3.51	0.95	-0.46	*3.23
		GS-1-EM0040	-1.92	*-4.57	*2.14	-0.16	*2.55
		GS-1-EM0168	-1.97	-0.94	0.77	1.40	2.15
	200 µM	ʻSuperbʼ	3.85	1.80	0.62	-1.77	*4.34
		GS-1-EM0040	0.26	-0.44	-0.03	-1.91	*4.51
		GS-1-EM0168	-1.06	1.66	-0.71	*-2.34	*3.91
ETp	50 µM	ʻSuperbʼ	-0.15	0.35	-0.48	-0.73	0.50
		GS-1-EM0040	-1.38	-5.80	0.62	*-2.87	*4.51
		GS-1-EM0168	-0.23	-0.25	-0.28	*-4.29	-1.00
	100 µM	ʻSuperbʼ	1.14	0.20	-0.06	*-3.21	0.86
		GS-1-EM0040	-0.19	*-5.11	0.66	-0.88	*5.00
		GS-1-EM0168	-1.51	*-3.32	-1.54	*-2.96	-0.72
	200 µM	ʻSuperbʼ	0.80	-0.47	-0.05	*-2.81	*3.19
		GS-1-EM0040	-3.76	*-5.64	-0.06	-1.69	*5.50
		GS-1-EM0168	-0.11	0.72	-1.23	*-4.41	-2.23

Table 6.2 Time-course expression of wheat *PR-1.2* transcripts in three wheat genotypes in response to SA, MeJA, ETp and water

	Treatment	Line	3 h	6 h	8 h	12 h	24 h
Salicylic Acid	50 µM	ʻSuperbʼ	*2.00	-1.06	-0.12	-1.50	-1.75
		GS-1-EM0040	-1.06	-1.64	0.53	-0.17	*3.27
		GS-1-EM0168	*5.09	*-3.69	-0.98	-0.04	1.08
	100 µM	ʻSuperbʼ	0.65	*3.27	-2.18	-1.73	-1.43
		GS-1-EM0040	-1.37	-0.04	*2.37	*-3.83	*3.21
		GS-1-EM0168	*-3.98	-1.77	0.01	-1.02	-0.84
	200 µM	ʻSuperbʼ	-1.36	*2.32	-1.49	*-2.98	1.36
		GS-1-EM0040	0.69	-0.78	-0.67	-1.59	1.90
		GS-1-EM0168	-0.81	1.79	0.29	*-2.71	-1.32
Methyl-Jasmonate	50 µM	ʻSuperbʼ	-0.07	*3.00	*-3.27	*-3.75	1.57
		GS-1-EM0040	-0.65	-1.92	-0.11	*-2.50	4.53
		GS-1-EM0168	-2.55	*-3.69	-0.17	-0.04	*2.35
	100 µM	ʻSuperbʼ	-0.72	*3.53	-1.79	*-2.97	1.50
		GS-1-EM0040	-0.85	-1.22	1.51	-1.59	1.52
		GS-1-EM0168	-1.51	-1.77	3.32	-0.55	1.45
	200 µM	ʻSuperbʼ	-1.53	1.82	*-2.79	*-3.26	1.72
		GS-1-EM0040	-1.58	-1.21	*-2.50	*-2.49	*3.47
		GS-1-EM0168	*-3.02	1.79	-2.01	-2.05	*2.93
ETp	50 µM	ʻSuperbʼ	1.20	*2.39	-0.12	-1.06	*2.60
		GS-1-EM0040	0.88	-1.93	*2.48	*-2.62	*5.96
		GS-1-EM0168	-0.68	0.13	0.41	-4.80	-2.64
	100 µM	ʻSuperbʼ	1.26	*3.03	*2.11	*-2.50	*3.29
		GS-1-EM0040	-0.45	-0.03	-1.33	-1.92	*6.59
		GS-1-EM0168	-1.88	1.69	*-2.30	*-3.38	1.40
	200 µM	ʻSuperbʼ	*2.22	*2.33	0.05	*-3.30	*3.48
		GS-1-EM0040	-0.52	1.40	*2.18	*-2.80	*5.54
		GS-1-EM0168	-2.06	1.57	0.33	*-5.38	-0.44

Table 6.3: Time-course expression of wheat *PR-2 Glu.2* transcripts in three wheat genotypes in response to SA, MeJA, ETp

Treatment		Line	3 h	6 h	8 h	12 h	24 h
Salicylic Acid	50 µM	‘Superb’	1.38	0.49	-0.55	0.72	-1.18
		GS-1-EM0040	-1.51	-0.38	-0.59	0.53	0.68
		GS-1-EM0168	4.11	1.47	-0.62	-1.81	-0.42
	100 µM	‘Superb’	-0.52	*-2.62	-0.63	-0.28	*-2.10
		GS-1-EM0040	-1.15	-1.93	0.56	-1.69	1.10
		GS-1-EM0168	*-7.15	1.22	-0.23	0.02	-0.97
	200 µM	‘Superb’	-0.81	*-2.14	-1.77	-0.72	0.77
		GS-1-EM0040	-0.80	-2.39	-1.03	0.02	0.76
		GS-1-EM0168	1.33	1.30	-0.42	-0.45	-1.70
Methyl-Jasmonate	50 µM	‘Superb’	-1.40	0.21	*-2.17	-0.63	-0.87
		GS-1-EM0040	-1.14	-3.15	-0.99	-0.65	1.02
		GS-1-EM0168	-2.79	-0.55	-1.19	-1.06	0.29
	100 µM	‘Superb’	-0.88	-1.92	*-2.28	0.22	-0.59
		GS-1-EM0040	*-3.23	-2.67	-1.38	0.75	-0.82
		GS-1-EM0168	-1.11	0.83	3.03	0.85	-0.28
	200 µM	‘Superb’	*-3.10	-1.14	*-2.11	-1.32	-0.56
		GS-1-EM0040	*-4.57	-1.21	*-2.44	-1.10	*2.21
		GS-1-EM0168	*-5.00	0.92	-1.56	-1.279	-0.89
ETp	50 µM	‘Superb’	0.86	0.90	0.20	-0.32	1.05
		GS-1-EM0040	-0.15	-4.11	-0.22	-1.64	*2.90
		GS-1-EM0168	-0.94	0.55	-0.59	-1.49	-1.40
	100 µM	‘Superb’	1.95	-0.51	-0.11	-1.05	0.30
		GS-1-EM0040	*-2.76	-3.22	*-2.30	0.16	*2.84
		GS-1-EM0168	0.11	-0.35	-1.41	-0.72	0.36
	200 µM	‘Superb’	1.08	-0.53	-0.13	-0.88	3.13
		GS-1-EM0040	-1.69	-3.18	-0.58	-1.14	0.86
		GS-1-EM0168	0.06	1.04	-0.28	*-2.72	-1.10

Table 6.4 Time-course expression of wheat *PR-3 CHI1* transcripts in three wheat genotypes in response to SA, MeJA, ETp and water

	Treatment	Line	3 h	6 h	8 h	12 h	24 h
Salicylic Acid	50 µM	ʿSuperb'	1.31	-0.13	2.86	-1.65	-0.67
		GS-1-EM0040	0.64	0.16	0.53	-0.69	*2.48
		GS-1-EM0168	4.46	-0.51	0.57	-1.81	1.41
	100 µM	ʿSuperb'	1.35	-0.21	-1.23	*-2.49	0.10
		GS-1-EM0040	0.58	*-4.03	0.08	*-3.49	*2.27
		GS-1-EM0168	*-4.21	-0.02	-0.57	-1.00	-0.38
	200 µM	ʿSuperb'	-0.87	0.42	*-2.16	*-2.23	-0.48
		GS-1-EM0040	-1.20	*-4.68	-0.72	-0.64	1.85
		GS-1-EM0168	-1.42	0.08	-1.17	*-3.04	-0.34
Methyl-Jasmonate	50 µM	ʿSuperb'	-1.49	-0.05	-1.41	*-2.77	*2.32
		GS-1-EM0040	0.57	*-3.64	-0.18	-1.83	*3.10
		GS-1-EM0168	-3.80	1.23	0.36	*-2.32	2.80
	100 µM	ʿSuperb'	-0.99	*2.70	-1.01	-1.57	*3.08
		GS-1-EM0040	-0.42	-2.67	0.45	-0.59	-1.38
		GS-1-EM0168	-1.69	-0.41	0.16	0.91	*2.40
	200 µM	ʿSuperb'	*-2.69	*2.15	0.06	-1.85	*3.74
		GS-1-EM0040	-0.57	0.75	0.09	*-2.68	*3.81
		GS-1-EM0168	-2.26	0.48	-0.40	-1.30	*4.22
ETp	50 µM	ʿSuperb'	-0.08	-0.48	1.53	-0.72	1.19
		GS-1-EM0040	1.69	*-2.79	0.70	*-2.07	*3.39
		GS-1-EM0168	-2.44	0.94	-0.71	-2.93	-0.10
	100 µM	ʿSuperb'	1.91	-0.18	0.07	*-3.08	0.00
		GS-1-EM0040	0.13	*-2.82	0.66	-1.22	*3.74
		GS-1-EM0168	-2.73	1.15	-0.51	*-3.10	-0.01
	200 µM	ʿSuperb'	1.04	*2.76	0.09	*-3.01	*3.54
		GS-1-EM0040	*-3.26	-0.92	1.13	-1.94	3.34
		GS-1-EM0168	-0.30	1.47	-0.28	*-4.79	-1.13

Table 6.5 Time-course expression of wheat *PR-3 CHI4* transcripts in three wheat genotypes in response to SA, MeJA, ETp and water

	Treatment	Line	3 h	6 h	8 h	12 h	24 h
Salicylic Acid	50 µM	‘Superb’	3.00	-1.38	1.16	-1.33	-0.78
		GS-1-EM0040	-1.34	-0.10	0.61	-0.39	*2.72
		GS-1-EM0168	3.78	0.46	-0.27	0.50	0.70
	100 µM	‘Superb’	2.59	-0.26	-1.32	-0.14	-0.66
		GS-1-EM0040	0.03	-1.73	0.21	-1.12	1.89
		GS-1-EM0168	1.91	-0.46	-0.32	-0.58	-1.17
	200 µM	‘Superb’	1.28	-1.00	-0.69	-0.77	0.13
		GS-1-EM0040	0.49	*-2.38	0.80	-0.42	1.43
		GS-1-EM0168	0.26	0.53	-0.20	-0.19	0.08
Methyl-Jasmonate	50 µM	‘Superb’	3.84	0.62	-0.45	-0.25	0.48
		GS-1-EM0040	-0.94	*-3.10	0.60	-0.48	1.73
		GS-1-EM0168	-0.39	0.75	-0.24	-1.17	1.75
	100 µM	‘Superb’	2.57	0.10	0.01	0.06	1.83
		GS-1-EM0040	-1.93	-2.38	0.03	-0.16	0.31
		GS-1-EM0168	-0.82	0.74	2.53	0.95	0.77
	200 µM	‘Superb’	3.76	-0.81	-0.25	-0.35	*2.40
		GS-1-EM0040	-0.56	-1.70	-0.05	-0.14	-1.73
		GS-1-EM0168	-0.57	0.16	-1.01	-0.13	*2.57
ETp	50 µM	‘Superb’	1.99	-0.92	0.01	-0.82	0.31
		GS-1-EM0040	-0.91	*-2.21	0.45	-0.48	1.75
		GS-1-EM0168	1.15	-0.23	-0.97	-0.43	-2.07
	100 µM	‘Superb’	2.45	-1.53	0.89	-1.36	2.25
		GS-1-EM0040	-0.77	-1.88	*-3.18	-0.16	*2.89
		GS-1-EM0168	0.23	-0.24	-0.01	-0.77	-1.51
	200 µM	‘Superb’	1.89	-1.24	0.99	-1.57	2.57
		GS-1-EM0040	-1.34	-1.08	0.62	-0.14	3.34
		GS-1-EM0168	0.77	0.30	-0.34	-1.49	-0.19

Table 6.6: Time course expression of wheat *PR-8 CHI3* transcripts in three wheat genotypes in response to SA, MeJA, ETp and water

Treatment		Line	3 hat	6 hai	8 hai	12 hai	24 hai
Salicylic Acid	50 µM	ʿSuperb'	*-0.87	-1.13	0.04	-1.59	-0.86
		GS-1-EM0040	*-3.66	3.12	*2.07	-0.16	1.60
		GS-1-EM0168	2.27	0.73	1.22	*-4.8	*4.02
	100 µM	ʿSuperb'	-0.18	*-2.71	0.47	*-2.25	1.62
		GS-1-EM0040	-0.36	-0.91	-0.22	*-3.22	1.74
		GS-1-EM0168	0.25	0.26	0.08	-1.25	0.35
	200 µM	ʿSuperb'	-1.27	-1.18	*-2.15	-0.85	-0.25
		GS-1-EM0040	-0.05	-1.15	0.50	0.35	*2.16
		GS-1-EM0168	0.04	1.31	-1.12	*-2.94	0.90
Methyl-Jasmonate	50 µM	ʿSuperb'	1.31	*1.62	1.91	-2.31	*2.81
		GS-1-EM0040	-0.61	0.24	1.60	-1.51	*4.07
		GS-1-EM0168	-0.58	2.92	*2.26	*-3.59	0.95
	100 µM	ʿSuperb'	-0.73	0.81	1.89	-1.02	*4.14
		GS-1-EM0040	*-5.01	0.63	*2.17	0.19	*4.87
		GS-1-EM0168	-0.80	*1.55	4.76	1.40	*2.98
	200 µM	ʿSuperb'	0.97	1.27	*2.29	-0.28	*5.21
		GS-1-EM0040	-0.82	*-5.31	1.70	-2.69	*2.76
		GS-1-EM0168	-0.48	1.960	1.53	-0.15	*4.58
ETp	50 µM	ʿSuperb'	0.11	-0.90	-1.13	-0.86	1.00
		GS-1-EM0040	-0.72	-1.36	0.71	*-2.17	-0.07
		GS-1-EM0168	0.78	-0.11	-1.11	*-3.87	0.31
	100 µM	ʿSuperb'	-0.68	-0.84	-0.26	*-3.73	0.47
		GS-1-EM0040	0.56	-0.87	-2.06	-0.42	1.87
		GS-1-EM0168	-0.15	0.20	-0.12	*-2.90	-0.52
	200 µM	ʿSuperb'	-1.54	-0.77	-0.26	*-2.97	*2.35
		GS-1-EM0040	-0.83	0.07	0.82	-1.83	*3.99
		GS-1-EM0168	-0.08	0.68	0.82	*-4.38	-0.27

Table 6.7 Time course expression of wheat *PR-9 PEROXIDASE* transcripts in three wheat genotypes in response to SA, MeJA, ETp and water

Treatment		Line	3 hat	6 hat	8 hat	12 hat	24 hat
Salicylic Acid	50 µM	ʻSuperbʼ	-0.87	-1.13	0.04	-1.59	-0.86
		GS-1-EM0040	*-3.66	3.12	*2.07	-0.16	1.60
		GS-1-EM0168	2.27	0.73	1.22	*-4.80	*4.02
	100 µM	ʻSuperbʼ	-0.18	*-2.71	0.47	*-2.25	1.62
		GS-1-EM0040	-0.36	-0.91	-0.22	*-3.22	1.74
		GS-1-EM0168	0.25	0.26	0.08	-1.25	0.35
	200 µM	ʻSuperbʼ	-1.27	-1.18	*-2.15	-0.85	-0.25
		GS-1-EM0040	-0.05	-1.15	0.50	0.35	*2.16
		GS-1-EM0168	0.04	1.31	-1.12	*-2.94	0.90
Methyl-Jasmonate	50 µM	ʻSuperbʼ	1.31	1.62	1.911	-2.31	*2.81
		GS-1-EM0040	-0.61	0.24	1.60	-1.51	*4.07
		GS-1-EM0168	-0.58	2.92	*2.26	*-3.59	0.95
	100 µM	ʻSuperbʼ	-0.73	0.81	1.89	-1.02	*4.14
		GS-1-EM0040	*-5.01	0.63	*2.17	0.19	*4.87
		GS-1-EM0168	-0.80	1.55	4.76	1.40	*2.98
	200 µM	ʻSuperbʼ	0.97	1.27	*2.29	-0.28	*5.21
		GS-1-EM0040	-0.82	*-5.30	1.70	-2.69	*2.76
		GS-1EM0168	-0.48	1.96	1.53	-0.15	*4.58
ETp	50 µM	ʻSuperbʼ	0.11	-0.90	-1.13	-0.86	1.00
		GS-1-EM0040	-0.72	-1.36	0.71	*-2.17	-0.07
		GS-1-EM0168	0.78	-0.11	-1.11	*-3.87	0.31
	100 µM	ʻSuperbʼ	-0.68	-0.84	-0.26	*-3.73	0.47
		GS-1-EM0040	0.56	-0.87	-2.06	-0.42	1.87
		GS-1-EM0168	-0.15	0.20	-0.12	*-2.90	-0.52
	200 µM	ʻSuperbʼ	-1.54	-0.77	-0.26	*-2.97	*2.35
		GS-1-EM0040	-0.83	0.07	0.82	-1.83	*3.99
		GS-1-EM0168	-0.08	0.68	0.82	*-4.38	-0.27

Table 6.8: Time course expression of wheat *PR-13 THIONIN* transcripts in three wheat genotypes in response to SA, MeJA, ETp and water

	Treatment	Line	3 hat	6 hat	8 hat	12 hat	24 hat
Salicylic Acid	50 µM	ʿSuperbʾ	1.31	1.46	1.56	-2.47	0.28
		GS-1-EM0040	*-2.71	-1.36	*-2.18	1.26	0.97
		GS-1-EM0168	2.95	*4.34	*-2.46	2.22	-0.37
	100 µM	ʿSuperbʾ	1.04	*-2.67	-1.89	-0.02	0.10
		GS-1-EM0040	-0.13	-0.35	0.62	-2.57	1.70
		GS-1-EM0168	-2.00	*3.48	*-2.12	0.23	1.69
	200 µM	ʿSuperbʾ	*2.28	*-2.23	*-2.46	-1.63	-0.32
		GS-1-EM0040	-1.20	-2.98	-0.19	-2.60	0.36
		GS-1-EM0168	*-3.81	*3.06	0.15	*-5.11	0.06
Methyl-Jasmonate	50 µM	ʿSuperbʾ	0.94	1.76	*-3.57	*-3.33	0.20
		GS-1-EM0040	-0.19	-0.65	*4.57	-0.63	0.72
		GS-1-EM0168	-1.89	-6.64	-1.58	*-4.35	*-2.50
	100 µM	ʿSuperbʾ	*2.25	-2.06	*-2.04	-2.63	*-2.27
		GS-1-EM0040	-1.90	-1.80	0.90	0.41	2.23
		GS-1-EM0168	*-2.81	*5.61	2.16	2.04	1.14
	200 µM	ʿSuperbʾ	*-2.04	-1.97	-1.37	-1.91	0.99
		GS-1-EM0040	-0.61	-0.09	0.28	-1.50	-0.84
		GS-1-EM0168	*-4.50	*7.61	*-2.59	-1.57	-0.66
ETp	50 µM	ʿSuperbʾ	*2.23	0.33	*-2.23	*-3.79	-1.32
		GS-1-EM0040	-0.23	-3.80	0.81	-3.16	1.36
		GS-1-EM0168	*-3.85	*3.98	*-3.31	-0.76	-0.37
	100 µM	ʿSuperbʾ	*2.11	2.48	-1.78	-1.28	0.14
		GS-1-EM0040	-0.39	-2.46	-2.05	*-5.32	*2.24
		GS-1-EM0168	-2.53	*2.37	-0.11	*-4.87	-0.25
	200 µM	ʿSuperbʾ	1.96	0.11	-1.46	1.00	1.61
		GS-1-EM0040	-0.39	-3.63	*-2.62	-0.65	*3.51
		GS-1-EM0168	-2.13	*2.91	-1.86	0.50	1.17

Table 6.9 RIN numbers of RNA samples for qRT-PCR. 3, 6, 8, 12 and 24 H represent hours after hormone treatment at which the sample was harvested.

RNA sample	RIN number	RNA sample	RIN number
"ETp50,GS-1-EM0040 ,3H,R1"	9.1	"ETp50,GS-1-EM0040 ,6H,R1"	8
"ETp50,GS-1-EM0040 ,3H,R2"	9.4	"ETp50,GS-1-EM0040 ,6H,R2"	9.1
"ETp50,GS-1-EM0040 ,3H,R3"	9.3	"ETp50,GS-1-EM0040 ,6H,R3"	9.2
"ETp50,GS-1-EM0168,3H,R1"	9.1	"ETp50,GS-1-EM0168,6H,R1"	9.1
"ETp50,GS-1-EM0168,3H,R2"	8	"ETp50,GS-1-EM0168,6H,R2"	9.2
"ETp50,GS-1-EM0168,3H,R3"	9.2	"ETp50,GS-1-EM0168,6H,R3"	9.1
"ETp50,SUPERB,3H,R1"	8.2	"ETp50,SUPERB,6H,R1"	8.6
"ETp50,SUPERB,3H,R2"	8	"ETp50,SUPERB,6H,R2"	8
"ETp50,SUPERB,3H,R3"	9.1	"ETp50,SUPERB,6H,R3"	8
"ETp100,GS-1-EM0040 ,3H,R1"	9.2	"ETp100,GS-1-EM0040 ,6H,R1"	8
"ETp100,GS-1-EM0040 ,3H,R2"	9.1	"ETp100,GS-1-EM0040 ,6H,R2"	8.3
"ETp100,GS-1-EM0040 ,3H,R3"	9.2	"ETp100,GS-1-EM0040 ,6H,R3"	8.5
"ETp100,GS-1-EM0168,3H,R1"	9.1	"ETp100,GS-1-EM0168,6H,R1"	7.8
"ETp100,GS-1-EM0168,3H,R2"	9	"ETp100,GS-1-EM0168,6H,R2"	8.9
"ETp100,GS-1-EM0168,3H,R3"	9	"ETp100,GS-1-EM0168,6H,R3"	9.1
"ETp100,SUPERB,3H,R1"	8	"ETp100,SUPERB,6H,R1"	9.1
"ETp100,SUPERB,3H,R2"	8	"ETp100,SUPERB,6H,R2"	9.1
"ETp100,SUPERB,3H,R3"	8.3	"ETp100,SUPERB,6H,R3"	8.9
"ETp200,GS-1-EM0040 ,3H,R1"	8.5	"ETp200,GS-1-EM0040 ,6H,R1"	8.7
"ETp200,GS-1-EM0040 ,3H,R2"	7.8	"ETp200,GS-1-EM0040 ,6H,R2"	8.7
"ETp200,GS-1-EM0040 ,3H,R3"	8.9	"ETp200,GS-1-EM0040 ,6H,R3"	8.8
"ETp200,GS-1-EM0168,3H,R1"	9.1	"ETp200,GS-1-EM0168,6H,R1"	9.2
"ETp200,GS-1-EM0168,3H,R2"	9.1	"ETp200,GS-1-EM0168,6H,R2"	8.8
"ETp200,GS-1-EM0168,3H,R3"	9.1	"ETp200,GS-1-EM0168,6H,R3"	8.8
"ETp200,SUPERB,3H,R1"	8.9	"ETp200,SUPERB,6H,R1"	9.1
"ETp200,SUPERB,3H,R2"	8.7	"ETp200,SUPERB,6H,R2"	8.7
"ETp200,SUPERB,3H,R3"	8.7	"ETp200,SUPERB,6H,R3"	8.1
"JA50,GS-1-EM0040 ,3H,R1"	8.8	"JA50,GS-1-EM0040 ,6H,R1"	8.4
"JA50,GS-1-EM0040 ,3H,R2"	9.2	"JA50,GS-1-EM0040 ,6H,R2"	8.7
"JA50,GS-1-EM0040 ,3H,R3"	8.8	"JA50,GS-1-EM0040 ,6H,R3"	9.1
"JA50,GS-1-EM0168,3H,R1"	8.8	"JA50,GS-1-EM0168,6H,R1"	8.4
"JA50,GS-1-EM0168,3H,R2"	9.1	"JA50,GS-1-EM0168,6H,R2"	8
"JA50,GS-1-EM0168,3H,R3"	8.7	"JA50,GS-1-EM0168,6H,R3"	8.2
"JA50,SUPERB,3H,R1"	9	"JA50,SUPERB,6H,R1"	8
"JA50,SUPERB,3H,R2"	9	"JA50,SUPERB,6H,R2"	8.3
"JA50,SUPERB,3H,R3"	8.7	"JA50,SUPERB,6H,R3"	9
"JA100,GS-1-EM0040 ,3H,R1"	9.1	"JA100,GS-1-EM0040 ,6H,R1"	8.3

"JA100,GS-1-EM0040 ,3H,R2"	8.4	"JA100,GS-1-EM0040 ,6H,R2"	8.9
"JA100,GS-1-EM0040 ,3H,R3"	8	"JA100,GS-1-EM0040 ,6H,R3"	8.9
"JA100,GS-1-EM0168,3H,R1"	9	"JA100,GS-1-EM0168,6H,R1"	8.7
"JA100,GS-1-EM0168,3H,R2"	8	"JA100,GS-1-EM0168,6H,R2"	8.7
"JA100,GS-1-EM0168,3H,R3"	8.3	"JA100,GS-1-EM0168,6H,R3"	8.8
"JA100,SUPERB,3H,R1"	9	"JA100,SUPERB,6H,R1"	9.2
"JA100,SUPERB,3H,R2"	8.3	"JA100,SUPERB,6H,R2"	8.8
"JA100,SUPERB,3H,R3"	8.9	"JA100,SUPERB,6H,R3"	8.8
"JA200,GS-1-EM0040 ,3H,R1"	8.9	"JA200,GS-1-EM0040 ,6H,R1"	9.1
"JA200,GS-1-EM0040 ,3H,R2"	8.7	"JA200,GS-1-EM0040 ,6H,R2"	8.7
"JA200,GS-1-EM0040 ,3H,R3"	8.7	"JA200,GS-1-EM0040 ,6H,R3"	9
"JA200,GS-1-EM0168,3H,R1"	8.8	"JA200,GS-1-EM0168,6H,R1"	8.4
"JA200,GS-1-EM0168,3H,R2"	9.2	"JA200,GS-1-EM0168,6H,R2"	8.7
"JA200,GS-1-EM0168,3H,R3"	8.8	"JA200,GS-1-EM0168,6H,R3"	9.1
"JA200,SUPERB,3H,R1"	8.8	"JA200,SUPERB,6H,R1"	8.4
"JA200,SUPERB,3H,R2"	9.1	"JA200,SUPERB,6H,R2"	9
"JA200,SUPERB,3H,R3"	8.7	"JA200,SUPERB,6H,R3"	8.7
"SA50,GS-1-EM0040 ,3H,R1"	9	"SA50,GS-1-EM0040 ,6H,R1"	9.1
"SA50,GS-1-EM0040 ,3H,R2"	9	"SA50,GS-1-EM0040 ,6H,R2"	8.4
"SA50,GS-1-EM0040 ,3H,R3"	8.7	"SA50,GS-1-EM0040 ,6H,R3"	8
"SA50,GS-1-EM0168,3H,R1"	9.1	"SA50,GS-1-EM0168,6H,R1"	9
"SA50,GS-1-EM0168,3H,R2"	8.4	"SA50,GS-1-EM0168,6H,R2"	8
"SA50,GS-1-EM0168,3H,R3"	9	"SA50,GS-1-EM0168,6H,R3"	8.3
"SA50,SUPERB,3H,R1"	8.7	"SA50,SUPERB,6H,R1"	9
"SA50,SUPERB,3H,R2"	9.1	"SA50,SUPERB,6H,R2"	8.3
"SA50,SUPERB,3H,R3"	8.4	"SA50,SUPERB,6H,R3"	8.9
"SA100,GS-1-EM0040 ,3H,R1"	8	"SA100,GS-1-EM0040 ,6H,R1"	8.9
"SA100,GS-1-EM0040 ,3H,R2"	9	"SA100,GS-1-EM0040 ,6H,R2"	8.7
"SA100,GS-1-EM0040 ,3H,R3"	8	"SA100,GS-1-EM0040 ,6H,R3"	8.7
"SA100,GS-1-EM0168,3H,R1"	8.3	"SA100,GS-1-EM0168,6H,R1"	8.7
"SA100,GS-1-EM0168,3H,R2"	9	"SA100,GS-1-EM0168,6H,R2"	8.7
"SA100,GS-1-EM0168,3H,R3"	8.3	"SA100,GS-1-EM0168,6H,R3"	8.8
"SA100,SUPERB,3H,R1"	8.9	"SA100,SUPERB,6H,R1"	9.2
"SA100,SUPERB,3H,R2"	8.9	"SA100,SUPERB,6H,R2"	8.8
"SA100,SUPERB,3H,R3"	8.7	"SA100,SUPERB,6H,R3"	8.8
"Water, GS-1-EM0040 ,3H,R1"	8.7	"Water, GS-1-EM0040 ,6H,R1"	9
"Water, GS-1-EM0040 ,3H,R2"	8.7	"Water, GS-1-EM0040 ,6H,R2"	8
"Water, GS-1-EM0040 ,3H,R3"	8.7	"Water, GS-1-EM0040 ,6H,R3"	8
"Water, GS-1-EM0168,3H,R1"	8.8	"Water, GS-1-EM0168,6H,R1"	8.3
"Water, GS-1-EM0168,3H,R2"	9.2	"Water, GS-1-EM0168,6H,R2"	8.5

"Water, GS-1-EM0168,3H,R3"	8.8	"Water, GS-1-EM0168,6H,R3"	7.8
"Water, SUPERB,3H,R1"	8.8	"Water, SUPERB,6H,R1"	8.9
"Water, SUPERB,3H,R2"	9.1	"Water, SUPERB,6H,R2"	9.1
"Water, SUPERB,3H,R3"	8.7	"Water, SUPERB,6H,R3"	9.1
"ETp50,GS-1-EM0040 ,8H,R1"	8.5	"ETp50,GS-1-EM0040 ,12H,R1"	8.2
"ETp50,GS-1-EM0040 ,8H,R2"	8.7	"ETp50,GS-1-EM0040 ,12H,R2"	8.7
"ETp50,GS-1-EM0040 ,8H,R3"	8.9	"ETp50,GS-1-EM0040 ,12H,R3"	8.4
"ETp50,GS-1-EM0168,8H,R1"	8.1	"ETp50,GS-1-EM0168,12H,R1"	8.1
"ETp50,GS-1-EM0168,8H,R2"	8.7	"ETp50,GS-1-EM0168,12H,R2"	8.7
"ETp50,GS-1-EM0168,8H,R3"	9.1	"ETp50,GS-1-EM0168,12H,R3"	9.1
"ETp50,SUPERB,8H,R1"	8.4	"ETp50,SUPERB,12H,R1"	8.4
"ETp50,SUPERB,8H,R2"	8	"ETp50,SUPERB,12H,R2"	8
"ETp50,SUPERB,8H,R3"	8.2	"ETp50,SUPERB,12H,R3"	8.2
"ETp100,GS-1-EM0040 ,8H,R1"	8	"ETp100,GS-1-EM0040 ,12H,R1"	8
"ETp100,GS-1-EM0040 ,8H,R2"	8.3	"ETp100,GS-1-EM0040 ,12H,R2"	8.3
"ETp100,GS-1-EM0040 ,8H,R3"	9	"ETp100,GS-1-EM0040 ,12H,R3"	8.2
"ETp100,GS-1-EM0168,8H,R1"	8.3	"ETp100,GS-1-EM0168,12H,R1"	8.3
"ETp100,GS-1-EM0168,8H,R2"	8.9	"ETp100,GS-1-EM0168,12H,R2"	8.9
"ETp100,GS-1-EM0168,8H,R3"	8.9	"ETp100,GS-1-EM0168,12H,R3"	8.9
"ETp100,SUPERB,8H,R1"	8.7	"ETp100,SUPERB,12H,R1"	8.7
"ETp100,SUPERB,8H,R2"	8.7	"ETp100,SUPERB,12H,R2"	8.7
"ETp100,SUPERB,8H,R3"	8.8	"ETp100,SUPERB,12H,R3"	8.8
"ETp200,GS-1-EM0040 ,8H,R1"	9.2	"ETp200,GS-1-EM0040 ,12H,R1"	9.2
"ETp200,GS-1-EM0040 ,8H,R2"	8.8	"ETp200,GS-1-EM0040 ,12H,R2"	8.8
"ETp200,GS-1-EM0040 ,8H,R3"	8.8	"ETp200,GS-1-EM0040 ,12H,R3"	8.8
"ETp200,GS-1-EM0168,8H,R1"	9.1	"ETp200,GS-1-EM0168,12H,R1"	9.1
"ETp200,GS-1-EM0168,8H,R2"	8.7	"ETp200,GS-1-EM0168,12H,R2"	8.7
"ETp200,GS-1-EM0168,8H,R3"	8.4	"ETp200,GS-1-EM0168,12H,R3"	8.1
"ETp200,SUPERB,8H,R1"	9	"ETp200,SUPERB,12H,R1"	8
"ETp200,SUPERB,8H,R2"	8.7	"ETp200,SUPERB,12H,R2"	8.7
"ETp200,SUPERB,8H,R3"	9.1	"ETp200,SUPERB,12H,R3"	9.1
"JA50,GS-1-EM0040 ,8H,R1"	8.4	"JA50,GS-1-EM0040 ,12H,R1"	8.4
"JA50,GS-1-EM0040 ,8H,R2"	9	"JA50,GS-1-EM0040 ,12H,R2"	8.2
"JA50,GS-1-EM0040 ,8H,R3"	8.7	"JA50,GS-1-EM0040 ,12H,R3"	8.7
"JA50,GS-1-EM0168,8H,R1"	8	"JA50,GS-1-EM0168,12H,R1"	8
"JA50,GS-1-EM0168,8H,R2"	8.1	"JA50,GS-1-EM0168,12H,R2"	8.4
"JA50,GS-1-EM0168,8H,R3"	8	"JA50,GS-1-EM0168,12H,R3"	8
"JA50,SUPERB,8H,R1"	8.3	"JA50,SUPERB,12H,R1"	8.3
"JA50,SUPERB,8H,R2"	9	"JA50,SUPERB,12H,R2"	0.5
"JA50,SUPERB,8H,R3"	8.3	"JA50,SUPERB,12H,R3"	8.3

"JA100,GS-1-EM0040 ,8H,R1"	8.9	"JA100,GS-1-EM0040 ,12H,R1"	8.9
"JA100,GS-1-EM0040 ,8H,R2"	8.9	"JA100,GS-1-EM0040 ,12H,R2"	8.9
"JA100,GS-1-EM0040 ,8H,R3"	8.7	"JA100,GS-1-EM0040 ,12H,R3"	8.7
"JA100,GS-1-EM0168,8H,R1"	8.7	"JA100,GS-1-EM0168,12H,R1"	8.7
"JA100,GS-1-EM0168,8H,R2"	8.8	"JA100,GS-1-EM0168,12H,R2"	8.8
"JA100,GS-1-EM0168,8H,R3"	9.2	"JA100,GS-1-EM0168,12H,R3"	9.2
"JA100,SUPERB,8H,R1"	8.8	"JA100,SUPERB,12H,R1"	8.8
"JA100,SUPERB,8H,R2"	8.8	"JA100,SUPERB,12H,R2"	8.8
"JA100,SUPERB,8H,R3"	9.1	"JA100,SUPERB,12H,R3"	9.1
"JA200,GS-1-EM0040 ,8H,R1"	8.7	"JA200,GS-1-EM0040 ,12H,R1"	8.7
"JA200,GS-1-EM0040 ,8H,R2"	8.7	"JA200,GS-1-EM0040 ,12H,R2"	8.9
"JA200,GS-1-EM0040 ,8H,R3"	8.4	"JA200,GS-1-EM0040 ,12H,R3"	8.1
"JA200,GS-1-EM0168,8H,R1"	8.7	"JA200,GS-1-EM0168,12H,R1"	8.7
"JA200,GS-1-EM0168,8H,R2"	9.1	"JA200,GS-1-EM0168,12H,R2"	9.1
"JA200,GS-1-EM0168,8H,R3"	8.4	"JA200,GS-1-EM0168,12H,R3"	8.4
"JA200,SUPERB,8H,R1"	9	"JA200,SUPERB,12H,R1"	8.3
"JA200,SUPERB,8H,R2"	8.7	"JA200,SUPERB,12H,R2"	8.7
"JA200,SUPERB,8H,R3"	8	"JA200,SUPERB,12H,R3"	8
"SA50,GS-1-EM0040 ,8H,R1"	9	"SA50,GS-1-EM0040 ,12H,R1"	8.3
"SA50,GS-1-EM0040 ,8H,R2"	8	"SA50,GS-1-EM0040 ,12H,R2"	8
"SA50,GS-1-EM0040 ,8H,R3"	8.3	"SA50,GS-1-EM0040 ,12H,R3"	8.4
"SA50,GS-1-EM0168,8H,R1"	9	"SA50,GS-1-EM0168,12H,R1"	8
"SA50,GS-1-EM0168,8H,R2"	8.3	"SA50,GS-1-EM0168,12H,R2"	9
"SA50,GS-1-EM0168,8H,R3"	8.9	"SA50,GS-1-EM0168,12H,R3"	8
"SA50,SUPERB,8H,R1"	8.9	"SA50,SUPERB,12H,R1"	8.3
"SA50,SUPERB,8H,R2"	8.7	"SA50,SUPERB,12H,R2"	8.1
"SA50,SUPERB,8H,R3"	8.7	"SA50,SUPERB,12H,R3"	8.3
"SA100,GS-1-EM0040 ,8H,R1"	8.8	"SA100,GS-1-EM0040 ,12H,R1"	8.9
"SA100,GS-1-EM0040 ,8H,R2"	9.2	"SA100,GS-1-EM0040 ,12H,R2"	8.9
"SA100,GS-1-EM0040 ,8H,R3"	9	"SA100,GS-1-EM0040 ,12H,R3"	8.7
"SA100,GS-1-EM0168,8H,R1"	8.3	"SA100,GS-1-EM0168,12H,R1"	8.7
"SA100,GS-1-EM0168,8H,R2"	8.9	"SA100,GS-1-EM0168,12H,R2"	8.8
"SA100,GS-1-EM0168,8H,R3"	8.9	"SA100,GS-1-EM0168,12H,R3"	8
"SA100,SUPERB,8H,R1"	8.7	"SA100,SUPERB,12H,R1"	8.8
"SA100,SUPERB,8H,R2"	8.7	"SA100,SUPERB,12H,R2"	8.8
"SA100,SUPERB,8H,R3"	8.8	"SA100,SUPERB,12H,R3"	8
"Water, GS-1-EM0040 ,8H,R1"	9.2	"Water, GS-1-EM0040 ,12H,R1"	8.7
"Water, GS-1-EM0040 ,8H,R2"	8.8	"Water, GS-1-EM0040 ,12H,R2"	9
"Water, GS-1-EM0040 ,8H,R3"	8.8	"Water, GS-1-EM0040 ,12H,R3"	8.1
"Water, GS-1-EM0168,8H,R1"	9.1	"Water, GS-1-EM0168,12H,R1"	8.7

"Water, GS-1-EM0168,8H,R2"	8.7	"Water, GS-1-EM0168,12H,R2"	9.1
"Water, GS-1-EM0168,8H,R3"	8.9	"Water, GS-1-EM0168,12H,R3"	8.4
"Water, SUPERB,8H,R"	8.6	"Water, SUPERB,12H,R3"	8.9
"Water, SUPERB,8H,R2"	8.7	"Water, SUPERB,12H,R3"	8.7
"Water, SUPERB,8H,R3"	9.1	"Water, SUPERB,12H,R3"	8
"ETp50,GS-1-EM0040 ,24H,R1"	9.1	"JA50,GS-1-EM0168,24H,R2"	8.7
"ETp50,GS-1-EM0040 ,24H,R2"	8.7	"JA50,GS-1-EM0168,24H,R3"	8
"ETp50,GS-1-EM0040 ,24H,R3"	8.1	"JA50,SUPERB,24H,R1"	8.3
"ETp50,GS-1-EM0168,24H,R1"	8	"JA50,SUPERB,24H,R2"	8
"ETp50,GS-1-EM0168,24H,R2"	8.7	"JA50,SUPERB,24H,R3"	8.4
"ETp50,GS-1-EM0168,24H,R3"	9.1	"JA100,GS-1-EM0040 ,24H,R1"	8
"ETp50,SUPERB,24H,R1"	8.4	"JA100,GS-1-EM0040 ,24H,R2"	9
"ETp50,SUPERB,24H,R2"	8.2	"JA100,GS-1-EM0040 ,24H,R3"	8
"ETp50,SUPERB,24H,R3"	8.7	"JA100,GS-1-EM0168,24H,R1"	8.3
"ETp100,GS-1-EM0040 ,24H,R1"	8	"JA100,GS-1-EM0168,24H,R2"	8.1
"ETp100,GS-1-EM0040 ,24H,R2"	8.4	"JA100,GS-1-EM0168,24H,R3"	8.3
"ETp100,GS-1-EM0040 ,24H,R3"	8	"JA100,SUPERB,24H,R1"	8.9
"ETp100,GS-1-EM0168,24H,R1"	8.3	"JA100,SUPERB,24H,R2"	8.9
"ETp100,GS-1-EM0168,24H,R2"	0.5	"JA100,SUPERB,24H,R3"	8.7
"ETp100,GS-1-EM0168,24H,R3"	8.3	"JA200,GS-1-EM0040 ,24H,R1"	9.1
"ETp100,SUPERB,24H,R1"	8.9	"JA200,GS-1-EM0040 ,24H,R2"	8.4
"ETp100,SUPERB,24H,R2"	8.9	"JA200,GS-1-EM0040 ,24H,R3"	8.3
"ETp100,SUPERB,24H,R3"	8.7	"JA200,GS-1-EM0168,24H,R1"	8.7
"ETp200,GS-1-EM0040 ,24H,R1"	8.7	"JA200,GS-1-EM0168,24H,R2"	8
"ETp200,GS-1-EM0040 ,24H,R2"	8.8	"JA200,GS-1-EM0168,24H,R3"	8.3
"ETp200,GS-1-EM0040 ,24H,R3"	9.2	"JA200,SUPERB,24H,R1"	8
"ETp200,GS-1-EM0168,24H,R1"	8.8	"JA200,SUPERB,24H,R2"	8.4
"ETp200,GS-1-EM0168,24H,R2"	8.8	"JA200,SUPERB,24H,R3"	8
"ETp200,GS-1-EM0168,24H,R3"	9.1	"SA50,GS-1-EM0040 ,24H,R1"	9
"ETp200,SUPERB,24H,R1"	8.7	"SA50,GS-1-EM0040 ,24H,R2"	8
"ETp200,SUPERB,24H,R2"	8.9	"SA50,GS-1-EM0040 ,24H,R3"	8.3
"ETp200,SUPERB,24H,R3"	8.1	"SA50,GS-1-EM0168,24H,R1"	8.1
"JA50,GS-1-EM0040 ,24H,R1"	8.7	"SA50,GS-1-EM0168,24H,R2"	8.3
"JA50,GS-1-EM0040 ,24H,R2"	9.1	"SA50,GS-1-EM0168,24H,R3"	8.9
"JA50,GS-1-EM0040 ,24H,R3"	8.4	"SA50,SUPERB,24H,R1"	8.9
"JA50,GS-1-EM0168,24H,R1"	8.3	"SA50,SUPERB,24H,R2"	8
"SA50,SUPERB,24H,R2"	9	"SA100,SUPERB,24H,R3"	8.4
"SA100,GS-1-EM0040 ,24H,R1"	8	"Water, GS-1-EM0040 ,24H,R1"	8.3
"SA100,GS-1-EM0040 ,24H,R2"	8.3	"Water, GS-1-EM0040 ,24H,R2"	8.7
"SA100,GS-1-EM0040 ,24H,R3"	8.1	"Water, GS-1-EM0040 ,24H,R3"	8

"SA100,GS-1-EM0168,24H,R1"	8.3	"Water, GS-1-EM0168,24H,R1"	8.3
"SA100,GS-1-EM0168,24H,R2"	8.9	"Water, GS-1-EM0168,24H,R2"	8
"SA100,GS-1-EM0168,24H,R3"	8.9	"Water, GS-1-EM0168,24H,R3"	8.4
"SA100,SUPERB,24H,R1"	8.7	"Water, SUPERB,24H,R1"	8
"SA100,SUPERB,24H,R2"	9.1	"Water, SUPERB,24H,R2"	9
"SA50,SUPERB,24H,R3	8.6	"Water, SUPERB,24H,R3"	9