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Exploring novel green, high-tech and molecular mechanisms for the control and early detection of Fusarium head blight in durum wheat

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Ph.D. candidate

Dr. Sara Francesconi

Ph.D. coordinator

Prof. Roberta Bernini

Supervisor

Prof. Giorgio M. Balestra

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Abstract

Wheat is the world's most economically important cereal crop, grown across millions of hectares. Fusarium head blight (FHB) disease is considered a major threat to durum (*Triticum turgidum* subsp. *durum* (Desfontaines) Husnache) and bread wheat (*T. aestivum* L.) and *Fusarium graminearum* is the most damaging FHB pathogen. Infection is linked to host anthesis, and symptoms include necrosis, bleaching of heads and shrivelled kernels. FHB is mainly managed by the application of fungicides at anthesis. Nevertheless, no fully effective fungicides are available for FHB control, so utilization of other mitigation measures, such as the use of resistant cultivars, is necessary for FHB management. Resistance to FHB is quantitative and multigenic and five components of resistance (type I, II, III, IV and V) have been described. The main problem in testing for FHB resistance is reproducibility, so necessary tools for breeding resistant cultivars are reliable inoculation methods and the testing for different FHB-associated characteristics. In the first part of the work, we screened three Italian wheat genotypes, Palesio, Claudio and Marco Aurelio, for type I, type II, and, in part, for type V resistances, with both phenotypic (% of disease incidence and severity, thousand kernel weight (TKW) and molecular (quantification of fungal biomass with Real-Time *q*PCR) approaches, using spray and point inoculation protocols. Results underlined that Palesio bread wheat showed type I tolerance to initial infection, and Marco Aurelio durum wheat showed an important type II resistance to disease spread when spray-inoculated (27% disease severity). Quantification of fungal biomass showed that differentiation among the three wheat cultivars was best visualized when spray inoculation was used. TKW data showed that % yield loss was greater after point inoculations, except in Marco Aurelio, which was not affected by inoculation method. This study has highlighted the complexity of testing for FHB resistance, and demonstrated the necessity to use as many resistance screening protocols as possible.

Several evidences shown that stomatal closure is involved in the first line of defence against plant pathogens. However, there is very little evidence to show that photosynthetic parameters change in inoculated plants. The aim of the second part of the research was to study the role of stomatal regulation in wheat after *F. graminearum* inoculation and explore its possible involvement in FHB resistance. RT-*q*PCR revealed that genes involved in stomatal regulation are induced in the resistant Sumai3 cultivar but not in the susceptible Rebelde cultivar. Seven genes involved in the positive regulation of stomatal closure were up-regulated in Sumai3, but it is most likely, that two genes, *TaBG* and *TaCYP450*, involved in the negative regulation of stomatal closure, were strongly induced, suggesting that FHB response is linked to cross-talk between the genes promoting and inhibiting stomatal closure. Increasing temperature of spikes in the wheat genotypes and a decrease in

photosynthetic efficiency in Rebelde but not in Sumai3, were observed, confirming the hypothesis that photosynthetic parameters are related to FHB resistance.

The application timing of chemicals to control FHB is not optimized. In fact, fungicides are applied when FHB symptoms are clearly visible and the spikes are almost entirely bleached (% of diseased spikelets > 80%), by when it is too late to control FHB disease. For this reason, farmers usually perform repeated fungicide treatments, causing a waste of money and posing significant risks to the environment and non-target organisms. In the third part of the study, we used unmanned aerial vehicle (UAV)-based thermal infrared (TIR) and red-green-blue (RGB) imaging to early detect FHB in *T. turgidum* (cv. Marco Aurelio) under natural field conditions. TIR and RGB data correlated with ground-based measurements such as spike's temperature, photosynthetic efficiency and molecular identification of FHB pathogens, demonstrating that these high-throughput phenotyping methodologies were able to early detect FHB at anthesis half-way (Zadoks stage 65, ZS 65), when the % of diseased spikelets ranged between 20% and 60%. Moreover, in greenhouse experiments the transcripts of the key genes involved in stomatal closure were mostly up-regulated in *F. graminearum*-inoculated plants, demonstrating that the physiological mechanism behind the spike's temperature increase and photosynthetic efficiency decrease could be attributed to the closure of the guard cells in response to *F. graminearum*. In addition, preliminary analysis revealed that there is differential regulation of genes between drought-stressed and *F. graminearum*-inoculated plants, suggesting that there might be a possibility to discriminate between water stress and FHB infection. This study shows the potential of UAV-based TIR and RGB imaging for field phenotyping of wheat and other cereal crop species in response to environmental stresses. This is anticipated to have enormous promise for the early detection of FHB disease, especially when combined with molecular diagnostics, and tremendous implications for optimizing the application of fungicides, since global food crop demand is to be met with minimal environmental impacts.

A consistent related issue linked to the use of conventional fungicides regards the fact that they accumulate in the soil and are dangerous for animal and human health. This fourth part of the study assayed the antifungal ability of chitosan hydrochloride against *Fusarium graminearum*. Chitosan reduced *F. graminearum* growth and downregulated the transcript of the major genes involved in the cell growth, respiration, virulence, and trichothecenes biosynthesis. Chitosan promoted the germination rate, the root and coleoptile development, and the nitrogen balance index in two durum wheat genotypes, Marco Aurelio (FHB-susceptible) and DBC480 (FHB-resistant). Chitosan reduced FHB severity when applied on spikes or on the flag leaves. FHB severity in DBC480 was of 6% at 21 dpi after chitosan treatments compared to *F. graminearum* inoculated control (20%). The elicitor-like property of chitosan was confirmed by the up-regulation of *TaPAL*, *TaPR1* and *TaPR2* (around

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1. Introduction

1.1 The wheat plant

Wheat is the world's foremost crop plant, followed by rice and maize. Bread wheat accounts for about 95% of world wheat production, while durum wheat the other 5%. Wheat is the first cereal to be produced in the world and accounts for more than 20% of the total human food calories. Wheat is extensively grown on 17% of all crop areas, in the temperate, Mediterranean-type and subtropical parts of both hemispheres. Furthermore, it is the staple food for 40% of the world's population, mainly in Europe, North America and the western and northern parts of Asia, and it is humans' most important source of protein. (www.faostat.fao.org; www.croptrust.org).

Wheat cultivars are superior to most of the other cereals in their nutritive value. Besides the grain containing from 60% to 80% of starch, it also contains from 7% to 22% of storage protein. The gluten proteins in the seed endosperm impart unique bread-baking qualities to wheat dough. Only minor amounts of wheat are occasionally used as animal feed, with the amount being highly dependent on wheat prices compared with other feed grains (Gustafson *et al.*, 2009).

In the future, the world wheat supply will become even more critical as the demand will exponentially grow while the wheat stocks are decreasing. The world population was projected to reach 8.3 billion by the 2030 and 9.3 billion by 2050. Income growth and urbanizations are also expected to increase in many developed and developing countries. For such these reasons, world demand is projected to require about a 66% increase in agricultural production by 2040. In addition, our ability to bring more land into wheat cultivation is rapidly diminishing due to the population growth, environmental pressures and the limited availability of arable land (www.fao.org). The success of wheat improvement programs to meet future demands will require the complementation of traditional breeding programs with innovative, non-traditional methodologies that will enhance the genetic variation in wheat.

1.1.1 Evolution of wheat

Modern wheat cultivars refer to two species, hexaploid bread wheat, *Triticum aestivum* L. ($2n=6x=42$, A^uA^uBBDD), and tetraploid, hard or durum-type wheat, *T. durum* (Desfontaines) Husnache ($2n=4x=28$, A^uA^uBB) used for pasta and low-rising bread, which are characterized by a huge genome sizes (15.961 Mb for bread wheat and 11.660 Mb for durum wheat) and complexity of the genomes (Peng *et al.*, 2011).

Wheat is a superb model organism for the evolutionary theory of allopolyploid speciation adaptation and domestication in plants (Gustafson *et al.*, 2009).

The family *Poaceae* evolved 50-70 million years ago and the sub-family *Pooideae* diverged around 20 million years ago (Inda *et al.*, 2008). The wild diploid wheat (*T. urartu*, $2n=2x=14$), donor of the A^uA^u genome, hybridized with the B genome ancestor, *Aegilops speltoides* ($2n=2x=14$) 300.000-500.000 years ago to produce the wild emmer wheat *T. dicoccoides*, $2x=4x=28$, genome A^uA^uBB (Dvorak and Akhunov, 2005). Around 10.000 years ago, hunter-gatherers began to cultivate wild emmer and the subconscious selection gradually created the cultivated emmer, *T. dicoccum* ($2n=4x=28$, genome A^uA^uBB) that spontaneously hybridized with another goat grass, *Ae. Tauschii* ($2n=2x=14$, genome DD) to produce an early spelt, *T. spelta* ($2n=6x=42$, genome A^uA^uBBDD). This cross took place after emmer wheat cultivation expanded from the Fertile Crescent to the natural habitat of *Ae. Tauschii*, probably south and west of the Caspian Sea. About 8.500 years ago, natural mutation changed the ears of both emmer and spelt to a more easily threshed type that evolved into the free-threshing ears of durum wheat (*T. durum*) and bread wheat (*T. aestivum*) (Figure 1.1) (Gustafson *et al.*, 2009; Peng *et al.*, 2011; Feldman and Levy, 2012).

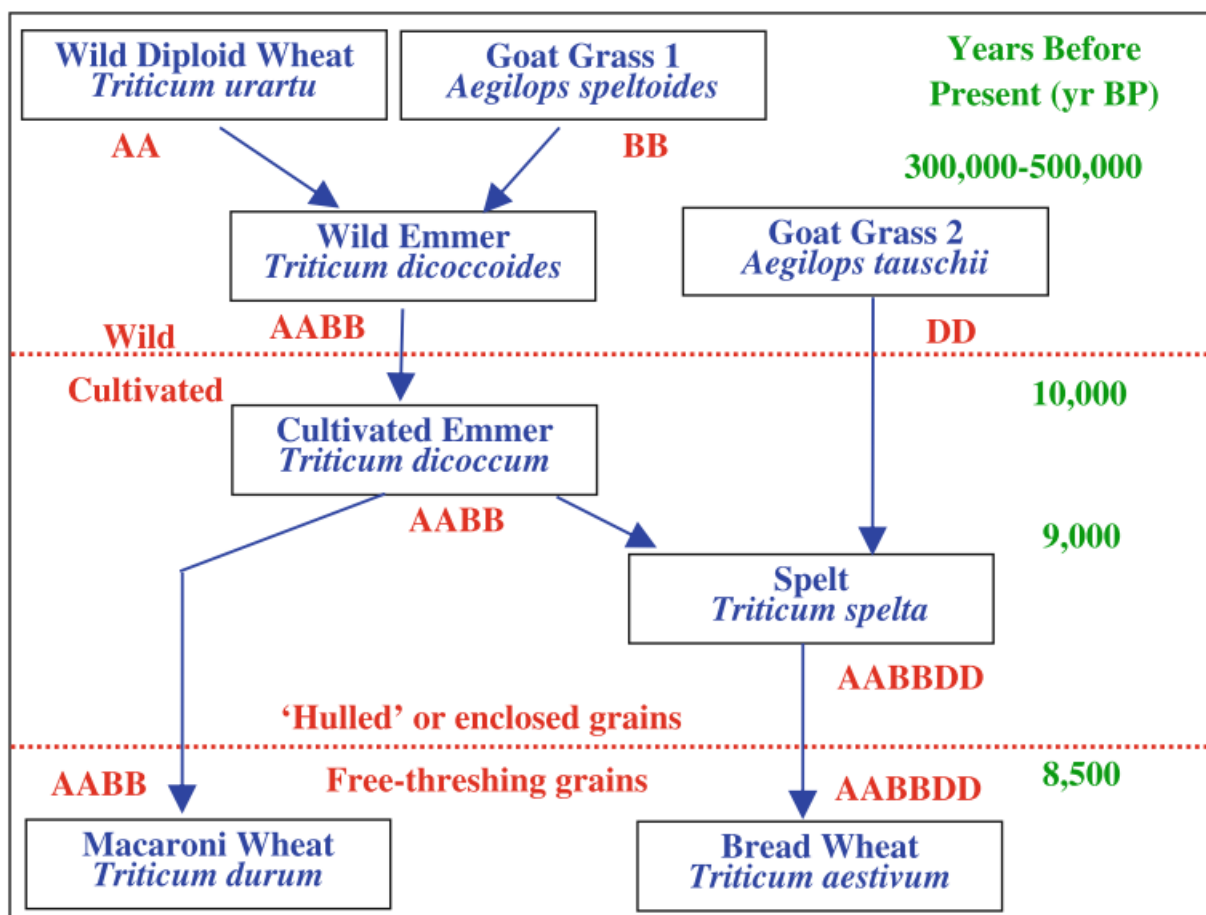


Figure 1.1 The evolution of wheat from the prehistoric Stone Age grasses to modern durum and bread wheat (Peng *et al.*, 2011).

The domestication of *Triticum* species occurred 10.000 years ago in a small core area within the Fertile Crescent-northern Syria, near the upper reaches of the Tigris and Euphrates rivers, and was

critical to have transformed humanity to more centralized, sedentary farming societies (Lev-Yadun *et al.*, 2000). The genetic changes required for wheat domestication were relatively straightforward and rapid, including selection for non-shattering, free-threshing (*tg*), non-brittle rachis (*br*) and hull-less spike (*Q*) characteristics and for higher yield. In fact, wild and primitive wheat forms have hulled grains and brittle ears that disarticulate at maturity into individual spikelets, with each spikelet having a wedge-shaped rachis internode at its base, and an arrowlike device that inserts the seed into the ground. By contrast, all cultivated wheat forms have non-brittle ears that stay intact after maturation, thus depending on humans for reaping, threshing, and sowing (Figure 1.2) (Gustafson *et al.*, 2009). Thus, the gene complexes for ease of harvest, yield, and suitability for long-term storage have been critical for domestication of cereals (Gustafson *et al.*, 2009).

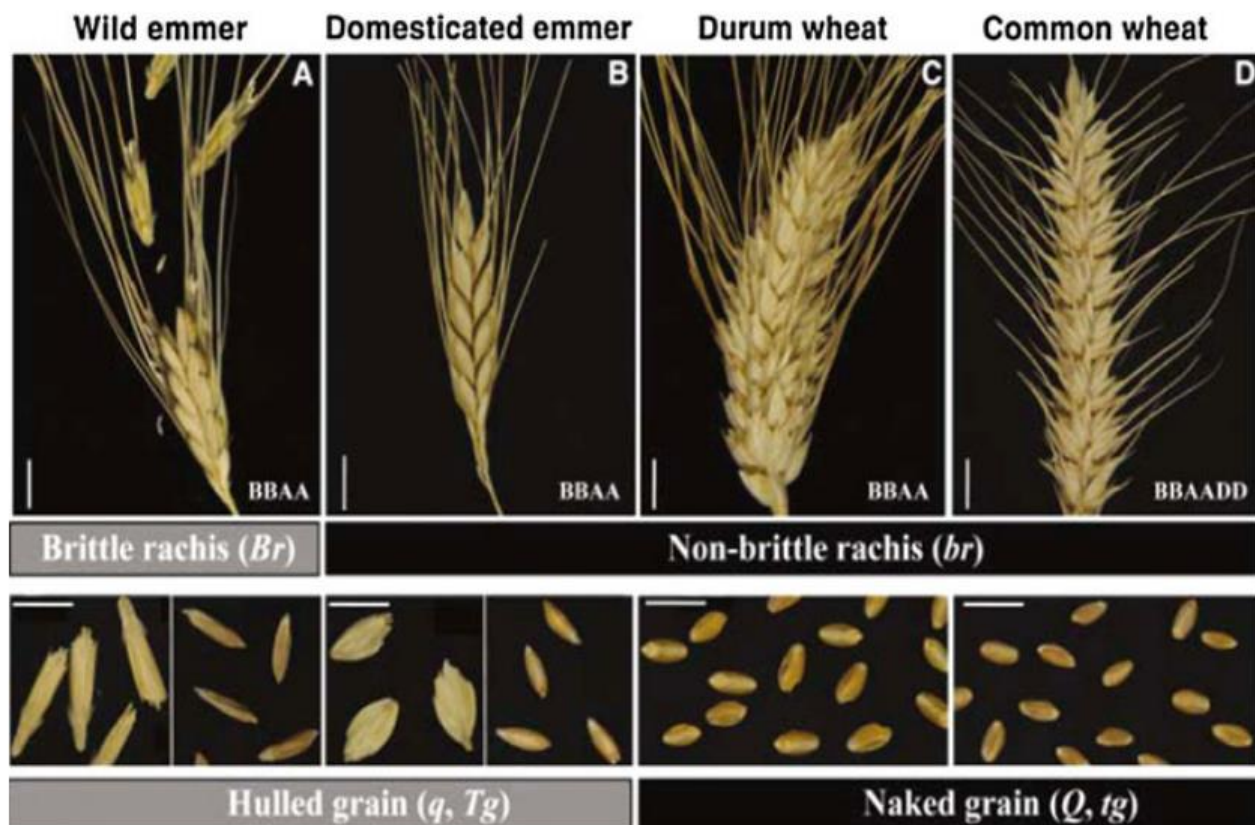


Figure 1.2 Wheat spikes showing (a) brittle rachis, (b-d) non-brittle rachis, (a) and (b) hulled grain, and (c) and (d) naked grain. White scale bars represent 1 cm. Letters at the lower right corner indicate the genome formula of each type of wheat. Gene symbols: *Br* brittle rachis, *Tg* tenacious glumes, and *Q* square head (Peng *et al.*, 2011).

As previously mentioned, the most important and best-characterized group of allopolyploids is the wheat genus; nevertheless, the *Triticum* spp. are not the only plant species evolved as polyploids. In fact, polyploidy represents more than 70% of the plant species, and it is defined as the presence of more than one genome per cell (Gustafson *et al.*, 2009). Polyploidy is one of the most important evolutionary events leading to a massive increase of genetic diversity, thus allowing species to adapt to varying environments (Feldman, 2001). This has clearly been the case for hexaploid wheat, which

combined the D genome from *Ae. tauschii* with the AB genomes from tetraploid wheat. In fact, hexaploid wheat has broader adaptability to different photoperiod and vernalization requirements, improved tolerance to salt, low pH, aluminium, and frost, better resistance to several pests and diseases, and extended potential to make different food products (Dubcovsky and Dvorak, 2007). The origin of polyploid wheat is complex because its evolution has involved a long-established massive intervention of human and environmental selection pressures. Studies of natural and synthetic wheat and related allopolyploids, as well as genome sequencing data, indicate that a broad range of DNA rearrangements occurred during, immediately, or within a few generations following allopolyploidization. These rapid changes contribute to increased diversity at the intra-specific level and were rapid and extensive, including loss of coding and non-coding DNA sequences, transposon activations, and gene silencing or duplication and pseudogenization. These rapid modifications allowed to achieve a genomic plasticity that resulted in increased fitness of the wheat allopolyploids species compared to their parents (Feldman and Levy, 2009, 2012).

1.1.2 Morphology and phenology

Wheat is a monocot species that germinates forming a coleoptile, following the generation of the primary root system. At the first leave stage, buds are formed that stretch in shoots, which repeat the structure of the leading shoot; each secondary bud in turn emits adventitious roots and can also develop new shoots. The leaf is composed of four parts: the sheath, the auricles, the ligule and the lamina. The sheath envelops the culm and, at the base of each internode at the meristematic region, it swells and extends beyond the point of insertion of the foil with a small expansion, the ligule. The foil presents to the point of insertion where the sheath forms two small sickle appendices, the auricles. In the culm, the hollow stem of the cereal plant, there are the nodes and the internodes, in which the leaves are inserted. At the end of the tillering, the internodes lengthen forming the culms that will bring an apical inflorescence. The inflorescence is a composite, distich spike, whose primary axis, the rachis, carries two opposite rows of lateral spikelets and a single terminal spikelet. The spikelets are placed on a short axis, the rachilla, which joins them to the rachis; each spikelet leads from 3 to 8 flowers. At the base of each spikelet there are two glumes and each flower has a lower glume, the *lemma*, and an upper glume, the *palea*. The flower has three stamens with bilobed anthers and a bifid pistil at the base of the *lodicule*, membranous pads that are located outside of the stamens, which swell at anthesis pulling back the glumes and allowing the stamens and the stigmas to protrude out (Figure 1.3) (Fratini and Valvassori, 1976; Lersten, 1987; Anderson and Garlinge, 2000; Khan and Shrewry, 2009).

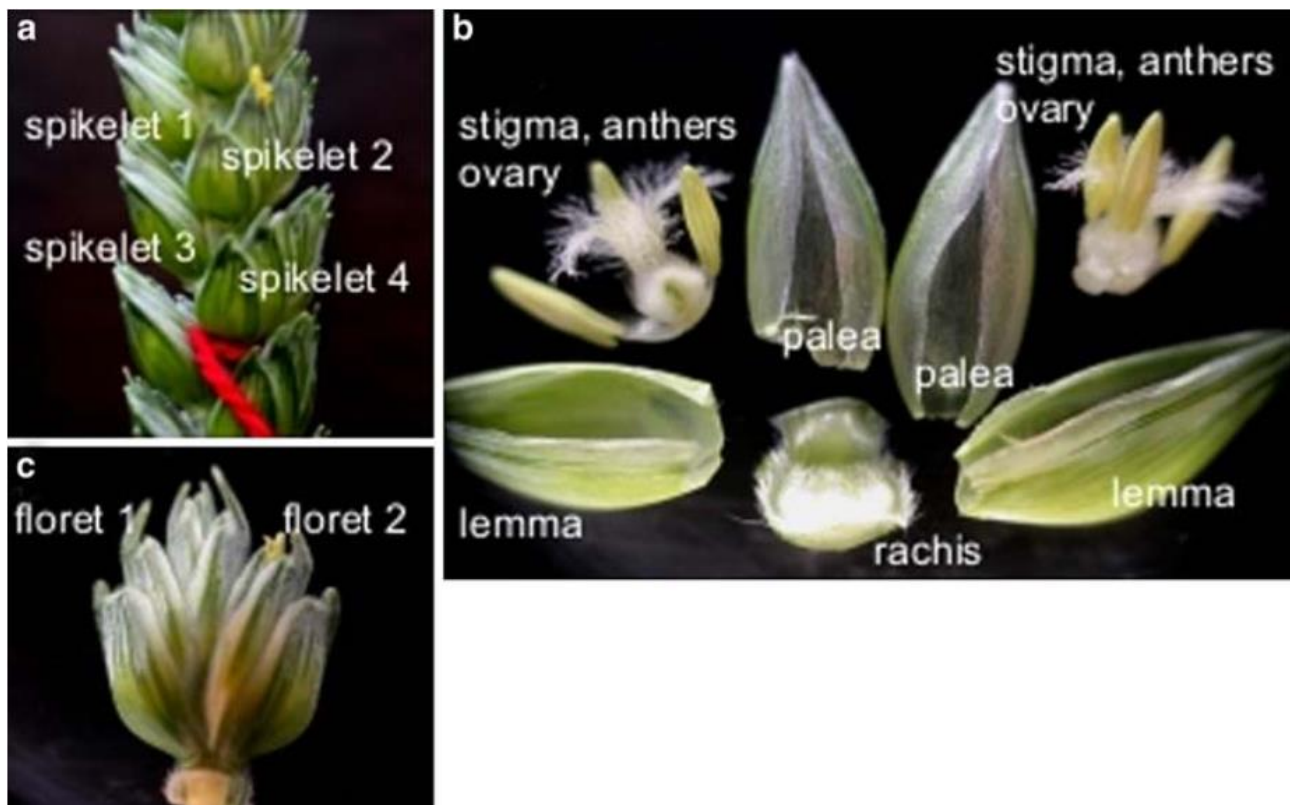


Figure 1.3 Illustration of (a) the spikelets, (b) the floral tissues separated in the reproductive part (ovary, stigma, anthers) and the lemma, palea and the subtending section of the rachis, and (c) the two florets per spikelets (Steiner *et al.*, 2009).

The grain is a caryopsis (or kernel), a fruit in which the pericarp strictly adheres to the seed, that is enclosed; in the caryopsis are distinguished a dorsal side and a ventral side, the latter crossed by a longitudinal groove. The granule is composed of an embryo (2-4%), an endosperm (87-89%) and the wrappers (8-10%). The embryo is located at one end of the caryopsis and is separated from the endosperm by the scutellum, an epithelial formation, whose function is to transfer the nutrients from the endosperm to the embryo. The embryo contains mostly fat, nitrogenous substances, enzymes, vitamins and hormones, while the endosperm consists of cells rich in starch, where the protein component tends to decrease gradually from the periphery to the central part of the granule. The consistency and aspect of the endosperm may vary according to the nitrogen content, assuming amber-coloured and corneas, vitreous or mealy characteristics, also depending on the cultivar and on the environment conditions (Figure 1.4) (Fratini and Valvassori, 1976; Lersten, 1987; Anderson and Garlinge, 2000; Khan and Shrewry, 2009).

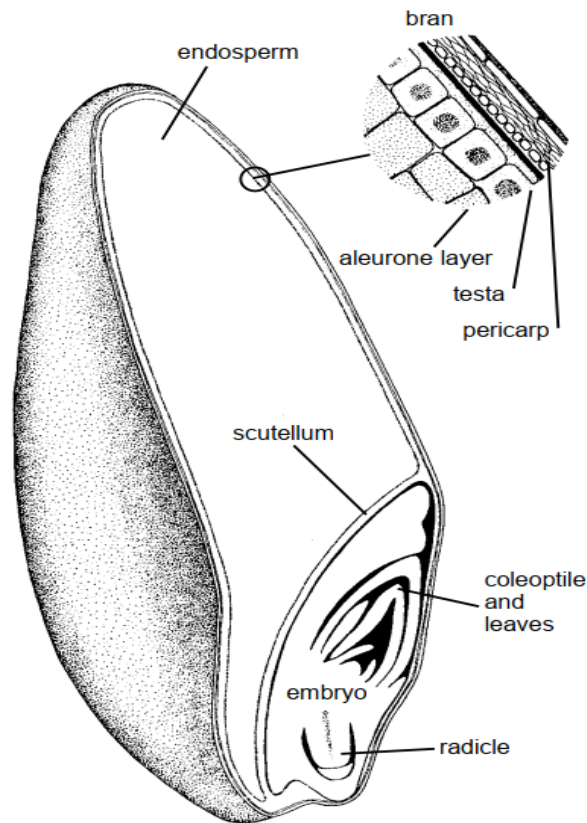


Figure 1.4 Structure of the wheat grain (Anderson and Garlinge, 2000).

The life cycle of wheat, autumn-winter or spring cereal, can be represented into different phenological phases: germination and emergence, tillering, rising, flowering and ripening. The germination begins when water is available for the caryopsis, which absorbs the 35-40% of its weight and, if the temperature and oxygenation are favourable, it germinates. At around 4°C, germination occurs with regularity. The first organs that protrudes from the kernel is the central embryonic radicle, followed by the coleoptile and the primary roots. After the emergence, the first leaf breaks the coleoptile and expands, followed by the leakage of the remaining leaves. The tillering is a phenomenon existing only in the microthermal cereals and consists in the development of the secondary shoots. At the stage of 3-4 leaves, the development of a sprout near the armpit of the first leaf is observed. This phenomenon is also observed in the second, third and fourth leaf; these shoots of tillering are called “first order shoot” and it is also possible that additional ones appear, of higher order, if, from their basal nodes, more shoots develop. The tillering should be considered as a branch of the culm. At the same time of tillering, the development of the radical secondary system starts, through the issue of adventitious roots. At the moment of the induction of flowering, the cauline apex stops differentiating new leaves and the differentiation of the future spikelets starts. The plant is now in the so-called toning stage, which consists in the appearance of double bulges on the cauline apex. At about 12°C, the plants start the rising phase: nodes and internodes, which previously were not far from each other, starting to distance thanks to the proliferation of meristematic tissue at their base. When all the

internodes are developed, the spike, already fully formed, is pushed through the sheath of the last leaf, resulting in an enlargement, which identifies the boot stage and, subsequently, the earing phase. A few days after the emergence of the ear, the plant is in the flowering stage, that, typically, occurs at around 15°C. The husk encloses the flower, where, at full anthesis, the yellow stamens and the swollen ovary, overflowed by a feathery stigma, are present. This morphology identifies the occurrence of fertilization, that is self-pollinating-cleistogamy. In general, the flowering starts from the middle spikelets, moving then upwards and downwards. The flowering occurs first in the main culm, followed by the primary tillering culm and proceeding in higher order culms. The ripening, or so-called caryopsis filling, occurs at around 18-20°C. In the endosperm there is a rapid accumulation of secondary starch granules, which allow the grain to reach the maximum of its volume and a humidity of around 70%. The kernels start to appear yellow and, due to the progressive accumulation of starch, assume a waxy-like morphology. At this phenological stage, the water content decreases to 40-45%. The maturation of the caryopsis is completed when the starch granules entirely fill the cells of the endosperm, the moisture content is 30% and the plant is almost completely yellowed. This stage is called physiological or yellow maturation and, at this stage, the endosperm stops accumulating reserve substances. Finally, a gradual and further loss of water occurs and the moisture content reaches 13%, determining the full maturation of the grain. The phenological stages of wheat are illustrated in Figure 1.5 (Fowler, 2018).

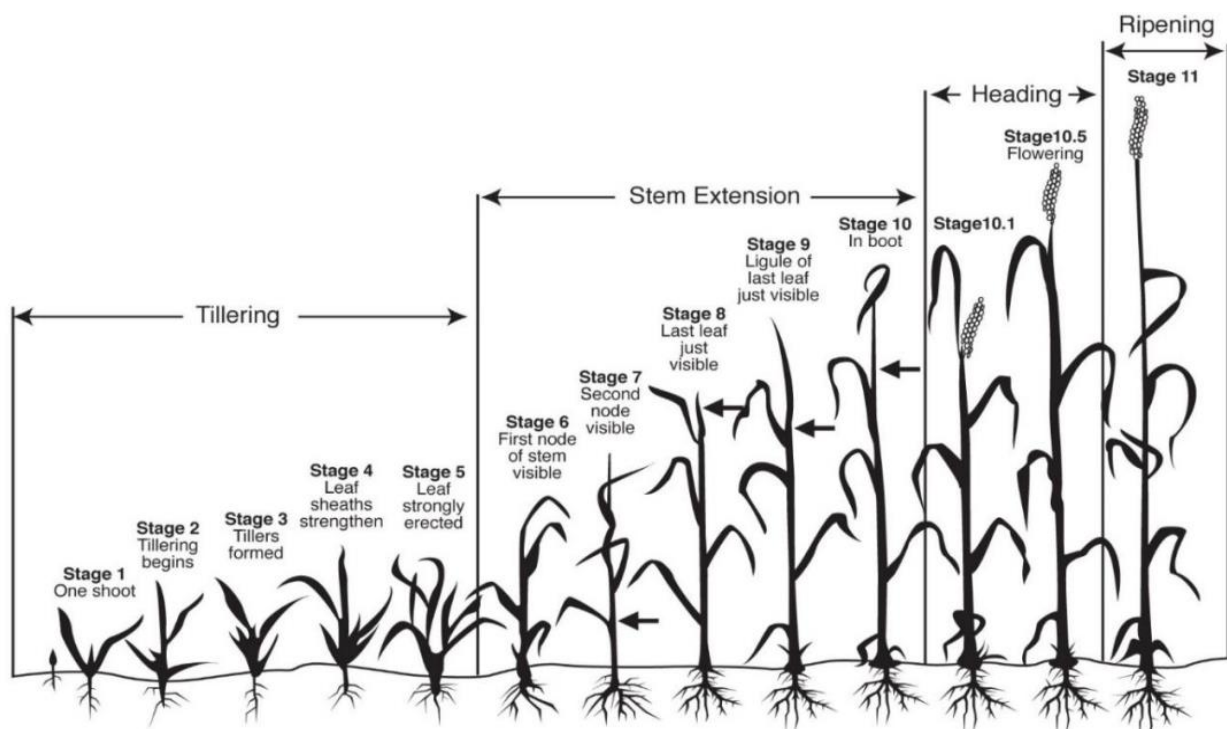


Figure 1.5 Wheat life cycle and phenological stages (New Mexico University, College of Agricultural, Consumer and Environmental Science, ACES, http://aces.nmsu.edu/pubs/_a/A415/).

1.2 Fusarium head blight of wheat

The wheat production has been challenged by the increasing abiotic (heat, cold, drought, etc.) and biotic (diseases, pests, weeds, etc.) stresses. Fusarium head blight (FHB), or scab, stands out as the most devastating wheat disease owing to lack of resistant cultivars, significant yield loss and grain quality reduction in epidemic years, and health threat of wheat food and feed made from grains contaminated with mycotoxins produced by the causal agents. FHB was firstly documented in England in 1884, but FHB epidemics have occurred regularly in the majority of the wheat-growing regions around the world, including Asia, North America, South America and Europe (Ma *et al.*, 2020). The main reasons behind the severe FHB epidemics are related to the decreased level of resistance in cultivars, changes in weather conditions and modification in crop management strategies (McMullen *et al.*, 2012a; Khan *et al.*, 2020). However, in recent years, FHB has become more prevalent in Asia, Europe, and South America, due to the continuously changing environment and the increasing threat of global warming have led to an increase in the FHB epidemic (Shah *et al.*, 2005), thereby resulting in increased economic losses (Parry *et al.*, 1995; Bai and Shaner, 2004; van der Lee *et al.*, 2015). The FHB epidemics have been reported to lead to a 10-70% of production loss during the epidemic years. Indeed, according to the International Maize and Wheat Improvement Centre (CIMMYT), FHB has been considered as one of the most destructive diseases impacting the production of wheat globally. Even under moderate disease pressure, quality and economic losses, particularly due to the contamination of the harvested grains with mycotoxins, are of great concern for the whole cereal production chain and may render a healthy, nice-looking harvest downgraded and devalued (McMullen *et al.*, 2012a; Khan *et al.*, 2020; Ma *et al.*, 2020; Mielniczuk and Skwaryło-Bednarz, 2020).

1.2.1 Causal agents, epidemiology and symptoms

The FHB species complex includes more than 16 species such as *F. chlamydosporum*, *F. boothii*, *F. scirpi*, *F. arthrosporioides*, *F. poae*, *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. verticillioides*, *F. asiaticum*, and *F. cortaderiae* (O'Donnell *et al.*, 2004; van der Lee *et al.*, 2015; Dweba *et al.*, 2017). The predominant species vary from year to year, and location to location, depending on temperature, moisture and crop rotations. *Fusarium graminearum* Schwabe is considered the most aggressive species, it is graded among the four crucial plant fungal pathogens (Dean *et al.*, 2012), and was thought to be a single cosmopolitan species (Ma *et al.*, 2020). The prevalence of the individual *Fusarium* species is determined by the capacity of local adaptation and by virulence, which is associated with trichothecene chemotypes of the pathogenic species (Ma *et al.*, 2020; Mielniczuk and Skwaryło-Bednarz, 2020). *Fusarium* species are hemi-biotrophic pathogens that obtain nutrients from both living and dead tissues (Dweba *et al.*, 2017; Khaledi *et al.*, 2017). In field conditions, the inocula

survive primarily on plant residues and soil, with local dissemination of the ascospores and conidia promoted by water splash or by wind for long-distance migration. The FHB infection are favoured when humid and warm conditions persist for a long duration and when plants are at the flowering stage. The infection cycle of *F. graminearum* in wheat starts with the settling of airborne spores on wheat spikelets, which, after germination, enter the plants via degenerated anther tissues or minute natural openings under the lemma. Further, the growth of fungus occurs between the cells and it passes from the xylem and the pith and colonizes with the tissue followed by necrosis. At the cellular level, the cell wall, mitochondria, chloroplasts, and membranes are also damaged. This leads to the water soaking in chlorenchyma tissues leading to the production of shrivelled kernels and premature bleaching that affects photosynthesis. During the infection, genes for deoxynivalenol (DON) biosynthesis are expressed by the fungus, which facilitates the spreading of the fungus from spikelet to rachis. There is an association between the DON biosynthesis and the colonization of developing tissues leading to shrivelled grains. The life cycle of *F. graminearum* consists of both sexual and asexual stages and haploid mycelial structures are formed in both stages. *Fusarium* species possess three forms of mitotic (asexual) spores, chlamydospores from hyphae, macroconidia from sporodochium, and microconidia from conidiophores. The anamorph (asexual stage) and teleomorph (sexual stage) of this pathogen are *F. graminearum* and *Gibberella zeae*, respectively. In *F. graminearum*, asexual spores are called macroconidia, whereas sexual spores are called ascospores. Generally, *F. graminearum* is haploid during its life cycle. Macroconidia formed on hyphae, called sporodochia, develop on infected crop residues under humid conditions and are largely responsible for short-distance dispersion. However, their sexual lifecycle is triggered by warm, humid, and wet conditions. As an ascomycota, its sexual life cycle consists of a prolonged dikaryotic phase that is homothallic and the two nuclei are genetically similar. These dikaryotic cells produce coiled cells, leading to the formation of ascus-filled perithecia. These asci consist of ascospores that are released outside via the mouth of the perithecium; these ascospores are the main inoculum of the infection. The general life cycle of *F. graminearum* is represented in Figure 1.6.

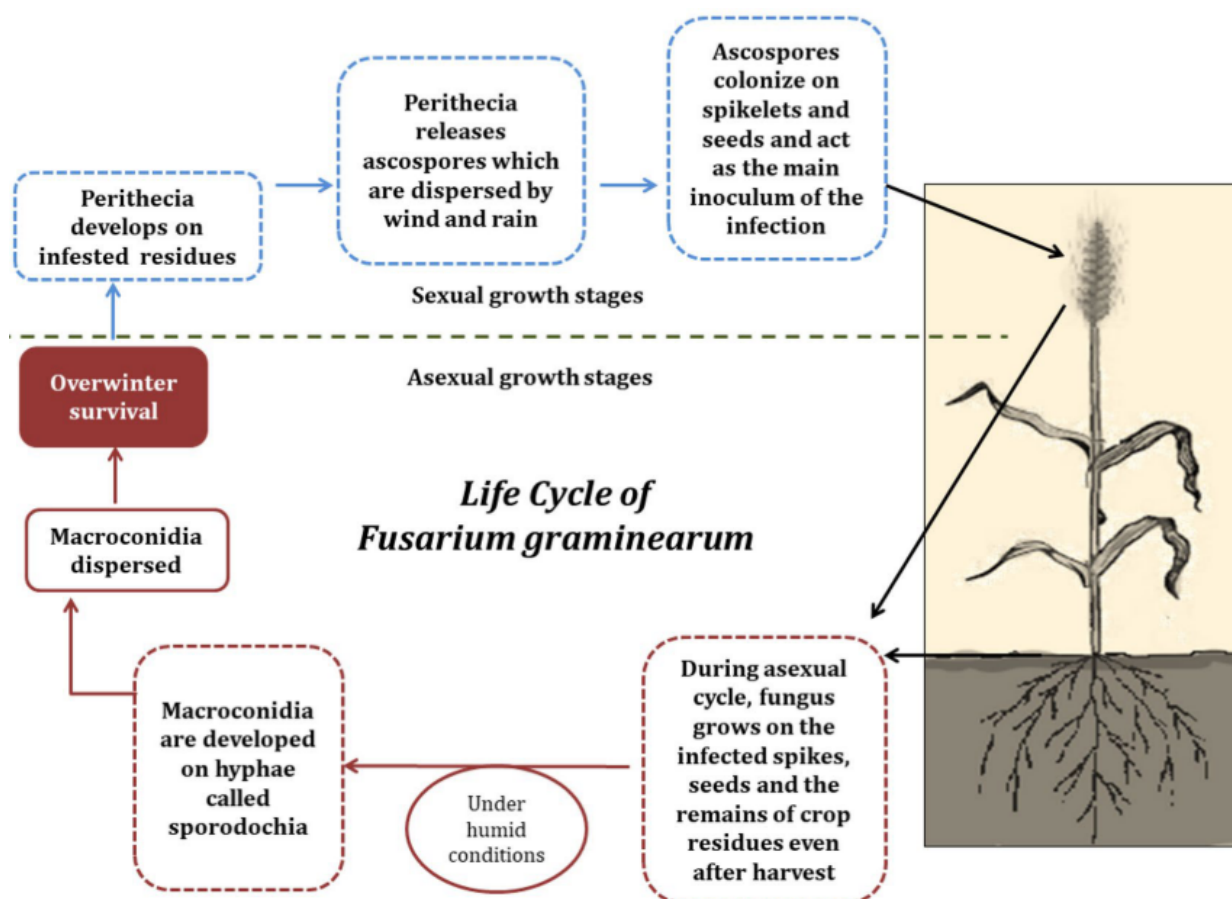


Figure 1.6 Life cycle of *Fusarium graminearum* (red and blue outlined stages represent asexual and sexual growth stages, respectively) (Khan *et al.*, 2020).

Genetic diversity of the pathogen and its adaptive response to the environment is usually enhanced due to sexual reproduction as it allows genetic exchange in *F. graminearum* populations via recombination. The disease symptoms on infected heads (Figure 1.7) are visible during the milk maturity stage of the grain. The infected spikes rapidly bleach and pink or salmon-coloured sporodochia with conidial spores, as well as mycelium layer, appear on infected chaff during persistent high humidity, after a few days of infection. The bleaching of infected spikelets inhibits the development of kernels, which causes grain number reduction in the spikes. The remaining kernels developing in infected spikes are smaller, grey, shrivelled, with a loose consistency and often covered with sporodochia and mycelium. Damage to starch granules and changes in storage protein composition occur in infected kernels. More importantly, the grains may contain high concentrations of trichothecene mycotoxins produced by the *Fusarium* species, such as deoxynivalenol (DON), making them unsuitable for human consumption and as livestock feed (Liu *et al.*, 1997; Miller *et al.*, 1998; Gilbert and Tekauz, 2000; McCallum and Tekauz, 2002; Jansen *et al.*, 2005; Beyer *et al.*, 2006; Vaughan *et al.*, 2016; Gunupuru *et al.*, 2017).



Figure 1.7 FHB symptoms. (a) and (b) head blight symptoms; (c) brown/purplish discoloration of the rachis; (d-f) orange sporodochia on spikelets (Scherin *et al.*, 2013).

1.2.2 Mycotoxins produced by *Fusarium* spp.

Mycotoxins are secondary metabolites of fungi including *Fusarium* spp., which commonly contaminate cereal grains (wheat, barley, oat, rye, maize and rice) and derived-cereal products (Desjardins and Proctor, 2007). Mycotoxins can also be found in animal feed. This topic is particularly important because the mycotoxins remain in food products obtained from infected animal organisms. Therefore, meat, milk, eggs and its products may also pose a serious threat to human health. Mycotoxins exert various effects on the human and animal bodies, among others they could show mutagenic, teratogenic and estrogenic effects (Anttila *et al.*, 2002; Bottalico and Perrone, 2002; Maresca, 2013). They also have a significant impact on the economy because the presence of mycotoxins at a certain level results in the exclusion of agricultural crops, feed and food products from commercial trade (European Commission, 2006). Secondary metabolites of these fungi, such as

DON, zearalenone (ZEA) and fumonisin (FUM) are among five most important mycotoxins on a European and world scale (Bai *et al.*, 2002; Bottalico and Perrone, 2002; Fagnano *et al.*, 2012). The level of contamination of cereal grain with *Fusarium* mycotoxins depends on many factors, such as weather conditions, cultivation system, method and date of grain harvest, as well as the degree of resistance of cultivated varieties to *Fusarium* spp. infection (Del Ponte *et al.*, 2009; Gorczyca *et al.*, 2018; Yan *et al.*, 2020). Each species of the genus *Fusarium* has a specific profile of toxic secondary metabolites that have phytotoxic and zootoxic effects (Pestka, 2010; Yli-Mattila, 2010). The following chemotypes were distinguished in *Fusarium* spp. based on the profile of produced secondary metabolites: chemotype I includes the strains producing DON and/or its acetyl derivatives (3-ADON and 15-ADON); chemotype II includes the strains producing nivalenol (NIV) and/or 4-acetylnivalenol (4-ANIV). DON-producing chemotypes of *F. graminearum* are widespread around the world, while NIV-producing chemotype is more commonly found in Asia and Europe, although the occurrence of individual chemotypes is also modified by weather conditions (Covarelli *et al.*, 2015; Pasquali *et al.*, 2016). Furthermore, another chemotype was distinguished consisting of strains capable of producing zearalenone (ZEA) (Batur-Ciesniewska and Suchorzynska, 2011). The effect of mycotoxins on the body can be chronic or acute depending on the dose and duration of exposure to toxins (Hussein and Brasel, 2001).

Trichothecene compounds are classified as tetracyclic sesquiterpenes (Foroud *et al.*, 2019). Sixteen genes involved in the synthesis of trichothecenes have been distinguished, most of them form the group of *Tri* genes, which contains genes that show a specific effect on trichothecene formation, such as *Tri5*, *Tri7*, *Tri13* and *Tri3* (Kimura *et al.*, 2007; Suchorzynska and Misiewicz, 2009). Trichothecene compounds are classified into four main groups (A, B, C and D) depending on chemical properties (functional groups) and fungi producing them. Trichothecenes produced by *Fusarium* spp. are included in groups A and B. Group A of trichothecenes includes: T-2 toxin and HT-2 toxin, diacetoxyscirpenol (DAS), scirpentriol (STO), 4-monoacetoxyscirpenol (MAS), neosolaniol (NEO). These metabolites are mainly produced by *F. sporotrichioides*, *F. sambucinum*, *F. poae*, *F. langsethiae* and *F. equiseti* (Thrane *et al.*, 2004; Kiecana *et al.*, 2012; Adhikari *et al.*, 2017). Group A trichothecenes show cytotoxic and immunosuppressive effects. T-2 toxin induces oxidative stress, causing DNA damage, inhibiting protein synthesis and damaging lipids. In addition, it affects the mucous membrane of the gastrointestinal tract, causes blistering of the skin, swelling, irritation, necrosis, and even cell apoptosis (Adhikari *et al.*, 2017). Group B trichothecenes include DON and its acetyl derivatives, 3 acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) as well as NIV and its acetylated form 4-ANIV, which are produced in cereal grains by *F. culmorum*, *F. graminearum*, *F. crookwellense*, *F. poae* and *F. equiseti* (Golinski *et al.*, 2010;

Kiecana and Mielniczuk, 2013; Covarelli *et al.*, 2015). Among *Fusarium* mycotoxins, DON generally most frequently contaminates cereal grains (barley, wheat, oat) and food products as well as animal feed (Golinski *et al.*, 2010; Spanic *et al.*, 2018). High DON doses have been found to cause vomiting, lack of appetite, weight loss and diarrhoea, necrosis of certain tissues, such as the gastrointestinal wall, bone marrow or lymphoid tissue. Acting at the cellular level, trichothecenes cause mitosis and chromosome separation disorders, they can also induce programmed cell death of normal cells (Goyarts *et al.*, 2007; Pestka, 2010).

Fumonisin in cereal grains are mainly produced by *F. fujikuroi* and *F. proliferatum*. The listed species of fungi are common maize pathogens, hence the highest level of fumonisin contamination is generally found in maize grain and its products, although they are also found in other cereals and food products (Kamle *et al.*, 2019). The production of fumonisin is dependent on *FUM1* gene which expresses an enzyme complex known as polyketide synthase that catalyses the initial step for fumonisin biosynthesis (Bojja *et al.*, 2004). Research has shown that fumonisin has neurotoxic, hepatotoxic and nephrotoxic effects in animals. In humans, it is classified as a potential carcinogen. Exposure to fumonisin may contribute to nervous system defects, probably due to sphingolipid metabolism disruption (Anttila *et al.*, 2002).

Zearalenone (ZEA) is one of the most common non-steroidal estrogenic mycotoxins produced by *Fusarium* spp. (Zhang *et al.*, 2018). ZEA is mainly produced by *F. graminearum* and *F. culmorum*; this metabolite can also be produced by some strains of *F. poae*, *F. equiseti* and *F. crookwellense* (Perkowski *et al.*, 1997; Bottalico and Perrone, 2002; Mielniczuk *et al.*, 2016). ZEA is produced in the acetate-polymalonate pathway by genes encoding for the polyketide synthases (PKSs). ZEA is present in particular in maize, wheat, barley, oat, rye, sorghum, millet and rice, which is why its distribution has a worldwide range (Zinedine *et al.*, 2007). In the body of animals, ZEA is metabolized in the liver to α -zearalenol, which is more estrogenic than ZEA (Fink-Gremmels and Malekinejad, 2007; Zinedine *et al.*, 2007).

In the populations of individual species, both non-pathogenic and highly-virulent strains are found on the infected plants, leading to rapid tissue necrosis, which is often associated with the presence of genes encoding mycotoxin formation in the genome of these fungi (Osborne and Stein, 2007; Mielniczuk and Skwaryło-Bednarz, 2020). It has been shown that cereal virulence of many species of the genus *Fusarium* largely depends on the synthesis of trichothecene compounds, especially DON and its acetyl derivatives and enzymes degrading cell walls of the host plant, mainly cellulases, chitinases and xylanases (Wanjiru *et al.*, 2002; Gilbert *et al.*, 2010). In wheat, when the fungus is unable to produce trichothecenes the disease is restricted to the inoculated spikelet(s) (Bai *et al.*, 2002). *Fusarium* disruption mutants in the *TRI5* gene do not produce trichothecenes and are unable

to gain access to the wheat rachis through infected spikelets (Bai *et al.*, 2002; Jansen *et al.*, 2005; Maier *et al.*, 2006).

1.2.3 FHB occurrence in Italy

In Italy, FHB has been permanently present since 1995, especially in the Northern and Central regions, with variable incidence and severity in relation to the year, cultivation area and wheat variety (Pancaldi *et al.*, 2010). Many studies conducted in the last twenty years highlighted that *Fusarium* incidence decreases from Northern to Southern Italian areas, mainly attributed to the great rainfalls occurring in Northern areas during the stage of anthesis. Particularly, many authors found that the genus *Fusarium* was the second most prevalent after the *Alternaria* spp. in all cultivation environments, even if regional differences in species composition were detected. Despite *F. poae* resulted the predominant species causing FHB in the research studies conducted around twenty years ago, nowadays, a shift from *F. poae* to *F. graminearum* is observed, mainly due to climate changes. Indeed, *F. graminearum sensu strictu* is the main FHB agent followed by *F. poae*, *F. avenaceum* and *F. culmorum*, especially when weather conditions are not suitable for *F. graminearum*. The amount of *Fusarium* biomass and mycotoxins detected and quantified are in accordance with the increasing incidence of FHB from the South to the North of Italy. With a special reference to the *Fusarium* associated mycotoxins, DON, NIV and their acetylated forms are the most frequently found mycotoxins in Italy and their detected amount is strongly correlated with an increasing gradient from the South to the North of Italy. This suggests that environmental conditions at the regional level play a remarkable effect of *Fusarium* incidence in durum wheat grains, also confirming that the gradual expansion of durum wheat from Southern to Northern areas of Italy might have increased the risk of FHB infections (Shah *et al.*, 2005; Fagnano *et al.*, 2012; Infantino *et al.*, 2012; Somma *et al.*, 2014; Amato *et al.*, 2015; Beccari *et al.*, 2017, 2020). However, Scala *et al.* (2016) suggested that environmental conditions at the local level might drive FHB outbreaks even in areas suitable for growing durum wheat, such as Southern Italy. However, in association with climatic parameters also crop rotation, fungicide application and cultivated varieties may play a role in *Fusarium* incidence in wheat grains (Beccari *et al.*, 2020).

1.3 Host-pathogen interactions

Plant pathogens use diverse life strategies to colonize the host tissues. For example, fungi can directly enter the plant epidermal cells, or extend the hyphae on top of, between, or through the plant cells. Symbiotic fungi can invaginate the feeding structures (haustoria) into the host cell plasma membrane. The haustorial extracellular matrix and the host plasma membranes form an intimate interface at which the interaction is determined. Despite the different mode of interactions between pathogens

and the host, the diverse pathogens all deliver effector molecules, called virulence factors, into the plant cell to enhance their fitness. Plants lack mobile defender cells and a somatic adaptive immune system; instead, they rely on the innate immunity of each cell and on systemic signals occurring from the infection sites. Particularly, two defence systems in plants are well described: the first is represented by transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs), while the second acts largely inside the cells, using polymorphic nucleotide-binding site leucine-rich (NB-LRR) protein products encoded by *R* genes (Dangl and Jones, 2001; Zipfel and Felix, 2005; Jones and Dangl, 2006). The current view of the plant immune system is represented as a zigzag model (Figure 1.8).

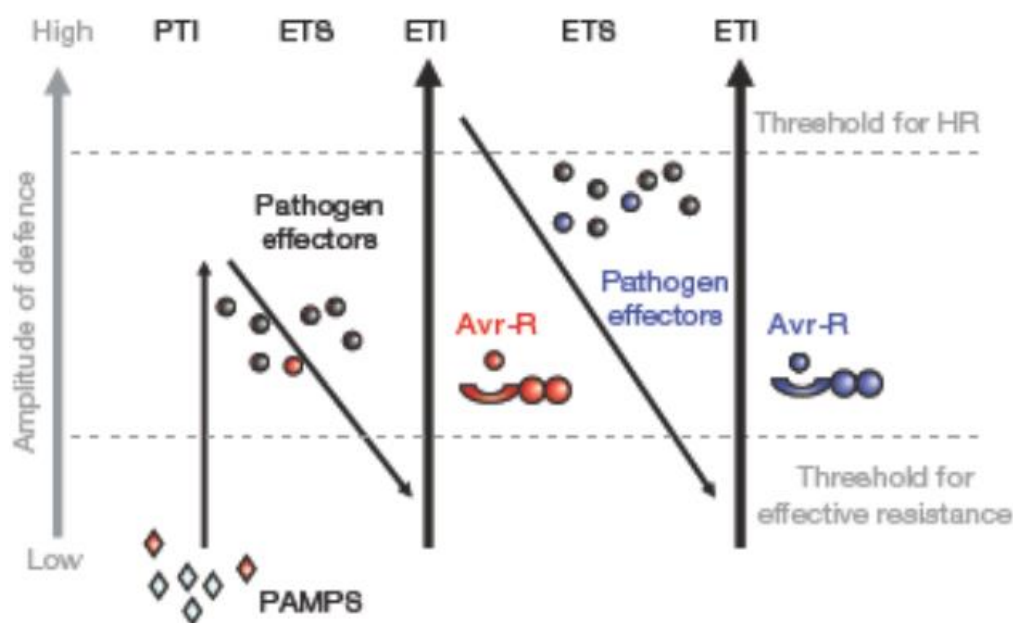


Figure 1.8 The zigzag model illustrating the quantitative output of the plant immune system (Jones and Dangl, 2006).

PAMPs (or MAMPs) are recognized by PRRs, resulting in PAMP-triggered immunity (PTI) that can stop further colonization. Nevertheless, successful pathogens deploy effectors that contribute to their virulence; the effectors interfere with PTI, resulting in effector-triggered susceptibility (ETS). A certain receptor can be recognized by a specific NB-LRR protein, resulting in effector-triggered immunity (ETI). ETI is an accelerated and amplified PTI response, resulting in disease resistance and, usually, it generates the hypersensitive cell death response (HR) at the infection site. The natural selection drives pathogens to avoid the ETI by diversifying the recognized effector or by acquiring additional effectors that can suppress ETI. By way of contrast, natural selection also drives the host to evolve new specific *R* genes, thus ETI can be triggered again (Jones and Dangl, 2006; Bentham *et al.*, 2020; Wang and Chai, 2020).

1.3.1 Wheat-*Fusarium* interactions and types of resistances against FHB

The genome of *F. graminearum* encodes 84 putative protein-coupled receptors (Cuomo *et al.*, 2007), which are essential in the early infection process, being involved in appressorium formation (Walter *et al.*, 2010). Particularly, the transducing beta-like gene *FTL1* is essential for pathogenesis on wheat heads (Ding *et al.*, 2009). *Fusarium* spp. enter into the cereal florets passively through the stomata or actively by penetration (Bushnell, 2001); the latter is facilitated by the vast array of hydrolysing enzymes secreted by *Fusarium* spp., such as cutinases, lipases, pectinases, xylanases, hemicellulases and cellulases (Walter *et al.*, 2010). By way of contrast, the host possesses diverse physical barriers to counteract the fungal penetration, such as the cuticle, the middle lamellae and the cell wall. The cuticular wax may reduce the water availability and thus inhibit fungal germination and penetration (Walter *et al.*, 2010). The phenylpropanoid and phenolics contained in the cuticular wall and cutin may offer some protection against penetrating *Fusarium* and the cuticle could be strengthened by the incorporation of fatty acids (Jetter *et al.*, 2006). Additionally, also phenylpropanoids and structural proteins reinforce the primary cell wall network; indeed, FHB-resistant wheat showed an increased accumulation of such compounds compared to FHB-susceptible genotypes (Carpita, 1996; El-Gendy *et al.*, 2001; Kang and Buchenauer, 2003). Furthermore, the host plant employs a range of protein to recognize the pathogen and its activities; the host plant detects the chitin and glucan components of *Fusarium* cells by chitin-binding proteins, chitinases, glucanases and thaumatin-like proteins (Theis and Stahl, 2004). The encoded enzymes could directly (via degradation of the fungal structural barriers) or indirectly (via elicitor activity of the fungal cell wall degradation products) play a role in defence and resistance against *Fusarium*. The contribution of these enzymes to FHB disease resistance is gene-specific and genotype-dependent (Walter *et al.*, 2010). The wheat plant also protects its structural barriers by inhibiting the activity of fungal degradative enzymes and by reinforcing its own barriers. Polygalacturonase- and xylanase-inhibiting proteins have been shown to accumulate in FHB-resistant wheat cultivars (Walter *et al.*, 2010). Resistance of wheat to infection also correlates with thickening of the cell wall and formation of cell wall appositions, papillae and increased lignin content in order to restrict the influx of *Fusarium* toxins and the efflux of plant nutrients (Kang and Buchenauer, 2000). The host resistance to the initial infection has been described as type I resistance forming the first line of defence against FHB and it is determined by developmental, physiological and morphological traits and might be enhanced by biochemical properties (Schroeder and Christensen, 1963). Additionally, morphological and phenological traits such as tallness, absence of awns, loose spikelet distribution and staggered flowering time contribute to type I resistance (Mesterházy, 1995).

Following penetration, *Fusarium* hyphae can spread within the cell apoplast, which leads to significant cytological alterations and ultimately to cell death. DON contributes to cellular damage disrupting plasma membranes, chloroplasts and ribosomes; DON also triggers hydrogen peroxide production causing cell death in cereals (Miller and Ewen, 1997; Desmond *et al.*, 2008). In general, DON facilitates the necrotrophic nutrition, since the ability to synthesize trichothecenes has been linked to enhanced virulence of *F. graminearum* during the wheat infection (Proctor *et al.*, 1995). At the same time, the secretion of fungal degrading enzymes facilitates the cell colonization by releasing compounds from the plant cells that serve as fungal nutrients, such as amino acids, sugars, fatty acids and ions (Divon and Fluhr, 2007). Plants can resist to fungal spread by modifying DON, by hindering toxin-target interactions or by interfering with downstream signalling cascades (Johal *et al.*, 1995). FHB-resistant varieties are able to transform DON to the less toxic DON-3-glucoside (Lemmens *et al.*, 2005) probably due to the enzymatic activity of an UDP-glucosyltransferase (UGT) (Poppenberger *et al.*, 2003). Since DON also induces the production of hydrogen peroxide, wheat may defend itself by counteractive oxidative stress through the activity of superoxide dismutase, catalase and ascorbate peroxidase, which neutralize free radicals (Lamb and Dixon, 1997; De Gara *et al.*, 2003). Additionally, various plant defence compounds are derived via the phenylpropanoid/shikimic acid pathway (Dixon and Paiva, 1995). Such compounds were found to be more abundant in heads of FHB-resistant than in those of susceptible wheat varieties following *F. graminearum* inoculation (Hamzehzarghani *et al.*, 2005; Paranidharan *et al.*, 2008). Particularly, the phenylpropanoid pathway plays a key role in the resistance of wheat to *Fusarium* spp. and DON; indeed, enhanced transcription of the phenylalanine ammonia-lyase (*PAL*), which catalyses the first step in the phenylpropanoid synthesis, has been associated to FHB resistance (Steiner *et al.*, 2009). The up-regulation of pathogenesis-related proteins (*PR*) is also higher in FHB-resistant cultivars, such as Sumai3, than in susceptible ones (Makandar *et al.*, 2006). The ability of cereals to retard or even stop the spread of *Fusarium* pathogens within head tissues and thus to display some degree of systemic resistance has been classified as type II FHB resistance (Schroeder and Christensen, 1963). In particular, type II resistance inhibits the fungal movement from spikelet to spikelet via colonization of the rachis. Many factors are associated to type II resistance, such as physical and chemical barriers previously described, induced chemical defence and detoxification of DON. Anatomical features are also associated to type II resistance, such as smaller diameter vessels, denser vascular bundles in the rachis, stronger thickened cortical sclerenchyma and cell walls, and shorter internodes in the upper part of the rachis joints (Ribichich *et al.*, 2000; Walter *et al.*, 2010; Gorash *et al.*, 2020). Different Quantitative Trait Loci (QTLs) contributing to type I and II resistance have been cloned in the wheat germplasm. *Fhb1* is the major QTL for type II resistance and has been localized in the distal

segment of chromosome 3BS in the genome of Sumai3 (bread wheat) (Cuthbert *et al.*, 2006). By now *Fhb1* is the most extensively studied, best validated and the most widely deployed FHB resistance QTL in wheat breeding programmes worldwide (McMullen *et al.*, 2012a). Several independent research team reported on the cloning of *Fhb1* with contradictory results regarding the causative gene. Rawat *et al.* (2016) identified a pore-forming toxin-like gene, where Su *et al.* (2019) and Li *et al.* (2019) concluded that the identity of the gene is a histidine-rich calcium-binding protein, but they disagree on its mode of action. In particular, Su *et al.* (2019) state that the wild type allele causes susceptibility and the resistance allele in Sumai3 results from a deletion embracing the start codon in the gene, while Li *et al.* (2019) report that the allele confers resistance in an active manner. However, further efforts are needed to clarify the contradictory results revealed by the three papers reporting the *Fhb1* gene isolation (Lagudah and Krattinger, 2019). *Fhb2* and *Qfhs.ndsu-3AS* are other QTLs conferring type II resistant and have been mapped in a Sumai3-derived line (BW278) and in wild emmer wheat, respectively (Cuthbert *et al.*, 2007; Zhu *et al.*, 2016). The wheat cultivar Wangshuibai is an important source of type I resistance and carries two major QTLs responsible for this trait: *Qfhi.nau-4b* and *Qfhi.nau-5a* designated as *Fhb4* and *Fhb5*, respectively (Xue *et al.*, 2010, 2011). *Qfhs.ifa-5A* has been identified in the CM82036 wheat line conferring type I resistance (Buerstmayr *et al.*, 2018; Steiner *et al.*, 2019).

Type I and II resistances are the most widely studied and described; nevertheless, Miller *et al.* (1985) proposed also type III resistance referring to the variations of DON accumulation in resistant and susceptible cultivars, which could result from the differential capacities of some lines to degrade DON (Miller and Arnison, 1986). Additionally, some cultivars have significant differences in kernel infection rate and yield reactions even at almost the same levels of head blight. Therefore, Mesterházy (1995) proposed type IV resistance for lower kernel infection rate and type V resistance for host tolerance indicated as preservation of grain yield.

1.3.2 Role of stomatal conductance in response to FHB

Stomata are specialized epidermal structure essential for plant survival and productivity, consisting of two guard cells around a pore. Stomata act in gas exchange, mainly CO₂ and O₂, which is necessary for optimal photosynthesis and which restrict water loss by modulating the transpiration level. The mode of action of stomata depends on the integration of environmental and intracellular signals. Indeed, CO₂ concentration, biotic and abiotic stresses, plant hormones can modulate stomatal movements. As an example, for plants that encounter dehydration stress or the presence of a pathogen, the most essential factor is the ability of stomata to close and thus prevent excess water loss or the invasion by pathogens. The opening and closing are achieved by the swelling and shrinking of the guard cells, which is driven by ion exchange, cytoskeleton reorganization and metabolite production,

modulation of gene expression and post-translational modification of proteins (Kim *et al.*, 2010; Daszkowska-Golec and Szarejko, 2013; Devireddy *et al.*, 2020; Gupta *et al.*, 2020; Nunes *et al.*, 2020). Stomatal closure, which is the earliest plant response to many stresses, is regulated by a complex network of signalling pathways (the genetic regulation is described in deep in Chapter 3), in which the major player is abscisic acid (ABA), which acts in concert with jasmonate (JA), ethylene, auxins and cytokinins (Nemhauser *et al.*, 2006; Huang *et al.*, 2008). ABA and JA are positive regulators of stomatal closure, while auxin and cytokinins are positive regulators of stomatal opening, while the mode of action of ethylene is still ambiguous (Nemhauser *et al.*, 2006; Huang *et al.*, 2008; Gietler *et al.*, 2020).

A wide range of pathogens takes advantages of stomata as natural openings on the tissue surface to gain access to the interior. Some pathogenic fungi possess the ability to directly penetrate the surface, but others are specialised in using stomata as an entry route (Melotto *et al.*, 2006; Grimmer *et al.*, 2012). Additionally, some fungi and oomycetes utilise stomata as a means of dispersing spores after successful colonisation of the leaf (Schulze-Lefert and Robatzek, 2006; Grimmer *et al.*, 2012). *Fuarium* spp. penetrates into the host both directly and indirectly (Kang and Buchenauer, 2000), but the role of stomatal movements in wheat in response to FHB as a possible mechanism of resistance is still unknown. Moreover, besides its well-established role in physiological processes and acclimation to abiotic stresses, the role of ABA as a regulator of plant biotic-resistance is ambiguous. In most of the described cases, ABA acts as a disease susceptibility factor in wheat in relation to pathogenic fungi (Asselbergh *et al.*, 2008), but deviations from that observations have been observed (Shamanin *et al.*, 2019). The influence of ABA on the resistance of cereals to fungus is dependent on a few factors, such as the intensity of increasing ABA, the type of pathogens, the source of ABA (endogenous or exogenous), or the method of application; however, it appears that ABA may influence the pathogen penetration and spread, as well as the expression of genes encoding proteins associated with plant response to biotic stresses (Gietler *et al.*, 2020). Several authors presented some data about the role of ABA during the interaction between wheat and *F. graminearum*. Qi *et al.* (2016) observed that inoculation of heads of a susceptible cultivar of *T. aestivum* with *F. graminearum* significantly increased the ABA levels. They also observed that *F. graminearum* is also able to synthesize low amounts of ABA. Therefore, these results suggest that ABA can act as an effector during pathogenesis. The authors also observed the effects of exogenous ABA application on FHB progression in wheat plants and they found that ABA treatments significantly increased the development of FHB symptoms. Additionally, another research (Buhrow *et al.*, 2016) showed that susceptible and resistant wheat cultivars have different responses regarding ABA. An increased amount of ABA was found in the spikes of the FHB-resistant cultivar after *F. graminearum* infection,

while ABA catabolites were found to be increased in the FHB-susceptible cultivar, suggesting that ABA may play a role in FHB resistance. On the other hand, the application of ABA enhanced the rate of FHB symptoms, especially from symptomatic tissues to neighbouring asymptomatic ones. Another recent study (Buhrow *et al.*, 2020) evaluated the transcriptomic responses of an FHB-susceptible wheat cultivar upon treatment with ABA and *F. graminearum*. The *F. graminearum* inoculation induced the expression of an ABA-biosynthetic cytochrome P450, while ABA treatment elicited 32% of the wheat differentially expressed genes common to those of the *F. graminearum* challenged samples. The elicited genes are involved in defence through phytohormone signalling and polyphenolic metabolism. Based on these results, a conclusion can be formulated that exogenous ABA enhances the susceptibility of wheat to FHB, while the molecular mechanisms of endogenous ABA may modulate the defence response against FHB.

1.4 Detection of FHB

After observing disease symptoms on plants, the presence of the pathogen is verified using disease detection techniques, which can be broadly classified into direct and indirect methods (Figure 1.9).

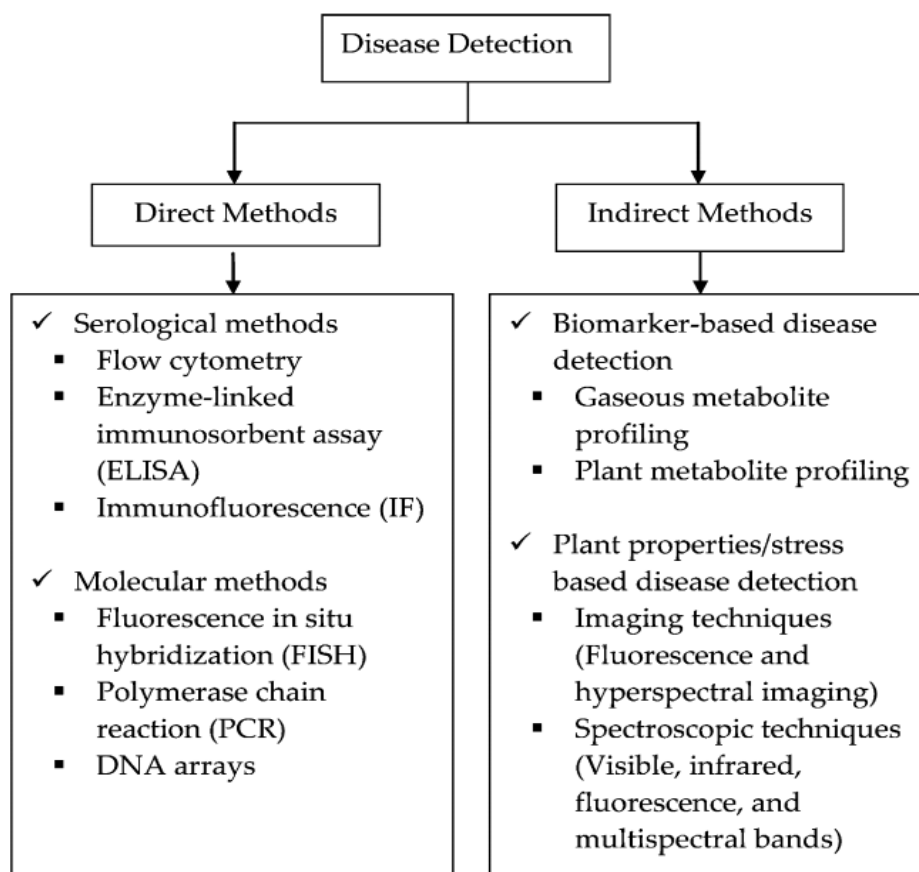


Figure 1.9 Methods of plant disease detection (Sankaran *et al.*, 2010).

The trend in the European Union for detecting plant pathogens, outlined in the European and Mediterranean Plant Protection Organization (EPPO) protocols, integrates phenotypic, serological,

and molecular techniques. Although serological and nucleic acid-based techniques are sensitive, accurate, and effective, they require detailed sampling procedures, expensive infrastructures, and are time-consuming. An advanced plant disease detection technique must provide rapid, accurate, and reliable detection in the early stages of disease for economic, production, and agricultural benefits (Sankaran *et al.*, 2010). To develop an innovative detection method, the ground-based disease detection could be possibly integrated with an automated agricultural vehicle. Ground-based disease detection methods include both field-based and laboratory-based experiments. The laboratory-based experiments provide a strong background knowledge for the field-based applications, such as spectroscopic and imaging techniques, which could be easily integrated with an unmanned vehicle for a fast, reliable, and real-time disease monitoring for control and management. The early detection of plant diseases (before the onset of symptoms) could be a valuable source of information for executing proper management strategies to prevent the spread of diseases and to apply pesticides in a cost-effective manner (Sankaran *et al.*, 2010; Martinelli *et al.*, 2015).

1.4.1 Molecular techniques

During the last thirty years, molecular techniques for plant disease detection have been well established and the sensitivity of such techniques refers to the minimum amount of microorganism that can be detected in the sample. The commonly used molecular techniques for disease detection are based on the polymerase chain reaction (PCR) and its variants (nested PCR, cooperative PCR, multiplex PCR, real-time PCR and loop-mediated isothermal amplification), where the genetic material of the disease-causing microorganism is extracted, purified, and amplified before performing its visualisation. The presence of the targeted amplicon confirms the presence of the pathogen. All of these methods can overcome uncertain diagnosis or pathogen taxonomy, enabling a rapid and accurate detection and quantification of pathogens, and the potential to detect unculturable pathogens. Some of the limitations of the molecular techniques are that they are time-consuming and labour-intensive, and require an elaborate procedure, especially during the sample preparation (collection and extraction) to obtain reliable and accurate results. In addition, these techniques require consumable reagents that must be tailored to detect each specific pathogen (sequence-specific primers or probes) (Sankaran *et al.*, 2010; Martinelli *et al.*, 2015). Molecular protocols for the detection of the causing agents of *Fusarium* head blight are well established, as reported by Mulè *et al.* (2005), Ur Rahman *et al.* (2020), Villafana *et al.* (2020). The translation elongation factor 1-alpha (*TEF*) sequence, which encodes an essential part of the protein translation machinery, is highly useful for the detection and identification of *Fusarium* spp., because it is informative at the species level, with an excellent representation of type-B trichothecene toxin producers (Geiser *et al.*, 2004). Knowledge of the molecular genetic basis of the production of trichothecenes provides the information to develop

assays based on the toxin biosynthetic genes for the generic identification of species of even isolates. Specific primers pair and probes have been designed targeting the *Tri* cluster, especially the *tri4*, *tri5* and *tri6* genes, as these genes are present in all trichothecene-producing *Fusarium* spp. (Mulè *et al.*, 2005; Ur Rahman *et al.*, 2020; Villafana *et al.*, 2020).

1.4.2 Plant phenotyping

The genotype-to-phenotype gap is one of the most important problems in modern plant breeding (Houle *et al.*, 2010; Großkinsky *et al.*, 2015). Despite genomics research has provided much information about the genetic structure of various plant species, the data they generate far outstrip our current capacity for plant phenotyping (Yang *et al.*, 2014). Conventional plant phenotyping tools are based on manual measurement of selected traits from a small sample of plants; for such reason, they have very limited throughput and prevent comprehensive analysis of traits within a single plant and across cultivars. The so-called phenotyping bottleneck (Furbank and Tester, 2011) limits the research on how expressed phenotypes correlate with underlying genetic factors and environmental conditions (Großkinsky *et al.*, 2015). With increasing options in image capture and open-source analysis tools, the field of high-throughput plant phenomics is exponentially growing, but have also revealed the necessary improvements of hardware, software, and community resources required to advance the field. A key improvement in high-throughput phenotyping platforms is the ability to non-destructively capture plant traits. This advance permits time-series measurements in order to follow the progression of growth and stress on individual plants. Eliminating destructive measurements also increases the experimental capacity for genotypes, treatments, and biological replicates by reducing the required replicate sampling sets. The main goal of phenomics is to automate the acquisition of plant images that allow to quantify genotype by phenotype by environment interactions. Plant imaging and analysis are employed to measure the physiological, growth, development, and other phenotypic properties of plants through automated processes. Digital camera technology has become relatively inexpensive and ubiquitous, leading to a recent surge in high-throughput phenotyping systems that utilize plant imaging to capture data (Fiorani and Schurr, 2013; Li *et al.*, 2014; Fahlgren *et al.*, 2015; Mahlein, 2016; Ubbens and Stavness, 2017; Qiu *et al.*, 2018; Pieruschka and Schurr, 2019).

The aim of plant imaging is to quantitatively measure a phenotype through the interaction between light and plants such as reflected, absorbed, or transmitted photons. Each component of plant cells and tissues has wavelength-specific absorbance, reflectance, and transmittance properties (Li *et al.*, 2014). A stressed plant reacts with protection mechanisms that lead to suboptimal growth which shows up as changes in parameters such as leaf area index, chlorophyll content, or surface temperature; thus, producing a spectral signature that is different from the signature of healthy,

unstresses vegetation. When plants are exposed to pathogens, they activate defence responses whose molecular mechanisms are very complex. Plants react to a certain pathogen with changes in physiological mechanism such as the reduction of the photosynthesis rate, which induces an increase of fluorescence and heat emission (Figure 1.10) (West *et al.*, 2003).

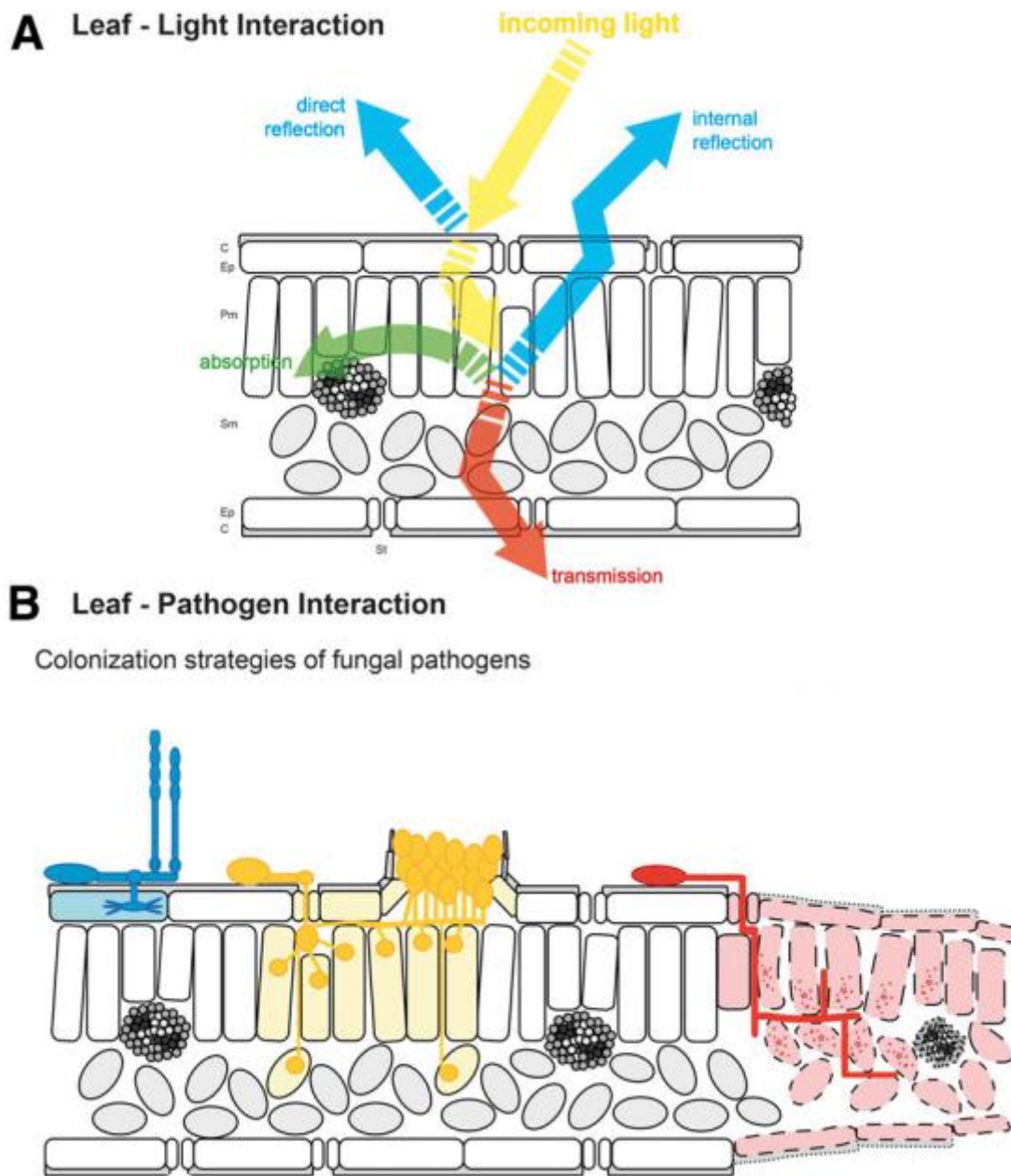


Figure 1.10 (a) The interaction of leaf tissue with light depends on structural and leaf chemical properties. (b) During pathogenesis, fungal pathogens influence leaf structural and chemical properties, and by this the leaf optics are altered (Mahlein, 2016).

For such that reasons, spectroscopic and imaging techniques are unique monitoring methods that have been used as proximal sensors to detect diseases in several plant hosts. Such techniques could be integrated with an autonomous agricultural vehicle that can provide information on disease detection

at early stages to control the spread of the pathogen. Pathogens cause a reduction of leaf plant chlorophyll content due to necrotic or chlorotic lesions that increase reflectance in the visible range and cause a shift of the red-edge position in the spectrum (Sankaran *et al.*, 2010).

Information about plant's metabolic status can be obtained by the artificial excitation of the plant photosystems and observation of the relevant responses. As an example, fluorescence spectroscopy refers to the measurement of emitted fluorescence from the object of interest after excitation with a beam of light (usually ultraviolet spectra). Particularly, chlorophyll fluorescence in about 650-800 nm range has been used to monitor diseases in plants (Li *et al.*, 2014). Irradiating the chloroplast with blue light will result in some re-emission of the absorbed light by the chlorophyll. The proportion of re-emission light compared with the irradiation is variable and depends on the plant's ability to metabolise the harvested light. The re-emitted light is the fluorescence and may be used to estimate the plant's efficiency of photo-assimilation, non-photochemical quenching and other physiological plant parameters. During disease infection, metabolic changes occurring from photosynthesis to respiration and nutrient flow derivation are the first to be affected. Fluorescence imaging can estimate photosynthesis to monitor the effects of plant pathogens and diagnose early stress responses to biotic factors before a decline in growth can be measured (Balachandran *et al.*, 1997; Chaerle *et al.*, 2007a, 2007b; Baker, 2008). Combining fluorescence imaging with image analysis techniques has been useful for discrimination and quantification of fungal infections (Konanz *et al.*, 2014). One disadvantage of current chlorophyll fluorescence imaging systems is that the preparation of the plants has to follow a strict protocol, and thus it is difficult to implement in normal agricultural greenhouses or field environments (Li *et al.*, 2014; Mahlein, 2016).

The simplest imaging method consists of the construction of growth profiles of the canopy using cameras sensitive to the visible range (400-700 nm) of the electromagnetic spectrum (Tackenberg, 2007; Hartmann *et al.*, 2011). The projected canopy area is extracted following image pre-processing and segmentation in the RGB (red, green, blue) space. A visible image is based on digital images and is intended to mimic human perception to provide information or input to systems that need data for plant phenotyping applications to trait-based physiological breeding. Typically, the raw data of an image is presented in spatial matrices of intensity values corresponding to photon fluxes in the red (~600 nm), green (~550 nm), and blue (~450 nm) spectral bands of visible light. Visible images have been widely used in plant science for its low cost and its ease of operation and maintenance. The visible imaging of plant growth over a period can be used to estimate the sum of stress response mechanisms and offers the opportunity to tease apart many of these responses. In the field, visible images provide information on the canopy cover and canopy colour (Casadesús *et al.*, 2007; Li *et al.*, 2014). Several research groups have used pattern recognition methods and machine learning to detect

and to identify plant diseases from RGB images (Camargo and Smith, 2009; Neumann *et al.*, 2014). Special attention must be given to the image acquisition step. Uniform focus, sharpness, and illumination are crucial for high accuracy and reliable results from automated image analysis. The most important principle for success is a sound standardized imaging procedure that yields repeatable results. Problems with RGB imaging are most commonly caused by the overlapping of leaves and by background soil noise. Consequently, new assessments should be performed on a case-by-case basis during phenology for the developmental stage of interest (Mahlein, 2016).

Measurements of leaf and canopy temperature by thermal imaging (3-14 μm spectral range) have been introduced in the lab and in the field to evaluate leaf water status (Jones *et al.*, 2009; Munns *et al.*, 2010). Thermal imaging allows for the visualization of infrared radiation, indicating an object as the temperature across the object's surface. Thermal imaging is used to measure leaf surface temperatures to study plant water relations, and specifically for stomatal conductance, because a major determinant of the leaf temperature is the rate of evaporation or transpiration from a leaf. Biotic stresses often result in decreased rates of photosynthesis and transpiration and the remote sensing of the leaf temperature by thermal imaging can be a reliable way to detect changes in the physiological status of plants in response to different biotic stresses (Nilsson, 1995; Chaerle and Van Der Straeten, 2000). Whereas many foliar pathogens, such as leaf spots or rusts, induce local changes, systemic infections (e.g., *Fusarium* spp.) often influences the transpiration rate and the water flow of the entire plant or organs. Thermal cameras offer several benefits, such as spatial resolution; however, it is often subjected to environmental factors such as environmental temperature, sunlight, rainfall, or wind speed (Mahlein, 2016).

The increasing availability of small, inexpensive, high resolution spatial and spectral sensors has enhanced the operational capabilities of UAV-mounted spectral sensors for crop disease monitoring at the farm scale. Methods are being improved through integrating different technologies, such as coupling spectroscopy and fluorescence data (Martinelli *et al.*, 2015). The availability of UAV-mounted sensors and the integration of spectroscopy, fluorescence, and potentially thermal imaging techniques, used in combination with data from non-phenotyping-based methods, could constitute an interesting and fruitful approach to study plant diseases in the near future. However, a key requirement for image-based phenotyping tools is to automatically transform those pictures into reliable and accurate phenotypic measurements. Most of the sensors do not measure plant physiological parameters directly but record a spectrum that is the sum of reflectance attributed to various plant characteristics and conditions (Fahlgren *et al.*, 2015; Ubbens and Stavness, 2017; Pieruschka and Schurr, 2019). Additionally, the current quality and quantity of data available from plant sensors has dramatically increased. These data require the use of advanced methods of handling,

analysis, and interpretation. Task-specific algorithms have to be developed to effectively handle these data. Supervised and unsupervised classification methods and clustering from remote sensing and data mining can be effective for detection, identification, and quantification of plant diseases from sensor data (Camargo and Smith, 2009; Martinelli *et al.*, 2015; Qiu *et al.*, 2018). Principal component analysis has been successfully applied to monitor processes during pathogenesis in wheat infected with *F. graminearum* (Bauriegel *et al.*, 2011; Bauriegel and Herppich, 2014).

Plant phenotyping aims to realise real-time, robust mapping systems for crop, soil, and environment variables to facilitate a management decision. By way of contrast, traditional agricultural management practices assume that parameters in crop fields are homogeneous, thereby leading to pesticide application and crop health management that is not always related to the existing disease management situation (Mahlein *et al.*, 2018). Since the occurrence of plant disease depends on specific environmental factors and that diseases often exhibit a heterogeneous distribution in fields, plant phenotyping by optical sensing is useful to identify primary disease foci and areas differing in disease severity in fields (Mahlein *et al.*, 2019). For such reasons, these techniques, in combination with advanced methods of data analysis, can be used for targeted pest management programs in sustainable crop production. Site-specific and targeted applications of pesticides according to precision crop protection strategies result in potential reduction in pesticide use and can thus reduce the economic expense and ecological impact in agricultural crop production systems (Berdugo *et al.*, 2014; Mahlein, 2016). With the case of Fusarium head blight, it should be possible to map affected locations of fields to allow segregation of more severely affected parts of fields from areas that are relatively unaffected. This can occur due to slight differences in crop growth stage in different parts of fields or nearby entire fields, causing some of the crop to escape disease, while other parts can be severely affected. In addition, where toxigenic *Fusarium* spp. are the main pathogen, optical sensing could allow segregation of harvesting severely-affected areas of fields from less-affected areas. It is also possible to use optical sensing to separate healthy and infected harvested grain (Arnal Barbedo, 2013; Al Masri *et al.*, 2017).

1.5 Management of FHB

There are several strategies for management of FHB in small grain production. Their effectiveness will vary depending on environmental conditions during the growing season, especially before, during, and after flowering, and cultivar susceptibility to FHB. They include cultural practices, including irrigation management, that reduce FHB intensity, planting resistant or tolerant cultivars, chemical control, biological control, disease forecasting, and harvesting strategies that minimize loss of grain quality and reduce DON. Integration of two or more management practices is more effective and therefore recommended (Wegulo *et al.*, 2015).

1.5.1 Chemical control by synthetic fungicides

Fungicide application is one of the strategies available for management of FHB and DON (Wegulo *et al.*, 2015). Because *F. graminearum* can establish itself in wheat heads both inter- and intracellularly, for a fungicide to be effective it needs to be persistent while being able to translocate within the tissue of the head (Brown *et al.*, 2010). Fungicides developed for FHB regulation should reduce the level of disease in the field as well as prevent the accumulation of DON (Mesterházy *et al.*, 2003). Ideally, fungicides should reduce field infection to less than 5% and FDKs should be minimal or absent. FHB incidence and DON accumulation can be radically decreased in field with proper management and timely application of fungicides (Lehoczki-Krsjak *et al.*, 2010). However, fungicide efficiency can be extremely variable and often inconsistent for controlling FHB in infected wheat (Lehoczki-Krsjak *et al.*, 2010; Mesterházy *et al.*, 2011). The timing of fungicide application is crucial for the control of FHB. The current classes of fungicides do not translocate quickly, thus it is essential that all the heads should have emerged before the fungicide application (Beyer *et al.*, 2006). Some farmers have difficulty with the timing of fungicide application because favourable weather for FHB infection frequently coincides with conditions that are unfavourable for ground spraying (Mesterházy *et al.*, 2003). Triazoles, a class of chemical fungicides in the demethylation inhibitor (DMI) group that obstructs sterol biosynthesis, such as tebuconazole, metconazole and prothioconazole, and benzimidazole fungicides are the most effective fungicides to decrease FHB infection and mycotoxin accumulation (Edwards *et al.*, 2001; Pirgozliev *et al.*, 2002; Paul *et al.*, 2008; Shah *et al.*, 2018). Strobilurin fungicides are generally ineffective in controlling FHB; application increases DON content as compared to nontreated controls (Pirgozliev *et al.*, 2002). Another fungicide, carbendazim, considerably decreased Fusarium disease occurrence as well as mycotoxin accumulation in grain (Shah *et al.*, 2018). The discovery of a tebuconazole-resistant isolate of *F. graminearum* in the state of New York (the first documentation of *F. graminearum* resistance to a DMI fungicide in the Americas) (Spolti *et al.*, 2014) and of a benzimidazole-resistant strain in China (Chen and Zhou, 2009) indicates the potential exists for fungicide resistance occurrence in the pathogen populations and poses a new challenge in the management of FHB and DON using conventional fungicides. This discovery highlights the need to develop fungicides that are effective in reducing FHB and DON, but with modes of action different from that of DMI fungicides. The new chemistries can then be used in rotation with DMI fungicides to mitigate the development of fungicide resistance in populations of *F. graminearum* (Bai and Shaner, 2004; McMullen *et al.*, 2012b). Fungicides not only affect the target organism, but the whole environment, including atmosphere, soil, groundwater, and surface water by flow, leaching, and pulverisation processes, thus contaminating the ecosystem (Taha *et al.*, 2014; Nsibande and Forbes, 2016). The presence of

fungicides in the ecosystems have adverse effects which vary with the contaminant concentration, amount, and exposure time (Margni *et al.*, 2002; Rice *et al.*, 2007). It is estimated that 10% of the chemical pesticides applied to soil and plants reach non-target areas (Schulz, 2004). Thus, the fauna and flora are continuously exposed to these substances, which are ingested and accumulated in the trophic chain and they may cause severe human and animal health problems (Srivastava *et al.*, 2019). Consequently, they may present detrimental effects to humans and can cause severe health problems such as cancer, infertility, malformation, and chromosomal changes as a consequence of oxidative stress (Sabarwal *et al.*, 2018). As an example, tebuconazole, one of the most common active compounds presents in FHB fungicides, has the potential to act as an endocrine disruptor (Taxvig *et al.*, 2008). In fact, severe restrictions have been posed on the use of compounds whose characteristics are persistence, bioaccumulation, toxicity, and long-range environmental transport (Mekonen *et al.*, 2016; de Souza *et al.*, 2020). For those reasons, these contaminants should be removed from the environment, especially from water.

1.5.2 Natural-derived fungicides with special reference to Chitosan

Natural pesticides are substances of natural origin (plant or animal) that limit the development of pathogens and insects. Natural bioactive compounds that have an impact on fungal pathogens are called natural fungicides. The action of these organic compounds is not specific, and their effect on pathogens is comprehensive (Balestra *et al.*, 1999; Quattrucci *et al.*, 2013; Canzoniere *et al.*, 2019; Fortunati *et al.*, 2019). Natural bioactive compounds used in plant protection kills fungi (fungicidal effect) or limit their development (fungistatic effect), as well as involve plant defence reactions (elicitors) (Jamiołkowska and Kopacki, 2020). Direct action of these compounds is based on inhibition of fungal sporulation, germination of spores, and reduction of hyphae growth. Natural secondary metabolites produced by plants under the influence of elicitors also have a protective role of plants in relation to pathogens. As a result of the indirect action of plant extracts in plant cells, there is the synthesis of phytoalexins, the accumulation of callose and the lignification of cell walls, which protect plants against pathogens (Jamiołkowska and Kopacki, 2020). The main classes of antimicrobial compound belong to alkaloids, flavonoids, terpenoids, phenolics, glycosides, tannins and fatty acids (Shuping and Eloff, 2017).

Recently, chitosan has been one of the most preferred biopolymers due to its biocompatibility, antioxidant, anticancer, biodegradability, antimicrobial, and non-toxic properties as well as being an economical material, produced from waste resources such as seafood shells (Dash *et al.*, 2011; Sharif *et al.*, 2018). Structurally, chitosan is a linear polymer, composed of two sub-units as D-glucosamine and N-acetyl-D-glucosamine, linked with each other through 1,4-glycosidic bonds (Rinaudo, 2006) (Figure 1.11).

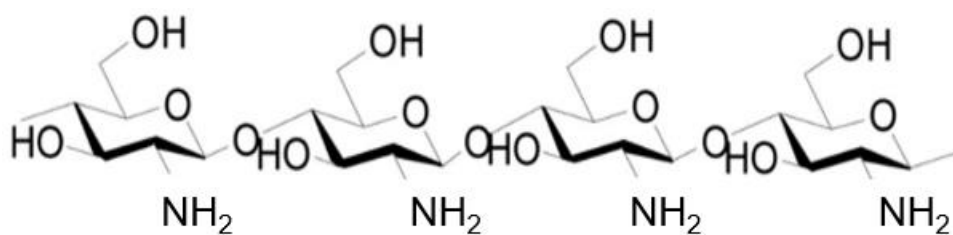


Figure 1.11 Chemical structure of chitosan (Modified from Ifuku, 2014).

of deacetylation and molecular weight are crucial parameters for chitosan bioactivity. Chitosan usually has a degree of acetylation of less than 10% (Ravi Kumar, 2000). Numbers of *N*-acetyl glucosamine and glucosamine subunits in the molecule define chitosan molecular weight (Mw) (Ravi Kumar, 2000). Large-Mid (70–100 kDa) Mw chitosan is soluble in weak acid solutions. Only chitosan oligosaccharides (<5000 Da) are water-soluble. Protonation of chitosan amino groups generates positive charges. Both positive charge and amenability to structural modifications confer chitosan numerous biological properties. Since the last decade, chitosan research is increasing due to its significant diverse uses in several fields of life (Rinaudo, 2006; Sharif *et al.*, 2018; Lopez-Moya *et al.*, 2019). On a commercial scale, chitin is extracted by following a classic, well-known acid-base method. The samples are demineralized using acids followed by a deproteinization with a base, respectively. However, the functionality of chitin increases when it is converted into chitosan. The presence of amine groups on chitosan makes it prone to structural modifications resulting in a functional derivative of chitin (Shamov *et al.*, 2002; Sharif *et al.*, 2018; Lopez-Moya *et al.*, 2019). Besides agriculture, chitosan has a large set of applications, such as in the food, cosmetic, textile, and biomedical industries (Cervera *et al.*, 2011; Akyuz *et al.*, 2017; Kaya *et al.*, 2017). In plants, chitosan is largely used to mimic biotic and abiotic stresses. The first study of using chitosan as an antipathogen in plants was reported by Allan and Hadwiger (1979), where they demonstrated the fungicidal effects of chitosan on different cell wall compositions of fungi. The improvement of the defence system after applying chitin and chitosan, both in monocotyledon and dicotyledons is the centre of addressing this biopolymer in multi-research area (Barber *et al.*, 1989). Chitosan has been a bio-fungicide, bio-bactericide, and bio-virucide, which spurs plant defence system against pathogens, thus inducing the immune system of plants, fruits, and vegetables (Allan and Hadwiger, 1979; Atabekov *et al.*, 1994; Li *et al.*, 2013; Kaya *et al.*, 2017).

Several studies have investigated the role of chitosan in the sensitivity of fungi (Palma-Guerrero *et al.*, 2009, 2010). The membranes of chitosan-sensitive fungi are highly-fluid (rich in polyunsaturated free fatty acid such as linolenic acid). On the contrary, chitosan-resistant fungi have low-fluidity membranes (enriched on saturated free fatty acids such as palmitic or stearic acid) (Palma-Guerrero *et al.*, 2010). Chitosan permeabilises the membrane of sensitive fungi, causing the trigger in

intracellular production of reactive oxygen species (ROS) and cell death (Lopez-Moya *et al.*, 2015). RNAseq data and gene ontology analysis revealed oxidoreductase activity, plasma membrane and transport as main proteins induced by chitosan (Lopez-Moya *et al.*, 2016). Therefore, chitosan causes an intracellular ROS burst which starts oxidizing the free fatty acids on the cell membrane. Increased membrane oxidation finally leads to full plasma membrane permeabilization and is perhaps responsible for the antifungal effect of chitosan (Lopez-Moya *et al.*, 2019).

This polymer also stimulates seed germination and growth of seedlings of ornamental plants (Ohta *et al.*, 1999; Kananont *et al.*, 2010). Chitosan is also an elicitor of plant defenses in economically important crops (Barber *et al.*, 1989; Gunupuru *et al.*, 2019). Chitin is a key structural component of fungal cell walls. Plant chitinases and glucanases release chitin and β -glucan oligomers from cell walls of fungal pathogens. These oligomers (MAMPs) are recognised by plant membrane PRRs triggering a PTI responses, including ROS production and alkalinisation, callose deposition and overexpression of PR proteins (Vander *et al.*, 1998; Bravo *et al.*, 2003; Jones and Dangl, 2006; Van Loon *et al.*, 2006; Beatrice *et al.*, 2017).

Interestingly, in agriculture chitosan has been employed also as a nanoparticle as growth enhancers and potent antimicrobial agents against pathogenic fungi and bacteria (Kong *et al.*, 2010). Alternatively, they can also act as nanocarriers for existing agrochemicals, which hence are referred to as chitosan-based agro-nanochemicals (Campos *et al.*, 2014; Kashyap *et al.*, 2015; Hernández-Téllez *et al.*, 2016). The nanocarrier system enables the agriculturally active ingredient to be encapsulated via ionic or covalent inter/intramolecular bonds or entrapped in a polymeric matrix of chitosan to develop an effective nano-delivery system formulation (Kashyap *et al.*, 2015; Maluin and Hussein, 2020).

1.6 References

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2. Different inoculation methods affect components of Fusarium head blight resistance in wheat

Sara Francesconi^{1*}, Angelo Mazzaglia¹, Giorgio Mariano Balestra^{1*}

¹*Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia, Via San Camillo de Lellis, 01100, Viterbo, Italy*

Corresponding authors: Sara Francesconi, francesconi.s@unitus.it; Giorgio Mariano Balestra, balestra@unitus.it

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Abstract: Fusarium head blight (FHB) is one of the most important fungal diseases of cereals, and *Fusarium graminearum* is the most damaging FHB pathogen. Infection is linked to host anthesis, and symptoms include necrosis, bleaching of heads and shrivelled kernels. No fully effective fungicides are available for FHB control, so utilization of other mitigation measures, such as the use of resistant cultivars, is necessary for FHB management. Resistance to FHB is quantitative and multigenic and five components of resistance (type I, II, III, IV and V) have been described. The main problem in testing for FHB resistance is reproducibility, so necessary tools for breeding resistant cultivars are reliable inoculation methods and the testing for different FHB-associated characteristics. We screened three Italian wheat genotypes, Palesio, Claudio and Marco Aurelio, for type I, type II, and, in part, for type V resistances, with both phenotypic (% of disease incidence and severity, thousand kernel weight (TKW) and molecular (quantification of fungal biomass with Real-Time qPCR) approaches, using spray and point inoculation protocols. Results underlined that Palesio bread wheat showed type I tolerance to initial infection, and Marco Aurelio durum wheat showed an important type II resistance to disease spread when spray-inoculated (27% disease severity). Quantification of fungal biomass showed that differentiation among the three wheat cultivars was best visualized when spray inoculation was used. TKW data showed that % yield loss was greater after point inoculations, except in Marco Aurelio, which was not affected by inoculation method. This study has highlighted the

complexity of testing for FHB resistance, and demonstrated the necessity to use as many resistance screening protocols as possible.

Keywords: *Fusarium graminearum*, *Triticum aestivum*, *Triticum durum*, spray inoculation, point inoculation.

2.1 Introduction

Wheat is one of the most cultivated crops, followed by rice and maize. Modern wheat cultivars include two species: hexaploid bread wheat, *Triticum aestivum* L. (AABBDD), and tetraploid, durum-type wheat, *T. turgidum* subsp. *durum* (Desfontaines) Husnache (AABB) used for pasta and low-rising bread (Doebley *et al.*, 2006; Dubcovsky and Dvorak, 2007; Charmet, 2011; Feldman and Levy, 2012). Bread wheat accounts for 95% of world wheat production, while durum wheat is the remaining 5%. Wheat accounts for more than 20% of total human food calories. Wheat crops are extensively grown, on 17% of all crop areas, and is the staple food for 40% of the world's population, mainly in Europe, North America and the western and northern parts of Asia (Peng *et al.*, 2011).

Fusarium head blight (FHB) is one of the most important fungal diseases of grain crops, including wheat, barley and maize (Goswami and Kistler, 2004; Osborne and Stein, 2007; van der Lee *et al.*, 2015). FHB is caused by the *Fusarium graminearum* Species Complex (FGSC), which comprises 16 different species. These produce various mycotoxins, including deoxynivalenol (DON) and zearalenone (ZEA), which are toxic to humans and animals (Desjardins and Proctor, 2007; Foroud and Eudes, 2009; Walter *et al.*, 2010; Darwish *et al.*, 2014).

During the past decade numerous FHB epidemics have been reported worldwide, causing significant economic losses (millions to billions of \$ US) (McMullen *et al.*, 2012; Wegulo *et al.*, 2015). The spectrum of *Fusarium* spp. causing FHB on wheat varies at the regional level depending on weather conditions, especially during host anthesis. Fungal growth is favoured by high temperatures and humidity, and abundant rain, during the growing season which favour pathogen infection, and can lead to significant yield losses. Given the current global warming associated with increased temperatures, major epidemics of the *Fusarium* diseases are likely (Vaughan *et al.*, 2016; Khaledi *et al.*, 2017).

Fusarium graminearum Schwabe is the predominant FHB pathogen, but its infection biology is yet to be fully understood. Airborne spores are transported by rain and wind to host floral tissues, where, at anthesis, they proliferate and spread rapidly intracellularly throughout the host spikelets, down into the rachial nodes and ultimately up and down the rachides until FHB symptoms are clear, involving necrosis and bleaching of heads causing shrivelled kernels (Nelson *et al.*, 1994; Dweba *et al.*, 2017).

In Italy, FHB on wheat has occurred each year since 1995, at varying levels of incidence and severity depending on the year, the region and the wheat genotype involved. (Pancaldi *et al.*, 2010). The disease has been reported mostly in the Northern-Central regions of Italy, and there is evidence indicating that the prevalent FHB species have shifted from *F. culmorum* (W. G. Smith) Saccardo to *F. graminearum* and *F. poae* (Peck) Wollenweber (Shah *et al.*, 2005). FHB incidence and severity increase from the South to the North of Italy, and is closely related to the amounts of precipitation during wheat anthesis (Covarelli *et al.*, 2015). Regarding mycotoxin production, DON is the most frequently found in Italy, and, as for disease incidence, occurrence of this mycotoxin increases from Southern to Northern Italian regions (Aureli *et al.*, 2015). Since durum wheat is grown more widely than bread wheat in Italy, but also is more susceptible to FHB than bread wheat, mycotoxin accumulation in kernels is of particular concern as a food safety issue (Boutigny *et al.*, 2008).

Chemical control of FHB using appropriate effective fungicides and correct application methods and timing are feasible for reducing disease severity (Blandino *et al.*, 2012). However, no fully effective FHB fungicide is available (Haidukowski *et al.*, 2012), and the application window is very narrow, spanning just a few days around host anthesis (Mesterházy *et al.*, 2003). Therefore, while new and eco-sustainable plant protection strategies are being developed (Fortunati *et al.*, 2019), the utilization of resistant genotypes remains important, and is possibly the most effective strategy for FHB control (D'Mello *et al.*, 1999).

Resistance to FHB in wheat and other cereals has quantitative and multigenic characteristics (Zhu *et al.*, 1999; Gervais *et al.*, 2003; Massman *et al.*, 2011). It is a non-trivial task for plant breeders to develop FHB-resistant and productive wheat cultivars, since plant breeding requires two essential pre-conditions: availability of genetic resources carrying positive alleles for the trait of interest and reliable testing methods that allow breeders to identify the desired genotypes (Buerstmayr *et al.*, 2014; Steiner *et al.*, 2017). Two main components of resistance have been described: type I resistance operates against initial infections and type II against the spread of symptoms induced by pathogens within their hosts (Schroeder and Christensen, 1963). Furthermore, type I and type II resistances vary independently among genotypes (Schroeder and Christensen, 1963). Three other types of FHB resistances have been described, but these are still not well understood. type III resistance is the host plant's ability to degrade DON (Miller and Arnison, 1986), type IV is the host's ability to tolerate high DON concentrations (Wang and Miller, 1988), and type V is resistance to kernel infection, evaluated by analysing grain samples post-harvest for incidence of diseased kernels (Mesterházy, 1995). Gilbert and Tekauz (2000) distinguished between resistance (host ability to prevent infection) and tolerance (host ability to mitigate the infection, with low impacts on yield), and attributed type IV and V resistances as FHB tolerance.

The most important goal in FHB resistance breeding is that resistant varieties should develop low symptom severity and simultaneously low mycotoxin contamination (Bai *et al.*, 2001; Snijders, 2004; Wilde *et al.*, 2007). In the second half of the 20th Century, large numbers of varieties, breeding lines and germplasm accessions were evaluated for FHB resistance. Quantitative variation in FHB susceptibility was detected, but no genotype was immune (Miller and Arnison, 1986; Wang and Miller, 1988; Buerstmayr *et al.*, 1999). Durum wheat was also more susceptible than bread wheat, where almost no variation in resistance to FHB has been found within historic and current *T. durum*, with most lines being susceptible, even among large germplasm collections of several thousand lines (Otto *et al.*, 2002; Stack *et al.*, 2002; Ghavami *et al.*, 2011; Prat *et al.*, 2014).

One of the main problems in testing for FHB resistance is the lack of reproducibility of results (Groth *et al.*, 1999; McCallum and Tekauz, 2002; Geddes *et al.*, 2008). The chief goal is to measure differences in genetic resistance, taking into account non-genetic factors, which can lead to errors in the results. Under natural conditions, infection pressure is usually not uniform in time and space, while in FHB resistance screenings, infection is achieved by applying uniform inoculum pressure over time (at flowering) and space (in greenhouses) (Campbell and Lipps, 1998). A necessary tool for breeding resistant lines is a reliable inoculation method enabling accurate quantitative disease assessment. Further, since FHB resistance is a complex quantitative trait, a single and simple method for measuring FHB resistance is sometimes insufficient (Buerstmayr *et al.*, 2014).

The objectives of the present study were to screen for type I and type II resistances in three prominent Italian wheat cultivars, whose FHB responses were unclear, by using phenotyping and molecular tools to assess FHB incidence and severity. Real-Time qPCR (FHB type II resistance) and measurement of thousand kernel weight (TKW) (FHB type V resistance) were carried out to quantify the fungal biomass in wheat chaff and rachides, and to assess the impacts of *F. graminearum* infection on yield loss.

2.2 Materials and methods

2.2.1 Plant material and growth conditions

Italian wheat genotypes Palesio (bread wheat), and Marco Aurelio and Claudio (durum wheat) were grown in a greenhouse, following the protocol developed by Watson *et al.* (2018), with modifications. Seeds were surface sterilized with sodium hypochlorite (0.5% v/v) for 20 minutes and then rinsed twice for 5 minutes in sterile distilled water. Seeds were then germinated in the dark on paper imbibed with sterile distilled water for 15 days at 4°C to break dormancy, followed by 2 days at room temperature. Subsequently, seedlings were transferred to 40×20 cm pots (20 plants for each pot), filled with TYPical Brill soil (Brill, Germany), and were grown at 16°–20°C until boot stage, 20–24°C during anthesis, and 24–29°C until maturity. The plants were fertilised to avoid nitrogen deficit,

by providing ammonium nitrate at the following proportions and plant stages: 20% at sowing, 40% at tillering and 40% at heading.

2.2.2 Fungal material, inoculum preparation and infection techniques

The highly virulent and DON-producing isolate of *F. graminearum*, wild type 3824 (Mandalà *et al.*, 2019), was cultured at 21°C on potato dextrose agar (PDA) and on synthetic nutrient poor agar (SNA) (Urban *et al.*, 2002) to obtain macroconidia for inoculum preparation. To prepare inocula, after a minimum of 10 days on SNA, conidia were scraped with a glass rod after pipetting 1 mL of sterile distilled water onto the surface of each Petri dish. The resulting conidium suspension was recovered, and the concentration measured using a Thoma Chamber (0.100 mm depth and 0.0025 mm²). Inocula were prepared in sterile distilled water supplemented with 0.05% (v/v) of Tween-20. Two inoculum methods and several conidium concentrations were tested: spray inoculation to evaluate type I FHB resistance, and point inoculation to evaluate type II resistance, and 500, 1500 or 2500 conidia per spike (c/s), to assess dependent disease pressure responses in symptom development. Conidium concentrations were prepared following the protocol of Stein *et al.* (2009). 10 µL of conidium suspension was applied to the central spike floret of each plant for point inoculations (using a laboratory pipette), or 100 µL of conidium suspension was applied to plants (using a manual nasal sprayer) for spray inoculations. Thus, 5×10⁴, 15×10⁴ and 25×10⁴ conidia/mL concentrations were prepared for point inoculations, while 5×10³, 15×10³ and 25×10³ conidia/mL were prepared for spray inoculation, in order to inoculate each test plant with 500, 1500 or 2500 conidia for each spike using the two both inoculation techniques. The spikes on the main culms (one spike per plant) were inoculated during anthesis (Zadok stage 69: Zadoks *et al.*, 1974), at greenhouse temperatures ranging from 20–24°C. Subsequently, the spikes were sprayed with sterile distilled water and covered with clear plastic bags for 48 hours to maintain (>80%) high humidity. Uninoculated control plants were treated with sterile distilled water supplemented with 0.05% (v/v) of Tween-20. Disease incidence (%) was determined for the spray inoculated plants by counting the numbers of bleached spikes at 3, 9, 15 and 21 days post infection (dpi). Disease severity (%) was determined for both spray and point inoculated plants by counting the numbers of bleached spikelets for each inoculated spike from 3 to 21 dpi. All inoculation trials were performed in three replicates, and each replicate contained 20 spikes for each variable (genotype × conidium concentration × inoculation technique).

2.2.3 Fungal biomass quantification

At 21 dpi, the 2500 c/s (the strongest disease pressure condition) point and spray inoculated spikes were collected and immediately stored in liquid nitrogen, for quantification of *F. graminearum* DNA in the chaff and rachis tissues. Fungal DNA quantification was performed following the protocol of

Horevaj *et al.* (2011) and Siou *et al.* (2014). Material to obtain the *F. graminearum* calibration curve (60 mg of fresh mycelium) and the wheat calibration curve (60 mg of uninoculated wheat material) and total inoculated wheat chaff and rachis (60 mg of inoculated wheat material), were finely ground using mortars and pestles plus liquid nitrogen, and were stored at -80°C until DNA extractions. Total wheat and fungal DNA extraction were performed following the protocol for the Invisorb® Spin Plant Mini Kit (Stratec Molecular GmbH, Germany), and DNA was quantified with a Qubit™ fluorometer 1.01 (Invitrogen, United States) using the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific, United States). DNA from inoculated samples was diluted to 10 ng/μL, while fungal and wheat calibration curves were obtained preparing three serial 1:10 dilutions from fresh fungal mycelium and uninoculated wheat material DNAs. Real-Time qPCR was performed following the instructions from Rotor Gene Q (Qiagen, Germany) and Xpert Fast SYBR (uni) Master Mix (GRiSP Research Solutions, Portugal). Real-Time qPCR amplification conditions included: an initial denaturation step of 3 minutes at 95°C; 35 cycles of 5 seconds denaturation at 95°C, 30 seconds of annealing at 61°C and 20 seconds of elongation at 72°C. A final melt cycle was performed to confirm the amplicons unicity. Real-Time qPCR was performed using the primer pair Tri6_10F/Tri6_4R for *F. graminearum* DNA quantification (Horevaj *et al.*, 2011), and Act_77F/Act_312R for wheat DNA quantification (Mandalà *et al.*, 2019). Three biological replicates were analysed for each quantification and from each of these, three technical replicates were obtained and tested. Results are reported as ng of fungal DNA per ng of plant DNA.

2.2.4 Thousand kernel weight (TKW)

At maturity, the 20 spikes from 2500 c/s (the greatest disease pressure condition) for point and spray inoculated plants, and the uninoculated control plants, were collected and stored at 4°C. The spikes were hand threshed to separate kernels from the chaff. Kernels were then weighed to determine and calculate the TKW. The TKW of inoculated plant kernels was then compared with the TKW of control plant kernels, to estimate the percent yield loss due to inoculations.

2.2.5 Statistical analyses

Data were subjected to analyses of variance (ANOVA). The following data were compared: disease incidence (%) among different conidium inoculation concentrations and genotypes for spray inoculated plants; disease severity (%) among different conidium inoculation concentrations, inoculation methods and genotypes; fungal DNA concentrations (ng of fungal DNA per ng of plant DNA); and yield loss (%) between 2500 c/s spray- and point-inoculated plants. Two levels of significance ($p < 0.05$ and $p < 0.01$) were computed to assess the significance of the F values. When significant F values were observed, a pairwise analysis was carried out using the Tukey Honestly

Significant Difference test (Tukey test) at the 0.95 or 0.99 confidence levels. Statistical analyses were performed using XLSTAT 2020.4 software (Addinsoft, France).

2.3 Results

Several conidium concentrations were tested to assess disease pressure responses in symptom development. The ANOVA test showed that there were no statistically significant differences in trends of FHB incidence (type I) or severity (type II) from the different conidium concentrations in the three cultivars tested. Incidence reached 100% between 15 and 21 dpi in all the three wheat cultivars for each conidium concentration tested; disease severity also reached 100% between 15 and 21 dpi, when the wheat spikes were point inoculated. From the spray inoculations, FHB severity for Marco Aurelio reached 44% at 21 dpi, while severity on Palesio and Claudio was also 100% at 21 dpi.

Figure 2.1 presents results obtained from the phenotypic evaluations of symptoms. Figures 2.1a, 2.1b and 2.1c show the genotype comparisons, for assessment of FHB differential responses connected to resistance genotype diversity, while Figures 2.1d, 2.1e and 2.1f show the inoculation method comparisons, for assessment of differences in symptom severity (type II). Figure 2.1a shows the variation in disease incidence at 3, 9, 15 and 21 dpi, for 2500 c/s, inoculated onto the three Italian wheat cultivars tested. Claudio was the most susceptible reaching 98% of symptomatic spikes at 9 dpi ($p<0.05$). At 15 dpi, Marco Aurelio showed reduced symptom progression ($p<0.05$), suggesting type I tolerance for most of the trial duration.

Figures 2.1b and 2.1c indicate the FHB severity trend comparisons between the genotypes at 2500 c/s. The spray inoculation technique (Figure 2.1b) gave disease severity at 9 dpi of 5% in Palesio and Marco Aurelio, and 57% in Claudio. This indicated the presence of initial type II resistance in Palesio and Marco Aurelio ($p<0.05$). From 10 dpi until the end of the trial, the statistically significant differences in symptoms between Claudio and Palesio disappeared, due to the high variability of results obtained with the spray inoculation method. In contrast, symptoms in Marco Aurelio reached a maximum of 27% at 21 dpi ($p<0.05$ for 15 and 19 dpi and $p<0.01$ for 20 and 21 dpi), compared to Claudio and Palesio.

Figure 2.1c shows the same genotype comparisons as previously described, but after point inoculations, to assess putative FHB type II resistance under more aggressive disease conditions. Between 3 and 13 dpi, no statistically significant differences were observed among the three wheat cultivars. Subsequently, Claudio again demonstrated high susceptibility, reaching 100% severity at 14 dpi, while significantly less symptom development was observed on Palesio and Marco Aurelio at 14-18 dpi ($p<0.05$). These results confirm that type II resistance was present in Palesio and Marco

Aurelio under harsh disease conditions. However, at 19–21 dpi, no symptom differences were detected among the three wheat genotypes.

Disease severity differences observed between the spray and point inoculation methods at 2500 c/s for the three wheat genotypes are shown in Figures 2.1d, 2.1e and 2.1f. Figures 2.1d and 2.1e show the severity progression, respectively, in Palesio and Claudio. Under both spray and point inoculation, these two wheat cultivars did not show any FHB resistance or tolerance. In contrast, Figure 2.1f shows the severity trend in Marco Aurelio, where, starting from 6 to 8 dpi, symptom progression was less after spray inoculation than point inoculation ($p<0.05$). These differences in symptom development were enhanced from 9 to 21 dpi ($p<0.01$), and at the end of the trial, disease severity reached 27% after spray inoculation, and 88% after point inoculation.

Additional estimations of FHB type II tolerance or resistance were made using fungal DNA quantification after spray and point inoculations with 2500 c/s. Figure 2 shows the Real-Time *qPCR* curves: Figures 2.2a, 2.2b, 2.2c and 2.2d show standard curves for DNA quantification of the *Actin* gene (Palesio, Claudio, Marco Aurelio), and the *Tri6* gene (*F. graminearum*). Figure 2.2e shows the amplifications results. Final results (Figure 2.3) showed that spray inoculation resulted in a less accumulation of fungal DNA in the wheat chaff and rachis than from point inoculation. Palesio (0.028 ± 0.00146 ng of fungal DNA ng per plant DNA) and Claudio (0.0025 ± 0.00012 ng) had less pathogen DNA than Marco Aurelio, (0.168 ± 0.0446 ng). After point inoculations, greater fungal DNA concentrations were detected, reflecting the aggressiveness of this inoculation method. Claudio again had reduced pathogen accumulation (0.342 ± 0.064 ng of fungal DNA/ng per plant DNA) compared to Marco Aurelio (0.447 ± 0.05 ng) and Palesio (0.532 ± 0.099 ng).

TKW was measured for kernels derived from 2500 c/s inoculated and uninoculated plants, to determine the pathogen impacts on potential wheat yields for the wheat genotypes. Figure 2.4 shows results reported as % yield losses. Point inoculations resulted in greater losses compared to spray inoculations ($p<0.05$), since losses from point inoculations reached 84% in Palesio, 91% in Claudio and 71% in Marco Aurelio. These differences were not significant with Marco Aurelio, where spray and point inoculation had similar effects. Claudio was more susceptible after spray (79% yield loss) or point (91%) inoculation, while Marco Aurelio was the least affected cultivar, under both spray (65% yield loss) and point inoculation (71%). However, all the yield losses were very high for all of the inoculation and cultivar treatments.

2.4 Discussion

FHB is normally a sporadic disease of wheat and other cereals, because infection and colonisation by *Fusarium* spp. are largely dependent on the prevailing weather conditions, which also determine disease severity (Xu, 2003; Burlakoti *et al.*, 2010). The risks of infection are associated with warm

and humid conditions (Xu and Nicholson, 2009). As a result, FHB incidence and severity usually vary from year to year (Sutton, 1982), and from region to region (Jelinek *et al.*, 1989).

To develop resistant cereal germplasm, artificial inoculation is essential to ensure disease development, to optimise host genotypic differentiation, and to reduce the influence of host morphological characters that can contribute to disease avoidance (Mesterházy, 1995; Vaughan *et al.*, 2016).

Evaluating FHB resistance using natural infections is often not possible as disease incidence and severity vary over time and space due to changes in environmental conditions such as temperature and precipitation that are difficult to control (Mesterházy *et al.*, 2003; Kriss *et al.*, 2012).

Obtaining consistent differentiation of FHB resistance levels relies on the use of comparative inoculation methods and different screening tests because of FHB's multigenic nature and complexity (Parry *et al.*, 1995; Browne, 2009). In the present study, type I and type II FHB responses were evaluated in three Italian wheat cultivars, to identify the presence of genotypic resistance/tolerance, and to assess two screening protocols, by testing different aspects and components of FHB resistance. Different conidium concentrations in inocula were assessed to evaluate disease incidence and severity responses connected to different disease pressure. FHB incidence increased in response to conidium concentration, but the differences observed were not statistically significant. Differences in disease severity were more pronounced after spray inoculations, but again, these differences were not statistically significant. These observations could be due to increases in disease development with increasing conidium concentration until a maximum was reached where additional inoculum does not increase the level of disease (Stein *et al.*, 2009). We also observed that results obtained from spray inoculations were characterized by high variability, compared to point inoculations. Kiecana and Mielniczuk (2013) explained that, despite spray inoculation resembling natural routes to infection by FHB pathogens, disease assessment could be arduous due to heterogeneity of conidium spatial location on wheat heads. When a pathogen is spray-inoculated, inoculum can also partially germinate, resulting in reduced symptom development (Parry *et al.*, 1995; Al Masri *et al.*, 2017). This could be due to close relationships between pathogen assessment and plant phenological stage. First establishment of FHB is related to host floret anthesis, which is not uniform within each spike: anthesis begins in the central floret, and then occurs in the upper and lower flowers (Dweba *et al.*, 2017; Kheiri *et al.*, 2019). Point inoculations, on the other hand, is reported to be more environmentally stable and results from this method are more reproducible, since the inoculum is applied directly into the central florets at anthesis. This ensures that equal amounts of inoculum are delivered to individual plants and reduces the chance of disease escape, which has been observed after spray inoculations (Engle *et al.*, 2007; Geddes *et al.*, 2008; Mesterházy *et al.*, 2015). Despite

this advantage, point inoculation does not represent the most natural source of *F. graminearum* inoculum and is more labour intensive and time-consuming to carry out. However, point inoculation likely mimics the fungal inoculum transferred onto cereal florets by tiny insects such as aphids and thrips that are often found in wheat crops (Usele *et al.*, 2013; Imathiu *et al.*, 2014; Sørensen *et al.*, 2016).

The genotype comparisons showed that Claudio durum wheat was the most type I and type II susceptible, Palesio bread wheat had initial type I and II resistance after spray inoculation, and type II tolerance after point inoculation. Marco Aurelio durum wheat showed type I tolerance and type II resistance after spray inoculation, and type II tolerance after point inoculation. These results are similar to those of Miedaner *et al.* (2003). They tested the covariation between spray and point inoculations, and compared host heritability of reactions to pathogens for the two methods. Point and spray inoculations resulted in similar mean disease severities among host genotypes, while the most important source of variance was observed between inoculation methods, reflecting the different disease severities achieved with the two methods.

We observed differential FHB responses within the diverse wheat genotypes analysed. To our knowledge, there are no published studies on the FHB reactions of Palesio and Marco Aurelio wheat cultivars, so our results cannot be compared to others. Amoriello *et al.* (2018) screened a number of durum wheat cultivars, including Claudio, for resistance to DON contamination, and Claudio was one of the most contaminated. The ability to degrade DON has been described as type III FHB resistance, and tolerance of high DON concentrations as type IV resistance. (Mesterházy, 1995; 2002; Gunupuru *et al.*, 2017). We cannot associate the susceptibility we observed as resulting from high DON accumulation, since we did not analyse mycotoxin content as an FHB resistance factor.

Regarding the type II tolerance observed in Palesio, it is known that bread wheat is naturally more resistant than durum wheat, because of its hexaploid nature (Buerstmayr *et al.*, 2014; Haile *et al.*, 2019). Nevertheless, the most interesting FHB type II responses were observed in Marco Aurelio durum wheat. Sources of resistance are limited in durum wheat (Stack *et al.*, 2002) and they reside mainly in other cultivated tetraploid wheat subspecies, such as ancient cereal crops (Oliver *et al.*, 2008). Some of these old cereal crops are *Triticum turgidum* subspecies, such as *T. turgidum* subsp. *turgidum*, which is native to Mediterranean countries, as is Marco Aurelio. It can therefore be assumed that the Italian wheat genotype Marco Aurelio possess some FHB resistance characteristics that also occur in the ancient cereals.

Results from fungal biomass quantification of the wheat chaff and rachides between the two inoculation techniques reflect the results obtained from the phenotypic evaluations of disease severity. When spray inoculated, wheat cultivars gave better contrast of pathogen spread, than when they were

point inoculated. Kumar *et al.* (2015) assessed a Real-Time *q*PCR technique to detect and quantify *F. graminearum* biomass in rachides of barley and wheat resistant and susceptible cultivars, using the *Tri6* gene for fungal quantification and the *Actin* gene for the normalization, as done here. They observed that disease severity could not discriminate resistance before 9 dpi, while spikelet resistance was discriminated in all the wheat and barley genotypes tested, based on *q*PCR quantification of the fungal biomass. We therefore conclude that the wheat cultivars used in our study possess levels of tolerance to pathogen colonisation when spray inoculated, but not when point inoculated. These results agree with those of Brennan *et al.* (2005), who showed that visual disease assessment clearly reflected yield losses, but that no significant relationship was present between symptom severity and fungal DNA content in grain.

The phenotypic FHB scoring after spray inoculation at 2500 c/s (disease incidence and severity) revealed that Claudio durum wheat reached 100% of diseased spikes and spikelets sooner than the other cultivars, and was also likely best limit pathogen spread. The rapid bleaching of spikes but low amounts of fungal DNA could be explained in several ways. During pathogen infection and FHB development, plant vessels become blocked, preventing water and nutrient supplies as a defence mechanism, and causing sudden spike wilting (Kang and Buchenauer, 2000; Kheiri *et al.*, 2019). head bleaching due to natural absence of water and nutrients could be confused with FHB symptoms (Zwart *et al.*, 2008). Regarding the low amount of fungal biomass present in the chaff and rachides, it is known that DON is an important virulence factor that facilitates infection spread (Bai *et al.*, 2002; Jansen *et al.*, 2005). Ilgen *et al.* (2009) observed that the *Tri5* gene, which controls the trichodeine synthase involved in DON synthesis, is highly induced in the transit zone of host rachis nodes, where the rachilla and rachides divide. It could be possible that wheat genotypes possessing type III or type IV resistances (ability to degrade DON and to tolerate high DON levels) will give contrasting pathogen spread into the host vessels. TKW evaluation revealed that Marco Aurelio was the least damaged wheat genotype, after spray and point inoculations. Resistance evaluation concentrates on visual head disease symptoms, since most QTL analyses have evaluated this trait. The TKWs were generally neglected in early wheat breeding programmes for resistance to FHB. Resistance to kernel infection arises from the premise that those genotypes should have a resistance type that does not affect levels, but their TKW values differ significantly. Therefore, it is not by chance that yield stability was developed as a major trait in plant breeding. However, FHB resistance, like yield, is governed by many QTLs, and infection severity has a strong impact on yield. It is therefore necessary to consider TKW in FHB resistance screening protocols (Canci *et al.*, 2004; Mesterházy *et al.*, 2015). Current wheat breeding programmes for FHB resistance focus more on type II than type I resistance (Buerstmayr *et al.*, 2003; Burlakoti *et al.*, 2010; Xiao *et al.*, 2016). type II resistance has also been

reported to be more genetically and environmentally stable than type I resistance, and provide a more reliable indication of cultivar resistance (Bai and Shaner, 2004). It is desirable, when possible, to replicate FHB resistance testing within and across environments (years and/or locations), in order to obtain meaningful results and to assess reproducibility of the data obtained (Buerstmayr *et al.*, 2014). Different FHB screening methods do not provide answer to the same question. FHB traits differ, and visual symptoms, fungal biomass and TKWs do not closely follow the similar patterns (Mesterházy *et al.*, 2015). No genotype in the present study was completely resistant to FHB. Thus, most genotypes probably quantitatively combine different levels of FHB resistances. This supports the assumption that FHB resistance types are probably governed by different loci and measure different resistance reactions, as has been suggested by Schroeder and Christensen (1963). For routine screening of FHB resistance in large breeding populations, a fast, cheap, and reliable inoculation method is desirable. Spray inoculation is advantageous over point inoculation. It is based on whole-plot inoculation (Martin *et al.*, 2017), which has similarity to natural disease situations and requires less time and labour for inoculation and disease assessments. Spray inoculation can also be useful to establish rapid and low cost assays to evaluate FHB resistance using wheat seedlings (Soresi *et al.*, 2015). However, spray inoculation is environmentally influenced, resulting in high variability and less replicable results. Point inoculation mimics infection through insects, but it is genetically and environmentally stable (Imathiu *et al.*, 2014). From our experience, we suggest, when possible, that both inoculation methods are used, and that as many FHB characteristics as possible are evaluated, since we observed differences in responses within inoculation techniques and the parameters assessed. In addition, screening different FHB parameters allowed us to recognize the FHB responses in Marco Aurelio, one of the most cultivated durum wheat cultivars in Italy.

2.5 References

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2.6 List of figures

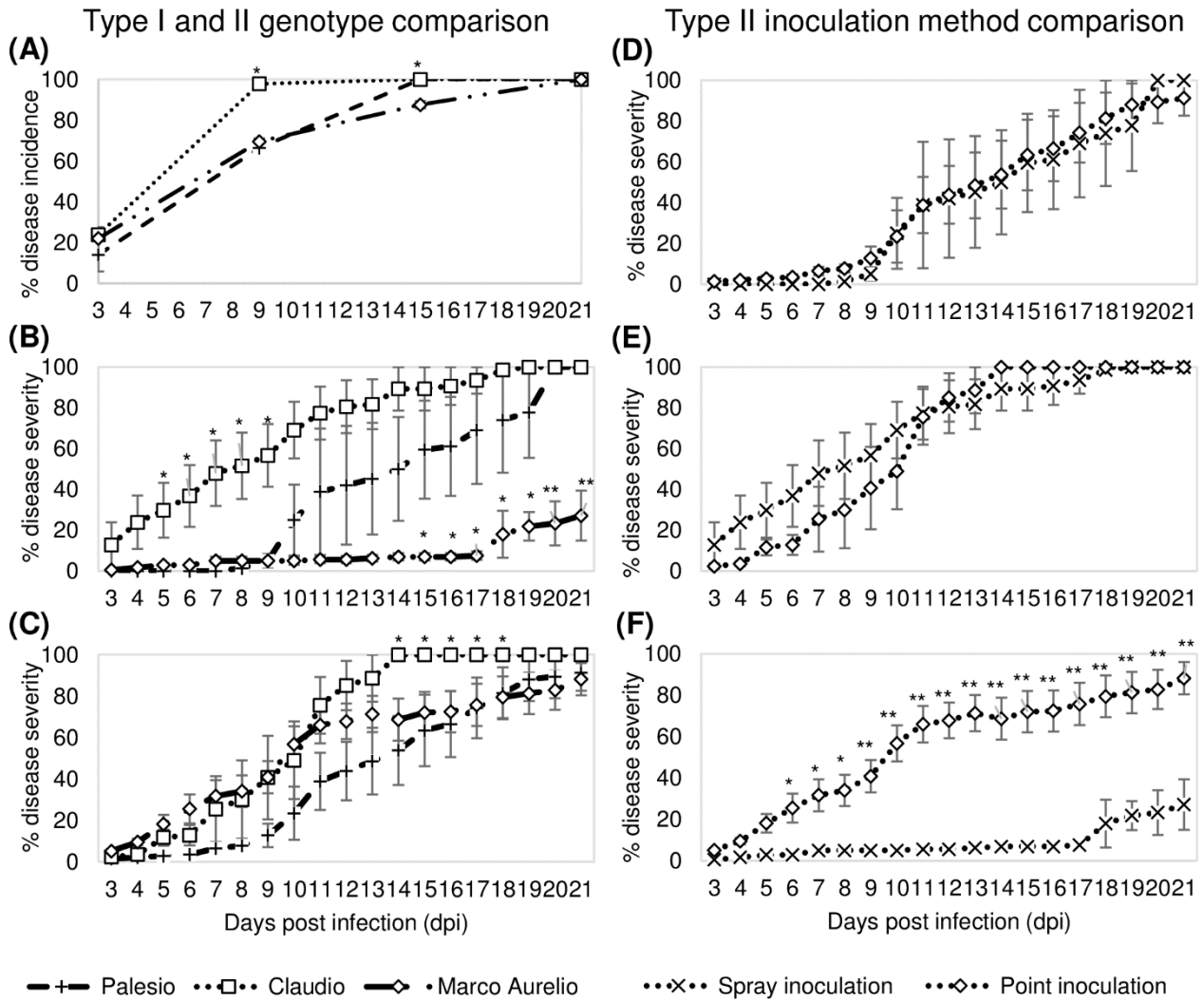


Figure 2.1 *Fusarium graminearum* symptom development during 21 dpi, following inoculation of three Italian wheat genotypes with 2500 conidia per spike. (A) % disease incidence (type I); (B) % disease severity after spray inoculation (type II); (C) % disease severity after point inoculation (type II); (D), (E) and (F) % disease severity (type II) between spray and point inoculation methods for, respectively, Palesio, Claudio and Marco Aurelio. Data represent averages and standard errors for three independent replicates with at least 20 plants for each genotype × inoculation combination. Statistical analyses were performed according to a one way analysis of variance (ANOVA) with the Tukey test at a 0.95 confidence level and (*) $p < 0.05$, and at a 0.99 confidence level and (**) $p < 0.01$.

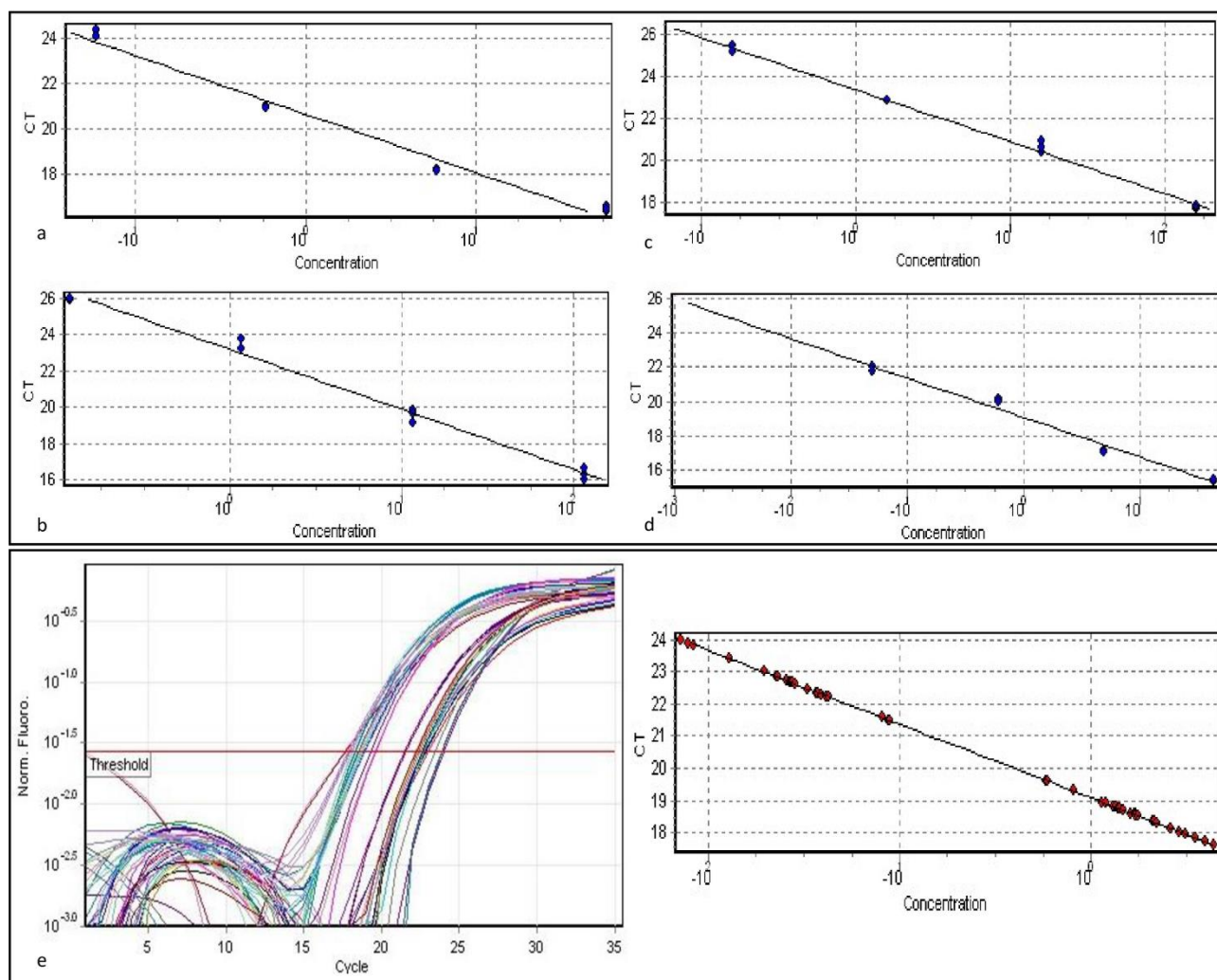


Figure 2.2 Standard curves resulting from Real-Time qPCR quantifications: (A), (B) and (C) show, respectively, *Act* standard curves for wheat genotypes Palesio, Claudio and Marco Aurelio pure DNAs. (D) shows the *Tri6* standard curve for *F. graminearum* pure DNA. (E) shows amplification curves of the *Tri6* gene (left) and interpolations with the standard curve (right).

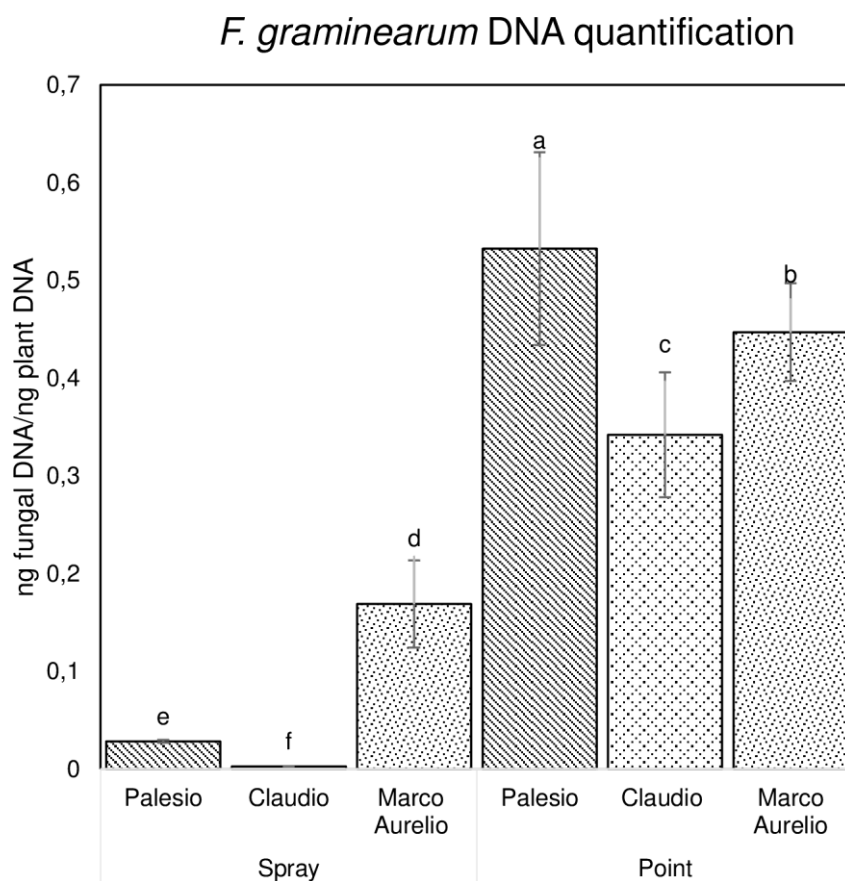


Figure 2.3 *Fusarium graminearum* biomass quantification from wheat chaff and rachides sampled from spikes that were either spray or point inoculated with 2500 conidia per spike. Data represents averages and standard errors for three independent technical replicates derived from three independent biological replicates for each genotype $\times$ inoculation method. Statistical analyses were performed according to two way analysis of variance (ANOVA), with Tukey test at 0.95 confidence level ($p < 0.05$).

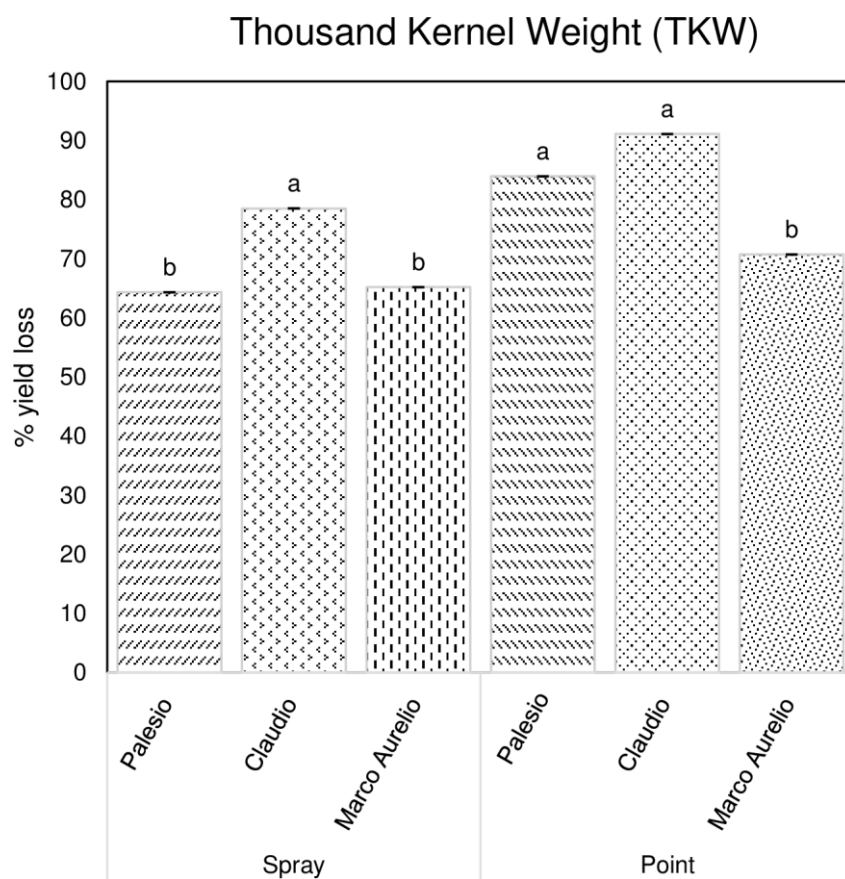


Figure 2.4 Yield losses (%) based on TKWs for inoculated and control plants after spray or point inoculations with *F. graminearum* at 2500 conidia per spike. Data represent averages and standard errors for three independent replicates performed with at least 20 plants for each genotype × inoculation combination. Statistical analyses were performed according to two way analysis of variance (ANOVA) with Tukey test at 0.95 confidence level ($p < 0.05$).

3. The modulation of stomatal conductance and photosynthetic parameters is involved in *Fusarium* head blight resistance in wheat

Sara Francesconi^{1*} and Giorgio Mariano Balestra¹

¹*Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia, Via San Camillo de Lellis, 01100, Viterbo, Italy*

Corresponding author: Sara Francesconi, francesconi.s@unitus.it

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Abstract: *Fusarium* head blight (FHB) is one of the most devastating fungal diseases affecting grain crops and *Fusarium graminearum* is the most aggressive causal species. Several evidences shown that stomatal closure is involved in the first line of defence against plant pathogens. However, there is very little evidence to show that photosynthetic parameters change in inoculated plants. The aim of the present study was to study the role of stomatal regulation in wheat after *F. graminearum* inoculation and explore its possible involvement in FHB resistance. RT-qPCR revealed that genes involved in stomatal regulation are induced in the resistant Sumai3 cultivar but not in the susceptible Rebelde cultivar. Seven genes involved in the positive regulation of stomatal closure were up-regulated in Sumai3, but it is most likely, that two genes, *TaBG* and *TaCYP450*, involved in the negative regulation of stomatal closure, were strongly induced, suggesting that FHB response is linked to cross-talk between the genes promoting and inhibiting stomatal closure. Increasing temperature of spikes in the wheat genotypes and a decrease in photosynthetic efficiency in Rebelde but not in Sumai3, were observed, confirming the hypothesis that photosynthetic parameters are related to FHB resistance.

Keywords: *Fusarium* head blight, Photosynthetic parameters, Resistance, Stomatal regulation, *Triticum aestivum*.

3.1 Introduction

Stomatal conductance is the physiological plant mechanism needed for gas exchange in order to achieve an optimal photosynthetic process by adjusting water transpiration. The movements of

stomata occur through the bumping and lessening of the guard cells and stomatal closure is the primary response of plants to water deficit. Absciscic acid (ABA), jasmonate (JA), ethylene, auxins and cytokinins interact in a composite network of cellular signals as modulators of stomatal closure. ABA functionality is strongly dependent upon synchronized activation of biosynthetic, catabolic, conjugating/deconjugating and transporting genes (Daszkowska-Golec and Szarejko, 2013). The positive regulation of stomatal closure starts in the plastids, where the first activated enzyme is the terpene synthase (TPS). During the following step the zeaxanthin epoxidase (ZEP) catalyses the conversion of zeaxanthin and antheraxanthin into violaxanthin. Violaxanthin is transformed into 9-cis-epoxycarotenoid by 9-cis-epoxycarotenoid dioxygenase (NCED). Xanthinin is transferred to the cytoplasm, where ABA-aldehyde is generated and the ABA-aldehyde oxidase (AAO) catalyses its conversion into ABA. The adjustment of stomatal movements requires also the activation of negative regulators, such as the cytochrome P450 family (CYP450) and the β -1,3-glucanase (BG), which can deconjugate or conjugate ABA. In the guard cells, phaseic acid binds the ABA receptor (REC) forming a complex that inhibits phosphatase (ABI). The inactivation of ABI permits the activation of downstream targets (Damour *et al.*, 2010; Kim *et al.*, 2010; Daszkowska-Golec and Szarejko, 2013; Merilo *et al.*, 2015). The signalling cascade begins with the activation of different kinases, such as Ca^{2+} -dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs). Moreover, the guard cells produce reactive oxygen species (ROS), for instance hydrogen peroxide (H_2O_2) and nitric oxide (NO). NADPH oxidase (RBOH) facilitates the generation of ROS in the stomata, which results to stomatal closure. CDPKs are linked to the JA-signalling pathway, which is positively implicated in stomatal closure. JA is subjected to metabolism by allene oxide synthase (AOS) and hydroperoxide lyase (HPL). The resulting signalling cascade activates several response-genes thanks to MYC and MYB domain transcription factors. (Schroeder *et al.*, 2001; Munemasa *et al.*, 2011; Mattos and Moretti, 2016).

Plants are incessantly subjected to an evolutionary pressure by the attack of diverse pathogens. Hence, plant physiology has evolved several defence strategies to counteract plant pathogens (Jones and Dangl, 2006). Plants evolved a specific type of recognition receptors, called pattern recognition receptors (PRRs), which are able to recognize conserved-elicitor domains, the pathogen-associated molecular patterns (PAMPs) (Jones and Dangl, 2006). Perception of PAMPs drives those basal defence responses, the PAMP-triggered immunity (PTI), which activates several physiological defence mechanisms: MAPK cascades, alkalinisation of the extracellular liquor, ROS and NO production, increases in Ca^{2+} influx, synthesis of salicylic acid (SA), ethylene, and induction of stomatal closure (Gu *et al.*, 2017). A wide range of foliar pathogens are known to disrupt stomatal movements in the presence of infection. Studies have shown that stomata are not just a passive gate

for pathogen entry but play an active role in the plant innate immune response, and the control of stomatal closure is one of the first lines of defence against pathogen invasion. Therefore, plants can restrict pathogen entry into the leaves by closing stomata or by inhibiting stomatal opening. Conversely, pathogens have evolved virulence factors to counteract host stomatal defences by inhibiting stomatal closure or promoting stomatal opening. In this regard, the physiological regulation of stomatal closure has a critical function during the plant-pathogen interaction, acting as a prompt barrier against invasion by pathogens (Melotto *et al.*, 2006; Schulze-Lefert and Robatzek, 2006; Mur *et al.*, 2013).

Among the fungal plant diseases, Fusarium head blight (FHB) of wheat (*Triticum aestivum* L. and *T. turgidum* subsp. *durum* (Desfontaines) Husnache), barley (*Hordeum vulgare* L.) and other cereals is one of the most devastating for grain crops (Parry *et al.*, 1995). *Fusarium graminearum* Schwabe is the predominant species which causes FHB in many countries (Gilbert and Tekauz, 2000). The wheat florets represent the first infection site for the fungal spores, which infect the head at anthesis, especially at warm and humid environmental conditions (Schöneberg *et al.*, 2019). Afterwards, the hypha of *F. graminearum* proliferates and invades the host tissues predominantly by direct penetration and passive entry, where plant stomata have a crucial role, because through them the fungus infects the spikelets internally by entering into the vascular bundles of the rachilla and rachis (Kang and Buchenauer, 2000). Spores germinate and initiate infection, generating typical symptoms, such as dark-brown, water-soaked spots on the infected spikes which bleach completely (Osborne and Stein, 2007). FHB is an extremely problematic disease because these pathogens produce mycotoxin deoxynivalenol (DON), toxic to human and animal safety (Oerke *et al.*, 2010). Resistance to FHB in wheat involves active and passive mechanisms, such as cell reinforcement, hypersensitive reaction (HR), production of phytoalexins and induction of defence-related genes (Mesterházy *et al.*, 1999). Resistance to FHB is quantitative and multigenic although the genetic function of many QTLs is still unknown (Ma *et al.*, 2020). Many studies support the hypothesis that resistance to FHB is capable of arousing different signalling cascades in the host plant, following recognition of pathogens, such as Ca^{2+} efflux, activation of JA and MeJA related genes, ROS production and activation of systemic-acquired resistance (SAR) (Makandar *et al.*, 2012; Antalová *et al.*, 2020). Additionally, few studies have highlighted the idea that there is phenotypic evidence that photosynthetic parameters change in order to counteract different fungal pathogens (Yang *et al.*, 2016).

The genetic mechanism of the regulation in wheat of stomatal conductance to FHB response remains still unclear and its elucidation would provide new information valuable to breeding programmes. In the present work we selected the main genes involved in stomatal closure in order to evaluate their response to FHB. The relative expression levels of the susceptible Italian bread wheat cultivar,

Rebelde, and the experimental resistant Sumai3 genotype bread wheat were compared. Furthermore, the relative expression gene levels of inoculated and drought-stressed plants were compared, to detect the existence of a putative differential gene response between FHB and water stress. Additionally, spike temperature, photosynthetic efficiency and morphology of stomata were evaluated as direct phenotypical parameters.

3.2 Materials and methods

3.2.1 Fungal material and preparation of the inoculum

The highly virulent and DON-producing isolate of *F. graminearum* wild type 3824 was isolated for the first time by the University of Pisa (Tomassini *et al.*, 2009). The isolate was cultured at 21°C on Synthetic Nutrient Poor Agar (SNA) to obtain macroconidia (Mandalà *et al.*, 2019). After 10 days on SNA, the conidia were scraped with a glass rod after pipetting 1 mL of sterile distilled water onto the surface of a Petri dish. The conidial suspension was recovered, and the concentration adjusted to 1×10^5 conidia/mL using a Thoma Chamber (0.100 mm depth and 0.0025 mm^2). The inoculum was prepared in sterile distilled water supplemented with 0.05% (v/v) of Tween-20.

3.2.2 Plant material, growth and inoculation conditions and experimental design

The FHB-susceptible Italian wheat Rebelde and the resistant Sumai3 (bread wheat) were grown in the greenhouse. The surfaces of the kernels were sterilized with sodium hypochlorite (0.5% v/v) for 20 minutes and then rinsed twice for 5 minutes in sterile distilled water. The kernels were germinated in the dark on paper soaked in sterile distilled water for 15 days at 4°C to break dormancy, followed by 2 days at room temperature. The seedlings were transferred to 40×20 cm pots, filled with TYPical Brill soil (Brill, Germany) and grown at 16°C–20°C up to the boot stage, 20°C–24°C during anthesis and 24°C–29°C up to maturity. The plants were fertilised using ammonium nitrate in the following proportions and at the following stages: 20% at sowing, 40% at tillering and 40% at heading (Francesconi *et al.*, 2019). The plants were subjected to three different treatments: i) drought stress (the plants did not receive water from the boot (Zadok stage 51) to flowering stages (Zadok stage 69) (Zadoks *et al.*, 1974; Tambussi *et al.*, 2000)); ii) artificial inoculation, (the plants were spray-inoculated at the flowering stage by applying a suspension of 100 µL of 1×10^5 conidia/mL in the central floret by using a manual nasal sprayer; the artificial inoculation lasted 10 days and the phenological stage of the plants was checked day by day, in order to inoculate at Zadok stage 69 (Stein *et al.*, 2009; Francesconi *et al.*, 2019)); iii) mock, (at the flowering stage the central floret was sprayed with 100 µL of Tween-20 0.05% resuspended in sterile distilled water). The spikes were homogenously sprayed with sterile distilled water and covered with clear plastic bags for 24 hours to maintain high humidity levels (>80%). The spikes subjected to drought and mock treatments were

sampled after removing the plastic bags, while the inoculated spikes were sampled 24, 48, 72 hours post inoculation (hpi) and 10 days post inoculation (dpi) to investigate an early or a late response to *F. graminearum*. The spikes collected were immediately stored in liquid nitrogen and at -80°C until the extraction of RNA. The disease severity of the FHB (%) was determined by counting the number of bleached spikelets for each inoculated spike from 3 to 21 dpi (Francesconi *et al.*, 2019). Data were obtained from three independent replicates with each replicate consisting of 20 spikes for each treatment.

3.2.3 Identification of *in-silico* sequences, homology and phylogenetic analysis and primer design

Sequence-similarity searches were performed using the URGI BLAST Database Wheat Genome (IWGSC_ref_v1) (<https://wheat-urgi.versailles.inra.fr/>). The similarity searches were carried out starting from known sequences of *Aegilops tauschii* Cosson, *H. vulgare*, *Oryza sativa* L. and *Zea mays* L. obtained from Gramene (www.gramene.org). Homologue sequences were extracted and aligned by using CLUSTAL MUSCLE (v3.8.31) configured for highest accuracy. Primers were designed to amplify the entire nucleotide sequences from the Rebelde and Sumai3 samples. Homology and phylogeny trees were generated using DNAMAN software (Lynnon Biosoft, Canada) using the maximum-likelihood method and setting the bootstrap values from a minimum of 1000 trials. The sequences were submitted to BLASTn (<https://blast.ncbi.nlm.nih.gov/>) analysis in order to extract the corresponding cDNA. Primers for Real-Time qPCR were designed inside the exonic regions. All the primers were designed using Primer3Plus 2.4.0. Primers for Glyceraldehyde-3-phosphate dehydrogenase (*TaGAPDH*), Pathogenesis Related Protein 1 (*TaPR1*), Actin, (*TaACT*), Tubulin (*TaTUB*) and Ferredoxin-NADP(H)-oxidoreductase (*TaFNR*) have been taken from the following literature references: *TaPR1* (Lu *et al.*, 2006), *TaACT* (Tundo *et al.*, 2016), *TaTUB*, *TaFNR* (Tenea *et al.*, 2011) and *TaGAPDH* (Jarošová and Kundu, 2010). The primer pair for *TaGAPDH* amplification has been adapted for *T. aestivum* since they have been originally designed for *H. vulgare* sequence. Supplementary table 3.1 shows the list of selected genes, their functions, the corresponding primer pairs and the amplicon lengths for the DNA and cDNA, while Supplementary table 3.2 reports the accession numbers of homologous sequences used for sequence-similarity searches.

3.2.4 DNA extraction and PCR

Wheat DNA was extracted from mature kernels of Rebelde and Sumai3. The kernels were ground to obtain a fine powder and 10 mg were subjected to DNA extraction using an extraction buffer composed of Tris 100 mM, EDTA 50 mM and NaCl 500 mM, SDS 10% (w/v), potassium acetate 5

M. The DNA was resuspended in 20 µL of DNase and RNase-free sterile distilled water and stored at -20°C. Total DNA was quantified with Qubit™ fluorometer 1.01 (Invitrogen, United States) using the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific, United States) and diluted to 10 ng/µL. A gradient PCR was performed by following the instruction of GoTaqGreen MasterMix (Promega, United States). The PCR was prepared in a total volume of 10 µL and conditions of amplification included: an initial denaturation step of 2 minutes at 95°C; 35 cycles of 30 seconds denaturation at 95°C; 40 seconds of annealing at 55-65°C; 60 seconds of elongation at 72°C; a final elongation step of 5 minutes at 72°C. Once the optimal annealing temperature (60°C) was obtained, the sequences were amplified following the PCR conditions described previously. The amplicon unicity was visualized on 1.5% agarose gel and confirmed by Sanger sequencing (Eurofins Genomics GmbH, Germany).

3.2.5 RNA extraction and cDNA synthesis

The RNA was extracted from entire wheat heads. Each head was ground in liquid nitrogen using a mortar and pestle until a fine powder was obtained. 100 mg of powder was subjected to RNA extraction following the instructions provided by InviTrap® Spin Plant RNA Mini Kit (Stratec Molecular GmbH, Germany). The RNA was resuspended in RNase-free sterile distilled water and immediately poured onto ice and quantified with Qubit™ fluorometer 1.01 (Invitrogen, United States) using the Qubit™ RNA BR Assay Kit (Thermo Fisher Scientific, United States). To confirm the total quality and integrity of the RNA, 5 µL of the extracted RNA was subjected to thermal shock (10 minutes at -80°C after 5 minutes at 65°C) and run on a 1.5% denaturing agarose gel. The synthesis of the cDNA was performed using 500 ng of RNA following the instructions provided by Xpert cDNA Synthesis Supermix with a gDNA eraser (GRiSP Research Solutions, Portugal) in a final volume of 20 µL. To ensure that the synthesis of the cDNA and the elimination of the gDNA had succeeded, a reverse transcriptase PCR (RT-PCR) of *TaACT* (containing an intron in the sequence amplified by the primer pair selected) was performed. The RT-PCR was performed by following the instruction provided by GoTaqGreen MasterMix (Promega, United States) in a total volume of 10 µL and conditions of amplification included: an initial denaturation step of 2 minutes at 95°C; 35 cycles of 30 seconds denaturation at 95°C; 40 seconds of annealing at 60°C; 30 seconds of elongation at 72°C; a final elongation step of 5 minutes at 72°C. The amplification run consisted also of a no-template control (NTC) and a genomic DNA (gDNA) control. The amplicons were visualised on a 1.5% agarose gel.

3.2.6 RT-qPCR

The efficiency of the RT-qPCR amplification (E) was determined for each primer pair and for each wheat genotype as follows: five 1:10 serial dilutions (1:1–1:10000) were obtained for each cDNA and amplified in four replicates. E and correlation coefficient (R^2) values were calculated by means of the slope of the standard curve obtained by plotting the fluorescence versus the serial dilution concentrations using the equation $E = 10^{\left(-\frac{1}{\text{slope}}\right)} - 1$. The most stable reference gene was chosen on the basis of the E value most similar to the E values of target genes, the highest R^2 and the lowest variability for the quantification cycles (Cq). The relative expression levels of the target genes were calculated on the basis of the Cq values of the four technical replicates derived from four independent biological replicates for each plant treatment by applying the equation $Relative\ expression = 2^{-\Delta\Delta Cq}$ using *TaACT* as the reference gene and the mock treatment to normalize the relative expression levels. The relative expression levels of *TaPR1* and *TaGAPDH* were quantified as internal control of the progression of the infection (Muthukrishnan *et al.*, 2001) and changes in photosynthesis (Yang *et al.*, 1993). The RT-qPCR was performed, following the instructions provided by the Rotor Gene Q (Qiagen, Germany) and Xpert Fast SYBR (uni) MasterMix (GRiSP Research Solutions, Portugal), in a final volume of 10 μ L. The amplification conditions included an initial denaturation step of 3 minutes at 95°C; 40 cycles of 5 seconds denaturation at 95°C; 30 seconds of annealing at 60°C and 20 seconds of elongation at 72°C. A final melt cycle (70–99°C) and a Sanger sequencing of the amplified cDNAs were performed to confirm the amplicons' unicity and identity. NTC controls were included and the amplification was considered negative when a value of $Cq \geq 38$ was detected (Bustin *et al.*, 2009).

3.2.7 The spike-temperature and photosynthetic efficiency measurements and optical microscopy

Spike-temperature and photosynthetic efficiency were measured as phenotypical parameters of stomatal regulation. The spike temperature was recorded using a portable thermometer (Extech 1200F Compact Laser Infrared Thermometer 42510A, United States), while the photosynthetic efficiency was measured using a portable fluorometer (V2.00f PAM 2000, Heinz Walz GmbH, Germany). Phenotypical parameters were measured on three independent replicates each one comprising of 20 individual spikes for each treatment and inoculation time-point. Each measurement was carried out at dawn. At the same time, three external glumes were randomly collected from the spikes for each treatment and inoculation time-point. A thin portion of the glumes was obtained and analysed with an optical microscope (Leitz Diaplan, Leica microsystems GmbH, Germany) using a 40X magnification in order to observe the stomata.

3.2.8 Statistical analyses

Data from the RT-qPCR were subjected to two-way variance analysis (ANOVA), where the two independent variables were the wheat genotype (Rebelde and Sumai3) and the treatments undergone by the plants (drought stress, 24 hpi, 48 hpi, 72 hpi and 10 dpi) while the dependent variable was the relative expression level. FHB disease severity and phenotypical data were subjected to one-way ANOVA analysis. Two levels of significance ($p < 0.05$ and $p < 0.01$) were computed to assess the significance of the F values. A pairwise analysis was carried out using the Tukey Honestly Significant Difference test (Tukey test) at the 0.95 or 0.99 confidence level. The Pearson's linear correlation was computed to establish the correlation between the genotypes and the photosynthetic parameters. Statistical analyses were performed using SYSTAT12 software (Systat Software Inc., United States). Principal component analysis (PCA) and heatmap were carried out using ClustVis software (Estonia) (Metsalu and Vilo, 2015).

3.3 Results

3.3.1 Evaluation of FHB severity in the susceptible bread wheat genotype Rebelde and in the resistant bread wheat Sumai3

FHB severity (%) is represented in Figure 3.1a. From 3 to 8 dpi no statistical differences between the two wheat genotypes were recorded. From 9 to 21 dpi, FHB severity differed significantly, since at 21 dpi it reached 28% and 96% in Sumai3 and Rebelde, respectively. Figure 3.1b shows the symptoms at 21 dpi, while the bleaching remained confined to the central florets in Sumai3 but not in the Rebelde variety. No FHB symptoms were observed on the mock plants.

3.3.2 Annotation, homology and phylogeny *in-silico* analyses of stomatal regulation genes

In the present study we isolated and annotated the genes involved in stomatal regulation from the bread wheat Rebelde and Sumai3 and we performed a homology and phylogeny analysis with related species. The amplification results shown that all the selected genes were successfully isolated from Rebelde and Sumai3 (Supplementary figure 3.1.). The sequencing results confirmed the unicity of the sequences from both genotypes. Supplementary figure 3.2 shows the homology and phylogeny trees of the genes selected. The closely-related sequences from Chinese Spring, *A. tauschii*, barley, Rebelde and Sumai3 mostly clustered together, while sequences from rice and maize represented the second major cluster. In particular, the genes of interest from Sumai3 and Rebelde showed a homology ranging from 87% to 100%. Supplementary figure 3.3 represents the alignment between Sumai3 and Rebelde of the genes having a homology equal or less than 95%. The major differences in the nucleotide sequence resides in SNPs mostly localized in intronic regions. A similar homology rate (87-100%) was also reported with Chinese Spring, *A. tauschii* and *H. vulgare*. *O. sativa* and *Z.*

mays resulted to be the most distance species, since the homology rate with Rebelde and Sumai3 was of 69-86%.

3.3.3 Establishment and validation of RT-qPCR

Supplementary figure 3.4 shows RNA integrity and *TaACT* amplifications from Rebelde and Sumai3, demonstrating that cDNA was successfully synthesized. The E and R² values (Supplementary table 3.3) for each primer pair were calculated: E ranged between 0.9056 to 1.1826 and 0.9759 to 1.2463, R² between 0.9907 to 0.9984 and 0.9891 to 0.9969 in the case of the amplification reactions obtained from Rebelde and Sumai3, respectively. E and R² were not calculated for *TaTUB* from Rebelde because Cqs were detected only for 1:1 (36.65) and 1:10 (39.51) serial dilution (Supplementary table 3.4) and the amplification obtained from the 1:10 dilution was considered negative (Cq>38). The E and R² values for *TaACT* were 1.1748 and 0.9954 for Rebelde and 1.1694 and 0.9961 for Sumai3, while E and R² for *TaFNR* were 0.8988 and 0.9835 for Rebelde and 0.9249 and 0.9661 for Sumai3 (Supplementary table 3.3). Standard errors (SE) among the Cq values for *TaACT* were ± 0.194 to ± 0.266 and ± 0.311 to ± 0.357 for Rebelde and Sumai3, respectively, while for *TaFNR* was ± 2.014 to ± 2.962 and ± 2.122 to ± 2.665 . Hence, *TaTUB* and *TaFNR* were not taken into account for normalization (Supplementary table 3.4).

3.3.4 Differential expression patterns of the genes involved in stomatal regulation

Figure 3.2 shows the heatmap for the relative expression values for each gene, genotype and type of stress applied to the plants, while Supplementary table 3.5 provides the relative expression values, SE and statistical analysis according to the Tukey test computed at 0.99 confidence level. Drought stress did not induce the selected genes, with the exception of *TaZEP* (2.638 and 1.533-fold in Rebelde and Sumai3), *TaBG* (4.313-fold) and *TaCYP450* (1.743-fold) in Sumai3. *TaPR1* was basally regulated in Rebelde (0.816-fold) but strongly up-regulated in Sumai3 (55.270-fold).

Between terminal drought stress and *F. graminearum* inoculation, the regulation of the selected genes was not extremely dissimilar within Rebelde, with the exception of *TaHPL*, which was strongly down-regulated at 10 dpi (0.009-fold), *TaKSL* slightly up-regulated at 48 hpi (1.617-fold), *TaREC* basally regulated at 24 hpi (1.048-fold) while down-regulated under drought stress (0.381-fold), *TaBG* up-regulated at 10 dpi (5.668-fold), *TaMAPK* basally regulated at 48 hpi (1.412-fold) and 72 hpi (1.098-fold) and up-regulated at 10 dpi (3.742-fold), *TaCYP450* progressively down-regulated from 48 hpi to 10 dpi (0.657-0.029-fold), *TaZEP* strongly down-regulated at 10 dpi (0.035-fold). Conversely, the expression pattern found among Sumai3 differed particularly when comparing drought stress and the different time of inoculation. Most of the selected genes were up-regulated at 24 and 48 hpi while *TaBG* and *TaCYP450* were considerably up-regulated (70.644 and 50.534-fold

at 24 hpi and 39.768 and 53.172-fold at 48 hpi, respectively). At 72 hpi and 10 dpi some genes were down-regulated with the exception of *TaHPL*, *TaZEP*, *TaAOS*, *TaBG*, *TaMAPK* and *TaCYP450*. As a result, the difference between the two genotypes was obvious, since the genes involved in stomatal regulation promptly responded to *F. graminearum* in Sumai3 but not in Rebelde, suggesting their involvement in FHB resistance. Furthermore, *TaHPL*, *TaKSL*, *TaAAO*, *TaREC*, *TaCDPK*, *TaNCED*, *TaABI*, *TaPIMP* and *TaZEP* seemed to be involved in early response, while *TaAOS*, *TaBG*, *TaMAPK* and *TaCYP450* in long-term response to *F. graminearum*. Additionally, principal-component analysis (PCA) was carried out to gain in-depth understanding of the role of the genes selected and Figure 3.3 shows that two main clusters have been identified as deriving from the PCA. Relative expression values for Sumai3 at 24 and 48 hpi clustered together, confirming the hypothesis that most of the genes are involved in an early response to *F. graminearum*. *TaPR1* was progressively up-regulated in both the genotypes, as expected. *TaGAPDH* was basal regulated under drought-stress condition in both the wheat genotypes and under *F. graminearum* infection in Rebelde, while progressively up-regulated in Sumai3 at 24, 48, 72 hpi and 10 dpi.

3.3.5 Phenotypical parameters of stomatal regulation

Figures 3.4a-e show the spike temperature reached after the plants were subjected to drought (a), 24 hpi (b), 48 hpi (c), 72 hpi (d) and 10 dpi (e). Spike temperatures after drought stress did not increase (15.60°C and 15.49°C, while the mock plants reached 16.88°C and 16.34°C respectively in the Rebelde and Sumai3). Spike temperatures increased gradually from 24 hpi to 72 hpi in Sumai3 (19.03°C, 19.63°C and 20.05°C at 24, 48 and 72 hpi, while the mock plants reached 17.01°C, 17.79°C and 17.98°C). The temperature increased significantly also in Rebelde at 48 (18.40°C, while the mock plants reached 17.27°C) and 72 hpi (19.53°C, while the mock plants reached 18.09°C), suggesting that the few slightly up-regulated and basally regulated genes in the susceptible wheat genotype may contribute to stomatal closure. Figures 3.4f-j show the photosynthetic efficiency of the spikes after the plants were subjected to drought (f), 24 hpi (g), 48 hpi (h), 72 hpi (i) and 10 dpi (j). Their photosynthetic efficiency was not significantly affected by drought and did not vary at 48 hpi, while the two genotypes differed significantly among themselves at 24 and 72 hpi but not compared to their respective mock controls. Sumai3 maintained a higher photosynthetic efficiency than Rebelde at 24 (0.784 and 0.735, respectively) and 48 hpi (0.778 and 0.702, respectively). Moreover, the observations performed by using the optical microscopy demonstrated that stomata remained open under mock and drought conditions and at 10 dpi in both the wheat genotypes, they were partly open at 24, 48 and 72 hpi in Rebelde and closed at the same time-points in Sumai3 (Supplementary figure 3.5). Additionally, the Pearson's correlation (Figures 3.5a-b) revealed that there is a moderate positive correlation between the temperature variation and the genotypic effect (Figure 3.5a) and a strong

positive correlation between the photosynthetic efficiency variation and the genotypic effect (Figure 3.5b) under the inoculation condition. These results confirmed that the increasing of temperature and photosynthetic efficiency are closely related to resistance to FHB.

3.4 Discussion

In the present study we isolated and annotated the genes involved in stomatal regulation from the bread wheat Rebelde and Sumai3 and we performed a homology and phylogeny analysis with related species. TPS is the enzyme involved in terpenoid biosynthesis, the precursors of ABA. TPS enzymes were largely studied in rice, where it is well known that they accumulate strongly in plant tissues after mechanical damage, but very little is known in other cereal-related species. In this study, a *TPS* sequence annotated as *ent*-kaurene synthase *KSL* was identified (Zhou *et al.*, 2012). The next reaction modulating stomatal movements is catalysed by *ZEP*. *ZEP* was the first gene isolated from *Arabidopsis* among the genes involved in stomatal regulation and was up-regulated during water stress (Audran *et al.*, 2001). The signalling pathway continues with the activation of *NCED*. *NCED* has been extensively characterized in *Arabidopsis*. In bread wheat, two genes have been cloned, *TaNCED1* and *TaNCED2*, responding to low temperature, drought, NaCl and ABA application (Tong *et al.*, 2017). AAOs catalyses the final step of carotenoid catabolism. AAOs are involved in the last reaction for positively regulating the stomatal closure. In wheat, three AAO isoforms were annotated, showing a central role in the accumulation of carotenoids in kernels (Colasuonno *et al.*, 2017). The CYP450 superfamily is the largest enzymatic protein family found in plants and is involved in the biosynthesis of diverse plant pigments, such as carotenoids and anthocyanins, as well as the regulation of hormone metabolism (Xu *et al.*, 2015). In bread wheat, two genes annotated as *TaCYP78A3* and *TaCYP707A1B* have been characterized; the functionality of *TaCYP78A3* is strongly correlated with kernel size, while *TaCYP707A1B* affected ABA level and dormancy in seed (Son *et al.*, 2016). BG plays a key role both as positive and negative regulator of stomatal closure by conjugating and deconjugating ABA. BG was up-regulated by drought stress and its knockdown caused defective stomatal movement, early germination and abiotic stress-sensitive phenotypes (Seiler *et al.*, 2011). BG has been also widely investigated for its antifungal activity, since it has been classified as a pathogenetic-related protein (Balasubramanian *et al.*, 2012). Among the large family of plant receptors, the *REC* family was amply characterized in *Arabidopsis* and rice as a class of membrane-localized receptors (Park *et al.*, 2009; He *et al.*, 2014). In wheat, two *RECs* were identified and one of them, *Ta_PYL4AS_A*, seemed to be associated with FHB susceptibility (Gordon *et al.*, 2016). *RECs* are able to interact with ABI to modulate the stomatal movements. ABIs are protein phosphatase type 2C class (PP2Cs) and have been investigated for their functions regarding plant growth, development and stress responses in *Arabidopsis* (Park *et al.*, 2009). The resulting signalling pathway generated

from the different modulation of the previous genes activates the cascade of CDPKs and MAPKs. The CDPKs represent a class of Ca^{2+} sensors capable of modulating its intracellular levels in response to hormones, light, abiotic stress and pathogen elicitors (Ding *et al.*, 2013). In wheat, 20 CDPKs have been identified, responding to cold, hydrogen peroxide, ABA, gibberellic acid (GA), powdery mildew and sharp eyespot (Wei *et al.*, 2016). MAPK pathways are relevant actors in signal transduction events, functioning downstream as sensors/receptors and converting signals into cellular responses (Danquah *et al.*, 2014). In bread wheat, 54 MAPKs have been identified and 84% of these were up-regulated by heat, cold, drought or salt stress (Goyal *et al.*, 2018). Activation of MAPKs as modulator of stomatal movements is linked to the production of ROS, leading to an establishment of a positive feedback loop involving the up-regulation of ROS-producing and antioxidant genes (Lin *et al.*, 2009). ROS have been identified as key molecules positively regulating stomatal closure and several studies in Arabidopsis, maize and wheat have identified RBOHs as a source of ROS in guard cells. The roles of ROS in plant defence have been studied in detail, since exposure of plant cells to avirulent pathogens or elicitors triggers a rapid and prolonged oxidative burst (Desikan *et al.*, 2004). In bread wheat, 46 RBOHs were identified as participants in cell-wall biosynthesis, defence responses and signal transduction (Navathe *et al.*, 2019). JA and MeJA play a key role in stomatal regulation. AOS, HPL and MYB represent the core of the JA cascade. In bread wheat, three copies of AOS have been cloned (Wu *et al.*, 2004). AOS is strictly related to HPL activity (Liu *et al.*, 2012). AOS and HPL were first attributed to the same gene family, until HPL was characterized in Arabidopsis, rice and maize as a protein strongly accumulated in the inflorescence and localized in plastids (Gao *et al.*, 2008). MYB transcription factors are regulatory proteins implicated in plant growth, secondary metabolism, hormone signal transduction, disease and abiotic stress resistance were identified (Xiong *et al.*, 2014; Yu *et al.*, 2016). In bread wheat, 23 gene fragments and 6 nearly complete open-reading frames (ORFs) encoding putative MYBs, implicated in providing to tolerance to salt, in lignin biosynthesis, fructan accumulation and increased spike and grain weight were isolated. In the present study, we identified a MYB sequence previously annotated as *TaPIMP* (Kooiker *et al.*, 2013).

Transcriptional analysis revealed that drought stress did not induce the selected genes. Although we hypothesised that the stomatal closure promoting genes were up-regulated because of drought stress, many studies are in agreement with our results which support the hypothesis that hydric conditions induce stomatal closure in drought-sensitive wheat varieties. By way of contrast, drought-tolerant cultivars correlate with lower level of closure-inducing genes and higher expressions of genes negatively regulators of stomatal closure (Ji *et al.*, 2011). Therefore, since we observed a basal regulation of *TaBG*, *TaCYP450* and *TaGAPDH*, while only *TaZEP* was up-regulated in Rebelde, and a slight up-regulation of the same genes in Sumai3, we can hypothesise that the two wheat genotypes

could be drought-tolerant, especially due to the fact that GAPDH is particularly sensitive to changes in photosynthesis by abiotic and biotic stresses (Yang *et al.*, 1993; Zhang *et al.*, 2013). These results could be supported by three studies, whereas in drought-tolerant wheat cultivars the up-regulation of *CYP* and *BG* and the down-regulation of *ZEP*, *NCED* and *AAO* have been demonstrated (Xue *et al.*, 2006; Rampino *et al.*, 2012; Gallé *et al.*, 2013).

Many transcriptomic research studies have embraced the concept of comparing the response of a resistant and susceptible genotype to *Fusarium* spp. infection, in order to explore and uncover the plant-pathogen molecular responses. Most of the transcriptomic studies pointed out that the establishment of an early and prompt defence response is a decisive feature that contributes to FHB resistance (Pritsch *et al.*, 2000; Ding *et al.*, 2011; Foroud *et al.*, 2012). Soresi *et al.* (2015) found that several stomatal movements responsive elements were induced in the resistant Langdon wheat line but not in its susceptible parental line (Soresi *et al.*, 2015). Dhokane *et al.* (2016) established a transcriptome analysis revealing that MYB transcription factors were up-regulated only in the resistant wheat genotype carrying the *Fhb2* (Dhokane *et al.*, 2016).

Much evidence supported our results indicating that the *CYP450* gene family plays an active role in wheat resistance against *F. graminearum* and DON. In actual fact, strong *CYP450* accumulations were found in resistant but not in susceptible wheat cultivars challenged with *F. graminearum* and DON (Li *et al.*, 2010; Gunupuru *et al.*, 2018). *CYP450*s are involved in the detoxification of xenobiotic substances. Hence, the induction of *CYP450* by *F. graminearum* in the resistant wheat genotype might denote a resistant physiological mechanism to FHB toxic metabolites, such as the mycotoxin DON. This hypothesis was confirmed by several studies demonstrating that *CYP450*s were able to detoxify DON *in vitro* (Ito *et al.*, 2013) and *in vivo* (Gunupuru *et al.*, 2018). De Zutter *et al.* (2017) investigated plant-response in wheat ears to a combined attack of *F. graminearum* and aphids and observed that *PR1* and *PR2*, the latter encoding a β -1,3-glucanase, were consistently up-regulated (De Zutter *et al.*, 2017). Our results are in agreement with those obtained by the previously cited authors. Of the negative regulator genes, *TaREC* was mainly down-regulated. This may be due to the possibility that *TaREC* may be involved in FHB susceptibility. Gordon *et al.* (2016) found that *REC* silencing in bread wheat resulted in lower FHB symptoms progression and decreased DON content in wheat heads (Gordon *et al.*, 2016). JA and its derivate MeJA, are positive regulators of FHB resistance in wheat and CDPKs are the enzymes favouring ABA and JA crosstalk. There is some evidence that *CDPKs* may be involved in wheat responses to some fungal diseases, like powdery mildew and sharp eyespot, but further information regarding FHB is not available (Li *et al.*, 2008; Wei *et al.*, 2016). We also investigated the role of *TaMAPK*, which was induced in Sumai3 at 24 and 48 hpi. In wheat, a gene labelled as *TaFLRS*, turned out to be a MAP kinase transcriptionally up-

regulated in *F. graminearum* inoculated spikes (Gao *et al.*, 2011). As far as we know, no information regarding the role or the mode of action of *TaKSL*, *TaZEP*, *TaNCED*, *TaAAO*, *TaABI* and *TaHPL* as responsive genes during wheat-*F. graminearum* interaction is available. Therefore, this work provided evidence for the first time that these genes are induced by *F. graminearum* in Sumai3, suggesting its role in FHB resistance.

Our research seems to make clear that the induction of stomatal regulation genes is a characteristic of the resistant Sumai3 genotype. The two QTLs most effective in contrasting FHB, *Fhb1* and *Qfhs.ifa-5A*, are derived from the genome of Sumai3, playing a key role in the regulation of FHB-responsive genes. Both QTLs contribute to FHB resistance by inducing several genes including *MYC*, *MYB* transcription factors and AOS (Gunnaiah *et al.*, 2012; Zhuang *et al.*, 2013; Ma *et al.*, 2020).

Despite the fact that the function of ABA as a response to abiotic stress has been well documented, its role in plant defence is more obscure. For instance, ABA seems to have an ambivalent role during the pathogen defence mechanisms; hence, its role as positive or negative regulator during the pathogen infection has not already been disclosed (Asselbergh *et al.*, 2008). Nevertheless, the bulk of evidence is leaning more toward ABA as a susceptible factor, at least with respect to fungal pathogens (Mauch-Mani and Mauch, 2005; Asselbergh *et al.*, 2008; Wang *et al.*, 2018). The role of ABA and *TaREC* in mediating susceptibility to FHB might explain why the genes involved in the negative regulation of stomatal closure (*TaCYP450* and *TaBG*) were importantly up-regulated in Sumai3, while *TaREC* was mainly down-regulated in the resistant wheat line.

After drought stress, neither spike temperature nor photosynthetic efficiency were negatively affected, reflecting the results obtained by transcript analysis, supporting the hypothesis that the two genotypes tolerate hydric stress. A morphological explanation for wheat tolerance to drought was provided by Ding *et al.* (2017), who observed that stomata were equally present on the abaxial and adaxial sides of glumes and lemmas, providing the first evidence of amphistomatous traits of wheat ears, which might explain how wheat spikes can maintain high photosynthetic rates even under conditions of drought (Ding *et al.*, 2017). Several plant metabolic pathways like photosynthesis change as an infection is established (Hill-Ambroz *et al.*, 2006; Pérez-Clemente *et al.*, 2015; Jaime *et al.*, 2019). Hence, chlorophyll fluorescence has been already used with success to estimate the fungal and bacterial disease severity, suggesting that changes in the chlorophyll metabolism and photosynthesis-related parameters are associated to the infection establishment (Wagner *et al.*, 2006; Bueno *et al.*, 2017). However, to date, the specific connection between *F. graminearum*-wheat interaction and photosynthetic parameters has not been sufficiently demonstrated. One consistent research study has evaluated the effect of *F. graminearum* infection on the photosynthetic rate of wheat flag leaves in resistant and susceptible lines. The authors observed that FHB caused more

significant reduction in the net photosynthetic rate of flag leaves in the resistant line (Yang *et al.*, 2016). These results are not in agreement with those observed by us in the present study, but this discordance may be due to several factors affecting the experimental settings. In actual fact, the authors in question evaluated the parameters at 7 and 21 dpi on the flag leaves while we considered earlier time-points in spikes. Hence, the present work represents the first evidence that connects the increasing spike temperatures with putative FHB resistance. Therefore, we might hypothesize that increases in spike temperatures at 24, 48 and 72 hpi and in photosynthetic efficiency at 24 and 72 hpi in Sumai3 compared to Rebelde, may be linked to a differential rate of stomatal conductance regulation mediated by an early FHB resistance response. This physiological mechanism could lead to an enhanced photosynthesis related to FHB resistance, as supported by the induction of transcript levels of *TaGAPDH* in the present study and as already observed by Zhang *et al.* (2013), who reported the accumulation of the protein after 72 hpi (Zhang *et al.*, 2013).

3.5 References

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3.6 List of tables

Supplementary table 3.1 A list of selected genes, genomic location, accession numbers, gene functions, primer pairs and amplicon lengths from DNA and cDNA. Primer pairs used in Real-Time qPCR are identified by the letter “q” in their names, with the exception of primer pairs for *TaGAPDH*, *TaPR1*, *TaACT*, *TaTUB* and *TaFNR*, where the same primer pairs were used both for PCR and Real-Time qPCR. The genomic location derived from the IWGSC database (IWGSC_ref_v1) (<https://wheat-urgi.versailles.inra.fr/>), while the accession numbers refer to the queries that matched on BLASTn (<https://blast.ncbi.nlm.nih.gov/>).

Gene, genomic location and accession number	Function	Primer name	Primer pairs (5'-3')	bp (DNA)	bp (cDNA)
<i>TaAOS</i> 4B:495147007-495149759 BLASTn: AY196004	Allene oxide synthase	TaAOS_F TaAOS_R TaAOS_qF TaAOS_qR	GTTAGCTGCTCGTGATCGAT TGCAGCAGCTTGCTTCTCTC TCGGGCGTATTGCTGAGG TGCAGCAGCTTGCTTCTCTC	1270 352	352
<i>TaHPL</i> 6D:13059842-13062314 BLASTn: AK335301	Hydroperoxide lyase	TaHPL_F TaHPL_R TaHPL_qF TaHPL_qR	GCAGCTGGCCAGTGAGCT GTGAAGGCGGTGCCGTCGCA GGACCACAGCATGACCGACA GAACCCGACCTCGCCGTT	1520 190	190
<i>TaKSL</i> Un:12441012-12441346 BLASTn: AB597957	Terpene synthase	TaKSL_F TaKSL_R TaKSL_qF TaKSL_qR	GGAACGGGATGCTAGAATACG TCTCTAACAACCATCCTTAG GGAACGGGATGCTAGAATACG CCTCTCCTTATGTGGTCGGA	2075 282	282
<i>TaAAO</i> 7B:687590126-687591793 BLASTn: AK331622	ABA-aldehyde oxidase	TaAAO_F TaAAO_R TaAAO_qF TaAAO_qR	TCGACGTTGCACAACACGAT GTAGTCTACAGTTCATCACA CCTGAGCAATCAAAGCATCCC TCAGGCTGACACGCTGAAC	2575 182	182
<i>TaREC</i> 2D:37645390-37646533 BLASTn: AK335719	ABA receptor	TaREC_F TaREC_R TaREC_qF TaREC_qR	GCTCCGACCTCGAGCCGATG ATCTTATCATCATCTTAC GCTGGAGATCCTGGACGAC GTTGCACTTGACGATGGTGT	1020 217	217
<i>TaBG</i> 3B:771171542-771172472 BLASTn: Y18212	β -1,3-glucanase	TaBG_F TaBG_R TaBG_qF TaBG_qR	GCGTGATCGGCAACAACCTC CGAAGCTCCTCTCCGTGGCG AACGTGCGCCCTACTACC GCGTGAACAGGCTCGTGTA	855 398	398
<i>TaMAPK</i> 4A:120605915-120606435 BALSTn: AF079318	Mitogen activated protein kinase	TaMAPK_F TaMAPK_R TaMAPK_qF TaMAPK_qR	ATCATCAGCTCAGCCTCAGC TACAGACAGTTTATAACCGA CATCGACGTCGTGTCGGT GTCTCGTTCCGGATGAATC	2618 154	154
<i>TaCDPK</i> 2D:161373811-161376953 BLASTn: KU516994	Calcium dependent protein kinase	TaCDPK_F TaCDPK_R TaCDPK_qF TaCDPK_qR	GGGAGCCTCGTGTGTTGGCG GTACTTGTATCTGCAACGA CTTCTTTGTGGTGTCCTCC GCTGTCAAACGCCTCCTT	1647 436	436
<i>TaCYP450</i> 7D:258236253-258238309 BLASTn: XM_020311851	Cytochrome P450	TaCYP_F TaCYP_R TaCYP_qF TaCYP_qR	GTATTGGTGGACGAGGAAGG ACTGTCACACTCACTTCCTG GTATTGGTGGACGAGGAAGG ATCTGGCGTGCGATCACT	1810 411	411
<i>TaNCED</i> 5A:572021505-572023279 BLASTn: KX711891	Epoxycarotenoid dioxygenase	TaNCED_F TaNCED_R TaNCED_qF TaNCED_qR	CGGTGGAGAGGCAGGAGAA CCGTGGAAGCCGTACGGCAC CGGTGGAGAGGCAGGAGAA CGCGCGTAGAACAGAGCA	1534 472	472
<i>TaABI</i> 3B:378687185-378688024 BLASTn: AB238930	Phosphatase	TaABI_F TaABI_R TaABI_qF TaABI_qR	ATGGAGGACGTGGCCGTGGC TGTGCAATGGTAGTATAG TAGCAGATTACTGTCGGGATCGG GAGCAGATGACAGCGACCA	2450 283	283
<i>TaPIMP</i> 1B:615731797-615736596 BLASTn: KX683396	MYB domain transcription factor	TaPIMP_F TaPIMP_R TaPIMP_qF TaPIMP_qR	TGCCTAGCTCGTGGGAGTAG ACTAATGGTATCAATGTTCA GTCACAGATCGCGTCGCAC GCATCCGAACCTGGCCGTACA	1307 325	325
<i>TaRBOH</i> 3A:509419957-509424756 BLASTn: AY561153	NADPH oxidase	TaRBOH_F TaRBOH_R TaRBOH_qF TaRBOH_qR	CTTCTCAATTACTTCAGCAC TAGTTCCAGAGACAACATCG TTGTTGGATTAGGAATTGGTGCT TGATCCATGTCGGCAATCTC	1010 441	280

<i>TaZEP</i> 2A:542649865- 542654664 BLASTn: AF384103	Zeaxanthin epoxidase	TaZEP_F TaZEP_R TaZEP_qF TaZEP_qR	AAGGTCGTGTCACCTTGCTT GGCACAACAATGTACTGTACTAGG CTTGTCACCAGCCACTGTAC GGCACAACAATGTACTGTACTAGG	2590 236	236
<i>TaGAPDH</i> – KR029493.1	Glyceraldehyde-3- phosphate dehydrogenase	TaGAPDH_F TaGAPDH_R	AGTTCATGCCATGACTGCAA CCAGTGCTGCTTGGAATGATG	105	105
<i>TaPR1</i> - AJ007348	Pathogenesis related protein-1	TaPR1_F TaPR1_R	ACTACGACTACGGGTCCAACA TCGTAGTTGCAGGTGATGAAG	145	145
<i>TaACT</i> - AB181991	Actin	TaACT_77F TaACT_312R	TCCTGTGTTGCTGACTGAGG GGTCCAAACGAAGGATAGCA	350	236
<i>TaTUB</i> - TAU76745	β -tubulin2	TaTUB_F TaTUB_R	CGAGGAGGGCGAGTACGA AGCAAAGCACGACATGGACAT	79	79
<i>TaFNR</i> - AJ457980	Ferredoxin - NADP(H)- oxidoreductase	TaFNR_F TaFNR_R	CACCGGCCCCAGTGATCTT AAGGGCGTCTGCTCCAAC	259	69

Supplementary table 3.2 Genomic location of homologous sequences used to obtain homology and phylogeny trees. The full-length sequences were extracted from Gramene (www.gramene.org).

Species/gene	TaAOS	TaHPL	TaKSL	TaAAO	TaREC	TaBG	TaMAPK
<i>T. aestivum</i> cv.	4B:49514700	6D:13059842	Un:12441012	7B:68759012	2D:37645390	3B:77171542	4A:12060591
<i>Chinese spring</i>	7-495149756	-13062314	-12441346	6-687591793	-37646533	-771172472	5-120606435
<i>A. tauschii</i>	4D:40891258	6D:12439081	2D:11789605	7D:61034659	2D:39227325	3D:58883492	4D:35287390
	4-408914208	-12441898	-11789886	8-610348258	-39228468	2-588835850	2-352874421
<i>H. vulgare</i>	4H:55155354	6D:12439081	/	7H:63869282	4H:46307633	3H:66923156	4H:48089422
	4-551555019	-12441898	/	6-638694028	1-463076591	4-669232045	9-480894749
<i>O. sativa</i>	3:6608878- 6609307	2:555800- 556066	4:5430598- 5430721	/	3:10419228- 10419521	1:41365909- 41366298	3:9848881- 9849220
<i>Z. mays</i>	1:29125887- 29126242	4:245807235- 245807497	/	/	1:46498895- 46499188	3:153225680- 153225857	9:141028451- 141028896

Species/gene	TaCDPK	TaCYP450	TaNCED	TaABI	TaPIMP	TaRBOH	TaZEP
<i>T. aestivum</i> cv.	2D:16137381	7D:25823625	5A:57202150	3B:37868718	1B:61573179	3A:50941995	2A:54264986
<i>Chinese spring</i>	1-161376953	3-258238309	5-572023279	5-378688024	7-615736596	7-509424756	5-542654664
<i>A. tauschii</i>	2D:16287739	7D:26007997	5D:46159456	3D:29155543	1D:45662831	3D:39593271	2D:40335229
	4-162880536	2-260082028	7-461596180	4-291556273	0-456628952	7-395933514	0-403353197
<i>H. vulgare</i>	2H:18643731	7H:24384397	5H:58966265	3H:36009716	1H:52219373	3H:52860960	2H:57091798
	3-186437726	3-243844915	9-589664370	4-360097638	9-522194042	0-528610140	3-570918742
<i>O. sativa</i>	7:22861699- 22861962	8:274222124- 27422381	3:24959628- 24960996	1:22618746- 22618897	5:26988130- 26988258	1:30632735- 30632942	4:22374018- 22374264
<i>Z. mays</i>	2:212773115- 212773455	1:210345242- 210345520	1:255021702- 255023080	3:224588858- 224589031	6:166302733- 166302865	3:203440771- 203441051	2:46298949- 46299167

Supplementary table 3.3 Amplification efficiency (E) and R² values for each selected gene amplified taken from each wheat genotype (Rebelde and Sumai3). The E and R² values were calculated as follows: five 1:10 serial dilutions (1:1–1:10000) were obtained for each sample of cDNA and amplified in four technical replicates obtained from four independent biological replicates. E and R² values were calculated by the slope of the standard curve obtained by plotting fluorescence versus serial dilution concentrations using the equation $E = 10^{\left(-\frac{1}{\text{slope}}\right)} - 1$. The E and R² values for the *TaTUB* taken from Rebelde were not calculated (x), because amplification did not occur at 1:100, 1:1000 and 1:10000 cDNA dilutions.

Gene	Rebelde		Sumai3	
	E	R ²	E	R ²
<i>TaAOS</i>	1.0633	0.9972	1.1191	0.9967
<i>TaHPL</i>	1.0336	0.9970	0.9759	0.9920
<i>TaKSL</i>	1.1207	0.9972	1.1094	0.9985
<i>TaAAO</i>	1.0747	0.9967	1.0643	0.9953
<i>TaREC</i>	0.9787	0.9982	1.2463	0.9911
<i>TaBG</i>	1.0967	0.9907	1.1484	0.9965
<i>TaMAPK</i>	0.9022	0.9955	0.9763	0.9954
<i>TaCDPK</i>	1.0815	0.9967	0.9606	0.9949
<i>TaCYP450</i>	1.0485	0.9925	1.0854	0.9925
<i>TaNCED</i>	1.1080	0.9944	1.0873	0.9922
<i>TaABI</i>	1.1746	0.9972	0.9012	0.9957
<i>TaPIMP</i>	1.1826	0.9934	1.0508	0.9939
<i>TaRBOH</i>	0.9844	0.9954	0.9462	0.9969
<i>TaZEP</i>	1.1052	0.9984	1.0785	0.9891
<i>TaGAPDH</i>	1.0249	0.9842	1.0354	0.9857
<i>TaPR1</i>	0.9056	0.9956	1.0893	0.9965
<i>TaACT</i>	1.1748	0.9954	1.1694	0.9961
<i>TaTUB</i>	x	x	1.0448	0.9849
<i>TaFNR</i>	0.8988	0.9835	0.9249	0.9661

Supplementary table 3.4 Quantification cycles (Cq) and relative standard errors (SE) obtained from five 1:10 serial dilutions of cDNA and used to evaluate the stability of each reference gene (*TaACT*, *TaTUB* and *TaFNR*) chosen from among the different plant treatments (mock, drought stress, 24 hpi, 48 hpi, 72 hpi and 10 dpi). Cq values were calculated from four technical replicates derived from four independent biological replicates. *TaTUB* from Rebelde did not occur at 1:100, 1:1000 and 1:10000 cDNA dilutions, so no Cq values were detected (nd), thus, the SE was not calculated (x).

Reference gene	Dilution	Rebelde		Sumai3	
		Cq	SE (±)	Cq	SE (±)
<i>TaACT</i>	1:1	19.51	0.194	19.63	0.348
	1:10	22.20	0.211	22.38	0.342
	1:100	24.98	0.242	25.15	0.343
	1:1000	27.79	0.266	28.00	0.311
	1:10000	31.53	0.246	31.69	0.357
<i>TaTUB</i>	1:1	36.65	0.638	19.54	1.366
	1:10	39.51	0.770	23.08	1.794
	1:100	nd	x	25.22	1.440
	1:1000	nd	x	30.11	1.570
	1:10000	nd	x	32.12	1.966
<i>TaFNR</i>	1:1	12.07	2.563	13.45	2.122
	1:10	14.05	2.014	16.69	2.665
	1:100	17.73	2.962	18.26	2.135
	1:1000	22.62	2.136	22.91	2.352
	1:10000	25.74	2.336	27.92	2.235

Supplementary table 3.5 Relative expression levels and standard errors of each gene selected for each genotype (Rebelde and Sumai3) and for each plant-treatment mode (drought stress, 24 hpi, 48 hpi, 72 hpi and 10 dpi). Relative expression values were obtained by using the equation $2^{-\Delta\Delta C_q}$ with *TaACT* as a reference gene and mock treatments used to normalize the relative expression levels. The data represent averages and standard errors for the four independent biological replicates and the four technical replicates examined. The data were subjected to two-way analysis of variance (ANOVA), where the two independent variables were the wheat genotype (Rebelde and Sumai3) and the plant treatment (drought stress, 24 hpi, 48 hpi, 72 hpi and 10 dpi) while the dependent variable was the relative expression level. Different letters among the plant treatments and wheat genotypes were assessed by means of significant F values by applying the Tukey Honestly Significant Difference test (Tukey test) at 0.99 confidence level ($p < 0.01$).

		Drought stress		24 hpi		48 hpi		72 hpi		10 dpi	
Gene		Rebelde	Sumai3	Rebelde	Sumai3	Rebelde	Sumai3	Rebelde	Sumai3	Rebelde	Sumai3
<i>TaAOS</i>	Relative expression	0.298	0.481	0.293	2.672	0.392	3.178	0.181	1.567	0.161	1.603
	Standard error ($\pm$)	0.028	0.093	0.032	0.783	0.061	0.219	0.029	0.285	0.034	0.306
	$p < 0.01$	cd	cd	cd	ab	d	a	d	bc	d	bc
<i>TaHPL</i>	Relative expression	0.510	1.091	0.515	7.895	0.319	12.556	0.268	3.246	0.009	0.717
	Standard error ($\pm$)	0.016	0.038	0.012	1.550	0.026	0.602	0.025	0.131	0.003	0.204
	$p < 0.01$	d	cd	d	b	d	a	d	c	d	cd
<i>TaKSL</i>	Relative expression	0.372	0.279	0.592	2.621	1.617	2.021	0.719	0.710	0.401	1.025
	Standard error ($\pm$)	0.084	0.091	0.190	0.593	0.344	0.357	0.023	0.230	0.069	0.592
	$p < 0.01$	b	b	b	ab	ab	ab	b	b	b	b
<i>TaAAO</i>	Relative expression	0.036	0.266	0.038	1.702	0.036	1.290	0.026	0.178	0.013	0.202
	Standard error ($\pm$)	0.005	0.017	0.003	0.460	0.005	0.085	0.009	0.023	0.007	0.045
	$p < 0.01$	c	b	c	a	c	a	c	bc	c	bc
<i>TaREC</i>	Relative expression	0.381	0.221	1.048	1.300	0.361	4.197	0.322	0.259	0.213	0.172
	Standard error ($\pm$)	0.075	0.009	0.091	0.346	0.017	1.263	0.014	0.015	0.044	0.054
	$p < 0.01$	b	b	b	b	b	a	b	b	b	b
<i>TaBG</i>	Relative expression	0.987	4.313	1.302	70.644	0.941	50.534	0.919	25.137	5.668	8.452
	Standard error ($\pm$)	0.056	0.418	0.154	19.553	0.013	10.159	0.317	2.994	1.974	4.575
	$p < 0.01$	b	b	b	a	b	a	b	b	b	b
<i>TaMAPK</i>	Relative expression	0.713	0.754	0.555	7.376	1.412	6.129	1.098	1.993	3.742	1.572
	Standard error ($\pm$)	0.088	0.107	0.072	1.113	0.113	0.313	0.042	0.021	0.821	0.214
	$p < 0.01$	c	c	c	a	c	a	c	bc	b	bc
<i>TaCDPK</i>	Relative expression	0.623	0.808	0.614	2.321	0.650	2.494	0.545	0.465	0.458	0.588
	Standard error ($\pm$)	0.052	0.026	0.079	0.471	0.073	0.129	0.030	0.240	0.091	0.094
	$p < 0.01$	b	b	b	a	b	a	b	b	b	b
<i>TaCYP450</i>	Relative expression	0.920	1.743	0.885	39.768	0.657	53.172	0.372	7.589	0.029	5.126
	Standard error ($\pm$)	0.069	0.517	0.095	11.314	0.073	6.406	0.066	0.958	0.014	1.594
	$p < 0.01$	b	b	b	a	b	a	b	b	b	b
<i>TaNCED</i>	Relative expression	0.663	0.597	0.636	2.732	0.721	3.315	0.702	0.730	0.105	0.663
	Standard error ($\pm$)	0.071	0.106	0.032	0.441	0.044	0.615	0.034	0.026	0.012	0.115
	$p < 0.01$	b	b	b	a	b	a	b	b	b	b
<i>TaABI</i>	Relative expression	0.661	0.378	0.522	5.791	1.044	5.979	0.604	1.175	0.070	1.094
	Standard error ($\pm$)	0.044	0.222	0.094	1.399	0.034	0.701	0.031	0.176	0.011	0.084
	$p < 0.01$	b	b	b	a	b	a	b	b	b	b
<i>TaPIMP</i>	Relative expression	0.916	0.004	0.818	1.918	1.221	2.409	0.462	0.352	0.019	0.368
	Standard error ($\pm$)	0.046	0.000	0.092	0.469	0.117	0.008	0.048	0.020	0.003	0.124
	$p < 0.01$	bc	d	bc	a	b	a	cd	cd	d	cd
<i>TaRBOH</i>	Relative expression	0.371	1.073	0.547	1.007	0.472	1.152	0.199	0.226	0.038	0.441
	Standard error ($\pm$)	0.021	0.031	0.026	0.021	0.040	0.097	0.009	0.026	0.026	0.074
	$p < 0.01$	bc	a	b	a	b	a	cd	cd	d	b
<i>TaZEP</i>	Relative expression	2.638	1.533	0.838	7.708	1.314	14.446	1.032	1.657	0.035	1.310
	Standard error ($\pm$)	0.232	0.107	0.049	2.757	0.126	2.672	0.085	0.236	0.003	0.073
	$p < 0.01$	c	c	c	b	c	a	c	c	c	c
<i>TaGADPH</i>	Relative expression	1.238	1.462	0.899	1.579	1.025	2.284	1.147	2.467	1.053	3.027
	Standard error ($\pm$)	0.410	0.157	0.014	0.324	0.346	0.237	0.411	0.222	0.154	0.263
	$p < 0.01$	b	b	b	b	b	a	b	a	b	a
<i>TaPR1</i>	Relative expression	0.816	55.270	1.444	23.705	2.133	65.303	4.337	1.426	9.392	38.874
	Standard error ($\pm$)	0.055	1.910	0.286	1.201	0.132	7.020	0.756	0.154	1.155	2.036
	$p < 0.01$	e	b	e	d	e	a	e	e	e	c

3.7 List of figures

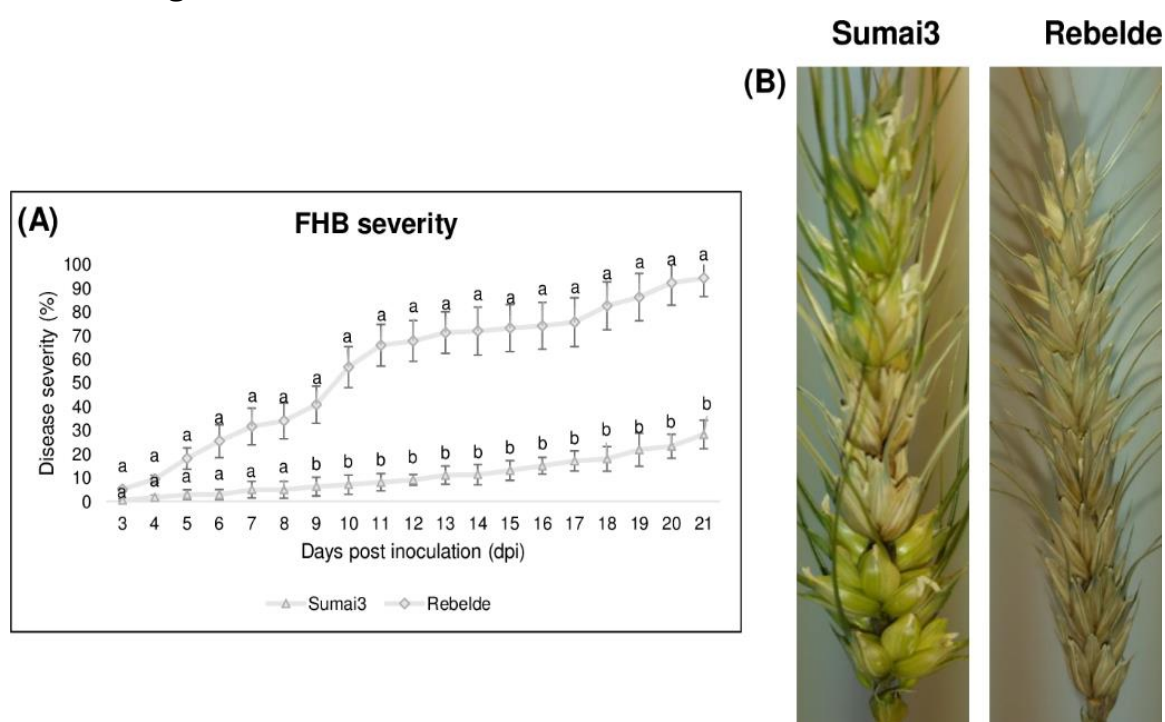


Figure 3.1 (A) Fusarium head blight (FHB) severity (%) in Sumai3 and Rebelde from 3 to 21 days post inoculation (dpi). (B) FHB symptoms in Sumai3 and Rebelde at 21 dpi. The data represent averages and standard errors from three independent replicates with at least 20 plants for each genotype. Different letters refer to the statistical analysis performed using one-way analysis of variance (ANOVA) with the Tukey test at a confidence level of 0.99 and $p < 0.01$.

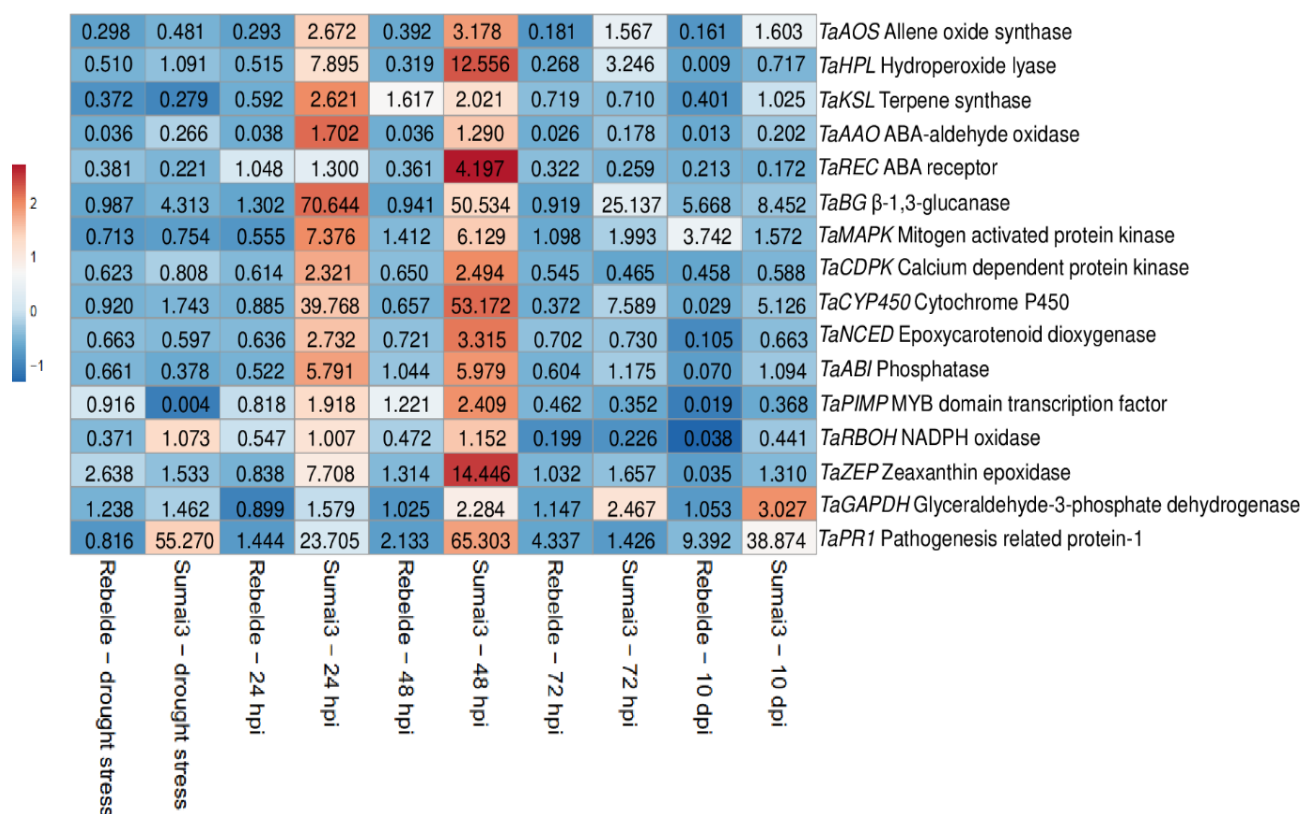


Figure 3.2 Heatmap related to the relative expression level of the genes selected. The relative expression levels are referred to the different plant treatments (drought stress, 24, 48, 72 hours post inoculation and 10 days post inoculation) and to the two wheat genotypes Sumai3 and Rebelde. Relative expression values were obtained by using the equation $2^{-\Delta\Delta C_q}$ with *TaACT* as reference gene and mock treatment used to normalize the relative expression levels. The heatmap was constructed with the z-score by analysing data with ClustVis software (Estonia).

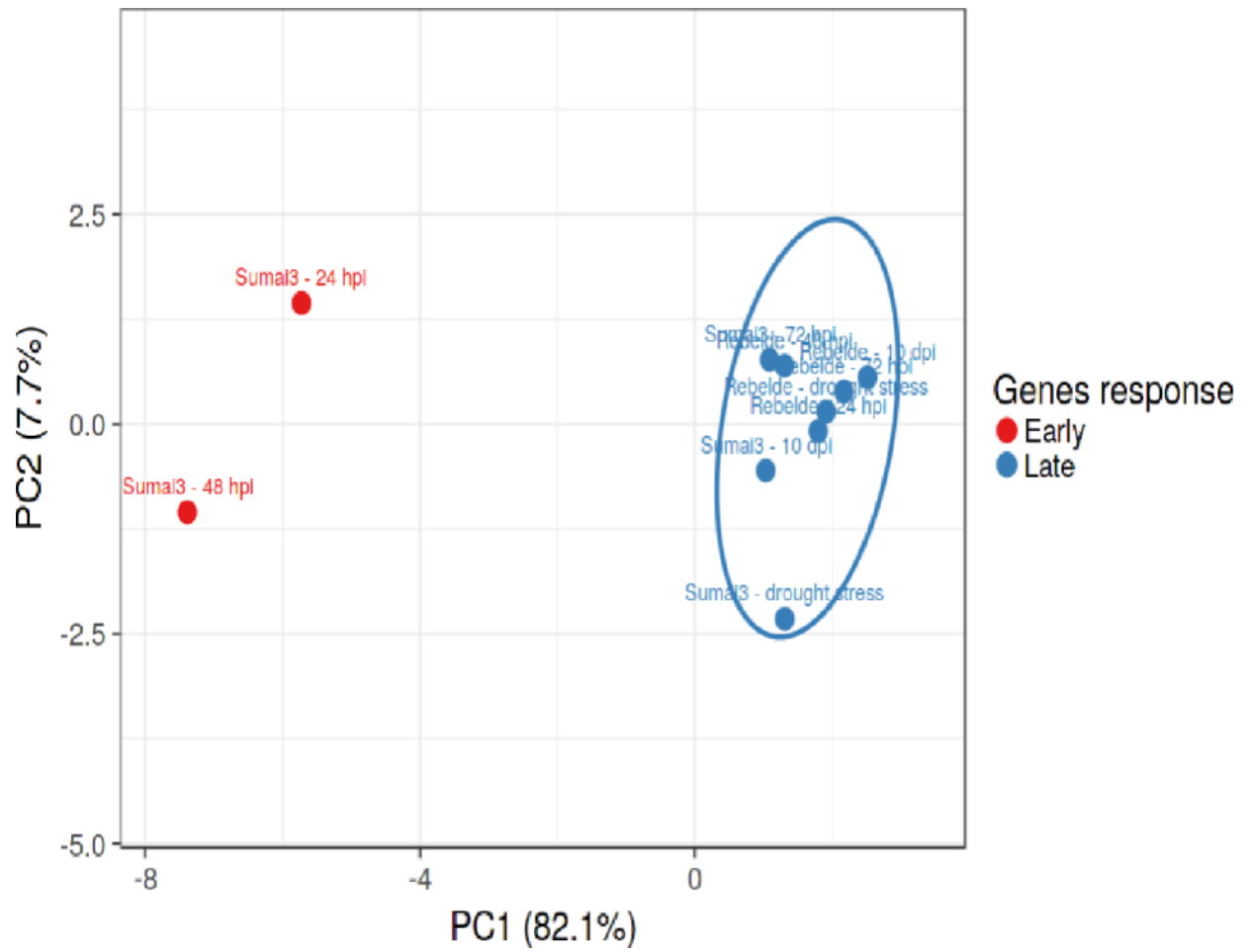


Figure 3.3 Principal component analysis (PCA) of the different plant treatments (drought stress, 24, 48, 72 hours post inoculation and 10 days post inoculation) and wheat genotypes (Sumai3 and Rebelde). The principal components 1 and 2 explain the 82.1% and 7.7% variability levels. PCA was conducted by using ClustVis software (Estonia).

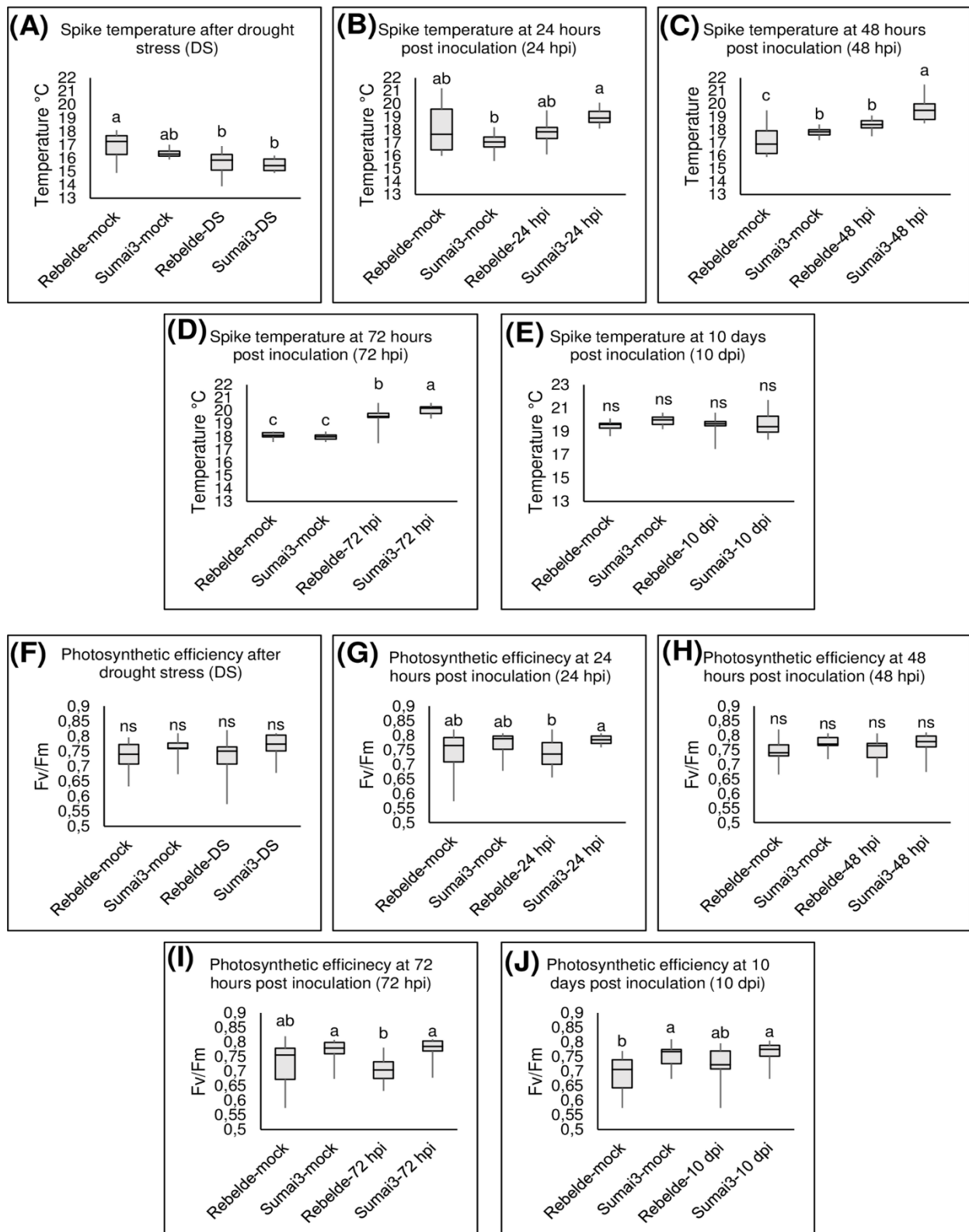


Figure 3.4 Box-plots of photosynthetic phenotypic parameters measured for each wheat genotype (Sumai3 and Rebelde) and each different plant treatment. a-e represent spike temperatures (°C) from plants subjected to drought stress (A), 24 hours post inoculation (B), 48 hours post inoculation (C), 72 hours post inoculation (D) and 10 days post inoculation (E). F-J represent the photosynthetic efficiency (F_v/F_m) of plants subjected to drought stress (F), 24 hours post inoculation (G), 48 hours post inoculation (H), 72 hours post inoculation (I) and 10 days post inoculation (J). F_v/F_m represents the variable-to-maximum-fluorescence ratio. The data represent averages and standard errors for three independent replicates with at least 20 plants for each genotype. Different letters refer to the statistical analysis performed using one-way analysis of variance (ANOVA) with the Tukey test at 0.95 or 0.99 confidence level and $p < 0.05$ or $p < 0.01$, while “ns” refers to not significant differences.

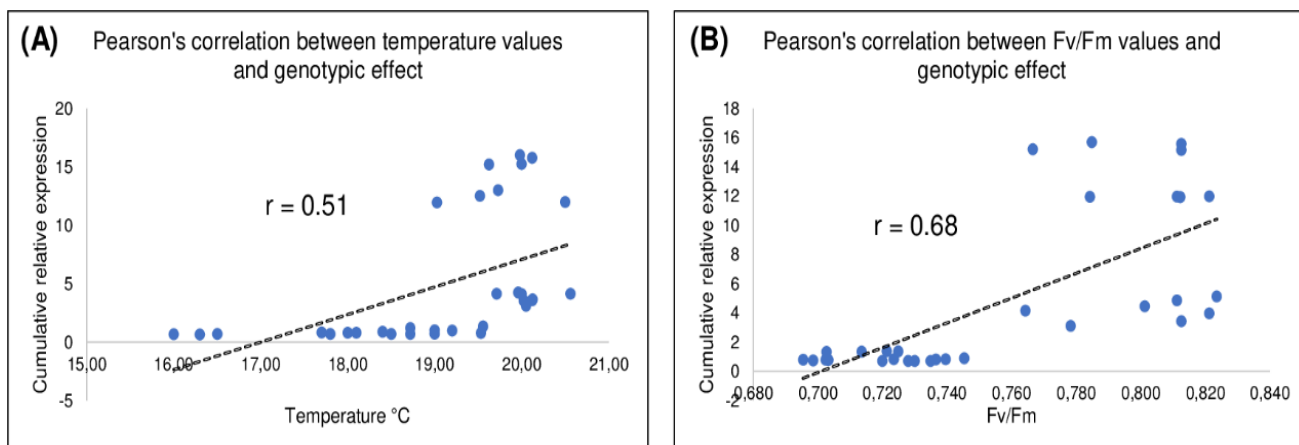
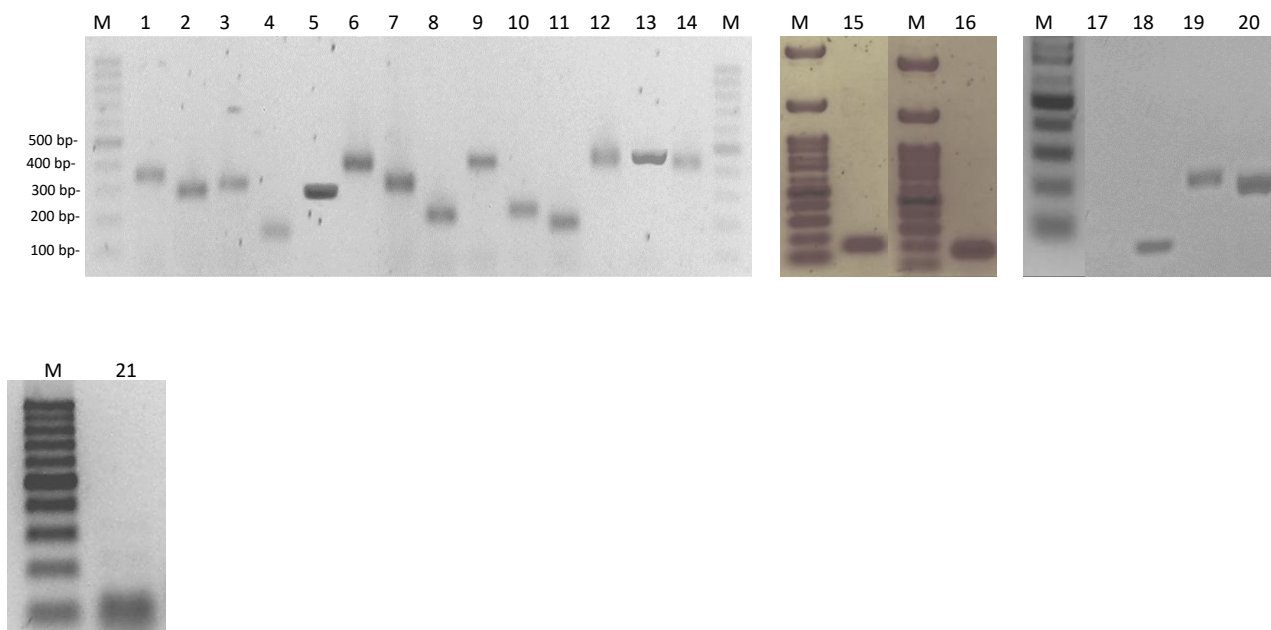


Figure 3.5 Pearson's correlation between (A) temperature values and genotype; (B) Photosynthetic efficiency (F_v/F_m) and genotype. Data show that higher temperature and photosynthetic efficiency values are positively correlated with an increasing of transcript levels in Sumai3 under *F. graminearum* inoculation. The Pearson's correlation was computed for $p < 0.05$.



Supplementary figure 3.1 1.5% agarose gel of selected genes amplified by using primer pairs for Real-Time qPCR reported in S1 Table. M) ExcelBand™ 100 bp + 3K DNA Ladder (Smobio), 1) *TaAOS*, 2) *TaABI*, 3) *TaACT*, 4) *TaPR1*, 5) *TaKSL*, 6) *TaBG*, 7) *TaPIMP*, 8) *TaREC*, 9) *TaCYP450*, 10) *TaZEP*, 11) *TaHPL*, 12) *TaCDPK*, 13) *TaNCED*, 14) *TaRBOH*, 15) *TaAAO*, 16) *TaMAPK*, 17) *TaTUB* from Rebelde (not detected), 18) *TaTUB* from Sumai3, 19) *TaFNR* from Rebelde, 20) *TaFNR* from Sumai3, 21) *TaGAPDH* from Rebelde. The figure represents the original picture of the gel.

TaKSL

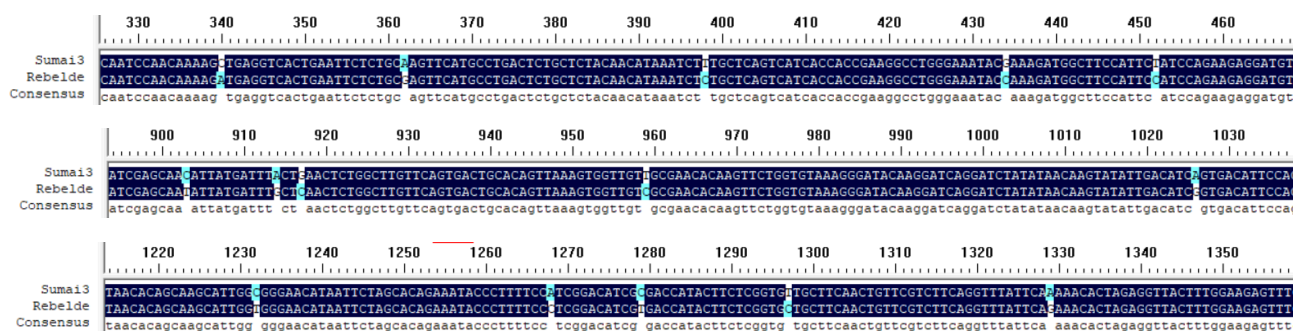
	340	350	360	370	380	390	400	410	420	430	440	450	460	470
Rebelde	ACGGAGGCTAGAAATACAAACCATCTTGGAAACCTGAAATCTCGCCATCTGCTATGACACGATGGGTGGCTATGGTGCATTGCCGACCTGATCGCAGGCTCCATGCTTCCCTCAGTGTGTTGAATGGATATTG													
Sumai3	ACGGAAAGCTAGAAATACAAAACATCTTCAPAAAACCTGAAATCTCGCCATCTGCTATGACACGATGGGTGGCTATGGTGCATTGCCGACCTGATCGCAGGCTCCATGCTTCCCTCAGTGTGTTGAATGGATATTG													
Consensus	acgg a gctagaatac aaa catctt aaa cctgaa tctcgcc tctgc tatgacac gcatgggtggctatgggtgccattgcc gac c gatc gtaggtgccatgcttccctcagtggtgtgaaatggatattg													
	520	530	540	550	560	570	580	590	600	610	620	630	640	650
Rebelde	ATTGGCTTATTAGCAACAAAGGATATTGTTATCTACCTTGGCTTGTATCATTGCATTTCGAAATGGAAAGCTTGGCTCCGACCACATAGTGGAGGTAGAAATTTATTGGAAGAAATCTCCACTGTATGGATATGCA													
Sumai3	ATTGGCTTATTAGCAACAAAGGATATTGTTATCTACCTTGGCTTGTATCATTGCATTTCGAAATGGAAAGCTTGGCTCCGACCACATAGTGGAGGTAGAAATTTATTGGAAGAAATCTCCACTGTATGGATATGCA													
Consensus	att ggcttattagc aacaaggatattt gtttattc ac ttggc tgtatcattgcattt aa ttggaacgttgggtccgaccacataag agagga tagaatttattggaag aat tctccactgt atggat a ca													
	1160	1170	1180	1190	1200	1210	1220	1230	1240	1250	1260	1270	1280	1290
Rebelde	AAATATCTCAAGATGTAGAAACATGTGCAATGGCTTTCGCCCTTTACCGATGAATGGTATGATGCTTCGTCAGATGAATGTGCCCATGTTCTCAAGCCTCCACTTTCATGCTCACTGGAAGGATATTTAAATCAACAA													
Sumai3	AAATATCTCAAGATGTAGAAACATGTGCAATGGCTTTCGCCCTTTACCGATGAATGGTATGATGCTTCGTCAGATGAATGTGCCCATGTTCTCAAGCCTCCACTTTCATGCTCACTGGAAGGATATTTAAATCAACAA													
Consensus	a a at caagatgtagaaacatgtgcaatggcttccgcttttcacggatgaatgg tatgatgt tgcgcagatga ttgtcccatggt ct aagcctccactttccat ctact gaaggatatttaaatca aca													
	1640	1650	1660	1670	1680	1690	1700	1710	1720	1730	1740	1750	1760	1770
Rebelde	CTCGTCACCTCGAGAGCTGGGAGAAAGAAACAGGCTGCAACAGCTTAATATTCTACGCAAGGGGAGCTGTGATAAATTGTATCTCTCTGCTGCTCAACCTATCTCTCATGAATCTCTGATGCTCGCATTCGATGTCGGAA													
Sumai3	CTCGTCACCTCGAGAGCTGGGAGAAAGAAACAGGCTGCAACAGCTTAATATTCTACGCAAGGGGAGCTGTGATAAATTGTATCTCTCTGCTGCTCAACCTATCTCTCATGAATCTCTGATGCTCGCATTCGATGTCGGAA													
Consensus	ctc g cacctcgagag tgggagaaagaaacaggctg ca cagct a attctacgcaagggagctgtgataaattgttatctctctgctgctg acc tacc ctcaatgaa tctctgatgctcgcatgcatgctgagaa													
	1890	1900	1910	1920	1930	1940	1950	1960	1970	1980	1990	2000	2010	2020
Rebelde	ACGAAGTTGAGTTCTCTCGAGCAAGTAGAAATAGTATTTTCTGCTTTTATATCCAGCAGTTAAACAGCTTGGAGAAATGGCTTCGAGCTACAAACCTGGCATGTGTACAAACACCTTGATGAACATGGCTACATTACGTAG													
Sumai3	ACGAAGTTGAGTTCTCTCGAGCAAGTAGAAATAGTATTTTCTGCTTTTATATCCAGCAGTTAAACAGCTTGGAGAAATGGCTTCGAGCTACAAACCTGGCATGTGTACAAACACCTTGATGAACATGGCTACATTACGTAG													
Consensus	ac aagttgagttct ctc gagcaagtagaa tagtattttctgcttttata acagttaa ca ttggagaaatggcttc gcagt ca aa gcatgttacaac cac tg atgaacatggctacattacc tgagi													

TaZEP

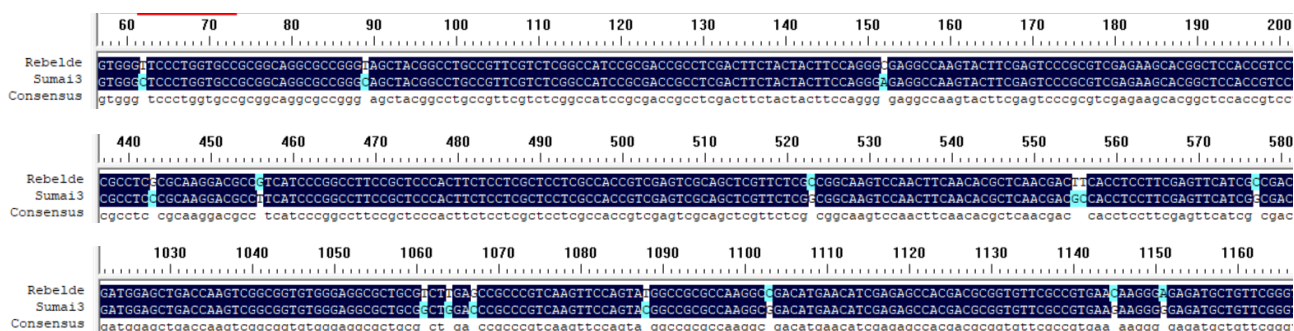
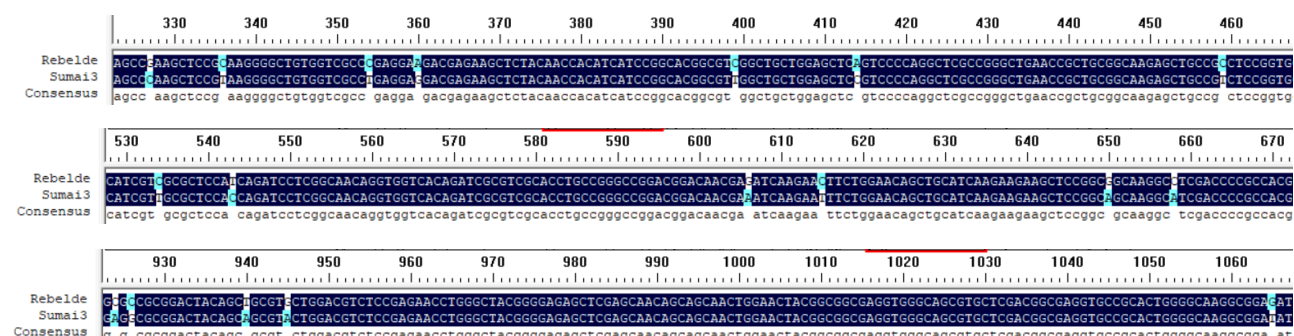
	1170	1180	1190	1200	1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
Sumai3	TGGTACCTGATCTGAGAATGGCAAAAGAAAGAGCTGCTCGAGATATTTAGCGGTGGTGTGATAATGTCATGATCTGCTAAATGCAACTGAAGGAGAGGATTCTCGCCGGGATATATACGACGGCCACCTACTATCA													
Rebelde	TGGTACCTGATCTGAGAATGGCAAAAGAAAGAGCTGCTCGAGATATTTAGCGGTGGTGTGATAATGTCATGATCTGCTAAATGCAACTGAAGGAGAGGATTCTCGCCGGGATATATACGACGGCCACCTACTATCA													
Consensus	tggttac gatctcgagaatggcaaaagaaagactgctcg agatatttagcgggtggtgtgataatgcat gatctgctaataatgcaactga gaggaagc attct cgccgggatatataga cggccacactactatca													
	1350	1360	1370	1380	1390	1400	1410	1420	1430	1440	1450	1460	1470	1480
Sumai3	TGTTCATGCTATGAGCCAAATCTGGGACAGGGTGGCTGCTAGGCTATTGAGGATGGTTACCACTGGCTGTAGAGCTTGAGAGGGCTGGGAAGAGAGTGTCAAGCTTAGAACTCCTGTGGAGGTATTATTCTCTCTGAGGAGTT													
Rebelde	TGTTCATGCTATGAGCCAAATCTGGGACAGGGTGGCTGCTAGGCTATTGAGGATGGTTACCACTGGCTGTAGAGCTTGAGAGGGCTGGGAAGAGAGTGTCAAGCTTAGAACTCCTGTGGAGGTATTATTCTCTCTGAGGAGTT													
Consensus	tgt catgctatgcagccaaatctgggacagggtggctgcatggctattgaggaatggttaccagctggctgtagagcttgagaagggc tgggaagagagtgtcaagcttagaactcctgtgga gttatttctccttgaggagtt													
	1640	1650	1660	1670	1680	1690	1700	1710	1720	1730	1740	1750	1760	1770
Sumai3	GAGTTGGCGCAGATTTTTCATCAAGGTTGCTATGCTTTGATGCTGAGCTGGCTTTTAGGTGGCAACAGCTCAAAAGCTAGAAGGAAGACCGTTAGTTGCGGGCTTTTCTGCAAGGCAACACCAACAGCTTGGTCGATGTTCTGAC													
Rebelde	GAGTTGGCGCAGATTTTTCATCAAGGTTGCTATGCTTTGATGCTGAGCTGGCTTTTAGGTGGCAACAGCTCAAAAGCTAGAAGGAAGACCGTTAGTTGCGGGCTTTTCTGCAAGGCAACACCAACAGCTTGGTCGATGTTCTGAC													
Consensus	gagttggc ggcagatttttcatcaaggttgct atgcttttgatgctgagctgg ttttaggtggcaacagctcaaaagctagaaggaagaccggt agttgcccggcttttctga aaggcaa c accagcttggtcgatggtt ag													
	2000	2010	2020	2030	2040	2050	2060	2070	2080	2090	2100	2110	2120	2130
Sumai3	AGAAAGAGGTTTCTATTGACCGATCTGGAAGCGAGCACGGTACATGGTTTAAAGCAACGAAGGAGCGCTACCGGTTACCTCCAAACTTCCCATTCTGTTTACACCTTTCGATGCCATCGAATTGGCTCAGACAAGAG													
Rebelde	AGAAAGAGGTTTCTATTGACCGATCTGGAAGCGAGCACGGTACATGGTTTAAAGCAACGAAGGAGCGCTACCGGTTACCTCCAAACTTCCCATTCTGTTTACACCTTTCGATGCCATCGAATTGGCTCAGACAAGAG													
Consensus	agaa aagggtttctatttgaccgatct ggaagcgagcacgggtacatggtttaa gacaagaagg ag cgtcacgggttacctccaacttccc gttcgttt cacccttc gatgccatcga ttggctcagacaagaag													

TaNCD

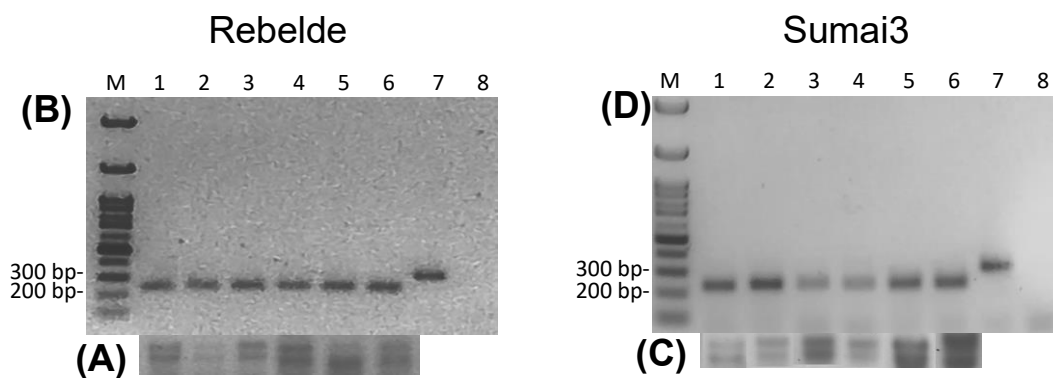
	390	400	410	420	430	440	450	460	470	480	490	500	510	520	530
Rebelde	AAGAAGCTCAACTTCTTCCAGCGCGCGCGCGCGCGCTTTCGAGGAGGGCTTCTGCGCAACGTGCTGAGCGCCACAGCGCTCTCTCGAGCGGTGACCCCGCGGTGAGATCTGCGCCCGG														
Sumai3	AAGAAGCTCAACTTCTTCCAGCGCGCGCGCGCGCGCTTTCGAGGAGGGCTTCTGCGCAACGTGCTGAGCGCCACAGCGCTCTCTCGAGCGGTGACCCCGCGGTGAGATCTGCGCCCGG														
Consensus	aagaagctcaacttcttccagcgcgcg ggcgct gacgc ttgcagagagggcttctgcgcaacgtgct gag cc caccg cttccc ggcaggtgcaccccgcggtgagatcgc gg aa ttcgc cccg														
	560	570	580	590	600	610	620	630	640	650	660	670	680	690	
Rebelde	TGCACGCTGCGCGTCCCGCGCATCCCGCTTCATCAACGGCGCTTACCGCGCAACGGCGCCAAACCGCACTTCGACCCCGTGGCGGCACCACTTTTCAGCGCGACGGCATGGTGACGCCCTGCGGATCCGCAAC														
Sumai3	TGCACGCTGCGCGTCCCGCGCATCCCGCTTCATCAACGGCGCTTACCGCGCAACGGCGCCAAACCGCACTTCGACCCCGTGGCGGCACCACTTTTCAGCGCGACGGCATGGTGACGCCCTGCGGATCCGCAAC														
Consensus	tgca c ctgcggt ccgg cgcattccc ct ttcatcaacggcgt tacgc cgcacggcgccaaccccgcaacttcgaccccggt gcgg caccacct ttgcagcggcagcgcatggtgtgcagccctgcg atccgcaac														
	680	690	700	710	720	730	740	750	760	770	780	790	800	810	
Rebelde	GCACGCCCTGCGCATCCGCAACCGGTCGCCCGAGACCTACGCCCTCCGCTTACGAGACGAGCGGCTCCACAGGAGCGCGGCTGGCGCGCCCATGTTCCCAAGGCCATCGGGCGAGCTCCACGGCACTTCGGGATCG														
Sumai3	GCACGCCCTGCGCATCCGCAACCGGTCGCCCGAGACCTACGCCCTCCGCTTACGAGACGAGCGGCTCCACAGGAGCGCGGCTGGCGCGCCCATGTTCCCAAGGCCATCGGGCGAGCTCCACGGCACTTCGGGATCG														
Consensus	gcacgccctgcg atccgcaacgg gtgcgcgagac tacgcctcc g ttac ga acggagcgct c caggagcgcgct ggcgg cccatgttcccaaggccatcgcgagctccagggccactccgggatcg														
	1400	1410	1420	1430	1440	1450	1460	1470	1480	1490	1500	1510	1520	1530	
Rebelde	TGGGAGGAGGAGGACACGAGAGGTTGTTATCGGTTCTCTGATGACCTCCCGGAGCTTCTTCAACGAGTCTGACGAGTGGCTGAGAGCGTGTCTCAGGAGATCCCGCTCAACACGGCGACCGGAGTCCACCGCGC														
Sumai3	TGGGAGGAGGAGGACACGAGAGGTTGTTATCGGTTCTCTGATGACCTCCCGGAGCTTCTTCAACGAGTCTGACGAGTGGCTGAGAGCGTGTCTCAGGAGATCCCGCTCAACACGGCGACCGGAGTCCACCGCGC														
Consensus	tgggagaggaggaa ac ga gagggtgt gt atcgg tctgtcatgac cccgag gacttcattctcaacgagtc gacgagtgctt gaggagctgtctcaggagatcccgctcaaacggcgacccgg gactccacgcgc														
	1570	1580	1590	1600	1610	1620	1630	1640	1650	1660	1670	1680	1690	1700	
Rebelde	GGTAACCTGAGGTGCGATGTGTAACCTGAACTCTGGGCGCAAGACCGGTACGCTTACTGCGCGGTGGCGAGCCGTGGCCCAAGGTGTCCGGCTTCGCCAAGGTGAACTCGCCACGGGAGGCTCACCAGGTTTCAAT														
Sumai3	GGTAACCTGAGGTGCGATGTGTAACCTGAACTCTGGGCGCAAGACCGGTACGCTTACTGCGCGGTGGCGAGCCGTGGCCCAAGGTGTCCGGCTTCGCCAAGGTGAACTCGCCACGGGAGGCTCACCAGGTTTCAAT														
Consensus	ggt aacct gaggtcg atgtt aactc a cctcctgggcgcaagac cggtaacg taactggcgctggc gaggc tggcccaaggtgtccggcttcgcccaaggt gaactcgccacggg gagctcaccaggttcga t														

$TqAAO$ 

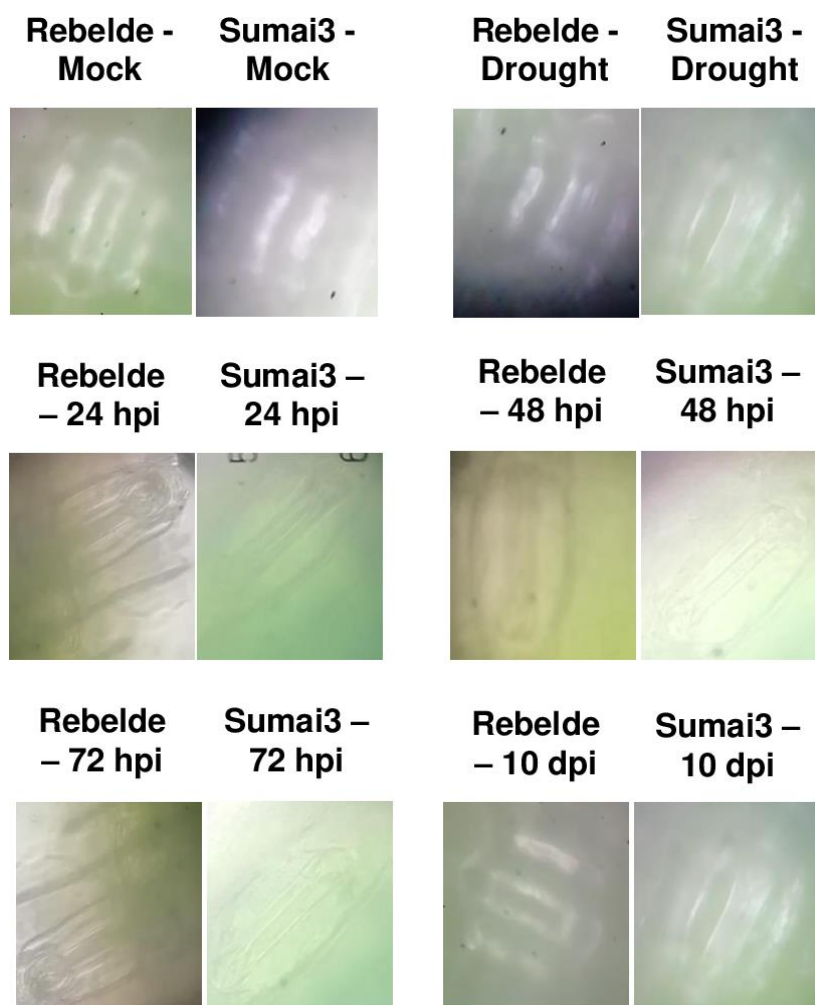
TqAOS

 $TqPIMP$ 

Supplementary figure 3.3 Alignment of sequences from Rebelde and Sumai3. The sequences with a homology equal or less than 95% (*TaKSL*, *TaZEP*, *TaNCED*, *TaAAO*, *TaAOS* and *TaPIMP*) were aligned in order to observe the major pieces of the sequences characterized by SNPs.



Supplementary figure 3.4 1.5% agarose gel of total extracted RNA (A, C) and RT-PCR (B, D) from Rebelde and Sumai3, respectively. M) ExcelBand™ 100 bp + 3K DNA Ladder (Smobio), 1) Mock, 2) Drought stress, 3) 24 hpi, 4) 48 hpi, 5) 72 hpi, 6) 10 dpi, 7) Genomic DNA (gDNA) control, 8) No template control (NTC). The figure represents the original picture of the gel.



Supplementary figure 3.5 Stomata from Sumai3 and Rebelde. The pictures were obtained by observing a slice of the external glume with an optical microscope (Leitz Diaplan) and by using a magnification of 40 $\times$.

4. UAV-based thermal, RGB imaging and gene expression analysis allowed detection of Fusarium head blight and gave new insights into the physiological responses to the disease in durum wheat

Sara Francesconi^{1*}, Mauro Maesano², Antoine Harfouche², Giorgio Mariano Balestra^{1*}

¹Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia, Via San Camillo de Lellis, 01100, Viterbo, Italy

²Department for Innovation in Biological, Agro-food and Forest systems (DIBAF), University of Tuscia, Via San Camillo de Lellis, snc, 01100, Viterbo, Italy

Corresponding authors: Sara Francesconi, francesconi.s@unitus.it; Giorgio Mariano Balestra, balestra@unitus.it

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Abstract: Wheat is one of the world's most economically important cereal crop, grown on 220 million hectares. Fusarium head blight (FHB) disease is considered a major threat to durum (*Triticum turgidum* subsp. *durum* (Desfontaines) Husnache) and bread wheat (*T. aestivum* L.) cultivars and is mainly managed by the application of fungicides at anthesis. However, fungicides are applied when FHB symptoms are clearly visible and the spikes are almost entirely bleached (% of diseased spikelets > 80%), by when it is too late to control FHB disease. For this reason, farmers often react by performing repeated fungicide treatments that, however, due to the advanced state of the infection, cause a waste of money and pose significant risks to the environment and non-target organisms. In the present study, we used unmanned aerial vehicle (UAV)-based thermal infrared (TIR) and red-green-blue (RGB) imaging for FHB detection in *T. turgidum* (cv. Marco Aurelio) under natural field conditions. TIR and RGB data coupled with ground-based measurements such as spike's temperature, photosynthetic efficiency and molecular identification of FHB pathogens, detected FHB at anthesis half-way (Zadoks stage 65, ZS 65), when the percentage (%) of diseased spikelets ranged between 20% and 60%. Moreover, in greenhouse experiments the transcripts of the key genes involved in stomatal closure were mostly up-regulated in *F. graminearum*-inoculated plants, demonstrating that the physiological mechanism behind the spike's temperature increase and photosynthetic efficiency decrease could be attributed to the closure of the guard cells in response to *F. graminearum*. In

addition, preliminary analysis revealed that there is differential regulation of genes between drought-stressed and *F. graminearum*-inoculated plants, suggesting that there might be a possibility to discriminate between water stress and FHB infection. This study shows the potential of UAV-based TIR and RGB imaging for field phenotyping of wheat and other cereal crop species in response to environmental stresses. This is anticipated to have enormous promise for the detection of FHB disease and tremendous implications for optimizing the application of fungicides, since global food crop demand is to be met with minimal environmental impacts.

Keywords: Fusarium head blight; durum wheat; high-throughput plant phenotyping; thermal imaging; RGB imaging; gene expression; early disease detection.

4.1 Introduction

Wheat is one of the most cultivated crops worldwide, grown on 220 million hectares and its annual production is estimated to account for more than 700 million tons, providing more than 20% of total human food calories (Khan *et al.*, 2020; Ma *et al.*, 2020). Modern wheat cultivars derive from two species: bread wheat (*Triticum aestivum* L.) and durum-type wheat (*T. turgidum* subsp. *durum* (Desfontaines) Husnache) used for pasta and low-rising bread (Peng *et al.*, 2011; Feldman and Levy, 2012).

The current food demand will double with a projected population of 9 billion in 2050. In response, farmers must grow more on their lands through sustainable intensification of agriculture, an approach to increase yield production without having negative effects on the environment or expanding the agricultural footprint (Giller *et al.*, 2015).

Wheat production is challenged by several abiotic and biotic stresses. Among the plant fungal diseases, Fusarium head blight (FHB) is one of the most destructive diseases leading to 10-70% of yield loss during the epidemic years (McMullen *et al.*, 2012). FHB is caused by the *Fusarium graminearum* species complex (FGSC), which embraces 16 different species. It is considered the most devastating wheat disease due to the lack of resistant cultivars, the significant yield loss and grain quality reduction, and the health risks associated with the contamination of crops with mycotoxin such as deoxynivalenol (DON) and zearalenone (ZEA), produced during the infection progress (Yang *et al.*, 2013; Dweba *et al.*, 2017). The spectrum of *Fusarium* spp. causing FHB on wheat varies at the regional level, depending on weather conditions. Fungal growth is favoured by high temperatures and humidity during the growing season. *F. graminearum* Schwabe is the predominant species that causes FHB in many countries, in Asia, North and South America and Europe (Dweba *et al.*, 2017; Khan *et al.*, 2020). In field conditions, the inoculum occurs primarily from plant residues and soils while the dissemination of the ascospores and conidia is mainly

promoted by water splash and wind. Anthesis is the most susceptible stage to infection. With a warm and humid climate at this stage, airborne spores proliferate and spread intracellularly into the rachial nodes and through the rachis until FHB symptoms are clearly visible. The symptoms include necrosis, premature bleaching of spikes, and shrivelled kernels that negatively affect photosynthesis. At favourable conditions, FHB develops rapidly within 3-6 days after the infection. Given the current global warming associated with increased temperatures, major epidemics of FHB are occurring. Thus, proper cultivation schemes and field management are essential to alleviate its threat (Bai and Shaner, 2004; Vaughan *et al.*, 2016; Ma *et al.*, 2020; Rod *et al.*, 2020).

Since 1995, *Fusarium* spp. infect wheat in Italy at various incidence (percentage of spikes showing symptoms) and severity (percentage of areas infected in spikes) depending on the year, the region, and the wheat cultivar involved (Pancaldi *et al.*, 2010). Its incidence and severity are closely related to the amounts of precipitation during wheat anthesis, increasing from the South to the North of Italy, being mostly reported in the Northern-Central regions (Shah *et al.*, 2005; Infantino *et al.*, 2012). Between the two major species, *T. turgidum* is the most widely grown in Italy, but is also more susceptible to FHB (Oliver *et al.*, 2008). Consequently, being mostly used for human consumption, the risk of mycotoxin-contaminated grain entering the food chain is a major concern (Boutigny *et al.*, 2008).

Chemical control of FHB using appropriate effective fungicides and correct application methods and timing can reduce the severity of the disease (Blandino *et al.*, 2012). However, no fully effective FHB fungicide is available (Haidukowski *et al.*, 2012), and the application window is very narrow, spanning just a few days around anthesis (Mesterházy *et al.*, 2003). Recently, an organic strategy to reduce FHB incidence and severity was proposed, but now it needs to be confirmed on wide areas (Francesconi *et al.*, 2020). For these reasons, early detection and control of FHB is a key factor to gain maximum protection of yield (Mahlein *et al.*, 2019) from fungal spread and mycotoxin accumulation; in fact, there is a strong evidence base in the research literature that a prompt and early application of fungicides instead of applying fungicides at late stages of infection drastically reduce the FHB spread and DON accumulation inside the grains, instead of applying fungicides at late stage of infection (Freije and Wise, 2015; Feksa *et al.*, 2019; Bolanos-Carriel *et al.*, 2020).

Recent advances in biological and bioanalytical research enabled genome-scale capturing of biological processes at the molecular level in plants (Weckwerth, 2011). Though transcripts evaluation is fundamental to understand plant responses to pathogens, these techniques are labour-intensive, time-consuming, destructive and slow-down the acquisition of phenotype parameters related to the gene responses, contributing to the phenotyping bottleneck (Furbank and Tester, 2011). Molecular data obtained in greenhouse or field trials combined with phenotypic and environmental

data discloses a wealth of information that can be used to improve field management (Alexandersson *et al.*, 2014).

For plant-pathologists, coupling transcriptomics and phenomics to agricultural practices is likely to have a large impact on the understanding of induced plant defenses and pathogen spread. In fact, phenomics and transcripts analysis can reveal important physiological changes in plants in response to pathogens which can help detecting infections before the appearance of their visible symptoms. Hence, these associated techniques have the potential to be a powerful weapon that optimizes fungicide spraying regimes for plant pathogen management (Alexandersson *et al.*, 2014).

To relieve the phenotyping bottleneck, phenotypic traits should be turned into quantifiable, objective, and consistent measures (Ludovisi *et al.*, 2017). Automated and high-throughput phenotyping (HTP) provides measurements that can track the development of a plant through its life stages and its interaction with the environment, establishing methodologies to detect crucial physiological traits and identify effective genotype-phenotype relationships (Goggin *et al.*, 2015). These methods speed up the phenotyping process and maximize the number of studied plants per experiment (Goggin *et al.*, 2015). Furthermore, they enable automated, non-destructive, and non-invasive screening of large populations and high dimensionality data (Li *et al.*, 2014; Fahlgren *et al.*, 2015).

With regards to FHB, *Fusarium* spp. infects spikelets within wheat spikes, decreasing stomatal conductance, and thus, affecting the chlorophyll and water content (Yang *et al.*, 2016; Francesconi and Balestra, 2020). This allows its early detection by colour imaging and by measuring the temperature of spikes and photosynthetic efficiency of plants using thermometers and fluorometers, respectively (Cambaza *et al.*, 2019; Mahlein *et al.*, 2019).

Red-green-blue (RGB) cameras are designed to emulate human vision by sensing light in the visible range of the electromagnetic spectrum (wavelengths from 390 to 700 nm). In this range, the reflectance is predominantly influenced by plant pigments (e.g., chlorophyll) (Mahlein, 2016). This allows the calculation of different vegetation indices (VIs) by computing the reflectance of a certain band of the green and red zone of the electromagnetic spectrum (Barbosa *et al.*, 2019; Brunori *et al.*, 2020). Photosynthetic response of green vegetation to incident light is the basis of VIs where healthy plants exhibit low red reflectance due to absorption of red light by chlorophyll resulting in a high index value, whereas unhealthy, stressed plants with reduced chlorophyll pigment display a low index value (Khan *et al.*, 2018). Therefore, RGB image analysis can serve as an important tool that detects physiological changes in plants caused by pathogen infections (Simko *et al.*, 2016). On the other hand, thermal infrared (TIR) sensors capture images containing information about the energy emitted from body surfaces, such as plant canopies. Plant pathogens affect the loss of water in plants regulated by stomata (Fang and Ramasamy, 2015), altering plant temperature where high values indicate areas

with closed stomata (Oerke *et al.*, 2006; Chaerle *et al.*, 2007). Thermography can serve as a tool to measure these changes towards an early detection of plant infections, ideally before symptoms appear (Al Masri *et al.*, 2017).

Unmanned aerial vehicles (UAVs) equipped with cameras and sensors have become advanced field phenomics tools that provide data with high spatial and temporal resolution to bridge the gap between time consuming ground-based measurements and satellite observations (Xie and Yang, 2020; Pineda *et al.*, 2021). UAVs allow rapid and non-destructive measurements and offer much quicker turnaround times than satellites at competitive costs (Ludovisi *et al.*, 2017).

The application of UAV-based imaging techniques has been broadening in several areas of agricultural sciences thanks to their ability to analyse plant temperature and colour discrepancies between distinct biological samples (Padmavathi and Thangadurai, 2016; Cambaza *et al.*, 2019). Many recent studies have exploited UAV-based sensors to monitor, detect and phenotype plant stresses in forestry (Sagan *et al.*, 2019), as well as to estimate leaf nitrogen concentration (Lu *et al.*, 2021), water and nitrogen use efficiencies (Yang *et al.*, 2020), and salinity stress (Johansen *et al.*, 2019) in different crops. Plant pathologists are recently also benefiting from the application of UAV-based sensors; in fact, UAV multispectral and hyperspectral imaging have been used to detect *Xylella fastidiosa* (Castrignanò *et al.*, 2021) and FHB (Liu *et al.*, 2020), respectively. Moreover, these techniques are relatively easy to use and are becoming cheaper (Dammer *et al.*, 2011; Cambaza *et al.*, 2019). Their exploitation to monitor FHB can contribute significantly to secure the cereal production systems (Mahlein *et al.*, 2019). In light of these advantages, UAV-mounted cameras and sensors are expected to enable new applications in field-based phenotyping of plant stress traits in large populations rapidly, precisely and accurately (Berni *et al.*, 2009; White *et al.*, 2012; Yang *et al.*, 2017).

In this study, we exploited plant physiological and molecular changes in response to *Fusarium* spp. infection to detect FHB in *T. turgidum* (cv. Marco Aurelio) fields through UAV-based TIR and RGB imaging. Particularly, i) two VIs as well as spike temperature were calculated using RGB and TIR images, from both uninfected and infected areas and were correlated to photosynthesis perturbation caused by the infection. Additionally, ii) transcripts of key genes involved in stomatal conductance regulation were investigated in greenhouse experiments, to explore the genotypic changes behind the observed phenotypic perturbations (increase in spike temperature and decrease in photosynthetic efficiency). Furthermore, iii) transcripts of *F. graminearum* inoculated plants were compared to those obtained from drought-stressed ones in order to investigate a differential response between these two types of stresses.

4.2 Materials and methods

4.2.1 Plant material and experimental design of the field experiments

The FHB-susceptible *T. turgidum* cv. Marco Aurelio was the cultivar of interest in the present work for both field and greenhouse experiments. This genotype, grown in Central and South Italy, is extensively used for pasta production and it is characterized by excellent protein content and high productivity, thus it is of high economic importance. The experimental fields were located in Amelia (Central Italy, 42°31'22.9''N, 12°25'15.5''E, Umbria Region) and Avigliano Umbro (Central Italy, 42°40'41.1''N, 12°27'44.6''E, Umbria Region). The *T. turgidum* field experiments were carried out during two consecutive years (2019 and 2020), controlled and drip-irrigated with a nutrient solution containing nitrogen, potassium, phosphorus, and small amounts of other compounds. Therefore, abiotic stresses (nutrient deficiencies and drought stress), diseases, or pests, which cause the same symptoms as FHB, were significantly minimized. On the other hand, fungicides for FHB management (tebuconazole and azoxystrobin) were not applied in order to favour the FHB natural infection. The fields were investigated periodically (one time per day) by experts and farmers to prevent other diseases, pests, or abiotic stress. An experimental plot was allocated within each field, and individuated by a 20 m grid by positioning 16 ground control points (GCPs) to be used for georeferencing. During March, April, and May 2019 and 2020 weather data (minimum, maximum and average temperature, and precipitation) were recorded daily by two meteorological stations installed at 100 m distance from each field to monitor the climate factors influencing the establishment of FHB infection. Historical weather data (from 2010 to 2018) were obtained from the Hydrographic service of Umbria Region (<https://annali.regione.umbria.it/>) to monitor the climatic trend of the last eleven years and to compare minimum, average and maximum temperature values (°C) and average precipitations (mm) recorded during the nine-year seasonal average (2010-2018), 2019 and 2020. The historical weather data were collected from two meteorological stations located in Amelia (42°33'25.0''N, 12°25'01.0''E) and Avigliano Umbro (42°40'39.0''N, 12°26'13.0''E). The experimental design for the field experiments is illustrated in Figure 4.1a and 4.1b.

4.2.2 UAV campaigns

An unmanned DJI Matrice 600 multi-copter (DJI, China) equipped with a Zenmuse X5 RGB camera (DJI, China) and a Zenmuse XT TIR camera (DJI, China) has been used in this study (Supplementary figure 4.1). DJI Matrice 600 is a hexa-copter (110 cm diagonal size) with a highly resistant carbon fiber frame, offering a 15 kg take-off weight. Its maximum transmission distance is 5 km and its maximum flight time is 40 minutes (Supplementary figure 4.1).

The Zenmuse XT was equipped with a 9 mm f1.4 lens. Its thermal sensitivity is less than 50 milliKelvins and the camera enables measurements in the range -25°C to +135°C. The image sensor

is a focal plane array (FPA) based on uncooled microbolometers with a spectral band response in the range of 7.5 to 13 μm . The camera field of view is equal to 69° (horizontally) $\times$ 56° (vertically), its resolution to 640×512 pixels at 30 frames per second (fps), and its spatial resolution to 1.889 milliradians (mrad). The camera captures images at an acquisition frequency of 30 Hz. The camera was radiometrically calibrated to further increase temperature measurement precision by setting external parameters during the flight planning such as air temperature and flat field correction (FFC). This is an offset calibration usually performed at power up, when the camera changes temperature, and periodically during the operation. This calibration compensates for certain errors that build up during the camera operation. During the data acquisition phase, the auto gain mode parameter was applied; the camera automatically selected the optimal gain mode according to the temperature range of the image (Deery *et al.*, 2016; Gómez-Candón *et al.*, 2016; Ludovisi *et al.*, 2017) and pictures were stored as 14-bit digital raw images.

The Zenmuse X5 is a 16-megapixel RGB camera equipped with an M4/3 sensor enabling it to capture detailed images at a resolution of 4608×3456 pixels and an ISO range of 100-25600.

The Zenmuse X5 and XT cameras were mounted on a highly reliable gyrostabilized 3-axis gimbal (DJI, China) that automatically stabilizes them in flight. The gimbal constantly communicates with the UAV, and quickly compensates for every minor movement with a precision accuracy of 0.02° .

The 16 GCPs were used to georeference RGB images. For calibration of thermal images, four 60×60 cm ground reference panels (GRPs) consisting of two plastic panels covered by black vinyl tape and two white Teflon® were positioned along the borders of each of the two study areas. A real-time kinematic (RTK) global navigation satellite system (GNSS) CS10 model (Leica Geosystems, Switzerland) with an accuracy of 1 mm was used for capturing GCPs and GRPs locations.

UAV campaigns were conducted at the following phenological stages: (i) three-quarters of inflorescence emerged (Zadoks stage 57, ZS 57) (Zadoks *et al.*, 1974) on 6 May 2019 and 7 May 2020; (ii) anthesis half-way (ZS 65) on 17 May 2019 and 18 May 2020; and (iii) kernel watery ripe (ZS 71) on 28 May 2019 and 29 May 2020. Two flights were performed during each campaign. The flight missions have been planned using Ground Station Pro app (DJI GS Pro, China). Each flight lasted 11 minutes covering an area of 1 ha at a nominal speed of 4 m/s and an altitude of 20 m, during which both cameras acquired nadiral images with 90% frontal and side overlap. To ensure similar solar illumination angles flights were performed between 11:00 and 12:00 local time under stable cloudless and low-wind conditions (Figure 4.1d and 4.1e).

4.2.3 Ground measurements

For each of the 16 GCPs, a circumference of 1 m of diameter was individuated and denominated as sampling area. During the UAV campaigns, we manually measured the spike temperature of sixteen

randomly selected plants in each of the 16 sampling areas using a portable infrared thermometer (Fluke 568, Fluke Corporation, United States) with an accuracy of $\pm 1\%$ or $\pm 1.0^{\circ}\text{C}$ (whichever is greater), positioned at 10 cm distance from the spike. At the same time, the FHB severity was calculated by counting the number of diseased spikelets and the total number of spikelets for each of the sixteen plants. Furthermore, the flag leaves of the selected plants were sampled and dark-adapted for 1 hour before measuring their photosynthetic efficiency, by quantifying F_v/F_m reflecting the potential quantum efficiency of photosystem II (Maxwell and Johnson, 2000), with a portable fluorometer (V2.00f PAM 2000, Heinz Walz GmbH, Germany). Moreover, the spikes were also sampled to distinguish FHB positive (FHB+) from FHB negative (FHB-) areas by molecular identification of *Fusarium* spp. (Figure 4.1c).

4.2.4 Molecular diagnostics of *Fusarium* pathogens

Spikes were stored in a portable fridge immediately after sampling. Afterward, different tissues (palea, lemma, glume, rachis, kernel when present) from each collected spike were plated on Petri dishes containing potato dextrose agar (PDA) and incubated at 21°C for 72 hours. The different morphotypes were subsequently isolated and cultured on PDA for one week. After each morphotype filled the Petri dish, the produced mycelium was gently scraped with a glass rod. 100 mg of mycelium were grinded and DNA was extracted using 80 μL of extraction buffer composed of Tris 100 mM, EDTA 50 mM and NaCl 500 mM; after that, 32 μL of SDS 10% (w/v) were added and the samples were incubated at 65°C for 10 minutes. 27 μL of potassium acetate 5 M were added and the samples were placed on ice for 20 minutes, then centrifuged for 20 minutes at 13.000 rpm. The supernatant was recovered and 80 μL of cold (-20°C) isopropanol were added to each sample; then, the samples were placed on ice for 10 minutes and centrifuged for 5 minutes at 13.000 rpm. The supernatant was discarded and 150 μL of cold (-20°C) ethanol (70% v/v) were added to the samples. Samples were centrifuged for 3 minutes at 13.000 rpm, the supernatant was discarded and the DNA was resuspended in 20 μL of DNase and RNase-free sterile distilled water and stored at -20°C (D'Ovidio and Porceddu, 1996). Total DNA was quantified with Qubit[™] fluorometer 1.01 (Invitrogen, United States) using the Qubit[™] dsDNA BR Assay Kit (Thermo Fisher Scientific, United States) and diluted to 10 ng/ μL . The molecular identification of *Fusarium* spp. was performed by amplifying the translational elongation factor 1- α (*TEF*) sequence using the primer pair EF1_F 5'-ATGGGTAAGGAGGACAAGAC-3'/EF2_R 5'-GGAAGTACCAGTGATCATGTT-3' designed to identify the FHB complex spp. (Geiser *et al.*, 2004). The polymerase chain reaction (PCR) was performed following the instructions of GoTaq[®] Green Master Mix (Promega, United States) and prepared in a total volume of 10 μL . The amplification conditions consisted of: (i) an initial denaturation step of 2 minutes at 95°C ; (ii) 35 cycles of 30 seconds denaturation at 95°C ; (iii) 40

seconds of annealing at 53°C; (iv) 60 seconds of elongation at 72°C; and (v) a final elongation step of 5 minutes at 72°C. The amplicon unicity was visualized on 1.5% agarose gel and sequenced by Sanger sequencing (Eurofins Genomics, Germany). The resulted sequences were submitted to BLASTn (<https://blast.ncbi.nlm.nih.gov/>) in order to identify the corresponding *Fusarium* spp.

4.2.5 RGB image processing and VIs calculation

250 RGB images were acquired using the UAV-mounted Zenmuse X5 camera. The camera exposure mode was set to shutter priority (S) with a shutter speed fixed 1/500 seconds to ensure minimization of motion blur, ISO of 100 and the white balance to sunny mode.

Geometric camera calibration, orthorectification, and mosaicking of the captured images were performed using Pix4Dmapper (Pix4D, Switzerland) software, specifically designed to process UAV images using techniques rooted in both computer vision and photogrammetry to match conjugate points in overlapped images and to define their relative positions and orientations using bundle block adjustments (Bollard-Breen *et al.*, 2015; Nishar *et al.*, 2016) (Figure 4.1f). The output of this step was the RGB orthomosaics of the study areas for each flight mission. No filtering process was applied to the images.

Finally, VIs from the orthomosaics were computed using quantum geographic information system (QGIS) software (version 3.4 Madeira - QGIS Development Team, Open Source Geospatial Foundation). The VIs carried out were: vegetative (VEG) (Hague *et al.*, 2006) and green leaf index (GLI) (Louhaichi *et al.*, 2001). VEG and GLI were calculated using the following formulas:

$$VEG = \frac{G}{R^a * B^{(1-a)}}$$

and

$$GLI = \frac{(2 * G - R - B)}{(2 * G + R + B)}$$

where R, G, and B are the reflectance of red, green and blue channels, respectively, and a is equal to 0.667.

Inside each sampling area, leaves and spikes were not separated in the images, because the upper part of the fields was uniformly composed by spikes (Figures 4.1a and 4.1b), since wheat canopies were very dense.

4.2.6 TIR image processing and temperature extraction

The UAV-mounted Zenmuse XT TIR camera captured 219 greyscale and georeferenced images. Thermal orthomosaics were generated for each flight, and the radiometric conversion was automatically performed using the Pix4Dmapper software (Figure 4.1f). The removal of bare soil pixels was not necessary, since the wheat planting were extremely dense, reducing the mixed pixel

problem. FLIR Tools software (2020[©] FLIR[®] Systems, United States) was used to calibrate temperatures in TIR images using the temperature values of GRPs measured immediately after the UAV flights using a portable infrared thermometer (Fluke 568, Fluke Corporation, United States). Calibration checks were performed by comparing the GRPs-measured to the UAV-derived temperatures. No filtering process was applied to the images. The extraction of temperature values was performed for each of the 16 sampling areas. Inside each sampling area, leaves and spikes were not separated in the images, because the upper part of the fields was uniformly composed by spikes (Figures 4.1a and 4.1b), since wheat canopies were very dense.

4.2.7 Plant growth, inoculation conditions and experimental design of the greenhouse experiments

Greenhouse experiments were conducted in a glasshouse located in Viterbo (Central Italy, 42°25'35.8''N, 12°04'49.3''E, Lazio Region). The surface of the *T. turgidum* kernels was sterilized with sodium hypochlorite (0.5% v/v) for 20 minutes and rinsed twice with sterile distilled water for 5 minutes. The kernels were germinated in the dark on a paper, soaked in sterile distilled water for 15 days at 4°C to break dormancy, followed by 2 days at room temperature. The seedlings were transferred to 40 × 20 cm pots, filled with TYPical Brill soil (Brill, Germany) and grown at 16°C-20°C up to the boot stage (ZS 51), 20°C-24°C during heading and anthesis (ZS 53-69), and 24°C-29°C up to maturity (ZS 71-99). The plants were fertilized using ammonium nitrate in the following proportions and at the following stages: 20% at sowing (ZS 00), 40% at tillering (ZS 20), and 40% at heading (ZS 49) (Francesconi *et al.*, 2019). The highly virulent and mycotoxin-producing isolate of *F. graminearum* wild type (WT) 3824 (Tomassini *et al.*, 2009) was cultured at 21°C on synthetic nutrient-poor agar (SNA) to obtain macroconidia (Mandalà *et al.*, 2019). After 10 days on SNA, the conidia were scraped with a glass rod after pipetting 1 mL of sterile distilled water onto the surface of a Petri dish. The conidial suspension was recovered, and the concentration was adjusted to 1×10⁵ conidia/mL using a Thoma chamber (0.100 mm depth and 0.0025 mm²). The inoculum was prepared in sterile distilled water supplemented with 0.05% (v/v) of Tween-20. Plants were subjected to three different treatments: (i) drought stress treatment where plants did not receive water from ZS 51 to 65 (Zadoks *et al.*, 1974; Tambussi *et al.*, 2000); (ii) artificial inoculation treatment where spikes were uniformly spray-inoculated at ZS 65 with a suspension of 1×10⁵ conidia/mL; and (iii) mock treatment where spikes were uniformly sprayed with a suspension of Tween-20 0.05% resuspended in sterile distilled water at ZS 65. The spikes were covered with clear plastic bags for 24 hours to maintain high humidity levels (>80%). Spikes subjected to drought and mock treatments were sampled after removing the plastic bags, while the inoculated spikes were sampled 24, 48 and 72 hours post inoculation (hpi) to investigate an early response to *F. graminearum*. Collected spikes were

immediately stored in liquid nitrogen at -80°C until the extraction of RNA. FHB severity (%) was monitored in the greenhouse by counting the number of bleached spikelets and the total number of spikelets for each spike from 3 to 21 days post inoculation (dpi). In addition, spike temperature and photosynthetic efficiency were recorded at 24, 48 and 72 hpi. The experimental design for the greenhouse trial is illustrated in Figure 4.1g. Data were obtained from three independent experiments, each experiment consisting of 20 spikes for each treatment.

4.2.8 RNA extraction and cDNA synthesis

Wheat spikes were ground with mortar and pestle in liquid nitrogen until a fine powder was obtained. The RNA was extracted from 100 mg of powder following the instructions provided by InviTrap® Spin Plant RNA Mini Kit (Stratec Molecular GmbH, Germany), resuspended in RNase-free sterile distilled water, immediately poured onto ice and quantified with Qubit™ fluorometer 1.01 (Invitrogen, United States) using the Qubit™ RNA BR Assay Kit (Thermo Fisher Scientific, United States). To confirm the total quantity and integrity of the RNA, 5 μL of the extracted RNA sample was subjected to a 10-minute thermal shock at -80°C , followed by 5 minutes at 65°C and run on 1.5% denaturing agarose gel. The synthesis of cDNA was performed using 500 ng of RNA following the instructions provided by Xpert cDNA Synthesis Supermix with a gDNA eraser (GriSP Research Solutions, Portugal) in a final volume of 20 μL . To ensure that the synthesis of the cDNA and the elimination of the gDNA had succeeded, a reverse transcription PCR (RT-PCR) of *T. aestivum* Actin (*TaACT*) (containing an intron in the amplified sequence) was performed following the instructions provided by GoTaq® Green Master Mix (Promega, United States) in a total volume of 10 μL . The amplification conditions consisted of: (i) an initial denaturation step of 2 minutes at 95°C ; (ii) 35 cycles of 30 seconds denaturation at 95°C ; (iii) 40 seconds of annealing at 60°C ; (iv) 30 seconds of elongation at 72°C ; and (v) a final elongation step of 5 minutes at 72°C . The amplification run included a no-template control (NTC) and a genomic DNA (gDNA) control. The amplicons were visualized on 1.5% agarose gel.

4.2.9 Gene expression by quantitative Real-Time PCR

Supplementary table 4.1 shows the list of target genes, their functions, and the corresponding primer pairs used to perform RT quantitative PCR (RT-qPCR) (Francesconi and Balestra, 2020). Briefly, the primer pair for *T. aestivum* glyceraldehyde-3-phosphate dehydrogenase (*TaGAPDH*) amplification is from Jarošová and Kundu (2010), for *T. aestivum* pathogenesis related protein 1 (*TaPR1*) from Lu *et al.* (2006), for *TaACT* from Tundo *et al.* (2016), and *T. aestivum* β -tubulin2 (*TaTUB*) and *T. aestivum* ferredoxin-NADP(H)-oxidoreductase (*TaFNR*) from Tenea *et al.* (2011). The remaining primers are from Francesconi and Balestra (2020). The amplification efficiency (E) of RT-qPCR was determined

for each primer pair as follows: five 1:10 serial dilutions (1:1-1:10000) were obtained for each cDNA and amplified in four replicates. E and coefficient of determination (R^2) values were calculated by means of the slope of the standard curve obtained by plotting the fluorescence versus the serial dilution concentrations using the equation (Bustin *et al.*, 2009)

$$E = 10^{\left(-\frac{1}{\text{slope}}\right)} - 1.$$

Reference genes with closest E values to target genes, highest R^2 , and lowest variability were selected for the quantification cycles (Cq). The relative expression levels of target genes were calculated on the basis of the Cq values of four independent biological replicates, each with four technical replicates, for each plant treatment by applying the equation (Bustin *et al.*, 2009)

$$\text{Relative expression} = 2^{-\Delta\Delta Cq}$$

using *TaACT*, *TaTUB*, and *TaFNR* as reference genes and the mock treatment to normalize the relative expression levels. Relative expression levels of *TaPR1* and *TaGAPDH* were quantified as internal control of the progression of the infection (Muthukrishnan *et al.*, 2001) and changes in photosynthesis (Zhang *et al.*, 2013). The RT-qPCR was performed following the instructions provided by Rotor-Gene Q (Qiagen, Germany) and Xpert Fast SYBR (uni) MasterMix (GRiSP Research Solutions, Portugal), in a final volume of 10 μ L. The amplification conditions consisted of: (i) an initial denaturation step of 3 minutes at 95°C; (ii) 40 cycles of 5 seconds denaturation at 95°C; (iii) 30 seconds of annealing at 60°C; and (iv) 20 seconds of elongation at 72°C. A final melt cycle (70-99°C) was performed to confirm the unicity of the amplicons. NTC controls were included and the amplification was considered negative when a value of $Cq \geq 38$ was detected (Bustin *et al.*, 2009).

4.2.10 Photosynthetic-related parameters measurements

Spike temperature and photosynthetic efficiency were measured for each plant treatment as performed during the field trial, described in section 2.3. Photosynthetic-related parameters were measured for three independent replicates, each consisting of 20 individual spikes, for every treatment.

4.2.11 Statistical analyses

One-way analysis of variance (ANOVA) was performed to analyse FHB severity, ground-measurements (temperature and photosynthetic efficiency) of FHB+ and FHB- spikes during the UAV-campaigns, UAV-based VEG, GLI and temperature of FHB+ and FHB- sampling areas during the UAV-campaigns, gene expression values, and temperature and photosynthetic efficiency of drought stressed and inoculated plants during the greenhouse experiments. One level of significance

($p < 0.01$) was computed to assess the significance of the F values. A pairwise analysis was carried out using Tukey's honest significant difference (HSD) test at 0.99 confidence level. Statistical analyses were performed using XLSTAT 2020.4 software (Addinsoft, France). Principal component analysis (PCA) was carried out to classify spike temperature, photosynthetic efficiency and VEG or GLI or UAV-based temperature values coming from FHB+ or FHB– areas and gene expression values, spike temperature and photosynthetic efficiency coming from drought stressed or *F. graminearum* inoculated plants. Heatmap was carried out by computing the z-score of the relative gene expression values. PCA and heatmap were computed by using ClustVis software (Tartu, Estonia) (<https://biit.cs.ut.ee/clustvis/>) (Metsalu and Vilo, 2015).

4.3 Results

4.3.1 Weather conditions influencing the FHB severity

Registered data were compared to historical data (2010-2018) (Figure 4.2a), showing that May 2020 was particularly hotter than 2010-2019 (the recorded average temperatures were 20°C, 14°C and 18°C in May 2020, May 2019 and May 2010-2018, respectively), while May 2019 was characterized by high daily average rainfall (7 mm) compared to May 2020 (2 mm) and the nine-year seasonal average (5 mm). These conditions favoured the naturally occurring FHB in the fields of interest. In fact, in 2019, FHB severity (Figure 4.2b) reached 59% and 92% at ZS 65 and 71, respectively, indicating that the wet season (mean relative humidity was >70%) was particularly favourable for the FHB to spread. Although April and May 2020 were not characterized by frequent rains (average precipitation of 2 mm), low precipitations favoured a moderate FHB infection with a severity of 27% and 68% at ZS 65 and 71, respectively.

4.3.2 Ground-based measurements during the UAV campaigns

Figure 4.3 illustrates the results obtained by ground-based measurements during the UAV campaigns. The molecular identification of FHB was performed by amplifying the *TEF* sequence from a bulk sample obtained from the samples collected in each sampling area, producing an amplicon of 700 bp. At ZS 57, all the sampling areas resulted FHB– in both 2019 and 2020; at ZS 65, 10 of the 16 sampling areas were FHB+ in 2019, while 6 sampling areas were FHB+ in 2020; at ZS 71, all the sampling areas were FHB+ in both 2019 and 2020 (Figure 4.3a). In 2019, thirty *Fusarium* morphotypes were isolated: ten morphotypes were *F. graminearum*, eleven were *F. poae*, eight were *F. avenaceum*, and one was *F. proliferatum*. In 2020, twenty-four morphotypes were identified: ten were *F. graminearum*, ten were *F. poae* and four were *F. avenaceum*. The isolated morphotypes and the data resulted from the BLASTn analyses are listed in Supplementary table 4.2. Ground-based spike temperature values recorded in 2019 and 2020 revealed that FHB+ spikes had a higher temperature

than the FHB⁻ ones at ZS 65 (Figure 4.3b and 4.3d). On the other hand, photosynthetic efficiency had an inverse relationship with the FHB severity: variable fluorescence/maximum fluorescence (F_v/F_m) demonstrated to be lower in FHB⁺ than in FHB⁻ at ZS 65 (Figure 4.3c and 4.3e).

4.3.3 UAV-based TIR and RGB imaging for FHB detection

Recorded weather data indicated that during the two UAV-campaigns conducted at ZS 65, the average daily air temperature and humidity values were 15°C (2019), 21°C (2020), and 67% (2019) and 72% (2020), respectively. Supplementary figure 4.2 shows the 16 sampling areas resulting from RGB (a and c) and thermal images (b and d) in Amelia (Supplementary figures 4.2a and 4.2b) and Avigliano Umbro (Supplementary figures 4.2c and 4.2d). PCA (Figure 4.4) demonstrated that VEG (a), GLI (b), and UAV-based temperatures (c) distinguished between FHB⁺ and FHB⁻ plants at ZS 65 for both the 2019 and 2020 campaigns. Moreover, box-plots indicate that VEG (d), GLI (e), and UAV-based temperature values significantly differed between FHB⁺ and FHB⁻ areas during the two UAV-campaigns (2019 and 2020). The average VEG values (d) recorded in FHB⁻ areas were 1.46 and 1.42, while in FHB⁺ areas were 1.00 and 1.02 in 2019 and 2020, respectively. The average GLI values (e) recorded in FHB⁻ areas were 0.21 and 0.13, while in FHB⁺ were 0.09 and 0.10 in 2019 and 2020, respectively. The average UAV-based temperature values (f) recorded in FHB⁻ areas were 18.32°C and 18.94°C, while in FHB⁺ were 21.06°C and 20.94°C in 2019 and 2020, respectively.

4.3.4 Monitoring of FHB in the greenhouse

The progress of FHB severity was monitored in *F. graminearum*-inoculated plants in the greenhouse from 3 to 21 dpi (Figure 4.5a). The severity gradually increased reaching values close to 100% at 17 dpi, confirming the susceptibility of *T. turgidum* cv. Marco Aurelio. Figure 4.5b shows the FHB symptoms at 21 dpi. While no symptoms were observed on the mock treatment, few non-necrotic bleached spikelets appeared on drought-stressed plants.

4.3.5 Monitoring spike temperature and photosynthetic efficiency between mock, drought, and inoculated treatments

Figure 4.6 shows the temperature of spikes (Figure 4.6a) and the photosynthetic efficiency (Figure 4.6b) after the three treatments. Compared to the mock treatment, the temperature increased and the photosynthetic efficiency decreased in drought-stressed and inoculated plants, confirming a perturbation of the photosynthetic activity. In fact, the more the infection progressed, the higher the differences in spike temperature and photosynthetic efficiency between the inoculated and mock plants: inoculated spikes reached a temperature of 17.89, 18.48, and 18.67°C while mock spikes measured 17.12, 16.44, and 16.19°C, at 24, 48 and 72 hpi, respectively; the photosynthetic efficiency measured 0.719, 0.695 and 0.618 F_v/F_m for the inoculated spikes and 0.794, 0.793 and 0.801 F_v/F_m

for the mock at 24, 48 and 72 hpi, respectively. Notably, *F. graminearum* infection perturbed the photosynthetic parameters more than drought stress, highlighting that, by using these measures, it is possible to distinguish between drought-stressed and FHB-infected plants.

4.3.6 Expression pattern of the genes regulating stomatal conductance by RT-qPCR

E, R^2 , and the stability of the reference genes were calculated to validate the RT-qPCR results. E ranged from 0.9652 to 1.2741 and R^2 from 0.9651 to 0.9954. Standard errors (SE) among the Cq values of the three reference genes ranged from 0.198 to 0.369 indicating their stable expression under the three different treatments.

Since metrics derived from TIR and RGB images allowed the detection of infected spikes at ZS 65, the greenhouse experiments were designed to investigate the differential stomatal regulation response in proximity of the same phenological stage.

Moreover, drought-stressed plants were studied to observe their gene expression differences with plants under *F. graminearum* inoculation. Figure 4.7a shows a heatmap of the relative expression values of plant genes under drought stress and *F. graminearum* inoculation at 24, 48, and 72 hpi. Supplementary table 4.3 provides the relative expression values, SE, and the HSD test computed at 0.99 confidence level. Under terminal drought stress, *T. aestivum* allene oxide synthase (*TaAOS*), *T. aestivum* terpene synthase (*TaKSL*), *T. aestivum* mitogen-activated protein kinases (*TaMAPK*), *T. aestivum* calcium dependent protein kinase (*TaCDPK*), *T. aestivum* phosphatase (*TaABI*), *T. aestivum* MYB domain transcription factor (*TaPIMP*), *T. aestivum* NADPH oxidase (*TaRBOH*), and *T. aestivum* zeaxanthin epoxidase (*TaZEP*) were slightly up-regulated showing expression values ranging from 1.254-fold to 1.892-fold. Among the different time-points of *F. graminearum* inoculation (24, 48, and 72 hpi), the relative expression values of *TaAOS*, *T. aestivum* abscisic acid (ABA) aldehyde oxidase (*TaAAO*), *T. aestivum* ABA receptor (*TaREC*), *T. aestivum* β -1,3-glucanase (*TaBG*), *TaMAPK*, *TaCDPK*, *T. aestivum* epoxycarotenoid dioxygenase (*TaNCED*), *TaRBOH*, and *TaZEP* gradually increased from 24 to 72 hpi, while *T. aestivum* hydroperoxide lyase (*TaHPL*) and *T. aestivum* cytochrome P450 (*TaCYP450*) were down-regulated. Notably, the expression patterns of *TaKSL*, *TaAAO*, *TaREC*, *TaBG*, *TaCYP450*, *TaNCED*, and *TaZEP* were different between drought-stressed and *F. graminearum*-inoculated plants. In particular, in the inoculation treatment, at 72 hpi *TaREC*, *TaBG*, *TaNCED*, and *TaZEP* were strongly up-regulated (5.729, 5.143, 4.988 and 4.256-fold change, respectively) while *TaPR1* and *TaGAPDH* were gradually up-regulated from 24 to 72 hpi, indicating that the *F. graminearum* infection perturbed the innate immunity and physiological photosynthesis of the plants. Figure 4.7b represents the PCA obtained by computing relative gene expression values, spike temperature and photosynthetic efficiency values from drought-stressed and

F. graminearum inoculated plants. PCA demonstrated that these data distinguished between hydric stress and *F. graminearum* inoculation.

4.4 Discussion

Changes in temperature and colour of spikes are a result of the physiological defensive response of *T. turgidum* to FHB. Indeed, a thickening of the vascular bundles occurs when the infection moves from the floret to the rachilla which causes an increase in temperature, a decrease in photosynthesis efficiency, and a reduced transpiration due to limited water supply and stomatal closure (Kang and Buchenauer, 2000; Kheiri *et al.*, 2019). These physiological changes allow remote sensing techniques to detect and quantify FHB in *T. turgidum* non-destructively.

Previous studies have revealed the potential of remote sensing methods in detecting and assessing plant diseases. For example, Oerke and Steiner (2010) detected FHB using thermal imaging only at a late infection stage, when it was too late for the disease to be controlled. Al Masri *et al.* (2017) studied the effect of the primary infection site by *F. graminearum* and *F. culmorum* using thermography under controlled conditions and they observed that FHB infection significantly increased the temperature of spikes as it progressed from 6 to 29 dpi. Mahlein *et al.* (2019) demonstrated that *Fusarium*-infected spikelets showed higher temperatures and lower F_v/F_m values compared to mock control using a digital thermo-camera and a chlorophyll fluorometer. The authors recorded temperature and F_v/F_m values in entire spikes to detect FHB infection at 5 and 7 dpi.

RGB imaging was widely employed to detect FHB-infected and FHB-damaged kernels (Jaillais *et al.*, 2015; Cambaza *et al.*, 2019; Abbaspour-Gilandeh *et al.*, 2020), but few studies explored this technique to detect FHB on spikes. Huang *et al.* (2020) proposed an FHB diagnostic model of disease severity based on the fusion of RGB and spectral imaging. The results showed that the model was able to identify FHB severity in plants with an accuracy of 92%, thereby providing a technical basis for timely and effective control of FHB. Dammer *et al.* (2011) made use of RGB imaging to detect FHB in the field. Experimental plants were artificially infected with a spore suspension and RGB images were captured and analysed to detect the disease symptoms. The authors found a linear correlation between RGB-derived and visually observed disease levels in plants. Qiu *et al.* (2019) accurately detected FHB in the field using RGB imaging. RGB-derived data correlated with the number of diseased spikes tallied by manual count. These results are in agreement with those obtained in our study since we observed an increase in temperature and a decrease in photosynthetic efficiency in FHB-infected spikes. Moreover, UAV-based measurements distinguished FHB+ and FHB- areas, confirming that TIR and RGB imaging are powerful tools for FHB detection. To the best of our knowledge, for FHB detection, TIR and RGB cameras have only been employed on ground-based phenotyping platforms, minimizing their portability and limiting the scale at which they can be used.

Thus, this is the first study demonstrating that UAV-mounted TIR and RGB cameras enable rapid characterization of *T. turgidum* and detection of FHB in the field, overcoming the limitations associated with ground-based phenotyping.

In the present study, the relative expression level of genes involved in stomatal regulation was evaluated at ZS 65 to elucidate the genetic mechanism responsible for the phenotypic response to perturbation of photosynthesis, and to establish whether or not a differential gene response exists between drought-stressed and FHB-infected *T. turgidum*. Stomatal closure is the primary response of plants to water deficit, controlled by abscisic acid (ABA), a key hormone involved in controlling many aspects of plant growth, development, and responses to a variety of biotic and abiotic stresses (Daszkowska-Golec and Szarejko, 2013; Duarte *et al.*, 2019). Our results are in agreement with the literature since the majority of stomatal closure positive regulating genes involved in ABA biosynthesis (*TaKSL*, *TaZEP*, *TaCDPK*, *TaMAPK*, *TaRBOH*, and *TaPIMP*) were induced, while the negative regulators (*TaCYP450*, *TaBG*, and *TaREC*) were down-regulated after being exposed to drought stress. In contrast, some positive regulators of stomatal closure (*TaNCED*, *TaAAO*, and *TaHPL*) were down-regulated while a negative regulator (*TaABI*) was induced. Our findings support the hypothesis stating that hydric stress conditions do not completely induce stomatal closure in drought-tolerant wheat varieties, which correlate with lower level of closure-inducing genes and higher expressions of genes negatively regulators of stomatal closure (Xue *et al.*, 2006; Ji *et al.*, 2011; Rampino *et al.*, 2012; Gallé *et al.*, 2013). Moreover, recorded temperature values of spikes in the greenhouse revealed that Marco Aurelio is moderately tolerant to drought stress since temperature measures of plants exposed to drought stress did not significantly differ from the mock. However, amongst the distinctive responses between drought-stressed and *F. graminearum*-inoculated plants, *TaAAO*, *TaREC*, *TaBG*, and *TaNCED* were down-regulated in the former and up-regulated in the latter. On the other hand, *TaMAPK* and *TaCDPK* were up-regulated in drought-stressed but not in *F. graminearum*-inoculated plants at 24 hpi. Spikes temperature and photosynthetic efficiency values of *F. graminearum*-inoculated plants differed significantly from the mock and notably, at 72 hpi, the photosynthetic efficiency allowed the distinction between *F. graminearum*-inoculated and drought-stressed plants. These observations can be extremely helpful to develop further methodologies aimed at distinguishing between drought-stressed and FHB-infected plants in the field. In this regard, biotic and abiotic stresses need to be distinguished in order to optimize practical field management. Shaik and Ramakrishna (2014) segregated biotic and abiotic stresses in rice by applying machine learning approaches to the expression levels of a set of stress-responsive genes. Focusing on the use of imaging, hyperspectral sensors are the most suitable to distinguish biotic and drought stresses in many crops (Jones, 2011; Susič *et al.*, 2018). To date, most of the research studies distinguish between

drought and disease infection applied separately, while Ramegowda and Senthil-Kumar (2015) amply reviewed experimental evidence suggesting that, under combined drought and biotic stress, plants exhibit tailored physiological and molecular responses. Such tailored responses occur only in plants exposed to simultaneous stresses and such information cannot be inferred from individual stress studies.

Additionally, in *F. graminearum*-inoculated plants, most of the positive regulators of stomatal closure (*TaAOS*, *TaKSL*, *TaAAO*, *TaNCD*, *TaPIMP*, *TaRBOH*, and *TaZEP*) were induced from 24 to 72 hpi while *TaMAPK* and *TaCDPK* were up-regulated at 48 and 72 hpi, confirming that early stomatal closure is the physiological mechanism behind the increasing temperature and decreasing photosynthetic efficiency in spikes. The negative stomatal closure regulators *TaBG* and *TaREC* were also remarkably up-regulated, while *TaCYP450* was down-regulated. Our results are in agreement with the literature data, reporting the induction of *TaBG* and *TaREC* and the down-regulation of *TaCYP450* in FHB-susceptible wheat cultivars. *TaBG* belongs to the pathogenesis-related proteins family (PR2) in wheat, which is known to be induced as a defense mechanism in response to biotic and abiotic stresses (Muthukrishnan *et al.*, 2001). Particularly, De Zutter *et al.* (2017) investigated the *T. aestivum* response to a combined attack of *F. graminearum* and *Sitobion avenae* aphids, and observed the consistent up-regulation of *PR1* and *PR2*. Another study (Francesconi and Balestra, 2020) demonstrated that *TaPR1* and *TaPR2* were induced in an *F. graminearum*-susceptible *T. aestivum* (cv. Rebelde) but not as much as in the FHB-resistant *T. aestivum* (cv. Sumai3). The up-regulation of *TaREC* could be explained by evidence supporting that it may be involved in FHB susceptibility, since Gordon *et al.* (2016) found that *REC* silencing in *T. aestivum* (cv. Chinese Spring) resulted in slower progression of FHB symptoms and decreased DON content in wheat heads. On the other hand, *TaCYP450* was down-regulated in *F. graminearum*-infected *T. turgidum*. In fact, much evidence indicated that *CYP450* plays an active role in wheat resistance against FHB and DON accumulation. Strong *CYP450* accumulations were found in *F. graminearum*- and DON-resistant but not in susceptible wheat cultivars (Li *et al.*, 2010; Gunupuru *et al.*, 2018; Francesconi and Balestra, 2020). Several studies also demonstrated that *CYP450* was able to detoxify DON *in vitro* (Ito *et al.*, 2013) and *in vivo* (Gunupuru *et al.*, 2018).

The present study proved that UAV-based TIR and RGB image analysis can detect FHB infections at ZS 65. This can improve different aspects of FHB management and plant breeding. For example, our methodology allows timely detection of FHB and mapping affected locations in the field, thus optimizing the application timing and amount of fungicides needed to control the disease (Oerke and Steiner, 2010). It can also provide valuable information about the severity of FHB and help meet future food traceability requirements. Indeed, the ability to monitor FHB severity before further

processing of harvested kernels can help determining whether the grains fit for human or animal consumption, with special regard to mycotoxin content (Dammer *et al.*, 2011). For such purpose, the image-assisted analysis coupled with prediction modelling could be a valuable method to predict and detect the accumulated mycotoxin in the grains (Battilani, 2016; Leplat *et al.*, 2018; Fernando *et al.*, 2021). Several research studies reported also the accumulation of mycotoxin in absence of macroscopic symptoms, while microscopic analysis revealed that the host cells drastically changed after the infection (Brown *et al.*, 2010; Peiris *et al.*, 2011; Alisaac *et al.*, 2021). For such reasons, the detection of mycotoxin in asymptomatic spikes could be successfully achieved by using multispectral imaging (Bauriegel *et al.*, 2011; Dammer *et al.*, 2011; Leplat *et al.*, 2018) to support the mycotoxin traceability performed by the costly techniques based on chromatography (Tittlemier *et al.*, 2021). Furthermore, the presented methodology can help quantifying host resistance to FHB in pre-breeding and commercial breeding trials (Yang *et al.*, 2017), speeding-up breeding programs.

4.5 References

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4.6 List of tables

Supplementary table 4.1 List of target genes, accession numbers, gene functions, and primer pairs. The forward (F) and reverse (R) primers used for amplifications of *Triticum aestivum* genes: primers for *TaGAPDH* are from Jarošová and Kundu (2010), for *TaPR1* from Lu *et al.* (2006), for *TaACT* from Tundo *et al.* (2016), and for *TaTUB* and *TaFNR* from Tenea *et al.* (2011). The remaining primers are from Francesconi and Balestra (2020).

Gene and Accession Number	Function	Primer name	Primer pairs (5'-3')
<i>TaAOS</i> AY196004	Allene oxide synthase	TaAOS_F TaAOS_R	TCGGGCGTATTGCTGAGG TGCAGCAGCTTGCTTCTCTC
<i>TaHPL</i> AK335301	Hydroperoxide lyase	TaHPL_F TaHPL_R	GGACCACAGCATGACCGACA GAACCCGACCTCGCCGTT
<i>TaKSL</i> AB597957	Terpene synthase	TaKSL_F TaKSL_R	GGAACGGGATGCTAGAAATACG CCTCTCCTTATGTGGTCGGA
<i>TaAAO</i> AK331622	ABA-aldehyde oxidase	TaAAO_F TaAAO_R	CCTGAGCAATCAAAGCATCCC TCAGGCTGACACGCTGAACT
<i>TaREC</i> AK335719	ABA receptor	TaREC_F TaREC_R	GCTGGAGATCCTGGACGAC GTTGCACTTGACGATGGTGT
<i>TaBG</i> Y18212	β -1,3-glucanase	TaBG_F TaBG_R	AACGTGCGCCCTACTACC GCGTCGAACAGGCTCGTGTA
<i>TaMAPK</i> AF079318	Mitogen activated protein kinase	TaMAPK_F TaMAPK_R	CATCGACGTCTGGTCCGT GTCCTCGTTCCGGATGAATC
<i>TaCDPK</i> KU516994	Calcium dependent protein kinase	TaCDPK_F TaCDPK_R	CTTCTTTGTGGTGTCCCTCC GCTGTCAAACGCCTCCTT
<i>TaCYP450</i> XM_020311851	Cytochrome P450	TaCYP_F TaCYP_R	GTATTGGTGGACGAGGAAGG ATCTGGCGTGCATCACT
<i>TaNCED</i> KX711891	Epoxy-carotenoid dioxygenase	TaNCED_F TaNCED_R	CGGTGGAGAGGCAGGAGAA CGCGCGTAGAACAGAGCA
<i>TaABI</i> AB238930	Phosphatase	TaABI_F TaABI_R	TAGCAGATTACTGTCTGGGATCGG GAGCAGATGACAGCGACCA
<i>TaPIMP</i> KX683396	MYB domain transcription factor	TaPIMP_F TaPIMP_R	GTCACAGATCGCGTCGCAC GCATCCGAACCTGGCCGTACA
<i>TaRBOH</i> AY561153	NADPH oxidase	TaRBOH_F TaRBOH_R	TTGTTGGATTAGGAATTGGTGCT TGATCCATGTCTGGCAATCTC
<i>TaZEP</i> AF384103	Zeaxanthin epoxidase	TaZEP_F TaZEP_R	CTTGTCAACAGCCACTGTAC GGCACAACAATGTACTGTACTAGG
<i>TaGAPDH</i> KR029493.1	Glyceraldehyde-3-phosphate dehydrogenase	TaGAPDH_F TaGAPDH_R	AGTTCATGCCATGACTGCAA CCAGTGCTGCTTGGAAATGATG
<i>TaPR1</i> AJ007348	Pathogenesis related protein-1	TaPR1_F TaPR1_R	ACTACGACTACGGGTCCAACA TCGTAGTTGCAGGTGATGAAG
<i>TaACT</i> AB181991	Actin	TaACT_77F TaACT_312R	TCCTGTGTTGCTGACTGAGG GGTCCAAACGAAGGATAGCA
<i>TaTUB</i> TAU76745	β -tubulin2	TaTUB_F TaTUB_R	CGAGGAGGGCGAGTACGA AGCAAAGCACGACATGGACAT
<i>TaFNR</i> AJ457980	Ferredoxin - NADP(H)-oxidoreductase	TaFNR_F TaFNR_R	CACCGGCCAGTGATCTT AAGGGCGTCTGCTCCAAC

Supplementary table 4.2 List of Fusarium morphotypes isolated during the two UAV campaigns (2019 and 2020). The code indicates the numerical code of the morphotype (1-30) and the year (2019 or 2020) indicates the year of isolation. The table represents the data derived from the BLASTn (<https://blast.ncbi.nlm.nih.gov/>) analyses.

Code	Scientific name	Max score	Total score	Query cover	E value	Percentage of identity	Accession Lenght	Accession
1_2019	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
2_2019	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
3_2019	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
4_2019	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
5_2019	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
6_2019	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
7_2019	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
8_2019	<i>F. graminearum</i>	717	717	97%	0	100%	658	HQ702570.1
9_2019	<i>F. graminearum</i>	702	702	96%	0	100%	658	HQ702570.1
10_2019	<i>F. graminearum</i>	704	704	97%	0	100%	658	HQ702570.1
11_2019	<i>F. poae</i>	1177	1177	99%	0	99.69%	644	KJ947335.1
12_2019	<i>F. poae</i>	1177	1177	99%	0	99.69%	644	KJ947335.1
13_2019	<i>F. poae</i>	1177	1177	99%	0	99.69%	644	KJ947335.1
14_2019	<i>F. poae</i>	1177	1177	99%	0	99.69%	644	KJ947335.1
15_2019	<i>F. poae</i>	1177	1177	99%	0	99.69%	644	KJ947335.1
16_2019	<i>F. poae</i>	1177	1177	99%	0	99.69%	644	KJ947335.1
17_2019	<i>F. poae</i>	1177	1177	99%	0	99.69%	644	KJ947335.1
18_2019	<i>F. poae</i>	1177	1177	99%	0	99.69%	644	KJ947335.1
19_2019	<i>F. poae</i>	1151	1151	98%	0	100%	658	HE802673.1
20_2019	<i>F. poae</i>	1151	1151	98%	0	100%	658	HE802673.1
21_2019	<i>F. poae</i>	1151	1151	98%	0	100%	658	HE802673.1
22_2019	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	727	MK577935.1
23_2019	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	727	MK577935.1
24_2019	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	727	MK577935.1
25_2019	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	727	MK577935.1
26_2019	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	727	MK577935.1
27_2019	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	727	MK577935.1
28_2019	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	727	MK577935.1
29_2019	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	727	MK577935.1
30_2019	<i>F. proliferatum</i>	1182	1182	98%	0	99.69%	703	MK952799.1
1_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
2_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
3_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
4_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
5_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
6_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
7_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
8_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
9_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
10_2020	<i>F. graminearum</i>	1201	1201	99%	0	99.85%	658	MK507898.1
11_2020	<i>F. poae</i>	1153	1153	98%	0	100%	627	MH582306.1
12_2020	<i>F. poae</i>	1153	1153	98%	0	100%	627	MH582306.1

13_2020	<i>F. poae</i>	1153	1153	98%	0	100%	627	MH582306.1
14_2020	<i>F. poae</i>	1153	1153	98%	0	100%	627	MH582306.1
15_2020	<i>F. poae</i>	1153	1153	98%	0	100%	627	MH582306.1
16_2020	<i>F. poae</i>	1153	1153	98%	0	100%	627	MH582306.1
17_2020	<i>F. poae</i>	1146	1146	99%	0	99.52%	664	MK629641.1
18_2020	<i>F. poae</i>	1146	1146	99%	0	99.52%	664	MK629641.1
19_2020	<i>F. poae</i>	1146	1146	99%	0	99.52%	664	MK629641.1
20_2020	<i>F. poae</i>	1146	1146	99%	0	99.52%	664	MK629641.1
21_2020	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	684	JX397840.1
22_2020	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	684	JX397840.1
23_2020	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	684	JX397840.1
24_2020	<i>F. avenaceum</i>	1199	1199	98%	0	99.85%	684	JX397840.1

Supplementary table 4.3 Relative expression levels and standard errors of each selected gene for every plant treatment (drought stress, 24 hours post inoculation (hpi), 48 hpi and 72 hpi) for *Triticum turgidum* (cv. Marco Aurelio). Relative expression values were obtained using the equation $2^{-\Delta\Delta Cq}$, where ΔCq is the difference between the quantification cycles (Cq) of the target and reference genes, and $\Delta\Delta Cq$ is the difference between ΔCq of the treatment and of the mock, with *TaACT*, *TaTUB*, and *TaFNR* as *Triticum aestivum* reference genes. The mock treatment was used to normalize the relative expression levels. Data represent averages and standard errors for the four independent biological replicates and the four technical replicates examined. Letters (a–d) represent different levels of statistical significance obtained by performing one-way analysis of variance (ANOVA) with the Tukey test at 0.99 confidence level and $p < 0.01$.

Gene	Function		Drought stress	24 hpi	48 hpi	72 hpi
<i>TaAOS</i>	Allene oxide synthase	Relative expression	1.528	1.643	2.042	2.312
		Standard error	0.052	0.048	0.053	0.030
		$p < 0.01$	b	b	a	a
<i>TaHPL</i>	Hydroperoxide lyase	Relative expression	0.647	0.752	0.423	0.312
		Standard error	0.023	0.015	0.018	0.022
		$p < 0.01$	a	a	b	b
<i>TaKSL</i>	Terpene synthase	Relative expression	1.892	2.631	2.574	2.481
		Standard error	0.069	0.099	0.047	0.044
		$p < 0.01$	b	a	a	a
<i>TaAAO</i>	ABA-aldehyde oxidase	Relative expression	0.648	1.872	2.549	2.999
		Standard error	0.002	0.014	0.039	0.047
		$p < 0.01$	b	a	a	a
<i>TaREC</i>	ABA receptor	Relative expression	0.472	1.573	3.751	5.729
		Standard error	0.067	0.150	0.238	0.275
		$p < 0.01$	d	c	b	a
<i>TaBG</i>	β -1,3-glucanase	Relative expression	0.814	2.572	2.694	5.143
		Standard error	0.048	0.123	0.247	0.367
		$p < 0.01$	c	b	b	a
<i>TaMAPK</i>	Mitogen activated protein kinase	Relative expression	1.782	0.518	1.591	2.347
		Standard error	0.150	0.027	0.268	0.254
		$p < 0.01$	b	c	b	a
<i>TaCDPK</i>	Calcium dependent protein kinase	Relative expression	1.647	0.627	1.617	2.426
		Standard error	0.255	0.046	0.990	0.271
		$p < 0.01$	b	c	b	a
<i>TaCYP450</i>	Cytochrome P450	Relative expression	0.027	0.157	0.235	0.314
		Standard error	0.003	0.014	0.036	0.085
		$p < 0.01$	b	a	a	a
<i>TaNCED</i>	Epoxycarotenoid dioxygenase	Relative expression	0.754	1.258	1.673	4.988
		Standard error	0.065	0.185	0.214	0.257
		$p < 0.01$	c	b	b	a
<i>TaABI</i>	Phosphatase	Relative expression	1.254	1.317	0.987	1.222
		Standard error	0.014	0.032	0.042	0.074
		$p < 0.01$	a	a	a	a
<i>TaPIMP</i>	MYB domain transcription factor	Relative expression	1.534	1.248	1.335	1.466
		Standard error	0.124	0.233	0.241	0.187
		$p < 0.01$	a	a	a	a
<i>TaRBOH</i>	NADPH oxidase	Relative expression	1.634	1.247	1.897	2.637
		Standard error	0.142	0.255	0.144	0.241
		$p < 0.01$	b	c	b	a
<i>TaZEP</i>	Zeaxanthin epoxidase	Relative expression	1.524	1.348	2.697	4.256
		Standard error	0.245	0.314	0.125	0.524
		$p < 0.01$	c	c	b	a
<i>TaGAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	Relative expression	1.854	1.476	2.587	5.016
		Standard error	0.124	0.314	0.417	0.237
		$p < 0.01$	c	c	b	a
<i>TaPR1</i>	Pathogenesis related protein-1	Relative expression	1.235	1.587	2.672	4.997
		Standard error	0.185	0.236	0.114	0.255
		$p < 0.01$	c	c	b	a

4.7 List of figures

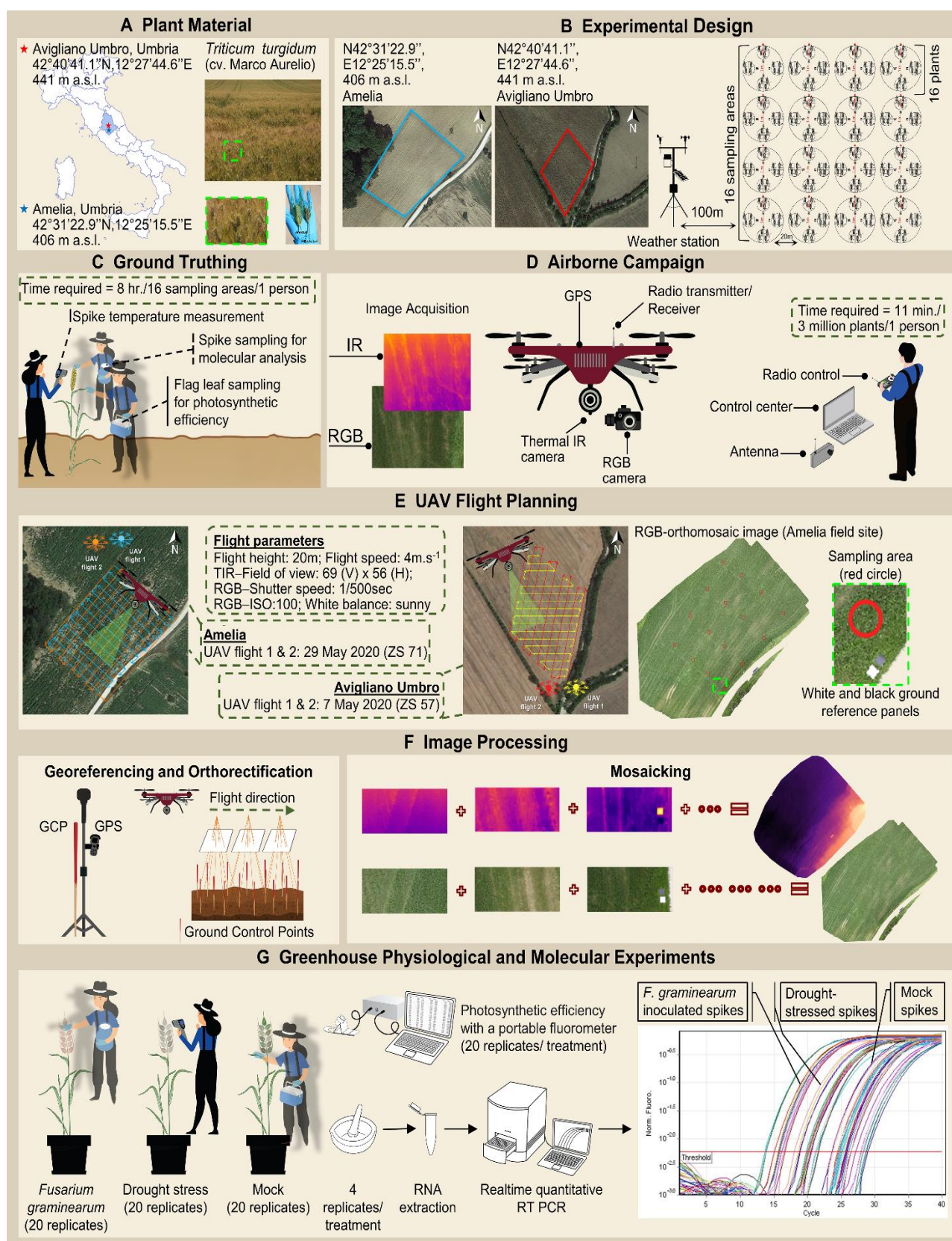


Figure 4.1 Workflow of the high-throughput field phenotyping methodology and the greenhouse experiment. (A) Plant material includes *Triticum turgidum* (cv. Marco Aurelio). Two fields were established in Amelia (Central Italy, 42°31'22.9"N, 12°25'15.5"E, Umbria Region, 406 m above sea level) and Avigliano Umbro (Central Italy, 42°40'41.1"N, 12°27'44.6"E, Umbria Region, 441 m a.s.l.) to host the *T. turgidum* plants. Sampled spikes show the

symptoms of a Fusarium head blight (FHB)-infected plant, compared to an uninfected plant. (B) An experimental plot was allocated within each field, and a 20 m grid was identified by positioning sixteen ground control points (GCPs) to be used for georeferencing. Around each of the GCPs, a circumference of 1 m of diameter was individuated and denominated as a sampling area. Weather data were recorded daily by two meteorological stations installed at 100 m distance from each field. (C) Within each sampling area, the spike temperature of sixteen randomly selected spikes was measured, and their flag leaves were sampled to measure their photosynthetic efficiency. Finally, the spikes were also sampled to distinguish FHB positive (FHB+) ones from FHB negative (FHB-) ones by molecular identification of *Fusarium* spp. (D) An unmanned DJI Matrice 600 hexacopter was equipped with Zenmuse X5 red-green-blue (RGB) and Zenmuse XT thermal infrared (TIR) sensors. The unmanned aerial vehicle (UAV) campaign allowed capturing RGB and TIR images of both experimental plots. (E) The flight missions were planned using Ground Station Pro app (DJI GS Pro, China). The UAV was flown in the autonomous mode at a nominal speed of 4 m/s, running parallel to plant rows to ensure complete coverage with good overlaps. (F) Image orthorectification, georeferencing, and mosaicking were performed using 16 GCPs captured with a global positioning system (GPS). (G) The greenhouse physiological and molecular experiments where plants were subjected to three different treatments: (i) drought stress treatment where plants did not receive water from Zadoks stage (ZS) 51 to 65; (ii) artificial inoculation treatment where spikes were uniformly spray-inoculated at ZS 65 with a suspension of 1×10^5 conidia/mL; and (iii) mock treatment where spikes were uniformly sprayed with a suspension of Tween-20 0.05% resuspended in sterile distilled water at ZS 65. Spikes temperature and photosynthetic efficiency of plants were recorded at 24, 48 and 72 hours post inoculation (hpi) from three independent experiments, each experiment consisting of 20 spikes for each treatment. Four spikes were sampled for each treatment, and were ground with mortar and pestle in liquid nitrogen until a fine powder was obtained, from which, RNA was extracted. Finally, quantitative real-time polymerase chain reaction (RT-qPCR) was performed to investigate transcripts of key genes involved in stomatal conductance regulation; transcripts of *F. graminearum*-inoculated plants were compared to those obtained from drought-stressed ones in order to investigate a differential response between these two types of stresses.

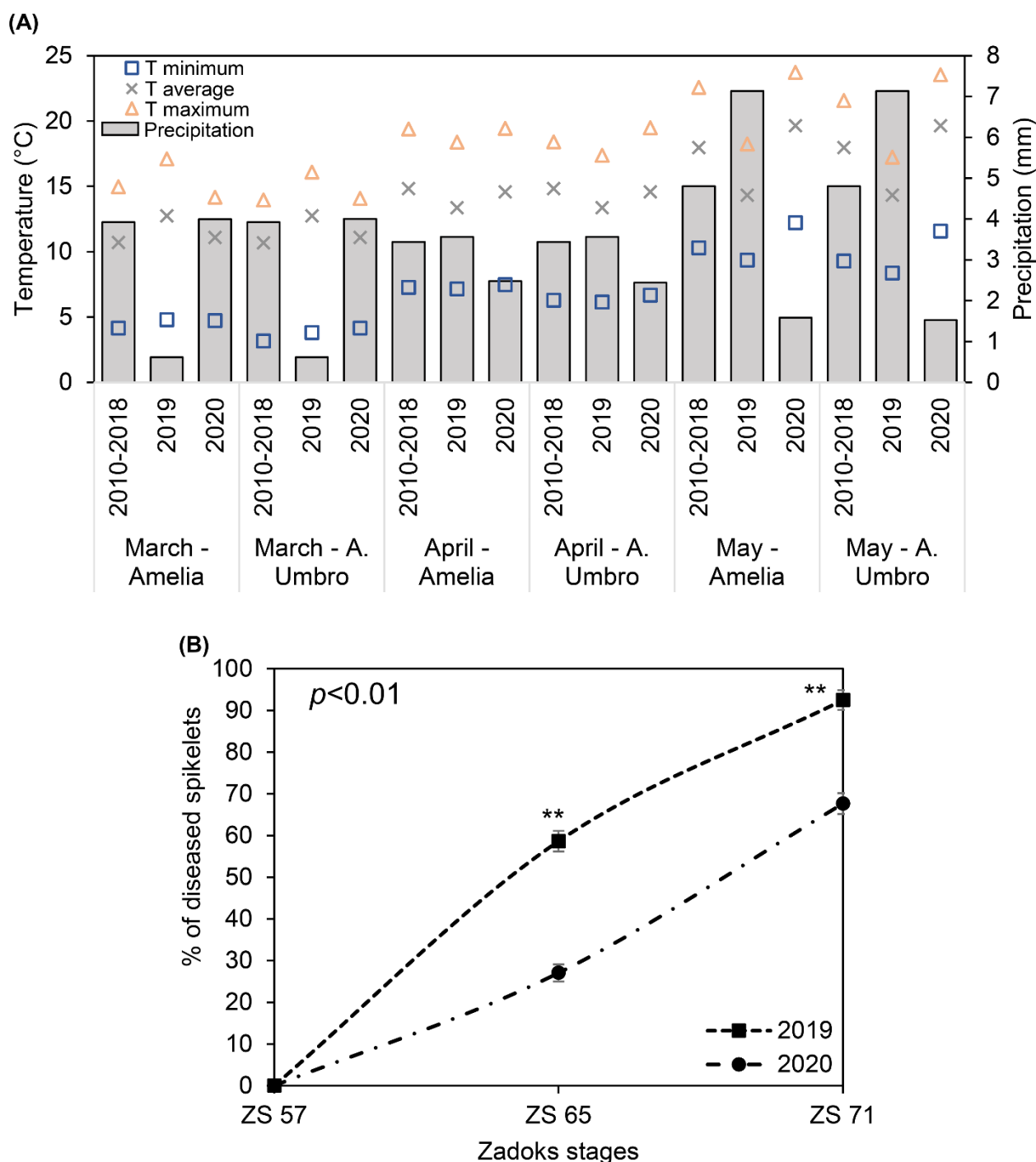


Figure 4.2 (A) Changes in maximum (yellow triangles), average (grey crosses) and minimum (blue squares) temperature (T), and precipitation (simple bar) of weather data recorded in 2019 in comparison with weather data recorded in 2020 and historical weather data from 2010 to 2018 for March, April, and May. The plotted values were obtained by averaging the mean daily values for each considered period. Weather data for 2019 and 2020 were recorded daily by two meteorological stations installed at 100 m distance from each of the experimental fields located in Amelia (Central Italy, 42°31'22.9''N, 12°25'15.5''E, Umbria Region) and Avigliano Umbro (Central Italy, 42°40'41.1''N, 12°27'44.6''E, Umbria Region). Historical weather data (2010-2018) were collected from the Hydrographic service of Umbria Region (<https://annali.regione.umbria.it/>). The historical weather data were collected from two meteorological stations located in Amelia (42°33'25.0''N, 12°25'01.0''E) and Avigliano Umbro (42°40'39.0''N, 12°26'13.0''E). (B) Severity percentage of Fusarium head blight (FHB) in *Triticum turgidum* (cv. Marco Aurelio) at Zadoks stage (ZS) 57, 65, and 71 in 2019 and 2020. Data represent averages and standard errors of 256 spikes (16 spikes for each of 16 visible targets). Asterisks (**) refer to the statistical analyses performed using one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) post hoc test at 0.99 confidence level and $p < 0.01$.

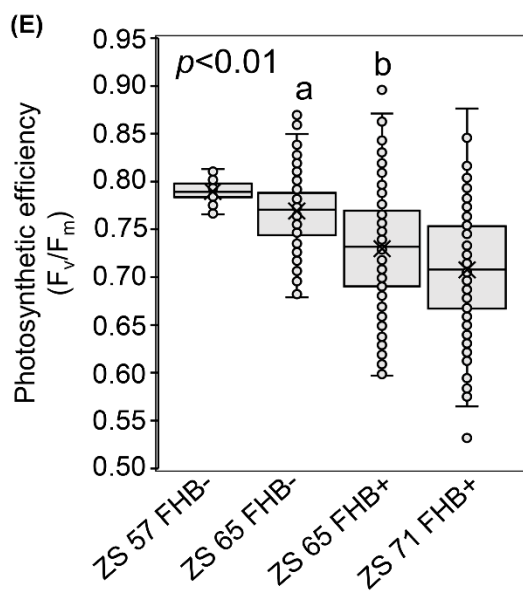
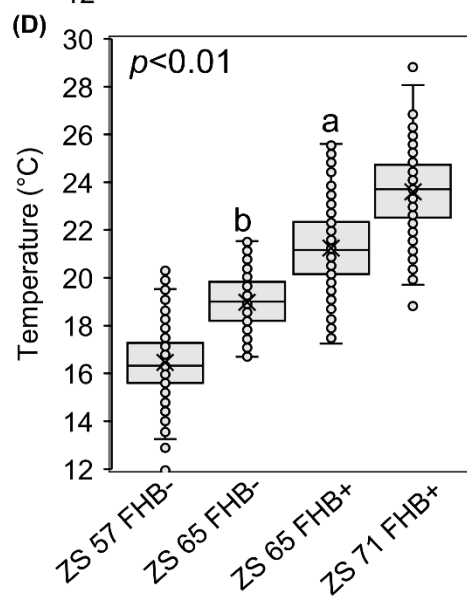
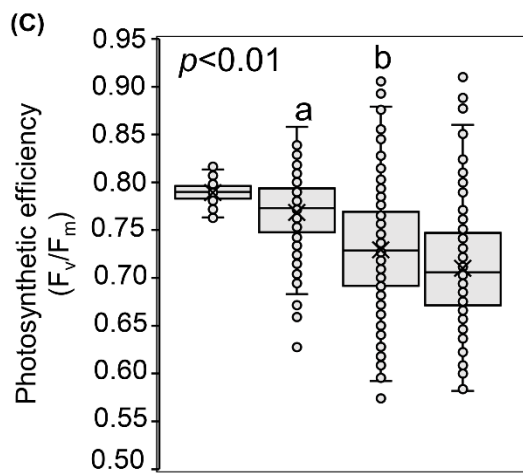
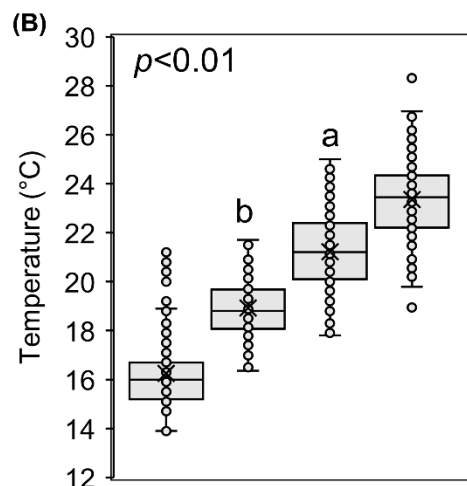
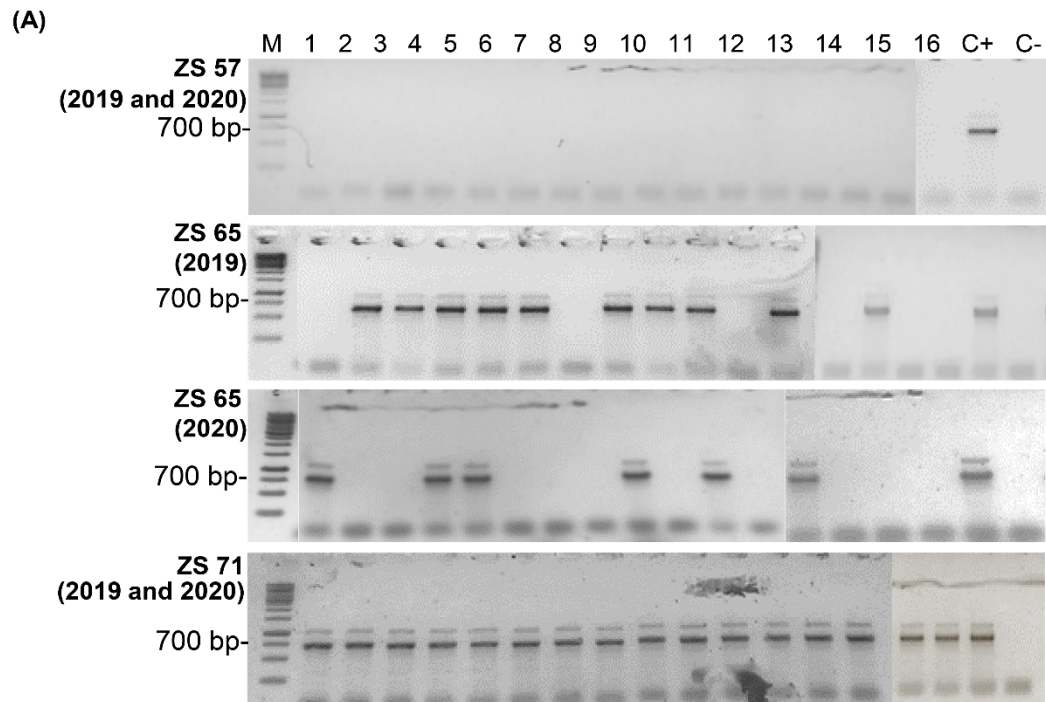


Figure 4.3 Molecular identification of Fusarium head blight (FHB) and ground-based temperature and photosynthetic efficiency measurements recorded in concurrence with unmanned aerial vehicle (UAV) campaigns conducted at Zadoks stage (ZS) 57, 65, and 71 of *Triticum turgidum* (cv. Marco Aurelio) in 2019 and 2020. (A) Molecular identification of *Fusarium* spp. using 1.5% agarose gel of the translational elongation factor 1- α (*TEF*) sequence (700 bp) in spikes of isolates obtained from the 16 sampling areas, sampled in 2019 and 2020 at ZS 57, 65, and 71. At ZS 57, none of the 16 sampling areas resulted FHB positive (FHB+) during both 2019 and 2020 UAV campaigns; at ZS 65, 10 and 6 sampling areas were FHB+ in 2019 and 2020, respectively; at ZS 71, all sampling areas were FHB+ during both 2019 and 2020 UAV campaigns. M represents a 100 bp DNA Ladder (Jena Bioscience); C- represents the negative control and C+ represents the presence of *F. graminearum* wild type (WT) 3824. The figure is obtained from four gels and the original pictures of the gels are available upon request. (B, D) Box-plot of the spikes' temperature ($^{\circ}\text{C}$) and (C, E) photosynthetic efficiency (F_v/F_m). The data represent averages and standard errors for 256 spikes (16 spikes for each sampling area) from 2019 (B, C) and 2020 (D, E). Different letters refer to the statistical analysis performed using one-way analysis of variance (ANOVA) with the Tukey's honest significant difference (HSD) post hoc test at 0.95 or 0.99 confidence level and $p < 0.05$ or $p < 0.01$.

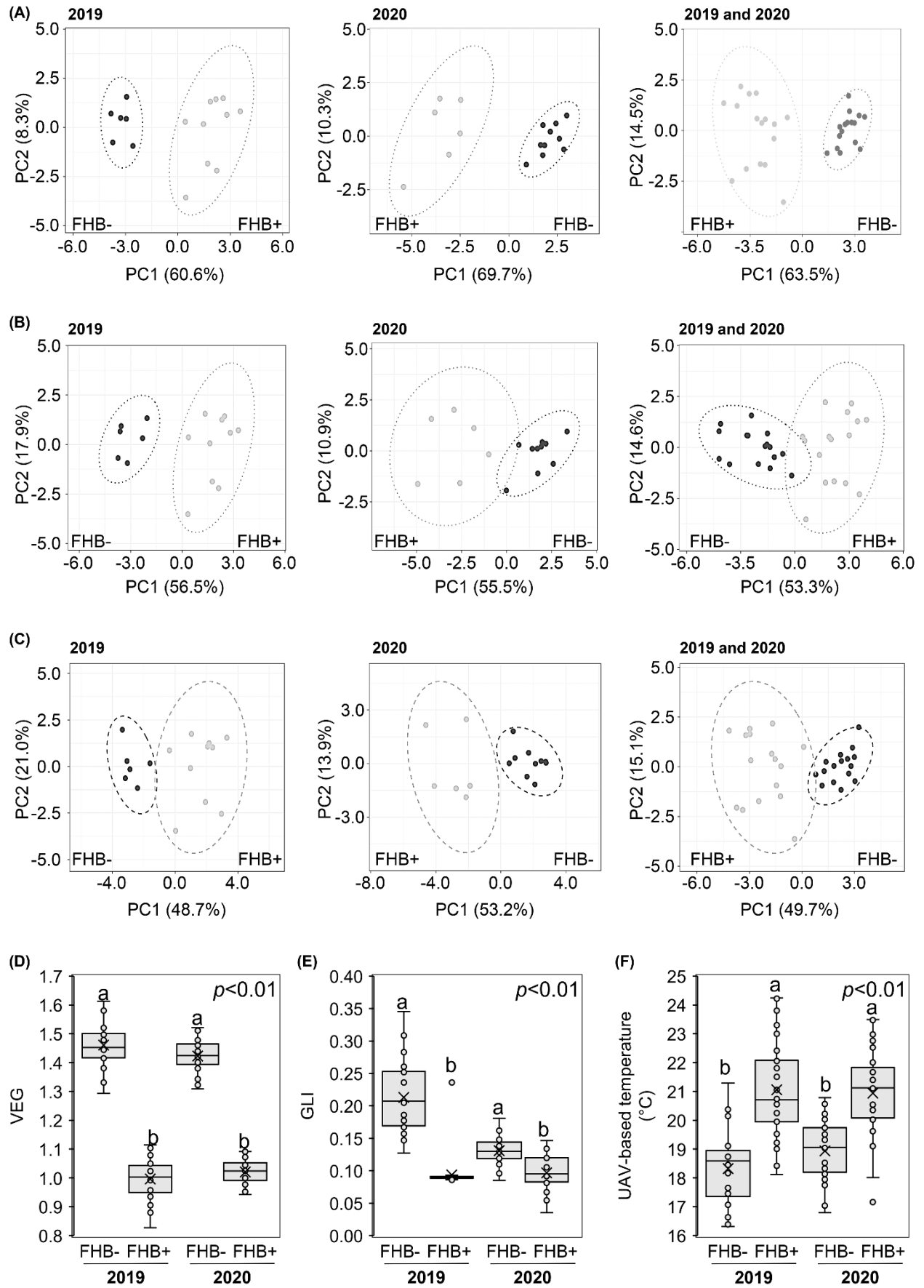


Figure 4.4 Principal component analysis (PCA) of unmanned aerial vehicle (UAV)-based (A) vegetative index (VEG), (B) Green leaf index (GLI), (C) Temperature at Zadoks stage (ZS) 65. PCA was performed by using ClustVis Software for $p < 0.05$ to distinguish between FHB+ and FHB- areas. Box-plot of UAV-based (D) VEG, (E) GLI, and (F) Temperature from FHB- and FHB+ areas. Data were recorded during 2019 and 2020. The data represent averages and standard errors for four measurements for each sampling area from 2019 (B, C) and 2020 (D, E). Different letters refer to the statistical analysis performed using one-way analysis of variance (ANOVA) with the Tukey's honest significant difference (HSD) post hoc test at 0.95 or 0.99 confidence level and $p < 0.05$ or $p < 0.01$. Only the data deriving from one year were compared.

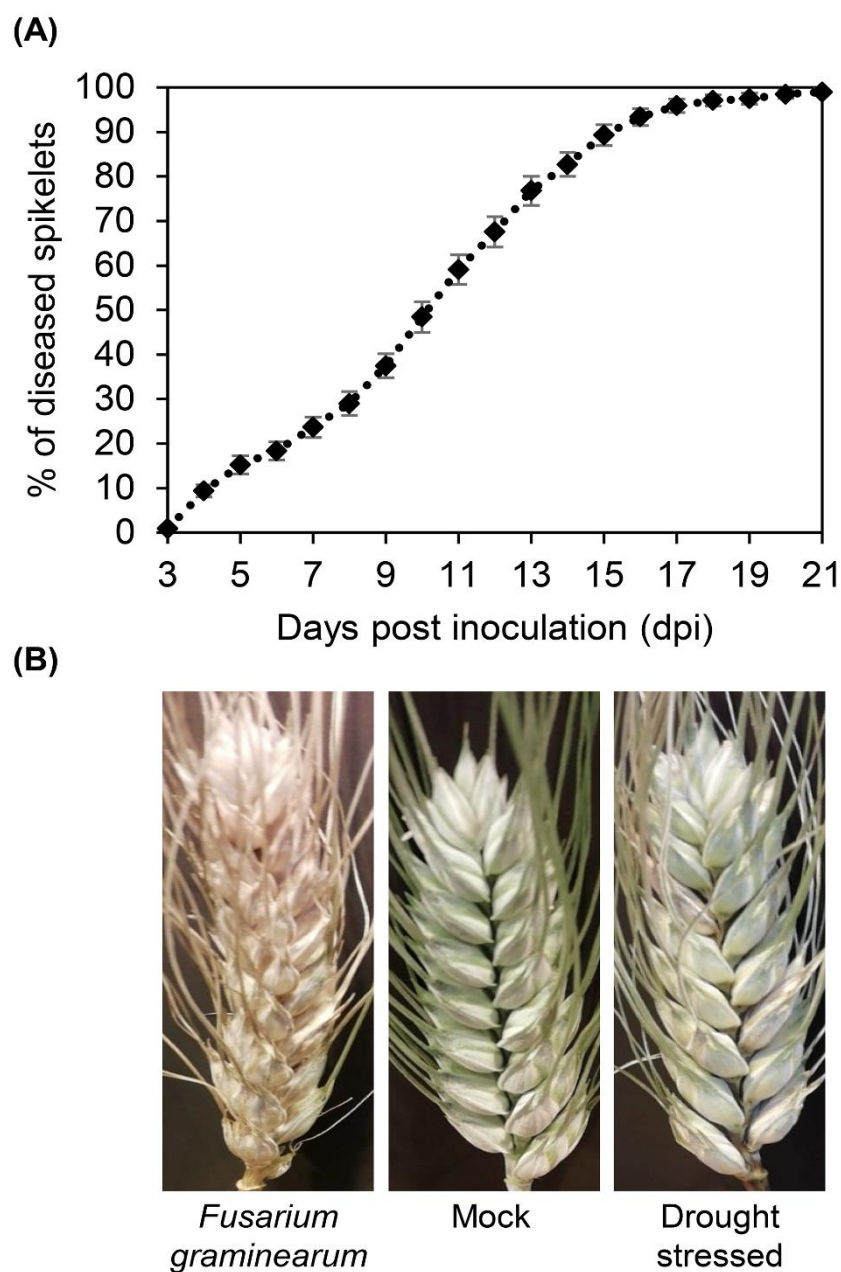


Figure 4.5 (A) Fusarium head blight (FHB) severity (%) in *Triticum turgidum* (cv. Marco Aurelio) from 3 to 21 days post inoculation (dpi) during the greenhouse experiments. (B) FHB symptoms in *T. turgidum* at 21 dpi. The data was derived from the averages and standard errors of three treatments (*F. graminearum*-inoculated, drought-stressed, and mock) with at least 20 plants for each, and three independent experiments for each treatment.

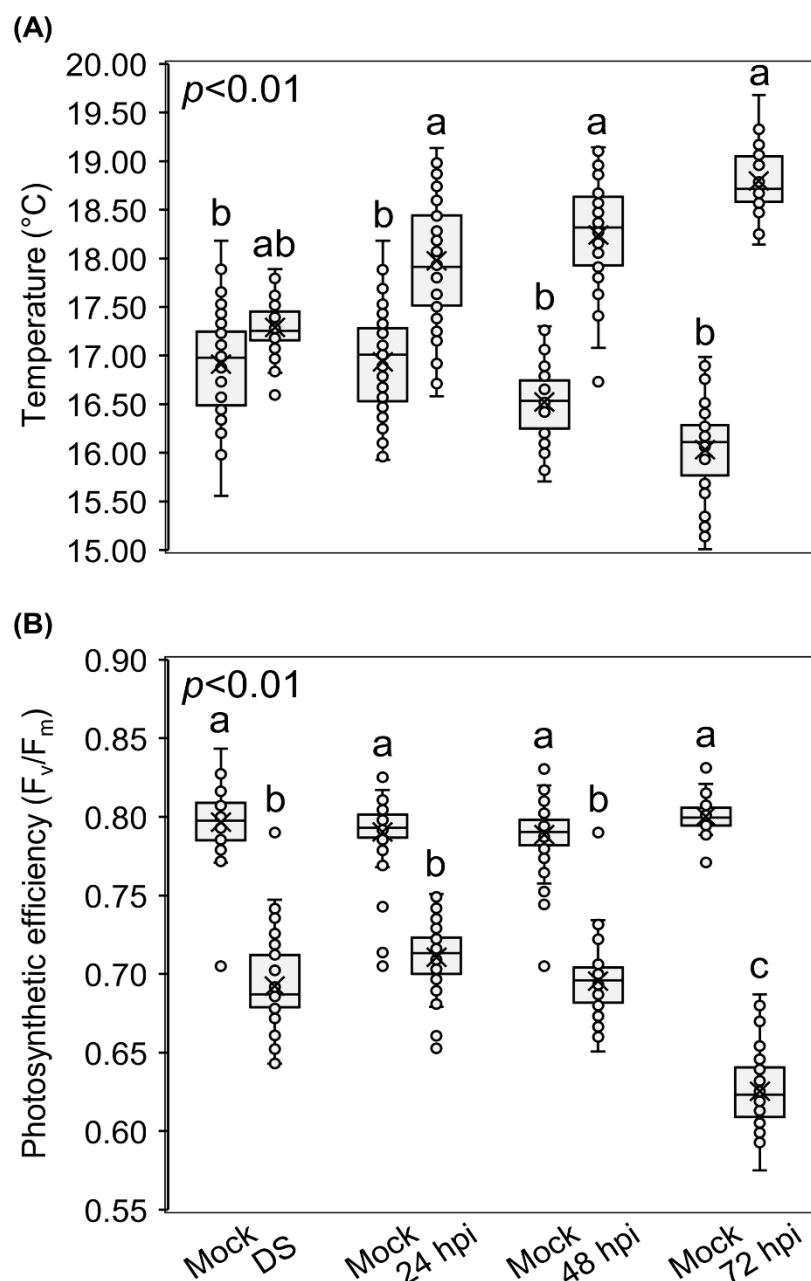


Figure 4.6 Box-plots of (A) spike temperatures (°C) and (B) photosynthetic efficiencies (F_v/F_m) of *Triticum turgidum* (cv. Marco Aurelio) plants subjected to mock (M), drought stress (DS) and *Fusarium graminearum* artificial inoculation (24, 48 and 72 hours post inoculation (hpi)) treatments during the greenhouse experiments. The data was derived from the averages and standard errors of three treatments (*F. graminearum*-inoculated, drought-stressed, and mock) with at least 20 plants for each, and three independent experiments for each treatment. Different letters refer to the statistical analysis performed using one-way analysis of variance (ANOVA) with the Tukey's honest significant difference (HSD) post hoc test at 0.99 confidence level and $p < 0.01$.

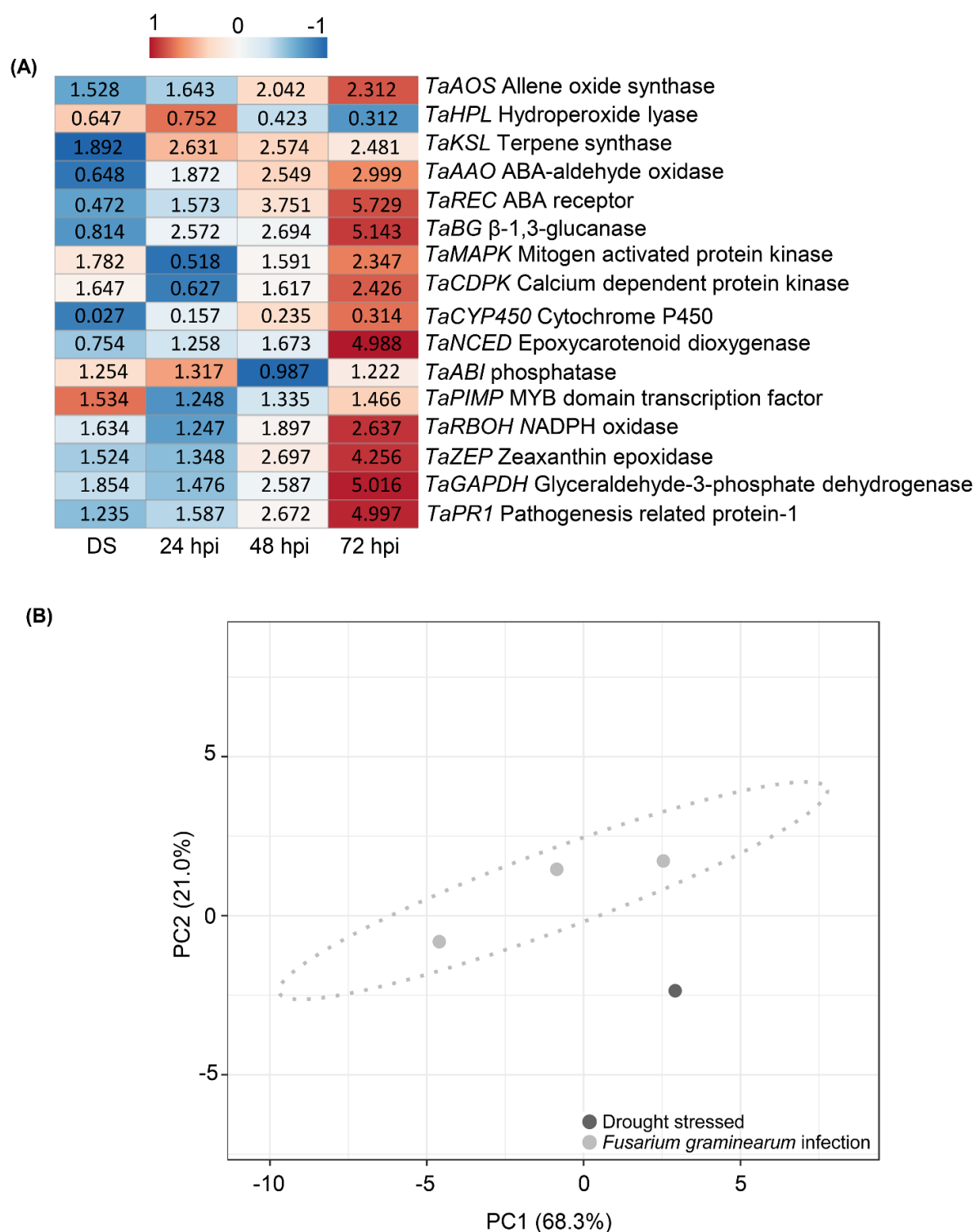
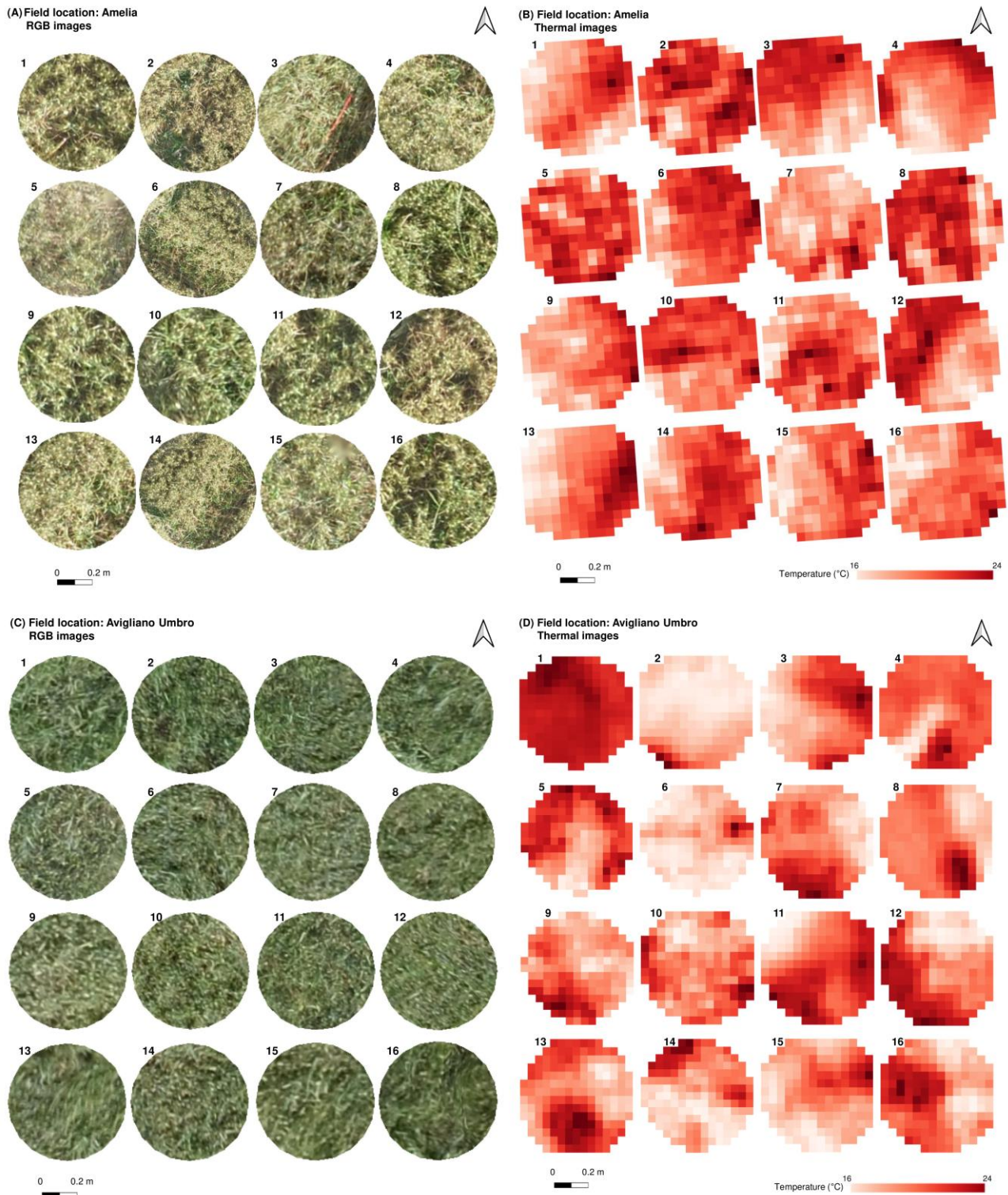


Figure 4.7 (A) Heatmap of relative expression level of the genes selected in *Triticum turgidum* (cv. Marco Aurelio) exposed to drought stress (DS) and *Fusarium graminearum*-inoculated at 24, 48, and 72 hours post inoculation (hpi) treatments. The expression values were normalized to the mock treatment and to *T. aestivum* actin (*TaACT*), *T. aestivum* β -tubulin2 (*TaTUB*) and *T. aestivum* ferredoxin-NADP(H)-oxidoreductase (*TaFNR*) as reference genes. The heatmap was constructed by plotting the z-score of the relative gene expression values and it was generated by analysing data with ClustVis Software. The red colour represents the up-regulated genes, while the blue colour the down-regulated genes. (B) Principal component analysis (PCA) of relative gene expression values, spike temperature and photosynthetic efficiency to distinguish between drought stress and *F. graminearum* infection during the greenhouse experiments. PCA was performed by using ClustVis Software for $p < 0.05$.



Supplementary figure 4.1 The DJI Matrice 600 unmanned aerial vehicle (UAV) platform used for field phenotyping of *Triticum turgidum* (cv. Marco Aurelio) on the ground, taking off, and in flight.



Supplementary figure 4.2 (a, c) Red-green-blue (RGB) and (b, d) thermal images of the 16 sampling areas in Amelia and Avigliano Umbro field sites, respectively. Images were calibrated and orthomosaicked using the Pix4Dmapper software (Pix4D, Switzerland). FHB+ areas had an average temperature of 21°C while FHB- areas had an average temperature of 19°C.

5. Chitosan hydrochloride decreases *F. graminearum* growth and virulence and boosts growth, development and SAR in two durum wheat genotypes

Sara Francesconi^{1*}, Barbara Steiner², Hermann Buerstmayr², Marc Lemmens², Michael Sulyok², Giorgio Mariano Balestra^{1*}

¹*Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia, Via San Camillo de Lellis, 01100, Viterbo, Italy*

²*Department of Agrobiotechnology Tulln (IFA-Tulln), University of Natural Resources and Life Sciences Vienna (BOKU), Konrad Lorenz Straße 20, A-3430 Tulln an der Donau, Austria*

Corresponding authors: Sara Francesconi, francesconi.s@unitus.it; Giorgio Mariano Balestra, balestra@unitus.it

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Abstract: Fusarium head blight (FHB) is a devastating disease for cereals. FHB is managed by fungicides at anthesis, but their efficacy is variable. Conventional fungicides accumulate in the soil and are dangerous for animal and human health. This study assayed the antifungal ability of chitosan hydrochloride against *Fusarium graminearum*. Chitosan reduced *F. graminearum* growth and downregulated the transcript of the major genes involved in the cell growth, respiration, virulence, and trichothecenes biosynthesis. Chitosan promoted the germination rate, the root and coleoptile development, and the nitrogen balance index in two durum wheat genotypes, Marco Aurelio (FHB-susceptible) and DBC480 (FHB-resistant). Chitosan reduced FHB severity when applied on spikes or on the flag leaves. FHB severity in DBC480 was of 6% at 21 dpi after chitosan treatments compared to *F. graminearum* inoculated control (20%). The elicitor-like property of chitosan was confirmed by the up-regulation of *TaPAL*, *TaPR1* and *TaPR2* (around 3-fold). Chitosan decreased the fungal spread and mycotoxins accumulation. This study demonstrated that the non-toxic chitosan is a powerful molecule with the potential to replace the conventional fungicides. The combination of a moderately resistant genotype (DBC480) with a sustainable compound (chitosan) will open new frontiers for the reduction of conventional compounds in agriculture.

Keywords: Chitosan; fusarium head blight; durum wheat; plant defence; systemic acquired resistance; fungicides.

5.1 Introduction

Wheat is one of the most cultivated crops worldwide and its annual production is estimated to be more than 700 million tons. Wheat accounts for more than 20% of total human food calories, it is extensively grown on 17% of all crop areas, and it is the staple food for 40% of the world's population, comprising Europe, North America, and the western and northern parts of Asia (Peng *et al.*, 2011; Feldman and Levy, 2012). Modern wheat cultivars refer to two species, the hexaploid bread wheat, *Triticum aestivum* L. and the tetraploid durum-type, *T. turgidum* subsp. *durum* (Desfontaines) Husnache (Charmet, 2011; Dubcovsky and Dvorak, 2007). Particularly, durum wheat is the most widespread crop in the Mediterranean area and around 77% of the Italian production of durum wheat comes from the Central and Southern regions, mainly employed for the production of pasta (Fagnano *et al.*, 2012).

Fusarium head blight (FHB) is caused by the *Fusarium* species and is considered as the most devastating wheat disease due to the lack of resistant cultivars, significant yield loss and grain quality reduction, and health threat of wheat food and feed contaminated with mycotoxins produced by the causal agents (Ma *et al.*, 2020; Khan *et al.*, 2020). FHB epidemics have occurred regularly in the wheat-growing regions such as Asia, North and South America, and Europe (McMullen *et al.*, 2012). *F. graminearum* Schwabe is the most aggressive FHB causal agents. *Fusarium* spp. are fungal hemi-biotrophic pathogens and in field conditions the inoculum arises primarily from plant residues and soils and its dissemination is mainly promoted by water splash and wind (van der Lee *et al.*, 2015; Darwish *et al.*, 2014). Anthesis is the most sensitive phenological stage to the infection and, with a warm and humid climate, FHB develops rapidly within 3-6 days after the infection. On wheat spikelets, airborne spores germinate and enter the plants via degenerated anther tissues or minute natural openings under the lemma. Further, the growth of the fungus occurs between the cells and it passes from the xylem and the pits and colonizes the tissue, forming necrosis. This leads to the water soaking tissues causing the production of shriveled kernels and premature bleaching that affects photosynthesis (Khaled *et al.*, 2017; Dweba *et al.*, 2017; Bai and Shaner, 2004; Parry *et al.*, 1995). After the infection, genes for deoxynivalenol (DON) biosynthesis are expressed by the fungus, facilitating the spreading of the pathogen from spikelets to rachis (Jansen *et al.*, 2005; Ilgen *et al.*, 2009).

Fungicide application is one of the strategies available for the management of FHB and DON (Wegulo *et al.*, 2015). Triazole fungicides containing tebuconazole are currently the most effective [18,19] and it is reported that the application of tebuconazole decreased the levels of DON in

artificially inoculated plants (Homdork *et al.*, 2000; Mesterházy and Bartok, 2003; Champeil *et al.*, 2004), but also conflicting results have been obtained (Simpson *et al.*, 2001). Another class of fungicides particularly in use in Italy for FHB control are strobilurins, comprising azoxystrobin, mixed with triazole compounds. Nevertheless, strobilurin fungicides are generally ineffective in controlling FHB, and their application tends to increase DON content as compared to nontreated control (Pirgozliev *et al.*, 2002). The efficacy of conventional chemical fungicides for FHB is variable and many researchers reported less than 50 to 60% of control (Mesterházy and Bartok, 2003; Shah *et al.*, 2018). Moreover, from recent studies, the occurrence of fungicide-resistant *Fusarium* strains, due to the over-use of the same types of compounds, has been reported. Notably, a *F. graminearum* isolate that is resistant to tebuconazole has been discovered in the USA (Spolti *et al.*, 2014) as well as some strains that are resistant to benzimidazole-based fungicides in China (Chen and Zhou, 2009). Fungicides not only affect the target organism, but the whole environment, including atmosphere, soil, groundwater, and surface water by flow, leaching, and pulverization processes, thus contaminating the ecosystem (Nsibande and Forbes, 2016; Taha *et al.*, 2014). The presence of fungicides in the ecosystems have adverse effects that vary with the contaminant concentration, amount, and exposure time (Margni *et al.*, 2002; Rice *et al.*, 2007). As a consequence, they may present detrimental effects to humans and can cause severe health problems such as cancer, infertility, malformation, and chromosomal changes as a consequence of oxidative stress (Sabarwal *et al.*, 2018). For those reasons, these contaminants should be removed from the environment, especially from water. In fact, severe restrictions have been posed on the use of compounds whose characteristics are persistence, bioaccumulation, toxicity, and long-range environmental transport (Mekonen *et al.*, 2016; de Souza *et al.*, 2020). To respond to the actual increasing demand of food and the concomitant need to reduce the use of conventional pesticides, the scientific research needs to focus on sustainable agricultural production systems (Oerke and Dehne, 2004; Shand *et al.*, 2006). In this scenario, natural-derived compounds represent a novel strategy for the replacement of conventional agrochemicals (Fortunati *et al.*, 2019; Morcia *et al.*, 2017; Gao *et al.*, 2016).

Chitosan has been one of the most interesting biopolymers due to its biocompatibility, antioxidant, anticancer, biodegradability, antimicrobial, and non-toxic properties as well as being an economical material, produced from waste resources such as seafood shells (Dash *et al.*, 2011; Sharif *et al.*, 2018). Chitosan is a derivate from chitin, and, besides agriculture, it has a large set of applications, such as in the food, cosmetic, textile, and biomedical industries (Kaya *et al.*, 2017; Akyuz *et al.*, 2017).

In plants, chitosan is largely used to mimic biotic and abiotic stresses. The first study of using chitosan as an antimicrobial agent in plants was reported by Allan and Hadwiger (1979), where they demonstrated the fungicidal effects of chitosan on different cell wall compositions of fungi. The

improvement of the defence system after applying chitin and chitosan has been observed in both monocotyledons and dicotyledons (Barber *et al.*, 1989). Chitosan has been a bio-fungicide, bio-bactericide, and bio- virucide, which boosts plant defence systems against the pathogen, thus inducing the natural defence system of plants, fruits, and vegetables (Ni *et al.*, 2020; Mania *et al.*, 2019; Fortunati *et al.*, 2017). It was also found to be involved in altering the growth of fungi and to reduce toxin production (Bhaskara *et al.*, 1998). Moreover, chitosan use is recommended to enhance the photosynthetic activity, vegetative growth, antioxidant activities, fruit quality attributes, and overall growth and yield of the crop (Sharif *et al.*, 2018). This body of research suggests that chitosan can be used as biostimulant of the plant immune system to challenge diverse hostile environments. In addition, many research studies stated that pathogenesis-related (PR) genes and several defence-related enzymes such as phenylalanine ammonia-lyase (PAL) are induced by chitosan (Sharif *et al.*, 2018; Hadwiger, 2013; Lopez-Moya *et al.*, 2019; Maluin and Hussein, 2020).

In this context, the focus of the present research study was to test the inhibitory effect of chitosan hydrochloride against *F. graminearum* by in vitro and in vivo assays. Four different in vitro assays were performed to study the mode of action of chitosan against *F. graminearum*, while two durum wheat genotypes (Marco Aurelio, FHB susceptible, and DBC480, FHB resistant) were employed as plant host during in vivo experiments.

5.2 Materials and methods

5.2.1 Fungal, chemical and plant materials

The highly virulent and mycotoxin-producing isolate of *F. graminearum* IFA66 was originally isolated by the IFA-Tulln (Tulln an der Donau, Austria) (Pastirčák *et al.*, 2002), and was the reference strain used in this study. Chitosan hydrochloride (99% purity, molecular weight 60 kDa, degree of deacetylation 80–90%) (CAS number 70694-72-3), a water-soluble derivate of chitosan, tebuconazole (CAS number 107534-96-3), and azoxystrobin (CAS number 131860-33-8) were purchased from Merck (Germany). The FHB-susceptible Italian durum wheat cultivar Marco Aurelio was provided by the Agricultural Consortium of Perugia (Umbria Region, Italy). This genotype, grown in Central and South of Italy, is extensively used for pasta production and it is characterized by an excellent protein content and high productivity, thus it has a high economic importance. The FHB-resistant durum wheat experimental line DBC480, harbouring the resistant quantitative trait locus *Fhb1*, was provided by IFA-Tulln (Tulln an der Donau, Austria). DBC480 was developed at IFA-Tulln by four generations of marker-assisted backcrossing of the highly resistant *T. aestivum* cultivar Sumai3 into the background of the Austrian *T. durum* variety Semperdur (Prat *et al.*, 2017).

5.2.2 *In vitro* assays

Four different *in vitro* assays were performed in order to test the inhibitory effect of chitosan hydrochloride against *F. graminearum*. Five concentrations of chitosan hydrochloride have been assayed, 0.01–0.05–0.1–0.5, and 1% (w/v). Moreover, tebuconazole and azoxystrobin were tested alone or in combination as reference fungicides at the suggested field dose from commercial formulations (0.06% w/v for tebuconazole and 0.08% w/v for azoxystrobin).

5.2.2.1 96 microtiter plates assay and determination of the minimum inhibitory concentration (MIC)

Fresh mycelium of *F. graminearum* grown on Potato Dextrose Agar (PDA) was transferred on Synthetic Nutrient Poor Agar (SNA) and cultured at 21 °C to obtain macroconidia (Urban *et al.*, 2002). After 10 days on SNA, the conidia were scraped with a glass rod after pipetting 1 mL of sterile distilled water onto the surface of a Petri dish. The conidial suspension was recovered, and the concentration was measured by using a Thoma Chamber (0.100 mm depth and 0.0025 mm²). Chitosan hydrochloride, tebuconazole, and azoxystrobin were dissolved in sterile distilled water at the previously cited concentrations, the pH values were controlled and resulted to be around 5, and filtered by using a sterile syringe filter with a pore size of 0.2 µm. The obtained solutions were pipetted into the microtiter plates and the conidial suspension was added to each well in order to obtain a final concentration of 1×10^5 conidia/mL. The plates were incubated at 21°C in the dark for 24 hours. After that, 10 µL of the conidial-antimicrobial compound suspension were transferred into a new microtiter plate containing Potato Dextrose Broth (PDB) and incubated at 21°C in the dark for 48 hours. The absorbance from the fungal biomass was measured at OD₄₅₀ by using a DR-200B Microplate reader (Diatek instruments, China). Mock (untreated conidial suspension cultured on PDB) and blank (PDB) controls were also included. The % of growth inhibition was calculated for each tested substance and concentration by using the following equation (Granade *et al.*, 1958; Ncube *et al.*, 2008): % of growth inhibition = $100 \times (\text{Mock-Treated}/\text{Mock})$. Data were obtained from three independent experiments, each one consisting of four replicates for each substance and concentration tested.

5.2.2.2 Incorporated medium assay

Sterile PDA was supplemented with chitosan hydrochloride, tebuconazole, azoxystrobin, or a mixture of tebuconazole and azoxystrobin at the previously cited concentrations. The PDA incorporated with the compounds was poured on Petri dishes and allowed to solidify. Afterward, a 5 mm plug from a fresh culture of *F. graminearum* was placed at the centre of the Petri dish and cultured in the dark at 21°C for 7 days. Mock control was also included. After 7 days, the mycelial growth was evaluated

by measuring four fungal colony diameters from each plate. The % of growth inhibition was calculated for each tested substance and concentration by using the equation % of growth inhibition = $100 \times (\text{Mock-Treated}/\text{Mock})$ (Ncube *et al.*, 2008). Data were obtained from three independent experiments, each one consisting of four replicates for each substance and concentration tested.

5.2.2.3 Agar diffusion assay

A *F. graminearum* conidial concentration of 1×10^5 conidia/mL was obtained and prepared as described in the “96 microtiter plates assay and determination of MIC” paragraph. 1 mL of the conidial suspension was homogenously plated on the surface of PDA onto the Petri dishes and allow to dry. After that, a well of 5 mm diameter was created by punching the agar in the centre of the Petri dish and 100 μ L of the tested compounds at different concentrations were pipetted inside. Mock control was also included. The plates were incubated in the dark at 21°C for 48 hours. Afterward, the visible inhibition halo was evaluated by measuring four diameters from each plate. The % of growth inhibition was calculated for each tested substance and concentration by using the following equation: % of growth inhibition = $100 \times (\text{Treated-Mock}/\text{Mock})$ (Ncube *et al.*, 2008). Data were obtained from three independent experiments, each one consisting of four replicates for each substance and concentration tested.

5.2.2.4 Real-Time qPCR

The relative expressions of the genes regulating the cellular physiology, the virulence, and the trichothecenes production in *F. graminearum* were evaluated after treating the mycelium with chitosan hydrochloride, tebuconazole, azoxystrobin, or the mixture of tebuconazole and azoxystrobin. First, solutions of PDB supplemented with chitosan hydrochloride at 1% w/v, tebuconazole at 0.06% w/v, azoxystrobin at 0.08% w/v, or the mixture of tebuconazole at 0.06% w/v and azoxystrobin at 0.08% w/v were prepared in sterile glass flasks. Starting from these solutions, four serial dilutions (1:10, 1:100, 1:1000, 1:10,000) were prepared for each compound. The dilutions were performed in PDB, in order to identify the optimal concentration that allowed to obtain the same quantity of fungal biomass compared to the mock treatment. In this way, the detected transcripts amount was not influenced by the recorded fungal biomass. 1 mL of a conidial suspension at 1×10^5 conidia/mL of *F. graminearum* was pipetted in each flask and cultured in the dark at 21°C in an orbital shaker for 48 hours. After that, 1 mL of each suspension was measured spectrophotometrically (OD₄₅₀) (Onda Spectrophotometer, Italy), and the serial dilution (1:1000) allowed to reach the same absorbance value of the mock treatment was chosen for the transcript's analysis. Fresh PDB was used as blank control. The fungal biomass was recovered by using a vacuum pump from the 1:1000 dilutions for each antimicrobial compound and immediately stored in liquid nitrogen and at -80°C. 100 mg of mycelium

were subjected to RNA extraction following the instructions provided by InviTrap® Spin Plant RNA Mini Kit (Strattec Molecular GmbH, Germany). The RNA was resuspended in RNase-free sterile distilled water and immediately poured onto ice and quantified with Qubit™ fluorometer 1.01 (Invitrogen, United States) using the Qubit™ RNA BR Assay Kit (Thermo Fisher Scientific, United States). To confirm the total quantity and integrity of the RNA, 5 µL of the extracted RNA was subjected to thermal shock (10 minutes at -80°C after 5 minutes at 65°C) and run on 1.5% denaturing agarose gel. The synthesis of the cDNA was performed using 500 ng of RNA following the instructions provided by Xpert cDNA Synthesis Supermix with a gDNA eraser (GRiSP Research Solutions, Portugal) in a final volume of 20 µL. To ensure that the synthesis of the cDNA and the elimination of the gDNA had succeeded, a reverse transcriptase PCR (RT-PCR) of the translational elongation factor 1- α (*TEF*) (containing an intron in the amplified sequence) was performed. The RT-PCR was performed by following the instructions provided by GoTaq MasterMix (Promega, United States) in a total volume of 10 µL and conditions of amplification included: an initial denaturation step of 2 minutes at 95°C; 35 cycles of 30 seconds denaturation at 95°C; 40 seconds of annealing at 53°C; 30 seconds of elongation at 72°C; a final elongation step of 5 minutes at 72°C. The amplification run also consisted of a no-template control (NTC) and a genomic DNA (gDNA) control. The amplicons were visualized on 1.5% agarose gel. Supplementary table 5.1 shows the list of target genes, accession numbers, their functions, the corresponding primer pairs, and their references used to perform the RT-qPCR (Yun *et al.*, 2014; Carapito *et al.*, 2008; Heidtmann-bemvenuti *et al.*, 2016; Horevaj *et al.*, 2011; Brown *et al.*, 2001; Geiser *et al.*, 2004; Harris *et al.*, 2016; Lee *et al.*, 2014). The efficiency of the RT-qPCR amplification (E) was determined for each primer pair as follows: five 1:10 serial dilutions (1:1-1:10,000) were obtained for each cDNA and amplified in four replicates. E and correlation coefficient (R^2) values were calculated by means of the slope of the standard curve obtained by plotting the fluorescence versus the serial dilution concentrations using the equation: $E = 10^{(-1/\text{slope})} - 1$. The relative expression levels of the target genes were calculated on the basis of the Cq values of the four technical replicates derived from four independent biological replicates for each compound by applying the equation: Relative expression = $2^{-\Delta\Delta Cq}$, using *TEF*, *TUB*, and *GAPDH* as the reference genes and the mock treatment to normalize the relative expression levels (Bustin *et al.*, 2009). The RT-qPCR was performed following the instructions provided by the Rotor Gene Q (Qiagen, Germany and Xpert Fast SYBR (uni) MasterMix (GRiSP Research Solutions, Portugal), in a final volume of 10 µL. The amplification conditions included an initial denaturation step of 3 minutes at 95°C; 40 cycles of 5 seconds denaturation at 95°C; 30 seconds of annealing at 60°C; and 20 seconds of elongation at 72°C. A final melt cycle (70–99°C) was performed to confirm

the amplicons' unicity. NTC controls were included and the amplification was considered negative when a value of $C_q \geq 38$ was detected.

5.2.3 Coating of kernels and evaluation of the biostimulant effect

The evaluation of the biostimulant effect of chitosan hydrochloride was conducted in a glass house located in Viterbo (Experimental Farm of the University of Tuscia, Viterbo, Italy), in order to verify if chitosan was able to boost the plant grow. For this assay, chitosan hydrochloride at 0.5% was tested, since this was the most promising concentration from the previous in vitro experiments. The solution was prepared in sterile distilled water as previously described. The surface of the kernels of the durum wheat genotypes Marco Aurelio and DBC480 were sterilized with sodium hypochlorite (0.5% v/v) for 20 minutes and then rinsed twice for 5 minutes in sterile distilled water. After that, the kernels were soaked for 1 hour in the solution containing chitosan and in distilled water as mock control under constant stirring and allowed to dry for 1 hour. Then, the kernels were transferred to 40 × 20 cm pots, filled with TYPical Brill soil (Brill, Germany), and grown at 18/12°C (day/night) with 12–14 hours of light for 14 days. At the end of the experiment, the germinated kernels were counted, and a kernel was considered as germinated when the coleoptile was visible. Nitrogen Balance Index (NBI) (DUALEX Scientific+™) related to the chlorophyll and flavonoid ratio was measured by positioning the instrument in the centre of the leaf and the meter was shielded from direct sunlight. Afterward, the seedlings were removed from the soil and gently washed with distilled water and the following growth parameters were measured: Coleoptile length (mm), main root length (mm), and number of developed roots. The % of growth promotion was calculated for each growth parameter measured (kernel germination, NBI, coleoptile and main root length, and number of developed roots) and tested substance by using the following equation: % of growth promotion = $100 \times (\text{Treated-Mock/Mock})$ (Leather and Einhellig, 1988). Data were obtained from three independent experiments organized as a complete randomized block, each one consisting of 20 plants for each experimental group (wheat genotype × compound).

5.2.4 In vivo antifungal activity evaluation

The artificial inoculation trials were conducted in a glass house located at the University of Natural Resources and Life Sciences, Vienna, Department of Agrobiotechnology Tulln, Austria. Seeds of the durum wheat genotypes Marco Aurelio and DBC480 were germinated into pots (17 cm diameter, 20 cm height) containing a mixture of peat, compost, and sand and placed in the greenhouse. Temperature in the greenhouse was on average 18/12°C (day/night) from tillering to heading with 12–14 hours of light. During flowering time, the conditions in the greenhouse were controlled and set at 21°C, 55% relative humidity during daytime and 17°C, 55% humidity during night with a 16 hours photoperiod. Chitosan hydrochloride at 0.5%, tebuconazole at 0.06%, azoxystrobin at 0.08%, and the

mixture of tebuconazole 0.06% and azoxystrobin 0.08% were prepared in distilled water as previously described. Macroconidia of the single-spore *F. graminearum* IFA66 were produced in liquid mungbean medium as described by Buerstmayr *et al.* (2000, 2002). The mungbean medium was removed from the conidial suspension by multiple centrifugation steps in double-distilled water. Aliquots of conidia stock solutions were stored at -80°C then thawed at 37°C and diluted with distilled water to achieve the desired final spore concentration just prior to inoculation. The antifungal compounds were sprayed on plants (wetted until runoff) 48 hours before inoculation in two different ways: on the spikes, to test the effective antifungal efficacy of the compounds, or on the flag leaves, to test the ability of boosting the SAR on durum plants. Plants subjected only to artificial inoculation and mock treatment were sprayed with distilled water. Plants were subjected to artificial inoculation at anthesis (Zadoks stage 69) (Zadoks *et al.*, 1974) by homogeneously spraying a conidial suspension of 25,000 conidia/mL, while mock plants were sprayed with distilled water (wetted until runoff). The wheat heads were covered with plastic bags for 48 hours to maintain high humidity levels (>80%). The disease incidence and severity of the FHB (%) were determined by counting the number of diseased spikes and the number of bleached spikelets in diseased spikes as well as the total number of spikes and spikelets, respectively, from 3 to 21 days post inoculation (dpi). Data were obtained from three independent experiments organized as a complete randomized block, each one consisting of 10 plants for each experimental group (wheat genotype × compound × mode of application).

5.2.5 Evaluation of induction of the Systemic Acquired Resistance (SAR) by Real-Time qPCR

The relative expressions of the *TaPR1*, *TaPR2* (pathogenesis related protein 1 and 2), and *TaPAL* (phenylalanine ammonia lyase) were evaluated as involved in the biosynthesis of salicylic acid (*TaPAL*) and indicators of the induction of SAR (*TaPR1* and *TaPR2*). The flag leaves were pre-treated 48 hours before the inoculation. The spikes were harvested 48 hours after the inoculation and immediately stored in liquid nitrogen and at -80°C. 100 mg of fine powder were subjected to RNA extraction following the instructions provided by the RNeasy Plant Mini Kit (Qiagen, Germany). The RNA was resuspended in RNase-free sterile distilled water and immediately poured onto ice and quantified with NanoDrop™. To confirm the total quantity and integrity of the RNA, 5 µL of the extracted RNA was subjected to thermal shock (10 minutes at -80°C after 5 minutes at 65°C) and run on 1.5% denaturing agarose gel. The synthesis of the cDNA was performed using 5 µg of RNA following the instructions provided by the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, United States) in a final volume of 20 µL. To ensure that the synthesis of the cDNA and the elimination of the gDNA had succeeded, a reverse transcriptase PCR (RT-PCR) of the *TaACT* (containing an intron in the amplified sequence) was performed. The RT-PCR was performed as

previously described in the “Real-Time qPCR” paragraph of the *in vitro* assays section by using an annealing temperature of 60°C. The amplification run consisted also of a no-template control (NTC) and a genomic DNA (gDNA) control. The amplicons were visualized on 1.5% agarose gel. Supplementary table 5.2 shows the list of target genes, accession numbers, their functions, the corresponding primer pairs, and their references used to perform the RT-qPCR (Lu *et al.*, 2006; Mandalà *et al.*, 2019; Tenea *et al.*, 2011; Francesconi and Balestra, 2020). The efficiency (E) and correlation coefficient (R^2) of the RT-qPCR were determined as previously described. The relative expression levels of the target genes were calculated on the basis of the Cq values of the four technical replicates derived from three independent biological replicates for each wheat genotype and compound by applying the equation $\text{Relative expression} = 2^{-\Delta\Delta Cq}$, using *TaFNR*, *TaTUB*, and *TaACT* as the reference genes and the mock treatment to normalize the relative expression levels (Bustin *et al.*, 2009). The RT-qPCR was performed following the instructions provided by the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, United States) and iTaq™ Universal SYBR® Green Supermix (Bio-Rad, United States) in a final volume of 10 µL. The amplification conditions included an initial denaturation step of 30 seconds at 95°C; 40 cycles of 10 seconds denaturation at 95°C; 35 seconds of annealing and elongation at 60°C. A final melt cycle (70–99°C) was performed to confirm the amplicons’ unicity. NTC controls were included and the amplification was considered negative when a value of $Cq \geq 38$ was detected.

5.2.6 Quantification of fungal biomass, mycotoxins analysis and impact on yield

At the end of ripening, all the inoculated and mock spikes were collected in order to evaluate if the different compounds and modes of application (on the spike or on the flag leaf) influenced the amount of fungal biomass and mycotoxins inside the flour and the impact on grain yield in the two durum wheat genotypes.

5.2.6.1 Determination of the impact on grain yield

Thirty spikes from each experimental group (wheat genotype × compound × mode of application) (10 spikes from each independent replicate) were harvested and weighed (g). The % of yield reduction was calculated by using the following equation: $\% \text{ of yield reduction} = 100 \times (\text{Mock-Treated}/\text{Mock})$.

5.2.6.2 Quantification of fungal biomass by Real-Time qPCR

Ten spikes from each independent replicate of each experimental group (wheat genotype × compound × mode of application) were homogeneously milled into a fine flour. The isolate of *F. graminearum* IFA66 was re-isolated by plating 100 mg of the flour obtained from the inoculated and untreated spikes on PDA and cultured in the dark at 21°C for 7 days in purity. 100 mg of fresh mycelium and 100 mg of flour from Marco Aurelio and DBC480 samples were subjected to total DNA extraction

following the protocol for the Invisorb® Spin Plant Mini Kit (Stratec Molecular GmbH, Germany). DNA was quantified with a Qubit™ fluorometer 1.01 (Invitrogen, United States) using the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific, United States). DNA from inoculated samples was diluted to 10 ng/μL, while fungal and wheat calibration curves were obtained preparing four serial 1:10 dilutions (1:1, 1:10, 1:100, 1:1000) from fresh fungal mycelium and uninoculated wheat material DNAs. RT- qPCR was performed following the instructions from Rotor Gene Q (Qiagen, Germany) and Xpert Fast SYBR (uni) Master Mix (GRiSP Research Solutions, Portugal). RT-qPCR amplification conditions included: An initial denaturation step of 3 minutes at 95°C; 40 cycles of 5 seconds denaturation at 95°C, 30 seconds of annealing at 60°C; and 20 seconds of elongation at 72°C. A final melt cycle (70–99°C) was performed to confirm the amplicons unicity. RT-qPCR was performed using the primer pair Tri6_10F/Tri6_4R for *F. graminearum* DNA quantification and TaACT_F/TaACT_R for wheat DNA quantification (Supplementary tables 5.1 and 5.2). Data were obtained from four technical replicates derived from three biological replicates. Results were expressed as ng of fungal DNA per ng of plant DNA (Horevaj *et al.*, 2011; Francesconi *et al.*, 2019).

5.2.6.3 Detection and quantification of mycotoxins by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Five grams of homogenized sample were extracted with 40 mL of acetonitrile/water/acetic acid (79:20:1, v/v/v) solvent in a 50 mL tube for 90 minutes and 180 rpm shaking speed using a GFL 3017 rotary shaker (GFL 2017). The raw extract was diluted 1:20 using acetonitrile/water/acetic acid (20:79:1, v/v/v) and 5 μL of diluted extract were injected subsequently. Metabolite analysis was carried out using a 1290 Series HPLC System (Agilent Technologies, United States) coupled to a QTrap 5500 LC-MS/MS System (Applied Biosystems SCIEX, United States) equipped with Turbo Ion Spray electrospray ionization source. Chromatographic separation was performed at 25°C on a Gemini® C18-column, 150 × 4.6 mm i.d., 5 μm particle size, equipped with a C18 4 × 3 mm i.d. security guard cartridge (Phenomenex, United States). Confirmation of positive metabolite identification was carried out by the acquisition of two-time scheduled multiple reaction monitoring (MRMs), which yielded 4.0 identification points according to the European Commission decision 2002/657. In addition, retention time and ion ratio had to agree with the related values of authentic standard within 0.03 minutes and 30% rel., respectively. Results were corrected using apparent recoveries obtained during validation. The accuracy of the method is verified on a continuous basis by participation in a proficiency testing scheme organized by BIPEA (Gennevilliers, France) with a current rate of z-scores between -2 and 2 of >94% (>1300 results submitted) (Sulyok *et al.*, 2020).

5.2.7 Statistical analysis

Data from *in vitro* experiments, RT-qPCR of the *F. graminearum* genes, biostimulant evaluation, and FHB disease incidence and severity were subjected to one-way analysis of variance (ANOVA), while data from RT-qPCR of the durum wheat genes were subjected to two-way ANOVA where the two independent variables were the wheat genotypes (Marco Aurelio and DBC480) and the treatments applied to the plants (chitosan 0.5%, tebuconazole 0.06%, azoxystrobin 0.08%, tebuconazole 0.06% + azoxystrobin 0.08%, and *F. graminearum* inoculated plants) and the dependent variable was the relative expression level. Data from yield reduction, fungal biomass, and mycotoxins quantification were subjected to three-way ANOVA where the three independent variables were the wheat genotypes, the plant treatments, and the mode of application of the compounds on plants (application on the spike or on the flag leaf). One level of significance ($p < 0.01$) was computed to assess the significance of the F values. A pairwise analysis was carried out using the Tukey Honestly Significant Difference test (Tukey test) at the 0.99 confidence level. Statistical analyses were performed using SYSTAT12 software (Systat Software Inc., United States). The heatmaps were generated to represent the relative expression levels of *F. graminearum* and wheat genes after the plant treatments, while the principal component analysis (PCA) was carried out to evaluate the genotype $\times$ compound effects on controlling FHB by plotting the % FHB incidence and severity at 21 dpi, the % of yield reduction, the ng of fungal DNA/ng of plant DNA, and the $\mu\text{g/kg}$ of the analysed mycotoxins and *F. graminearum*-related compounds. PCA and heatmap were carried out using ClustVis software (Estonia) (Metsalu and Vilo, 2015).

5.3 Results

5.3.1 *In vitro* antifungal activity of chitosan hydrochloride

The minimum inhibitory concentration (MIC) value of chitosan hydrochloride was established by a 96 microtiter plates assay and the concentration at 1% resulted to inhibit completely *F. graminearum* growth. Notably, lower concentrations of chitosan also resulted in being effective in controlling *F. graminearum* growth, since the % of inhibition ranged from 53 to 87% after treating the conidia of *F. graminearum* with chitosan at 0.01–0.05–0.1 and 0.5%. Importantly, chitosan at 0.1–0.5 and 1% demonstrated the ability to inhibit *F. graminearum* as much as the reference fungicides (tebuconazole, azoxystrobin, and the mixture of tebuconazole and azoxystrobin) used at the recommended field doses (0.06% for tebuconazole and 0.08% for azoxystrobin) (Figure 5.1a). After seven days of culturing *F. graminearum* in chitosan-incorporated PDA, none of the chitosan concentrations tested was as effective as tebuconazole and the mixture of tebuconazole and azoxystrobin in limiting *F. graminearum* radial growth. However, chitosan at 0.05–0.1–0.5 and 1% showed remarkable fungal inhibition (from 55 to 75%), resulting in being more effective than azoxystrobin, which reduced the

F. graminearum growth of 32% (Figure 5.1b). Figure 5.1d evidences the radial growth of *F. graminearum* after seven days of incubation on incorporated PDA with chitosan at different concentrations, reference fungicides, and mock control. Finally, Figure 5.1c shows that only chitosan at 0.5 and 1% inhibited *F. graminearum* by agar diffusion (48 and 60% of inhibition, respectively) more efficiently than azoxystrobin but not as much as tebuconazole and the mixture of tebuconazole and azoxystrobin.

In the present study, we also evaluated the relative transcripts level of some major *F. graminearum* genes responsible for redox balance, cell respiration, nutrient transport, cell wall degrading enzymes, ergosterol biosynthesis, and trichothecenes production, in order to evaluate the mode of action of chitosan hydrochloride compared to the reference fungicides on the physiology and virulence of *F. graminearum*. Supplementary figure 5.1 shows RNA integrity (a) and *TEF* amplification (b) from *F. graminearum*, demonstrating that the cDNA was successfully synthesized. The reaction efficiency (E) and R^2 values ranged from 0.92 to 1.20 and from 0.97 to 0.99, respectively, from each primer pair. Figure 5.2 shows the heatmap and the relative expression values for each gene and compound treatments, while Supplementary table 5.3 also provides the standard errors and statistical analysis according to the Tukey test computed at 0.99 confidence level.

Chitosan hydrochloride only upregulated *Tri8* (3.34-fold) and *Tri101* (5.00-fold), while the rest of the genes were strongly downregulated. Tebuconazole demonstrated a remarkable induction of the *Tri* pathway, among which *Tri8*, *Tri11*, *Tri12*, and *Tri101* showed relative expression values of 19.94-, 26.84-, 24.95-, and 24.31-fold. Azoxystrobin also promoted the upregulation of the *Tri* genes, even if not as much as tebuconazole. The mixture of tebuconazole and azoxystrobin drastically reduced the induction of the *Tri* pathway, with the exception of *Tri8* and *Tri101* (16.81- and 31.40-fold, respectively). On the other hand, the genes involved in redox balance, cell respiration, nutrient uptake, cell wall degrading enzymes, and ergosterol biosynthesis were mostly down-regulated after each type of treatment, with the exception of *Erg3*, *Fg09943*, *Fg01572*, *Fg04196*, *Fg06619*, and *Fg10231* after tebuconazole treatment, *Erg3*, *Fg01572*, *Fg06619*, and *Fg10231* after azoxystrobin treatment, *Fg09443*, *Fg01572*, *Fg04196*, and *Fg10231* after the mixture treatment (Figure 5.2 and Supplementary table 5.3).

5.3.2 Biostimulant effects of chitosan hydrochloride on durum wheat

Kernels of Marco Aurelio and DBC480 were coated with chitosan hydrochloride at 0.5% or water in order to evaluate their effects on durum plants. As shown in Figure 5.3, chitosan promoted the germination rate (14%), the number of developed roots (12%), the length of the main root (4%), and coleoptile (6%) and the chlorophyll and flavonoids ratio expressed as NBI values (21%) both in Marco Aurelio and DBC480 seedlings. No significant differences were observed in the growth

between the two durum wheat genotypes after the chitosan coating. Supplementary figure 5.2 shows the growth of the durum seedling at 14 days after the coating with the different compounds.

5.3.3 *In vivo* antifungal activity of chitosan hydrochloride and its effectiveness on SAR induction in durum wheat

Plants of Marco Aurelio and DBC480 were preventively treated with chitosan hydrochloride at 0.5% or the reference fungicides at the previously cited concentrations 48 hours before the inoculation. The compounds were applied directly on the spikes or on the flag leaves. Figure 5.4 illustrates the FHB incidence and severity (%) from 3 to 21 dpi after applying the compounds on the spikes. Chitosan reduced the FHB incidence as much as the reference fungicides from 3 to 5 dpi in Marco Aurelio (a) (FHB incidence was of 75%, while it was of 92% in *F. graminearum* inoculated control plants), at 6 dpi its effect was similar to azoxystrobin, while from 7 to 9 dpi, only tebuconazole and the mixture of the reference fungicides reduced the FHB incidence. From 10 to 21 dpi, none of the compounds reduced the FHB incidence. In DBC480 plants (b), chitosan reduced the FHB incidence as much as the reference fungicides from 3 to 13 dpi (FHB incidence was of 50%, while it was of 80% in *F. graminearum* inoculated control plants), while from 14 to 21 dpi showed a similar effect in reducing FHB incidence to the mixture of the reference fungicides, demonstrating a higher efficacy than tebuconazole and azoxystrobin applied alone. At 21 dpi, chitosan and the mixture of fungicides-treated plants showed an FHB incidence of 60%, while it was of 69%, 80%, and 100% in tebuconazole, azoxystrobin, and *F. graminearum* control plants, respectively. Chitosan decreased FHB severity in Marco Aurelio (c) as much as the reference fungicides from 4 to 16 dpi, and from 17 to 21 dpi chitosan was more effective than azoxystrobin but not as much as tebuconazole and the mixture of tebuconazole and azoxystrobin. At 21 dpi, *F. graminearum* inoculated control and azoxystrobin-treated plants showed 100% of FHB severity, 85% after chitosan treatment, and 60% after tebuconazole and the mixture of the reference fungicides treatments. On DBC480 (d), chitosan reduced the FHB severity as much as the reference fungicides from 9 to 21 dpi (6% of FHB severity). FHB severity in *F. graminearum* inoculated control plants was 20% and in azoxystrobin-treated plants was 15%. Figure 5.4e shows FHB symptoms at 21 dpi on Marco Aurelio and DBC480 subjected to the different compounds' treatments at the spike level and mock plants, and no FHB symptoms were recorded.

Figure 5.5 shows the FHB incidence and severity from 3 to 21 dpi after applying the compounds on the flag leaves. In Marco Aurelio plants (a), chitosan reduced the FHB incidence as much as the reference fungicides from 3 to 5 dpi (FHB incidence of 60% in chitosan treated plants, while it was 90% in the *F. graminearum* inoculated control plants without pre-treatment), while at 6 dpi the FHB incidence of chitosan and tebuconazole treated plants was reduced but not as much as in azoxystrobin

and the mixture of tebuconazole and azoxystrobin treated plants. From 7 to 21 dpi, no FHB reduction was observed in chitosan and tebuconazole treated plants, while azoxystrobin and the mixture of the reference fungicides reduced the FHB severity from 6 to 8 dpi and from 6 to 9 dpi, respectively. From 10 to 21 dpi, the FHB severity was of 100% in all the experimental groups. In DBC480 plants (b), chitosan reduced the FHB incidence from 3 to 6 dpi as much as the reference fungicides; from 7 to 9 dpi chitosan was more effective than tebuconazole and from 10 to 11 dpi and from 18 to 21 dpi chitosan significantly reduced the FHB incidence more than tebuconazole and the mixture of the reference fungicides (FHB incidence of 70% in chitosan and 90% in tebuconazole and the mixture of the reference fungicides treated plants at 21 dpi, respectively). Interestingly, the azoxystrobin compound was the most effective in reducing FHB incidence from 10 to 20 dpi, while the FHB incidence of azoxystrobin treated plants at 21 dpi was similar to that observed in chitosan treated plants. FHB severity in Marco Aurelio plants (c) was not significantly reduced for the entire infection trial, with the exception of the mixture of the reference fungicides, which reduced FHB severity from 15 to 21 dpi (FHB severity of 65%, while it was of 90% in *F. graminearum* inoculated control plants). In DBC480 plants (d), all the treatments reduced the FHB severity from 8 to 13 dpi, while from 14 to 21 dpi only chitosan and azoxystrobin significantly reduced the FHB severity compared to *F. graminearum* inoculated control plants (FHB severity was of 6%, while it was of 21% in *F. graminearum* inoculated control plants). Figure 5.5e shows FHB symptoms at 21 dpi on Marco Aurelio and DBC480 subjected to the different compounds' treatments at the flag leaf level and mock plants, where no FHB symptoms were recorded.

From Marco Aurelio and DBC480 plants treated at the flag leaf level, the relative transcript levels of *TaPR1*, *TaPR2*, and *TaPAL* were evaluated, in order to assess the putative induction of SAR by the different plant treatments. Supplementary figure 5.3 shows RNA integrity (a) and *TaACT* amplification (b) from Marco Aurelio and DBC480, demonstrating that the cDNA was successfully synthesized. E and R² values ranged from 0.97 to 1.11 and from 0.97 to 0.99, respectively, from each primer pair. Figure 5.6 shows the heatmap and the relative expression values for each gene and compound treatments, while Supplementary table 5.4 also provides the standard errors and statistical analysis according to the Tukey test computed at 0.99 confidence level.

TaPAL was up-regulated after *F. graminearum* inoculation (2.89- and 2.62-fold in Marco Aurelio and DBC480, respectively) and chitosan treatments (1.76- and 2.35-fold in Marco Aurelio and DBC480, respectively). Notably, the upregulation of *TaPAL* in DBC480 plants after the chitosan treatment was significantly higher than in Marco Aurelio plants. *TaPAL* was also upregulated in DBC480 plants treated with azoxystrobin (2.29-fold) but basal-regulated in Marco Aurelio (1.09-fold). *TaPAL* was also basal-regulated after tebuconazole treatment in Marco Aurelio and slightly

downregulated in Marco Aurelio plants treated with the mixture of the reference fungicides and in DBC480 plants treated with tebuconazole and the mixture of the reference fungicides. *TaPR1* was promptly induced (3.48-fold) in *F. graminearum* inoculated Marco Aurelio plants and it was also induced in *F. graminearum* inoculated DBC480 (2.04-fold) and chitosan treated plants (2.91- and 2.16- fold in Marco Aurelio and DBC480, respectively). Tebuconazole, azoxystrobin, and the mixture of the two reference fungicides basal-regulated the genes in both Marco Aurelio and DBC480, with the exception of azoxystrobin in DBC480 plants, that slightly upregulated *TaPR1* (1.47-fold). *TaPR2* was promptly induced in *F. graminearum* inoculated and chitosan treated DBC480 plants (3.41- and 3.23- fold). The *F. graminearum* artificial inoculation, chitosan treatment in Marco Aurelio, and tebuconazole application in DBC480 also induced *TaPR2* (2.51-, 2.69-, and 1.79-fold, respectively), while the gene resulted basal-regulated after the application of azoxystrobin in DBC480 plants and down-regulated after tebuconazole, azoxystrobin and the mixture of the reference fungicides application in Marco Aurelio (Figure 5.6 and Supplementary table 5.4)

5.3.4 Chitosan hydrochloride effectiveness on maintaining grain yield, containing *F. graminearum* biomass spread and FHB-associated compounds accumulation

The impact of chitosan on maintaining grain yield was evaluated in the two durum wheat genotypes and compared to the reference fungicides, and data are expressed as % of yield reduction relative to untreated control plants (without pre-treatment and *F. graminearum* inoculation) (Figure 5.7). Chitosan applied on Marco Aurelio's spikes or flag leaves did not protect grain yield compared to *F. graminearum* inoculated plants. In fact, only tebuconazole applied directly on the spikes (62%) and the mixture of the reference fungicides applied on the spikes (59%) or on the flag leaves (65%) significantly contained the yield reduction compared to *F. graminearum* inoculated plants. On the other hand, chitosan applied on the flag leaves of DBC480 plants strongly contained the yield reduction (0.5%), as much as the azoxystrobin and the mixture of the reference fungicides applied on the spikes and the mixture of the reference fungicides applied on the flag leaf, compared to the *F. graminearum* inoculated DBC480 plants (20% of yield reduction).

Figure 5.8 shows the quantification of *F. graminearum* biomass measured by fungal DNA in the heads of Marco Aurelio and DBC480, where Figure 5.8a illustrates the three standard curves used for the quantification of durum wheat (*TaACT* gene) and *F. graminearum* (*Tri6* gene) biomass. E and R² values ranged from 1.04 to 1.73 and from 0.98 to 0.99, respectively, from each primer pair. Figure 5.8b shows the results from the biomass quantification expressed as ng of fungal DNA/ng of plant DNA. Chitosan or azoxystrobin applied on Marco Aurelio's spikes (1.74 and 2.41 ng of fungal DNA/ng of plant DNA, respectively) did not reduce the accumulation of the fungal biomass compared to the *F. graminearum* inoculated control (2.58 ng of fungal DNA/ng of plant DNA). On the other

hand, the application of chitosan on the flag leaves in Marco Aurelio plants reduced the accumulation of the fungal biomass as much as the reference fungicides (0.39, 1.22, 1.11, and 0.56 ng of fungal DNA/ng of plant DNA from chitosan, tebuconazole, azoxystrobin, and the mixture of the reference fungicides treated heads). In DBC480 plants, neither the added compounds nor the mode of applications influenced the accumulation of the fungal biomass compared to the *F. graminearum* inoculated plants (0.0025 ng of fungal DNA/ng of plant DNA), with the exception of the mixture of the two reference fungicides applied at the flag leaf level, which surprisingly increased *F. graminearum* biomass into the heads (0.028 ng of fungal DNA/ng of plant DNA). In the mock-treated control heads, only traces of fungal DNA were detected (3.6×10^{-4} and 4×10^{-5} ng of fungal DNA/ng of plant DNA in Marco Aurelio and DBC480, respectively).

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was applied to detect and quantify the FHB-associated mycotoxins and other FHB-related compounds in the durum wheat flours from the heads of Marco Aurelio and DBC480 subjected to the different compound treatments and modes of application (Figure 5.9). Deoxynivalenol (DON) (a) was not affected by the chitosan treatment in Marco Aurelio (297,000 $\mu\text{g/kg}$ and 243,000 $\mu\text{g/kg}$ by the spikes and flag leaves application, respectively), compared to the *F. graminearum* inoculated control (306,000 $\mu\text{g/kg}$). On the other hand, azoxystrobin applied on the spikes (395,000 $\mu\text{g/kg}$) favoured its accumulation. In DBC480, chitosan did not influence the amount of detected DON (29,000 $\mu\text{g/kg}$ and 20,700 $\mu\text{g/kg}$ by the spikes flag leaves application, respectively) compared to the *F. graminearum* inoculated control (21,300 $\mu\text{g/kg}$). The amount of detected deoxynivalenol-3-glucoside (D3G) (b) was not affected by the compounds' treatments and mode of applications in both Marco Aurelio and DBC480. The DON/D3G ratio (c) was calculated to evaluate the amount of DON transformed in D3G as a detoxification mechanism. The DON/D3G ratio was not influenced by the different compounds and mode of application in both the two durum wheat genotypes, but there was a significant difference between the two genotypes, since DBC480 showed lowest DON/D3G values compared to Marco Aurelio, after the following treatments: Azoxystrobin applied on the spikes (8.34 in Marco Aurelio and 5.17 in DBC480), chitosan applied on the flag leaves (8.41 in Marco Aurelio and 4.59 in DBC480), tebuconazole applied on the flag leaves (7.99 in Marco Aurelio and 3.17 in DBC480), and *F. graminearum* inoculated control plants (8.64 in Marco Aurelio and 4.35 in DBC480). The amount of 15-acetyldeoxynivalenol (15-ADON) (d) was reduced in Marco Aurelio after the application on the flag leaves of chitosan (25,600 $\mu\text{g/kg}$), azoxystrobin (26,300 $\mu\text{g/kg}$), and the mixture of the reference fungicides (17,500 $\mu\text{g/kg}$) compared to *F. graminearum* inoculated control (37,600 $\mu\text{g/kg}$). In DBC480, only the application of the reference fungicides on the flag leaves reduced the amount of 15-ADON, since the detected quantity was under the limit of detection (LOD) of the methodology,

compared to *F. graminearum* inoculated control (4740 µg/kg). The detected amount of 3-acetyldeoxynivalenol (3-ADON) (e) was lower in Marco Aurelio in the flours derived from the application of chitosan, tebuconazole, azoxystrobin, and the mixture of the reference fungicides on the flag leaves (3720, 5190, 4190, and 2370 µg/kg, respectively) compared to the *F. graminearum* inoculated control heads (6910 µg/kg). On DBC480, only the mixture of the reference fungicides applied on the flag leaves decreased the amount of accumulated 3-ADON (under the LOD) compared to the *F. graminearum* inoculated control plants (326 µg/kg). Chitosan and the mixture of the reference fungicides applied on the flag leaves reduced the amount of nivalenol (NIV) (f) in Marco Aurelio (385 and 332 µg/kg, respectively) compared to the *F. graminearum* inoculated control (595 µg/kg), while NIV was not detected in all the DBC480 treated heads. Zearalenone (ZEA) (g) was significantly reduced in Marco Aurelio by the application on the flag leaves of chitosan (31 µg/kg), while its accumulation was favoured by the application on the spikes of azoxystrobin (111 µg/kg) and the mixture of the reference fungicides (124 µg/kg) compared to the *F. graminearum* inoculated control (63 µg/kg). ZEA was not detected in all the DBC480 treated plants. The amount of detected culmorin (CUL) (h), of 5-hydroxyculmorin (5-HCUL) (i) and 15-hydroxyculmorin (15-HCUL) (j) was not reduced in Marco Aurelio and DBC480 after the application of chitosan compared to the *F. graminearum* inoculated plants. The application on the flag leaves of chitosan induced the accumulation of Butenolid (BUT) (k) in DBC480 heads (25500 µg/kg) compared to the *F. graminearum* inoculated control (7430 µg/kg). The accumulation of aurofusarin (AUR) (l) in Marco Aurelio was reduced by the application of chitosan on the flag leaves (1260 µg/kg) compared to the rest of the applied treatments. On the other hand, chitosan did not reduce the amount of accumulated AUR in DBC480, compared to the *F. graminearum* inoculated control (398 µg/kg). Chrysogin (CHR) (m) was significantly reduced in Marco Aurelio after chitosan and the mixture of the reference fungicides applied on the flag leaves (2200 and 166 µg/kg, respectively), compared to the *F. graminearum* inoculated control (3790 µg/kg). On DBC480, azoxystrobin applied on the spikes (25 µg/kg) and chitosan (45 µg/kg), azoxystrobin (50 µg/kg), and the mixture of the reference fungicides (21 µg/kg) applied on the flag leaf contained the accumulation of CHR compared to the *F. graminearum* inoculated control (89 µg/kg). The amount of detected Gibepyron D (GID) (n) in Marco Aurelio was not influenced by the treatments with the exception of azoxystrobin applied on the spikes or on the flag leaves, which favoured its accumulation (699 and 621 µg/kg, respectively) compared to the *F. graminearum* inoculated plants (336 µg/kg). In DBC480 GID was not detected (under the LOD).

Notably, the % of yield reduction, the amount of fungal biomass, and FHB-related molecules were significantly lower in DBC480 than in Marco Aurelio, with the exception of DON/D3G ratio for

chitosan, tebuconazole, and the mixture of the reference fungicides applied on the spike and azoxystrobin applied on the leaves, and the amount of detected BUT. In this regard, the interaction between the effect of the durum wheat genotypes and the control strategies in reducing FHB was evaluated by principal component analysis (PCA) (Figure 5.10). The two genotypes clearly clustered separately as susceptible (Marco Aurelio) and resistant (DBC480). Tebuconazole applied on the spikes and chitosan, azoxystrobin, and the mixture of the reference compounds applied on the flag leaves, contributed to FHB control in Marco Aurelio, while all the treatments, with the exception of tebuconazole applied on the flag leaves, contributed to FHB control in DBC480.

5.4 Discussion

The concerns regarding the environmental safety and the efficacy of fungicides in controlling FHB highlight the urgent need to develop green as much as possible and environmentally safe fungicides that are effective in reducing FHB and mycotoxins accumulation in kernels with different modes of action from that of conventional molecules (Wegulo *et al.*, 2015). In this regard, chitosan was extensively evaluated as a plant biostimulant and antimicrobial agents against different plant pathogens (fungi, bacteria, and viruses), and recently against different *Fusarium* spp. (Buzón-Durán *et al.*, 2020; Alvarez-Carvajal *et al.*, 2020; Lipsa *et al.*, 2020; Mejdoub-Trabelsi *et al.*, 2019), and the modes of action have been hypothesized to describe its ability to boost plant growth and defence mechanism (Sharif *et al.*, 2018; Maluin and Hussein, 2020). Many studies evaluated the antifungal ability of chitosan against FHB pathogens *in vitro* and *in vivo*. A recent study (Buzón-Durán *et al.*, 2020) evaluated *in vitro* and *in vivo* chitosan oligomers mixed with amino acids against *F. culmorum*, reporting that chitosan oligomers demonstrated a 90% maximal effective concentration (EC₉₀) of 2230.26 µg/mL (0.2%). These results are in agreement with those obtained in the present study because we also observed an inhibition very close to 90% at a similar chitosan concentration (0.1%). Interestingly, the authors obtained valuable data on the reduction of disease severity and DON content by mixing chitosan with tyrosine. Kheiri *et al.* (2016, 2017) tested chitosan and chitosan nanoparticles on *F. graminearum* growth and they observed the highest inhibition *in vitro* by supplementing PDA with chitosan at 0.1% (68% of growth inhibition) and with chitosan nanoparticles at 0.5% (85% of growth inhibition), compared to the reference tilt fungicides applied at the recommended field dose (72% of growth inhibition). Interestingly, the microscopic examination of the treated *F. graminearum* showed dehydration and deformation in mycelial growth. During the greenhouse experiments, by using as host plant the cultivar Falat (bread wheat) they observed a disease severity of 29% and 27% in chitosan and chitosan nanoparticles treated plants, while the plants treated with the tilt fungicides reached 0.33% of disease severity after three weeks of inoculation. Another study (Gunupuru *et al.*, 2019) combined chitosan with a plant biostimulant, the liquid seaweed extract (LSE) from a brown

macroalga and evaluated the efficacy of chitosan and LSE, alone or combined, against *F. graminearum* growth. *In vitro*, no synergistic effect of LSE and chitosan was observed on mycelial growth and chitosan at 0.001% showed 43% of growth inhibition. On the other hand, during the *in vivo* experiments (cultivar Helios, bread wheat), the authors observed a synergistic effect of LSE and chitosan in reducing FHB severity, while chitosan applied alone reduced FHB severity of 15% compared to the inoculated control plants. Notably, the authors also evaluated the induction of transcription of *TaPR1*, *TaPR2*, and *TaPR3* in wheat seedlings at 24, 48, and 72 hours post inoculation (hpi). *TaPR1* and *TaPR2* showed the greatest up-regulation at 48 hpi (25 and 500-fold, respectively) and were significantly induced in all the chitosan-based treatment compared to the plants subjected only to *F. graminearum* inoculation (10 and 200-fold, respectively) (Gunupuru *et al.*, 2019). These results are in agreement with data obtained from our experiments since we observed a consistent *F. graminearum* growth inhibition by applying chitosan *in vitro* at different concentrations (0.01–1%) and a significant reduction of FHB incidence and severity during the greenhouse experiments by applying chitosan at 0.5%. Moreover, we also observed a significant up-regulation of defence-related genes at 48 hpi (*TaPAL*, *TaPR1*, and *TaPR2*) in chitosan treated plants and *F. graminearum* inoculated spikes, even though we observed a transcript amount extremely low if compared to the fold-change detected by the previously cited authors. These differences could be attributed to the fact that the authors used as host plant a bread wheat cultivar while we performed the experiments by using two durum wheat genotypes; thus, the abundance of the transcript amounts assigned to the D genome of bread wheat are absent in the durum wheat genotypes. Furthermore, they evaluated the relative expression of the genes from treated seedlings, while we analysed the transcript on the spikes, which were not subjected directly to the treatments, since the application of the compounds was performed on the flag leaves to observe the putative indirect antifungal ability of chitosan as SAR inductor. Regarding the antimicrobial mechanism of chitosan, several researchers have presented their hypotheses on the direct and/or indirect effects against pathogens: The positive charge of the protonated chitosan enables electrostatic interaction with the negative charge of the pathogens surface; chitosan increases the permeability of the membrane by damaging the cells of the pathogens resulting in cell death (Liang *et al.*, 2018); chitosan is also able to chelate the essential elements for the growth of the pathogens, such as metal ions, minerals, and nutrients, preventing the normal growth of the pathogens; chitosan may also interact with DNA or RNA leading to the inhibition of the mRNA synthesis; the deposition of chitosan on plant and pathogen surface forms a biofilm that limits the nutrient availability for microorganisms (Helander *et al.*, 2001; Cruz-Romero *et al.*, 2013; Xing *et al.*, 2015); the ability of chitosan to induce the SAR in plants is associated with its elicitor-like properties, which boost the early activation of pathogenesis-related proteins and improve the plant

resistance. As a result, chitinase and β -1,3-glucanase (PR2) enzymes degrade the fungal cell wall and stop its growth on the host plant (Sharif *et al.*, 2018; Maluin and Hussein, 2020; Xing *et al.*, 2015). During the *in vivo* evaluation of the antifungal activity, a significant reduction of FHB incidence and severity was observed in Marco Aurelio and DBC480 plants treated at the flag leaves with azoxystrobin-based formulations. The ability of azoxystrobin applied on the flag leaves to protect the plants against FHB was also confirmed by the basal-regulation in Marco Aurelio of *TaPAL* and *TaPR1*, the up-regulation of *TaPAL* and *TaPR1*, and the basal-regulation of *TaPR2* in DBC480 and by the reduced amount of some types of mycotoxins accumulated in the flours. *TaPAL* is the key enzyme in the biosynthesis of salicylic acid (SA), which is the central player in the hypersensitive reaction (HR) and SAR, which are associated with early signalling events in response to pathogens, such as the activation of PR genes (Dong, 2001; Gruner *et al.*, 2013; Muthukrishnan *et al.*, 2001; Gao *et al.*, 2015). Many studies demonstrated that the fast activation of SAR by expressing diverse PR genes resulted in a significant enhancement of FHB resistance (Mackintosh *et al.*, 2007; Makandar *et al.*, 2012; Qi *et al.*, 2019). Moreover, it was demonstrated that strobilurins improve the hormonal balance in plants. In particular, a high concentration of strobilurins inhibits the biosynthesis of ethylene (ET) (Grossmann *et al.*, 1997) but improves the production of abscisic acid (ABA) (Grossmann *et al.*, 1999). It has been demonstrated that ET contributes to FHB resistance during early infection but then promotes the senescence of wheat heads that favours the fungal colonization in the dead tissues of the host (Wang *et al.*, 2018; Bönninghausen *et al.*, 2019). On the other hand, the accumulation of ABA quickly induces the stomatal closure, which prevents the pathogen entry into the host tissues (Daszkowska-Golec and Szarejko, 2013; Kang and Buchenauer, 2000). For such these reasons, the concomitant ability of azoxystrobin to induce the SAR pathway, to inhibit the ET biosynthesis, and favour ABA accumulation could have led to the reduction of FHB incidence and severity as much as the mycotoxin's accumulation in Marco Aurelio and DBC480 plants.

Chitosan was tested as a plant growth-promoter in many crops in several research studies. Indeed, the chitosan seeds coating resulted to boost the germination rate, the root apparatus, the aerial biomass, the photosynthetic rate, and the yield in maize, pearl millet, rapeseed, soybean, sunflower, peanut, rice, tomato, chickpea, and coffee (El Hadrami *et al.*, 2010; Katiyar *et al.*, 2015; Badawy and Rabea, 2016; Kumaraswamy *et al.*, 2018). In wheat, the kernels coated with chitosan (0.2–0.8%) significantly improved the germination rate, seedling growth parameters, and the synthesis of antioxidant enzymes (superoxide dismutase, peroxidase, and catalase) (Zeng and Luo, 2012). Ma *et al.* (2012) reported that wheat kernels treated with chitosan showed higher growth than control under salinity stress. These results are in agreement with the data presented in the present study, since chitosan positively influenced the growth parameters on Marco Aurelio and DBC480 after coating the kernels with

chitosan at 0.5%. The chitosan's ability to boost the germination rate in many plant species has been attributed to its excellent film forming property, allowing it to form a semipermeable film on the seed surface, which can maintain the seed moisture and absorb the soil moisture, promoting the seed germination (Zeng and Luo, 2012). Furthermore, the signalling mechanism of chitosan to regulate the development and growth in plants has been elucidated on various crops (El Hadrami *et al.*, 2010; Katiyar *et al.*, 2015; Badawy and Rabea, 2016; Kumaraswamy *et al.*, 2018). Chitosan helps in the activation of hydrolytic enzymes required for the degradation of reserve nutrients such as starch (Kumaraswamy *et al.*, 2018). Chitosan stimulates root cell division by the activation of auxin and cytokinin, which further contribute to the uptake of nutrients. Chitosan also significantly enhanced the crop yield by boosting the photosynthesis index by increasing the chlorophyll content and stomatal activity by switching on the genes related to the chlorophyll synthesis (Kumaraswamy *et al.*, 2018).

In the present study, we demonstrated that chitosan inhibited, *in vitro*, the *F. graminearum* growth, virulence, and trichothecenes synthesis by analysing the transcripts amount of the major genes involved in cell growth, respiration, establishment of virulence, and mycotoxins biosynthesis. To our knowledge, this is the first research study evaluating the *F. graminearum* genes response after treating the mycelium with chitosan and reference fungicides (tebuconazole and azoxystrobin). We also demonstrated that chitosan at 0.5% contained the fungal spread inside the head as much as the accumulation of various mycotoxins and *F. graminearum*-related compounds, especially if applied at the flag leaf level, in the two durum wheat genotypes (Marco Aurelio and DBC480). No reports regarding the quantification of the FHB-associated pathogens in the spikes after chitosan treatment have been performed before, thus this is the first study that assesses the ability of chitosan to control the *F. graminearum* spread in the heads applied directly on the spikes or on the flag leaf. On the other hand, two studies evaluated the efficacy of tebuconazole and azoxystrobin in controlling FHB-associated pathogens spread by preventively applying the compounds on the spikes. Both the research studies demonstrated that tebuconazole significantly reduced the amount of fungal DNA in the spikes, while azoxystrobin was completely ineffective (Edwards *et al.*, 2001; Zhang *et al.*, 2009). These results are in agreement with those obtained in the present study, where tebuconazole and the mixture of tebuconazole and azoxystrobin applied on the spikes of Marco Aurelio reduced the accumulated fungal biomass, while the amount of fungal DNA detected from azoxystrobin treated heads were similar to the *F. graminearum* inoculated control.

Khan and Doohan (2009) and Zachetti *et al.* (2019) quantified the amount of DON from inoculated kernels after treating the spikes with chitosan, which led to a significant reduction of the mycotoxin. Another study quantified DON, D3G, 15-ADON in spikes subjected to different chitosan- based

treatments and they also observed a reduction in the number of accumulated mycotoxins (Gunupuru *et al.*, 2019). These results agree with those obtained in the present study, since chitosan, especially when applied at the flag leaf level, reduced the amount of most of the mycotoxins analysed in the present work. Many studies also evaluated the effect of tebuconazole and azoxystrobin in the reduction of DON content, highlighting that only tebuconazole is effective in controlling the accumulation of DON in the spikes, while azoxystrobin resulted ineffective or favoured the accumulation of a higher quantity of DON (Simpson *et al.*, 2001; Pirgozliev *et al.*, 2002; Zhang *et al.*, 2009, Ramirez *et al.*, 2004; Blandino and Minelli, 2006). The present research study confirms the results previously obtained, since tebuconazole contributed to decrease the number of accumulated mycotoxins while azoxystrobin applied on the spikes did not influence it or in many cases favoured their accumulation. To our knowledge, no reports regarding the accumulation of 3-ADON, NIV, ZEA, CUL, 5-HCUL, 15-HCUL, BUT, AUR, CHR, and GID in response to chitosan or reference fungicides is present. Thus, this is the first research work that analysed the amount of these FHB-related compounds after chitosan, tebuconazole, and azoxystrobin treatments.

During all the experiments, there was a significant difference between Marco Aurelio and DBC480 in the ability to counteract FHB. *F. graminearum* inoculated control in DBC480 plants demonstrated a strong type II resistance (resistance to infection spread) to FHB, since FHB severity was of 25% at 21 dpi, the yield reduction was around 20%, and DBC480 inoculated plants accumulated a significantly lower amount of *F. graminearum* biomass and mycotoxins compared to Marco Aurelio. The resistant behaviour of DBC480 is partly attributed to the presence of *Fhb1*. *Fhb1* is one of the most effective QTL in contrasting FHB and derives from the Chinese Spring wheat of Sumai3. *Fhb1* is located on the short arm of the chromosome 3B and provides type II resistance. Nowadays, the identity and mode of action of *Fhb1* are not fully clarified, in fact, many research studies attributed the identity of *Fhb1* to diverse genes, but many evidences underlined that *Fhb1* is involved in DON detoxification (Lemmens *et al.*, 2005; Su *et al.*, 2019; Rawat *et al.*, 2016). This hypothesis is also supported by the present study on the evidence that the DON/D3G ratio was lower in DBC480 compared to Marco Aurelio. Interestingly, the application of chitosan on DBC480 plants decreased FHB severity to 6% at 21 dpi compared to 25% of FHB severity of the *F. graminearum* inoculated control plants, suggesting that the strategy of combining a resistant genotype with a novel and sustainable active ingredient shows additive effects and appears effective for the concomitant reduction of FHB and xenobiotic compounds in the environment.

5.5 References

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5.6 List of tables

Supplementary table 5.1 A list of *F. graminearum* selected genes, accession numbers, gene functions, primer pairs, amplicon length from DNA and cDNA and primer pairs references.

Gene and Accession number	Function	Primer pairs (5'-3')	bp (DNA)	bp (cDNA)	Reference
<i>Erg3</i> - XM_011320132.1	C5-sterol desaturase	ACCAACAACCCTGTCGTCAA TCCGTATAAGTGC GGTTGTCA	239	239	Yun <i>et al.</i> , 2014
<i>Fg03624</i> - XM_011323775.1	Xylanase	CTTCCGGTGCTCAGAAGAAGG CGGACCAGTACTGCTGGAAGG	87	87	Carapito <i>et al.</i> , 2008
<i>Fg03695</i> - XM_011323698.1	Endoglucanase	GCATCAACCTCAAGGTCACCTGG TGCGTAGAGGTTGAAGAGAATGC	127	127	Carapito <i>et al.</i> , 2008
<i>Fg07551</i> - XM_011329028.1	Exopolysaccharuronase	GGCTCTCTTGGCCAGTACAAGG AAGGTGACGTTGTTCGATGAAGC	126	126	Carapito <i>et al.</i> , 2008
<i>Fg09443</i> - XM_011329987.1	Pectin methyl esterase	TGTTGAGGAGAAGACCACCTTCC AGAAGAGCCACCGTTGCTACC	121	121	Carapito <i>et al.</i> , 2008
<i>Fg03725</i> - XM_011323666.1	Multidrug transporter	TGGTCGTCAATACCTCTTCATTGG TACCACCAGCGTTGGTAAGAGG	111	111	Carapito <i>et al.</i> , 2008
<i>Fg01572</i> - XM_011319083.1	Glutamate decarboxylase	GGCCAATACTACCAGCTCATCC CGAACGTTCTGACATGATGACG	115	115	Carapito <i>et al.</i> , 2008
<i>Fg04196</i> - XM_011323131.1	Succinate semialdehyde dehydrogenase	GCCGGATACTTCTTCTCCAAGG CGTAACCGCTCTCCTTGACACC	126	126	Carapito <i>et al.</i> , 2008
<i>Fg06619</i> - XM_011327936.1	NADP-dependent alcohol dehydrogenase	CTCAGCCTCTCGATATTACAG AAGTGAGACGTGCCGATGTAGC	122	122	Carapito <i>et al.</i> , 2008
<i>Fg10231</i> - XM_011320878.1	NADH-ubiquinone oxidoreductase	CCAAGGCCACTGCTACTGATCC CAGATCCTTCTTGTGGTCTCG	115	115	Carapito <i>et al.</i> , 2008
<i>FPP</i> - XM_011328132.1	Farnesyl pyrophosphate synthase	TTTGGCAAGCCCGAACACATT GCGGATCTGGCCAACAACCTTCT	234	234	Lee <i>et al.</i> , 2014
<i>Tri1</i> - AY339129.1	Cytochrome P450 monooxygenase	GCTCGCGAACTAATCACTCC TCCAAACATTGGCAGATGA	185	185	Heidtmann-Bemvenuti <i>et al.</i> , 2015
<i>Tri3</i> - XM_009265280.1	Trichothecene 15-O-acetyltransferase	CTTGACGGGATATCAAGAAATGTTACGA CTCGCCTGTTGTAGTTCGCTTGATTT	208	208	Lee <i>et al.</i> , 2014
<i>Tri4</i> - EF685280.1	Trichodiene oxygenase	TCGAGGCACAACAGAAGGGTATCC AATGTCGGCCTTGGTGGTGTC	237	237	Lee <i>et al.</i> , 2014
<i>Tri5</i> - XM_011323870.1	Trichodiene synthase	GGAGCGTATCGA GAATTTGC TGCCCAACTGTATACGACCA	168	168	Heidtmann-Bemvenuti <i>et al.</i> , 2015
<i>Tri6</i> - AB017495.1	Trichothecene transcriptional regulator	TCTTTGTGAGCGGACGGGACTTTA ATCTCGCATGTTATCCACCTGCT	245	245	Horevaj <i>et al.</i> , 2011
<i>Tri8</i> - HG970333.1	Trichothecene 3-O-esterase	GCTACTTTGGACTCAATTCG CATACTGTACCGCAAGTTCTG	139	139	Lee <i>et al.</i> , 2014
<i>Tri9</i> - LT222054.1	Unknown	AGCCGCTAAACTGATCGACTCATA GCTTTGGCTGCGACCCATAT	85	85	Lee <i>et al.</i> , 2014
<i>Tri10</i> - AF365969.1	Regulatory gene	GTGGCCGGGACGCTTCAAT ATCCGCTCAAGTCTTCCCATCTCAT	254	254	Brown <i>et al.</i> , 2001
<i>Tri11</i> - XM_009265275.1	Trichothecene C-15 hydroxylase	AAGTACTTCACCCGACAAACGAC CGGCAAGGCGAATGTCAAAC	192	192	Lee <i>et al.</i> , 2014
<i>Tri12</i> - XM_009265274.1	Trichothecene efflux pump	TCCACAGTCATCTTTCCCCAGTCT CTCCCAGTGCCATAGCGAAGTAGT	172	172	Lee <i>et al.</i> , 2014
<i>Tri14</i> - XM_009265272.1	Unknown	CTGGGAACCTACGCATCAAACATT CGAATGAGCTGCCCAATGATGT	165	165	Lee <i>et al.</i> , 2014
<i>Tri101</i> - AB011417.1	Trichothecene 3-O-acetyltransferase	GTGGGACTCTGGGATTACGACTTT GTCCACTCCTTATCCGCCTTCAA	130	130	Lee <i>et al.</i> , 2014
<i>TEF</i> - MH572267.1	Translational elongation factor 1- α	ATGGGTAAGGAGGACAAGAC GGAAGTACCAGTGATCATGTT	700	324	Geiser <i>et al.</i> , 2004
<i>TUB</i> - XM_011329885	β -tubulin	TTGCATTGGTACACTGGTGAGG AGGCAGCTCCTCCTGTA CTCC	110	110	Carapito <i>et al.</i> , 2008
<i>GAPDH</i> - XM_011326605.1	Glyceraldehyde-3-phosphate dehydrogenase	TGACTTGACTGTTCGCCTCGAGAA ATGGAGGAGTTGGTGTGCGGTTA	160	160	Harris <i>et al.</i> , 2016

Supplementary table 5.2 A list of wheat selected genes, accession numbers, gene functions, primer pairs, amplicon length from DNA and cDNA and primer pairs references.

Gene and Accession number	Function	Primer pairs (5'-3')	bp (DNA)	bp (cDNA)	Reference
<i>TaPAL</i> - X99705	Phenylalanine ammonia-lyase	CGATGCTCGTCCGAGTCAAT CATGACCTCACAGAAGACGC	407	407	<i>De novo</i> designed
<i>TaPR1</i> - AJ007348	Pathogenesis related protein-1	ACTACGACTACGGGTCCAACA TCGTAGTTGCAGGTGATGAAG	145	145	Lu <i>et al.</i> , 2006
<i>TaPR2</i> - Y18212	β -1,3-glucanase	AACGTGCGCCCCTACTACC GCGTCGAACAGGCTCGTGTA	398	398	Francesconi <i>et al.</i> , 2020
<i>TaFNR</i> - AJ457980	Ferredoxin-NADP(H)-oxidoreductase	CACCGGCCCAGTGATCTT AAGGGCGTCTGCTCCAAC	259	69	Tenea <i>et al.</i> , 2011
<i>TaTUB</i> - TAU76745	β -tubulin	CGAGGAGGGCGAGTACGA AGCAAAGCACGACATGGACAT	79	79	Tenea <i>et al.</i> , 2011
<i>TaACT</i> - AB181991	Actin	TCCTGTGTTGCTGACTGAGG GGTCCAAACGAAGGATAGCA	350	236	Mandalà <i>et al.</i> , 2019

Supplementary table 5.3 Relative expression levels and standard errors of each *F. graminearum* gene selected and compound (chitosan hydrochloride C, tebuconazole T, azoxystrobin A, and the mixture of tebuconazole and azoxystrobin T+A). Relative expression values were obtained by using the equation $2^{-\Delta\Delta C_q}$ with *TEF*, *TUB* and *GAPDH* as reference genes and mock treatment used to normalize the relative expression levels. The data represent averages and standard errors for the four independent biological replicates and the four technical replicates examined. The data were subjected to one-way analysis of variance (ANOVA). Different letters among the plant treatments were assessed by means of significant F values by applying the Tukey Honestly Significant Difference Test (Tukey test) at 0.99 confidence level ($p < 0.01$).

Gene		C	T	A	T+A
<i>Erg3</i>	Relative expression	0.01	2.19	2.58	0.92
	Standard error ($\pm$)	0.044	0.065	0.047	0.05
	$p < 0.01$	b	a	a	ab
<i>Fg03624</i>	Relative expression	0.33	0.59	0.61	0.87
	Standard error ($\pm$)	0.02	0.12	0.13	0.04
	$p < 0.01$	b	ab	ab	a
<i>Fg03695</i>	Relative expression	0.01	0.38	0.11	0.12
	Standard error ($\pm$)	0.06	0.38	0.01	0.09
	$p < 0.01$	c	a	bc	b
<i>Fg07551</i>	Relative expression	0.01	0.15	0.50	0.23
	Standard error ($\pm$)	0.07	0.10	0.13	0.01
	$p < 0.01$	b	b	a	ab
<i>Fg09443</i>	Relative expression	0.04	1.54	0.36	1.18
	Standard error ($\pm$)	0.06	0.06	0.18	0.11
	$p < 0.01$	c	a	bc	ab
<i>Fg03725</i>	Relative expression	0.01	0.57	0.08	0.88
	Standard error ($\pm$)	0.01	0.12	0.08	0.07
	$p < 0.01$	c	ab	bc	a
<i>Fg01572</i>	Relative expression	0.04	9.64	2.69	5.69
	Standard error ($\pm$)	0.05	0.07	0.03	0.03
	$p < 0.01$	c	a	bc	ab
<i>Fg04196</i>	Relative expression	0.01	1.30	0.64	1.58
	Standard error ($\pm$)	0.02	0.03	0.14	0.09
	$p < 0.01$	b	a	ab	a
<i>Fg06619</i>	Relative expression	0.04	1.23	2.44	0.93
	Standard error ($\pm$)	0.08	0.06	0.16	0.08
	$p < 0.01$	b	ab	a	ab
<i>Fg10231</i>	Relative expression	0.10	4.00	4.90	4.30
	Standard error ($\pm$)	0.01	0.03	0.03	0.11
	$p < 0.01$	b	a	a	a
<i>FPP</i>	Relative expression	0.11	3.25	1.33	4.38
	Standard error ($\pm$)	0.01	0.77	0.22	0.22
	$p < 0.01$	c	ab	bc	a
<i>Tri1</i>	Relative expression	0.02	0.03	1.30	0.31
	Standard error ($\pm$)	0.07	0.10	0.09	0.09
	$p < 0.01$	b	b	a	b
<i>Tri3</i>	Relative expression	0.01	0.82	1.33	0.30
	Standard error ($\pm$)	0.04	0.15	0.23	0.08
	$p < 0.01$	c	ab	a	bc
<i>Tri4</i>	Relative expression	0.02	0.07	0.21	0.03
	Standard error ($\pm$)	0.00	0.18	0.03	0.01
	$p < 0.01$	b	b	a	b
<i>Tri5</i>	Relative expression	0.02	1.67	2.76	0.60
	Standard error ($\pm$)	0.07	0.15	0.34	0.33
	$p < 0.01$	b	ab	a	b
<i>Tri6</i>	Relative expression	0.03	2.09	2.05	0.82
	Standard error ($\pm$)	0.08	0.39	0.16	0.16
	$p < 0.01$	c	a	ab	bc
<i>Tri8</i>	Relative expression	3.34	19.94	11.52	16.81
	Standard error ($\pm$)	0.09	5.23	2.29	2.94
	$p < 0.01$	b	a	ab	a
<i>Tri9</i>	Relative expression	0.01	4.98	2.04	0.52
	Standard error ($\pm$)	0.00	0.01	0.58	0.13
	$p < 0.01$	c	a	bc	bc

<i>Tri10</i>	Relative expression	0.02	2.73	4.19	0.18
	Standard error ($\pm$)	0.01	0.84	0.72	0.04
	$p<0.01$	b	ab	a	b
<i>Tri11</i>	Relative expression	0.15	26.84	4.90	1.44
	Standard error ($\pm$)	0.01	0.92	0.76	0.55
	$p<0.01$	c	a	b	c
<i>Tri12</i>	Relative expression	0.05	24.95	13.21	5.12
	Standard error ($\pm$)	0.03	0.37	1.95	0.76
	$p<0.01$	c	a	b	c
<i>Tri14</i>	Relative expression	0.10	3.39	10.62	2.90
	Standard error ($\pm$)	0.01	0.59	1.86	1.01
	$p<0.01$	b	b	a	b
<i>Tri101</i>	Relative expression	5.00	24.31	0.16	31.40
	Standard error ($\pm$)	1.76	3.01	0.01	0.65
	$p<0.01$	c	b	c	a

Supplementary table 5.4 Relative expression levels and standard errors of each wheat gene selected for each durum wheat genotype (Marco Aurelio and DBC480) and compound (chitosan C, tebuconazole T, azoxystrobin A, and the mixture of tebuconazole and azoxystrobin T+A). Fg represents the *F. graminearum* inoculated control. Relative expression values were obtained by using the equation $2^{-\Delta\Delta C_q}$ with *TaFNR*, *TaTUB* and *TaACT* as reference genes and mock treatment used to normalize the relative expression levels. The data represent averages and standard errors for the four independent biological replicates and the four technical replicates examined. The data were subjected to two-way analysis of variance (ANOVA). Different letters among the plant treatments were assessed by means of significant F values by applying the Tukey Honestly Significant Difference Test (Tukey test) at 0.99 confidence level ($p < 0.01$).

		Marco Aurelio					DBC480				
Gene		Fg	C	T	A	T+A	Fg	C	T	A	T+A
<i>TaPAL</i>	Relative expression	2.89	1.76	1.16	1.09	0.84	2.62	2.35	0.67	2.29	0.27
	Standard error ($\pm$)	0.22	0.29	0.22	0.27	0.23	0.84	0.66	0.10	0.80	0.07
	$p < 0.01$	a	b	b	b	c	a	a	c	a	c
<i>TaPR1</i>	Relative expression	3.48	2.91	0.87	0.98	1.05	2.04	2.16	1.21	1.47	0.84
	Standard error ($\pm$)	0.27	0.61	0.22	0.18	0.28	0.56	0.59	0.16	0.44	0.12
	$p < 0.01$	a	b	c	c	c	b	b	c	bc	c
<i>TaPR2</i>	Relative expression	2.51	2.69	0.47	0.49	0.40	3.41	3.23	1.79	1.04	0.61
	Standard error ($\pm$)	0.69	0.34	0.16	0.13	0.05	0.52	0.83	0.26	0.20	0.08
	$p < 0.01$	b	b	d	d	d	a	a	b	c	d

5.7 List of figures

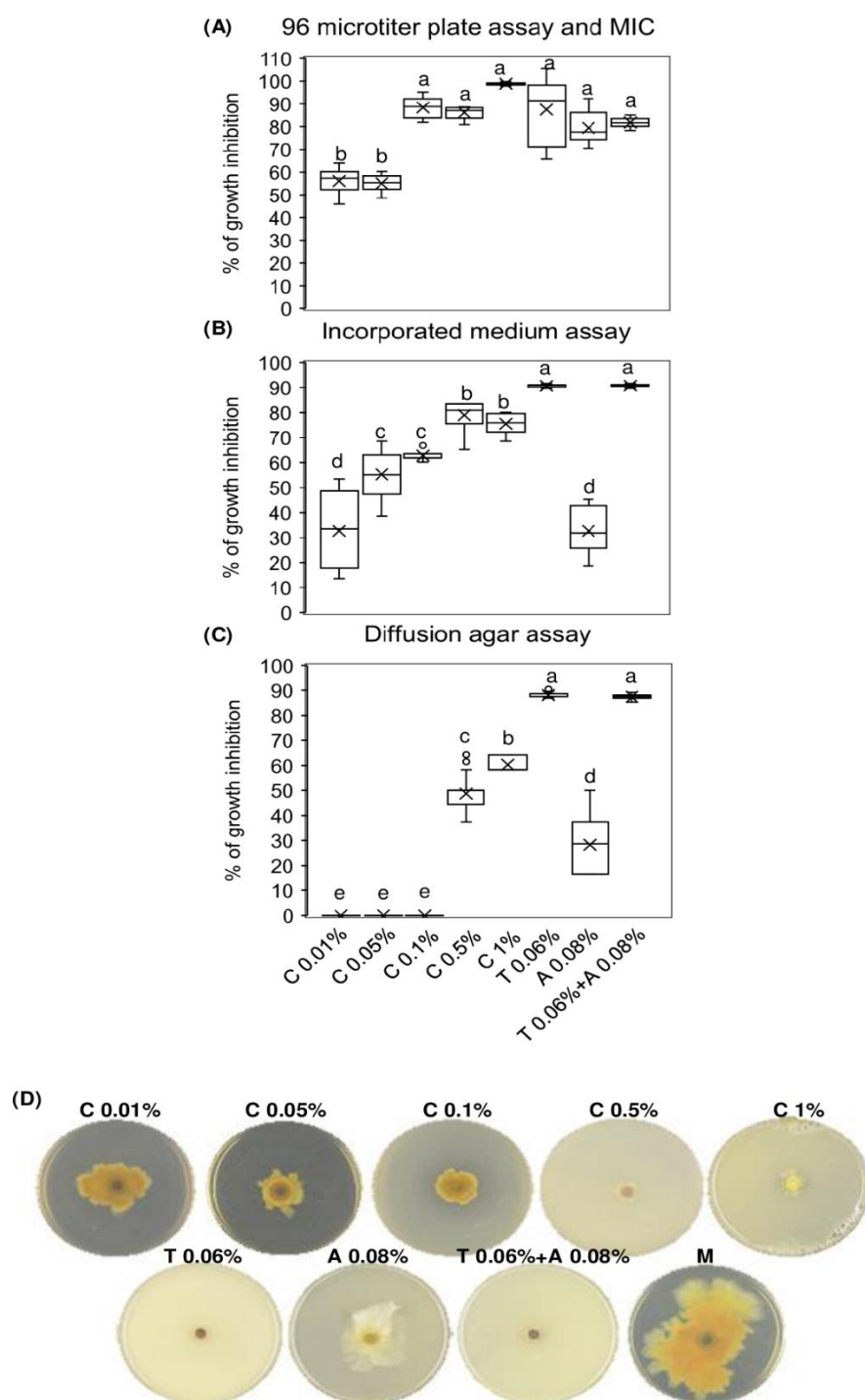


Figure 5.1 *In vitro* antifungal assays. Boxplots of (A) 96 microtiter plates assay and determination of the minimum inhibitory concentration (MIC); (B) incorporated medium assay; (C) diffusion agar assay. (d) *F. graminearum* growth on Potato Dextrose Agar (PDA) incorporated with the tested compounds. Chitosan hydrochloride was tested at 0.01–0.05–0.1–0.5 and 1%, tebuconazole and azoxystrobin were used as control fungicides at the recommended field doses of 0.06% and 0.08%, respectively, and were assayed alone or mixed. M represents the Mock control. Results are expressed as % of growth inhibition. The data represent averages and standard errors from three independent experiments, each one consisting of four replicates for each substance and concentration tested. Different letters (a, b, c, d, e) into the subfigures refer to the statistical analysis performed using one-way analysis of variance (ANOVA) with the Tukey test at a confidence level of 0.99 and $p < 0.01$.

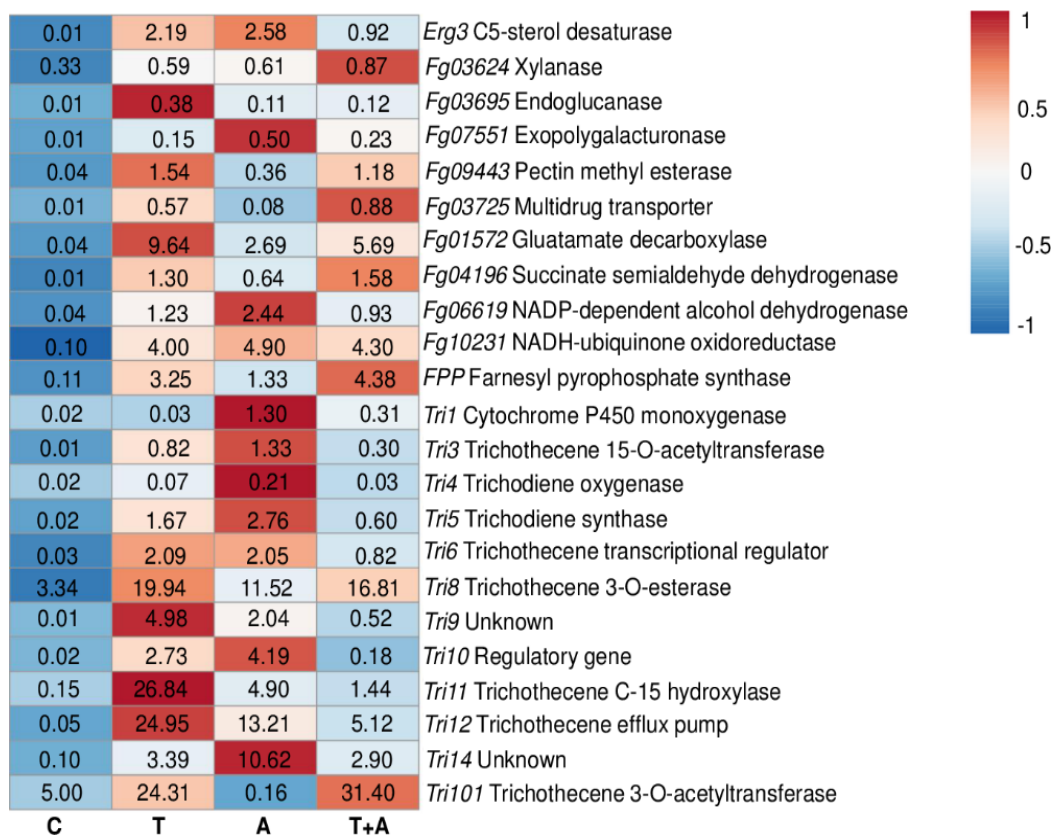


Figure 5.2 Heatmap and relative expression levels of the selected *F. graminearum* genes. The relative expression levels are referred to the different compounds' treatments (Chitosan hydrochloride C, Tebuconazole T, Azoxystrobin A, Tebuconazole+Azoxystrobin T + A). Relative expression values were obtained by using the equation $2^{-\Delta\Delta C_q}$ with *TEF*, *TUB*, and *GAPDH* as reference genes and mock treatment used to normalize the relative expression levels. The heatmap was constructed with the z-score by analysing data with ClustVis software (Estonia).

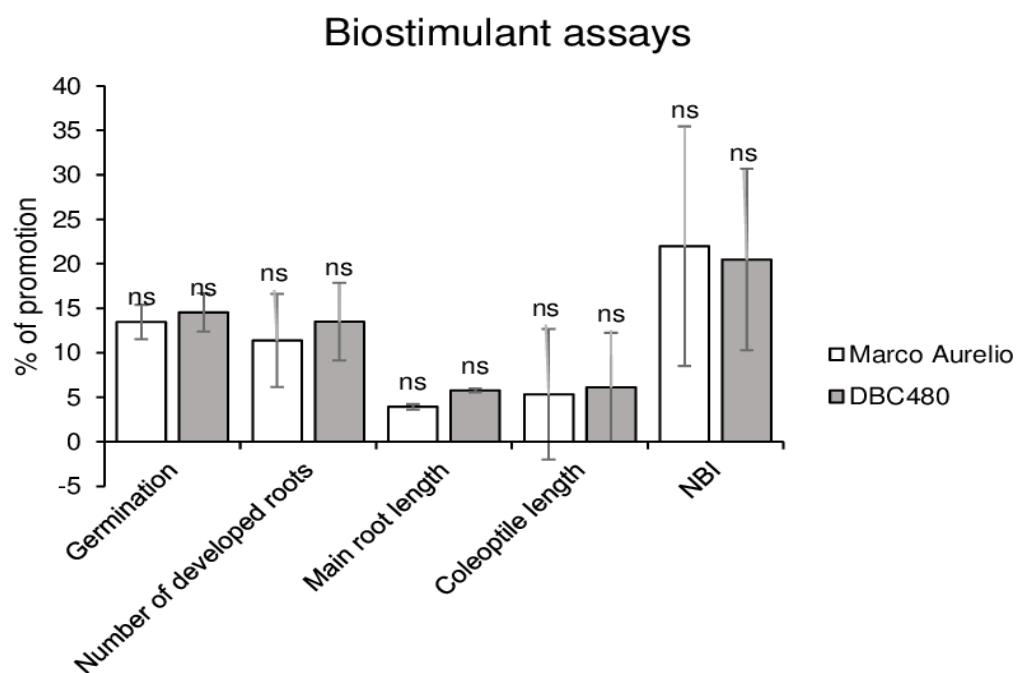


Figure 5.3 *In vivo* biostimulant assays. The biostimulant effect of chitosan hydrochloride at 0.5% was tested on Marco Aurelio and DBC480 by evaluating the kernels germination, number of developed roots, main root length, coleoptile length, and Nitrogen Balance Index (NBI). Results are expressed as % of growth inhibition. The data represent averages and standard errors from three independent experiments organized as a complete randomized block, each one consisting of 20 plants for the experimental group (wheat genotype × compound). The statistical analysis was performed using one-way analysis of variance (ANOVA); “ns” refers to “not significant”.

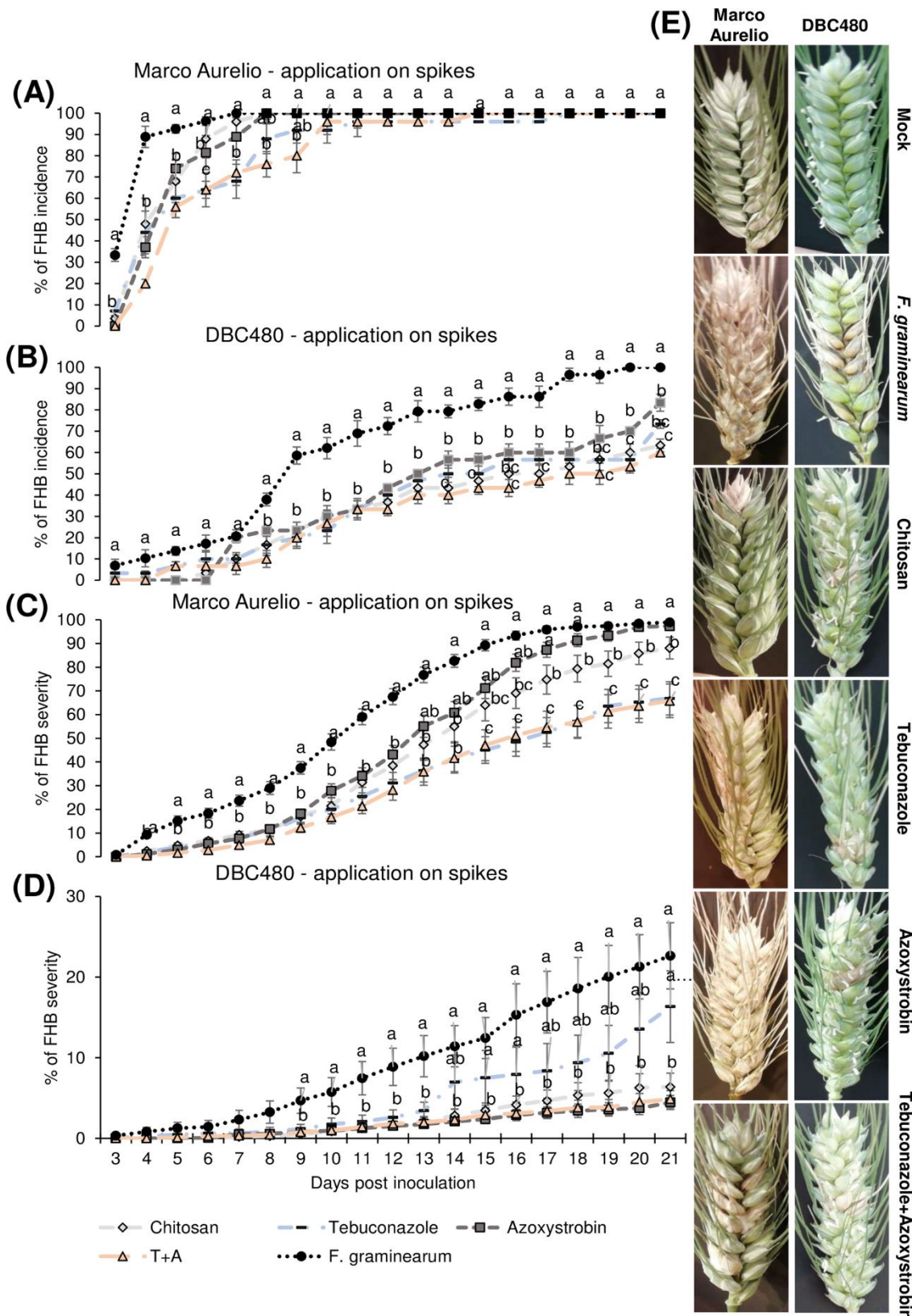


Figure 5.4 Fusarium head blight (FHB) incidence and severity (%) in (A, C) Marco Aurelio and (B, D) DBC480 from 3 to 21 days post inoculation (dpi) and (E) FHB symptoms at 21 dpi for plants treated 48 hours before the *F. graminearum* inoculation at the spike level with chitosan at 0.5%, tebuconazole at 0.06%, azoxystrobin at 0.08%, the mixture of tebuconazole at 0.06%, and azoxystrobin at 0.08%. Merely *F. graminearum* inoculated and mock plants were used as controls. The data represent averages and standard errors from three independent replicates with at least 10 spikes for each experimental group (wheat genotype × compound). Different letters (a, b, c) into the subfigures refer to the statistical analysis performed using one-way analysis of variance (ANOVA) with the Tukey test at a confidence level of 0.99 and $p < 0.01$.

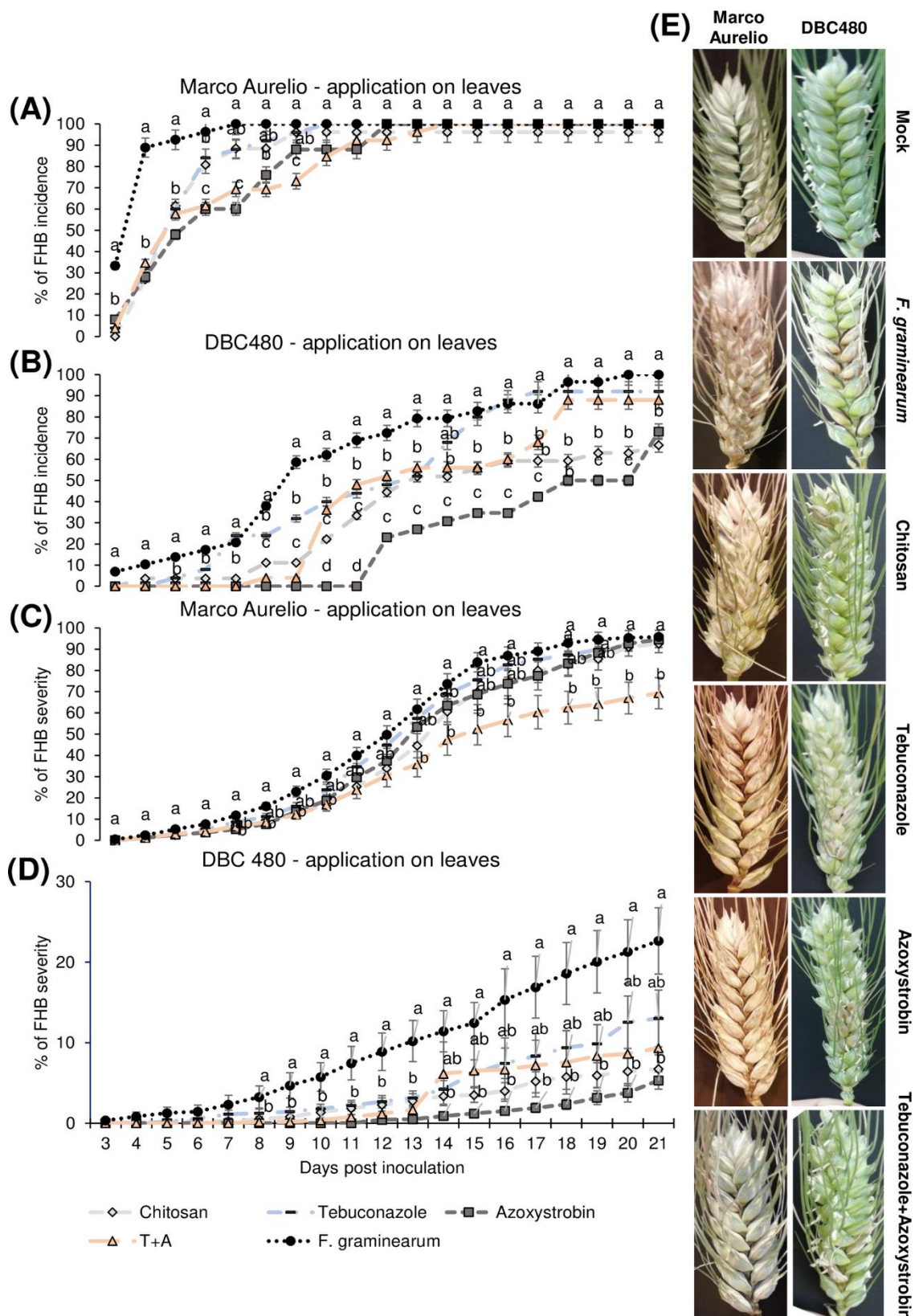


Figure 5.5 Fusarium head blight (FHB) incidence and severity (%) in (A, C) Marco Aurelio and (B, D) DBC480 from 3 to 21 days post inoculation (dpi) and (E) FHB symptoms at 21 dpi for plants treated 48 hours before the *F. graminearum* inoculation at the flag leaf level with chitosan at 0.5%, tebuconazole at 0.06%, azoxystrobin at 0.08%, the mixture of tebuconazole at 0.06%, and azoxystrobin at 0.08%. Merely *F. graminearum* inoculated and mock plants were used as controls. The data represent averages and standard errors from three independent replicates with at least 10 spikes for each experimental group (wheat genotype × compound). Different letters (a, b, c) into the subfigures refer to the statistical analysis performed using one-way analysis of variance (ANOVA) with the Tukey test at a confidence level of 0.99 and $p < 0.01$.

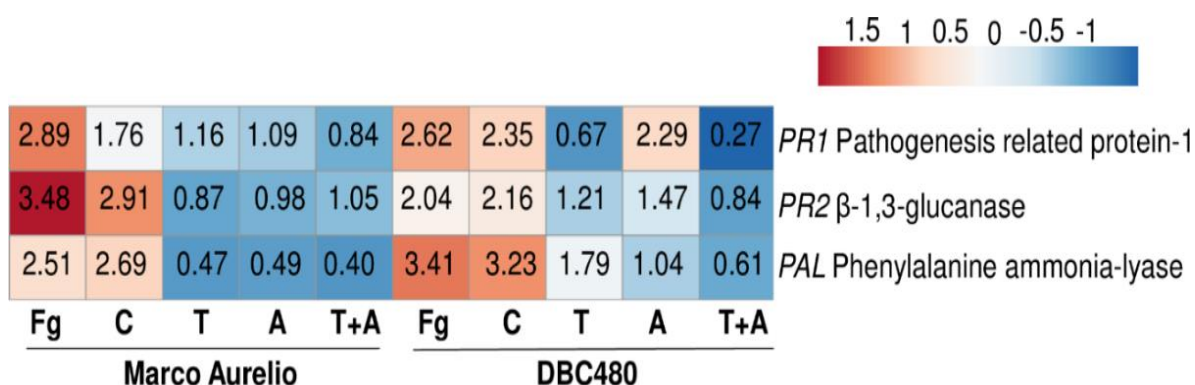


Figure 5.6 Heatmap and relative expression levels of the selected durum wheat genes. The relative expression levels are referred to the different compounds' pre-treatments (Chitosan C, Tebuconazole T, Azoxystrobin A, Tebuconazole + Azoxystrobin T + A) applied on the flag leaf and to the two wheat genotypes Marco Aurelio and DBC480. Fg represents the *F. graminearum* inoculated control. Relative expression values were obtained by using the equation $2^{-\Delta\Delta C_q}$ with *TaFNR*, *TaTUB*, and *TaACT* as reference genes and mock treatment used to normalize the relative expression levels. The heatmap was constructed with the z-score by analysing data with ClustVis software (Estonia).

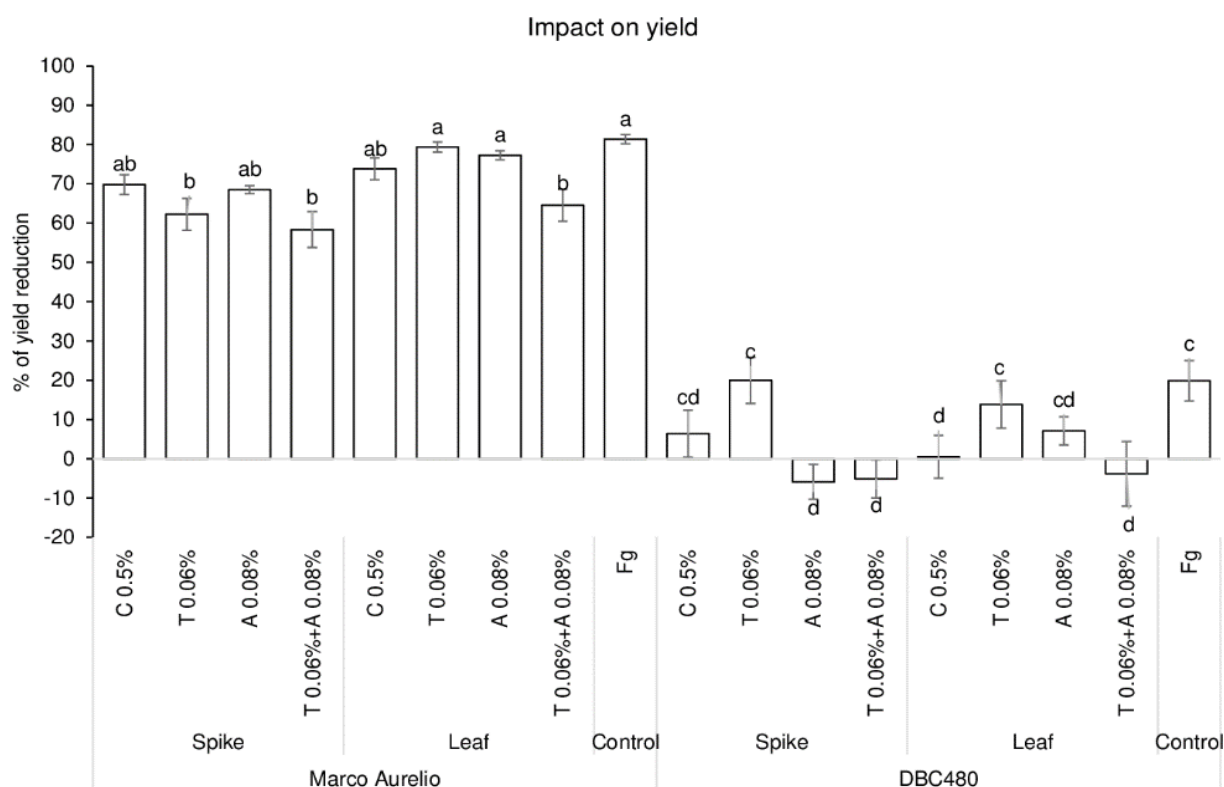


Figure 5.7 Percent yield reduction in Marco Aurelio and DBC480 due to *F. graminearum* infection after treating the plants at the spike or flag leaf level with Chitosan at 0.5% (C 0.5%), tebuconazole at 0.06% (T 0.06%), azoxystrobin at 0.08% (A 0.08%), the mixture of tebuconazole at 0.06% and azoxystrobin at 0.08% (T 0.06% + A 0.08%) prior to *F. graminearum* inoculation; *F. graminearum* inoculated spikes served as controls (Fg). The data represent averages and standard errors from three independent replicates with at least 10 plants for each experimental group (wheat genotype × compound × mode of application). Different letters (a, b, c, d) refer to the statistical analysis performed using three-way analysis of variance (ANOVA) with the Tukey test at a confidence level of 0.99 and $p < 0.01$.

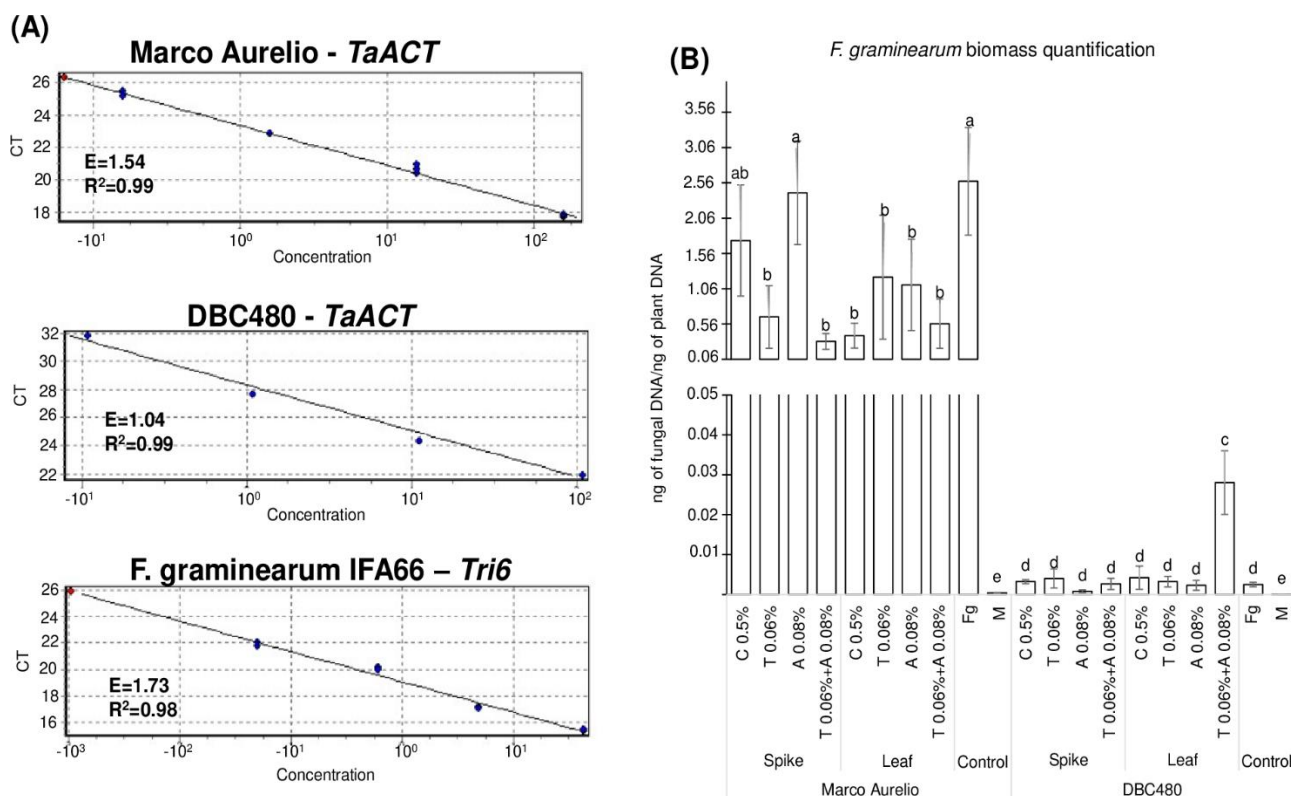


Figure 5.8 (A) Standard curves of the *TaACT* and *Tri6* genes for the Real-Time qPCR quantification of the fungal biomass. (B) *F. graminearum* biomass quantification expressed as ng of fungal DNA/ng of plant DNA from Marco Aurelio and DBC480 spikes recorded after treating the plants at the spike or flag leaf level with Chitosan at 0.5% (C 0.5%), tebuconazole at 0.06% (T 0.06%), azoxystrobin at 0.08% (A 0.08%), the mixture of tebuconazole at 0.06% and azoxystrobin at 0.08% (T 0.06% + A 0.08%) prior to *F. graminearum* inoculation, solely *F. graminearum* inoculated (Fg) and Mock (M) controls were included. The data represent averages and standard errors from four technical replicates derived from three biological replicates for each variable (wheat genotype $\times$ compound $\times$ mode of application). Different letters (a, b, c, d, e) into the subfigures refer to the statistical analysis performed using three-way analysis of variance (ANOVA) with the Tukey test at a confidence level of 0.99 and $p < 0.01$.

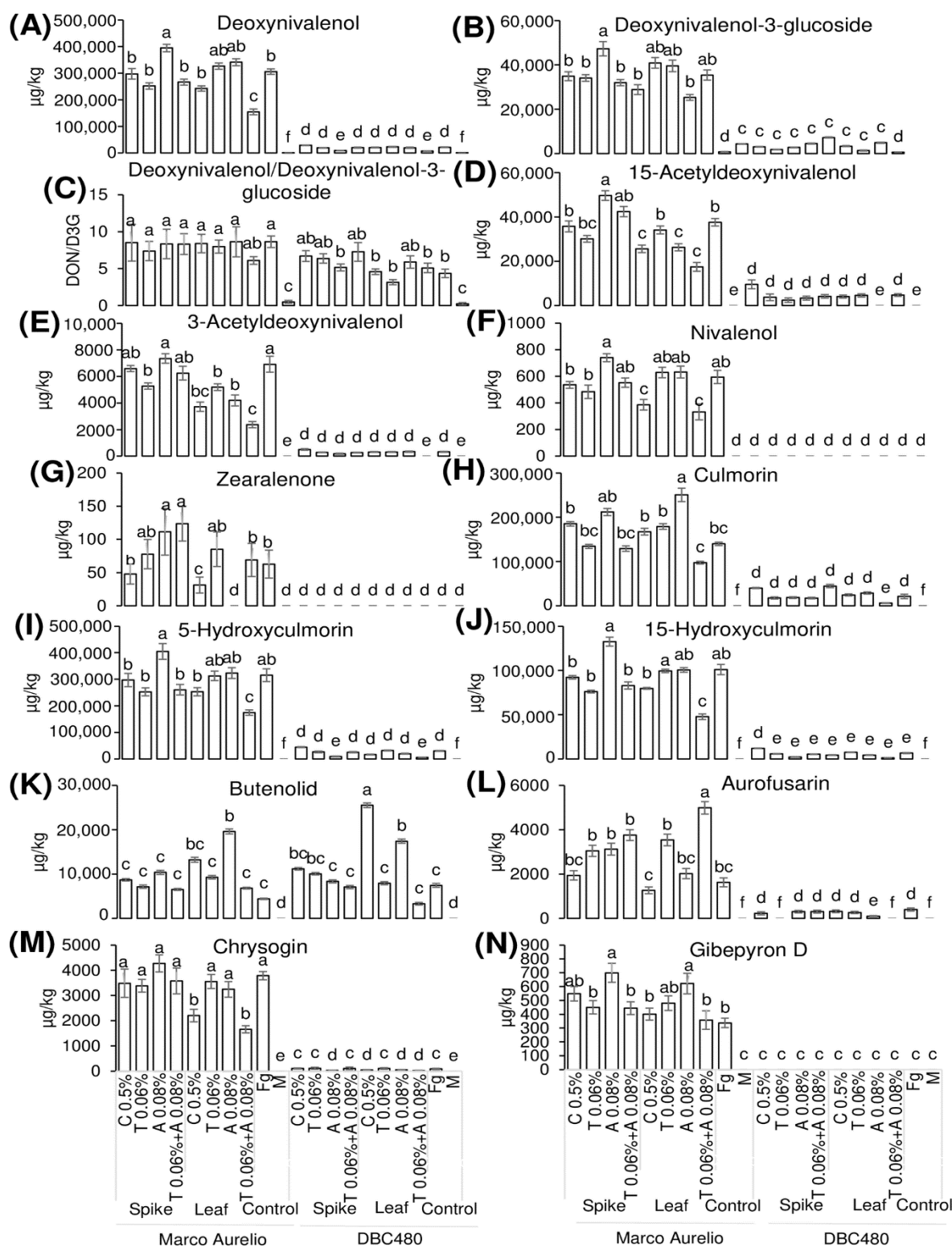


Figure 5.9 Detected and quantified mycotoxins and *F. graminearum*-associated molecules by using LC-MS/MS from Marco Aurelio and DBC480 spikes after treating the plants at the spike or flag leaf level with chitosan at 0.5% (C 0.5%), tebuconazole at 0.06% (T 0.06%), azoxystrobin at 0.08% (A 0.08%), the mixture of tebuconazole at 0.06% and azoxystrobin at 0.08% (T 0.06% + A 0.08%) prior to *F. graminearum* inoculation, solely *F. graminearum* inoculated (Fg) and Mock (M) controls were included as controls. (A) Deoxynivalenol; (B) Deoxynivalenol-3-glucoside; (C) Deoxynivalenol/Deoxynivalenol-3-glucoside; (D) 15-Acetyldeoxynivalenol; (E) 3-Acetyldeoxynivalenol; (F) Nivalenol; (G) Zearalenone; (H) Culmorin; (I) 5-Hydroxyculmorin; (J) 15-hydroxyculmorin; (K) Butenolid; (L) Aurofusarin; (M) Chrysogin; (N) Gibepyrone D. The data represent averages and standard errors from four technical replicates derived from three biological replicates for each experimental group (wheat genotype $\times$ compound $\times$ mode of application). Different letters (a, b, c, d, e, f) into the subfigures refer to the statistical analysis performed using three-way analysis of variance (ANOVA) with the Tukey test at a confidence level of 0.99 and $p < 0.01$.

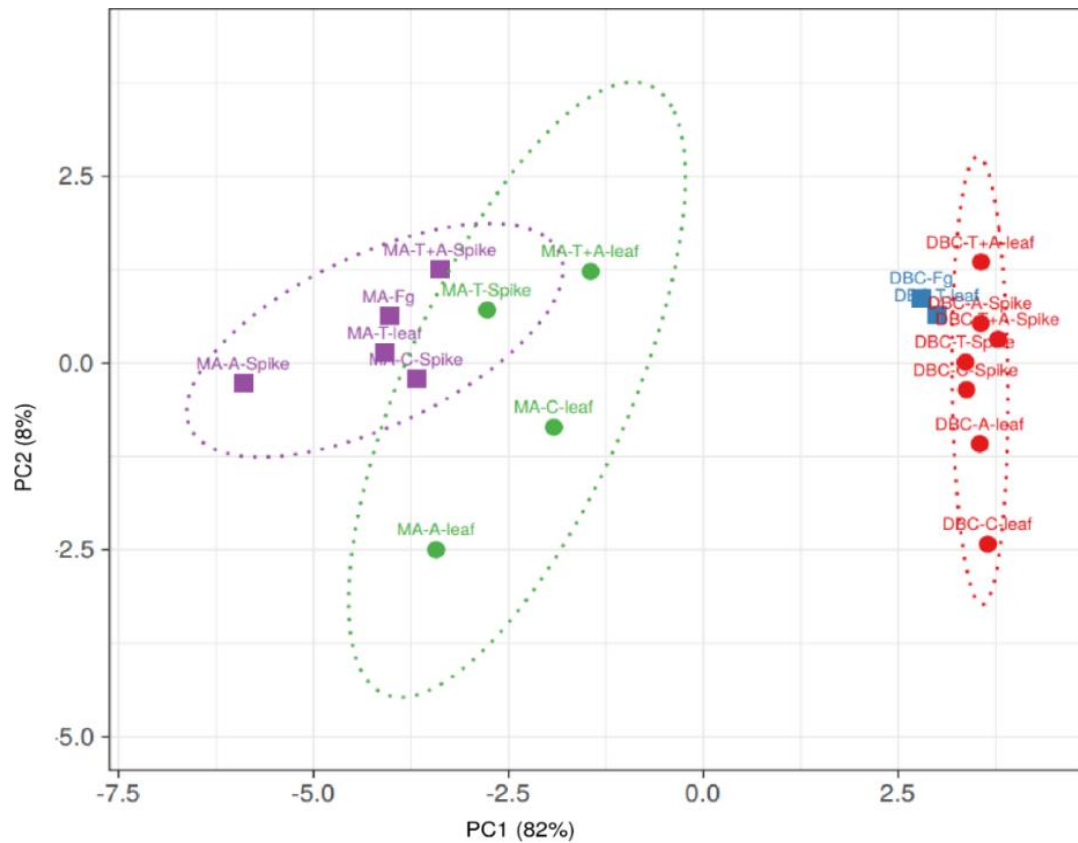
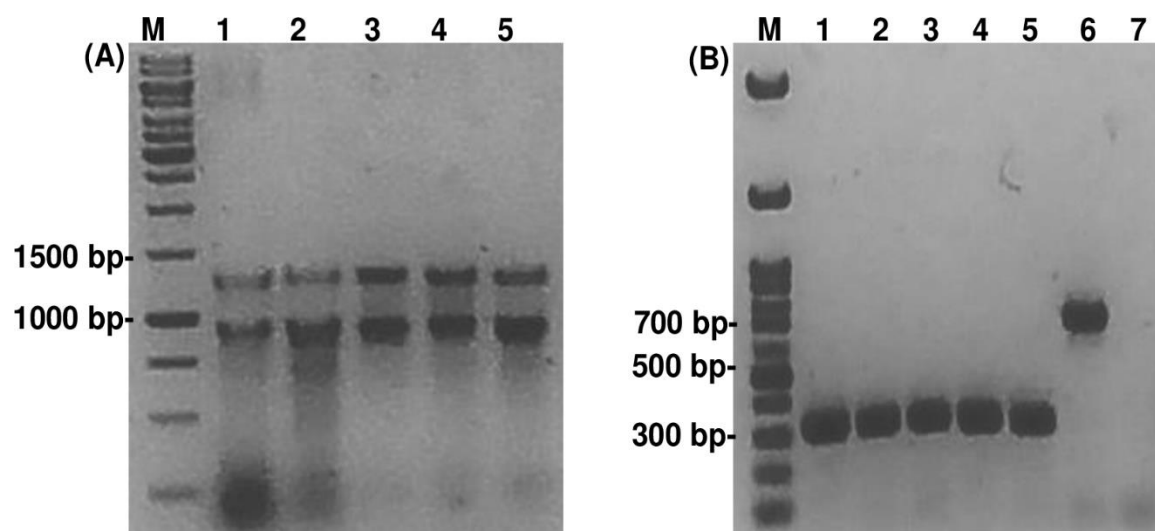
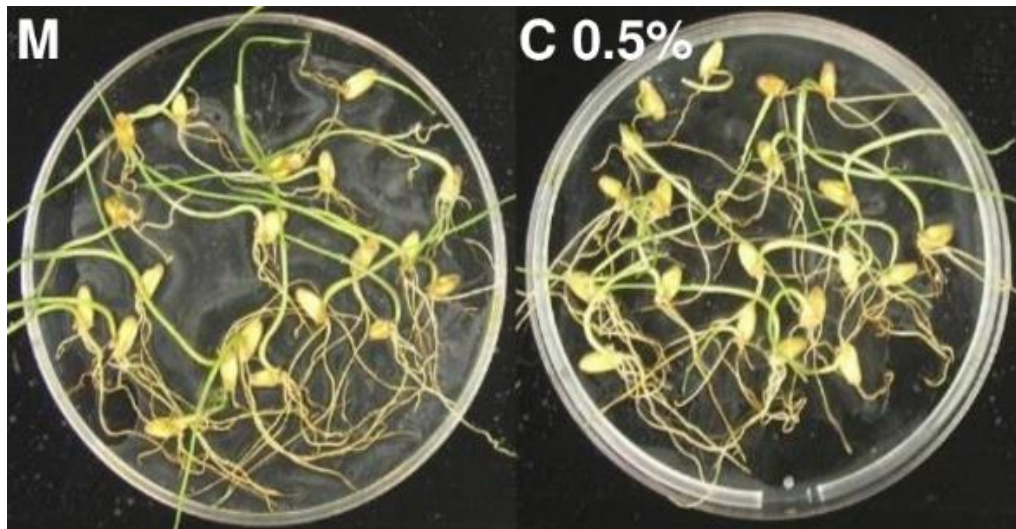


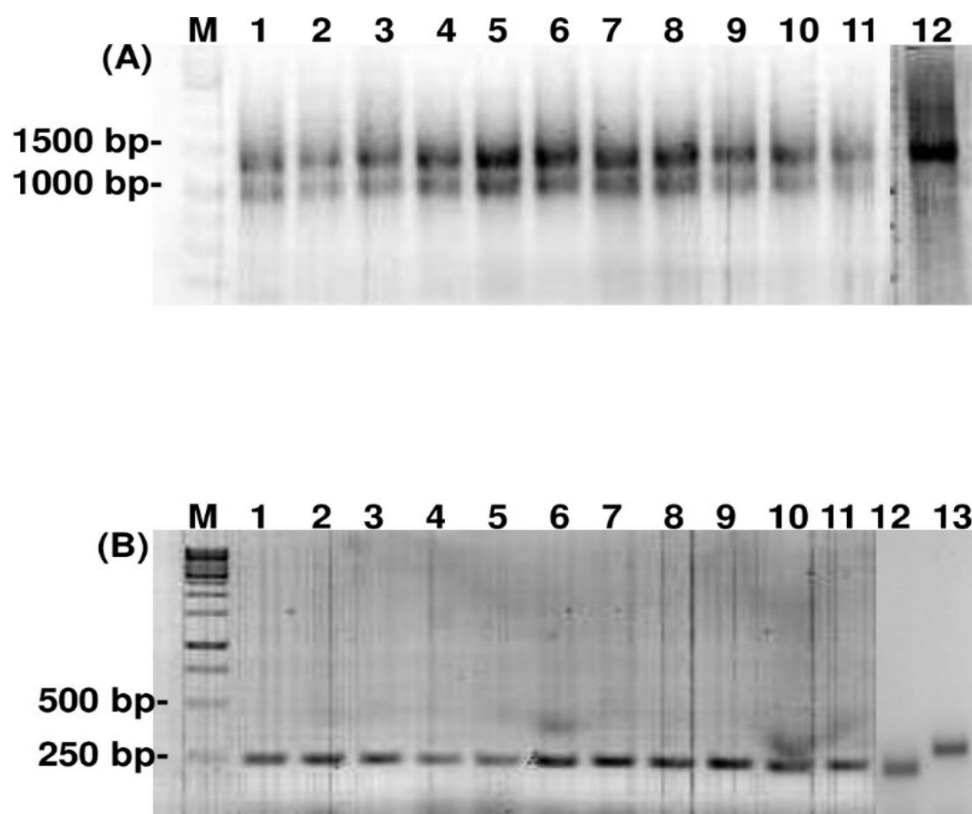
Figure 5.10 Principal Component Analysis (PCA) of the two durum wheat genotypes Marco Aurelio (MA) and DBC480 (DBC), plant treatments chitosan (C), tebuconazole (T), azoxystrobin (A), the mixture of tebuconazole and azoxystrobin (T + A), and the *F. graminearum* inoculated control (Fg), and the two modes of application (spike of leaf). The PCA was carried out to evaluate the genotype $\times$ compound effects on controlling FHB by plotting the % of FHB incidence and severity at 21 dpi, the % of yield reduction, the ng of fungal DNA/ng of plant DNA, and the $\mu\text{g/kg}$ of the analysed mycotoxins and *F. graminearum*-related compounds. PCA was conducted by using ClustVis software (Estonia).



Supplementary figure 5.1 1.5% agarose gel of total extracted RNA (A) and RT-PCR (B) from *F. graminearum*. M) GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific) and ExcelBand™ 100 bp + 3K DNA Ladder (Smobio); 1) Chitosan hydrochloride; 2) Tebuconazole; 3) Azoxystrobin; 4) Tebuconazole+Azoxystrobin; 5) Mock; 6) Genomic DNA (gDNA); 7) No template control (NTC). The figure represents the original pictures of the gels.



Supplementary figure 5.2 Durum seedlings at 14 days after the coating with chitosan hydrochloride at 0.5% (C). M represents the mock control.



Supplementary figure 5.3 1.5% agarose gel of total extracted RNA (A) and RT-PCR (B) from the spikes of Marco Aurelio and DBC480 subjected to the different compounds' treatments at the flag leaf level. M) GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific); 1) Marco Aurelio, chitosan; 2) Marco Aurelio, tebuconazole; 3) Marco Aurelio, azoxystrobin; 4) Marco Aurelio, tebuconazole+azoxystrobin; 5) Marco Aurelio, *F. graminearum* inoculated control; 6) Marco Aurelio, mock control; 7) DBC480, chitosan; 8) DBC480, tebuconazole; 9) DBC480, azoxystrobin; 10) DBC480, tebuconazole+azoxystrobin; 11) DBC480, *F. graminearum* inoculated control; 12) DBC480, mock control; 13) Genomic DNA (gDNA). The figure represents the pictures of three gels.

6. Conclusions

This study has firstly highlighted the complexity of testing for FHB resistance, and demonstrated the necessity to use as many resistance screening protocols as possible, such as the use of comparative inoculation methods and different tests because of FHB's multigenic nature and complexity. In the present study, we individuated an Italian durum wheat cultivar, Marco Aurelio, showing type II tolerance after artificial inoculation of *F. graminearum*. Nevertheless, the cultivar Marco Aurelio cannot be considered completely tolerant to *F. graminearum*, because the % of yield loss after the inoculation was consistent (more than 60%) and because we assayed the cultivars by using only one species and strain. It would be extremely interesting and informative to challenge the cultivar to several *Fusarium* species belonging to the Fusarium head blight species complex.

Secondly, the research study reported that most of the genes involved in the positive regulation of stomatal closure were induced in the resistant cultivar Sumai3 after *F. graminearum* inoculation. Nevertheless, *TaBG* and *TaCYP450*, negative regulators of stomatal closure, were even stronger up-regulated. Thus, we concluded that stomatal closure is involved in resistance to FHB in wheat by an intense cross-talk among positive and negative genes regulating the guard cells movements. The transcriptional changes also resulted in modification of photosynthetic parameters resulting in an increasing of spikes temperature, as an outcome of stomatal closure, in both of the wheat genotypes and in a decreasing of photosynthetic efficiency in Rebelde but not in Sumai3, confirming that photosynthetic parameters are related to FHB resistance. The role of stomatal movements as a resistant mechanism against bacterial plant pathogens is well known, characterized and amply described in literature. On the other hand, its role against fungal pathogens is mostly unknown. For such reason, this research contributed to disclose the genetic mechanism of stomatal conductance in wheat during *F. graminearum* infection. In the future, it would be informative to study in deep the role of stomatal conductance by using a microarray approach. In such way, not only the specific genes regulating the stomatal movements will be investigated but the entire transcriptome profile of wheat genotypes challenged by biotic and/or abiotic stresses. This technique will allow to identify a wide range of genes differentially expressed in untreated, inoculated and drought-stressed plants. Furthermore, since stomatal movements are regulated by the biosynthesis and catabolism of ABA and its role during the *F. graminearum* and wheat interaction is still ambiguous, it would be useful to study the amount of accumulated ABA during *F. graminearum* infection. Moreover, the clear evidences that stomatal closure and photosynthetic parameters are differentially regulated during FHB infection allow us to believe that this physiological response could be exploited to detect FHB in field by using phenomics-based approaches, which were investigated in the third part of our research study.

The narrow identification window of opportunity that allows effective control of FHB makes its early detection a key factor to gain maximum, environmentally sustainable protection of yield. To maximize FHB disease control efficiency, we explored the use of UAV-based RGB and TIR imaging combined with molecular diagnostics to early detect the presence of FHB in *T. turgidum* (cv. Marco Aurelio). The third part of the study revealed that: (i) stomatal closure is the physiological mechanism responsible for temperature increase and photosynthetic efficiency decrease in *T. turgidum* spikes during an FHB infection; (ii) VIs and temperatures extracted from RGB and TIR imaging data can detect these physiological changes; and (iii) different transcriptional regulations exist between drought-stressed and *F. graminearum*-inoculated plants. These findings provide mechanisms for early detection of FHB in *T. turgidum* and shed light into new valuable genomic information to further develop a phenotyping method able to distinguish between drought-stressed and FHB-infected plants in the field. Research in plant stress physiology is benefiting from new types of precision disease management technologies based on phenomics, genomics, and transcriptomics data. In the last decade, plant genomics and phenomics have matured to the point where, applied together, they can drastically reduce bottlenecks in phenotypic and genotypic evaluation of plant traits and when coupled with artificial intelligence and exascale computing, they can accelerate the development of new crop varieties with improved yield potential and enhanced tolerance to biotic and abiotic environmental stresses. Their implementation and application will become necessary to elucidate the architecture of plant physiological mechanisms to develop innovative tools to be applied in a new green revolution required to double the realized average annual yield increment. To date, no studies have been carried out attempting to use UAV-based TIR and RGB imaging data for early detection of FHB in *T. turgidum*. Developing trait measurement methodologies that combine phenomics and genomics to early detect plant diseases can provide a timely warning of their imminent threat. Advanced phenotyping technologies combined with molecular techniques and non-invasive sensors have a great potential to improve the control of diseases in plants, allowing decisions to be made in time for fungicides to be effective, reducing the costs and the negative environmental impacts of their unnecessary applications. More in-field studies are necessary to implement the great potentiality of such technologies, such as to test their efficacy and reproducibility under different environmental and geographic conditions. Despite the big advantages and power of phenomics applied for the early detection of pathogens to reduce the pesticides application, a huge limit resides in the data and image analysis which is quite labour-intensive and time-consuming. For this purpose, software for fast, sensitive and real-time image analysis should be developed to support the need for a timely decision system to optimize the application of pesticides.

Chemical fungicides accumulate in the soil, being part of the animal and human food chain, causing negative or unknown effects on the health. Furthermore, the efficiency of tebuconazole and azoxystrobin in protecting wheat plants from FHB-related pathogens is extremely variable and their constant use is favoring the selection of resistant strains. Chitosan is a natural, biodegradable, non-toxic, and eco-friendly compound with a wide range of positive properties for plants, such as biostimulator of growth and of defense systems. The present study demonstrated that chitosan hydrochloride, a water-soluble form of chitosan, boosted the wheat growth and development, reduced FHB severity, and the accumulation of the fungal biomass as much as the amount of several mycotoxins and FHB-related compounds in the spikes. The application of chitosan on the flag leaves contributed to decrease FHB in the susceptible Italian cultivar Marco Aurelio, but the combination of two strategies (resistant genotype $\times$ eco-friendly compound) was extremely promising, posing the basis for novel biotechnological approaches forward a sustainable agriculture. More research is needed forward a practical application of chitosan to control FHB, such as studies on production costs and its stability in different environments. From the present study, it was clearly obvious that the double use of a resistant cultivar and an efficient natural-derived compound was the most promising strategy to control FHB. For this reason, we believe that further researches should be focused on studying the application of different eco-friendly strategies showing synergistic effects to control plant pathogens. In such ways, the appearance of resistant strains will be contrasted (because the pathogens will not be subjected to only one and continuative control method) and it will be possible to employ a drastically reduced amount of conventional pesticides and/or innovative natural-derived compound with antimicrobial properties. Another interesting and promising strategy to reduce the amount of antimicrobial compounds (conventional or natural-derived) used for agricultural purpose resides in nanotechnology. This innovative field of science is opening great potentiality regarding the use of green nanoparticles as carriers for active molecules. In particular, cellulose and its derived nanoparticles (cellulose nanocrystals) are extremely versatile to vehicle active ingredient in plants. The functionalization of cellulose nanocrystal with chitosan will enhance its antimicrobial activity by optimizing its release, permeation and diffusion on and in the plant on a systemic level, thus reducing the concentration of employed chitosan.

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