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Engineering danger sensing and signaling in plant immunity: use of oligosaccharins to enhance durum wheat resistance to fusariosis

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Un abbraccio alla bambina che ero, timida e insicura.
Un abbraccio alla donna che sono diventata,
testarda e imperfetta ma sempre sincera,
guerriera ma con il cuore sempre troppo scoperto.
Per la donna che sono,
per tutto l'amore che ho dentro,
per le mie vittorie e le mie sconfitte,
per tutte le volte che mi sono rialzata,
per tutti i mostri che ho affrontato nel corso della vita.
Un abbraccio alla bambina che sono ancora e che sarò sempre,
con le favole nel cuore e mille sogni dentro gli occhi.

A me stessa.

ABSTRACT

Fusariosis causes substantial yield losses in wheat crop worldwide and compromises food safety because of the presence of toxins associated to fungal disease. Among the current approaches to crop protection, the use of elicitors, able to activate the natural defense mechanisms of plants, represents a strategy gaining increasing attention. Numerous studies indicate that local application of plant cell wall-derived elicitors, such as oligogalacturonides (OGs) derived from partial degradation of pectin, induces systemic resistance against pathogens in different plant species. The aim of this study was to establish the efficacy of OGs in protecting durum wheat, characterized by an extreme susceptibility to fusariosis caused by *Fusarium graminearum* (*Fg*).

To evaluate the functionality of OGs as elicitors of immunity and their ability to restrict phytopathogen fungal growth in durum wheat, seedlings and spikes of cv. Svevo were inoculated with OGs, *Fusarium graminearum* spores or a co-treatment of both. Chitosan (CHIT) was used as a positive control in the experimental setup. Results demonstrated that OGs are active elicitors of wheat defenses, able to trigger the expression of typical immune marker genes and resistance to *Fg*. Notably, the inhibition of fungal growth in the OG-cotreated durum wheat tissues was accompanied by a strong transcriptional downregulation of important positive regulators of mycotoxin biosynthesis. Furthermore, engineered durum wheat plants with potentially altered endogenous OG levels, i.e. OG-machine lines, were generated, characterized and used to evaluate the possible OG involvement in orchestrating wheat responses to *Fg* infection. Such lines express a chimera protein, composed by a *Phaseolus vulgaris* polygalacturonase-inhibiting protein and a *Fusarium phyllophilum* polygalacturonase, under the control of a pathogen-inducible promoter. The OG-machine lines displayed a higher resistance to *Fg* compared to wild type cv. Svevo and are now under further investigations for the elucidation of molecular mechanisms regulating defense activation upon sensing danger signals in cereals. Data described in my thesis hint that immune system engineering may be exploited to enhance durum wheat resistance to fusariosis, to diminish the usage of chemicals and for a sustainable yield increase.

Keywords

Plant immunity, oligogalacturonides (OGs), *Triticum durum*, Fusarium Head Blight, Wheat – *F. graminearum* interaction, elicitors of immunity, molecular mechanisms danger sensing and signaling, sustainable agriculture.

RIASSUNTO

La fusariosi causa sostanziali perdite di raccolto del grano in tutto il mondo e compromette la sicurezza alimentare a causa della presenza di tossine associate a malattie fungine. Tra gli attuali approcci alla protezione delle colture, l'uso di elicitori, in grado di attivare i naturali meccanismi di difesa delle piante, rappresenta una strategia che sta guadagnando sempre maggiore attenzione. Numerosi studi indicano che l'applicazione locale di elicitori derivati dalla parete cellulare vegetale, come gli oligogalatturonidi (OG) derivati dalla parziale degradazione della pectina, induce resistenza sistemica contro i patogeni in diverse specie vegetali. Lo scopo di questo studio è stato quello di stabilire l'efficacia degli OG nella protezione del grano duro, caratterizzato da un'estrema suscettibilità alla fusariosi causata da *Fusarium graminearum* (*Fg*).

Per valutare la funzionalità degli OG come elicitori di immunità e la loro capacità di limitare la crescita di funghi fitopatogeni nel grano duro, piantine e spighe di cv. Svevo sono state inoculate con OG, spore *di Fg* o un co-trattamento. Il chitosano (CHIT) è stato utilizzato come controllo positivo nel setup sperimentale. I risultati hanno dimostrato che gli OG sono elicitori attivi delle risposte di difesa in frumento poiché inducono l'attivazione di tipici geni marcatori dell'immunità e la resistenza a *Fg*. In particolare, l'inibizione della crescita fungina nei tessuti di grano duro trattati con OG è stata accompagnata da una forte inibizione a livello trascrizionale di importanti regolatori positivi della biosintesi delle micotossine. Inoltre, sono state generate, caratterizzate e utilizzate piante di grano duro ingegnerizzato con livelli di OG endogeni potenzialmente alterati, chiamate linee OG-machine, per valutare il possibile coinvolgimento degli OG nell'orchestrazione delle risposte del grano all'infezione da *Fg*. Tali linee esprimono una proteina chimerica, composta da un inibitore proteico della poligalatturonasi di *Phaseolus vulgaris* e una poligalatturonasi di *Fusarium phyllophilum*, sotto il controllo di un promotore inducibile da patogeni. Le linee OG-machine hanno mostrato una maggiore resistenza a *Fg* rispetto al controllo, cv. Svevo, e sono ora oggetto di ulteriori indagini per la delucidazione dei meccanismi molecolari che regolano l'attivazione della difesa a seguito della percezione di segnali di pericolo nei cereali. I dati descritti nella mia tesi suggeriscono che l'ingegnerizzazione del sistema immunitario può essere sfruttata per migliorare la resistenza del grano duro alla fusariosi, per diminuire l'uso di sostanze chimiche e per un aumento sostenibile della resa.

Parole chiave

Immunità vegetale, oligogalatturonidi (OG), *Triticum durum*, Fusariosi della Spiga, Interazione frumento – *F. graminearum*, elicitori dell'immunità, meccanismi molecolari che regolano il rilevamento e la segnalazione del pericolo, agricoltura sostenibile.

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List of abbreviations

A

15-ADON: 15-Acetyl DeOxyNivalenol.
3-ADON: 3-Acetyl DeOxyNivalenol.
4-ANIV: 4-Acetyl NIValenol.

B

BCAs: BioControl Agents.

C

CDPKs: Calcium-Dependent Protein Kinases.
CHIT: CHITosan.
COS: ChitoOligoSaccharides.
cv: cultivar.
CWDEs: Cell Wall Degrading Enzymes.

D

DA: Degree of Acetylation.
DAMPs: Danger-Associated Molecular Patterns.
DEGs: Differentially Expressed Genes.
DON: DeOxyNivalenol.
DP: Degree of Polymerization.
dpi: days post infection.

E

ET: Ethylene.
ETI: Effector Trigger Immunity.

F

FAO: Food and Agriculture Organization.
Fg: *Fusarium graminearum.*
***Fg* 3827:** *Fusarium graminearum* strain 3827.
***FgΔTri5*:** *F. graminearum Δtri5* or trichothecene- or DON- deficient mutant.
FHB: Fusarium Head Blight.
FpPG: *Fusarium phyllophilum* PolyGalacturonase.

G

GAXs: GlucuronoArabinoXylans.
GFP: Green Fluorescent Protein.
GHs: Glycoside Hydrolases.

H

HG: HomoGalacturonan.
HR: Hypersensitive Response.
hpi: hours post infection.

I

ICs: Infection Cushions.

J

JA: Jasmonic Acid.

L

LRR: Leucine-Rich Repeat.

M

MAMPs: Microbe-Associated Molecular Patterns.
MAPKs: Mitogen-Activated Protein Kinases.
MeJa: Methyl Jasmonate.
min: minute.
MS: Murashige and Skoog.
Mt: Million tonnes.
MW: Molecular Weight.

N

NIV: NIValenol.
NO: Nitric Oxide.

O

OGM: OG-Machine.
OGs: OligoGalacturonides.

P

PAMPs: Pathogen-Associated Molecular Patterns.
PCA: Principal Component Analysis.

PCD: Plant Cell Death.
PCR: Polymerase Chain Reaction.
PDA: Potato Dextrose Agar.
PGs: PolyGalacturonases.
PGIPs: PolyGalacturonase Inhibitor Protein.
PIMS: Pectin Integrity Monitoring System.
PMEs: Pectin-Methylesterases.
POXs: PerOXidases.
PR: Pathogenesis-Related.
PR1: Pathogenesis-Related 1 protein.
PRIs: Plant Resistance Inducers.
PRRs: Pattern Recognition Receptors.
PTI: PAMP-Triggered Immunity.
PvPGIP2: *Phaseolus vulgaris* PolyGalacturonase Inhibiting Protein 2.

Q

qRT-PCR: Quantitative Real-Time PCR.
QTLs: Quantitative Trait Loci.

R

RHs: Runner Hyphae.
RLKs: Receptors-Like Kinases.
RLPs: Receptor-Like Proteins.
ROS: Reactive Oxygen Species.

S

SA: Salicylic Acid.
SAR: Systemic Acquired Resistance.
SE: Standard Error.
spp.: species.
SNA: Synthetic Nutrient Agar.

T

TaPR1.1.: *Triticum aestivum* Pathogenesis-Related 1.1. protein.
TAXIs: *Triticum aestivum* Xylanase Inhibitors.
TLXIs: Thaumatin-Like Xylanase Inhibitors.

V

VIGS: Virus-Induced Gene Silencing.

W

WAKs: Wall-Associated Kinases.
wt*Fg*: *F. graminearum* wild-type.

X

XIPs: Xylanase inhibitor proteins.
XIs: Xylanase inhibitors.

1. INTRODUCTION

1.1. Wheat as a staple crop

The cultivation of wheat (*Triticum* spp.) and human history began since remote time. Indeed, wheat was one of the first cereals to be domesticated in the Fertile Crescent and has been the basic staple food of the major civilizations of Europe, West Asia and North Africa. Nowadays, wheat continues to be the most important food grain source for humans and is the most worldwide cultivated plant, being the third most produced cereal after maize and rice (FAOSTAT, 2020) (Figure 1).

FAO estimated that the global wheat production in 2020 reached about 760 million tonnes (Mt). The five major wheat producing countries in 2020 were China (134 Mt), India (107 Mt), Russia (86 Mt), United States of America (50 Mt), and Canada (35 Mt) (FAOSTAT, 2020).

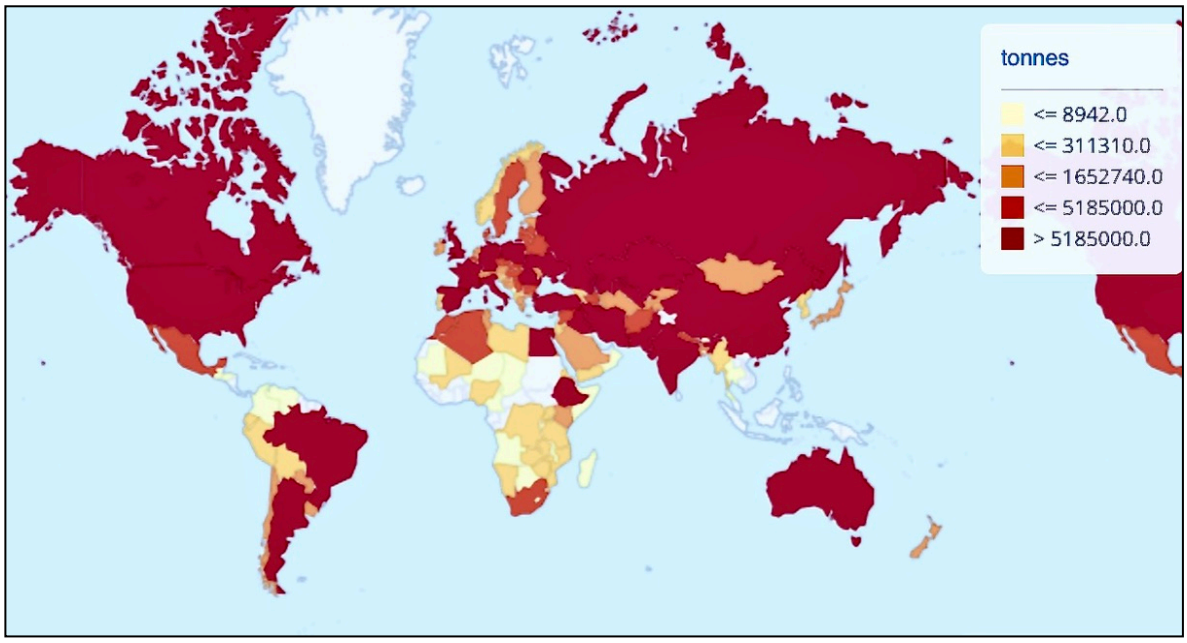


Figure 1. Production of wheat in 2020 (http://www.fao.org/faostat/en/#data/QC/visualize).

Wheat is a major diet component in many countries being primary source of energy, protein and dietary fiber. Wheat grains are composed by around 70% carbohydrates, 13% water, 10-13% proteins, vitamins and minerals. Even if wheat proteins have a low quality for human nutrition, due to deficiency of some essential amino acids, they confer to the gluten, made of wheat flour and water, exceptional viscoelastic proprieties, essential for

processing wheat in a broad range of products, from bread to pasta and many others satisfying foods (Shewry, 2009). This is one of the essential key factors for wheat success worldwide, together with its adaptability to a wide range of pedo-climatic conditions, the use of mechanical supports from production to transformation and the possibility to easily harvest and store grains.

Wheat is also an important source of animal feed, particularly in years where harvests are adversely affected by rain and significant quantities of the grain are made unsuitable for food usage. Such low-grade grain is often used by the industry to make adhesives, paper additives and several other products (Sarka et al., 2011). In addition, in the last few years there has been an increase in the use of wheat for biofuel and biodiesel production (Taghizadeh-Alisaraei et al., 2022).

Nowadays, the wheat cultivars that are mostly cultivated belong to two species, *Triticum durum* (durum wheat) and *Triticum aestivum* (bread wheat) with the latter one grown on over 95% of the wheat growing area (Shewry, 2009). Moreover, durum wheat is mainly involved in pasta making whereas bread wheat is mainly used in bread making industry.

1.1.2. Origin and phylogeny of wheat

Wheat (*Triticum* spp.) belongs to the Triticeae tribe of the Poaceae family of grasses, together with barley (*Hordeum* spp.) and rye (*Secale* spp.), from which it diverged 7 and 11 million years ago, respectively (Huang et al., 2002).

The genus *Triticum* originated in the Middle Eastern, in the flatlands between the Tigris and the Euphrates rivers, where archaeological excavations revealed that wheat cultivation began around 10000 years ago. In Western Europe, wheat was probably introduced during the Aryan population migrations. Afterwards, wheat expanded across the Mediterranean region, and then into Germany and Britain, after the occupation of Roman legions.

The domestication process favored the selection of genetic features that resulted in greater yield (e.g., bigger seeds and spikes), free-threshing state (seeds released from the glumes during threshing), and robust rachis (no disarticulation of dried inflorescence at maturity) (Kilian et al., 2007).

Cultivated wheat is an interesting example of natural allopolyploidy in which spontaneous interspecific hybridization between wild species belonging to *Triticum* and

Aegilops genera, followed by spontaneous doubling of chromosomes, generate the fertile allopolyploid *Triticum* species.

Schultz (1913) divided all the cultivated wheats belong to the genus *Triticum* into three major taxonomic groups (einkorn, emmer, and dinkel). This classification was supported by Sakamura (1918), who discovered that Schultz's three wheat groups also differed in their chromosome number; the einkorns were diploids (2n = 2x = 14), the emmers were tetraploids (2n = 4x = 28), and the dinkels were hexaploids (2n = 6x = 42), all with the genomic basic chromosome number x = 7.

Wheat classification has been and continues to be the topic of various debates and revisions; such difficulties are primarily related to the large number of species, both wild and cultivated, and to the high capacity of inter-specific crossing. In the past, several studies were carried out using different approaches to identify the wild progenitors of cultivated species and to establish a classification (Sax, 1921; Kihara, 1924).

Based on further cytogenetic analysis, Kihara (1924) designated the genome formulae for the cultivated einkorn (*Triticum monococcum* L., 2n = 2x = 14), emmer (*Triticum turgidum* L. 2n = 4x = 28), and dinkel (*Triticum aestivum*, 2n = 6x = 42) as AA, AABB, and AABBDD, respectively.

The diploid einkorn wheat, T. *monococcum* var. *monococcum* (2n = 2x = 14, A^mA^m), was domesticated directly from its wild form, T. *monococcum* var. *aegilopoides* (2n = 2x = 14, A^mA^m) (Heun et al., 1997). Similarly, the cultivated emmer wheat, *Triticum dicoccum* (2n = 2x = 28, BB A^uA^u), was domesticated from the wild emmer, *Triticum dicoccoides* (2n = 2x = 28, BB A^uA^u), which is an allopolyploid, derived from the amphiploidy between *Triticum urartu* (2n = 2x = 14, A^uA^u) and the B genome ancestor, *Aegilops speltoides* (2n = 2x = 14, SS) (Johnson and Dhaliwal, 1976; Dvorak and Akhunov, 2005) (Figure 2).

T. urartu has played a significant role in the wheat evolution by contributing the A^uA^u genome to all tetraploid and hexaploid wheats (Dvorak et al., 1993). There has been much controversy regarding the origin of the B genome of polyploid wheats (Riley et al., 1958). Recent molecular evidence, however, is convincing that the B genome of polyploid wheats were donated by *Ae. speltoides* (Petersen et al., 2006). Furthermore, the cytoplasmic genome heterogeneity within *Ae. speltoides* indicated that it may be the cytoplasmic donor of all polyploid wheats (Gill and Friebe, 2001).

For several decades, the evolution of *T. aestivum* (2n = 6x = 42, BBAADD) has been the topic of several studies and intense discussions. As illustrated in Figure 2, hexaploid

wheat was generated from a crossing between the tetraploid wheat species *T. dicoccum* (2n = 4x = 28, BBAA) and the diploid wheat species *Aegilops tauchii* var. *strangulata* (2n = 2x = 14, DD) (Dvorak et al., 1998).

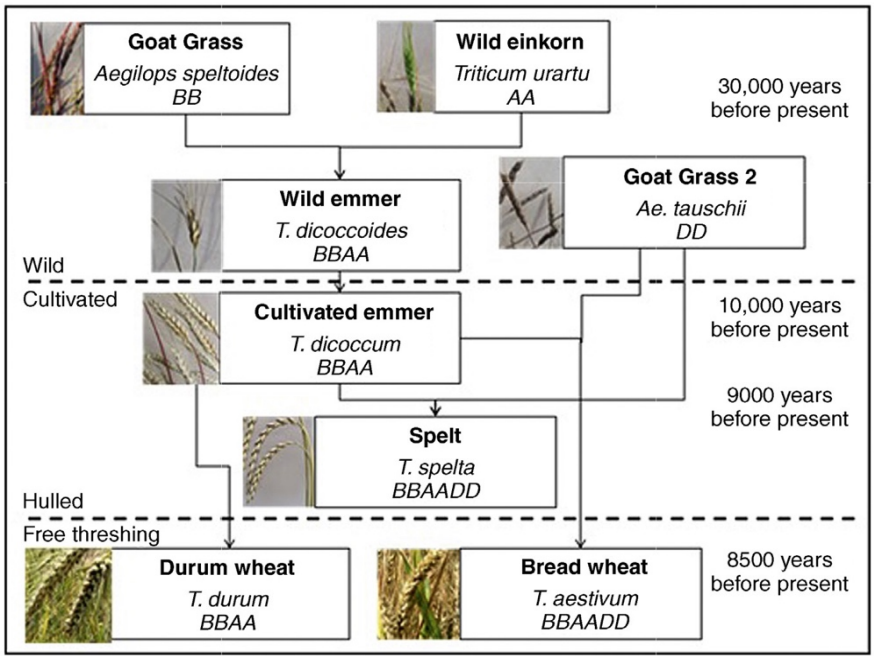


Figure 2. Evolution and genome relationship between wheat and its ancestors (Singh and Upadhyaya, 2015)

Chromosome pairing in polyploid *Triticum* species occurs between homologous chromosomes and not between homoeologous. This is due to the suppressor *Ph1* (Vega and Feldman, 1998) and *Ph2* (Dong et al., 2002) genes. Therefore, in plants lacking these genes, particularly the *Ph1* gene, it was observed the homoeologous gene pairing during meiosis, resulting in partial sterility of plants, indicating the crucial role of the *Ph1* gene for chromosome pairing and for the evolution of polyploid wheats and their domestication (Martinez-Perez et al., 2001).

In modern agriculture the cultivated species of wheat are bread wheat or common wheat (*T. aestivum* L.), durum wheat (*T. durum* Desf.) and spelt wheat (*T. spelta* L.).

1.1.3. Generalities and developmental stages systems of wheat

Wheat is a monocot and may be planted anywhere from the Arctic Circle to higher altitudes near the equator, although it optimally grows between the latitudes of 30° and 60°N and 27° and 40°S (Nuttonson, 1955). The optimum temperature of growing is around

25°C, with minimum and maximum growth temperatures, respectively, of 3° and to 32°C (Briggle, 1980).

Wheat is usually classified as winter or spring growth habit based on flowering responses to cold temperatures. Winter wheat development is promoted by exposing seedlings to temperature range from 3° to 8° C; it is usually planted in the autumn to germinate and develop into young plants that remain in the vegetative phase during the winter and resume growth in early spring. Instead, spring wheat does not require cold conditions for normal development and can be planted in spring. The growth cycle of wheat can be divided into different phenological phases: germination, seedling establishment and leaf production, tillering and head differentiation, stem and head growth, head emergence and flowering, grain filling, and maturity (Figure 3).

Growth Stage	Description of stage
	Seedling growth
GS10	First leaf through coleoptile
GS11	First leaf unfolded (ligule visible)
GS13	3 leaves unfolded
GS15	5 leaves unfolded
GS19	9 or more leaves unfolded
	Tillering
GS20	Main shoot only
GS21	Main shoot and 1 tiller
GS23	Main shoot and 3 tillers
GS25	Main shoot and 5 tillers
GS29	Main shoot and 9 or more tillers
	Stem elongation
GS30	Ear at 1 cm (pseudostem erect)
GS31	First node detectable
GS32	Second node detectable
GS33	Third node detectable
GS37	Flag leaf just visible
GS39	Flag leaf blade all visible
	Booting
GS41	Flag leaf sheath extending
GS43	Flag leaf sheath just visibly swollen
GS45	Flag leaf sheath swollen
GS47	Flag leaf sheath opening

Growth Stage	Description of stage
	Ear emergence
GS51	First spikelet of ear just visible above flag leaf ligule
GS55	Half of ear emerged above flag leaf ligule
GS59	Ear completely emerged above flag leaf ligule
	Flowering
GS61	Start of flowering
GS65	Flowering half-way
GS69	Flowering complete
	Milk development
GS71	Grain watery ripe
GS73	Early milk
GS75	Medium milk
GS77	Late milk
	Dough development
GS83	Early dough
GS85	Soft dough
GS87	Hard dough (thumbnail impression held)
	Ripening
GS91	Grain hard (difficult to divide)
GS92	Grain hard (not dented by thumbnail)
GS93	Grain loosening in daytime

Figure 3. Illustration of the Zadoks scale system to describe wheat growth stages. Adapted from Tottman (1987).

Among the numerous developmental classifications, the Freak, Zadoks, and Haun scales are usually employed (Zadoks et al., 1974; Haun, 1973; Large, 1954). The Zadoks method is the most frequently adopted system for describing wheat development because its stages are easier to recognize in the field and it is more detailed than other methods, allowing for accurate staging. This system is based on a two-digit code: the first digit indicates the principal stage of development, which begins with germination (stage 0) and ends with kernel ripening (stage 9), and the second digit, between 0 and 9, subdivides each principal growth stage (Figure 3).

Germination begins when water is available to the caryopsis. It absorbs 35-40% of its weight and germinates if temperature and oxygen levels are favorable. First, the central embryonic root begins to emerge from the seed, followed by the coleoptile and other primary roots. After emergence, the first leaf protrudes from the coleoptile tip and grows, followed by development of the remaining leaves (Baldoni and Giardini, 2000).

Tillering is a branching phenomenon that begins at the 4-5 leaf stage and consists of the formation of secondary shoots known as tillers. Lower leaves on the main shoot and the coleoptile are the points of attachment from which tillers are formed. Many factors influence the quantity of tillers, including genotype, cultivation conditions, sowing date, and temperatures. However, only two to four tillers are normally able to produce fertile spikes (Anderson et al., 1995).

Wheat inflorescence, the spike (head or ear), begins to differentiate before stem elongation, when internodes increase in length due to meristematic tissue proliferation at the base of each node. When all lower internodes are developed, the already fully formed spike is pulled out through the lamina of the last leaf, known as flag leaf, resulting in an enlargement that characterizes the boot stage. Booting is defined as the stage, when the spike can be felt within the whorl of leaf sheaths, but this is not visible (McMaster, 2009).

The flowering stage, or heading, begins occurs a few days after ear emergence, starting from the central spikelet and moving upward and downward. The rachis is the main axis of the spike and it includes two rows of spikelets in alternating order and a single terminal one (Figure 4A). Spikelets are grouped along a short axis, the rachilla, which connects them to the rachis. The spikelet can present from 3 to 8 florets, which are located between two external glumes at the base of each spikelet. Each floret is enclosed by two leaf-like organs called glumes; the outer known as lemma and the inner as palea (Figure 4B).

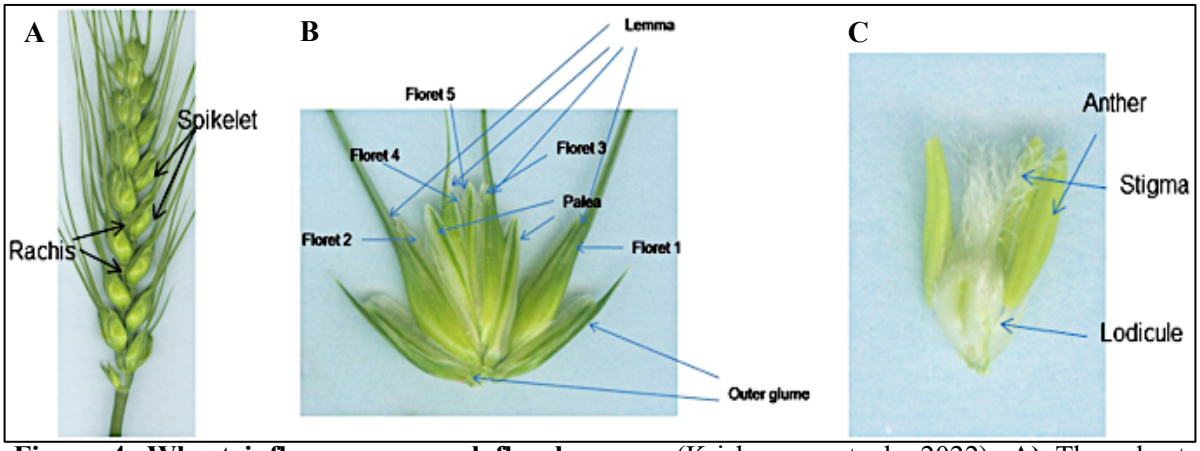


Figure 4. Wheat inflorescences and floral organs (Krishnappa et al., 2022). **A)** The wheat inflorescence (spike, ear, or head) is composed of spikelets attached at the nodes of a zigzag rachis; **B)** A spikelet removed from the rachis. The spikelet consists of multiple florets attached at the rachilla. Glumes enclose the spikelet. Each floret is enclosed by two leaf-like organs, the lemma and the palea; **C)** An image of an opened floret with pistil and anthers. Two membranous pads called lodicules are located at floret base.

The floret presents three stamens with bilobed anthers, and the ovary, with a bifid style and a feathery stigma. Two membranous pads called lodicules are located at floret base. Lodicules swell at anthesis, pulling back the glumes back and allowing the stamens and stigmas to protrude (Figure 4C). The yellow anthers and the swollen ovary with an open feathery stigma are visible in the closed flower during self-pollinating fertilization (Krishnappa et al., 2022).

The kernel development, or caryopsis filling, consists of three phases. First, in the milk phase, the endosperm cells accumulate secondary starch granules, reaching the maximum volume and a humidity of about 70%. Afterwards, in the dough phase, the kernel starts a progressive accumulation of starch and protein, becoming waxy and yellow. In the last phase, the ripening, water content decreases, reaching 30-45% (Sabelli and Larkins, 2009). Caryopsis is fully matured when the starch granules completely fill the endosperm cells, its moisture content is 30%, and the plant is almost entirely yellowed. Furthermore, maturity is characterized by the fact that endosperm stops accumulating reserve substances. The following gradual and further loss of water leads the grain moisture content to 13%, indicating the complete maturation.

The wheat caryopsis is a dry indehiscent fruit characterized by two sides, with respect to the spikelet axis: the upper or dorsal side is rounded, while the lower or ventral one presents a longitudinal groove (Meyer, 2022). The pericarp strictly adheres to the seed,

which is composed by two different parts: the embryo and the endosperm, the latter supporting the first growth of the embryo during germination (Figure 5A).

The embryo, or germ, is situated at the point of attachment of the spikelet axis and is composed by the plumule (the coleoptile), the radicle (primary root), and the scutellum, an epithelial formation whose function is to transfer the nutrients from the endosperm to the embryo (Figure 5B-C). The endosperm consists of cells rich in starch surrounded by the aleurone layer, made of metabolically active cells, the testa, or seed coat, and the pericarp, or fruit coat (Figure 5C).

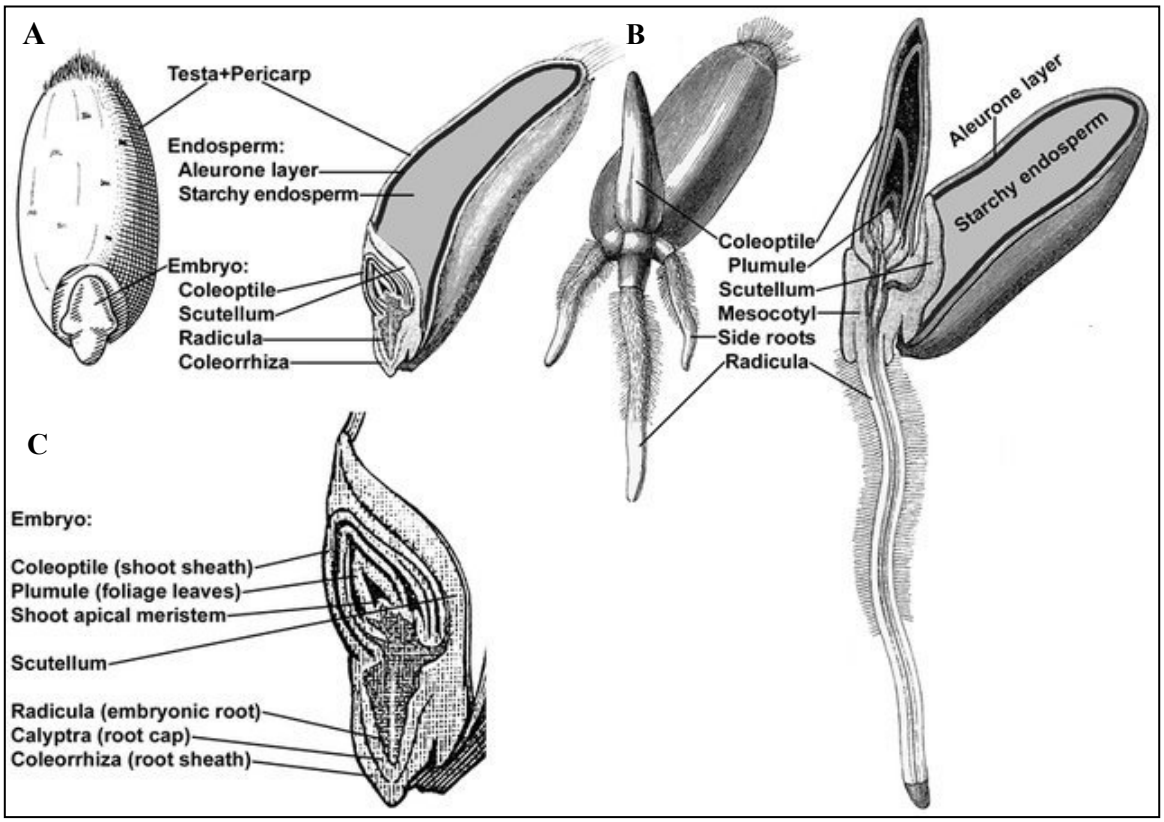


Figure 5. Structure and germination of a wheat grain. A) posterior view and longitudinal section of seed; **B)** germinating seed; **C)** Embryo longitudinal view. (http://www.seedbiology.de/structure.asp#caryopsis)

1.1.4. Disease impact on wheat yield production and quality

Plant diseases are globally causing substantial losses in staple crop production, undermining the urgent goal of a 70% increase needed to meet the food demand, a task made more challenging by the climate changes. Main consequences concern the reduction of food amount and quality. Crop diseases also compromise food safety due to the presence of pesticides and toxins (Bigini et al., 2021).

Wheat is regularly subject to numerous abiotic and biotic stresses which can strongly influence the plant growth and affect the yield and the quality traits of the wheat grain's derived products. The protein and starch content of the durum and bread wheat grain is the prime measure of wheat quality. Drought and heat stress during grain filling was recognized to alter quality characteristics of bread wheat doughs, as consequence of modified accumulation of gluten proteins that are responsible for their technological properties (Phakela et al., 2021). Moreover, the exposure to low temperature has a great impact on productivity of wheat plants, mostly because it alters their growth and development, metabolism and physiology.

Another serious and affecting damage in term of yield and quality losses that occurs on cereals is represented by biotic stresses, which can affect different plant organs (e.g., roots, leaves, flowers, grains), and can be derived from bacterial, viral, fungal, or even parasitic infestations.

On average, at least 20% of the global wheat production is lost due to diseases and pests every year (Savary et al., 2019). In Europe, the most harmful fungal pathogens for wheat crops are: *Fusarium graminearum* (Fusarium Head Blight - FHB), *Zymoseptoria tritici* (*Septoria tritici* Blotch), *Puccinia recondita* (Brown Rust), *Puccinia striiformis* (Yellow Rust) and *Blumeria graminis* (Powdery mildew) (Mehta, 2014). These pathogens can be obligate parasites which infect only living plant tissues, such as rusts and powdery mildew, or facultative parasites which can live in dead plant tissues, such as *Septoria tritici* Blotch and scab. All the diseases caused by fungal pathogens which can infect the plant throughout its development are mentioned in Figure 6.

Fusarium head blight (FHB), caused by *Fusarium* spp., is the most destructive disease affecting small grained cereals and leads to significant reduction of the grain yield and quality.

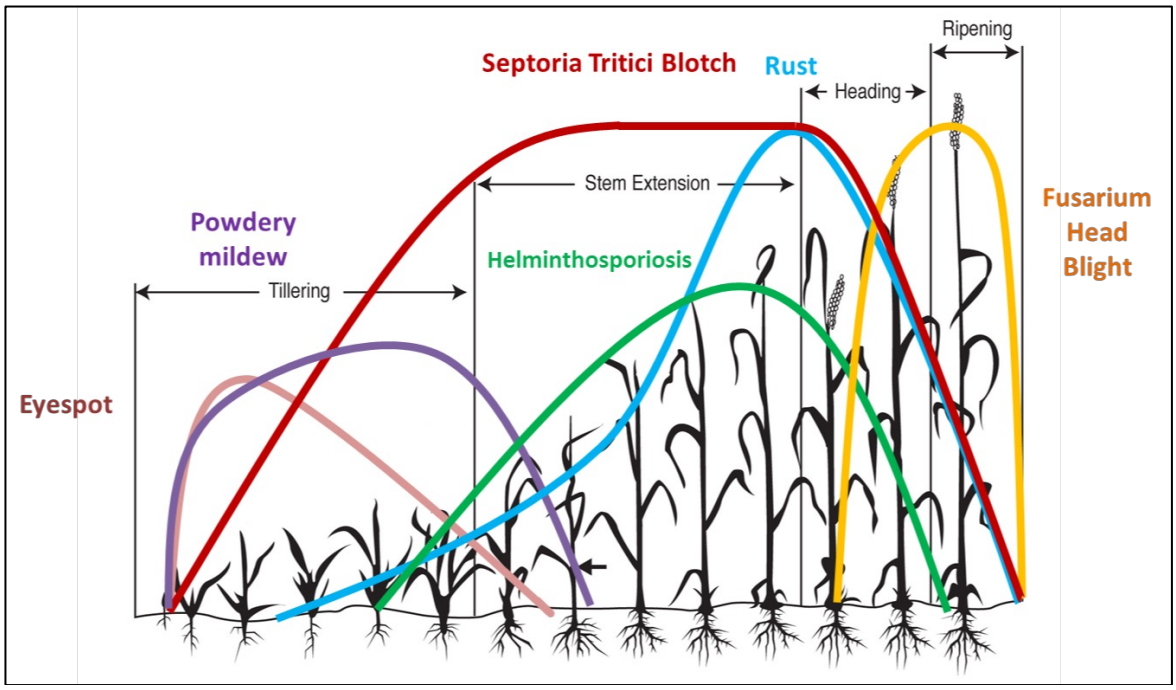


Figure 6. The phenological and growth stages in which the main wheat phytopathologies occur. (Source: BASF France Agro https://www.agro.basf.fr/)

One option to avoid yield losses caused by such diseases is the usage of fungicides. Nevertheless, repeated use of fungicides induces a considerable selection pressure on respective pathogens as well as fungicide resistance or tolerance as detected already in *B. graminis*, *Septoria* spp. or *Fusarium* spp. (Becher and Wirsel, 2012; Cools and Fraaije, 2013). Hence, cultivars carrying resistances are the most environment-friendly and cost-effective way of preventing yield losses in wheat. In particular, resistances against leaf rust, stripe rust, stem rust and powdery mildew infections result in complete resistance but are at risk to be overcome by virulent isolates due to the extensive spore production of these pathogens.

Besides fungal pathogens, viruses are also important pathogens for wheat, as *Triticum* species are natural hosts for more than 40 different viruses, among which some develop important diseases. These are viruses belonging to the genus Bymovirus (family Potyviridae) or the genus Furovirus (family Virgaviridae) transmitted by the root-infecting plasmodiophorid *Polymyxa graminis* as well as insect-transmitted viruses (family Luteoviridae and Geminiviridae) (Langridge, 2017). As virus infections cannot be directly contrasted by using chemicals, the only way of avoiding yield losses in this case is taking chemical measures to restrict their vectors, which in the case of soil-borne vectors, are inefficient, and growing of resistant cultivars is the only way of ensuring wheat cultivation in the growing area of infested fields.

1.2. Fusarium head blight

FHB or scab is one of the most important diseases in bread (*Triticum aestivum*) and durum (*Triticum durum*) wheat. Nineteen different *Fusarium* species cause wheat FHB, although *F. graminearum* (teleomorph *Gibberella zeae*) is the main causal agent of disease (Buerstmayr et al., 2012). *Fusarium culmorum*, *F. avenaceum*, *F. moniliforme*, *F. oxysporum* and *F. poae* are the other related species that play a minor role in FHB development.

FHB occurs in most parts of the world especially in the wheat-growing areas of the United States, Canada, Australia and Europe and primarily in regions with warm and humid conditions during flowering (Wegulo et al., 2015). The infection of the ear can cause high yield losses ranging from 50% to 70% during epidemics by reducing the number of grains per spike and thousand grain weight. Also, germination is affected, and the grain quality is reduced. *Fusarium* infection may also change the content of gliadins and glutenins (Spanic et al., 2019). Furthermore, *Fusarium* species can produce various mycotoxins that are toxic to humans and animals, respectively. Indeed, the contamination of grain cereals with mycotoxins, especially with deoxynivalenol, is a major problem in the wheat industry causing the alteration of the milling, baking and pasta making grain properties (Schmidt et al., 2016). Most mycotoxins are chemically and thermally stable during food processing, including cooking, boiling, baking, frying, roasting, pasteurization, and decontamination by physical and chemical methods. These compounds can also come to the human plate via animal products such as meat, eggs, milk as the result of the animal eating contaminated feed. Efforts were made in several countries to find an economically acceptable way of destruction of mycotoxins into non-toxic products. However, most methods used at present lead to a partial removal of mycotoxin levels and have major disadvantages, starting with limited efficacy to losses of important nutrients and generally with high costs (Kaushik, 2015).

FHB disease symptoms are confined to the head, grains, and sometimes the peduncle. Typically, the first noticeable symptom is bleaching of some or all the spikelets while healthy heads are still green (Figure 7A-B). As the fungus moves into the rachis, spikelets located above or below the initial infection point may also become bleached. When all conditions are highly favorable for FHB development, a growth of salmon pink to red colored mycelia can be seen on the base of the spikelet and spreads through the entire head. Wet and warm weather during crop growing and maturation may favor FHB

progression. Ultimately the infected grains become discolored, shrunken, and chalky white in appearance with black perithecia giving the name “scab” (Figure 7C).

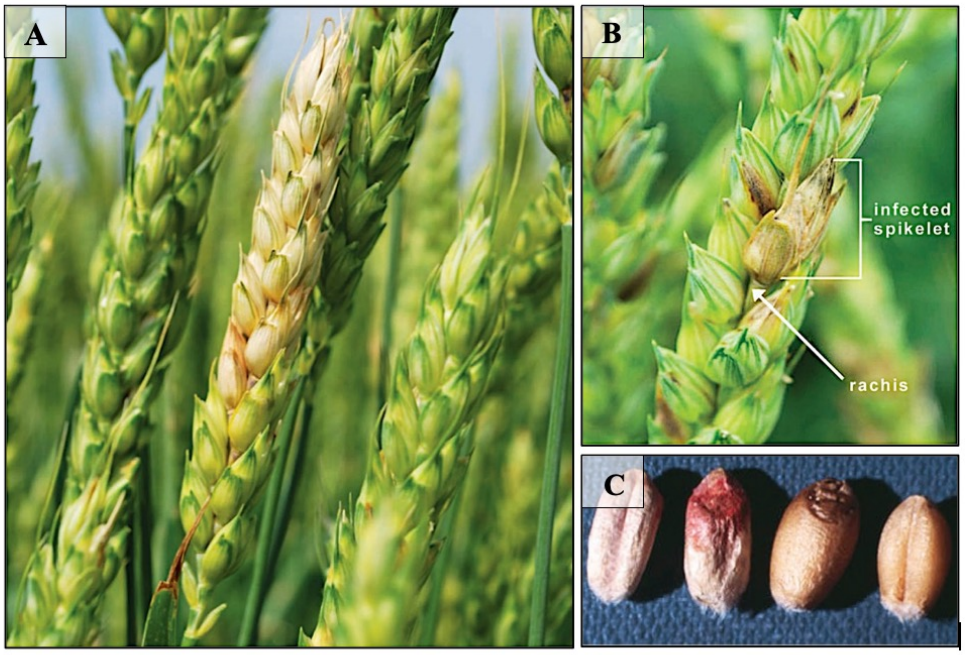


Figure 7. Typical symptoms of FHB on wheat. A) An individual spike infected by *Fusarium graminearum*; **B)** A symptomatic bleached wheat spikelet; **C)** Bleached and shriveled tombstone kernels (left) compared to healthy wheat kernels (right). (https://ag.purdue.edu/ department/btny/)

1.2.1. *Fusarium graminearum* species

Filamentous fungi within the *Fusarium graminearum* species complex (*Fg* complex) are the most important etiological agents of FHB on wheat and other cereal grains worldwide. Members of the *Fg* complex were considered a single cosmopolitan species, due to the failure of morphological species recognition to accurately assess species limits for this group. O’Donnell et al. (2000) first identified seven phylogenetic lineages within the *Fg* complex, employing genealogical concordance phylogenetic species recognition (GCPSR). Most recently, employing a high-throughput multilocus genotyping assay of portions of 13 housing keeping genes, combined with GCPSR and molecular marker technologies, 13 phylogenetically distinct, cryptic species have been identified within the *Fg* complex (Yli-Mattila, 2009). Nowadays, it includes 16 species: *F. graminearum, F. cortaderiae, F. meridionale, F. boothii, F. asiaticum, F. austroamericanum, F. pseudograminearum, F. gerlachii, F. vorosii, F. aethiopicum, F. nepalens, F. louisianense, F. ussurianum, F. brasilicum, F. mesoamericanum* and *F. acaciae- mearnsii* (Sarver et al., 2011).

In the surveys conducted worldwide to date, *F. graminearum* complex (*F. graminearum* spp.) is cosmopolitan in distribution and has been found in Asia, Africa, America, Europe, and Oceania, while another species, *F. asiaticum*, is widespread in Asia (Desjardins and Proctor, 2011; Qu et al., 2008).

1.2.2. Life cycle and infection process of *Fusarium graminearum*

Fusarium spp. grow and overwinter in the soil on plant debris of maize, wheat, and other cereals (Figure 8). The saprophytic mycelium of *F. graminearum* on stubble residues such as chlamydospores perithecia and macroconidia build the inoculum for the infection in the next growing season (Buerstmayr et al., 2012).

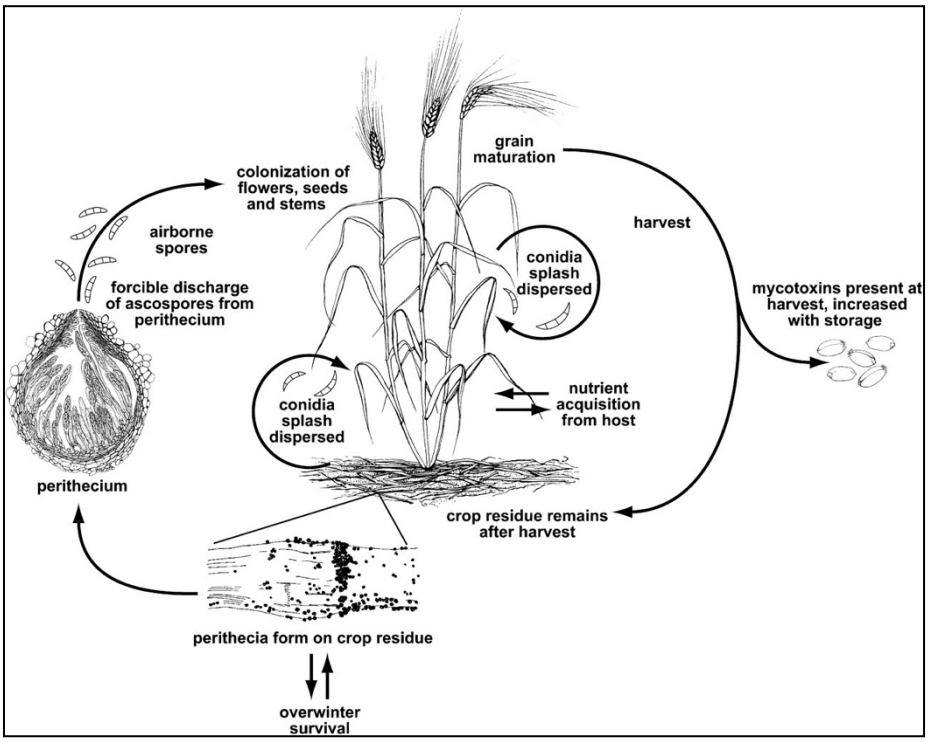


Figure 8. The life cycle of *F. graminearum* (sexual phase, *G. zeae*), causal agent of Fusarium head blight on wheat (Trail, 2009). Details of specific aspects of the cycle are discussed in the text.

During the flowering stage when climatic conditions, including warm (about 24 °C) and humidity, are favorable for the fungus growth, sexual ascospores or asexual conidiospores (conidia) are spread by wind, insects, rain, or irrigation, and land on flowering ears where they germinate. Extruded anthers during wheat anthesis (flowering) are thought to be the site of primary infection. If the anthers are infected just after their emergence, the fungus will colonize and kill the florets and kernels will not develop.

The fungal spores germinate on the surface of flowers and the hyphae of the fungus enter either passively via natural openings such as the stomata, the base of the lemma and palea or actively via the cell wall. *F. graminearum* forms different specialized epiphytical hyphal structures on the plant surface. Elongate non-invasive runner hyphae (RH) form an evenly distributed network covering the plant tissue (Figure 9A). Invasive cells can be divided into three morphological classes (Boenisch and Schäfer, 2011): foot structures, lobate appressoria, and infection cushions (IC).

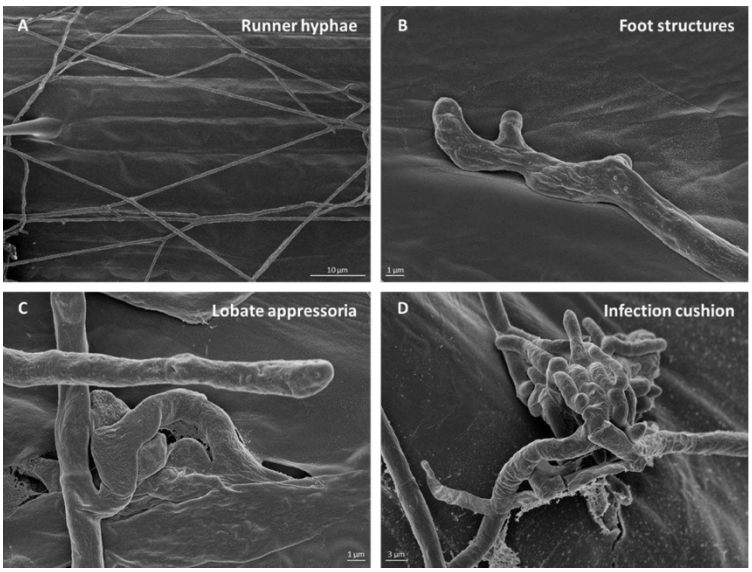


Figure 9. Scanning electron microscopy pictures of infection structures of *F. graminearum* on wheat palea (Boenisch and Schäfer, 2011). **A)** Mainly unbranched runner hyphae (RH) cover the plant surface. **B)** Small side branches form foot structures which are able to penetrate the plant cuticle. **C)** Lobate appressoria formed by hyphae aggregation. **D)** Infection cushions (ICs) constitute the most complex infection structure of *F. graminearum*.

Foot structures are formed when RH release short side branches which form small swellings directly on the plant surface and penetrate the cuticle (Figure 9B). They are the first infection structures formed by *F. graminearum* and can be observed during the initial colonization stage (infection stage I). Lobate appressoria are more complex multicellular infection structures formed by aggregation of hyphae (Figure 9C). The most complex multicellular infectious organs are ICs which are thought to be the fungus most important tools for host invasion. They are formed by highly branched and agglomerated hyphae and cause multiple penetration events underneath them (Figure 9D). Lobate appressoria and ICs belong to the class of compound appressoria and are formed during the main infection stage (infection stage II) (Boenisch and Schäfer, 2011; Bormann et al., 2014). Growing hyphae proceed rapidly growing inside the host, causing necrosis of host cells and using

the dead plant material as nutrition. At this stage, the fungus expresses genes involved in mycotoxin production, including DON, which causes shriveled, undersized grains known as “tombstones” (Trail, 2009). In late infection stages, also after harvest, the fungus develops sexual reproductive organs (perithecia) in which ascospores are produced. It overwinters as mycelia or spores in crop residues, seeds, or in the soil. During springtime, ascospores are produced in newly formed perithecia on crop residues which constitute the major portion of the primary inoculum during the infection period (Wegulo, 2012).

Although the way *F. graminearum* establishes infection in a single spikelet is well documented, how this pathogen spreads from spikelet-to-spikelet and ultimately colonizes the entire wheat head is the main focus of different studies in the last years. There is controversy over whether this fungal pathogen invades wheat floral tissue using a necrotrophic or a non-necrotrophic mode of nutrition (Leonard and Bushnell, 2003). Multiple investigations focused on different aspects of fungal pathogenicity in specific tissues of the ear, mainly a single floret (Ribichich et al., 2000; Wanjiru et al., 2002) or the spread of mycelium from the peduncle into the stem and culm tissue below the ear (Guenther and Trai, 2005). Brown et al. (2010) described a detailed microscopic investigation of the entire colonization of a susceptible wheat ear by the wild-type *F. graminearum* strain PH-1, tracking the infection pathways and hyphal networks generated by the pathogen from the initially inoculated floret into the rachis nodes and internodes and beyond into the neighboring spikelets. The pathways of spikelet-to-spikelet colonization in wheat has been visualized in depth using ultrastructural cellular morphology of wheat cells (Brown et al., 2010). Upon inoculation, invasive mycelia of the FHB spread throughout the spikelet, down into the rachial node and ultimately up and down the rachis until FHB symptoms are clear.

Although the pathogenesis of *F. graminearum* is widely investigated by molecular genetics approaches, detailed studies about its cellular and developmental processes at the initial stages of infection are very limited. Qiu et al. (2019) applied live-cell imaging approach to characterize the spatial and temporal development of growing hyphae and plant responses during *F. graminearum* and wheat coleoptile interactions. At early stages of the infection (5–8 hours post inoculation, hpi), conidia of *F. graminearum* germinate into epiphytic runner hyphae on the extracellular surface or intercellular space of the wheat coleoptiles. Usually, the RH do not infect host tissues immediately, but give rise to hyphae that grow and branch on host surfaces. Interestingly, after hyphal branching, multiple appressoria-like structures of the fungus are formed at the hyphal tips (Figure 10A).

Moreover, some infectious hyphae could be observed in the intercellular space between the wheat coleoptile cells. By 16 hpi, infectious hyphae arising from the surface hyphae begin to penetrate the host epidermal cell walls (Figure 10B).

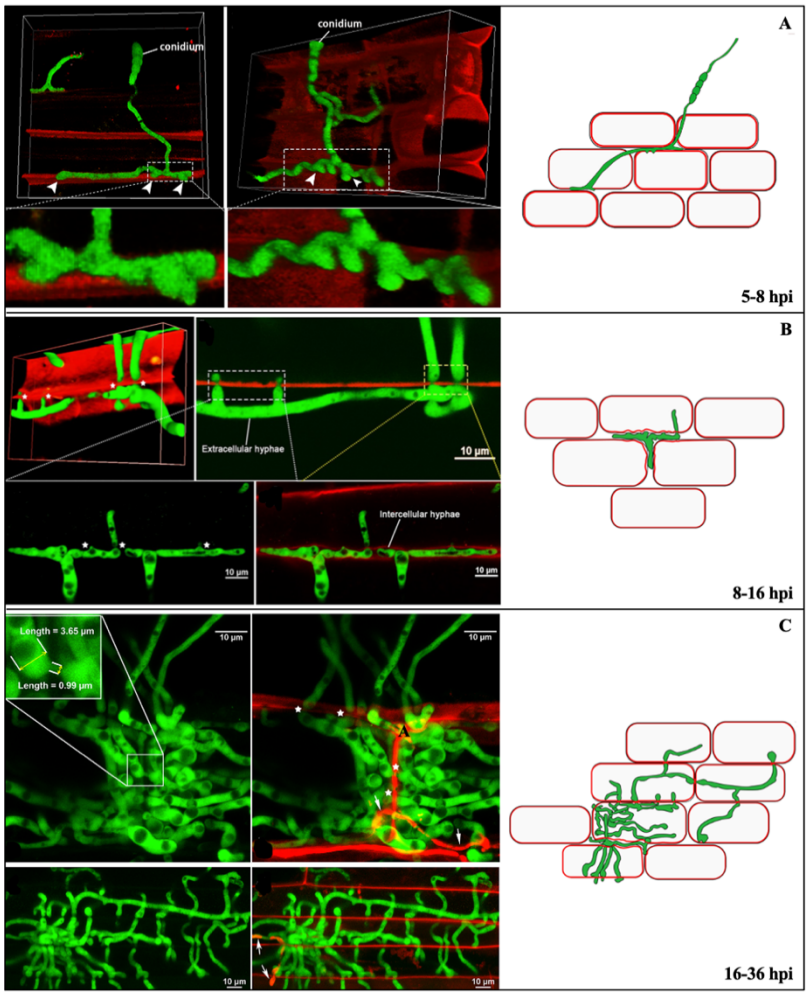


Figure 10. Laser confocal microscopy images of wheat coleoptiles after *F. graminearum* infection (Qiu et al., 2019). Tissue cells of wheat coleoptiles are red colored. GFP-tagged *F. graminearum* PH-1 strain is green colored. **A)** At the early stages (5–8 hpi) of infection a conidium of *F. graminearum* germinated into epiphytic RH on the extracellular surface of wheat coleoptiles. After hyphal branching, multiple appressoria-like structures are formed at the hyphal tip. The hypha continues to grow in the intercellular spaces. **B)** Extracellular hyphae begin to penetrate the cell wall of wheat coleoptiles at 10 hpi. Bulbous and vacuolar hyphae are formed in the intercellular space of wheat coleoptiles at 16 hpi. **C)** Cell-to-cell invasion using constricted invasive hypha pegs. Invasive hyphae in the first-invaded cell continue to spread to the adjacent cells. The pegs expand and grow as bulbous invasive hyphae in the newly invaded cells.

Interestingly, at the penetration site, the penetration peg was markedly constricted as it pierces through the wheat coleoptile cell walls. Upon reaching the epidermal cell lumen, the penetration peg expands to form a normal-sized filamentous hypha. Noteworthy, the infectious hyphae in the intercellular space of the wheat coleoptiles display significant

morphological changes when compared with those arising from the epiphytic surface. They are thicker and more bulbous, and filled up the intercellular space of the wheat coleoptile cells and subsequently break the wheat coleoptiles cell walls the to get access into the cells. After penetrating the wheat coleoptile cells at 16 hpi, invasive hyphae were observed to grow in the first invaded cell and often to fill it up. Like in the initial penetration, the invasive hyphae in the first-invaded cell continue to spread to adjacent cells by means of highly constricted invasive hyphal pegs, which, then, expand and grow as bulbous invasive hyphae (Figure 10C).

1.2.3. *Fusarium graminearum* - wheat interaction

As described above, *Fg* can enter cereal florets either passively through natural openings, such as stomata (Bushnell, 2001), or actively by direct penetration. An active route for entry of the fungus is the penetration of the epidermal cuticle and cell wall with short infection hyphae (Mary Wanjiru et al., 2002). As a barrier surrounding the cell, the cell wall represents the first obstacle for pathogen entry. *Fusarium graminearum* secretes a broad spectrum of proteins, including cell wall-degrading enzymes (CWDEs), proteases, lipases, oxidoreductases, and effectors into extracellular milieu to benefit their survival. The CWDEs play a significant role in the life cycle of a fungal pathogen as they are used to facilitate the assimilation of nutrients and to overcome the plant cell wall (Kubicek et al., 2014). In fact, the degradation of the plant cell wall components, such as cellulose, hemicelluloses and pectins, is required for fungal pathogens to penetrate and proliferate within host cells. The complexity of the plant cell wall is reflected by the high number of CWDEs secreted by pathogens, generally referred to as glycoside hydrolases (GHs), enzymes able to cleave hydrolytically glycosidic bonds in polysaccharides (Henrissat, 1991; Benedetti et al., 2019).

Studies have shown that *Fg* produces numerous enzymes or their corresponding transcripts to facilitate the degradation of celluloses and hemicelluloses. The hydrolysis of cellulose generally involves the synergic action of two different enzymes, one working in an endo-acting way (endoglucanase) while the other in an exo-acting way (exoglucanase). The activity of these enzymes is followed by the activity of a β-glucosidase that hydrolyze the cellodextrin oligomers to glucose. Cellulases are found in GH5, GH6, GH7, GH12 and GH45 families whereas β- glucosidases belong to GH1 and GH3 families (Zerillo et al., 2013).

The term 'hemicelluloses' is used to describe the non-cellulosic components of the plant cell wall, such as xyloglucans, xylans and galactomannans. Xyloglucan is breakdown by the activity of endo-β- 1,4- glucanases, enzymes belonging to GH5, GH12, GH16 and GH44 families. Xylans are depolymerized by the activity of endo-1,4-β-xylanases that cleave the glycosidic bond in the xylan backbone. Most fungal xylanases are classified into the GH10 and GH11 families. Galactomannans are degraded by enzymes comprising β-mannanase and β-mannosidase (Moreira and Filho, 2008). β-mannanase are found in GH5 and GH26 families, whereas β- mannosidase belong to GH1 and GH2 families. In grasses, pectin is a minor constituent of cell wall, with hemicellulose being the main one. In fact, monocot pathogens have relatively higher hydrolytic enzymes for hemicellulose (GAXs) (King et al., 2011). In particular, xylanases degrade the linear backbone of the predominant hemicellulose (arabino)xylan into xylose residues (Beliën et al., 2006). The xylanase encoding gene *XylA*, from *F. graminearum*, is involved in the degradation of D-xylose, the main component of cereal plant cell walls (Tini et al., 2020).

The degradation of pectin requires the action of different enzymes that act both in a hydrolytic way (polygalacturonases, PGs) and in a non-hydrolytic way, via a β-elimination (pectin lyases and pectate lyases). Pectin degrading enzymes, such as PGs, pectin and pectate lyases and PECTIN METHYLESTERASES (PMEs), modify the pectic homogalacturonan (HG) backbone. PME cooperates with PG to degrade highly methyl-esterified pectin and promote the infection (Sella et al., 2016). *F. graminearum*-generated endo-polygalacturonase PG and xylanase Xyr1 share a synergistic role during plant infection. In this context, PGs play a critical role in plant-microbe interaction, since the accumulation of oligomeric HG degradation intermediates, i.e. oligogalacturonides (OGs), triggers the immune system, after being perceived by plant specific pattern recognition receptors (PRRs), which monitor the integrity of pectin and activate downstream defenses (Ferrari et al., 2013).

In addition to CWDEs, the tomatinase-like enzyme FgTom, KP4-like proteins, the putative catalase-peroxidase KatG2 and Fca7, and cerato-platanins were verified to be involved in *Fg*-host interaction (Voigt et al., 2005; Carere et al., 2017; Lee et al., 2018; Guo et al., 2019; Lu and Faris, 2019; Quarantin et al., 2019; Eranthodi et al., 2022). Furthermore, the production of reactive oxygen species (ROS) generated by the host is suppressed by *Fg*- secreted exo- 1,5-a-L-arabinanase Arb93B (Hao et al., 2019).

Plant cells continuously monitor the status of their cell walls with various types of sensors and membrane receptors, which can detect mechanical deformations or changes in

its structure or composition, during pathogen invasion attempts. The presence of a fungus is recognized by means of highly conserved components, such as chitin and glucan in their cell wall; chitin can be detected by plant chitin-binding proteins and chitinases, and glucan can be detected as a result of the action of plant glucanases and thaumatin-like proteins (Theis and Stahl, 2004). The encoded enzymes could play a role in defense and resistance against *Fg* either directly (via degradation of the fungal structural barrier) or indirectly (via elicitor activity of fungal cell wall degradation products). Upon the detection of a fungal pathogen agent, plants can counter the cocktail of CWDEs by producing glycosidase inhibitors, in particular xylanase inhibitors (XIs) and PG inhibitor proteins (PGIPs). XIs have been studied less intensely in plant defense than other inhibitors. Three classes of XIs proteins are present in wheat: *Triticum aestivum* xylanase inhibitors (TAXIs, Debyser et al., 1999), xylanase inhibiting proteins (XIPs, McLauchlan et al., 1999) and thaumatin-like xylanase inhibitors (TLXIs, Fierens et al., 2007). XIs proteins are localized in the apoplast and their expression is induced during stress conditions and fungal infections (Dornez et al., 2010; Igawa et al., 2005). For example, *F. graminearum* xylanases have the capacity to cause host cell death, both in cell suspensions and in wheat spike tissue, and TAXI-III and XIP-I prevented the enzyme activity and host cell death (Tundo et al., 2015). Indeed, the constitutive expression in durum wheat transgenic plants of the *TAXI-III* gene delayed FHB symptoms (Moscetti et al., 2013). The wheat xylanase inhibitors *Taxi-I, Taxi-III* and *Taxi-IV* were up-regulated in heads of wheat cv. Norin 61 in response to *Fg* infection, and *Titicum aestivum* xylanase inhibitor I (TAXI-I) was inhibitory to *Fg* xylanases (Igawa et al., 2005; Beliën et al., 2006).

PIGPs are leucine-rich repeat (LRR) glycoproteins that physically interacts with fungal PGs, limiting cell wall degradation and fungal growth, but also promoting the formation of OGs, i.e. elicitors of host defense responses (Ridley et al., 2001; De Lorenzo et al., 2001; D'Ovidio et al., 2004; Ferrari et al., 2013). Different PGIPs show specific recognition capabilities against many PGs produced by fungi. Indeed, PGIPs from different plants can differ in their inhibitory activities, and PGIPs of the same species can inhibit PGs from different fungi or different PGs from the same fungus (De Lorenzo et al., 2001). Diploid and polyploid wheats contain a single copy of *PGIP* gene in each genome and only the *TaPGIP1* (genome B) and *TaPGIP2* (genome D) are expressed (Janni et al., 2006) in the latter. Transcripts of both genes accumulate in roots, stem and spikes during normal growth (Janni et al., 2006), probably contributing to wheat development. Moreover, *Tapgip1* and *Tapgip2* are up-regulated following both fungal infection and mechanical

wounding (Janni et al., 2013). The involvement of PGIP in plant defense has been demonstrated. For example, the constitutive (Ferrari et al., 2012; Janni et al., 2008) and floral tissue-specific (Tundo et al., 2016) expression of the bean *PvPGIP2* in wheat transgenic plants was found to limit symptom development following infection by *Bipolaris sorokiniana* (50% reduction) and *Fusarium graminearum* (20-30% reduction).

Appropriate activation of early defense signaling events leads to disease resistance, which is implemented by cellular activities such as synthesis of phytoalexins, detoxification enzymes, and cell wall modifications. Resistance of wheat to *Fg*-infection is correlated with thickening of the cell wall and the formation of cell wall appositions (Kang and Buchenauer, 2000; Ribichich et al., 2000). Cell wall appositions, formation of papillae and increased lignin content have been hypothesized to restrict the influx of Fusarium toxins and the efflux of plant nutrients (Kang and Buchenauer, 2000). Cell wall reinforcement and lignification in response to *Fusarium* can be achieved by polyphenol oxidases that catalyze the oxidation of polyphenols to quinones (Mohammadi and Kazemi, 2002), and by peroxidases (POXs) that promote the oxidation of phenols and lignification. Moreover, fungal infection usually activates plant immune responses such as the influx of calcium ion (Ca^{2+}), ROS burst, transcriptional reprogramming, antimicrobial substance production, and callose deposition (Xu et al., 2022).

Over the years, one contentious issue has been whether *Fg* exhibits a biotrophic lifestyle during the initial stages of infection of floral tissues (Brown et al., 2010; Trail, 2009). A recent detailed microscopic study of the *Fg* infection process in wheat heads found no indication of necrotrophy at the initial stages of infection, as the advancing *Fg* hyphae remained in the intercellular spaces of wheat rachis cells before subsequent intracellular growth, which presumably leads to subsequent cell death and necrosis (Brown et al., 2010). Therefore, *Fg* may be classified as a hemibiotrophic pathogen. In particular, Ding et al. (2011) demonstrated that resistance to *F. graminearum* infection is associated with coordinated and ordered expression of diverse defense signaling pathways and altered secondary metabolism. Therefore, it was proposed a model to illustrate the early cellular events leading to FHB resistance after *Fg* infection (Figure 11). There were two major phases of resistance reactions during the first 24 hpi. The first phase occurred within 6 hpi, probably corresponding to a transient biotrophic stage. In this stage, changes or activation of Ca^{2+} fluxes, Ca^{2+} signaling, salicylate (SA) signaling, PA signaling, and ROS production and scavenging were the major cellular activities. Since they are all related to hypersensitive response (HR) and programmed cell death (PCD) and in order to constrain

cell death that favors the *Fg* growth, multiple mechanisms controlling PCD were also activated during this stage (Balint-Kurti, 2019). The second phase occurred after 6 hpi and before 24 hpi, probably corresponding to the start of the necrotrophic stage. In this stage, the jasmonate (JA)/ethylene (ET) signaling pathways and ROS production via PAO were activated, leading to different resistance reactions (Ding et al., 2011). ET signaling activation occurred between the two major phases, facilitating the transition from SA to JA defense signaling, since they are usually antagonistic. The activating order of these signaling events could be critical in mounting resistance and genes, such as *EDS1, NPR1, BIK1*, and *ERF1*, were actively involved in the regulation processes. Important immune responses, such as PR protein production, cell wall enforcement, and synthesis of antimicrobial compounds as well as detoxification, were detected soon after the infection start and peak from 6 to 12 hpi. Both SA and JA/ET defense signaling pathways regulate such immune gene expression.

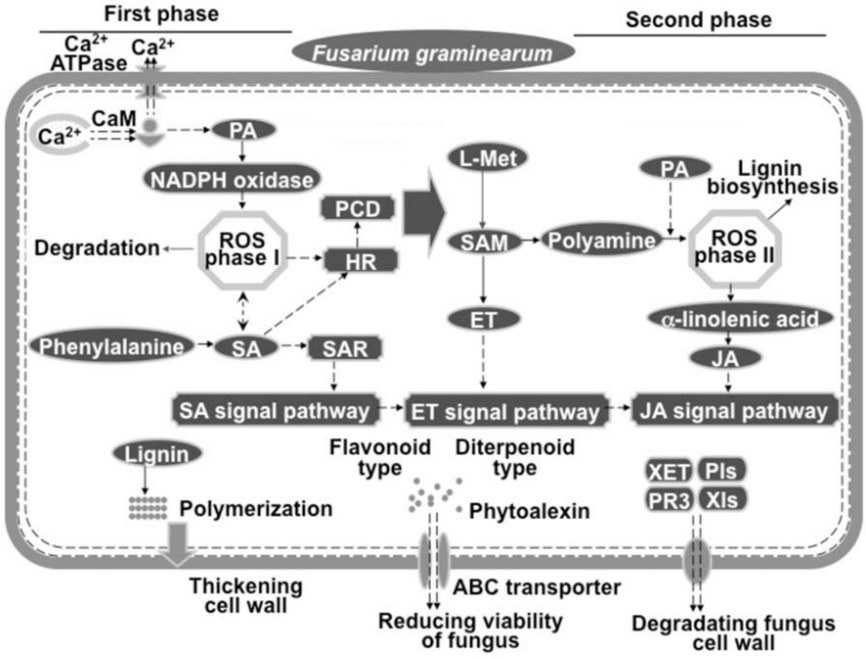


Figure 11. A simplified model of *Fusarium graminearum*–wheat interaction. *Fusarium graminearum* produces cell wall-degrading enzymes and toxins to colonize wheat. The plant responds to infection by producing defense-related hormones, pathogenesis-related (PR) proteins, reactive oxygen and proteins involved in cellular detoxification. PIs, protease inhibitors; XET, xyloglucan endotransglycosylase; Xls, xylanase inhibitors; PA, phosphatidic acid; SAM, S-adenosylmethionine (Adapted from Ding et al., 2011).

Furthermore, Zhang et al. (2012) observed that the genes associated with the fungus non-symptomatic stage are involved in primary metabolic pathways, whereas genes

involved in cell wall degradation dominate the later growth stage of the infection process. Nevertheless, once necrosis develops in the infected tissue, the pathogen further colonizes the dead tissue, most probably by switching to a saprophytic lifestyle. It is, therefore, expected that pathogen genes expressed during different phases of disease development might be different (Zhang et al., 2012). Indeed, the comparison of the transcriptomes of *Fg* feeding on living or dead tissues suggests that the fungus uses host signals to modulate the expression of several genes (Boedi et al., 2016). Similarly, some fungal genes are repressed by host signals (Boedi et al., 2016). On the other hand, host signals sensing is also required for the activation of DON biosynthesis. Wheat anthers are rich in various nutrients and have stimulating compounds to increase the virulence of *F. graminearum* (Jiang et al., 2019). Following infection of wheat florets, the fungus expresses genes for trichothecene biosynthesis almost immediately (Jansen et al., 2005). DON is a virulence factor in wheat, causing tissue necrosis and allows the fungus to spread into the rachis from florets in wheat (Jansen et al., 2005). Interestingly, DON is the only mycotoxin shown to be a virulence factor (Bai et al., 2002).

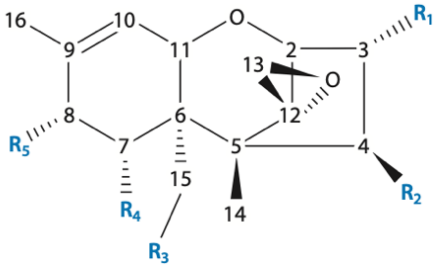
1.2.4. *Fusarium* trichothecenes

In addition to enormous yield losses, FHB pathogens have serious potential impacts on human and animal health via the contamination of wheat, barley, and maize with various mycotoxins (Bennett and Klich, 2003). Trichothecenes, a large family of sesquiterpenoid secondary metabolites (SMs), are considered the most common mycotoxins produced by *Fusarium* species. These compounds are characterized by a tricyclic ring structure containing a double bond at C-9, 10 and an epoxide group at C-12,13, that has been deemed crucial for toxicity (Desjardins et al., 1993) (Figure 12).

Regardless of the size and structural composition, trichothecenes are strongly associated with chronic and fatal toxicosis of humans and animals. They inhibit eukaryotic protein synthesis by interacting with the ribosomal protein L3 within the 60S subunit causing an inhibition of its peptidyl transferase activity (Rocha et al., 2005; Desjardins, 2006). Additional impacts of trichothecene toxicity include disruption of nucleic acid synthesis, mitochondrial function, membrane integrity, and cell division. Trichothecenes have also been shown to induce apoptosis in animal cells and may induce PCD in plants (Rocha et al., 2005).

Due to food safety problems caused by trichothecenes to animals and humans, the US Food and Drug Administration has set advisory levels for their presence in grains and finished products for human consumption and in animal feed (U.S. Food and Drug Administration, 2010). Similarly, the EU has set maximum levels for trichothecenes in cereals and their derived products, distinguish maximal concentration between animal feed and human/infant food (European Commission, 2006).

To date, more than 200 trichothecenes have been described and are divided into four groups (from A to D), based on the type of substitution at the C-8 position. Type C and D trichothecenes, which are characterized by a second epoxide (C-7,8 or C-9,10) or an ester-linked macrocycle (C-4,16), respectively, are not associated with FHB (Sudakin, 2003). Type A and B are more common in cereals. In general, type A trichothecenes tend to be more toxic than type B, but the latter ones occur more frequently and at higher concentrations (Foroud and Eudes, 2009). The major type A trichothecenes in *Fusarium* species include T-2 toxin and HT-2 toxin, both of which possess an isovalerate ester at C-8 (Mirocha et al., 2003). *F. sporotrichioides* and *F. poae* are some of the major type A trichothecene producers (Liddell, 2003). More recently, a new type A trichothecene called NX-2 and its derivatives were identified in *F. graminearum* (Varga et al., 2015). The type B trichothecenes, characterized by a C-8 keto group are produced by many different *Fusarium* species, especially *F. graminearum*, and include the important trichothecenes NIV, DON, and its 3-acetylated (3-ADON) or 15-acetylated (15-ADON) derivatives (McCormick et al., 2011).



Trichothecene	R_1	R_2	R_3	R_4	R_5
DON	–OH	–H	–OH	–OH	=O
3-ADON	–OAc	–H	–OH	–OH	=O
15-ADON	–OH	–H	–OAc	–OH	=O
NIV	–OH	–OH	–OH	–OH	=O
4-ANIV	–OH	–OAc	–OH	–OH	=O
NX-2	–OAc	–H	–OH	–OH	–H
NX-3	–OH	–H	–OH	–OH	–H
NX-4	–OH	–H	–OAc	–OH	–H

Figure 12. Structures of trichothecenes produced by Fusarium graminearum. Structures of type A and B trichothecenes produced by F. graminearum, including deoxynivalenol (DON), nivalenol (NIV), and their acetylated derivatives as well as of the new trichothecene mycotoxin NX-2, NX-3, and NX-4 (Chen et al., 2019).

DON is the most frequently detected mycotoxin in cereal grains worldwide, with a 59% average incidence rate, ranging from 50% in Asia to 76% in Africa, but tends to occur at higher concentrations in Europe and Asia (Lee and Ryu, 2017). Furthermore, DON had a higher occurrence in feedstuffs, with a 72%–79% incidence rate in corn samples sourced from North America and Central Europe, and 17%–47% in corn samples from South America and Southern Europe.

Based on the economic importance of FHB and trichothecene toxicity, *Fg* was listed as one of the top ten fungal plant pathogens (Dean et al., 2012), and its mechanisms for pathogenicity and regulation of trichothecene biosynthesis have been extensively investigated during the past three decades. During this time, the availability of the complete genomic sequence of *Fg* and the development of molecular genetic tools for the fungus have led to a better understanding of the comprehensive regulation of trichothecene mycotoxin biosynthesis.

1.2.4.1. Trichothecenes biosynthesis

Trichothecene biosynthesis is a complex process that proceeds from farnesyl phosphate via the trichodiene, followed by a sequence of sesquiterpene cyclization, eight oxygenations and four esterifications (Desjardins, 2006). The biosynthetic enzymes required for trichothecene production are encoded by 15 *TRI* genes, which are located at three different loci on different chromosomes in *Fg*: a 12-gene core *TRI* cluster, two genes at the *TRI1-TRI16* locus, and the single-gene *TRI101* locus (Alexander et al., 2009). Most of the biosynthetic enzymes necessary for trichothecene production are in the core *TRI* cluster. Among them, the gene *TRI5* (formerly designated *TOX5*) encodes a trichodiene synthase, which cyclizes farnesyl pyrophosphate (FPP) to trichodiene (TDN), the first step in trichothecene biosynthesis (Hohn and Beremand, 1989). Because *Fusarium spp*. are haploid and the *TRI5* gene occurs as a single copy in the genome, it has been found that, disruption of the *TRI5* gene interrupts the biosynthesis of trichodiene and all the other trichothecenes (Bai et al., 2002). Fungal loss of the *TRI5* function mutants had provided opportunity to examine the involvement of trichothecenes in plant-*Fusarium* interaction (Desjardins, 2006). Five genes are important to determine the basis for the type of produced trichothecene: *TRI16* is functional only in the T-2 toxin *Fusarium* strains (Brown et al., 2004); *TRI7* and *TRI13* are functional only in NIV-producing strains and not in DON-producing ones (Lee et al., 2002); a sequence variation in *TRI8* determines the 3-

ADON and 15-ADON chemotypes (Alexander et al., 2011); allelic variants of *TRI1* are responsible for an important structural difference in type A and type B trichothecenes (McCormick et al., 2006). *TRI6* and *TRI10* are transcription factors essential for the coordination of expression of genes at the three loci (Seong et al., 2009). Instead, *TRI101* encodes an acetyltransferase that reduces the toxicity of trichothecenes, thus representing a self-protection mechanism for the fungus (McCormick et al., 1999).

The trichothecene biosynthesis pathway and genes associated with trichothecene production are presented in Figure 13. Trichodiene (TDN) is converted to calonectrin (CAL) by nine reactions sequentially catalyzed by TRI4, TRI101, TRI11, and TRI3 (Alexander et al., 2009). *TRI4* encodes a key multifunctional CYP58 family cytochrome P450 monooxygenase for four consecutive oxygenation steps in trichothecene biosynthesis, converting TDN to isotrichotriol (McCormick et al., 2006). The enzymes TRI101 (C-3 acetyltransferase) (McCormick et al., 1999), TRI11(C-15 hydroxylase) (Alexander et al., 1998), and TRI3 (15-O-acetyltransferase) (McCormick et al., 1999) catalyze in sequence to produce CAL from isotrichotriol. All these reaction steps catalyzing farnesyl pyrophosphate (FPP) to CAL are shared among *Fusarium* species that produce type A trichothecenes (T-2 toxin and NX-2) and type B trichothecenes (NIV and DON) (Figure13A). In DON producers, CAL is hydroxylated at both the C-7 and C-8 positions by cytochrome P450 monooxygenase TRI1 and deacetylated by TRI8, leading to the formation of either 3-ADON or 15-ADON followed by DON (Brown et al., 2003) (Figure 13B). Two alternative pathways for 4-ANIV and NIV biosynthesis exist for *Fg* NIV producers, either the TRI13-TRI7-TRI1-TRI8 pathway using the CAL as a substrate or the TRI13-TRI7-TRI8 pathway using the 3,15-acetyl DON as the initial substrate (McCormick et al., 2011) (Figure 13C). TRI1 enzyme catalyzes hydroxylation at both C-7 and C-8 in *Fg* DON and NIV producers, leading the formation of NX-2 and derivatives thereof (Lofgren et al., 2018) (Figure 13D).

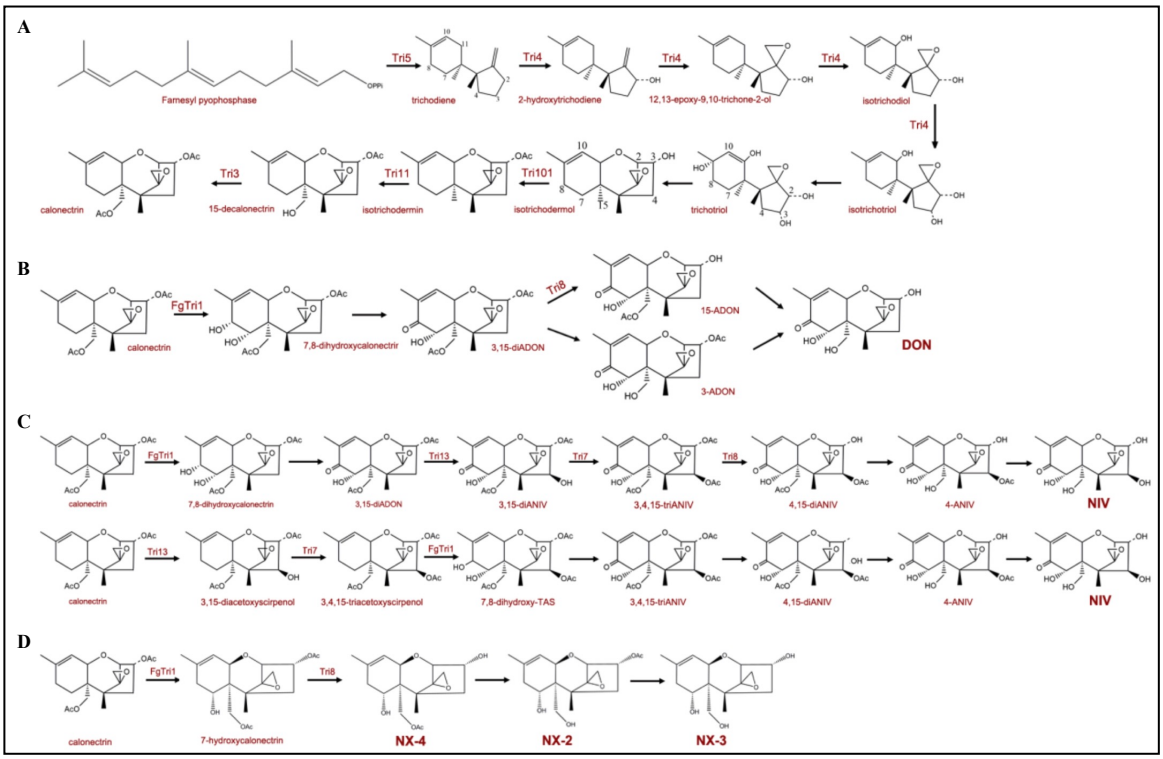


Figure 13. Biosynthetic pathways of different trichothecenes in *F. graminearum*. A) Conserved biosynthetic pathway of type A and type B trichothecenes; **B)** Biosynthetic pathway of DON; **C)** Alternative biosynthetic pathway of NIV; **D)** Biosynthetic pathway of NX-2 (Adapted from Chen et al., 2019).

1.2.4.2. Role of trichothecenes in *Fusarium*-wheat interaction

Although DON is of particular concern for human and animal health, the fungus produces DON to overcome plant defense and potentially "win" the plant-pathogen "battle". During early stages of the FHB infection, the fungus germinates and grows biotrophically into the intercellular spaces. The role of DON seems not to be important during these stages. However, DON pathway is already active and low *Tri5* expression can be detected in *Fg* (Mudge et al., 2006; Ilgen et al., 2009). Very low concentration of DON (10 ppm) was described to inhibit PCD in Arabidopsis cells (Diamond et al., 2013), probably to facilitate the initial spread of the fungus during the biotrophic stage. Afterward, the necrotrophic switching begins, and the fungus produces larger amount of DON, particularly at the rachis node (Mandalà et al., 2019). It was demonstrated that high doses of DON (100 - 200 ppm) infiltrated in wheat leaves cause H_2O_2 production within 6 hours, followed by cell death (Desmond et al., 2008). H_2O_2 accumulation, besides triggering plant defense mechanisms and, ultimately, HR, also triggers further the DON production in the fungus (Ponts et al., 2007), leading to a positive feedback to increased DON and H_2O_2.

As mentioned before, DON is considered a virulence factor, essential for fungal spread along the spike from the inoculated floret, although not essential for the initial establishment of the infection. Indeed, Bai et al. (2002) demonstrated that *Fg* DON-nonproducing mutants (i.e. *FgΔTri5*) are able to produce the initial infection but unable to determine the spread of the disease symptoms in inoculated spikes of both susceptible and resistant genotypes. A further confirmation was given by Jansen et al. (2005). Their work allowed to observe the growth of wild type *Fg* (wt*Fg*) and *FgΔTri5* mutant by transforming the fungi with the constitutively expressed green fluorescent protein (GFP) reporter gene. After injection of the two strains in the wheat spikelet, *FgΔTri5* infection was stopped at the rachis node, where heavy cell wall thickening was formed by the plant defense mechanisms. This fortification was not built up during wt*Fg* colonization, where hyphal movement was observed through the rachis. Finally, Ilgen et al. (2009) monitored the trichothecene pathway during plant infection by developing a *Fg* strain expressing the *GFP* gene under the control of the endogenous promoter of *TRI5*, and in the meantime localizing hyphal growth with the constitutive expression of the *dsRed* gene. They found that the most extensive GFP fluorescence was observed at the rachis node, confirming the high induction of the TRI pathway and mycotoxin production at this stage of the disease.

1.2.5. Control strategies for FHB

DON contamination of infected grains is closely linked to the severity of FHB disease in the field. Thus, the best way to prevent food and feed contamination by DON is to protect plants during crop cultivation. Several reviews have summarized the available strategies for FHB management, including the use of fungicides, biological control, and the use of resistant genotypes (Chulze et al., 2015; Wegulo et al., 2015; Dweba et al., 2017). During the FHB disease cycle, debris, fungal spore release, and weather at anthesis are critical variables for controlling the pathogen spread. As regards the integrated management of the disease, the best chance to success comes from combining two or more different strategies.

1.2.5.1. Chemical control

Chemical control is one of the main practices in an integrated FHB management approach. Fungicides are currently used both before and during the flowering stage to reduce yield loss and mycotoxin contamination (Mullenborn et al., 2008). Effective

fungicides should be safe products with short pre-harvest interval and have high efficacy in reducing FHB and DON. To date, many fungicides with different active ingredients are being used to manage FHB. Demethylation inhibitor (DMI) class of fungicides, especially tebuconazole, metconazole, and prothioconazole, block fungal sterol biosynthesis and are the most widely used and effective fungicides for the suppression of FHB and reduction of mycotoxin accumulation (Paul et al., 2008). Triazole fungicide application, usually tebuconazole or prothioconazole, can reduce FHB incidence, disease severity and DON accumulation. Application is most effective during anthesis, but application after or prior to anthesis also can reduce disease severity and DON accumulation (Paul et al., 2018). Tebuconazole, one of the most widely tested products, reduced FHB severity 25-77% and DON content 32-89% in field trials (Paul et al., 2007). Another older fungicide, the benzimidazole carbendazim, is also effective against FHB, with approximately 70% disease reduction (Liu et al., 2014). Fungicides in the quinone inhibitor class have been shown to increase DON levels in grain and therefore are not recommended for FHB and DON control (Ellner, 2005). Many fungicides have been used to reduce FHB, including triazoles, carbendazim, mancozeb, benomyl, prochloraz, propiconazole and triadimenol. None of these chemicals, however, sufficed by themselves to completely control FHB in wheat crops (Dweba et al., 2017; Spolti et al., 2013), maybe because several factors, such as level of inoculum, cultivar resistance, climatic conditions, crop sensitivity and yield potential, affect the success of the fungicide application in controlling FHB.

The two main critical factors in the usage of fungicides to control FHB are the timing and rate of application. To be effective, fungicide application is usually timed at the anthesis growth stage or up to 6 days after anthesis because susceptibility to FHB infection is highest at this stage (D'Angelo et al., 2004). Application of fungicides several weeks before anthesis may be more harmful for non-toxigenic microorganisms and can promote subsequent spread of toxigenic Fusarium species in the field (Henriksen and Elen, 2005). Fungicides should be applied from both sides of the plots as partial coverage reduces the control of FHB. The rate of application may vary with the type of fungicides applied and comes with the fungicide label. It has been found that the concentration of fungicides was highest in the glumes and gradually decreases when moving to lemma and the embryo (Mesterházy et al., 2018).

Another issue for the chemical control of FHB in the field is represented by the resistance to fungicide. Repeated and intensive application of the same fungicides, indeed, can lead to development of resistance to the compound in pathogen populations. Resistant

F. graminearum strains to azoles and carbendazim have been already characterized (Liu et al., 2014; Spolti et al., 2014). What is worse is that the carbendazim-resistant strains can produce higher amounts of mycotoxins during infections (Zhang et al., 2009). Thus, the use of mixtures of multiple triazoles and/or triazoles with strobilurins are recommended for a more effective control (Gilbert and Haber, 2013). In addition, it is always recommended to use fungicides together with other management strategies such as tillage, crop rotation and resistant cultivars. The combined effect of several strategies would provide a better control with higher yield and less infection (Richard et al., 2022).

1.2.5.2. Biological control

Biological control is defined as an environmentally friendly strategy using living microorganisms or their derivatives to reduce the growth of a targeted pathogen. Utilization of the biocontrol agents (BCAs) alone or as part of an integrated management program is an alternative approach and a very promising strategy for the control of FHB and DON accumulation.

BCAs can be applied to stubble to limit pathogen growth on residues of previously harvested crops (Palazzini et al., 2013; Wegulo et al., 2015), or on wheat heads during anthesis to inhibit fungal infection (Schisler et al., 2002). BCAs can interact with the pathogen either directly, e.g. parasitism or antibiosis, or indirectly, e.g. induction of resistance, competition, or plant growth promotion (Legrand et al., 2017). Among the achieved results of the biological control could be aborting, curtailing, or delaying the germination of the pathogen spores in the infection court of the head (Fernando et al, 2000).

Biocontrol of FHB has been intensively studied, and many BCAs have been isolated from various environmental conditions. Successful reduction of FHB incidence and severity in the field has already been achieved by involving bacterial strains, mainly belonging to *Bacillus* spp., *Pseudomonas* spp., *Lysobacter enzymogenes*, and *Streptomyces* spp., as well as fungal strains, including *Cryptococcus* spp., *Trichoderma* spp., *Clonostachys rosea*, and *Aureobasidium pullulans*. These BCAs can be applied directly to spikes to slow disease progression or applied to straw residues to suppress production of perithecia. Moreover, since 2002 a number different viruses – dsRNA (Li et al., 2019; Wang et al., 2013), negative strand RNA (Wang et al., 2018), and positive single-stranded RNA (Chen et al., 2016) viruses – have been tested in the FHB biocontrol. The results

obtained hinted that infected fungal strains often have few or no morphological differences respect to the uninfected strains (Li et al., 2016). Some viruses, however, are reported to cause major changes in internal fungal metabolism (Bormann et al., 2018) and/or to be able to reduce their pathogenicity (Darissa et al., 2012). These mycoviruses appear to have potential for the *Fg* biocontrol, even if more experiments are needed to develop a biocontrol approach involving such viruses.

Despite more than 70 years of intensive research and some promising results, few BCAs are currently available on the market. Lack of commercialized BCAs for FHB may be due to their unstable biocontrol efficacy in the field, to their strict storage and transport conditions, to the unidentified biocontrol mechanisms, and to the complexity of the product registration. Greenhouse applications often are more effective than field applications. Experimental conditions vary considerably and direct comparisons of results from different studies usually cannot be made. Effects can be measured only in terms of reduction of fungal growth/sporulation, toxin production or disease severity.

Furthermore, multiple antagonist microorganisms are available and can control FHB pathogens both *in vivo* and *in vitro*. Successful antagonism *in vitro*, however, is not always a good predictor of successful *in vivo* activity (Whitaker and Bakker, 2019). Combination between chemical and biological applications may be possible, but the data, to date, are relatively limited (Palazzini et al., 2018). Overcoming these challenges will promote BCA usage for managing FHB and DON contamination in the future.

1.2.5.3. Breeding

Deploying disease-resistant cultivars is, nowadays, considered the most economical and durable method for controlling FHB. However, most wheat cultivars are susceptible or moderately susceptible to the *F. graminearum* species complex, and highly resistant genotypes are not yet available (Bai and Shaner, 2004). Furthermore, the polygenic nature of FHB resistance, the effect of environment on resistance phenotype and the complex disease evaluation procedures make it complicated to increase resistance through breeding (Bai and Shaner, 2004). Moreover, resistance to FHB and DON in small grains is complex and inherited quantitatively.

Nowadays, five types of plant resistance to FHB are known (Mesterházy, 1995): type I – resistance to pathogen penetration and the onset of disease; type II – resistance to spread of the pathogen in the plant once the disease is established; type III – resistance to grain

infection; type IV – tolerance of the disease, i.e. infection occurs but grain yield is not reduced; and, type V – toxin degradation or inhibition of toxin activity. Bread wheat is generally less susceptible to FHB than durum wheat.

Because of the quantitative nature of resistance, development of resistant cultivars has been slow, and to date only a few cultivars with moderate resistance have been released worldwide (Brar et al., 2019). Efforts to breed for resistance using traditional and molecular techniques are continuing, and both native and exotic sources of resistance have been identified in the FHB-prone regions of the world (Kollers et al., 2013; Yu et al., 2008).

To date, more than 250 QTLs for FHB resistance have been described from various wheat genotypes (Bai et al., 2018; Cainong et al., 2015). QTLs associated with both reduced FHB severity and lower DON content include: *Fhb1* (chromosome 3BS), *Qfhs.nau-2DL* (2DL), *Qfhs.ifa- 5A* (5A), and *Fhb7AC* (7A) (Buerstmayr and Lemmens, 2015). Among them, *Fhb1* derived from Chinese cultivar Sumai 3 on chromosome 3BS is the most important QTL and has been reported to provide a moderately high level of resistance against various isolates and species of Fusarium (Jia et al., 2018). FHB resistant cultivars and breeding lines, including Sumai-3, accumulate very low levels of DON (<2 mg/kg) and have fewer than 10% infected spikelets (Bai et al., 2001). Incorporation of QTLs into commercial lines can be even more difficult as gene pyramiding usually is required for an effective level of resistance to be obtained and both doubled haploids and molecular mapping with linked markers may be required to select genetic material with commercial potential (Lemes da Silva et al., 2019).

In the U.S., breeding efforts supported by funding from the U.S. Wheat and Barley Scab Initiative (USWBSI) (http://scabusa.org/) have resulted in the development and release of wheat and barley cultivars with moderate resistance to FHB and DON. In Europe, efforts similar to those of the USWBSI have been undertaken to breed for resistance to FHB and DON. Notably, among these are the “FUCOMYR” and “MYCOTOCHAIN” projects in which concerted efforts by multiple European Union nations involving public and private institutions and breeding and food processing companies identified genotypes with resistance to FHB and DON (Ruckenbauer et al., 2007). Thanks to the breeders increasing effort to enhance the resistance level of small grain cereal crops to FHB, more and more moderately resistant cultivars are becoming available to producers.

1.2.5.4. Biotechnological resources

Over the past few decades, several tactics have emerged as enhancements or as an alternative to, traditional breeding methods. Traditional breeding is a long and tedious process, requiring many generations of screening, which can be costly and time consuming. Breeders often screen thousands of lines, narrowing them down every year, for up to ten years, before they are ready to apply for the registration of one or two cultivars. Nowadays, biotechnology represents our best resource both for protecting crop yield and for a science-based increased sustainability in agriculture (Bigini et al., 2021). Over the last decades, agricultural biotechnologies have made important progress based on the diffusion of new, fast, and efficient technologies, offering a broad spectrum of options for understanding plant molecular mechanisms and breeding. This knowledge is accelerating the identification of key resistance traits to be rapidly and efficiently transferred and applied in wheat breeding programs to developing FHB-resistant cultivars.

Efficient protocols are available for genetic transformation of a few wheat cultivars, which are suitable for tissue culture regimes, in particular via biolistic gene transfer of bread (Tian et al., 2019) and durum (Wang et al., 2022) wheat and via Agrobacterium-mediated transformation (Kumar et al., 2019). Moreover, new breeding techniques such as cisgenesis, intragenesis, site direct mutagenesis, RNA interference (RNAi), genome editing using zinc finger nucleases, TALENs and CRISPRs/Cas9 methods, were developed to improve and finely modify cereal genomes (Barabaschi et al., 2016; Dunwell, 2014) (Figure 14).

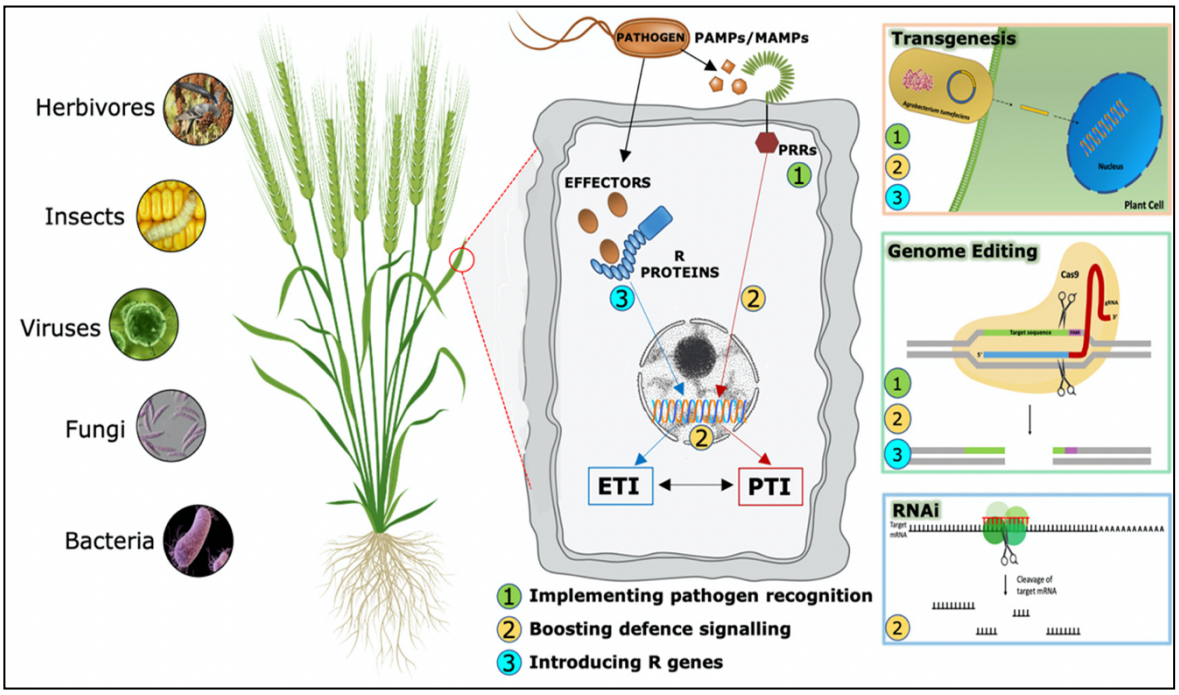


Figure 14. Biotechnological approaches and their possible involvement to enhance cereal resistance to pathogens (Bigini et al., 2021).

The plant complex and multilayered immune system offers different levels on which researchers could act through biotechnological approaches in order to enhance or implement plant resistance. The transgenic engineering to achieve FHB resistance in wheat can be grouped based on the types of candidate genes used for: implementing pathogen recognition; interfering with fungal growth, by using PR and anti-fungal proteins; antagonizing fungal virulence factors, such as trichothecenes; manipulating natural resistance mechanisms, by counteracting CWDEs or modifying/boosting host defense response pathways and responses (Figure 14).

Significant levels of FHB resistance can be achieved through introducing foreign genes with major effects into elite genotypes. Several genes have either been shown or suggested to contribute to FHB resistance owing to their wide capacity to encode proteins involved in fusariosis suppression (Xue et al., 2011). Some of these genes were incorporated from non-*Triticum* genomes, yielding non-negative physiological effects when expressed within the wheat genome (Han et al., 2012). Target genes for genetic engineering include encoding enzymes which detoxify DON, and genes responsible for the biosynthesis of antifungal proteins (Ferrari et al., 2012; Hou et al., 2015). Constitutive overexpression of plant genes encoding defense signaling pathway–related proteins, cell wall– degrading enzyme inhibitors, or detoxification proteins results in greatly enhanced resistance to *F. graminearum* (Bigini et al., 2021).

Faster activation of defense responses in the presence of the fungus also increases plant resistance. For instance, transgenic wheat lines expressing *Arabidopsis thaliana NPR1*, PGIP or UDP-glycosyltransferase showed increased resistance to FHB (Ferrari et al., 2012; Makandar et al., 2006; Xing et al., 2018).

Strengthening of plant cell wall by inhibitors of CWDEs was proved effective in reducing FHB disease symptoms in wheat, for example by overexpressing the *PvPGIP2* (Ferrari et al., 2012), *AcPMEI* (Volpi et al., 2011) or *TAXI-III* (Moscetti et al., 2013) genes.

Modification by a transgenic approach of plant defense pathways to better respond to Fusarium attack is also an efficient mechanism, probably the one with better results so far. For instance, over-expression of some resistance genes existing within the wheat genome under pathogen attack including those encoding stress responsive hormones such as MeJA, ET and SA (Makandar et al., 2012) have the potential to improve FHB resistance.

In addition, overexpression of genes encoding important transcription factors and signaling molecules in plants under FHB attack have been shown to enhance FHB resistance in wheat (Bahrini et al., 2011). A class of DNA-binding transcription regulators connected to pathogen defense mechanisms is represented by the WRKY family. The constitutive overexpression of the *TaWRKY45* conferred an enhanced type II resistance against FHB to transgenic wheat plants, under greenhouse conditions (Bahrini et al., 2011). Recently, the wheat *TaWRKY70* gene, located at the QTL-2DL was identified (Kage et al., 2017). Its silencing by VIGS, in wheat, decreased expressions of resistance genes and resistance related induced metabolites and increased fungal biomass after *Fg* infection (Kage et al., 2017).

1.3. Use of elicitors to increase disease resistance to FHB

As describe above, the indiscriminate utilization of pesticides in crop protection leads to several related issues towards both target and non-target organisms, as well as to environmental pollution. In addition, the trend of consumers today is inclined towards foods that are produced in a way that is environmentally sustainable, using fewer pesticides in a way that balances the economic, environmental and quality of life benefits not only for farmers but also for consumers (Aung and Chang, 2014). For this purpose, resistant genetic lines of wheat turn out to be a convenient option for the environment. However, the development cost is high, and the viability of their resistance is approximately three to five years, which renders them limited as a long-term solution (Huerta Espino et al., 2011).

Therefore, new approaches, preferably based on green methods, are needed to control fungal pathogens. To find harmless control strategies for crop disease management, we need to exploit the plant innate immunity that, if timely activated, can efficiently contrast, and restrict plant infection by microorganisms. In fact, although plants face many types of biotic stresses caused by various organisms including fungi and bacteria, they generally resist most pathogens, and plant infection is usually the exception, not the rule (Pandey et al., 2017). The innate immunity represents the first step in defense against invaders and can be activated within a few minutes after pathogen sensing (Abdul Malik et al., 2020). The faster pathogen detection occurs, the sooner proper immune responses are mounted by plants, with a consequent higher probability to restrict or block the tissue invasion. Only pathogens with an evolved ability to evade recognition or suppress host defense mechanisms, or both, are successful. Immunogenic signals are detected by cell surface localized PRRs, transmembrane proteins that are classified according to their structure in Receptors-Like Kinases (RLKs) and Receptor-Like Proteins (RLPs) (Roudaire et al., 2021). The ability of plants to recognize different classes of "non-self" and/or "self/altered self" danger signals, respectively known as Microbe-Associated Molecular Patterns (MAMPs) and Damage-Associated Molecular Patterns (DAMPs), guarantees the establishing of effective defense responses even in the presence of potential pathogens not recognized through MAMPs, and, at the same time, the fine regulation of immune reactions in the presence of commensal and beneficial microorganisms that belong to the same taxa as the pathogenic ones (Tanaka and Heil, 2021; Boller and Felix, 2009; Dodds and Rathjen, 2010; Maffei et al., 2012) (Figure 15).

These defense mechanisms remain inactive or latent until they are activated after exposure or contact with inducing agents or when an inductor is applied (Mandal et al., 2010). Among the several defense responses of plants, there are changes in the ion flow that promote the depolarization of the plasma membrane, production of ROS, nitric oxide (NO), activation of calcium-dependent protein kinases or mitogen-activated protein kinases (CDPKs and MAPKs) (Boller and Felix, 2009; Saed-Moucheshi et al., 2014; Boudsocq et al., 2010; Wu et al., 2014). These signaling events, when combined, modulate the expression of transcription factors, which leads to massive transcriptional reprogramming related to defense, which in turn results in the activation of various genes involved in the biosynthesis of various antioxidant enzymes (Davar et al., 2013; Ge and Guest, 2013) and metabolites related to the specific stress detected. These include, for example, PR proteins such as β-1,3-glucanases and chitinases, compounds with antimicrobial activity such as

phytoalexins and deposition of callose and lignin for cell wall strengthening (Burketova et al., 2015; Mandal and Mitra, 2007; Xie et al., 2015).

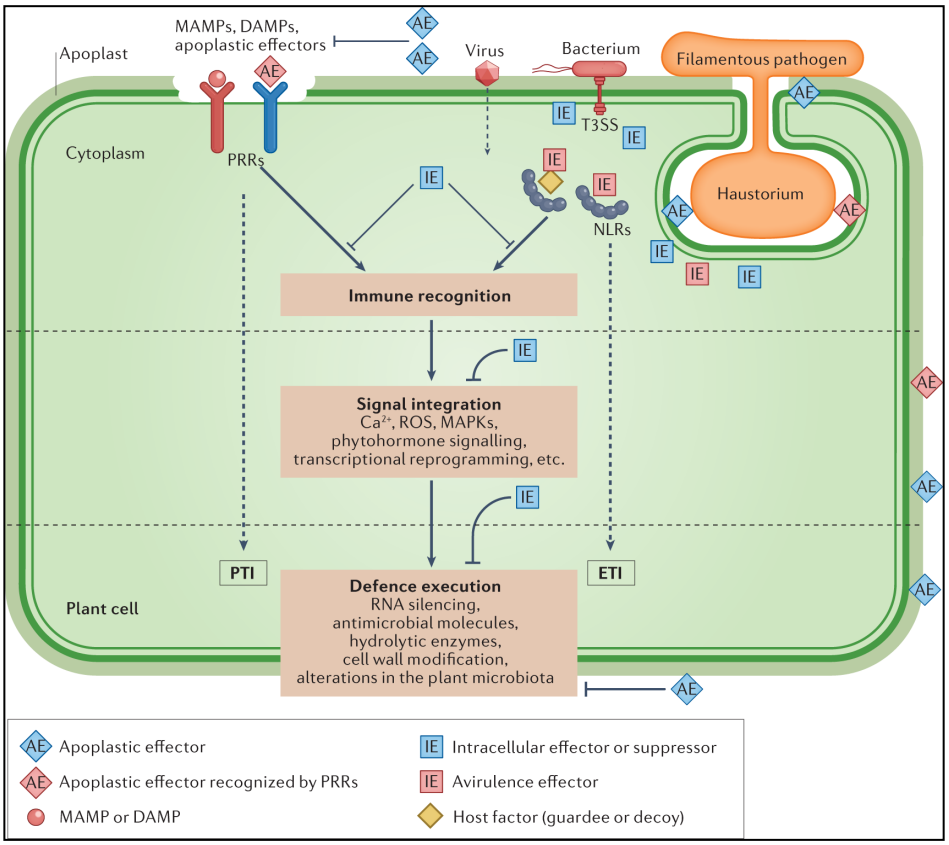


Figure 15. Stepwise activation of plant innate immunity (Wang et al., 2022).

All the previously mentioned defense mechanisms are activated once the plant recognizes PAMPs and/or DAMPs. PAMPs can be components of the cell wall, including chitin, β-glucans, ergosterol, and mannan, in the case of fungi. At the same time, bacteria derived PAMPs include lipoic acid, peptidoglycans, and flagellin, which are highly conserved molecules essential for these microorganisms' physiology and life cycle (Molina et al., 2007; Yang et al., 2019). Many MAMPs have been described and can be (glyco) type proteins, carbohydrates or lipids. In addition to the signals related to microorganisms, there are also DAMPs, host derived, which are structurally very diverse and include, for example, pectin-derived OGs. To circumvent PTI, pathogens deliver effector proteins inside host cells, where they interfere with defense responses. Plants perceive effectors through resistance (R) genes and activate a more robust and faster defense response, termed as effector-triggered immunity (ETI). In the zigzag model, PTI and ETI are viewed as two separate sequential branches of plant immunity (Jones and Dangl, 2006). However,

there are several examples where effectors fulfill the criteria to be designated as PAMPs (Thomma et al., 2011) and PAMPs as effectors. Based on several plant-pathogen interaction examples, Wang et al. (2022) proposed a continuum between PTI and ETI, in which the strict boundaries of the zigzag model cannot be maintained (Figure 15).

In addition to the innate immune system, plants can also acquire immunity against the perception of specific biotic and abiotic stimuli, a process mediated by the priming of inducible defenses, which causes plants to show an activation of various rapid and/or strong cellular defense responses (Mauch-Mani et al., 2017). Systemic acquired resistance (SAR) is a state of defense induced by a local infection with pathogens or by the application of a chemical substance that confers resistance to a broad spectrum of pathogens and is dependent on SA (Mishra et al., 2012). SAR induction results in accumulation of the signal molecule SA, which induces the expression of a wide set of defense genes (Miura and Tada, 2014). Therefore, when this process occurs, greater resistance is observed in the distal parts as a consequence of mobile signals in the plant belonging to the SA, which is biologically active in tissues far from the point of origin from where the response began (Manosalva et al., 2010). This response can present as a HR or local necrotic lesion caused by a virulent pathogen (Walters et al., 2014).

Exogenous plant resistance inducers (PRIs) application is interesting since it can be incorporated into integrated disease management programs. These inducers are also called elicitors, although the terms are sometimes confused: elicitors, in a broad sense, are chemicals or biofactors from various sources that can induce physiological and morphological changes in the target organism. These include abiotic elicitors such as inorganic compounds and metal ions, as well as biotic elicitors such as bacteria, fungi, viruses, plant cell wall components, and chemical compounds released at the site of infection by plants following the emergence of diseases (Thakur and Sohal, 2013). In the same way, other authors define that an elicitor or inducer is a molecule, or molecules, present in an organism or produced by the plant itself, whose function includes the generation of defense responses (Mandal et al., 2010).

It is important to note that in the field of crop protection, PRIs offer many advantages, because they are not directly toxic to pathogens, like pesticides, so they do not generate resistance; however, they are recognized by the PRRs and induce innate immunity (Boller and Felix, 2009). Moreover, PRIs can potentially protect against multiple pathogens, leading to a broad-range resistance (Oliveira et al., 2014).

On the other hand, plants have the adaptability of priming; this prepares them to respond faster and more vigorously to the stress caused by the attack of pathogens. Elicitors generate the priming state, availed to activate plant responses such as the production of phytohormones and phytoalexins, an increase in the expression of defense-related genes, and enhanced synthesis of enzymes with antioxidant activity (Moreno-Escamilla et al., 2018).

1.3.1. Oligosaccharides

Among the most studied PAMPs and DAMPs are oligosaccharides (Sobhy et al., 2014). Indeed, it has been shown that some oligosaccharides can act as elicitors of immunity and consequently activate the defense responses in different plant species. In addition, many studies that have compared oligosaccharides with chemical elicitors highlighted that the triggered responses shown in plants were similar; therefore, it is possible to consider the oligosaccharides as elicitors that can be used in agriculture (Zheng et al., 2020).

Regarding the origin, the oligosaccharides involved in plant-pathogen interactions are produced by enzymatic degradation of polysaccharides that are structural constituents of the cell wall of fungi or plants (Zhang et al., 2015). β-glucans, xyloglucan, chitin, chitosan, peptidoglycan, oligogalacturonides, laminarin, alginates, fucans, carrageenans, and ulvans are common carbohydrate elicitors. Their elicitor activities are influenced by different structural features, including degree of polymerization (DP) or molecular weight (MW), branching characteristics, functional groups, conformation, features of glycosidic linkages and monosaccharide composition (Ferreira et al., 2016; Ma et al., 2017; Xu et al., 2017).

In recent years, the structures of many poly- or oligosaccharides have been determined. Defense signaling induced by several carbohydrate elicitors has also been studied intensively. However, the structure-activity relationships of oligosaccharide elicitors are still not well understood. So, the activity and structure of several common carbohydrate elicitors such as chitin, chitosan and oligogalacturonides will be introduced below.

1.3.1.1. Chitin and Chitosan

Chitin and chitosan predominantly exist as a structural component in the cell walls of fungi and in the exoskeletons of insects, nematodes, and crustaceans (Liaqat and Eltem, 2018). Chitin, a heteropolymer of β-(1–4)-linked N-acetylglucosamine with a varying percentage of deacetylated glucosamine, is one of the most abundant natural

polysaccharides, second only to cellulose (Trouvelot et al., 2014). Chitosan is the deacetylation product of chitin, which is a heterogeneous polysaccharides consisting of β-1,4-linked GlcNAc and 2-amido-2-deoxy-D-glucopyranose (GlcN) residues (Rinaudo, 2006). It is reported that the presence of (acetyl)amino groups would be highly beneficial for chemical modifications to construct sophisticated molecular architectures. Special emphasis has thus been put on the structural transformations of chitin and chitosan to explore their full potential (Kurita, 2006). Chitin and chitosan are broad-spectrum elicitors that act on many plant species (Katiyar et al., 2015).

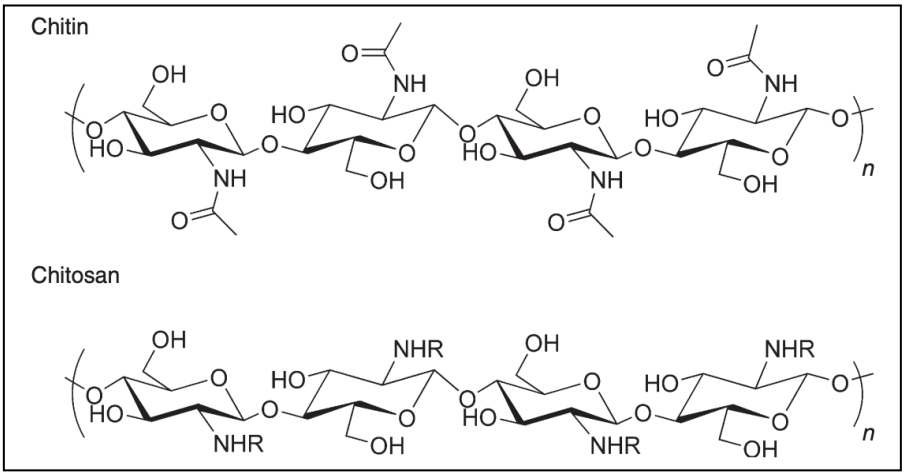


Figure 16. Chemical structures of chitin and chitosan (Song et al., 2012).

Plants do not contain chitin but could efficiently digest it by chitinases that degrade chitin into oligosaccharide fragments. These chitin fragments could be recognized as stress signals by plant cells and elicit defense reactions (Eckardt, 2008). The cell surface receptor CHITIN ELICITOR RECEPTOR KINASE 1 of Arabidopsis (AtCERK1) directly binds chitin through its lysine motif (LysM)-containing ectodomain (AtCERK1-ECD) to activate immune responses. The crystal structure of an AtCERK1-ECD complexed with chitin fragments was solved, revealing that chitin octamer, but not tetramer or pentamer, can act as a bivalent ligand to induce AtCERK1-ECD dimerization that is critical for its activation (Liu et al., 2012). The molecular basis of the specific recognition of chitin oligosaccharides was also identified in rice (*Oryza sativa*), which clarified the formation and activation of the receptor CEBiP (chitin-elicitor binding protein) complex (Shimizu et al., 2010). Moreover, chitin fragments have been reported to induce a set of defense reactions, including depolarization of membrane potential (Kuchitsu et al., 1993), ion fluxes

(Kuchitsu et al., 1997), oxidative burst (Ning et al., 2004), phytoalexin synthesis (Yamada et al., 1993), defense gene induction and enzyme activation (Takai et al., 2001).

Compared with chitin, chitosan can induced a series of biochemical and molecular changes in plants, including calcium influx (Zuppini et al., 2003), plasma membrane H+-ATPase inhibition (Amborabé et al., 2008), ROS burst (Li and Zhu, 2013), MAPK activation (Yin et al., 2010), synthesis of phytohormones (Iriti and Faoro, 2008), defense enzyme activation (Guan et al., 2009; Li et al., 2013; Ma et al., 2013), PR proteins accumulation (Lin et al., 2005), production of phytoalexin (Aziz et al., 2006) and callose deposition (Iriti and Faoro, 2008). However, the recognition receptor of chitosan in plants has not yet been elucidated clearly. Liu et al. (2018) demonstrated an interaction of fluorescence-labeled chitosan fragments with wheat leaf cell and further purified a chitosan binding protein from wheat plasma membrane proteins, but they did not report the function of this chitosan binding protein. So far, whether the specific receptors of chitosan exist or not remains controversial.

Chitin and chitosan possess broad-spectrum antimicrobial effects against bacteria, fungi, and viruses. Generally, chitosan is more effective against fungi, and it often exhibits higher growth inhibition effects on Gram-positive bacteria than on Gram-negative ones (Malerba and Cerana, 2016). Therefore, Xing et al. (2015) proposed to use the chitosan antimicrobial and elicitor activities for pest control for agricultural purposes. The physiological properties and biological function of chitin and chitosan depend on their molecular weight (MW), degree of polymerization (DP), degree of acetylation (DA), and chemical derivative attached (Khan et al., 2017). To date, it is well known that the effect of chitin and chitosan on plants is strictly dependent on their MW. It has been reported that chitin oligosaccharides larger than pentose strongly induced the formation of phytoalexins in suspension-cultured rice cells at a very low concentration (10^{-9}–10^{-6} M) (Okada et al., 2007). In contrast, Takai et al. (2001) reported that the expression level of an elicitor-responsive gene (*EL5*) was enhanced by addition of chitin heptamer or octamer, whereas chitin hexamer or shorter oligosaccharides or nonacetylated oligosaccharides showed much weaker activity in suspension-cultured rice cells. Low MWs chitosan are more effective in general. For example, researchers have proved that chitosan with low MWs (1335Da) can induce defense responses more effectively than those with higher MWs (3350 Da or 50,000 Da) to resist rice blast pathogen infection in rice (Lin et al., 2005). Similarly, chitosan (MW from 2 to 17 kDa) with lower MWs possessed higher antiviral activity (Davydova et al., 2011). Furthermore, the antiviral activity of the chitosan (MW from 1.2

to 40.4 kDa) increases with the decrease of their MWs (Kulikov et al., 2006). Therefore, an exciting feature of chitosan is the excellent biological activity exerted by its fractions with a lower molecular weight called chitooligosaccharides (COS) (Ahmed et al., 2012).

In recent years, chitin oligosaccharides with different DP have also been widely studied. It is reported that soluble chitin oligosaccharides with DP ranging from 2 to 6 can be dissolved in neutral water, while the oligosaccharides with DP exceeding 6 are not easily dissolved (Yin et al., 2016). However, Hayafune et al. (2014) found that chitin oligosaccharides with DPs exceeding 6, especially the DPs reach to 7 and 8, showed the high elicitor activity in rice. Recently, Gubaeva et al. (2018) compared the ability of various chitosan oligomers with different DA and sequences to elicit an oxidative burst indicative of induced defense reactions in *Arabidopsis thaliana* seedlings. They found the elicitor activity of chitosan oligomers increased with increasing DA. Therefore, partially acetylated chitosan fragments required a minimum DP of 6 and at least four N-acetyl groups to trigger a response.

Different reports described plant growth-promotion effects triggered by chitin and chitosan and possible applications in agriculture, both for crop and horticulture benefits, are being developed. It has been reported that chitosan could enhance the photosynthesis rate of maize (Khan et al., 2002), promote nutrient uptake of beans (Chatelain et al., 2014) and increase the yield of wheat (Wang et al., 2015). It is worth noting that chitin and chitosan promote plant growth and development when applied in low quantities, which is characteristic for biostimulants, other than fertilizers (Du Jardin, 2015). Chitosan with different MWs have been tested to promote plant growth, with low MW recognized as being more active (Nge et al., 2006). Zhang et al. (2016) studied the chitosan fragment size effect of on wheat seedlings by foliar application using nine fully deacetylated chitooligomers, including chitobiose to chitooctaose, and two chitosan fragments with narrow DPs (DP8-10, DP10-12). As a result, chitotetraose to chitooctaose and two oligomer mixtures with DP 8-10 and DP 10-12 significantly enhanced the growth and photosynthesis of wheat seedlings, whereas the small chitobiose and chitotriose were inactive. Therefore, the plant-promotion effect of chitosan fragments also needed an essential chemical structure, and a DP of at least 4 (Zhang et al., 2016). Therefore, such fascinating carbohydrate polymers are applied in organic agriculture, not only as elicitor but also as fertilizer, biostimulants and signaling molecules to induce plant defense responses, to promote plant growth, to facilitate efficient uptake of nutrients, enhancing abiotic stress tolerance and improving shelf-life and quality of edible product

(Battacharyya et al., 2015; Bouissil et al., 2019). In addition, polysaccharides are also employed as safe release systems for agrochemicals, allowing active ingredient to be delivered slowly and continuously for longer duration to a specified target at a desired rate (Campos et al., 2015). In the European Union, chitosan was the first compound in the list of basic substances approved for plant protection purposes (Reg. EU 66 2014/563), for both organic agriculture and integrated pest management (Romanazzi et al., 2018).

1.3.1.2. Oligogalacturonides (OGs)

More than 30 years ago, phytoalexin accumulation in soybean cotyledons provided the first evidence that pectin fragments activate defensive responses (Hahn et al., 1981). These fragments, called endogenous elicitors, were later identified as oligomers of alpha-1,4-linked galacturonosyl residues (oligogalacturonides, OGs) that can be produced by partial hydrolysis of polygalacturonic acid (Nothnagel et al., 1983).

OGs are probably the best characterized plant DAMPs and elicit in several plant species a wide range of defense responses, similar to those of well-characterized PAMPs, such as the bacterial flagellin (Ferrari et al., 2013; Desaki et al., 2012). OGs trigger the accumulation of phytoalexins (Davis et al., 1986), glucanase and chitinase (Davis and Hahlbrock, 1987; Broekaert and Pneumas, 1988), the deposition of callose, the production of reactive oxygen species (ROS) (Bellincampi et al., 2000; Galletti et al., 2008), and nitric oxide (Rasul et al., 2012) (Figure 17).

OGs are thought to be released from plant cell walls upon partial degradation of homogalacturonan (HG) by microbial PGs during infections (Cervone et al., 1989) or by the action of endogenous PGs induced by mechanical damage (Orozco-Cardenas and Ryan, 1999). The signaling activity of OGs is clear evidence that plants have created mechanisms to detect HG degradation to quickly identify and react to tissue damage.

Pectin is one of the most exposed components of the cell and, therefore, is one of the first structures to be altered during the initial pathogen invasion or when the cell wall undergoes a stress rupture (De Lorenzo and Ferrari, 2002). Since plant cell wall integrity may be efficiently controlled by monitoring pectin status, De Lorenzo et al. (2011) proposed the existence of a system called "pectin integrity monitoring system," or PIMS. OGs are key elements in PIMS, since they may act as indicators of cell wall integrity, both in adverse conditions and during normal growth. Furthermore, because HG-degrading enzymes such as PGs are among the first enzymes secreted by pathogens during

host colonization, PIMS also includes fungal and insect PG inhibitors (PG-inhibiting proteins or PGIPs), which protect the cell wall by limiting HG degradation (De Lorenzo et al., 2001; De Lorenzo and Ferrari, 2002; Di Matteo et al., 2006). By inhibiting the enzymatic activity of PGs secreted by pathogens, a dual role in PIMS was proposed for PGIPs, which not only limit pectin degradation by inhibiting the PG activity but also favor the accumulation of elicitor-active OGs (De Lorenzo et al., 2001; De Lorenzo and Ferrari, 2002).

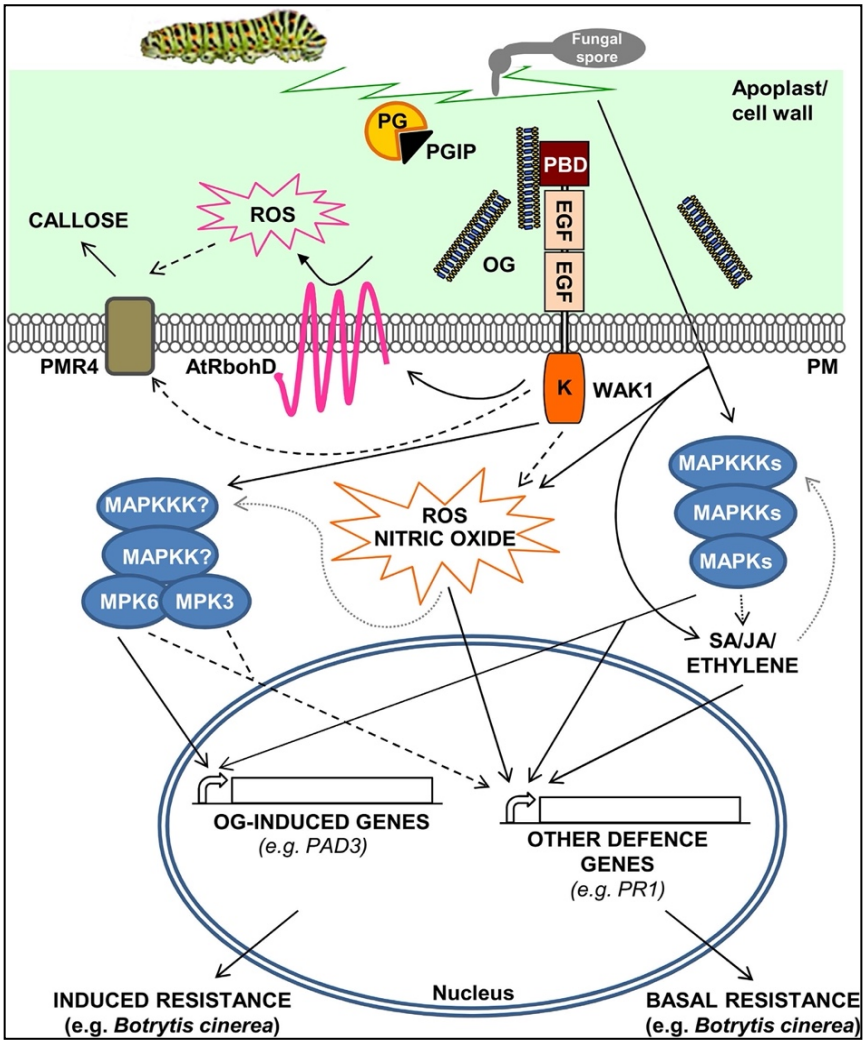


Figure 17. A model for the activation of *Arabidopsis thaliana* defense responses triggered by oligogalacturonides (Ferrari et al., 2013).

The adoption of *Arabidopsis thaliana* as a model plant has provided a useful tool to advance the knowledge of the OG biology. Early studies on plant response to OGs were often limited to specific molecular targets or processes, including the production of plant hormones, such as ET and JA, or the expression of specific defense-related genes (Doares

et al., 1995; Ellis et al., 2002). More recent transcriptomics and functional genetic analyses have enabled systems-level studies of plant responses to OGs and the characterization of the OG-responsive transcriptome in Arabidopsis (Moscatiello et al., 2006; Denoux et al., 2008). In Arabidopsis, OGs activate a set of responses that are independent of the signaling pathways involving ET, SA and JA (Zipfel et al., 2004; Ferrari et al., 2007) and induce the phosphorylation of two mitogen-activated protein kinases (MAPKs), namely AtMPK3 and AtMPK6 (Denoux et al., 2008; Galletti et al., 2011). AtMPK6 appears necessary for the early expression of defense genes and for the induced resistance against *Botrytis cinerea* triggered by these elicitors (Galletti et al., 2011) (Figure 17). Furthermore, OGs trigger a robust oxidative burst mediated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase AtRbohD, which is at least partially responsible for the subsequent deposition of callose (Zhang et al., 2007; Galletti et al., 2008).

Understanding how OGs are perceived is necessary to elucidate their role *in vivo*, but the identification of an OG receptor has been daunting for a long time. Wall-associated kinases (WAKs) were indicated as interesting candidates because of their ability to bind OGs and polygalacturonic acid (Anderson et al., 2001; Decreux and Messiaen, 2005). WAKs are receptor-like kinases, with an extracellular domain containing epidermal growth factor motifs, a transmembrane domain and an intracellular Ser/Thr kinase domain (Anderson et al., 2001). WAKs were first identified in Arabidopsis as pectin-bound proteins and, subsequently, WAK1 was shown to carry a N-terminal pectin binding domain that interacts with non-methylesterified HG and OGs in a Ca^{2+}-dependent manner (Lally et al., 2001; Wagner and Kohorn, 2001; Decreux and Messiaen, 2005).

An important aspect to be considered is that major end products of the pectin degradation OGs with different degrees of polymerization (DP). As describe above for chitin and chitosan, the DP determines the physiological properties and biological function of OGs too. Recent studies have suggested that long OGs (DP > 10) are the most effective in modulating signaling involved in plant defense responses, while short OGs having little or no effect (Ferrari et al., 2007; Denoux et al., 2008). However, other studies have suggested that also short OGs (DP < 10) impact plant defense (Simpson et al., 1998) and development (Miranda et al., 2007). For instance, short OGs (DP4-6, DP2 and DP1-7, respectively) induced genes involved in pathogen response and defense in potato and tomato and the synthesis of phytohormones in tobacco and tomato (Montesano et al., 2001; Simpson et al., 1998; Norman et al., 1999). Furthermore, Davidsson et al. (2017) investigated the role of trimers (DP3) in DAMP signaling transcriptomic analysis of

Arabidopsis thaliana exposed to these compounds. The transcriptomic data obtained by RNA sequencing suggested that trimers clearly induced the expression of a number of genes involved in plant defense, while genes involved in growth, biosynthetic pathways and development were downregulated. The up-regulation of defense-associated and OG-responsive genes identified in this study was comparable to previous studies in which in vitro-grown Arabidopsis seedlings have been treated with long OGs (Ferrari et al., 2007; Denoux et al., 2008). However, the modulation of gene expression induced by trimers was generally not as strong as that induced by long OGs. Just few studies were made to study the effects of OGs in wheat plants (Randoux et al., 2010; Ochoa-Meza et al., 2021).

1.3.2. Engineering danger sensing and signaling in plant immunity

Based on the current knowledge and the new fast and precise molecular techniques, the manipulation of plant immune system to render them more reactive or sensitive in responding to microbial challenges is becoming a feasible strategy to combat diseases. Thus, new biotechnological strategies are expected to be developed soon to strengthen immune responses and improve health in both plants and animals.

As describe above, activation of immunity mediated by OGs occurs in many plants and is effective against many microbes. The generation of elicitor-active oligogalacturonides is promoted by plant-encoded PGIPs, which may favor the *in vivo* accumulation of elicitor-active oligomers (Ferrari et al., 2013). To test the hypothesis that the accumulation of OGs *in vivo* is a consequence of the interaction of microbial PGs with plant PGIPs, Benedetti et al. (2015) produced Arabidopsis transgenic plants engineered to accumulate equimolar levels of a fungal PG and a plant PGIP and, consequently, endogenous OGs that would activate defense-related responses. Authors constructed a chimeric protein by fusing PG from the fungal pathogen *Fusarium phyllophilum* (*Fp*PG) to *Pv*PGIP2, a PGIP from common bean (*Phaseolus vulgaris*). *Pv*PGIP2 is the only known effective inhibitor of FpPG (Benedetti et al., 2011). *Pv*PGIP2 and *Fp*PG were linked by three alanine residues. The chimera was named the “OG-machine” (Figure 18).

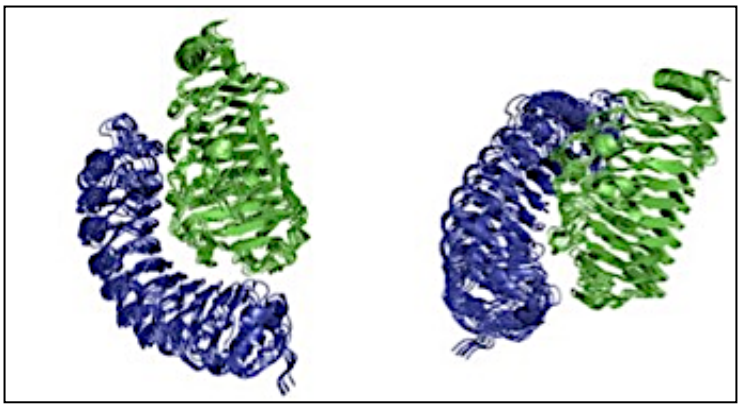


Figure 18. Three-dimensional structure of the chemically cross-linked complex between *Fp*PG (in green) and *Pv*PGIP2 (in blue). Two orthogonal viewpoints are reported (Adapted from Benedetti et al., 2011).

The rationale for constructing the OG-machine chimera and expressing it in transgenic plants was based on the hypothesis that PGIPs evolved not only to inactivate pathogen-encoded PGs, but also to regulate the activity of PGs so that they generate OGs of specific DP that function as elicitors of the plant defense response. Benedetti et al. (2015) demonstrated that the expression of the chimeric PGIP–PG protein in Arabidopsis plants causes the *in vivo* release of OG fragments, which triggered a defense response similar to that activated by exogenously applying purified OGs (Figure 19).

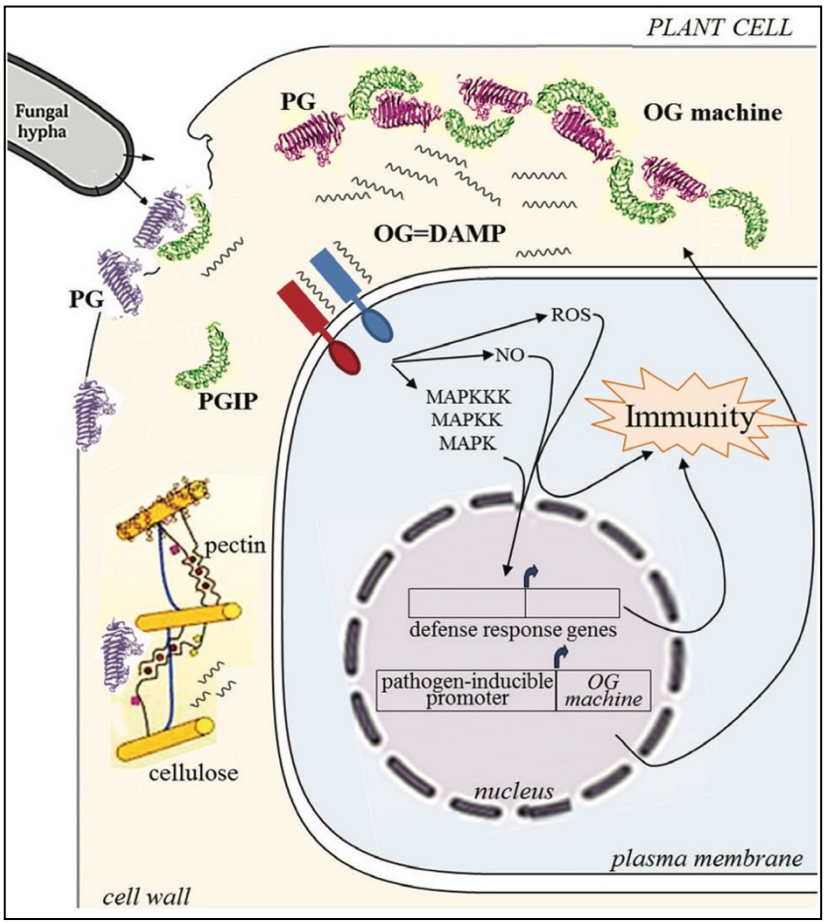


Figure 19. Schematic representation of the events occurring in the cell wall during a fungal attack of a plant tissue. Polygalacturonase (PG) secreted by the pathogen breaches the wall by degrading the pectin component. In the presence of the inhibitor PGIP degradation is slowed down and the formation of signal molecules (OGs) for the activation of immunity is favored. Expression under a pathogen-inducible promoter of a PG-PGIP chimera protein named OG-machine enhances the accumulation of OGs and immunity (Cervone et al., 2015).

However, when the OG-Machine was induced at high levels, plant growth was significantly reduced or arrested, suggesting that high concentrations of endogenous OGs interfere with normal developmental programs and lead to a deleterious hyperimmune response (Benedetti et al., 2015). This finding is consistent with the current notion that trade-off occurs between growth potential and the capacity for defense. Maintenance of immunity is costly and immune responses are typically counterbalanced by decreasing the allocation of resources to biomass production (Todesco et al., 2010).

However, by using a pathogen-inducible promoter, it is possible to engineer plants for the release of OGs only in the infection site, avoiding undesirable changes in growth and development. In fact, it was observed that the expression of the PGIP–PG chimeric protein under the control of the *AtPR1* gene resulted in increased resistance to infection (Benedetti et al., 2015), pointing to a possible biotechnological strategy to protect plants against

microbial disease by exploiting the plant innate immunity. A strategy that uses the OG-Machine to generate OGs, functioning as DAMPs, could potentially be used to engineer crops to be more resistant to diseases.

2. AIM OF THE THESIS

One of humanity's greatest challenges is satisfying the food demand for a rapid increase in global population, which is expected to exceed 9 billion by 2050 (Fróna et al., 2019). Cereals are cultivated in greater amounts for their edible caryopses and provide more food energy to humans than any other crop; wheat, maize, and rice are the most important crops worldwide (Bigini et al., 2021). Nowadays, worldwide crop losses from phytopathogens range from 17% to 30% for major agricultural crops, undermining the urgent goal of a 70% increase required to satisfy the food demand, directly affect food quality and human health (Botticella et al., 2021; Savary et al., 2019).

FHB is a destructive fungal disease mainly caused by *Fusarium graminearum* and can lead to a 50-70% loss of marketable grain. Besides reducing grain weight and quality during the infection, Fusarium species produce several trichothecene mycotoxins, including deoxynivalenol and nivalenol, which are toxic for humans and animals (Häggblom and Nordkvist, 2015) and, therefore, represent a significant hazard in the food chain (Magan and Aldred, 2007). On top of this, climate changes are predicted to positively impact on the frequency and severity of FHB epidemics (Jung et al., 2022).

FHB control strategies are still limited: in bread wheat, genetic variation for FHB resistance is large and a multitude of resistance sources from "foreign" and "native" wheat germplasm are known (Steiner et al., 2017). On the contrary, durum wheat is notorious for its extreme susceptibility to FHB (Miedaner et al., 2017) and breeding for FHB resistance is difficult due to the lack of resistance sources (Miedaner et al., 1997) and difficulties in efficiently combining the numerous small-effect resistance quantitative trait loci (Steiner et al., 2019). Therefore, new, harmless, and sustainable control strategies for FHB disease management are urgently required.

Given that i) sustainable increase in crop yield and resilience to biotic (and abiotic) stresses as well as the improvement in nutritional and healthiness values represent the main concomitant targets to be pursued in agriculture in the shortest period and ii) diminishing the usage of chemicals and toxic compounds would be very difficult to succeed with conventional breeding, the role of plant sciences and biotechnology becomes crucial to guide sustainable increases in agricultural production to feed the world’s population. Therefore, to find harmless control strategies for crop disease management, we need to exploit the plant innate immunity that, if timely activated, can efficiently contrast, and restrict plant infection by microorganisms. The faster pathogen detection occurs, the

sooner proper immune responses are mounted by plants, with a consequent higher probability to restrict or block the tissue invasion. The innate immunity represents the first step in defense against invaders and can be activated within a few minutes after sensing immunogenic signals derived both from pathogens or plant tissue damage (Abdul Malik et al., 2020). Among the current approaches to crop protection, the use of elicitors of immunity, able to activate the natural defense mechanisms of the plant, as alternatives/complementary to pesticides and genetic resistances, is a strategy rising increasing attention. Numerous studies indicate that local application of cell wall-derived elicitors, such as COS and OGs induce broad-spectrum, long-lasting, and systemic resistance against pathogens in different plant species, such as Arabidopsis, rice, grapevine and tomato (Ferrari et al., 2007; Aziz et al., 2004; Moscatiello et al., 2006). Based on the knowledge that OGs may activate a wide range of defense responses, manipulation of plant innate immunity by engineering OG sensing and signaling could be a valid approach to strengthen plant immune responses against a broad spectrum of microorganisms. During microbial infections, plant PGIP activity may favor the *in vivo* accumulation of elicitor-active OG oligomers by limiting the degradation of homogalacturonan (Ferrari et al., 2013). Indeed, the *in vivo* accumulation of OGs detected in engineered Arabidopsis transgenic plants expressing the OG-Machine under the control of a pathogen-inducible promoter contributed to enhance the resistance to a variety of pathogens, providing direct evidence for the function of OGs as elicitors of the plant defense response (Benedetti et al., 2015). Thus, the expression of OG-Machine to produce OGs acting as DAMPs could be a possible biotechnological strategy to engineer crops to be more resistant to microbial diseases.

On these bases, the aim of my study was to exploit the OG biology in triggering resistance against *F. graminearum* in durum wheat. Such objective was achieved by firstly investigating if OGs are perceived as danger signals capable of inducing immune responses and resistance to FHB in durum wheat plants. Moreover, I generated, characterized, and analyzed transgenic durum wheat lines expressing the OG-Machine chimera under the control of a pathogen-inducible promoter, i.e. *TaPR1.1*. The aim was to engineer wheat responses to *Fusarium graminearum* infection and to facilitate the elucidation of molecular mechanisms regulating plant defense activation upon sensing danger signals in cereals.

3. MATERIALS AND METHODS

3.1. Evaluation of OGs as elicitors of immunity effective in triggering resistance against *F. graminearum* in durum wheat

3.1.1. Preparation of OGs

Oligogalacturonides were prepared according to the protocol described in Pontiggia et al. (2015). High molecular weight un-methylated polygalacturonic acid (PGA; Alfa Aesar) was solubilized in 100 ml of sodium acetate 50 mM, pH 5.0 to a concentration of 2% (w/v). The solution was digested for 180 min with 0.018 RGU of *A. niger* endoPG, and the enzyme was inactivated by boiling the digest at 100°C for 10 min in a water bath. After enzyme inactivation, the sample was diluted with 50 mM sodium acetate to a concentration of 0.5% PGA. To precipitate OGs, ethanol was added to the digest to a final concentration of 17% (v/v); the sample was incubated overnight at 4°C with shaking and then centrifuged 30 min at 30000 g. OGs, recovered in the pellet, were re-dissolved in water, dialyzed against water in a dialysis tube with a molecular mass cut off (MWCO) of 1000 Da (Spectra/Por®), and lyophilized.

3.1.2. Influence of OGs on wheat morphological parameters

To assess OG-related effects on plant morphological parameters, durum wheat seeds cv. Svevo were surface sterilized with 75% (v/v) ethanol for 2 min followed by incubation in 40% (v/v) sodium hypochlorite for 15 min. Following sterilization, seeds were washed three times with sterile water and then placed in Petri dishes (90 mm diameter) containing sterile paper. Three days after germination, seedlings were transferred in sterile culture tubes containing half-strength Murashige and Skoog medium (2.245 g L^{-1} MS powder), supplemented with sucrose 0.5% (w/v) (Sigma-Aldrich, Burlington, MA, USA) and solidified with agar 0.8% (w/v) (Duchefa Biochemie, Haarlem, The Netherlands) at pH 5.8, in the absence or presence of different concentrations (10, 100, 250, 500 μg/ml) of purified OGs. Chitosan (100 μg/ml) was used as known compound affecting wheat growth in the experimental setup (Liu et al., 2021). Culture tubes were incubated vertically in a growth chamber at 24 °C with a 16 h light/8 h dark photoperiod. After seven days, fresh

weight, root length, first leaf length and stomatal density were measured for each seedling. The root length and the first leaf length of all plantlets were measured by using the freely available ImageJ software (http://rsbweb.nih.gov/ij/). To count stomata, I acquired fluorescence microscopic images of the first leaf central portion, between midrib and margin, and manually measured stomatal density. For each treatment, at least 10 seedlings were used, and three images were evaluated. Stomata density is intended as n° stomata/mm^2.

3.1.3. *Fusarium graminearum* infection assay in wheat seedlings

3.1.3.1. Plant and fungal growth conditions

Plant material was prepared according to the protocol described in Jia et al. (2017). Wheat seeds cv. Svevo were rinsed with running water and then soaked overnight at 4 °C in a 500 ml flask containing sterile water to help seeds to break dormancy and germinate faster. The imbibed seeds were placed in Petri dishes (90 mm diameter) containing 2 layers of sterile paper and germinated in the dark for 2 days at 25 °C in a growth chamber. Germinated seeds were then transplanted to 24-well cell culture plates, one seed per well, and grown in the growth chamber for 1 day under controlled environment conditions at 25 °C with a 18 h light/6 h dark photoperiod.

The fungal pathogen *Fusarium graminearum* strain 3827 was cultured on potato dextrose agar (PDA) medium (AppliChem GmbH; Darmstadt, Germany). To induce macroconidia production, the fungus was cultured at 25 °C on synthetic nutrient agar (SNA) medium (Urban et al., 2002) containing 0.1% KH_2PO_4, 0.1% KNO_3, 0.1% $MgSO_4*7H_2O$, 0.05% KCl, 0.02% glucose, 0.02% sucrose, 2% bactoagar (Becton Dickinson; New Jersey, USA). Macroconidia were harvested from 90 mm SNA agar plates after 10 days of incubation by adding 1 ml sterile water and scraping off conidiospores with a spatula. Conidia concentration was estimated by Thoma chamber, adjusting the concentration to the proper concentration for the infection assays. For long-term storage in -80 °C, conidiospore suspensions were prepared to a density of 10^6 spores ml^{-1} in 10% glycerol.

3.1.3.2. Wheat coleoptile inoculation

Inoculation assays on wheat coleoptiles cv. Svevo were conducted as reported by Jia et al. (2017). Three-day-old wheat seedlings were used. The top 1-2 mm of three-day-old wheat coleoptiles were cut off and inoculated with OGs (500 µg/ml), *Fusarium graminearum* spores (2 x 10^3 conidia) and a co-treatment of both OGs and fungus spores. Sterile water was used as mock treatment and chitosan (CHIT; 100 µg/ml) as a positive control in the experimental setup. The inoculated seedlings in a growth chamber for 7 days under controlled environment conditions at 24 °C with a 18 h light/6 h dark photoperiod and 95% relative humidity. Lesion size on coleoptiles of inoculated wheat seedlings were measured at different days post-infection and by photographing them with a ruler as reference. The final measurement was taken at 7 dpi. The longitudinal length of brown lesions on wheat coleoptiles was measured as the lesion size at the indicated time by using ImageJ software (http://rsbweb.nih.gov/ij/). For each experiment, at least 15 seedlings for treatment were used. For the expression analyses of plant immune-related marker genes and fungal genes, inoculated wheat seedlings were collected at 6, 24 and 48 hpi, frozen in liquid nitrogen and stored at -80 °C.

3.1.3.3. RNA sequencing

Three-day-old wheat seedlings were inoculated with OGs (10 and 500 µg/ml), Fusarium graminearum spores (2 x 10^3 conidia) and a co-treatment of both OGs and fungus spores. Sterile water was used as mock treatment and CHIT (100 µg/ml) as a positive control in the experimental setup. Three biological replicates, for each condition, were selected 48 hpi. For each sample, fresh plant material (approximately 100 mg) was frozen by using liquid nitrogen, ground in 2-ml tubes by using steel beads (diameter 0.4 mm) and homogenized through a TissueLyser II (Qiagen, Valencia, CA, USA). Total RNA was isolated by using a CTAB-based lysis buffer following the 'pine tree method' (Chang et al. 1993). The RNA pellet was resuspended in DEPC-treated water and quality of the extracted RNA was determined by using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation and RNA sequencing were performed by GENARTIS s.r.l. laboratories located in Verona (Italy). In total, 24 Illumina RNA-seq libraries were generated using the TruSeq stranded mRNA ligation kit (Illumina) from 700 ng of RNA samples, after poly(A) capture and according to manufacturer's

instructions. Using the Agilent 4150 Tape station (Agilent), capillary electrophoretic analysis was carried out to measure the quality and size of the RNAseq libraries. Libraries were quantified by real-time PCR against a standard curve with the KAPA Library Quantification Kit (KapaBiosystems, Wilmington, MA, USA) and sequenced with Illumina technology in 150PE mode on a Novaseq6000 sequencer.

The RNA-sequencing obtained reads were aligned to the transcriptome of durum wheat (Maccaferri et al., 2019) available at https://plants.ensembl.org/ using the *Salmon* software v1.4.0 (Kingsford, 2017). *Salmon* uses a quasi-mapping approach to align the reads, which allows for more sensitive and accurate alignment compared to traditional methods. This software estimates the relative abundance of different transcripts as transcripts per million (TPM), a normalization method computed considering the library size, number of reads and the effective length of the transcript (Kingsford, 2017). Raw read count and TPM data were generated from each *Salmon* output file, by combining isoform counts to obtain counts at gene level. The outputs including transcript lengths, count of aligned reads and abundance estimates were imported in DESeq2 by using the R package *tximport* (Soneson et al., 2015) using the *lengthScaledTPM* method. The data were used to identify differentially expressed genes (DEGs) using the *DESeq2* package (Love et al., 2014). The variance on reads count was calculated based on the three biological replicates per condition by applying a negative binomial distribution to model the count data and identify genes with significant changes in expression between the different conditions. The DEG identification was performed after normalization of the count data and correction for multiple testing, both accounted by DESeq2, through Wald test. Variance on reads count was calculated based on the three biological replicates per condition. A threshold of adjusted p-value <0.05 were used to identify DEGs. Both the identified DEGs and all transcripts of the *T. durum* Svevo transcriptome were annotated through Blast2GO (Conesa et al., 2005) to obtain an updated functional annotation and to assign their associated Gene Ontology (GO) terms. The identified DEGs were then analyzed for functional enrichment using Blast2GO to reveal the biological processes, pathways, or other functional categories that are enriched among the DEGs. To perform GO enrichment analysis and to provide a summary of the functions and pathways associated to the obtained sets of DEGs, ShinyGO v0.77 online tool was used (Ge et al., 2020).

3.1.4. *Fusarium graminearum* infection assay on wheat spikes

3.1.4.1. Plant and fungal growth conditions

Wheat seeds cv. Svevo were surface sterilized with 75% (v/v) ethanol for 2 min, 40% (v/v) sodium hypochlorite for 15 min and then rinsed thoroughly in sterile water. Seed germination was performed in Petri dishes (90 mm diameter) containing 2 layers of sterile paper. When seminal roots and the hypocotyls emerged, around 10 days post-germination, seedlings were transferred in jiffy pots with soil, and vernalized at 4°C for 1 month. Afterwards, plants were grown in a climatic chamber at 18 to 23°C with a 18 h light/6 h dark photoperiod. When plants presented the second/third leaf, they were two by two transferred in 14x14 cm pots.

PDA (AppliChem GmbH; Darmstadt, Germany) was used for the growth and maintenance of *Fusarium graminearum* strain 3827, and SNA medium was used to promote sporulation, as described previously in section 3.1.3.1.

3.1.4.2. Wheat spikes inoculation

Inoculation assays on wheat spikes cv. Svevo were conducted as reported by Makandar et al. (2012). At anthesis stage, inoculation of wheat plants was performed by single-spikelet inoculation. Briefly, the glumes of two opposite central florets of a wheat head were inoculated with OGs (500 µg/ml), *Fusarium graminearum* spores (2 x 10^4 conidia) and a co-treatment of both OGs and fungus spores. Sterile water was used as mock treatment and CHIT (100 µg/ml) as a positive control in the experimental setup. High humidity conditions were maintained for 3 days by covering the inoculated spikes with a plastic bag. Over time, the fungal infection spread out to the other spikelets within each spike. FHB disease symptoms were assessed by counting the number of visually diseased spikelets at different days post-infection (dpi) and by relating them to the total number of spikelets of the respective head, resulting in a percentage of symptomatic spikelets. The final count was taken at 21 dpi. For each experiment, at least 15 plants for treatment were used. For the expression analyses of plant immune-related marker genes and fungal genes, inoculated spikes were collected at 6, 24 and 48 hpi, frozen in liquid nitrogen and stored at -80 °C.

3.1.5. Quantification of fungal biomass in wheat seedlings and spikes

Genomic DNA from different inoculated seedlings and spikes was extracted with Nucleospin Plant II kit (Macherey-Nagel, Oensingen, Switzerland) according to manufacturer's instructions. After a quality control on electrophoresis gel and quantity check of gDNA through the spectrophotometer, a quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed. According to the manufacturer's instructions, 50 ng of gDNA were amplified in a 20-µL reaction mixture containing 1X SsoAdvanced Universal SYBR green Supermix (Biorad, Hercules, CA, USA) and 0.5 µM of each primer. qRT-PCR analysis was performed by using a CFX96 Real-Time System (Biorad, Hercules, CA, USA) and data analysis was done using LinRegPCR software. The DNA content of the *Fusarium graminearum* β-tubulin gene (*Fg β-tubulin*), relative to the wheat *TdACTIN* gene, was determined in wheat seedlings (7 dpi) and spikes (6, 24 and 48 hpi) inoculated with *Fg* spores and the co-treatment of both OGs and fungus spores. Data were determined using a modification of the Pfaffl method (Pfaffl, 2001) and expressed in arbitrary units. Primer sequences are shown in Table 1.

3.1.6. Gene expression analyses

RNA was extracted from different inoculated seedlings and spikes with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's instruction (Chomczynski and Sacchi, 2006). The optical density (OD) of 2 µL of extracted RNA was measured at 230, 260 and 280 nm with Thermo Scientific™ Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Oslo, Norway). The RNA concentration was converted from OD at 260 nm, and the purity was evaluated by the OD ratio at 260/280 nm for protein contamination and the OD ratio at 260/230 nm for polysaccharide, phenol, and other contaminations. RNA integrity was checked on 2% agarose gel by electrophoresis.

The reverse transcription was performed using the iScript gDNA clear cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. All reaction volumes were 20 µL, contained 1 µg of RNA. The mix was first processed on T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) at 25 °C for 5 min, 75 °C for 5 min to remove any genomic DNA contaminations. The iScript Reverse Transcription Supermix

was added, and the reverse transcription reaction was performed at 25 °C for 5 min, 46 °C for 20 min, 95 °C for 1 min.

Table 1. List of primers employed for qRT-PCR analysis.

Primer name	Sequence 5' -> 3'	Annealing temperature	Amplicon size
TdACTIN F	TCCTGTGTTGCTGACTGAGG	58 °C	236 pb
TdACTIN R	GGTCCAAACGAAGGATAGCA		
TdPR1 F	GAGAATGCAGACGCCCAAGC	58 °C	217 bp
TdPR1 R	CTGGAGCTTGCAGTCGTTGATC		
PvPGIP2 F	CCTCACCGGGAAGATTCCG	58 °C	238 bp
PvPGIP2 R	TTAGCTGCGTCAGTCCCTGC		
Fg β-tubulin F	ACTTCCGTCTGTTCCGTG	58 °C	171 bp
Fg β-tubulin R	TTCCATCTCGTCCATACCCT		
FgTRI5 F	TGAGCAGTACAACTTTGGAGG	58 °C	130 bp
FgTRI5 R	ATGCTTCCGCTCATCAAACAG		
FgTRI6 F	TCTTTGTGAGCGGACGGGACTTTA	58 °C	245 bp
FgTRI6 R	ATCTCGCATGTTATCCACCCTGCT		

According to the manufacturer's instructions, cDNA was amplified from 50 ng of total RNA in a 20-µL reaction mixture containing 1× SsoAdvanced Universal SYBR green Supermix (Biorad, Hercules, CA, USA) and 0.5 µM of each primer. qRT-PCR analysis was performed by using a CFX96 Real-Time System (Biorad, Hercules, CA, USA). Three technical replicates were performed for each sample, and data analysis was done using LinRegPCR software. The expression of plant immune-related marker gene *TdPR1* was normalized to *TdACTIN* housekeeping gene, while the expression of *FgTRI5* and *FgTRI6* genes was normalized to *Fg β-tubulin* gene. The expression levels of each gene were determined using a modification of the Pfaffl method (Pfaffl, 2001) and expressed in arbitrary units. Primer sequences are shown in Table 1.

3.1.7. Impact of OGs on wheat agronomical and growth parameters

To assess the impact of OGs on wheat agronomical and growth parameters of *Fg* infected plants, at the end of infection experiment we evaluated the number of seeds per spike, the number of seeds per spikelet, the primary spike weight, the weight of seeds per spike and the weight of 1000 seeds. The number of seeds per spike and per spikelet were

calculated as the number of fully filled seeds per spike and per spikelet, respectively. The primary spike weight was measured in grams as well as the weight of seeds per spike and the weight of 1000 seeds. For each treatment, 15 plants were chosen for the evaluation and average number of the measurements was reported for each parameter considered. In addition, I also measured the length and the width of seeds by photographing them with a ruler as reference and using ImageJ software (http://rsbweb.nih.gov/ij/).

3.1.8. Statistical Analysis

Data were analyzed with Student's t-test or ANOVA by using the SYSTAT12 software (Systat Software Incorporated, San Jose, CA, USA). When significant F values were observed ($p < 0.05$), a pairwise analysis was carried out by the Tukey Honestly Significant Difference test (Tukey test).

3.2. Analysis of engineered durum wheat plants with altered endogenous OG levels

3.2.1. Preparation of construct

The pAHC17_TaPr1.1::OGM construct (Figure 20) was prepared by inserting the complete coding region of OGM into the BamHI site of pAHC17 (Christensen and Quail, 1996) under control of the *Triticum aestivum* Pr1.1 promoter and NOS terminator.

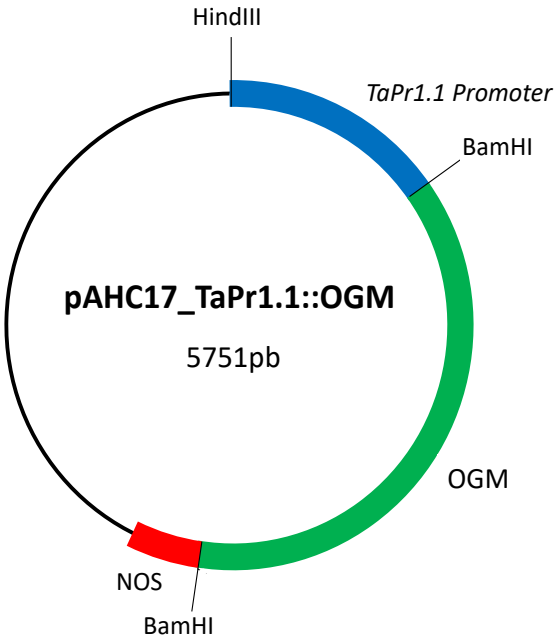


Figure 20. Schematic representation of pAHC17_TaPr1.1::OGM construct with main restriction sites.

3.2.2. Transgenic plants production

3.2.2.1. Media

Modified Murashige and Skoog (MS) media for wheat cellular cultures were used (Sears and Deckard, 1982) with MS Salt (Duchefa Biochemie; Haarlem, Netherlands), Maltose, Thiamine-HCl, L-asparagine at pH 5.85 and Phytagel as gelling agent. Media composition varied in the different phases of plant production (Dissection, Bomb, Regeneration and Rooting) as indicated in Weeks et al. (1993). The different medium compositions are reported are reported in Table 2. After autoclaving at 121°C for 20 min, 2,4-dichlorophenoxyacetic acid (2,4-D) was added and the medium poured in 100 mm x 15 mm Petri dishes for Dissection and Regeneration media, in 60 mm x 15 mm Petri dishes for Bomb medium, and in Magenta vessels for Rooting medium. Selection marker phosphinotricin (PPT; Duchefa Biochemie) is present only in regeneration and rooting media.

Table 2. Culture media and compositions.

Medium	Composition
Dissection	• Murashige & Skoog Salt mixture 4.3 g/l • Maltose 40 g/l • Thiamine-HCl (25 mg/500 ml) 10 ml/l • L-asparagine 0.15 g/l • 0.25% v/v phytagel • 2,4 D (0.5 mg/ml) 2 ml/500 ml
Recovery	• Murashige & Skoog Salt mixture 4.3 g/l • Maltose 40 g/l • Thiamine-HCl (25 mg/500 ml) 10 ml/l • L-asparagine 0.15 g/l • 0.25% v/v phytagel • 2,4 D (0.5 mg/ml) 2 ml/500 ml
Bombardment (Bomb sucrose)	• Murashige & Skoog Salt mixture 4.3 g/l • Maltose 40 g/l • Thiamine-HCl (25 mg/500 ml) 10 ml/l • L-asparagine 0.15 g/l • 0.25% v/v phytagel • 2,4 D (0.5 mg/l) 2 ml/500 ml • Sucrose 171.5 g/l
Regeneration	• Murashige & Skoog Salt mixture 4.3 g/l • Maltose 40 g/l • Thiamine-HCl (25 mg/500 ml) 10 ml/l • L-asparagine 0.15 g/l • 0.25% v/v phytagel • 2,4 D (0.5 mg/ml) 0.2 ml/500 ml • PPT (5 mg/l)
Rooting	• Murashige & Skoog Salt mixture 2.15 g/l • Maltose 20 g/l • Thiamine-HCl (25 mg/500 ml) 5 ml/l • L-asparagine 0.075 g/l • 0.25% v/v phytagel • PPT (5 mg/l)

3.2.2.2. Embryo isolation

Caryopses at 10 to 18 days post-anthesis from *T. durum* cv Svevo plants, grown in the field, were collected and surface-sterilized using sodium hypochlorite 1% (10 min), 70% ethanol (15 min) followed by three times washing (5 min) with distilled sterile water.

Immature embryos of 0.5 to 1 mm long were aseptically removed under the stereo microscope and placed with the scutella exposed on the Dissection medium (Figure 21A). Collected embryos were stored in the dark at 23° C for 5 days to induce callus formation.

3.2.2.3. DNA-coating of gold particles

To perform a bombardment experiment for the genetic transformation, gold nanoparticles coating with the plasmidic DNA was required. Thirty mg of gold particles (0.6 μm) (Bio-Rad Laboratories; Segrate, Italy) were resuspended in 500 μL of 100% ethanol. Thirty-five μL of the suspension were aliquoted into 1.5 ml tubes, quickly centrifuged and the ethanol removed. The microprojectiles were then washed with cold sterile water (200 μL), spun and the supernatant discarded. Afterwards, plasmidic DNA pAHC17_TaPr1.1::OGM and the pUBI::BAR marker plasmid, carrying the BAR gene resistance for the phosphinotricin (PPT) herbicide, were added to the gold particles for the co-bombardment, using a 3:1 molar ratio. The following formulas were used to calculate the μL of plasmid DNA needed for coating the gold particles, using 15 μg of pUBI::BAR:

- Gene plasmid (pAHC17_TaPr1.1::OGM) (μL): bp gene/bp marker x 15 μg x (1/ plasmid concentration) x 3.
- Marker plasmid (μL): 15 μg x (1/plasmid concentration).

The microprojectiles pellet was resuspended in a solution containing 2.5 M CaCl2 (250 μL), spermidine (50 μL), plasmid DNA and water (250 μL - μL of plasmid). The tubes were shaken with a vortex mixer at 4° C for 20 min and briefly centrifuged. The supernatant was removed, and the pellet was washed with 600 μL of 100% ethanol. The DNA-coated gold pellets were resuspended in 36 μL of 100% ethanol and stored on ice until they were used.

3.2.2.4. Bombardment

Constructs were introduced into immature embryos-derived calli by biolistic bombardment. About 100 embryos-derived calli were transferred in each of the Bomb medium plate 4 h before the bombardment. For each bombardment, 10 μL of the DNA-gold suspension were placed in the center of a macrocarrier disk and bombarded using a Model PDS-1000/He Biolistic particle delivery system (Bio-Rad Laboratoires) (Figure

21B) as described in Weeks et al. (1993). The distance between the macrocarrier disk and the target was 13 cm, and the rupture disc strength was 1100 psi. Immediately after the bombardment, calli were kept in the bombardment medium and stored in the dark at 23°C for 24 h. The day after the bombardment, calli were transferred in the recovery medium for 4 weeks and sub-cultured in a fresh medium every 2 weeks. During the recovery stage, calli were kept at 23°C in the dark.

3.2.2.5. Plant regeneration

After 4 weeks, calli were transferred to the regeneration medium containing the PPT herbicide as selective agent, where they were kept for 6 weeks and sub-cultured in a fresh medium every 2 weeks. During the regeneration stage, calli were kept at 23°C with 16 hours light/8 hours dark photoperiod. Usually starting from the third week, resistant calli regenerated new shoots (Figure 21C).

After 6 weeks, the regenerated shoots were individually transferred to Magenta vessels containing the rooting medium, under herbicide selection, for additional 2-3 weeks with 16 hours light/8 hours dark photoperiod. At this stage, plants capable to form long, highly branched roots in the selective medium were considered resistant (Figure 21C). Sensitive plantlets exhibited yellow necrosis, and reduced vigor within 1 week and did not produce roots, whereas resistant plantlets thrived in the rooting media.

Once the regenerated plantlets had sufficient roots and leaves, they were transferred into pots and kept in a growth chamber for 5-10 days at 23°C with 16 hours light/8 hours dark photoperiod. Plants were covered with plastic bags to maintain a high humidity and to allow them to acclimatize to the *ex-vitro* conditions (Figure 21D). After 5-10 days, plants were transferred to bigger pots; these primary regenerants are called T0 plants (Figure 21D).

Figure 21. Production of transgenic wheat plants. **A)** Dissection of embryos from caryopsis placed in dissection media for callus induction; **B)** Model PDS-1000/He Biolistic particle delivery system (Bio-Rad Laboratories) used for bombardment; **C)** Plant regeneration: calli in regeneration medium (left) and plantlet in magenta vessels with rooting medium (right); **D)** Plantlets in soil for acclimatation (left) and regenerated plants (right).

3.2.3. Screening of regenerated wheat plants

Genomic DNA was extracted from the second fully expanded leaf of regenerated plants and wild type plants cv. Svevo with Nucleospin Plant II kit (Macherey-Nagel, Oensingen, Switzerland) according to manufacturer's instructions.

After a quality control on electrophoresis gel and quantity check of gDNA through the spectrophotometer, a polymerase Chain Reaction (PCR) was conducted in order to verify the presence of transgene in the regenerated wheat plants. To avoid any interference with the endogenous wheat PGIPs, specific primer pairs were designed on the sequence of *Phaseolus vulgaris* polygalacturonase-inhibiting protein 2 (*Pv*PGIP2; accession number AJ786409.1). Wheat *TdACTIN* gene was used to verify the presence of gDNA for each line. The PCR reaction was performed using 50 ng of the extracted DNA in a 20-µL reaction mixture containing 2X GoTaq PCR Master Mix (Promega, Madison, Wisconsin, USA) and 0.5 µM of each primer. The following amplification conditions were employed:

1 cycle at 95°C for 2 min; 35 cycles at 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec; a final step at 72°C for 5 min.

At the end of the screening, three OG-Machine (OGM) transgenic lines (#12, #29, #43) were selected and analyzed.

A quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was also performed to identify the number of transgene insertions in each OGM line considered. The DNA content of the *PvPGIP2* gene, relative to the wheat *TdACTIN* gene, was determined in OGM and Svevo lines. Data were determined using a modification of the Pfaffl method (Pfaffl, 2001) and expressed in arbitrary units. Primer sequences are shown in Table 1.

3.2.4. *Fusarium graminearum* infection assay on wheat OG-machine seedlings

Plant material was prepared according to the protocol described in Jia et al. (2017). Wheat seeds cv. Svevo and TaPR1.1::OGM transgenic lines were rinsed with running water and then soaked overnight at 4 °C in a 500 ml flask containing sterile water to help seeds to break dormancy and germinate faster. The imbibed seeds were placed in Petri dishes (90 mm diameter) containing 2 layers of sterile paper and germinated in the dark for 2 days at 25 °C in a growth chamber. Germinated seeds were then transplanted to 24-well cell culture plates, one seed per well, and grown in the growth chamber for 1 day under controlled environment conditions at 25 °C with a 18 h light/6 h dark photoperiod.

The fungal pathogen *Fusarium graminearum* strain 3827 was cultured on potato dextrose agar (PDA) medium (AppliChem GmbH; Darmstadt, Germany). In order to produce macroconidia, the fungus was cultured at 25 °C on synthetic nutrient agar (SNA) medium (Urban et al., 2002) containing 0.1% KH_2PO_4, 0.1% KNO_3, 0.1% $MgSO_4*7H_2O$, 0.05% KCl, 0,02% glucose, 0.02% sucrose, 2% bactoagar (Becton Dickinson; New Jersey, USA). Macroconidia were harvested from 90 mm SNA agar plates after 10 days of incubation by adding 1 ml sterile water and scraping off conidiospores with a spatula. Conidia concentration was estimated by Thoma chamber, adjusting the concentration to the proper concentration for the infection assays. For long-term storage in -80 °C, conidiospore suspensions were prepared to a density of 10^6 spores ml^{-1} in 10% glycerol.

3.2.4.1. Plant and Fungal growth conditions

Plant material was prepared according to the protocol described in Jia et al. (2017). Wheat seeds cv. Svevo and TaPR1.1::OGM transgenic lines were rinsed with running water and then soaked overnight at 4 °C in a 500 ml flask containing sterile water to help seeds to break dormancy and germinate faster. The imbibed seeds were placed in Petri dishes (90 mm diameter) containing 2 layers of sterile paper and germinated in the dark for 2 days at 25 °C in a growth chamber. Germinated seeds were then transplanted to 24-well cell culture plates, one seed per well, and grown in the growth chamber for 1 day under controlled environment conditions at 25 °C with a 18 h light/6 h dark photoperiod.

The fungal pathogen *Fusarium graminearum* strain 3827 was cultured on potato dextrose agar (PDA) medium (AppliChem GmbH; Darmstadt, Germany). In order to produce macroconidia, the fungus was cultured at 25 °C on synthetic nutrient agar (SNA) medium (Urban et al., 2002) containing 0.1% KH2PO4, 0.1% KNO3, 0.1% MgSO4*7H2O, 0.05% KCl, 0,02% glucose, 0.02% sucrose, 2% bactoagar (Becton Dickinson; New Jersey, USA). Macroconidia were harvested from 90 mm SNA agar plates after 10 days of incubation by adding 1 ml sterile water and scraping off conidiospores with a spatula. Conidia concentration was estimated by Thoma chamber, adjusting the concentration to the proper concentration for the infection assays. For long-term storage in -80 °C, conidiospore suspensions were prepared to a density of 10^6 spores ml^{-1} in 10% glycerol.

3.2.4.2. Wheat coleoptile inoculation

Inoculation assays on wheat coleoptiles cv. Svevo and TaPR1.1::OGM transgenic lines were conducted as reported by Jia et al. (2017). Three-day-old wheat seedlings were used. The top 1-2 mm of three-day-old wheat coleoptiles were cut off and inoculated *Fusarium graminearum* spores (2 x 10^3 conidia). Sterile water was used as mock treatment. The inoculated seedlings in a growth chamber for 7 days under controlled environment conditions at 24 °C with a 18 h light/6 h dark photoperiod and 95% relative humidity. Lesion size on coleoptiles of inoculated wheat seedlings were measured at different days post-infection and by photographing them with a ruler as reference. The final measurement was taken at 7 dpi. The longitudinal length of brown lesions on wheat coleoptiles was measured as the lesion size at the indicated time using ImageJ software

(http://rsbweb.nih.gov/ij/). For each experiment, at least 15 seedlings for each line were used. For the expression analyses of plant immune-related marker genes and fungal genes, inoculated seedlings were collected at 48 and 72 hpi, frozen in liquid nitrogen and stored at -80 °C.

3.2.5. *Fusarium graminearum* infection assay on wheat spikes

3.2.5.1. Plant and Fungal growth conditions

Wheat seeds cv. Svevo and TaPR1.1::OGM transgenic lines were surface sterilized with 75% (v/v) ethanol for 2 min, 40% (v/v) sodium hypochlorite for 15 min and then rinsed thoroughly in sterile water. Seed germination was performed in Petri dishes (90 mm diameter) containing 2 layers of sterile paper. When seminal roots and the hypocotyls emerged, around 10 days post-germination, seedlings were transferred in jiffy pots with soil, and vernalized at 4°C for 1 month. Afterwards, plants were grown in a climatic chamber at 18 to 23°C with a 18 h light/6 h dark photoperiod. When plants presented the second/third leaf, they were two by two transferred in 14x14 cm pots.

PDA (AppliChem GmbH; Darmstadt, Germany) was used for the growth and maintenance of *Fusarium graminearum* strain 3827, and SNA medium was used to promote sporulation, as described previously in section 3.2.4.1.

3.2.5.2. Wheat spikes inoculation

Inoculation assays on wheat spikes cv. Svevo and TaPR1.1::OGM transgenic lines were conducted as reported by Makandar et al. (2012). At anthesis stage, inoculation of wheat plants was performed by single-spikelet inoculation. Briefly, the glumes of two opposite central florets of a wheat head were inoculated with *Fusarium graminearum* spores (2 x 10^4 conidia). Sterile water was used as mock treatment. High humidity conditions were maintained for 3 days by covering the inoculated spikes with a plastic bag. Over time, the fungal infection spread out to the other spikelets within each spike. FHB disease symptoms were assessed by counting the number of visually diseased spikelets at different days post-infection and by relating them to the total number of spikelets of the respective head, resulting in a percentage of symptomatic spikelets. The final count was taken at 21 dpi. For each experiment, at least 15 plants for each line were used. For the expression analyses of

plant immune-related marker genes and fungal genes, inoculated spikes were collected at 48 and 72 hpi, frozen in liquid nitrogen and stored at -80 °C.

3.2.6. Quantification of fungal biomass in wheat seedlings and spikes

Genomic DNA from different inoculated seedlings and spikes of each line was extracted with Nucleospin Plant II kit (Macherey-Nagel, Oensingen, Switzerland) according to manufacturer's instructions. After a quality control on electrophoresis gel and quantity check of gDNA through the spectrophotometer, a quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed. According to the manufacturer's instructions, 50 ng of gDNA were amplified in a 20-µL reaction mixture containing 1X SsoAdvanced Universal SYBR green Supermix (Biorad, Hercules, CA, USA) and 0.5 µM of each primer. qRT-PCR analysis was performed by using a CFX96 Real-Time System (Biorad, Hercules, CA, USA) and data analysis was done using LinRegPCR software. The DNA content of the *Fusarium graminearum* β-tubulin gene (*Fg β-tubulin*), relative to the wheat *TdACTIN* gene, was determined in wheat seedlings and spikes inoculated with *Fusarium graminearum* spores (2 x 10^3 conidia) at 48 and 72 hpi. Data were determined using a modification of the Pfaffl method (Pfaffl, 2001) and expressed in arbitrary units. Primer sequences are shown in Table 1.

3.2.7. Gene expression analyses

RNA was extracted from inoculated seedlings and spikes with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's instruction (Chomczynski and Sacchi, 2006). The optical density (OD) of 2 µL of extracted RNA was measured at 230, 260 and 280 nm with Thermo Scientific™ Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Oslo, Norway). The RNA concentration was converted from OD at 260 nm, and the purity was evaluated by the OD ratio at 260/280 nm for protein contamination and the OD ratio at 260/230 nm for polysaccharide, phenol and other contaminations. RNA integrity was checked on 2% agarose gel by electrophoresis.

The reverse transcription was performed using the iScript gDNA clear cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. All reaction volumes were 20 µL, contained 1 µg of RNA. The mix was first processed on

T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) at 25 °C for 5 min, 75 °C for 5 min to remove any genomic DNA contaminations. The iScript Reverse Transcription Supermix was added, and the reverse transcription reaction was performed at 25 °C for 5 min, 46 °C for 20 min, 95 °C for 1 min.

According to the manufacturer's instructions, cDNA was amplified from 50 ng of total RNA in a 20-µL reaction mixture containing 1× SsoAdvanced Universal SYBR green Supermix (Biorad, Hercules, CA, USA) and 0.5 µM of each primer. qRT-PCR analysis was performed by using a CFX96 Real-Time System (Biorad, Hercules, CA, USA). Three technical replicates were performed for each sample, and data analysis was done using LinRegPCR software. The expression of *PvPGIP2* gene and plant immune-related marker gene *TdPR1* were normalized to *TdACTIN* housekeeping gene, while the expression of *FgTRI5* and *FgTRI6* genes was normalized to *Fg β-tubulin* gene. The expression levels of each gene were determined using a modification of the Pfaffl method (Pfaffl, 2001) and expressed in arbitrary units. Primer sequences are shown in Table 1.

3.2.8. Phenotypical characterization of OG-Machine durum wheat transgenic lines

In order to characterize the phenotype of TaPR1.1::OGM transgenic lines, the plant height and the numbers of spikes per plant were evaluated at the end of flowering. Wheat cv. Svevo was used as the wild type control. The plant height was measured by a tape measure, while the numbers of spikes per plant was obtained by counting the number of spikes per plant. For each line, 15 plants were chosen for the evaluation and average number of the measurements was reported for each parameter considered.

3.2.9. Impact of OG-Machine on wheat agronomical and growth parameters

To assess the impact of OG-Machine on wheat agronomical and growth parameters of *Fg* infected plants, at the end of infection experiment we evaluated the number of seeds per spike, the number of seeds per spikelet, the primary spike weight, the weight of seeds per spike and the weight of 1000 seeds. The number of seeds per spike and per spikelet were calculated as the number of fully filled seeds per spike and per spikelet, respectively. The primary spike weight was measured in grams as well as the weight of seeds per spike and

the weight of 1000 seeds. For each line, 15 plants were chosen for the evaluation and average number of the measurements was reported for each parameter considered. In addition, we measured also the length and the width of seeds by photographing them with a ruler as reference and using ImageJ software (http://rsbweb.nih.gov/ij/).

3.2.10. Statistical Analysis

Data were analyzed with Student's t-test or ANOVA by using the SYSTAT12 software (Systat Software Incorporated, San Jose, CA, USA). When significant F values were observed ($p < 0.05$), a pairwise analysis was carried out by the Tukey Honestly Significant Difference test (Tukey test).

4. RESULTS

4.1. OG evaluation as elicitors of immunity in durum wheat - *F. graminearum* interaction

4.1.1. Effects of OG treatments on durum wheat morphological parameters

To assess OG-related effects on plant morphological parameters, durum wheat seedlings, cv. Svevo, were grown on MS medium in the absence or presence of different concentrations (10, 100, 250, 500 µg/ml) of purified OGs for 7 days. CHIT (100 µg/ml) was used as a positive control (Liu et al., 2021).

None of the OG treatments altered fresh weight, root length and first leaf length. Conversely, CHIT strongly inhibited all growth-related parameters considered (Figure 22A-C). Indeed, in the presence of CHIT, seedling fresh weight, root length and first leaf length were inhibited by 57.4%, 83.2% and 29,7%, respectively.

Interestingly, seedlings treated with CHIT also displayed a significant stomatal density increase by 16,6% compared to the mock treated ones (Figure 22D). A similar behavior was also observed in seedlings treated with both OG 10 µg/ml and 100 µg/ml, which displayed an increased stomatal density by 14,7% and 22,4%, respectively, compared to the control condition. On the contrary, higher concentrations of OGs, i.e. 250 µg/ml and 500 µg/ml, caused a significant stomatal density decrease by 9,7% and 34,2%, respectively (Figure 22D). It is worth noting that such dose-dependent response to OGs recalls plant response to phytohormones, like auxins (Figure 23A-B).

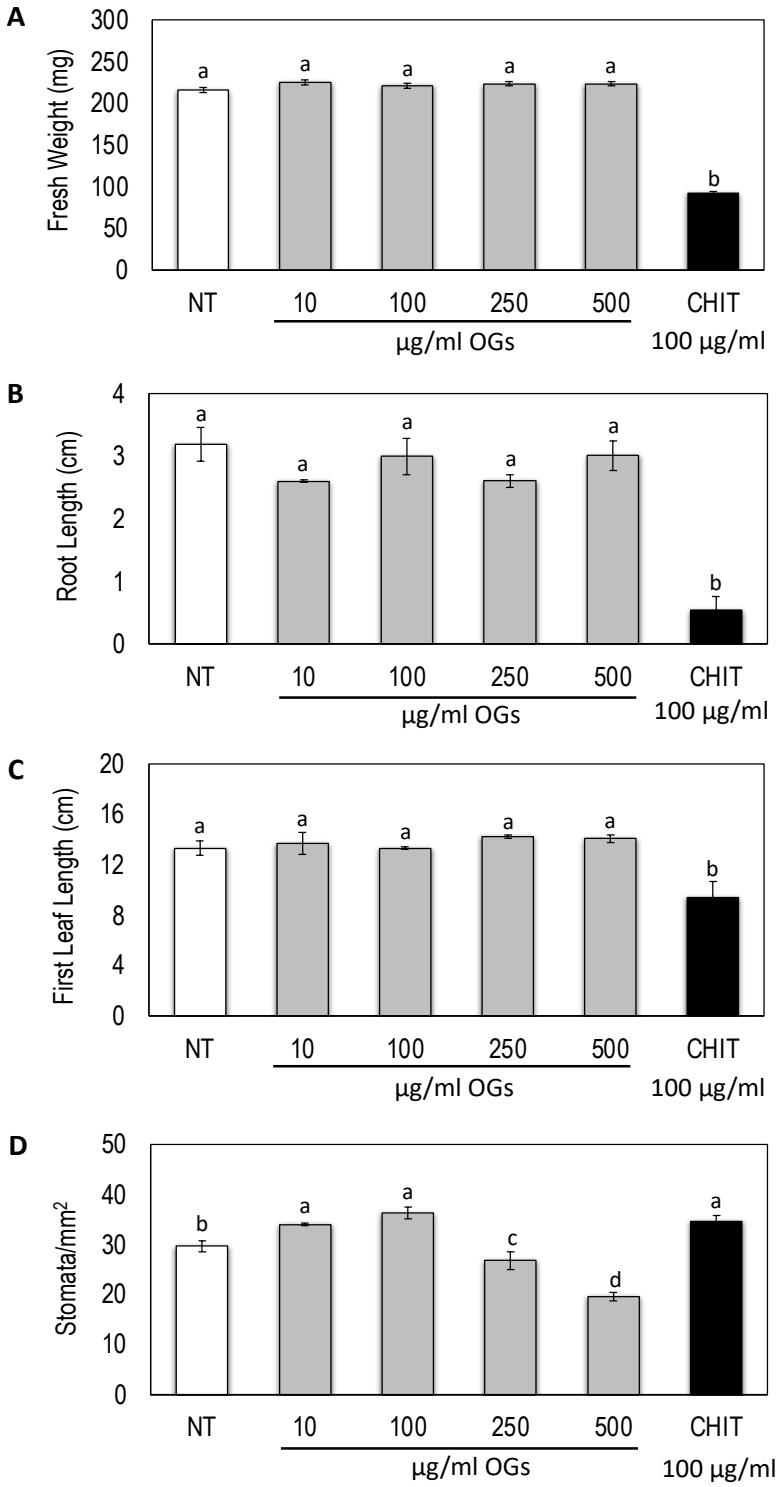


Figure 22. Effects on morphological parameters of wheat seedlings grown in MS medium in the absence or presence of different concentrations of purified OGs or CHIT 100 µg/ml. A) Fresh weight; **B)** Root length; **C)** First leaf length; **D)** Stomatal density expressed as stomata/mm^2. The measurements were carried out 7 days after transplanting in MS medium. The statistical significance was tested by means of ANOVA followed by Tukey test. Different letters indicate statistically different values ($p < 0.05$).

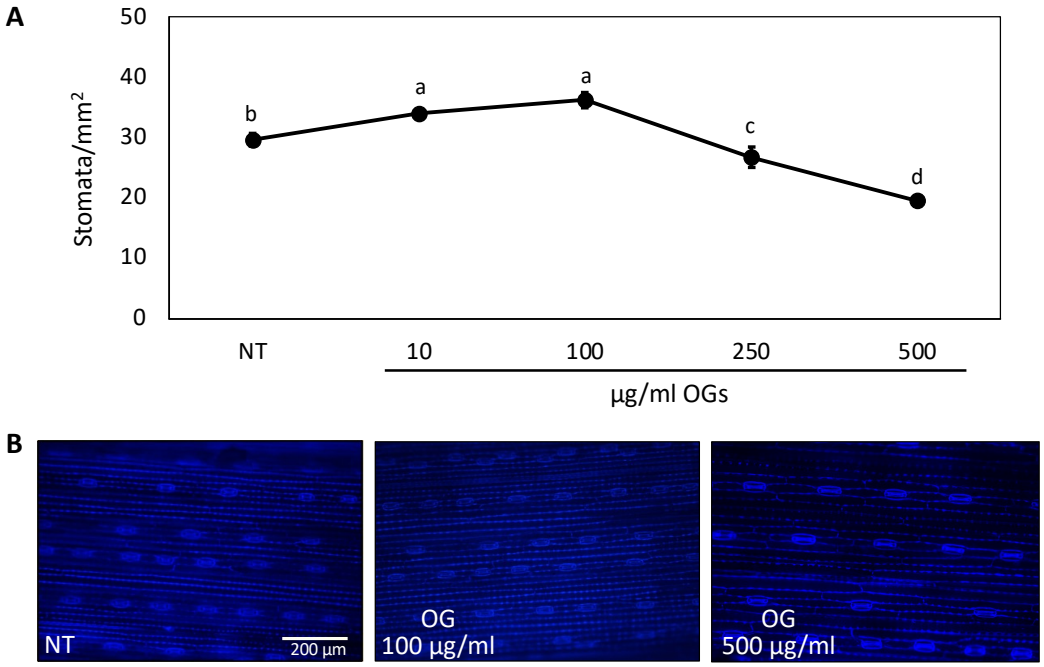


Figure 23. Oligogalacturonide treatments affect stomatal density in a dose-dependent manner. **A)** Variation of stomatal density in relation to dose-dependent response to OGs; **B)** Images showing stomata distribution in untreated and OG-treated wheat first leaf. The measurements and images were taken 7 days post seedlings transplanting in MS medium. The statistical significance was tested by means of ANOVA followed by Tukey test. Different letters indicate statistically different values ($p < 0.05$).

4.1.2. OG-triggered resistance to *Fusarium graminearum* in durum wheat seedlings

To evaluate the functionality of OGs as elicitors of immunity and their ability to restrict phytopathogen fungal growth in durum wheat coleoptiles, three-day-old seedlings were inoculated either with OGs at different concentrations (10, 100, 500 µg/ml), *Fusarium graminearum* spores (2 x 10^3 conidia) or a co-treatment of both OGs and fungus spores. Sterile water was used as mock treatment and CHIT (100 µg/ml) as a positive control in the experimental setup.

In the presence of co-treatment with both OGs or CHIT and the fungus spores, disease lesion size and fungal accumulation, evaluated at different dpi, were significantly lower compared to seedlings treated with the fungus spores only (Figure 24A-C).

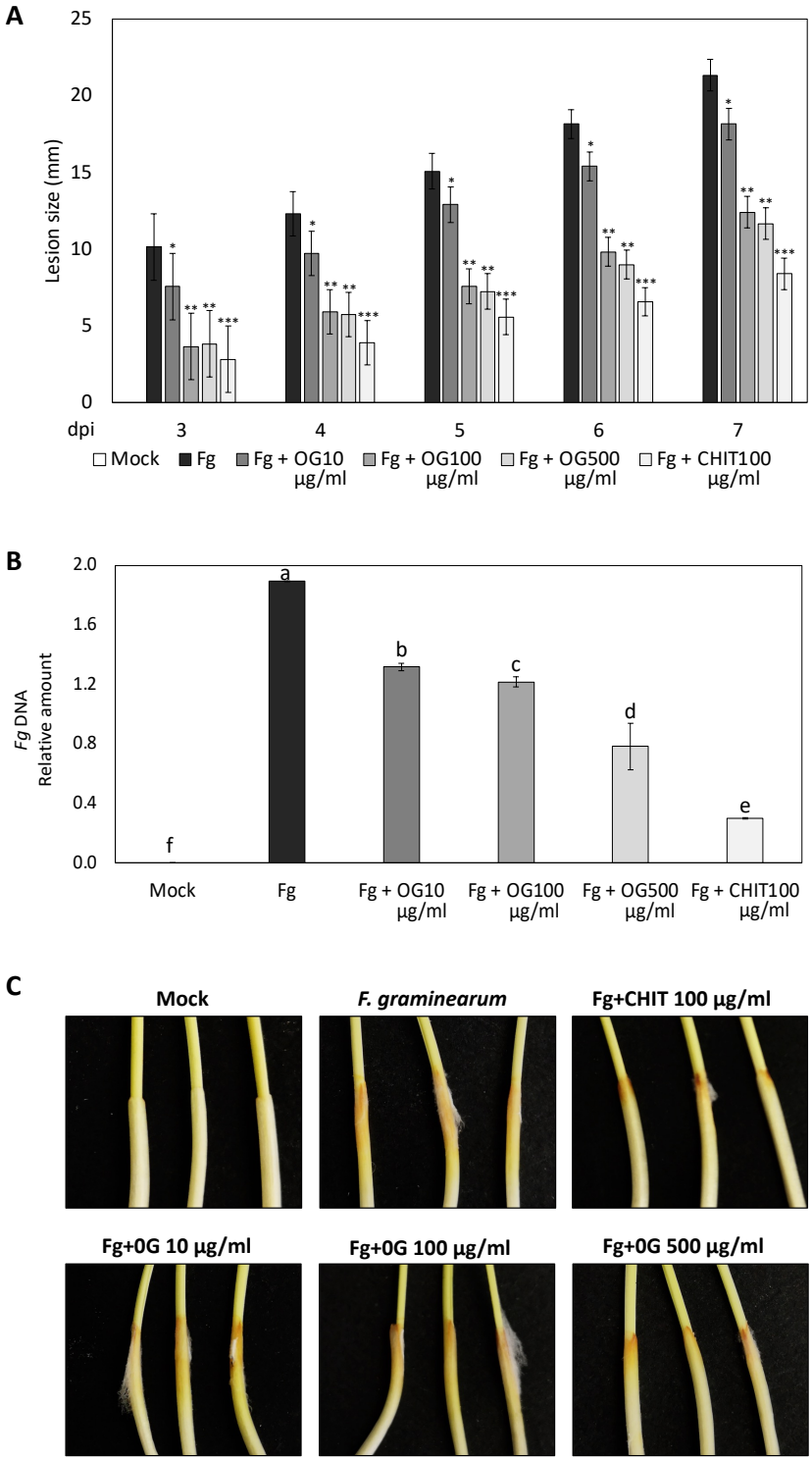


Figure 24. OGs trigger resistance to FHB in a dose-dependent manner. **A)** Coleoptile lesion size in the cv. Svevo inoculated with *Fg* and *Fg* + OG co-treatment. Disease was monitored for 7 days post inoculation (dpi); **B)** Real- time PCR analysis of DNA content of Fg *β-tubulin* gene relative to the wheat *TdACTIN* gene in wheat coleoptiles at 7 dpi; **C)** Photographs showing disease spread in representative wheat coleoptiles for each treatment condition. Photographs were taken at 7 dpi. All values are the means ± SE (n = 10). Asterisks above the bars indicate values that are significantly different from seedlings inoculated with the *Fg* alone (*** $p < 0.01$, student t-test). The statistical significance was tested by means of ANOVA followed by Tukey test. Different letters indicate statistically different values ($p < 0.05$).

Interestingly, OGs trigger resistance to FHB in a dose-dependent manner, with the higher OG concentration used, displaying the greater reduction in symptoms as well as in fungal accumulation. In particular, at 7 dpi, seedlings co-treated with OG 10, 100 and 500 µg/ml showed a significant disease lesion size reduction of 14.8%, 41.8% and 45.3%, respectively. Seedlings inoculated with both CHIT and *Fg* displayed a reduction of 60.6% compared to seedlings treated with the fungus alone. Moreover, results showed that the exogenous application of OG or CHIT strongly limited the fungal growth and accumulation. Indeed, in the presence of OG 10, 100 and 500 µg/ml, the fungal amount was decreased by 30,4%, 35,7% and 58,6% compared to seedlings treated with the fungus alone, whereas co-treatment with CHIT and *Fg* restricted the fungal amount by 84%.

4.1.2.1. Gene expression reprogramming by OGs in durum wheat seedlings

Given the high similarity between the results obtained in seedlings and spikes infected with *Fg* in presence or absence of immune elicitors (see section 4.1.3.) and to obtain a higher grade of reproducibility, we adopted the protocol published by Jie et al. (2017) for large-scale analyses. In this regard, RNAseq was carried out to obtain a general view of the transcriptome in durum wheat seedlings inoculated with OGs at different concentrations (10 and 500 µg/ml), *Fusarium graminearum* spores (2 x 10^3 conidia) or a co-treatment of both OGs and fungus spores. Sterile water was used as mock treatment and CHIT (100 µg/ml) as a positive control in the experimental setup.

Sequencing of RNA samples produced on average 36,994,174 of reads per sample (ranging from 24,463,053 to 55,619,350). Reads mapped to the durum wheat transcriptome using *Salmon* resulted in an average mapping rate of 78.67% (ranging from 67.26 to 88.34%). The data distribution is evident by observing results from multidimensional analysis (Principal component analysis, PCA). The PCA of count data, transformed by the 'vst' function of DESeq2, showed that RNA samples of *F. graminearum* inoculated plants clustered far from the uninoculated ones, except for samples of plants inoculated with CHIT and *Fg* (Figure 25), that grouped in between the two groups. A limited variability was observed for samples of uninoculated plants, while biological replicates of plants affected by the pathogen resulted relatively dispersed.

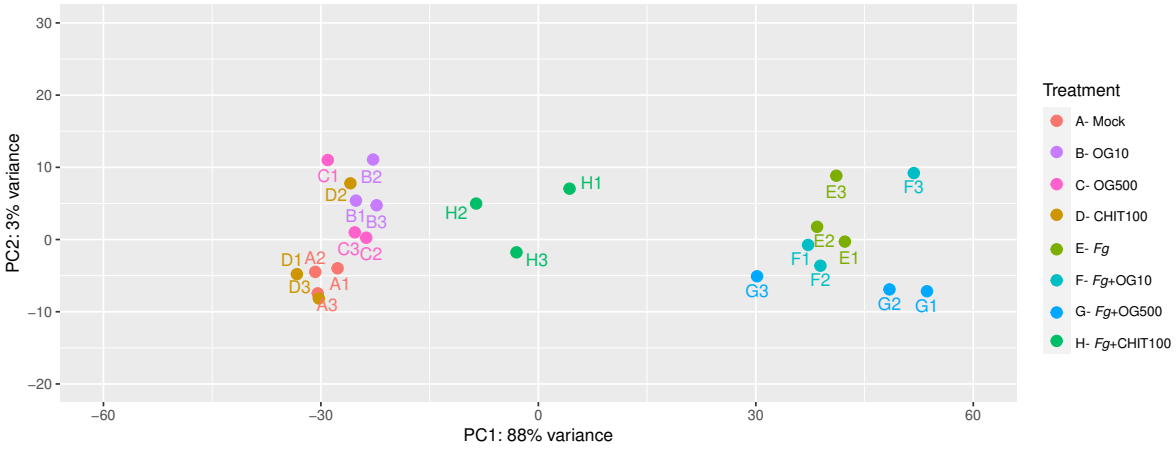


Figure 25. Principal components analysis (PCA) of normalized read counts (regularized log transformation of normalized data) of all samples used in transcriptomic experiment.

A total of 4345 genes (out of 66073 *T. turgidum* total genes) were found to be differentially regulated in all samples. A cut-off of the p-adjusted value ≤ 0.05 was used to classify a gene as differentially expressed (DEG) in comparison with the control. Looking at the three treatments (OG10, OG500 and CHIT100), 84 genes were exclusively regulated in OG10, 83 in OG500 and 133 in CHIT100 (Figure 26A). Shared and exclusive genes were found among *Fg*, *Fg*+OG500 and OG500 alone (Figure 26B). Particularly, 113 genes were regulated in OG500 and, among them, 47 were exclusively differentially regulated in OG500, while 62 were in common with *Fg* and *Fg*+OG500 (Figure 26B), although with a different expression level.

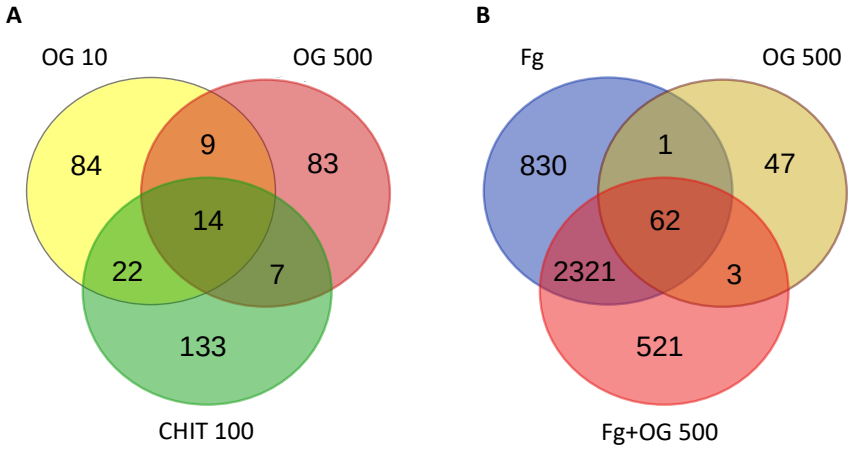


Figure 26. Venn diagrams illustrating the number of differentially expressed genes (DEGs) in the three conditions OG10, OG500 and CHIT100 (**A**) and in the three conditions (*Fg, Fg*+OG500, OG500) (**B**).

Considering the up- and down-regulated genes, the sample with most up-regulated genes was *Fg*+OG500 (2728 out of 2906 regulated genes) while the sample with most down-regulated genes was *Fg* (494 out of 3214 regulated genes). Among the common genes in the three conditions, several genes coding transcription factors belonging to diverse groups as well as (Figure 27).

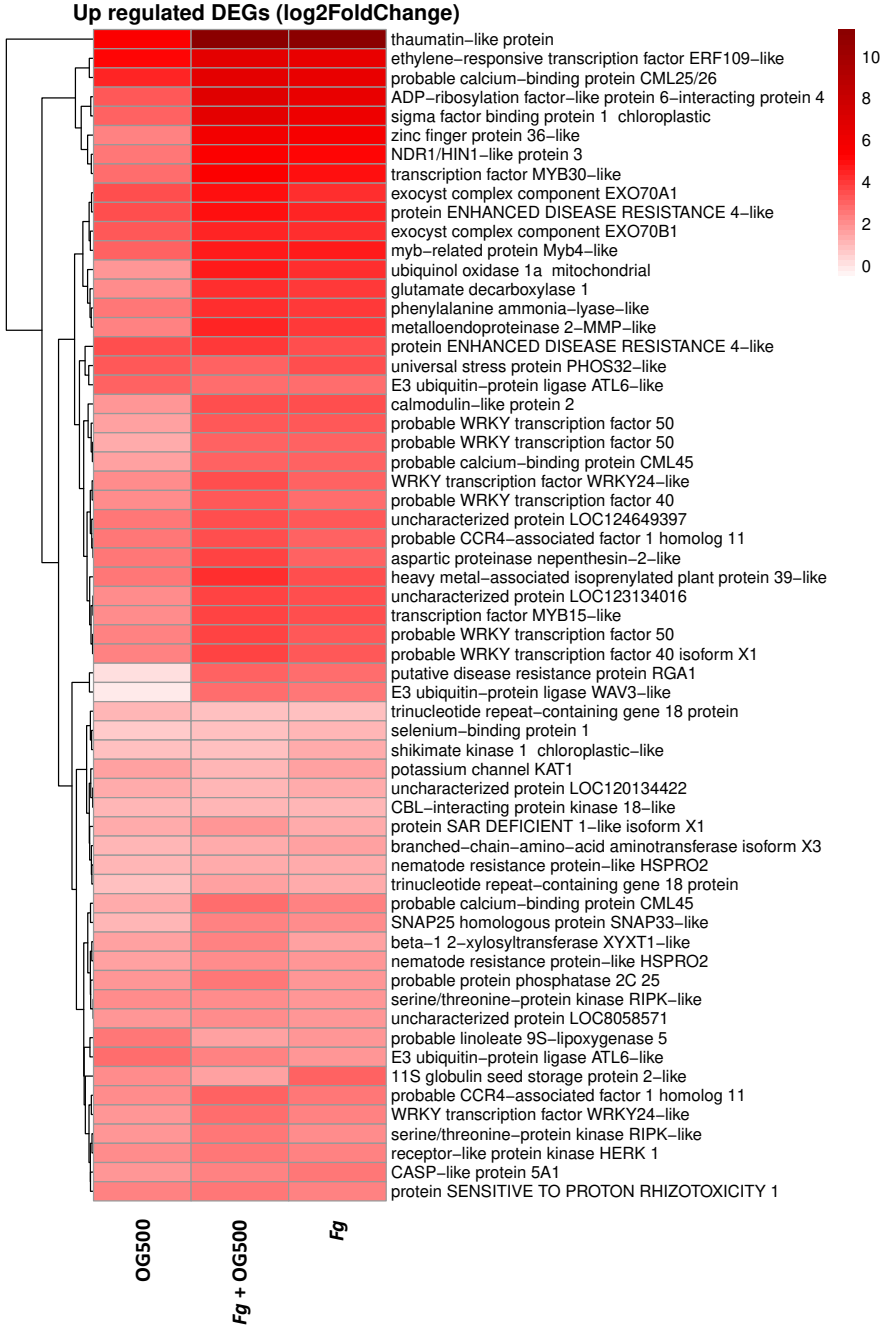


Figure 27. Heatmap depicting expression of 59 up-regulated DEGs shared by condition *Fg*, *Fg*+OG500 and OG500. These shared DEGs may be related to a common response mediated by *F. graminearum* and OG 500 µg/ml. Different color intensity indicates different levels of expression (log2 Fold Change).

Particularly, a gene coding for a thaumatin-like protein (*TRITD6Bv1G225300*) is one of the upregulated genes in the three conditions, although with a high regulation in the presence of the pathogen compared to the OG500 alone. It is worth noting that the gene with the highest upregulation among these genes, *i.e.* a gene coding for a peroxidase 5 like (*TRITD0Uv1G116360*) resulted to be downregulated in OG500 sample. Results suggested that the presence of the pathogen is dominant in the plant response, independently from the OG treatment (Figure 27).

To have an overview of the regulation of the main metabolic processes and signaling pathways involved in the different stress conditions, a GO enrichment analysis was carried out for DEGs in the diverse conditions (Figure 28 and 29). Differentially expressed transcripts were grouped in functional classes, based on the specific biological process in which they are involved. Results showed the presence in CHIT100 of different terms related to “Sulfur compound metabolic process”, “Cellular amino acid metabolic process”, “Signaling” and “Cellular response to stimulus” (Figure 28A). Relevant terms found in OG10 transcriptome are “Response to toxic substance”, “Glutamate metabolic process” and “Hexose metabolic process” (Figure 28B). It is worth noting the presence in OG500 of different term related to defense such as “Regulation of defense response to fungus”, “Regulation of response to external stimulus”, “Response to fungus”, “Defense response to fungus”, “Defense response”, “Chitin catabolic process” and “Lignin metabolic process” (Figure 28C).

These last, together with “Defense response to fungus”, have been also found in the enrichment analysis of *Fg* transcripts. Other relevant terms found in *Fg* transcriptome are “Sulfur compounds metabolic proc.”, “Hydrogen peroxide metabolic process”, “Hydrogen peroxide metabolic process”, “Reactive oxygen species metabolic process” and “Response to oxidative stress” (Figure 29A). “Response to oxidative stress” are present also in the *Fg* and OG-inoculated plants (*Fg*+OG500 and *Fg*+OG10) (Figure 29C-D). Results showed the presence in *Fg*+CHIT100 of different terms related to “Cell wall metabolic process”, “Cellular amino acid metabolic process”, “Cellular amide metabolic process” and “Peptide metabolic process” and “Carboxylic acid metabolic process” (Figure 29B).

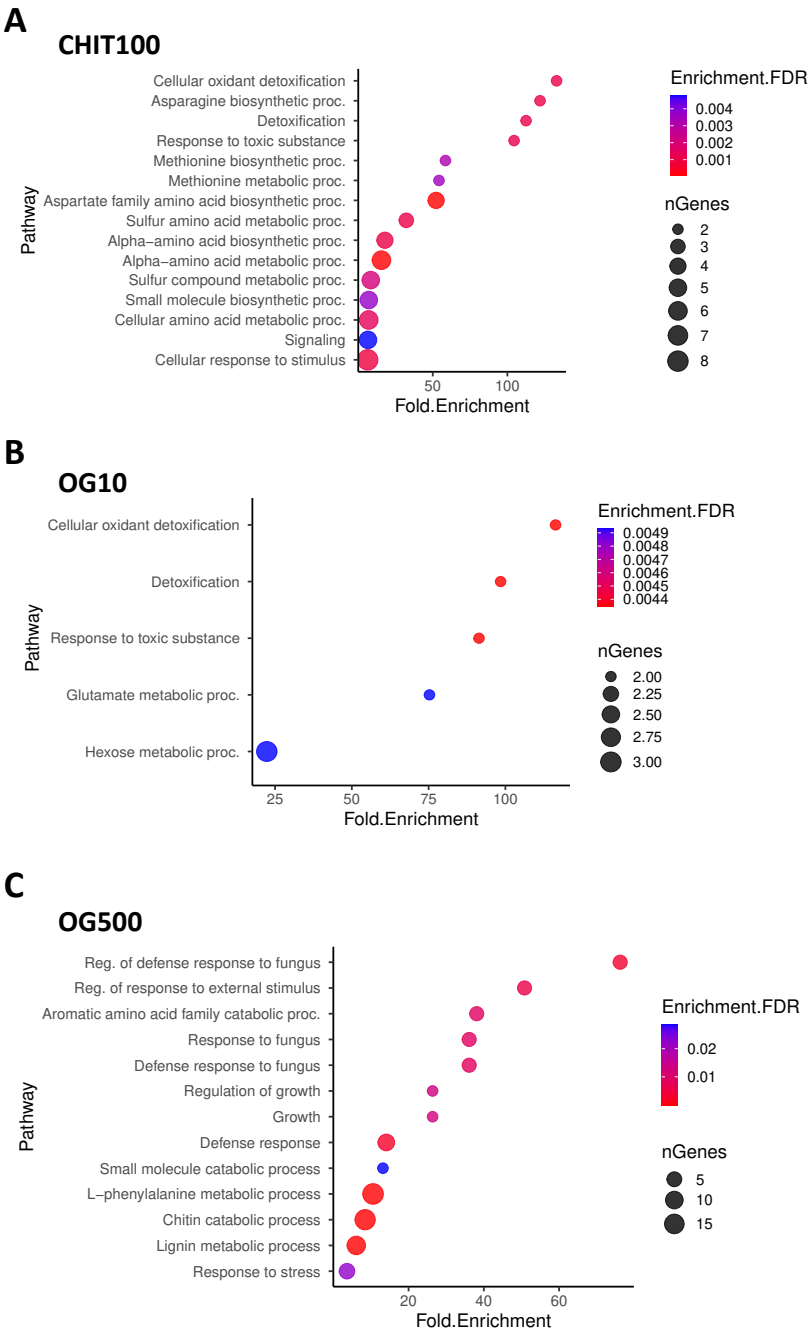


Figure 28. Bubble plots showing GO-enriched terms classified as Biological Process (BP) in detected DEGs. In detail, enriched terms in CHIT100 (**A**), in OG10 (**B**) and in OG500 (**C**) are reported. The x-axis shows the fold enrichment values, *i.e.*, the percentage of genes in the selected DEG list belonging to a pathway divided by the corresponding percentage in the all reference gene list, and the y-axis reports the GO terms. Sizes of bubbles are proportional to the number of genes assigned to the related GO term, while bubbles color indicates the significance of the enriched term (False Discovery Rate values) as calculated by the enrichment analysis by Blast2GO.

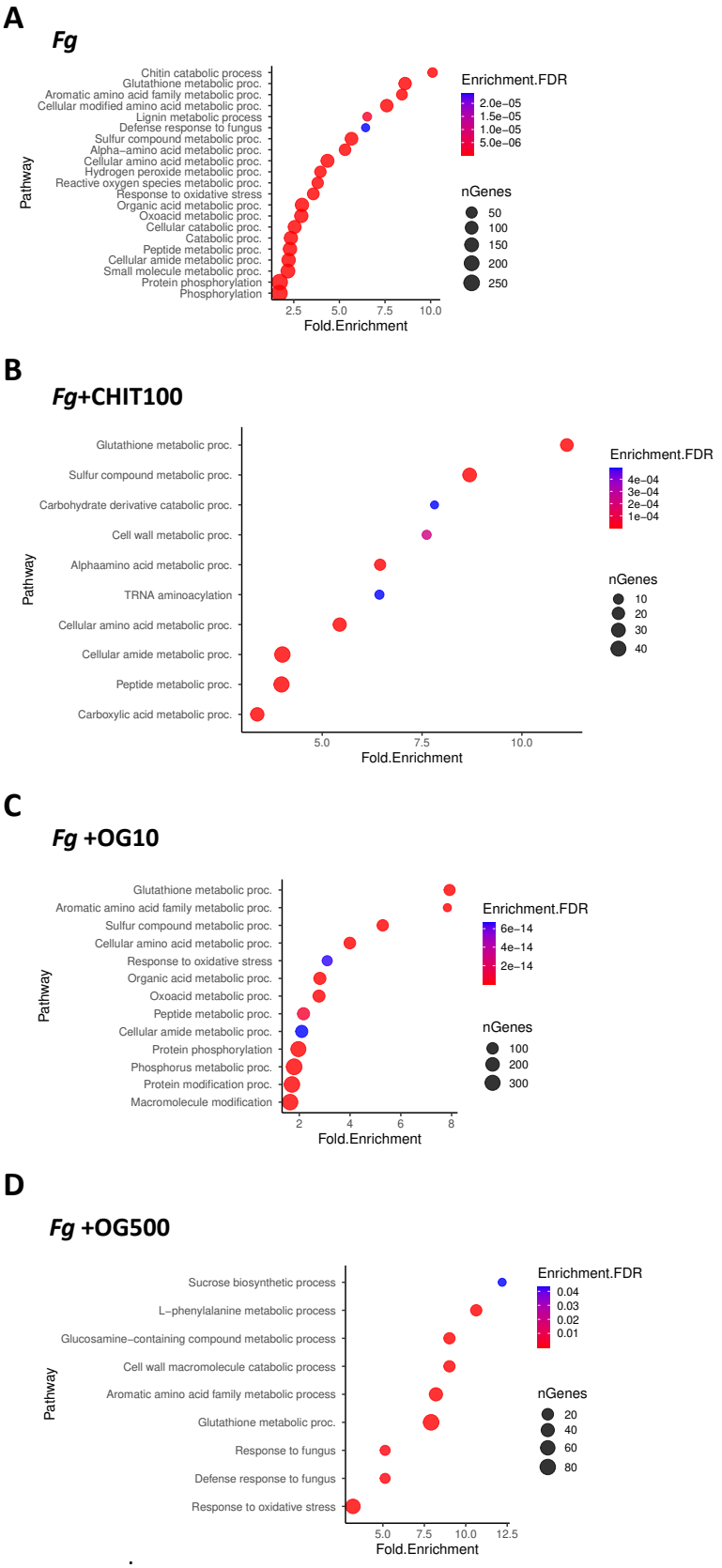


Figure 29. Bubble plots showing GO-enriched terms classified as Biological Process (BP) in detected DEGs. In detail, enriched terms in *Fg* (**A**), in *Fg*+CHIT100 (**B**), *Fg* + OG10 (**C**) and *Fg* + OG500 (**D**) are reported. The x-axis shows the fold enrichment values, i.e., the percentage of genes in the selected DEG list belonging to a pathway divided by the corresponding percentage in the all

reference gene list, and the y-axis reports the GO terms. Sizes of bubbles are proportional to the number of genes assigned to the related GO term, while bubbles color indicates the significance of the enriched term (False Discovery Rate values) as calculated by the enrichment analysis by Blast2GO.

To better describe the effect of the treatments, specific subsets of analyses were performed. Interestingly, the most up-regulated gene in all the treatments was a peroxidase 5 like gene (*TRITD0Uv1G116360*) with the exception to OG500, where the most up-regulated genes is a thaumatin-like protein (*TRITD6Bv1G225300*), followed by an ethylene-responsive transcription factor ERF109-like (*TRITD1Bv1G202640*) and a probable calcium-binding protein CML25/26 (*TRITD4Av1G142800*). Notably, the same thaumatin-like gene was also found among the up-regulated genes in *Fg* treatment, suggesting a role in plant defense response. Moreover, infection marker genes, such as pathogenesis-related (*PR*) genes, were up regulated in all the *Fg* treated samples. In my analyses, the expression of 12 *PR1* genes, among the 24 paralogs in durum wheat genome, was differently regulated in the different treatments, with some differences among them. Particularly, only one gene resulted significantly up-regulated in *Fg*+CHIT100 (Log_2 FC = XXX), while all the *PR1* gene set was regulated with a Log_2 FC average of 7.40, 5.22 and 7.61 in *Fg*, *Fg*+OG10 and *Fg*+OG500, respectively.

4.1.2.2. Gene expression validation by qRT-PCR

To validate the obtained transcriptomic data, analyses of the expression of two genes, *TdPR1* and *FgTRI6,* were carried out by qRT-PCR by using total RNA extracted from 3-day-old wheat seedlings. The expression of the wheat immune-related marker gene *TdPR1* was normalized to *TdACTIN*, used as a housekeeping gene, while the expression of *FgTRI6* gene was normalized to the *Fg β-tubulin* gene. In the presence of CHIT, the expression of *TdPR1* was largely induced at 24 hpi and further increased at 48 hpi (Figure 30A). Similar results were also observed in seedling treated with OGs. Interestingly, the *TdPR1* expression was significantly induced at 6 hpi only in response to OG treatment.

In seedlings treated with both fungus and elicitors, the *TdPR1* expression was higher than that observed in tissues treated with the fungus alone, at both 24 and 48 hpi (Figure 30B). Noteworthy, in tissues cotreated with *Fg* and OGs, a more robust induction of *TdPR1* gene was observed, which resulted 3- and 1.9-fold higher than that detected in tissues treated only with *Fg*, at 24 hpi and 48 hpi, respectively. Therefore, an OG-triggered

priming effect on the induction of typical immune marker genes, such as *TdPR1*, involved in the salicylate signaling pathway, was observed.

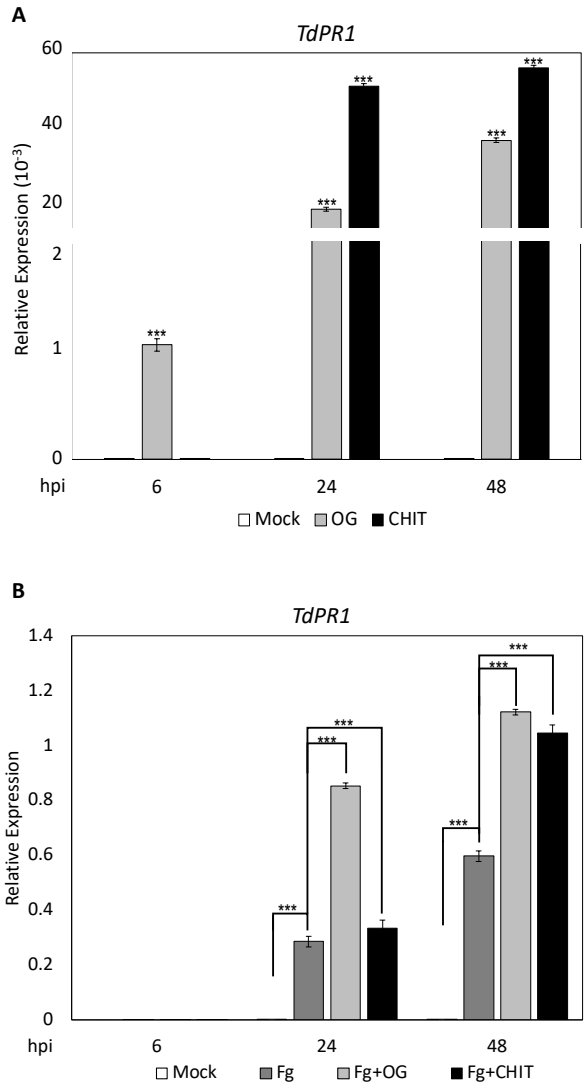


Figure 30. *TdPR1* gene expression in seedlings of the wheat cv. Svevo inoculated with OGs (500 µg/ml), *Fusarium graminearum* spores (2 x 10^3 conidia) and a co-treatment of both OGs and fungus spores. Gene expression was evaluated by qRT-PCR. Sterile water was used as mock treatment and CHIT (100 µg/ml) as a positive control in the experimental setup. Samples were collected at 6, 24 and 48 hpi. Gene expression was normalized to expression of *TdACTIN*. All values are the means ± standard error (SE) (n = 4). Asterisks above the bars indicate values that are significantly different from mock and *Fg* treatment (* $P < 0.05$; t-test).

In order to assess the fungal pathogenicity, I analyzed the expression of *FgTRI6* gene that is involved in the biosynthesis of fungal mycotoxins. A considerably lower expression of such gene was observed in cotreated tissues with either OGs or CHIT compared to tissues inoculated with the fungus alone, both at 24 and 48 hpi (Figure 31). Therefore, both

CHIT and OGs seem to limit the spread of the fungus in vegetal tissues and consequently to decrease the abundance of mycotoxins.

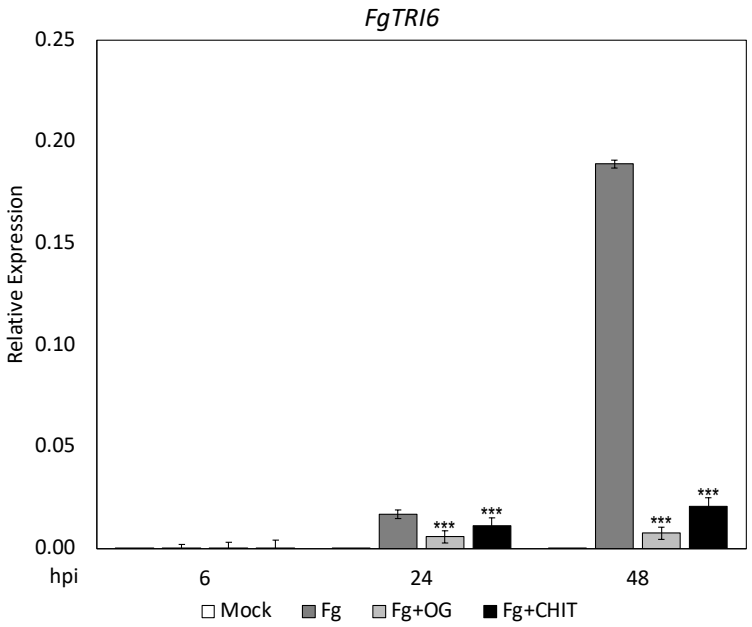


Figure 31. Evaluation of the *FgTRI6* gene expression in inoculated seedlings with *Fusarium graminearum* spores (2 x 10^3 conidia) and a co-treatment of both OGs and fungus spores. Gene expression was evaluated by qRT-PCR. Sterile water was used as mock treatment and chitosan (100 µg/ml) as a positive control in the experimental setup. Samples were collected at 6, 24 and 48h post inoculation (hpi). Gene expression was normalized to the expression of the *Fgβ-tubulin* gene. All values are the means ± standard error (SE) (n = 4). Asterisks above the bars indicate values that are significantly different from Fg treatment (* $P < 0.05$; t-test).

4.1.3. OG-triggered resistance to *Fusarium graminearum* in durum wheat spikes

To evaluate the functionality of OGs as elicitors of immunity and their ability to restrict phytopathogen fungal growth in durum wheat spikes, at anthesis stage two opposite central florets of a wheat head were inoculated with OGs (500 µg/ml), *Fusarium graminearum* spores (2 x 10^4 conidia) and a co-treatment of both OGs and fungus spores. Sterile water was used as mock treatment and CHIT (100 µg/ml) as a positive control in the experimental setup.

Following spike point–inoculation with *F. graminearum*, symptom progression was slower in spikes co-treated with OGs and fungus spores than in spikes inoculated with the fungus alone. The maximal reduction of 25% in symptom severity was detected between 7 and 13 dpi (Figure 32A).

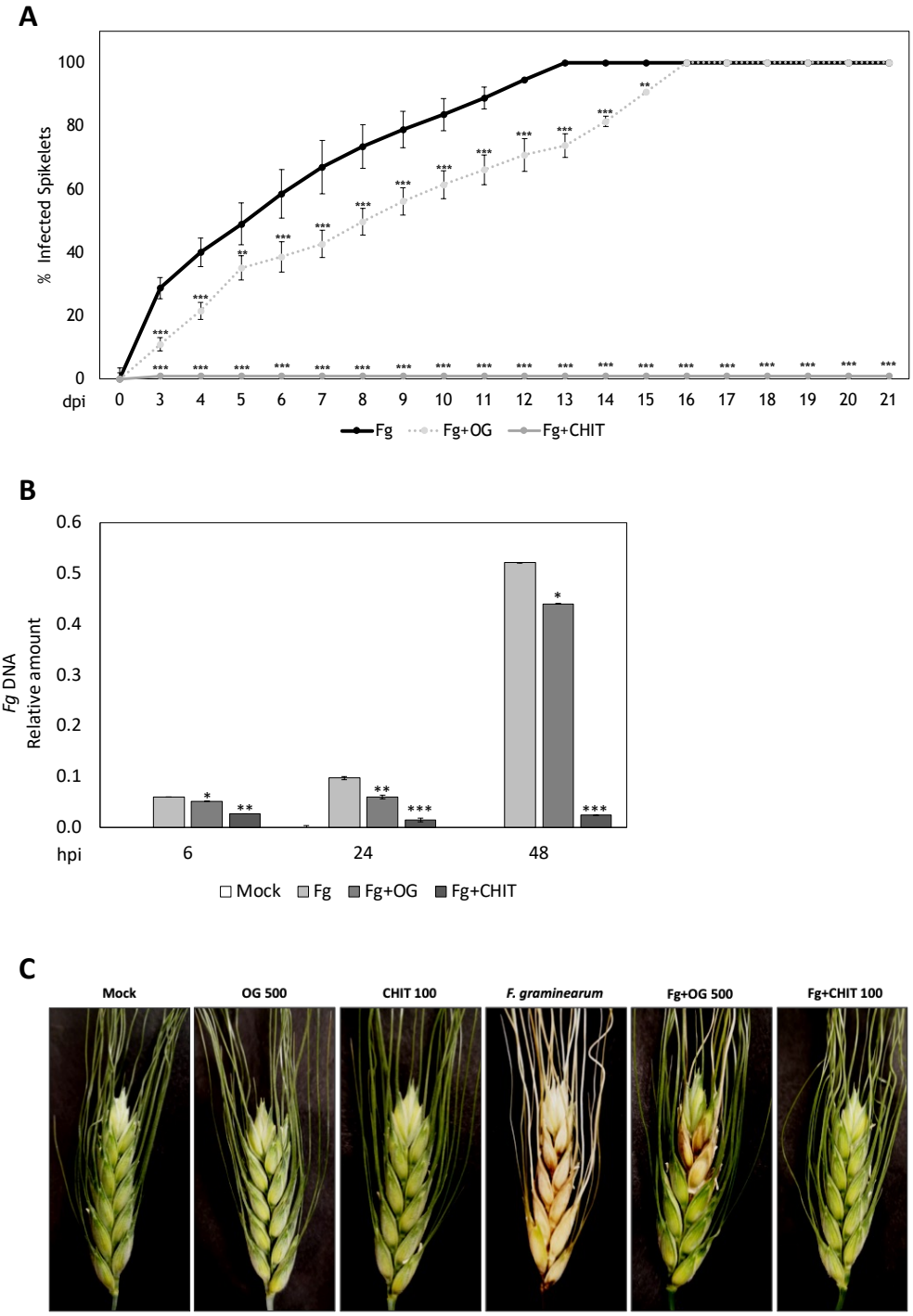


Figure 32. OGs trigger resistance to FHB in cv. Svevo spikes. **A)** Disease severity in the cultivar Svevo inoculated with Fg and Fg + OG or Fg + CHIT co-treatment. Disease progression was monitored for 21 dpi; **B)** Real-time PCR analysis of DNA content of *Fg β-tubulin* gene normalized to the wheat *TdACTIN* gene in wheat coleoptiles at 6, 24, 48 hpi; **C)** Images showing disease spread in representative wheat spikes for each treatment condition. Images were taken at 10 dpi. All values are the means ± SE (n = 15). Asterisks above the bars indicate values that are significantly different from seedlings inoculated with the *Fg* alone (*** $p < 0.01$, student t-test).

At this infection stage, several OG-cotreated inoculated spikelets showed a temporary block of the infection. Subsequently, the infection progression restarted and the difference in symptoms between OG-cotreated and *Fg*-inoculated plants progressively decreased until it was no longer significant at 16 dpi. The spikes cotreated with CHIT and *Fg* did not show symptoms of the fungal disease (Figure 32A), likely because of the inhibitory effect of CHIT on spore germination and hyphal development of *F. graminearum* (Deshaies et al. (2022).

The fungal abundance, monitored by comparing among treatments the DNA accumulation quantified through quantitative PCR amplifying the *Fgβ-tubulin* gene, was significantly lower in spikes of plants cotreated with fungus and elicitors. In presence of OG-cotreatment, a fungal amount of 84.6%, 61.5% and 84.4% was observed compared to spikes inoculated with the fungus alone at 6, 24 and 48 hpi, respectively (Figure 32B). Interestingly, in presence of co-treatment of CHIT and *Fg*, results show that the fungal amount was only of 4.6% compared to the *Fg* control condition. As shown in Figure 32C, at 10 dpi the severity of the symptoms is particularly evident in the spikelets inoculated with the fungus alone that show 100% of the infected spikelets. On the contrary, spikes cotreated with OGs and fungus show a considerably lower severity of the disease at this point.

4.1.3.1. Expression analyses of *TdPR1, FgTRI6* and *FgTRI5* genes in durum wheat - *F. graminearum* interaction

To follow the expression of wheat defense related genes as well as the mycotoxin biosynthetic genes and how they correlate with the reduction of the FHB symptoms in spikes, three genes, *TdPR1, FgTRI6* and *FgTRI5*, were considered and qRT-PCR was carried out by using total RNA extracted from flowering wheat heads. In the OGs or CHIT pretreated spikes, the expression of *TdPR1* was significantly higher compared to untreated spikes at 6, 24 and 48 hpi (Figure 33A). Notably, a different kinetic of induction was observed between the two pretreatments: in the presence of OGs, the expression of *TdPR1* was largely induced at 6 hpi and gradually decreased at 24 and 48 hpi (Figure 33A); conversely, in the CHIT pretreated spikes the higher accumulation of *TdPR1* transcripts was detected at 48 hpi.

In co-treatment experiments with both fungus and elicitors, the expression of *TdPR1* resulted to be lower than that observed in spikes inoculated only with *Fg* (Figure 33B).

Noteworthy, contrasting results o0f the analyses on the levels of *TdPR1* transcripts were observed in wheat seedlings and spikes in the presence of the fungus and elicitors. Indeed, in seedlings cotreated with both *Fg* and elicitors the *TdPR1* expression was higher than that observed in tissues treated with the fungus alone, while a lower accumulation of *TdPR1* transcripts was detected in co-treated spikes, both at 24 and 28 hpi. These results could represent an evident example of tissue-specific regulation of immune marker gene expression.

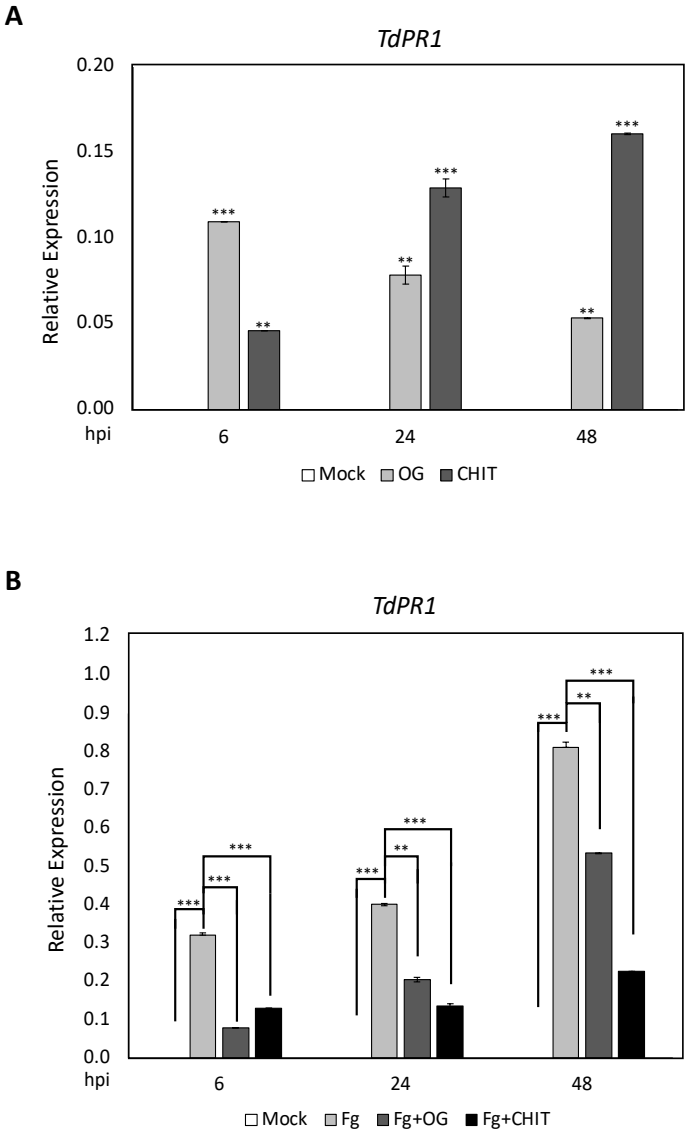


Figure 33. *TdPR1* gene expression in spikes of the wheat cv. Svevo inoculated with OGs (500 µg/ml), *Fusarium graminearum* spores (2 x 10^4 conidia) and a co-treatment of both OGs and fungus spores. Gene expression was evaluated by qRT-PCR. Sterile water was used as mock treatment and CHIT (100 µg/ml) as a positive control in the experimental setup. Samples were collected at 6, 24 and 48 hpi. Gene expression was normalized to expression of *TdACTIN*. All

values are the means ± standard error (SE) (n = 4). Asterisks above the bars indicate values that are significantly different from mock and *Fg* treatment (* P < 0.05; t-test).

A lower fungus abundance, accompanied by the downregulation of *FgTRI6* and *FgTRI5* biosynthetic genes, were detected in elicitor co-treated spikes at 6, 24 and 48 hpi (Figure 34A-B).

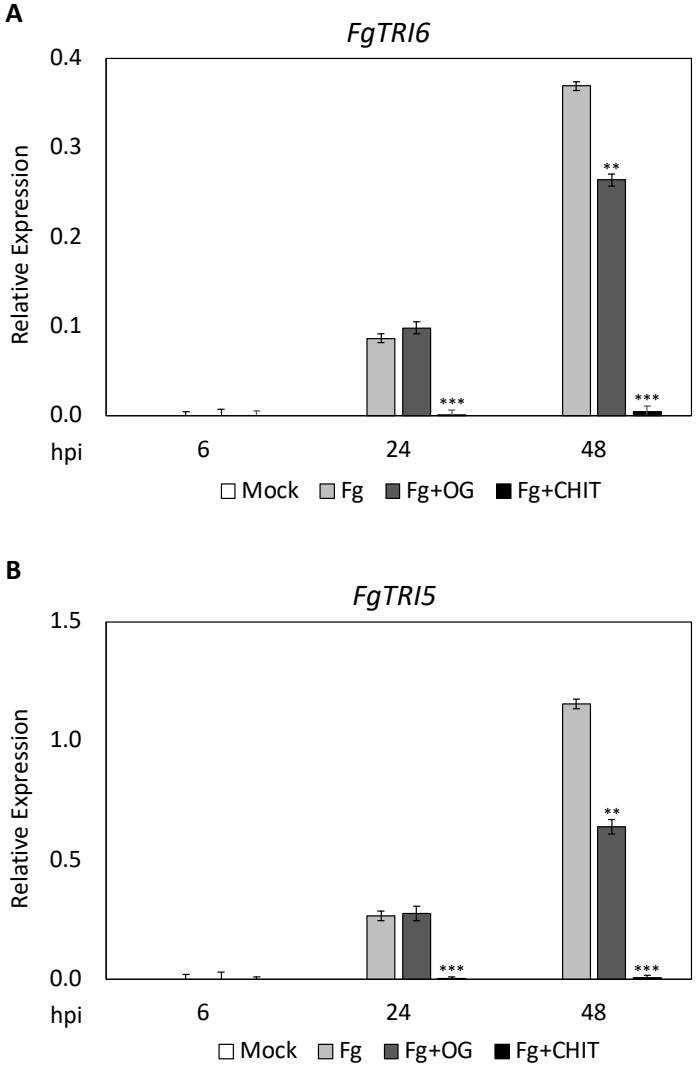


Figure 34. Evaluation of the *FgTRI6* (A) and *FgTRI5* (B) gene expression in inoculated spikes with *Fusarium graminearum* spores (2 x 10^4 conidia) and a co-treatment of both OGs and fungus spores. Gene expression was evaluated by qRT-PCR. Sterile water was used as mock treatment and chitosan (100 µg/ml) as a positive control in the experimental setup. Samples were collected at 6, 24 and 48h post inoculation (hpi). Gene expression was normalized to the expression of the *Fgβ-tubulin* gene. All values are the means ± standard error (SE) (n = 4). Asterisks above the bars indicate values that are significantly different from *Fg* treatment (* P < 0.05; t-test).

4.1.4. Impact of OGs on wheat agronomical and growth parameters

After verifying that OGs and CHIT limit the infection spread of *F. graminearum*, we evaluated the impact of the elicitors on important wheat agronomical and growth parameters after *Fg* infection. In the analysis the number of seeds per spike, the number of seeds per spikelet, the primary spike weight, the weight of seeds per spike, the weight of 1000 seeds and the yield loss were considered (Figure 35A-F).

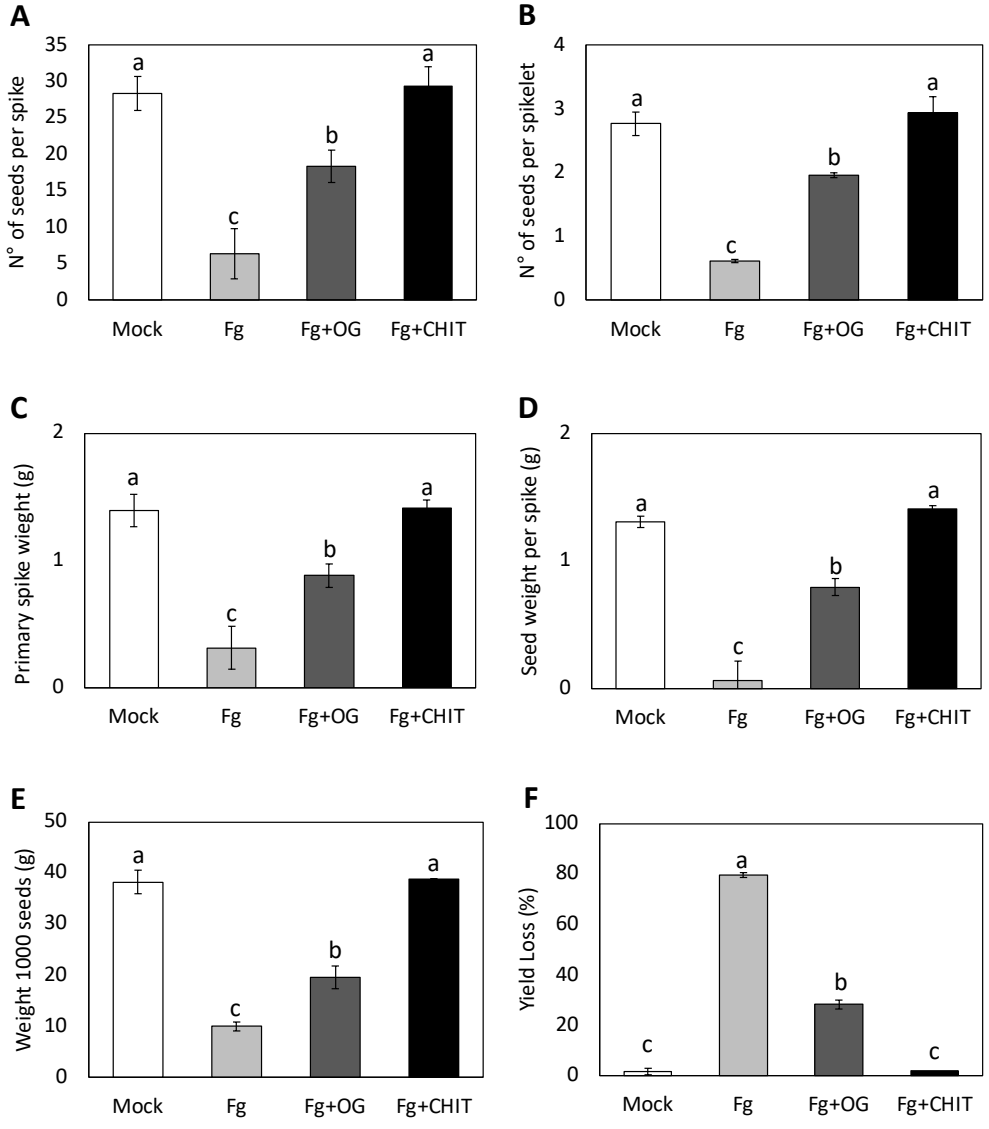


Figure 35. Agronomic and growth parameters in OG- and CHIT-cotreated wheat plants after *F. graminearum* infection. A) Number of seeds per spike; **B)** Number of seeds per spikelet; **C)** Primary spike weight (g); **D)** Seed weight per spike (g); **E)** Weight 1000 seeds (g); **F)** Yield loss (%) for the wheat cv. Svevo. A minimum of 10 plants per treatment condition were analyzed to obtain data. All values are the means ± standard error (SE). The statistical significance was tested by means of ANOVA followed by Tukey test. Different letters indicate statistically different values ($p < 0.05$).

In *Fg*-inoculated plants, significantly lower yield values were observed for all the parameters considered. The number of seeds per spike and per spikelet were inhibited of 77.6% and 76.9%, respectively, compared to untreated plants (Figure 35A-B). Moreover, results showed an inhibition of the primary spike weight, the weight of seeds per spike and the weight of 1000 seeds of 77.3%, 95.1% and 73.8%, respectively (Figure 35C-E). Interestingly, for plants cotreated with chitosan and the fungus, no significant differences were observed compared to mock condition.

In presence of OG cotreatment, higher yield values were observed compared to *Fg*-inoculated plants. The inhibition of the number of seeds per spike and per spikelet were only of 35.3% and 29.2%, respectively, compared to untreated plants (Figure 35A-B). For the primary spike weight, the weight of seeds per spike and the weight of 1000 seeds, a significant reduction of 36.6%, 38.9% and 48.6%, respectively, was observed (Figure 35C-E). Therefore, OGs limit the infection spread of *F. graminearum* as well as the yield loss associated to fungal disease. The loss of production was then calculated and expressed as percentage (Figure 35F). Interestingly, *Fg*-inoculated plants displayed a production loss of 79.7%, while OG-cotreated plants exhibited a yield reduction of only 28.3% compared to the mock condition. No significant differences were observed between CHIT-cotreated and untreated plants.

The quality and the filling of kernels were also evaluated, considering the length and the width of wheat seeds, after *Fg* infection. As shown in Figure 36A-B, OG and CHIT treatments did not affect wheat seed length and width. On the contrary, the fungus *Fg* negatively impacted on both parameters considered by compromising the good filling of kernels and causing their shriveling.

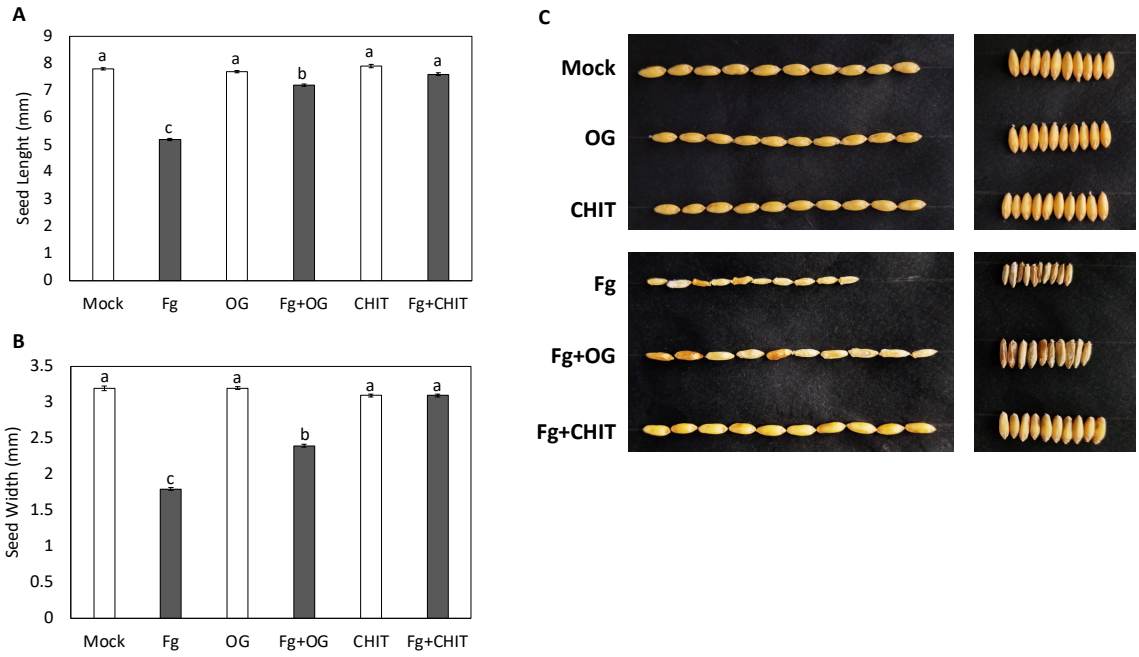


Figure 36. Seed quality and filling in OG- and CHIT-cotreated wheat plants after *F. graminearum* infection. A) Seed length (mm); **B)** Seed width (mm); **C)** Photographs showing length and width of representative seeds for each treatment condition. Ten seeds per treatment were analyzed to obtain data. All values are the means ± standard error (SE). The statistical significance was tested by means of ANOVA followed by Tukey test. Different letters indicate statistically different values ($p < 0.05$).

Seeds derived from *Fg*-inoculated spikes displayed an inhibition of seed length and width of 33,4% and 43,75%, respectively, compared to untreated condition. Interestingly, for seeds derived from CHIT-cotreated plants no significant differences were observed compared to mock condition. Moreover, we observed that in the presence of OG-cotreatment, seeds displayed a reduced shriveling and an inhibition of length and width of only 6.5% and 25%, respectively (Figure 36C).

4.2. Analysis of engineered durum wheat plants with altered endogenous OG levels

4.2.1. Generation and screening of OG-Machine expressing durum wheat lines

Biolistic transformations of durum wheat immature embryos cv Svevo were performed with co-bombardment of the pAHC17_TaPr1.1::OGM and the pUBI::BAR as selectable marker. The pAHC17_TaPr1.1::OGM construct was prepared by inserting the complete

coding region of OG-Machine into pAHC17 vector under control of the *Triticum aestivum Pr1.1.* promoter and NOS terminator. The transformation experiments were carried out following the procedure reported by Janni et al. (2008). In total, 2150 immature wheat embryos were co-transformed using pAHC17_TaPr1.1::OGM and pUBI::BAR. Two separate bombardment experiments were performed. Six independent transgenic regenerated lines were obtained. Such lines were analyzed for the presence of the transgene by PCR using specific oligonucleotides (*PvPGIP2F/PvPGIP2R*, see Table 1) which produce a 238 bp long amplicon. Genomic DNA, extracted from leaves of the transgenic lines (T_0), was used as template. Svevo was used as negative control. Three T_0 lines, #12, #29, #43, displayed a positive PCR result hinting the presence of the OGM construct (Figure 37A) and were selected for further characterization in subsequent generations. A quantitative Real-time PCR was also performed to detect differences among the transgenic lines in terms of number of the OGM construct insertions in their genome. The results shown in Figure 37B indicated that the line #12 carries a higher number of insertions compared to #43, which displayed a slightly higher amount of amplicons respect to #29.

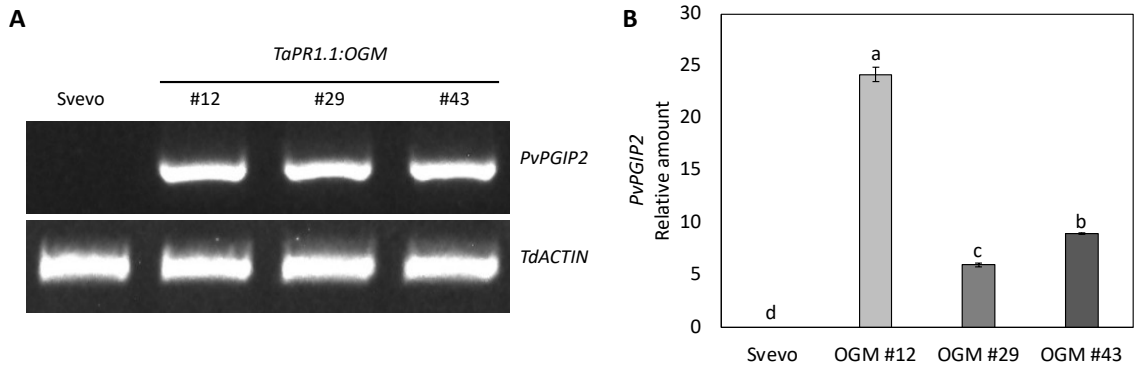


Figure 37. Generation and screening of OG-Machine expressing durum wheat lines. **A)** PCR analysis on genomic DNA of Svevo wild-type and TaPR1.1::OGM transgenic wheat lines #12, #29 and #43. The expected PCR products using *PvPGIP2 F* and *PvPGIP2 R* primers indicate the presence of the transgene only in the OG-Machine lines. **B)** Real- time PCR analysis of DNA content of *PvPGIP2* gene relative to the wheat *TdACTIN* gene. All values are the means ± standard error (SE) (n = 4). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test).

4.2.2. Phenotypical characterization of the OG-Machine durum wheat transgenic lines

Phenotypic analyses of the OG-Machine transgenic plants have been performed. The phenotypic features analyzed were the plant height and the number of spikes per plant

(Figure 38A-C). As control, cv. Svevo wild type was used. As shown in Figure 38A, transgenic lines #12, #29 and #43 were, respectively 3.9%, 3.4% and 5.8%, taller than the non-transgenic Svevo plants. All OG-Machine transgenic lines displayed a significant inhibition of the number of spikes per plant (Figure 38B). Line #29 plants showed a reduction of the number of spikes by 30.7%, while the line #12 and #43 plants by 46.1% and 38.4%, respectively, compared to cv. Svevo wild type.

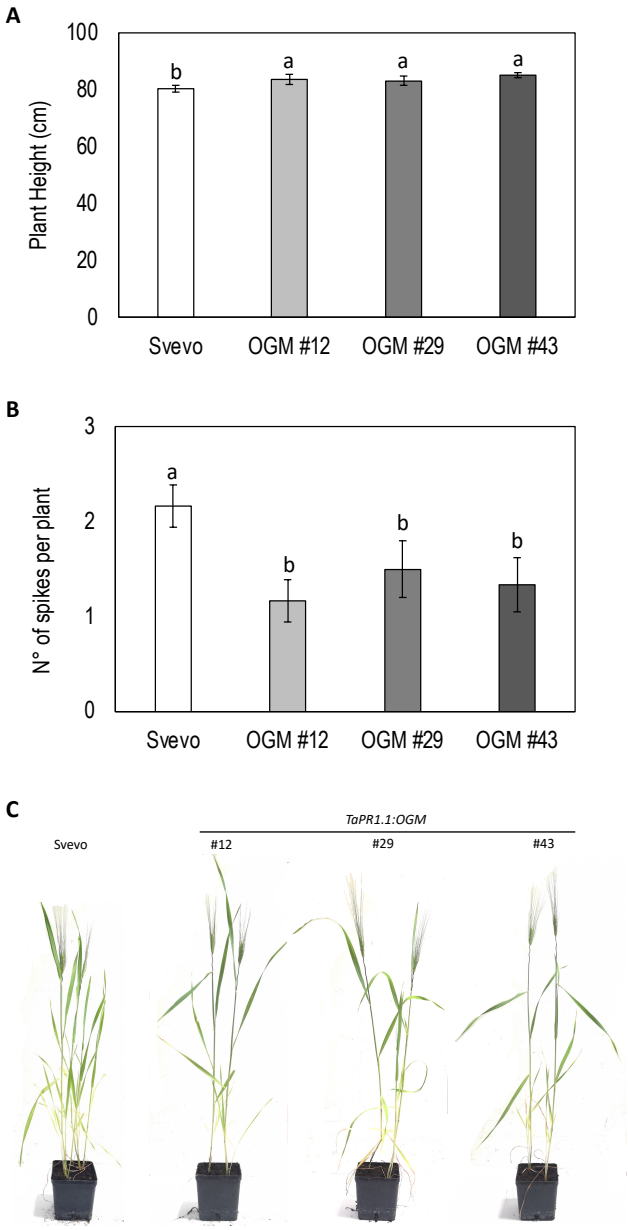


Figure 38. Phenotypic analyses of OG-Machine transgenic lines and Svevo wt plants. **A)** Plant height; **B)** Number of spikes per plant; **C)** Photograph showing phenotype in representative plants of wheat cv. Svevo and the TaPR1.1:OGM transgenic wheat lines #12, #29 and #43. A minimum of 10 plants per line were analyzed to obtain data. All values are the means ± standard error (SE) (n = 10). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test).

4.2.3. Analysis of fusariosis disease severity in durum wheat seedlings expressing the OG-Machine chimera protein

In order to evaluate the possible involvement of OG-Machine in limiting the infection process of *Fusarium graminearum* in durum wheat, three-day-old seedlings of wild type cv. Svevo and TaPR1.1::OGM transgenic lines were inoculated with *Fusarium graminearum* spores (2 x 10^3 conidia), as reported by Jia et al. (2017). Symptom progression was visually scored for a period of seven days and evaluated by measuring brown disease lesion extension on coleoptiles.

As shown in Figure 39A, three OG-Machine transgenic lines show a very similar trend of infection which is significantly slower than the wild type control. Moreover, they showed less severe and extended symptoms compared to the untransformed control. Importantly, symptom reduction was consistent throughout the time of infection for all three transgenic lines. At 7 dpi, seedlings of line #12, #29 and #43 displayed a significant disease lesion size reduction of 23.8%, 18.6% and 21.9%, respectively, compared to wild type cv. Svevo (Figure 39B).

The fungal abundance was detected in *Fg*-inoculated seedlings of wild type cv. Svevo and TaPR1.1::OGM transgenic lines at 48 and 72 hpi. The results show that *Fg* amount was lower in OG-Machine lines compared to untransformed control. Interestingly, at 72 hpi in the OG-Machine lines #12, #29 and #43 it was observed a fungal amount of 3.5%, 5.3% and 6.7% compared to seedlings of cv. Svevo (Figure 39C).

These results demonstrate that the induction of OG-Machine construct could strongly limits the fungal growth and accumulation during infection process in wheat.

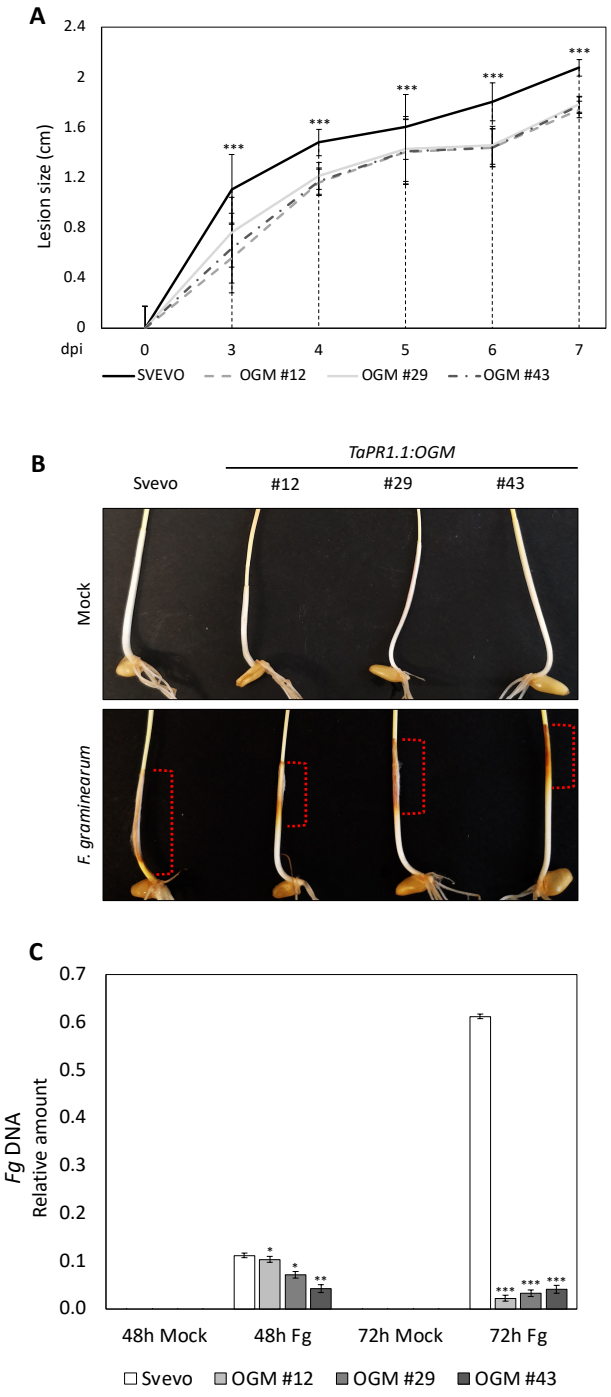


Figure 39. The OG-Machine transgenic lines are more resistant to FHB in seedling infections. **A)** Leaf lesion size in the cv. Svevo and the TaPR1.1::OGM transgenic lines inoculated with *Fg*. Disease was monitored for 7 dpi. All values are the means ± SE (n = 4). Asterisks above the bars indicate values that are significantly different from transgenic plants (*** P < 0.01, t-test); **B)** Photograph showing disease spread. Photographs were taken at 7 dpi. The lesion size is indicated with a red square bracket; **C)** qRT-PCR analysis on the DNA content of *Fg β-tubulin* gene relative to the wheat *TdACTIN* gene in wheat seedlings at 48h and 72h post inoculation with Fg. All values are the means ± SE (n = 4). Asterisks above the bars indicate values that are significantly different from Svevo (*P < 0.05; t-test).

4.2.3.1. Expression analyses of *TdPR1, FgTRI6* and *FgTRI5* genes in seedlings of OG-Machine transgenic lines

To assess the inducibility of the OG-Machine construct, we considered treatment with *F. graminearum*. Accumulation of *PvPGIP2* transcripts in #12, #29 and #43 transgenic lines was verified by qRT-PCR on cDNA. Svevo was used as negative control. This analysis was performed three times. Results showed that *PvPGIP2* transcripts were detectable only in the transgenic lines and only in the presence of the pathogen. Noteworthy, the three selected OG-Machine lines displayed different expression levels of the transgene. Transcript levels of line #12 was 9-fold and 7-fold higher than in line #29 and #43, respectively, at 72 hpi (Figure 40A).

To evaluate the possible involvement of the OGM in regulating wheat responses to *Fg* infection, Svevo and OG-Machine seedlings were inoculated with the fungus. Expression analyses of plant immune marker genes and fungal toxin biosynthetic genes were carried out by qRT-PCR. Fungal infection resulted in significantly stronger induction of *TdPR1* in *TaPR1.1*::OGM plants than in Svevo wild type, therefore indicating the expression of OG-Machine promotes robust activation of defense responses. The expression of *TdPR1* in the transgenic lines #12, #29 and #43 was 4.5-fold, 5-fold and 4-fold higher, respectively, compared to cv. Svevo wild type plants at 72 hpi (Figure 40B). Interestingly, OGM lines displayed a downregulation of *FgTRI6* and *FgTRI5* suggesting a possible role of the OG-Machine in both limiting the spread of the pathogen and the expression of toxin biosynthetic genes in the fungus. The expression of *FgTRI6* in the transgenic lines #12, #29 and #43 was 3.5-fold, 2-fold and 2.1-fold lower, respectively, compared to cv. Svevo wild type plants at 48 hpi (Figure 40C). The same expression trend was observed at 72 hpi. Similarly, the expression of *FgTRI5* in the transgenic lines #12, #29 and #43 was 14-fold, 9-fold and 4.3-fold lower, respectively, compared to wild type plants at 72 hpi (Figure 40D).

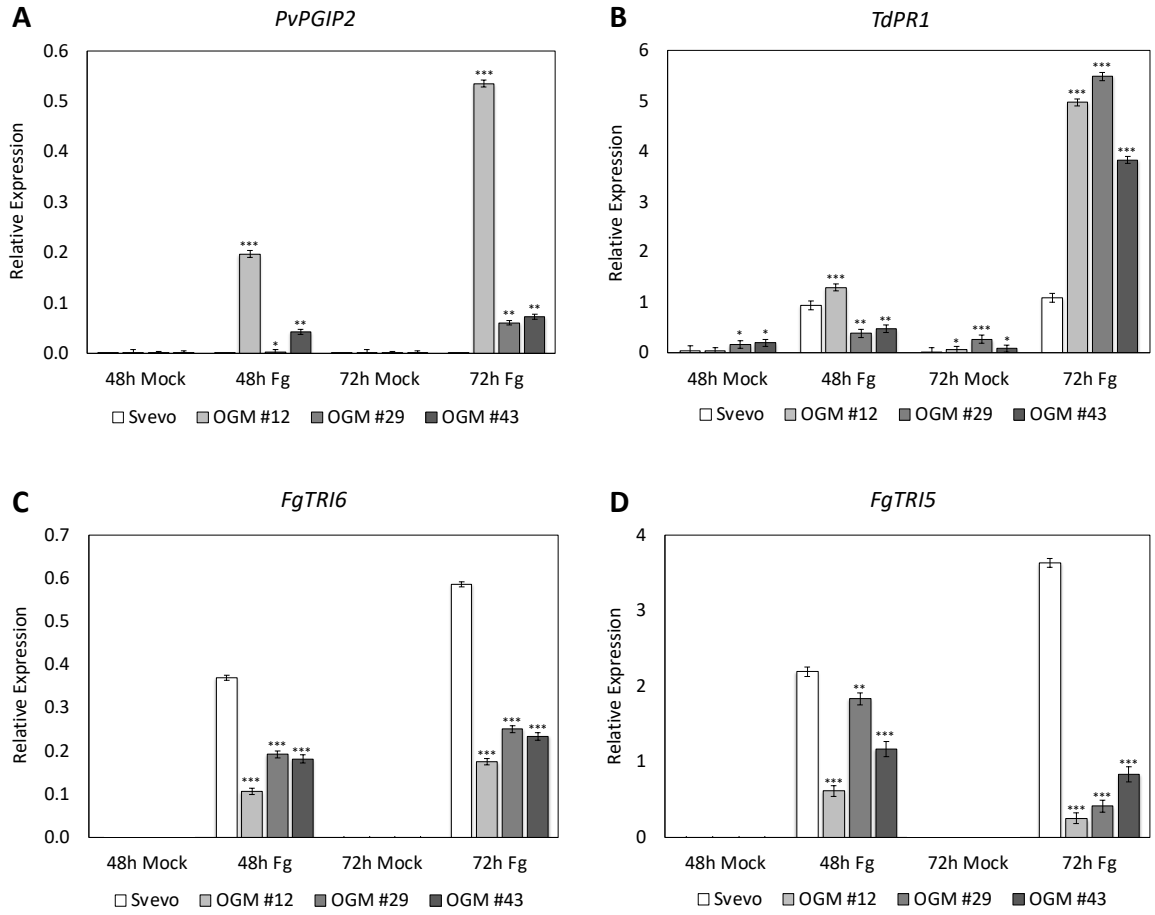


Figure 40. Evaluation of the *PvPGIP2* (A), *TdPR1* (B), *FgTRI6* (C) and *FgTRI5* (D) gene expression in *Fg*-inoculated seedlings of wheat cv. Svevo and TaPR1.1::OGM transgenic lines. Gene expression was evaluated by qRT-PCR. Sterile water was used as mock treatment. Samples were collected at 48 and 72 hpi. *PvPGIP2* and *TdPR1* gene expression was normalized to the expression of the *TdACTIN* gene. *FgTRI6* and *FgTRI5* gene expression was normalized to the expression of the *Fgβ-tubulin* gene. All values are the means ± standard error (SE) (n = 4). Asterisks above the bars indicate values that are significantly different from Fg treatment (* $P < 0.05$; t-test).

4.2.4. Analysis of fusariosis disease severity in durum wheat spikes expressing the OG-Machine chimera protein

In order to confirm that the expression of OG-Machine enhance resistance to *F. graminearum,* wheat spike tissue from *TaPR1.1::*OGM transgenic wheat lines and cv. Svevo were used for the infection experiments with the fungal pathogen. The opposite central spikelets in primary spikes were point inoculated and visually examined for the spread of disease for a period of 21 days. Three independent infection experiments on at least 15 plants of control Svevo and transgenic lines #12, #29 and #43 were performed in T_2 generation. FHB disease symptoms, manifested as spike bleaching, usually appeared 3

dpi and reached the maximum expansion 21 dpi. The OG-Machine transgenic lines exhibited a similar behavior and showed a significant reduction of symptoms starting from 3 to 17 dpi (Figure 41A). In particular, at 10 dpi, the symptom reduction in line #12 was by 57% compared to Svevo wild type, while the line #29 showed a symptom reduction by 34% compared to the same control. Also the line #43 displayed a significant reduction of symptoms by 46% compared to untransformed plants.

The fungal abundance, which was monitored by comparing the accumulation of *Fgβ-tubulin* gDNA at 48 and 72 hpi, was significantly lower in spikes of transgenic plants compared to Svevo wild type. At 72hpi, when the symptoms were already evident on inoculated spikelets, the transgenic lines #12, #29 and #43 displayed a fungal amount of 73.3%, 64.3% and 60.4%, respectively, compared to control Svevo (Figure 41B).

As shown in Figure 41C, at 10 dpi the severity of the symptoms was particularly evident in the spikes of Svevo wild type that showed 100% of the infected spikelets. On the contrary, spikes of OG-Machine lines showed a considerably lower severity of the disease.

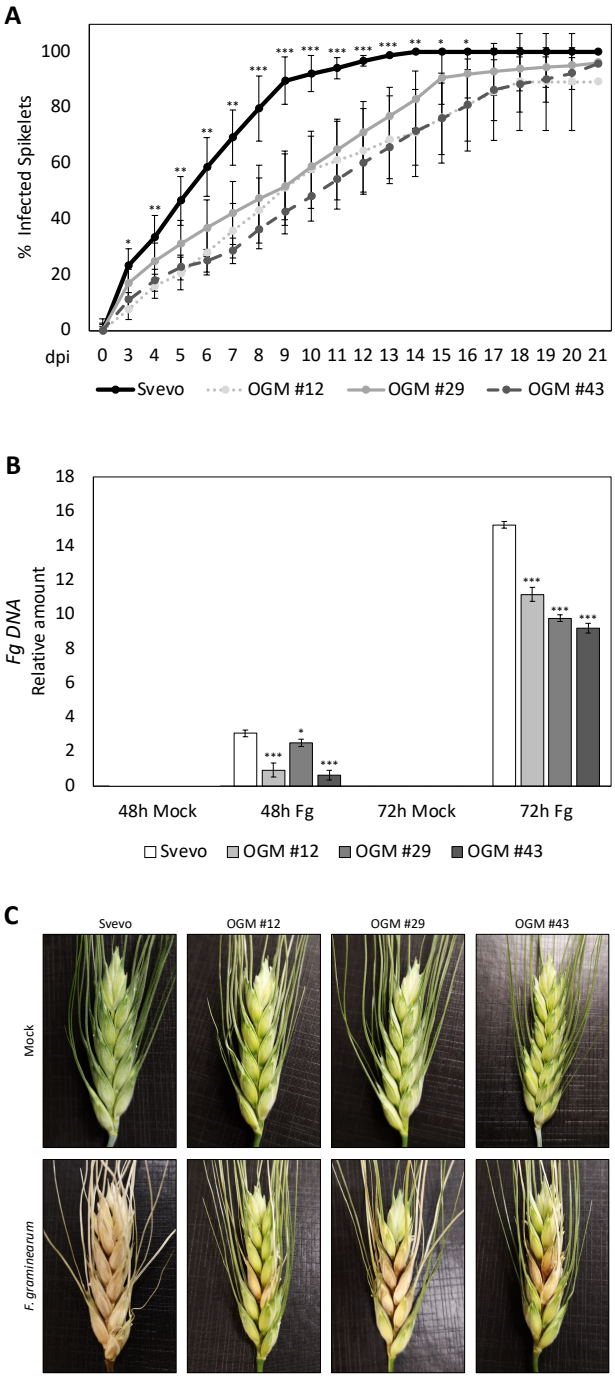


Figure 41. OG-Machine lines displayed a higher resistance to *F. graminearum.* A) Disease severity was express as percentage of infected spikelets. Disease progression was monitored for 21 dpi. All values are the means ± standard error (SE) (n = 10). Asterisks above the bars indicate values that are significantly different from transgenic plants for that time point (* $P < 0.05$; t-test); **B)** Real-time PCR analysis of the DNA content of the *Fg β-tubulin* gene relative to the wheat *TdACTIN* gene in wheat spikes with Fg at 48 and 72 hpi. All values are the means ± SE (n = 4). Asterisks above the bars indicate values that are significantly different from Svevo (*$P < 0.05$; t-test); **C)** Photograph showing disease spread in a representative spike from wheat cv. Svevo and TaPR1.1:OGM transgenic lines. Photographs were taken at 10 dpi.

4.2.4.1. Expression analyses of *TdPR1*, *FgTRI6* and *FgTRI5* genes in spikes of OG-Machine transgenic lines

To assess the correct inducibility of the OG-Machine construct also in spike tissue, accumulation of *PvPGIP*2 transcripts in #12, #29 and #43 transgenic lines was verified by qRT-PCR on cDNA. Samples were collected at 48 and 72 hpi with *F. graminearum*. Svevo was used as negative control. This analysis was performed three times. Results showed that *PvPGIP2* transcripts were detectable only in the transgenic lines and only in the presence of the pathogen. As already observed in seedlings, the three selected OG-Machine lines displayed different expression levels of the transgene at both 48 and 72 hpi. *PvPGIP2* transcript level in the line #12 was 2.8-fold and 1.6-fold higher than in line #29 and #43, respectively, at 72 hpi (Figure 42A).

To confirm that the induction of OG-Machine enhance resistance to *Fg* infection, expression analyses of plant immune marker genes and fungal toxin biosynthetic genes were carried out by qRT-PCR. Fungal infection resulted in significantly stronger induction of *TdPR1* in *TaPR1.1*::OGM plants than in Svevo wild type, therefore indicating the expression of OG-Machine promotes robust activation of defense responses. The expression of *TdPR1* in the transgenic lines #12, #29 and #43 was 3.8-fold, 2.8-fold and 3.6-fold higher, respectively, compared to cv. Svevo wild type plants at 72 hpi (Figure 42B). Interestingly, OGM lines displayed a downregulation of *FgTRI6* and *FgTRI5* suggesting a possible role of OG-Machine in both limiting the spread of the pathogen and the expression of toxin biosynthetic genes. The expression of *FgTRI6* in the transgenic lines #12, #29 and #43 was 4-fold, 2.6-fold and 3-fold lower, respectively, compared to cv. Svevo wild type plants at 72 hpi (Figure 42C). Similarly, the expression of *FgTRI5* in the transgenic lines #12, #29 and #43 was 3.1-fold, 2.1-fold and 2.2-fold lower, respectively, compared to wild type plants at 72 hpi (Figure 42D).

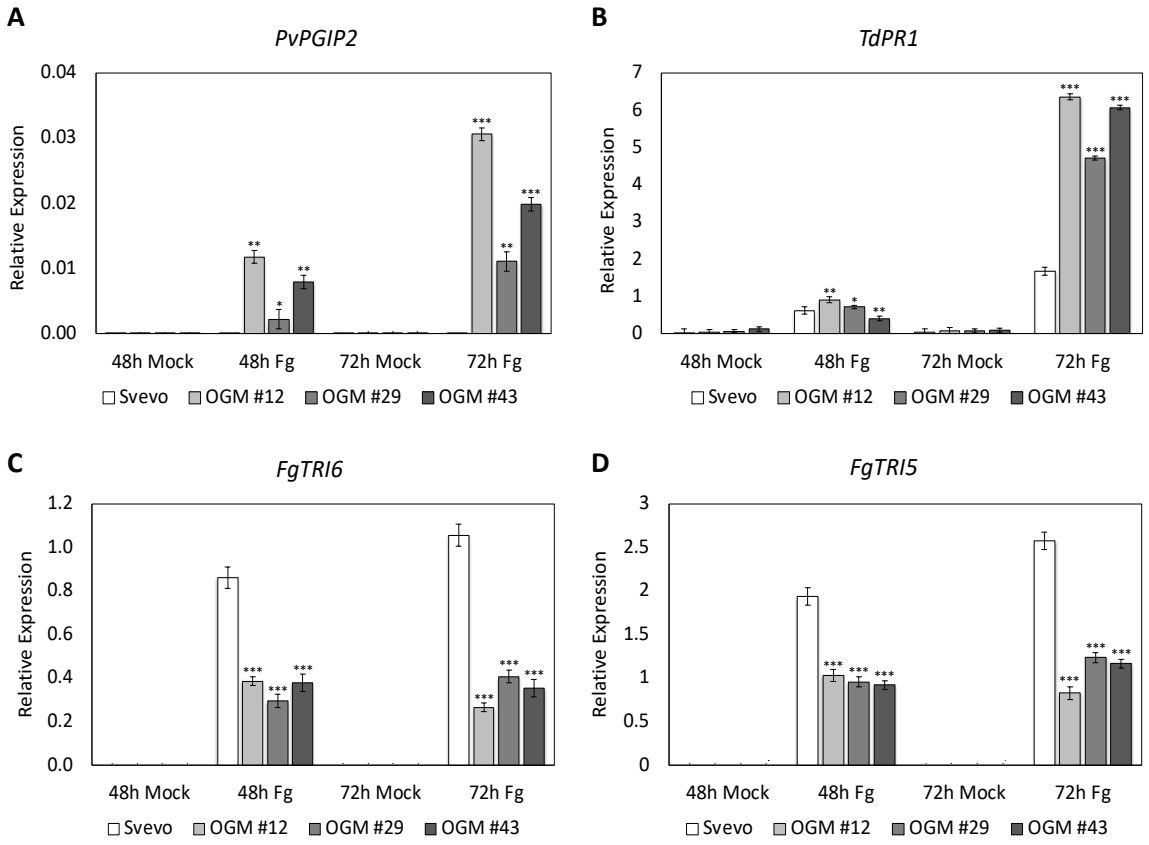


Figure 42. Evaluation of the *PvPGIP2* (A), *TdPR1* (B), *FgTRI6* (C) and *FgTRI5* (D) gene expression in *Fg*-inoculated spikes of wheat cv. Svevo and TaPR1.1::OGM transgenic lines. Gene expression was evaluated by qRT-PCR. Sterile water was used as mock treatment. Samples were collected at 48 and 72 hpi. *PvPGIP2* and *TdPR1* gene expression was normalized to the expression of the *TdACTIN* gene. *FgTRI6* and *FgTRI5* gene expression was normalized to the expression of the *Fgβ-tubulin* gene. All values are the means ± standard error (SE) (n = 4). Asterisks above the bars indicate values that are significantly different from Fg treatment (* $P < 0.05$; t-test).

4.2.5. Impact of OG-Machine on wheat agronomical and growth parameters

After verifying that the OG-Machine chimera limits the infection spread of *F. graminearum* and the mycotoxin biosynthesis, the impact of such chimera protein on important wheat agronomical and growth parameters after *Fg* infection was evaluated. In the analysis the number of seeds per spike, the number of seeds per spikelet, the primary spike weight, the weight of seeds per spike, the weight of 1000 seeds and the yield loss were detected in Svevo and *TaPR1.1*::OGM lines (Figure 43A-F).

The transgenic lines did not show any significant differences compared to Svevo wild type for any of the parameters analyzed in mock condition. In *Fg*-inoculated Svevo plants,

significantly lower yield values were observed for all the parameters considered. The number of seeds per spike and per spikelet were inhibited of 64.1% and 46.9%, respectively compared to untreated plants (Figure 43A-B). Moreover, results showed an inhibition of the primary spike weight, the weight of seeds per spike and the weight of 1000 seeds of 80.73%, 92,2% and 78,2%, respectively (Figure 43C-E). In *Fg*-inoculated OG-Machine plants, higher yield values were observed compared to Svevo plants inoculated with the fungus. Line #12 displayed an inhibition of the number of seeds per spike and per spikelet of 46.6% and 10.6%, respectively, compared to untreated #12 plants (Figure 43A-B). Similar results were observed for lines #29 and #43. Line #29 showed an inhibition of the number of seeds per spike and per spikelet of 43.5% and 14.3%, respectively, while line #43 of 43.2% and 19.4% for the same parameters considered (Figure 43A-B). For the primary spike weight, the weight of seeds per spike and the weight of 1000 seeds, a significant reduction of 52.7%, 63.9% and 62.2%, respectively, was observed in line #12 compared to untreated condition (Figure 43C-E). Similar results were observed for lines #29 and #43. Line #29 showed an inhibition of primary spike weight, the weight of seeds per spike and the weight of 1000 seeds by 57.1%, 65% and 65.9%, respectively, while line #43 by 57%, 55.4% and 57.7% for the same parameters considered (Figure 43C-E). Therefore, the expression of the OG-Machine seems to limit the infection spread of *F. graminearum* as well as the yield loss associated to fungal disease. The loss of production was then calculated and expressed as percentage (Figure 43F). Interestingly, *Fg*-inoculated Svevo plants displayed a production loss of 71.5%, while OG-Machine lines #12, #29 and #43 exhibited a yield reduction of only 40.7%, 41.2% and 40.2%, respectively, compared to untreated plants.

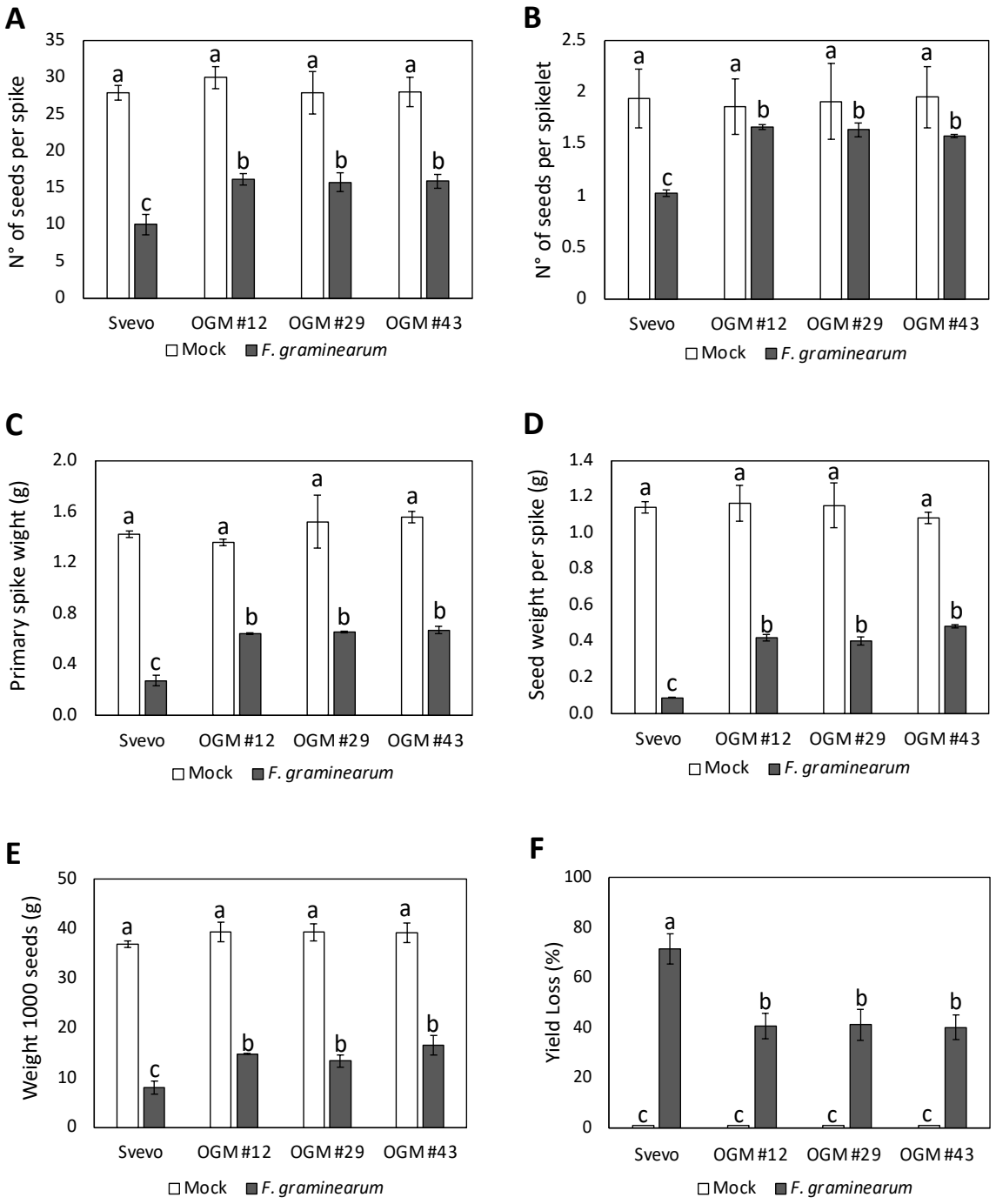


Figure 43. Agronomic and growth parameters in Svevo and OG-Machine transgenic plants after *F. graminearum* infection. A) Number of seeds per spike; **B)** Number of seeds per spikelet; **C)** Primary spike weight (g); **D)** Seed weight per spike (g); **E)** Weight 1000 seeds (g); **F)** Yield loss (%). A minimum of 15 plants per line were analyzed to obtain data. All values are the means ± standard error (SE). The statistical significance was tested by means of ANOVA followed by Tukey test. Different letters indicate statistically different values ($p < 0.05$).

The quality and the filling of kernels was also evaluated, considering the length and the width of wheat seeds, after *Fg* infection. As shown in Figure 44A-B, the untreated transgenic lines did not show any significant differences compared to Svevo wild type in terms of both seed

length and width in untreated condition. On the contrary, the presence of fungus *Fg* negatively impacted on both parameters considered by compromising the good filling of kernels and causing their shriveling. Seeds derived from *Fg*-inoculated Svevo spikes displayed an inhibition of seed length and width of 37,4% and 58,6%, respectively, compared to untreated condition. Interestingly, seeds derived from *Fg*-inoculated OG-Machine showed a more reduced shriveling and inhibition of length and width compared to Svevo control line (Figure 44C). Seed length of transgenic lines #12, #29 and #43 was inhibited by 19.4%, 28.4% and 7.2%, respectively, compared to untreated plants. Moreover, after *Fg* infection a reduction of seed width of 33.3%, 42.8% and 14.8% in line #12, #29 and #43, respectively, was observed.

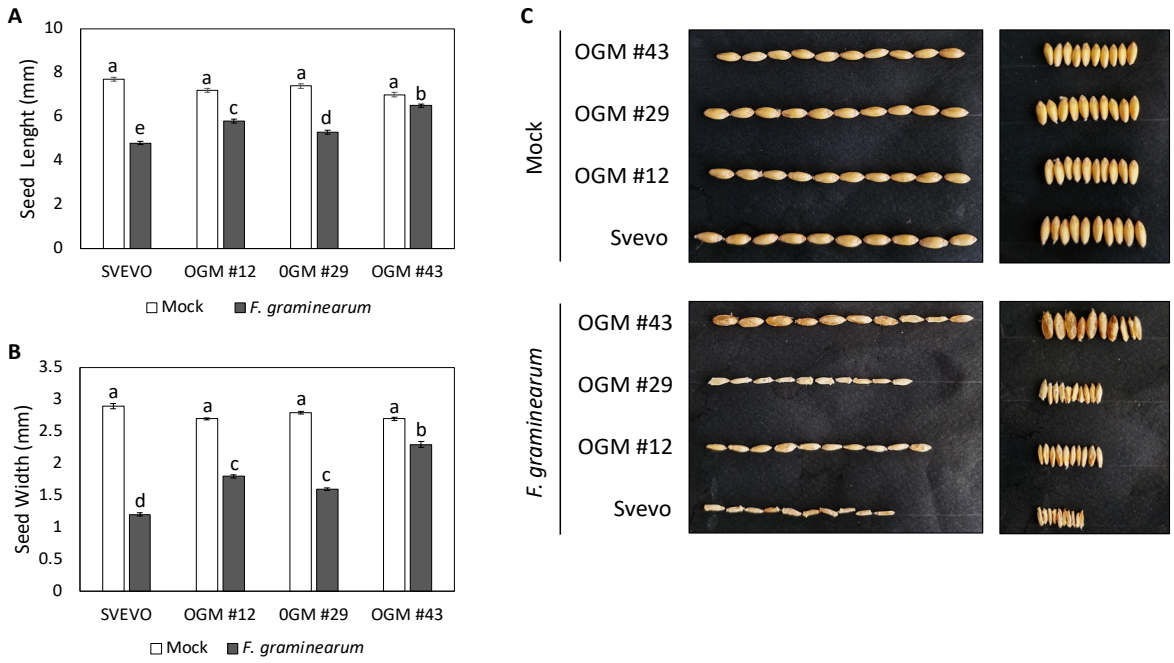


Figure 44. Seed quality and filling in Svevo and OG-Machine transgenic plants after *F. graminearum* infection. **A)** Seed length (mm); **B)** Seed width (mm); **C)** Photographs showing length and width of representative seeds for each line. Ten seeds per line and per treatment were analyzed to obtain data. All values are the means ± standard error (SE). The statistical significance was tested by means of ANOVA followed by Tukey test. Different letters indicate statistically different values ($p < 0.05$).

5. DISCUSSION

FHB causes substantial yield losses in wheat crop worldwide and compromises food safety because of the presence of toxins associated to fungal disease. Nowadays, chemical control represents the most used and effective strategy in crop protection. In the prospect of increased agricultural sustainability, new approaches, preferably based on green methods, are needed to control fungal disease, including FHB. In this *scenario*, the use of elicitors, able to stimulate the plant innate immune system, represents a novel and promising strategy in crop protection, as an alternative to conventional pesticides. Numerous studies indicate that local application of cell wall derived elicitors, such as COS and OGs induce broad-spectrum, long-lasting, and systemic resistance against foliar pathogens in different plant species (Ferrari et al., 2007; Aziz et al., 2004; Moscatiello et al., 2006). OGs are potent elicitors of immunity when applied exogenously as they are capable of triggering a wide range of defense responses including accumulation of phytoalexins in soybean (Davis et al., 1986), deposition of callose, production of ROS in Arabidopsis (Galletti et al., 2008) and NO (Rasul et al., 2012) in Arabidopsis, accumulation of SA in strawberry (Osorio et al., 2008), activation of defense related genes (Denoux et al., 2008). Nevertheless, so far, a clear demonstration of the OG capability to activate defense-associated responses and resistance against F. graminearum in wheat is lacking. Therefore, the aim of this study was to establish the efficacy of OGs in protecting durum wheat, characterized by an extreme susceptibility to fusariosis.

The results of my PhD thesis demonstrated that, when exogenously applied to different wheat tissues, OGs can induce the expression of typical defense-related genes (i.e. *TdPR1*) genes, and restrict FHB symptoms, therefore suggesting that they are sensed as danger signals. This was further confirmed by RNAseq analyses, carried out to obtain a general view of the transcriptome in durum wheat seedlings inoculated with OGs at different concentrations (10 and 500 µg/ml), *Fusarium graminearum* spores (2 x 10^3 conidia) or a co-treatment of both OGs and fungus spores. Out of the 66559 *T. turgidum* total genes 4577 genes were found to be differentially regulated in all samples with 84 genes exclusively regulated in OG10, 83 in OG500 and 133 in CHIT100 (used as positive control). Among the genes exclusively up-regulated in OG500 treatment is a putative polyamine oxidase-like. It has been suggested that the production of hydrogen peroxide (H_2O_2) deriving from polyamine oxidation might be correlated with cell wall

reinforcement during pathogen invasion, besides cell wall maturation and lignification during plant development. Moreover, H_2O_2 originated from polyamine oxidation may be involved as signal molecules in mediating cell death, the hypersensitive response and the expression of defense genes (Cona et al., 2006). Further, an *NHL* gene, involved in the activation of plant defense responses to biotic stresses, was found to be up-regulated in response to OG500, confirming the regulation of defense-related genes mediated by these elicitors, independently from the presence of the pathogen. Among the 113 genes deregulated in OG500 (47 were exclusive) many were related to defense processes, such as "Regulation of defense response to fungus", "Regulation of response to external stimulus", "Response to fungus", "Defense response to fungus". As in the case of stomatal density, a dose-dependent response in the induction of resistance to FHB was observed; the higher the concentration of OG was used, the greater the reduction of symptoms was observed. Interestingly, the most up-regulated gene in OG10 treatment was a *PEROXIDASE 5 LIKE* gene (*TRITD0Uv1G116360*), whereas it resulted to be more than 30-fold repressed in the case of OG500 treatment. It will be of great interest to study the possible involvement of such gene in determining specific responses to different doses of OGs, together to the other 83 genes specifically deregulated in one or the other treatment, respectively. Among these, regulators of stomata formation may be included, given that low concentration of OGs favor an increase in stomatal density, while higher doses caused a significant decrease. Noteworthy, this kind of response to OGs is very similar to the response of plant tissues to phytohormones and could be used as a tool to engineer plants to improve water-use efficiency and drought tolerance without yield reductions (Bertolino et al., 2019). In this context, the exogenous application of OGs 500 μg/ml may be a useful approach to increase drought tolerance, reduce water loss and limit pathogen penetration into the plant trough stomata.

It is worth noting that wheat seedlings grown *in vitro* and treated with OGs did not display phenotypic alteration compared to untreated plants in terms of fresh weight, root length and first leaf length. On the contrary, CHIT strongly inhibited plant morphological parameters. Even if not comparable, such findings seem to contradict other studies reporting that chitosan may promote growth and development of plants, enhancing the photosynthesis rate of maize (Khan et al., 2002), inducing early flowering and increasing the accumulative inflorescence number of orchids (Limpanavech et al., 2008), promoting nutrient uptake of beans (Chatelain et al., 2014), and increasing the yield of wheat (Wang et al., 2015).

As regards the evaluation of the OG capability to restrict *F. graminearum* growth in different durum wheat tissues, it was considered that the traditional wheat head infection assay is limited due to seasonal, temporal and spatial factors. The distinct structures (rachis, paleas, lemmas, caryopses and glumes) and diverse features of wheat florets also make it difficult to track the infection progress of *F. graminearum*. Therefore, a modified wheat coleoptile infection assay was used to study fungal infection inside wheat host tissue (Jia et al., 2017). Unlike the wheat head infection assay, there are few temporal and spatial constraints for the seedling infection system. The results showed that the exogenous application of OGs led to a significant reduction of disease lesion size on wheat coleoptiles. Relevant genes involved in the wheat response to pathogens, including several chitinases and β-1,3-glucanases, have been found in the *Fg* and elicitor-cotreated plants. Several genes coding for diverse chitinases have been found to be up-regulated in OG10 inoculated plants, while only one gene coding for a basic endochitinase-like (*TRITD7Av1G248080*) was up-regulated in OG500 treated seedlings. Several genes coding for endoglucanases and glucan endo-1,3-beta-glucosidase have been also present in *Fg*, *Fg*+CHIT100, *Fg*+OG10 and *Fg*+OG500 samples. Interestingly, two glucosidase genes (*TRITD5Av1G203860* and *TRITD7Bv1G020930*) were found to be up regulated in OG10 inoculated plants. Genes involved in cell wall metabolism were also affected. The expression of genes involved in plant cell wall metabolism was also altered, including genes involved in the phenylpropanoid pathway. Genes encoding for caffeoylshikimate esterase-like, an enzyme with a role in lignin biosynthesis (Vanholme et al., 2013) were also found to be regulated in plants cotreated with OGs and *Fg* and inoculated with the fungus alone. Particularly, *TRITD3Av1G215830, TRITD3Bv1G201050, TRITD1Bv1G138100* and *TRITD5Bv1G161840* were all up-regulated in *Fg* and *Fg*+OG500 inoculated plants, while *TRITD5Bv1G161840* resulted to be down-regulated in *Fg*+OG10 ones. Only *TRITD3Av1G215830* and *TRITD3Bv1G201050* were found to be up regulated in *Fg*+OG10 inoculated plants. This result confirms the diverse regulation in the presence of a different OG concentrations (OG10 vs OG500). The up-regulation of genes coding for a probable *PECTINESTERASE 53* (*TRITD2Av1G026410*) and a *PECTINESTERASE INHIBITOR 8-LIKE* (*TRITD2Av1G261160*) was also found in OG10 inoculated plants, but not in the presence of the pathogen. Among the down-regulated genes in *Fg*+OG500, genes coding for 3-*KETOACYL*-CoA *SYNTHASE 5-LIKE* (*TRITD7Bv1G046900* and *TRITD7Av1G031380*), an enzyme putatively involved in cuticular wax biosynthesis (Yang et al., 2021). The same genes were up-regulated in

Fg+OG10, while a third 3-ketoacyl-CoA synthase 5-like (*TRITD6Av1G004320*) resulted to be down-regulated in *Fg* inoculated plants. Two *EXPANSIN* genes (*TRITD1Av1G134890* and *TRITD5Av1G240040*) were also up regulated in *Fg* infected plants, independently from the OG treatment (*Fg*, *Fg*+OG10 and *Fg*+OG500). It is worth noting that any expansin genes was regulated by CHIT100, independently from the presence of the pathogen. Since the *PR1* gene is recognized as a marker gene for the SA activated defense responses (Delaney et al., 1994), and, in *A. thaliana* leaves, the expression of *AtPR1* was enhanced in response to *F. graminearum* infection (Chen et al., 2006), the expression analysis of *TdPR1* was carried out. In the presence of CHIT, the expression of *TdPR1* was largely induced at 24 and further increased at 48 hpi. Similar results were observed in seedling treated with OGs. Interestingly, the *TdPR1* expression was significantly induced at 6 hpi only in response to OG treatment, hinting that downstream shared and distinct signaling pathways may be activated in response to the PAMP and the DAMP, as confirmed by transcriptome analyses in section 4.1.2.1. and discussed above. In seedlings cotreated with both *Fg* and elicitors, the *TdPR1* expression was higher than that observed in tissues treated with the fungus alone, at both 24 hpi and 48 hpi. Noteworthy, in tissues cotreated with the fungus and OGs, a more robust induction of *TdPR1* gene expression was observed, 3- and 1.9-fold higher than in tissues treated only with *Fg*, at 24 hpi and 48 hpi, respectively. Therefore, OGs trigger both a priming effect and the induction of typical immune marker genes, such as *TdPR1*, known to be part of the SA signaling pathway. In order to assess the fungal pathogenicity, I analyzed the expression of *FgTRI6* gene. A considerably lower expression of such gene was detected in tissues cotreated with either OGs or chitosan compared to tissues inoculated with the fungus alone, both at 24hpi and at 48hpi. Therefore, both CHIT and OGs seem to limit the spread of the fungus in vegetal tissues and consequently to decrease the abundance of mycotoxins.

To confirm the results obtained in wheat seedlings, similar analyses were carried out also in wheat spikes. FHB disease severity in wheat flowering heads of cv. Svevo was expressed as percentage of infected spikelets and was monitored for 21 dpi. A slower disease progression was detected in wheat heads inoculated with the co-treatment *Fg* and OGs compared to the ears inoculated with the fungus alone. Previous studies demonstrated that *Fusarium graminearum* was highly sensitive toward chitosan since this compound inhibits fungal spore germination, mycelium growth and virulence (Luan et al., 2022; Francesconi et al., 2021). These results agree with the data presented in my present study, since it was observed that in presence of the cotreatment Fg and CHIT wheat heads did not

show symptoms of infection. Interestingly, the severity of the symptoms was particularly evident in the spikes inoculated with the fungus alone that show 100% of the infected spikelets, while the ears inoculated with *Fg* and OG cotreatment showed a considerably lower severity of the disease.

In the present study, it was demonstrated that OGs, like chitosan, limit the spread of the *Fg* in wheat plant tissues and, consequently, decrease the mycotoxin contamination, determined by analyzing the transcript amounts of the major genes involved in plant defense and mycotoxins biosynthesis. In the presence of pretreatment with OGs and chitosan, the expression of *TdPR1* was significantly higher compared to untreated spikes at 6, 24 and 48 hpi. However, different kinetics of induction were observed between leaves and spikes. In co-treatment experiments with both fungus and elicitors, the expression of *TdPR1* resulted to be lower than that observed in spikes inoculated with *Fg* only. Noteworthy, contrasting levels of *TdPR1* gene expression were observed in wheat seedling and spikes in presence of the fungus and elicitors. In according with other studies (Silva et al., 2019), these results suggest that mechanisms of defense could be differently regulated in different organs. A lower fungus abundance, accompanied by the downregulation of *FgTRI6* and *FgTRI5* biosynthetic genes, was detected in elicitor co-treated spikes.

Concerning the impact of elicitor treatment on agronomic and yield parameters of *Fg*-infected durum wheat plants, this study shows that exogenous application of OGs reduces the yield losses associated with *F. graminearum* infection by 51,34%. Moreover, spikes cotreated with fungus and OGs displayed higher values compared to ears treated with the fungus alone in terms of number of seeds per spike and spikelet, primary spike weight, seed weight per spike and weight of 1000 seeds. Furthermore, it was observed that OGs and chitosan limit the shriveling of *Fg*-infected durum wheat seeds. In particular, wheat spikes and seeds in presence of cotreatment with CHIT and *Fg* did not show differences compared to untreated condition.

Based on the knowledge that OGs may activate a wide range of defense responses, manipulation of plant innate immunity by engineering OG sensing and signaling could be a valid approach to strengthen plant immune responses against a broad spectrum of microorganisms. Therefore, I generated and analyzed transgenic durum wheat lines expressing the OG-Machine under the control of a pathogen-inducible promoter, *TaPR1.1*. The aim was to evaluate the possible involvement of the OG-Machine in regulating durum wheat responses to *Fusarium graminearum* infection. Analyses were conducted on seedling and spikes of three different OG-Machine transgenic lines, i.e. #12, #29 and #43.

The first investigation was carried out to evaluate the possible involvement of the OG-Machine in limiting *Fusarium graminearum* infection. *Fg*-inoculated OG-Machine seedlings displayed a significant reduction of disease lesion size on coleoptiles and a slower disease progression compared to Svevo wild type plants. Similar results were observed also on spikes after wheat head infection assay. Interestingly, at 10 dpi, the severity of symptoms was particularly evident in Svevo control line showing 100% of infected spikelets, while transgenic lines show a considerably lower percentage of infected spikelets.

Afterwards, the inducibility of the OG-Machine and its ability in regulating wheat responses to *Fusarium graminearum* infection were evaluated by analyzing the expression of *PvPGIP2* and *TdPR1* genes, respectively, both in seedlings and spikes. Similar results were obtained in two specific wheat organs. The induction of *PvPGIP2* was detected only in transgenic lines and only in presence of *Fg.* Interestingly, OG-Machine transgenic lines displayed different levels of expression of the transgene. Transcript level of the line #12 was 9-fold and 7-fold higher than in line #29 and #43, respectively, at 72 hpi. The different expression level of the transgene in three OG-Machine lines was demonstrated to be correlated to the number of the OGM construct insertions in their genome. Results indicated that the line #12 carries a higher number of insertions compared to #43, which displayed a slightly higher amount of amplicons respect to #29. OG-Machine chimera expression greatly increases the abundance of *TdPR1* transcripts in seedlings and spikes. In fact, the expression of *TdPR1* in line #12, #29 and #43 was higher compared to Svevo wild type plants at 72 hpi. Moreover, OG-Machine plants displayed a higher resistance to *Fg.* The fungal abundance was lower within the coleoptiles and spikes of transgenic lines compared to control cv. Svevo. In addition, the expression of *FgTRI6* and *FgTRI5* was found to be downregulated in OG-Machine *Fg*-infected lines, suggesting a possible role of OG-Machine in both limiting the spread of the pathogen and negatively affect the expression of toxin biosynthetic genes.

Concerning the impact of OG-Machine on agronomic and yield parameters of *Fg*-infected durum wheat plants, this study shows that the expression of the PvPGIP2-FpPG chimera reduces the yield losses associated with *F. graminearum* infection by 30,6%. Moreover, transgenic lines spikes inoculated with fungus displayed higher values compared to wild type Svevo ears in terms of number of seeds per spike and spikelet, primary spike weight, seed weight per spike and weight of 1000 seeds. Furthermore, it was observed that the induction of OG-Machine limits the shriveling of *Fg*-infected durum

wheat seeds. Interestingly, seeds of wheat transgenic lines derived from *Fg*-inoculated spikes, displayed a major length and width compared to Svevo seeds after fungal infection. It resulted particular evident for seeds of line #43. These results highlighted that there is not a correlation between the total amount of OG-Machine transcript and the resistance to *Fg* infection observed in three different lines. Based on the knowledge that transgenic Arabidopsis plants expressing the OG-Machine under the control of a pathogen-inducible promoter displayed *in vivo* production of OGs (Benedetti et al., 2015), the enhanced resistance against *F. graminearum* in wheat transgenic lines could be associated to potentially altered endogenous OG levels.

6. CONCLUSION AND PERSPECTIVES

The improvement of natural plant defense mechanisms to control pathogen attacks is one of the most attracting strategies to increase resistance in a sustainable manner. In this scenario, the use of cell wall derived elicitors, such as OGs, able to stimulate the plant innate immune system, represents a novel and promising strategy in crop protection, as an alternative to conventional pesticides.

The present work allowed firstly to establish the functionality of OGs as elicitors of immunity and their ability in protecting durum wheat, characterized by an extreme susceptibility to fusariosis. The improvement of disease response in a highly susceptible species, like durum wheat, must be considered as an important result. Concerning the impact on FHB disease, this is the first observation of direct involvement of OGs in enhancing resistance against *F. graminearum* phytopathogen.

This study demonstrated that the exogenous application of OGs led a significant decrease of FHB lesion sizes and disease severity on wheat coleoptiles and spikes, respectively. Moreover, it was observed that OGs are active elicitors of plant defenses in wheat, triggering the induction of typical immune marker genes in spikes characterized by a priming effect in seedlings. The reported results highlighted that both chitosan and OGs limit the spread of the fungus in wheat seedlings and spikes and, consequently, decrease the mycotoxin contamination due to lower expression of *FgTRI6* and *FgTRI5* biosynthetic genes. Furthermore, based on reported data it was demonstrated that exogenous application of OGs reduces the yield losses associated with *F. graminearum* infection by 51,34% and limit the shriveling of *Fg*-infected durum wheat seeds. An important aspect to be considered is that wheat seedlings and spikes did not display phenotypic alteration in terms of growth and development after elicitation with OGs. On the contrary, it was observed that chitosan is a promising elicitor of immunity in wheat against *Fusarium graminearum*, but strongly inhibits plant morphological parameters.

Based on the knowledge that engineering OG sensing and signaling could be a valid approach to strengthen plant immune responses, I generated and analyzed transgenic durum wheat lines expressing the OG-Machine under the control of a pathogen-inducible promoter, *TaPR1.1*. The aim was to evaluate the possible involvement of the OG-Machine in regulating durum wheat responses to *Fusarium graminearum* infection. Data demonstrated that the expression of the PvPGIP2-FpPG chimera is induced only upon

pathogen detection in all transgenic lines analyzed and leads to a significant decrease of FHB lesion sizes and disease severity on wheat coleoptiles and spikes, respectively. Moreover, it was observed that the OG-Machine lines display a more robust induction of *TdPR1* expression in response to FHB, accompanied by the downregulation of *FgTRI6* and *FgTRI5* genes. In addition, the fungus growth was lower within the coleoptiles and spikes of OG-Machine transgenic lines compared to control cv. Svevo. These findings suggest a possible role of OG-Machine in both limiting the spread of the fungus and negatively affect toxin biosynthetic genes. In this study, it was also evaluated the impact of OG-Machine expression on agronomic and yield parameters of *Fg*-infected durum wheat spikes. Noteworthy, it was observed that the expression of the PvPGIP2-FpPG chimera reduces the yield losses associated with F. *graminearum* infection by 30,6% and limits the shriveling of *Fg*-infected durum wheat seeds.

The enhanced resistance against *F. graminearum* in wheat transgenic lines could be associated to potentially altered endogenous OG levels. However, more studies are necessary to evaluate whether endogenously generated OGs accumulate to significant concentrations and function as signaling molecules in pathogen-infected wheat tissues. Therefore, a strategy that uses the OG-Machine to generate OGs that function as DAMPs could potentially be used to engineer crops to be more resistant to microbial pathogens.

Furthermore, this study demonstrate also that the activation of OG-Machine triggers a defense response similar to that activated when OGs are exogenously applied, pointing also to a possible biotechnological strategy to protect plants against microbial diseases.

In conclusion, based on data reported, this study provides the first evidence that wheat coleoptile infection assay is a rapid and highly reproducible method to perform large-scale phenotypical analysis and to study the infection process of *F. graminearum* without being limited due to seasonal, temporal and spatial factors.

A starting point for further analysis could be to test such approach in the field, involving different durum wheat genotypes and different pathogens.

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