“Controlling mycotoxin producing fungal pathogen *Fusarium graminearum* using beneficial microorganisms”

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Abstract

In the history of humankind, cereal crops are important as they are staple foods. It is calculated that about 29% of the world production of cereals corresponds to wheat, which was around 757,4 million of tones for the period of 2017-18. However, the worldwide population is estimated will reach 9.2 billion by 2050. Besides, the United Nations set a goal to reduce hunger to zero by 2030. It is thought that cereal crop production is not enough and it is necessary to find solutions to help to cope with the future demand. Nevertheless, the production of cereal crops is typically affected by cultural practices, germ lines selected, droughts, climate change and plant diseases and pests.

Fusarium graminearum (*Fg*) is an economically important fungal pathogen of the grass crops including wheat and maize and it causes Fusarium Head Blight (FHB) in wheat. Infected plants display symptoms such as shrunken grains and chalky white appearance in heads. Additionally, this fungus produces mycotoxins including deoxynivalenol (DON), the T-2 and fumorisin B1. Translocation of mycotoxins in grains leads to loss in yields and low quality of grains. Additionally, these mycotoxins can cause serious health problems on humans and livestock, mainly by the DON or vomitoxin.

Different approaches have been explored to control this fungus. Chemical fungicides were the first option some years ago. However, they damage the environment, fungal pathogens developed resistance to them and some of them including Carbendazim and Epoxiconazole have already been banned in the UK and other countries. On the other hand, resistance genes are used in plant breeding which have the disadvantage that it is possible for pathogens to overcome the resistance. Crop rotation is also challenging when farmers rotate with the same type of crops. Recently, there is an emergence of the use of RNA for silencing pathogenic genes in crop diseases.

Nevertheless, work still needs to be done to find better alternatives to suit the control of this pathogen. Here, it was proposed a strategy that uses microbial biological control agents (MBCA). The international market of MBCA is growing due to its advantages and nowadays there is better understanding of their general use and effects and substantial research is advocated to this area. Moreover, commercial strains have already been in the

market for several years, and they are not totally understood. Additionally, new strains are emerging and need to be studied. Hence, there is a lack of understanding to determine the specific mode of action of these MBCAs in *in vivo* assays and, more specifically, the gene expression of the *Fg* challenged with the BCA was not known.

Here it is reported the study of the commercial strains, QST713 (Serenade®) and FZB24 (TAEGRO®) and the non-commercial strain EU07 as MBCA which possesses antagonistic effect against *Fg*-K1-4 and as a plant growth promoter. It was found that this new strain has better performance controlling the *Fg*-K1-4, it challenges the growth-defence trade-off phenomenon and produces changes in morphology and gene expression of the *Fg*-K1-4.

Our results showed that EU07 and FZB24 bacterial broths at 2.5 and 5.0 µg/mL, treated with heat and proteinase K, showed significant reduction of the fungal growth (69.7 - 75.2%, n=36, $\alpha < 0.05$) compared with QST713. Infection head assays showed that Brachypodium distachyon line 21 (*Bd*-21) plants infected with *Fg*-K1-4 and treated with EU07 showed decreased %DSI compared with the plants non-treated (26% to 16%, $\alpha < 0.05$, n=330), in foliar treatments. EU07 significantly increases the defence responses on *Arabidopsis thaliana* (*At*) plants infected with the naturally infecting powdery mildew fungus ($F (4, 20) = 45.000$, $\alpha <.000$). EU07 treatment increases the biomass in leaves ($F (3, 20) = 3.763$, $\alpha = 0.027$) and decreases the flowering time ($F (4, 20) = 510629.69$, $p <.000$). Furthermore, EU07 increases the production of heads in *Bd*-21 significantly ($F (6, 65) = 4.36$, $p = .001$).

Comparative genomics showed that these *Bacillus* strains are all in the same Operational Group *B. amyloliquefaciens*. QST713 and EU07 are *B.* velezensis, two different strains variants with similar genome length (4.2 Mbp). FZB24 is a *B. amyloliquefaciens* has the same genome size as FZB42, but smaller genome size (3.9 Mbp) than QST713 and EU07. Moreover, same analysis revealed that EU07 possesses specific genes not found in QST713 (scf_25983_10, UPF0457 protein YnzG and scf_25983_44 and Aspartate racemase (EC 5.1.1.13)). More genes were found to be different between EU07 and QST713 at different grades of identical sites (%). Interestingly, a great proportion of genes, present in EU07 and QST713 but not found in FZB24 are classified to be Phages-prophages and RNA process modification.

Finally, in the transcriptomics analysis were found 709 significantly differentially expressed (DE) genes in a threshold FDR of 0.01 and logFC 1, which were related to ribosomal functions. A total of 2,232 transcripts were found to be significantly differentially expressed (DE) in the threshold FDR 0.05 and abs logFC 1 in which it was observed a decrease in the level of expression of genes related to mycotoxin production (aurofusarin and trichothecene clusters). Finally, it was also found a decrease in level of expression of some orphan secreted protein (OSP) genes, which has function as effectors, in this threshold.

Upon the completion of this research, altogether, it is demonstrated that the three *Bacillus* strains are competent antagonistic agents against *Fg*. More specifically, it was showed that the novel strain, EU07, it is an important antagonistic agent against *Fg* in both *in vitro* and *in vivo* due to the metabolites it produces. The PGP-activity assays demonstrated superiority of EU07 on biomass production, immune response and flowering time which is related to its gene content. More importantly, EU07 overcame the growth-defence trade-off phenomenon, stimulating the growth of the plant whilst increasing the defence response in *Arabidopsis thaliana* and *Brachypodium distachyon*. It is reported the sequencing of the novel strain EU07. It is provided the first transcriptomic results of the interaction of *Fg* with a *B. velezensis*, obtaining transcripts of the *Fg*-K4-1. It is provided data in regards of the expression of genes related to pathogenesis and mycotoxin production affected by the presence of EU07. Finally, future work needs to be done to study EU07 and its use as a potential commercial product has been discussed.

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Declaration of the authors rights

I declare that the thesis here presented is my sole own work and generated by myself during my research work.

I confirm that the work was done wholly while in candidature for the PhD degree at this University and have not been submitted to any other qualification or institution. Where any published work of others was used, it was clearly attributed or stated in the written work. When join contributions of other authors were made, acknowledgement was expressed.

Abbreviations

18S	Small component of the structural ribosomal RNA in Svedberg units.
28S	Large component of the structural ribosomal RNA Svedberg units.
At	*Arabidopsis thaliana*
atm	Atmosphere -pressure unit-
Bd	*Brachypodium distachyon*
Bd-21	*Brachypodium distachyon* line 21
BME	2-Mercaptoethanol (also β-mercaptoethanol, 2-ME or β-met)
bp	Base pair
BSA	Bovine Serum Albumin
˚C	Degree Celsius
cDNA	Complementary DNA
CDS	Coding sequence
CMC	Carboxymethylcellulose agar
Col-0	Columbia accession (*At*)
Cp	Cross point
Cq	Quantification cycle
d	Day
D	Dark regime
dai	Days after inoculations
DNA	Deoxyribonucleic acid
DEPC	Diethyl Pyrocarbonate
EDTA	Ethylenediaminetetraacetic acid

EDS1	Enhanced disease susceptibility 1
EPPO	European and Mediterranean Plant Protection Organization
EU07	*Bacillus velezensis* var
FBI	*Fungal-bacteria interaction*
Fg	*Fusarium graminearum*
Fg-K1-4	*Fg* strain used in this study
FG	Fungal growth
FHD	*Fusarium* head blight
Fo	*Fusarium oxysporum*
FPKM	Fragments per kilobase per million
Fv	*Fusarium verticillioides*
FZB24	*Bacillus amyloliquefaciens* var
gDNA	Genomic DNA
GUS	β-glucuronidase Reporter system
h	Hour
HCl	Hydrochloric acid
Hpa	*Hyaloperonospora arabidopsidis*
HSE	Health and Safety Executive
kb	Kilobase
LB	Luria-Bertani broth
L	Light regime
M	Molar
mg	Milligrams
min	Minute
ml	Millilitre
mm	Millimetre

mM	Millimolar
mRNA	Messenger RNA
N	Normal
NA	Nutrient agar
NaCl	Sodium chlorate
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometre
nt	Nucleotide
n.d.	Not date
OD	Optical density
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PD	Potato dextrose broth
PDA	Potato dextrose agar
ppm	Parts per million
PR-Gus	Pathogen-related GUS
QC	Quality control
qPCR	Quantitative polymerase chain reaction
QST713	*Bacillus velezensis* var
RIN	RNA integrity number
RLK	Receptor like kinases
RPM	Revolutions per minute
RNA	Ribonucleic acid
RPKM	Reads per kilobase per million
RT	Room temperature

RT-PCR	Reverse transcription polymerase chain reaction
RNA-seq	High-throughput RNA sequencing
s	Second
sdH_2O	Distilled water
SAR	Systemic Acquired resistance
SNA	Spezieller Nährstoffarmer Agar
Tm	Melting temperature or annealing temperature
TPM	Transcripts per million
UBQ	Ubiquitin gene
µg	Micrograms
µl	Microliter
µm	Micrometre
UI	Enzymatic activity
V	Volts
v/v	Volume / Volume
w/v	Weight/ Volume
WA	Water agar
WGS	Whole genome sequencing

Chapter 1

General Introduction

1.1 Introduction

Since the last century, chemical pesticides have been used heavily to increase the availability of food for humankind. The rise in the production of crops came along with concerns in the environmental and health issues since the discovery of most powerful and synthesised pesticides. From a few decades ago, the tendency to use alternatives have been progressed, enhanced by the discovery of microorganism capable of suppressing crop pests and pathogens (Matthews, 2015). Nonetheless, the pressure for the increase of the production of crops is driven by the necessity of supply by the demand of the growing world population.

1.2 Food Security

The world population has been increasing at a high rate. By the year 2050, it is projected that the population will rise up to 9.1 billion people, this is 30% more than nowadays. The aforementioned, represents a common preoccupation in the sense of, how to keep supplying the basic needs of the humankind. Food, which is probably the main necessity for the humans, takes special attention. Upon this, the United Nations (UN) started the program Millennium Development Goal, which aims

to tackle specific problems in human society by 2015. It is also named Sustainable Development Goals (SDGs), claims to reduce the hunger to zero, achieve food security and promote sustainable agriculture by 2030. The merit of this effort is promoting the continuous access to safe and nutritious food for all the people throughout the countries around the world (United Nations, n.d.). Besides that, the arable lands have been becoming less available. Additionally, the surge of climate change adds to the problem, displacing the areas suitable for food production and healthy crops (Tirado et al., 2010).

Same authors drew the attention to a specific factor that can threaten the food security efforts, which are reflected in the disease triangle of epidemiology (a host [susceptible plant], an agent [pathogenic organisms] and a favourable environment). They continue adding some other factors to the list, which include: the loss of ecosystems, the recent tendency of use of the land for fuel productions instead of food crops and the consequent increasing the prices of basic foods such as maize. Moreover, as mentioned above, climate change adds a high quote of importance to the food availability due to the contamination of food sources with non-infections hazards such as mycotoxins. These factors could worsen the availability, the safety and the access to food due to the lowering of the production and the increasing the market prices.

1.3 Importance of Wheat

Some of the important crops are wheat, barley, rice and maize as they are the primary foods worldwide due to their status as staples of the culinary preparations for humans for thousands of years. They all, belong to the grass family (*Poaceae*). The most economical representatives are distributed in three grass subfamilies: the Ehrhartoideae (rice), Pooideae (wheat, barley, rye) and the Panicoideae (maize, sorghum, sugarcane and millets). Wheat (*Triticum aestivum*) and their varieties are mostly polyploids, containing several copies of independent genomes (Vogel et al., 2010). The world production of the wheat was around 757.4 millions of tones for the period of 2017-18, that is about 29% of the total cereal production in the same period. Particularly, the UK's production was 13.55 millions of tones with a value of £2.1 billions (FAO, 2020).

1.4 Plant Diseases in Wheat

Wheat and other cereal crops are affected by many plant diseases that have been investigated for a long time. At the beginning of the XX century, the most remarkable examples of diseases included yellow rust (*Puccinia striiformis* var. *tritici*), bunt of wheat (*Tilletia caries*), leaf spot of oats (*Pyrenophora chaetomioides*), leaf stripe of barley (*Puccinia striiformis* f. sp*. hordei*), take-all (*Gaeumannomyces graminis* var. *tritici*), eyespot (*Kabatiella zeae*) and others ("Diseases of Cereals," 1945). Today, the most important diseases in wheat are: brown rust (*Puccinia triticina*), Fusarium

head blight (*Fusarium graminearum*), Fusarium foot rot (*Fusarium* spp.), mildew (*Blumeria graminis* f. sp.), *Septoria tritici* in wheat, true eyespot (*Tapesia yallundae*) and yellow rust (*P. striiformis* f.sp. *tritici*) (CropScience, n.d.).

Diseases produced by *Fusarium* species are some of the most studied but still producing new questions. For instance, much uncertainty still exists on their role in plant diseases and their control, especially *Fusarium* ear blight or head blight (Figure 1.1).

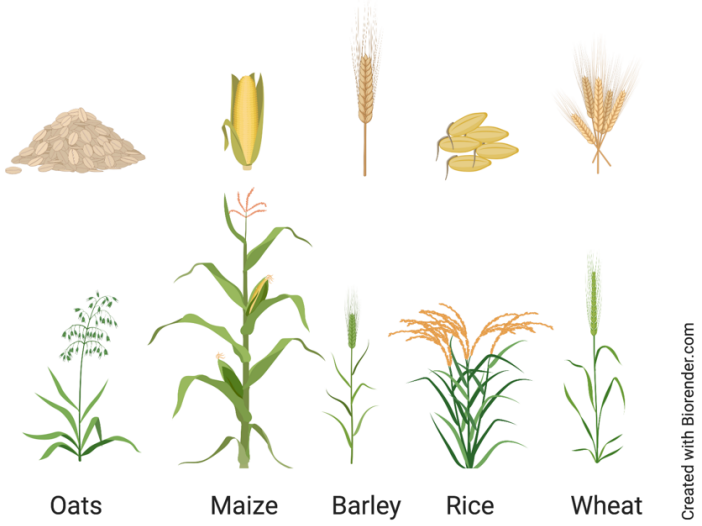


Figure 1.1 Cereal crops affected by Fusarium Head Blight (FHB).

FHB affects mainly small cereal crops such as oats, maize, barley, rice, wheat and rye at the floral area or heads; also known as head blights (scab) of small grain cereals or 'ear rot' of maize (Ferrigo et al., 2016). Fusariosis can affect other areas of the plant.

1.4.1 Fusarium evolution of and mycotoxin production

Genus *Fusarium* has been extensively studied by many scientists and has been represented by several species, according to their virulence on humans, livestock

and plants, as well as with their secondary metabolites with benefit/detrimental effects on the economy (Leslie and Summerell, 2006). Same authors pointed out that according with the American Phytopathological Society (circa 2005) over 81 of 101 economically important plants have associations with a *Fusarium* disease, revealing the importance of this genus for agriculture worldwide. Taxonomically, *Fusarium graminearum* is a species (formerly the *Gibberella zeae* (Schw.) Petch) in the *Fusarium* genus (formerly the *Gibberella* genus) of the Nectriaceae family, in the Hypocreales order within the Ascomycota phylum.

In a beautifully written paper with taxonomic and evolution point of view, O'Donnell et al. (2013) analysed the evolution of the *Fusarium* group, they dated the evolutionary events of the group and the genes responsible for secondary metabolites (pathogenic-related). They associated the evolution of plant-associated species complex with the angiosperm diversification. They focused on the species complex *sambucinum*, which includes *F. graminearum*, *F. culmorum*, *F. pseudograminearum* and others. Interestingly, they concluded that the ITS rDNA region could not resolve fusaria that diverged recently (8 Mya), on the ground that this group has almost no variation since the Pliocene in the *Fujikuroi* species complex (8.1 Mya) and in lineages such as *F. graminearum* (0.8Mya), for instance. Interestingly, they also concluded that the diversification of genes of the most toxicogenic and phytopathogenic fusaria occurred along with the appearance and

radiation of grasses and dicots. Finally, they concluded that the *F. graminearum* species complex diverged from the time of grasses diversification.

The modern studies of the *Fusarium* species started around 1821 by Fries, 110 years later Wollenweber & Reinking (cited by Summerel 2019) published Die Fusarien, a compendium which described 65 species, 55 varieties and 22 forms in 16 sections. After that, around 50 years later, in Europe and USA researchers defined separated own approaches for the study of *Fusarium*. However, in 1971, the CMI (Commonwealth Mycology Institute) published "*The genus Fusarium"* in UK, a milestone for the latest study of the *Fusarium* using morphology characteristics to distinguish between species. By 70's, taxonomy of the genus *Fusarium* started to evolve with the use of the term mating types, which derived in the use of phylogenetic species concept for the classification of *Fusarium* sp. Just before the 2000 year, the *Fusarium* species became widely documented (and their hosts) using DNA-based technology. Another milestone on the *Fusarium* taxonomy occurred in 2012 when, after the International Botanical Congress in Melbourne (2011), the International Code of Nomenclature for algae, fungi and plants (ICN) established the use of single nomenclature, contrary to the dual that used one name for the sexual (perfect or teleomorph) and another for the asexual state (anamorph) and finally agreed to use only the name *Fusarium* for the genus. Finally, taxonomy of the *Fusarium* grew bigger since the use of species complexes terminology by 1997 in *F. oxysporum* species complex (FOSC) firstly. Although, the use of this

nomenclature is not a rule for the ICN, the approach tries to group fungi by morphology and phylogenetic markers with some level of cryptic speciation (Summerell, 2019). Nowadays, species complex is widely used in the *Fusarium* taxonomy. For instance, the NBCI web page reported 20 species complex in the *Fusarium* group which agrees with Geiser et al. (2013) and Summerell (2019) (Figure 1.2).

In another work, Zhao et al. (2014) reflected in their findings about *F. graminearum* genome compared with the closely related *F. verticillioides* and *F. oxysporum*, determining that there were non-conserved regions, according to previous studies. However, they also found that 13 non-conserved regions were identified on *F. graminearum*, which they designated as Nc1 to Nc13. Interestingly, this study showed that, *F. graminearum*, a new evolved strain (0.8 Mya), possesses fewer chromosomes but similar gene content to those closely related species and with more history in the evolutionary tree (~8.0 Mya). *F. verticillioides* (Fv) possesses 7 more chromosomes but similar gene content and *F. oxsyporum* (Fo) showed more gene content in the chromosomes Fo3, Fo6, Fo14, Fo15, that is totally absent in *F. graminearum* (Sperschneider et al., 2015; C. Zhao et al., 2014) (Figure 1.3). Sperschneider et al. (2015) demonstrated that many of those related genes and chromosomes are associated to pathogenicity, which evolved rapidly.

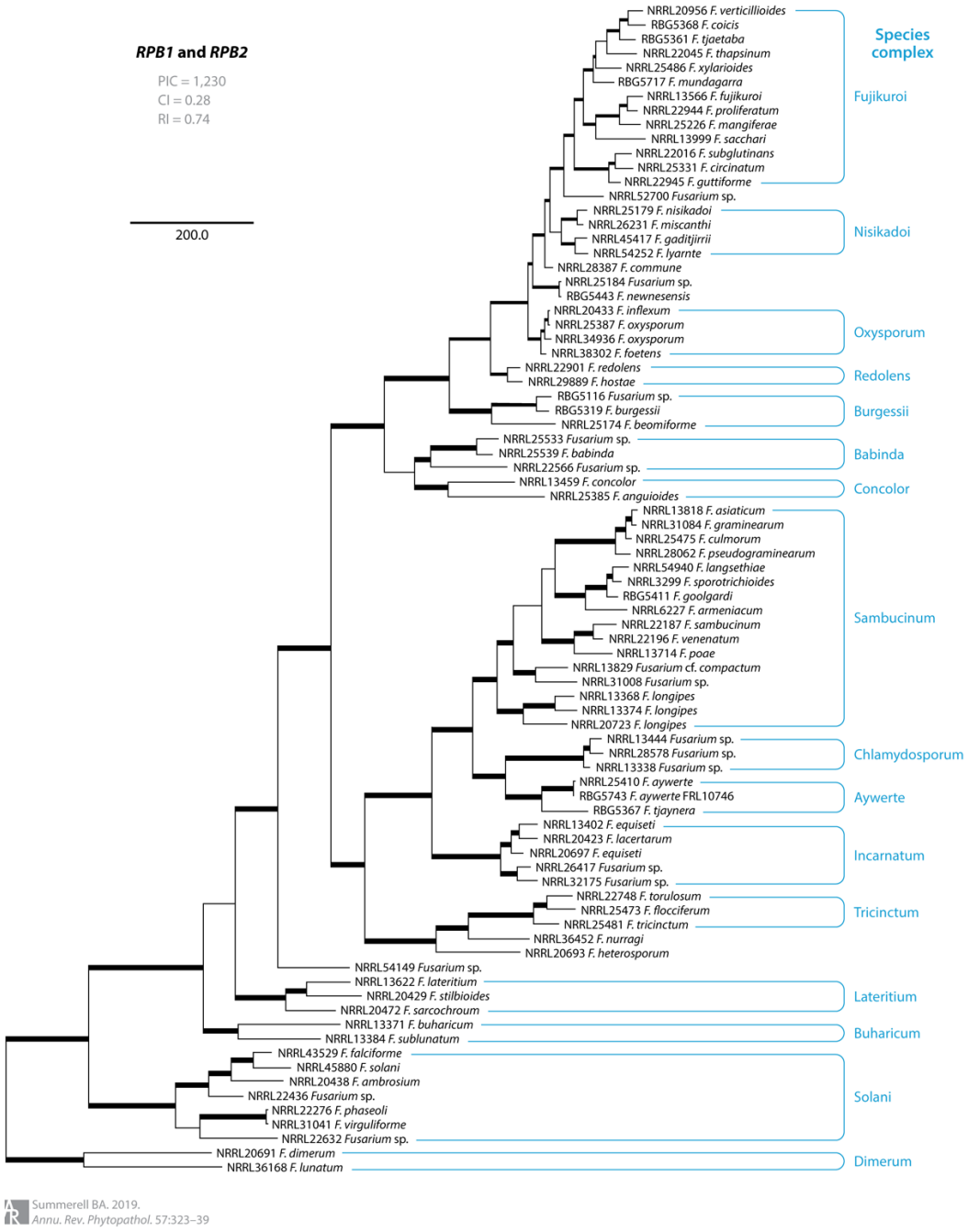


Figure 1.2 Phylogenetic relationship of major species of Fusarium.

Sixteen species complexes of *Fusarium* are represented in the phylogenetic tree based in RNA polymerase II subunit: RPB1 and RPB2. Abbreviations: CI, confidence interval; PIC, preinitiation complex; RI, repeat interval. (Taken from Summerell, 2019)

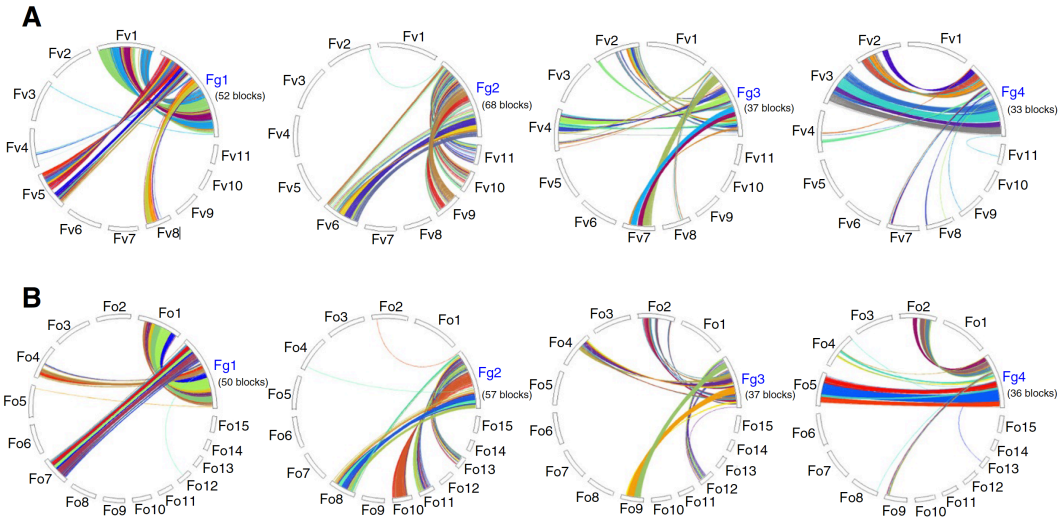
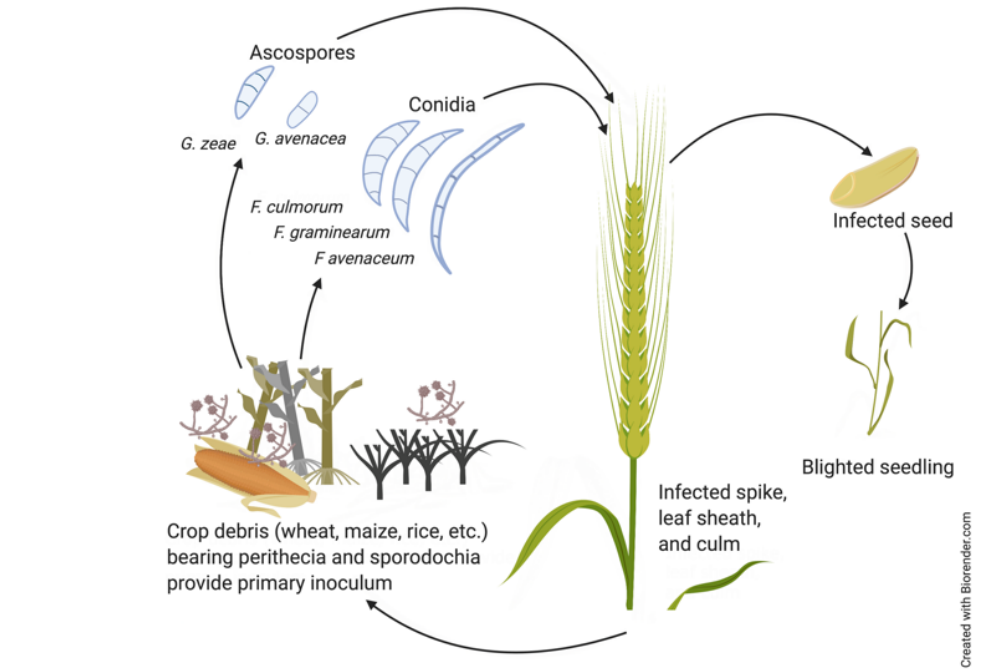


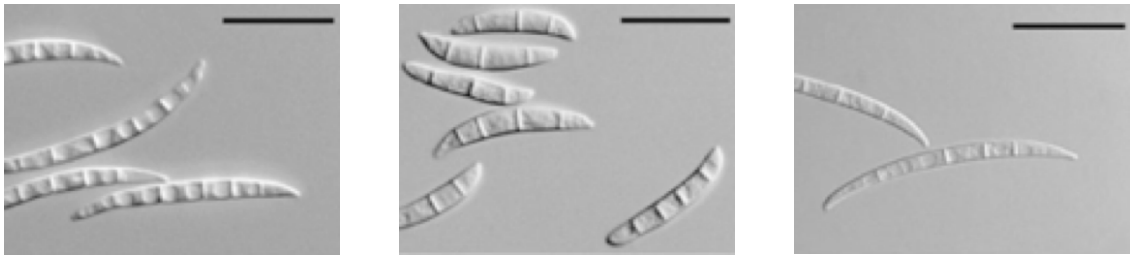
Figure 1.3 Synteny analysis blocks of Fusarium graminearum compared with F. verticillioides and F. oxysporum represented in cycle plots.

The four *F. graminearum*'s chromosomes (**Fg1-4**) gene content was compared with **a.** *F. verticillioides*' eleven chromosomes (**Fv1-11**) gene content and with **b.** *F. oxyporum*'s fifteen chromosomes (**Fo1-15**) gene content. All the genes in of *Fv* matched with *Fg*. The genes in chromosomes 3, 6, 14 and 15 of Fo did not match with any gene in *Fg*. Each bands' colour represents an independent synteny block (Adapted from Zhao et al., 2014).

1.4.2 Fusarium Head Blight (FHB)

By 1992, the fungal disease *Fusarium* head blight (FHB) or scab, arose as an emerging plant disease (Gale et al., 2005). It is caused by the fungi *Fusarium graminearum*, *Fusarium* spp., *Bipolaris sorokiniana* and *Drechslera avenacea*. However, the *F. graminearum* is believed to be the principal causal agent (Figure 1.4).





F. graminearum *F. culmorum* *F. avenacium*

Figure 1.4 Life cycle of FHB

Top: FHB is produced by different Fusarium species such as *F. culmorum*, *F. avenaceum* and *F. graminearum.* The spores of these fungi overwinter on the debris of the plants or in infected tissues, that are lifted up by the wind, or other weather conditions, towards the new spikelets in the next crop season; then the spores germinate and grow over the spikelets. Sometimes the spikelets are affected causing the decrease on the yield of the crops, but in some other cases, the fungus is infecting the plant, symptoms are not visible but the mycotoxins are present and carried onto the chain production ("Fusarium head blight," n.d.). **Bottom**: Microscopy view of macroconidia (scale bar 25µm) of *F. graminearum*, *F. culmorum*, *F. avenacium* (Leslie and Summerell, 2006).

The plants show symptoms such as shrunken grains and chalky white appearance (Figure 1.5). Additionally, the fungus produces mycotoxins. Translocation of mycotoxins in grains leads to the loss in yields and low quality of grains (Gunupuru et al., 2017).

Since the outbreak in 1992 in USA, the studies around the epidemiology, dispersion, control and characterisation have been increased notably. The interest in the knowledge of the behaviour of the causal agents, mainly *F. graminearum*, metabolism, the way of action, have been studied (Beccari et al., 2018; Champeil et al., 2004; Madgwick et al., 2011).

a. b. c.

Figure 1.5 FHB symptoms on wheat.

Symptoms of FHB include shrink of grains (**a**), straw light colour in spikes (**b**) and part or total infection of the spikes with some without visible symptoms (**c**) (Bayer CropScience, n.d.; Crop Protection Network, n.d.; Swire, 2018).

Champeil et al. (2004), proposed specific factors which trigger the attacks by the fungus in fields. They included humidity, precipitation and total radiation. Similar to any other plant pathogen interactions, the susceptibility of the plant and the virulence of the fungus affect the production of mycotoxins in fields. Therefore, the

epidemiology of the disease is driven by the climate, cultivars used, the source, production, maturation and the dispersion of the inoculum, infection of the heads and incubation-sporulation cycle (Champeil et al., 2004; Drakulic et al., 2017).

1.4.3 Mycotoxins: Deoxynivalenol

Besides, according to Bennett and Klich (2003), the mycotoxins are secondary metabolites produced by fungi, that are capable of causing disease and death in humans and other animals and are heat resistant. There are three relevant mycotoxins produced by FHB: the trichothecene deoxynivalenol (DON), the T-2 and fumorisin B1 (Figure 1.7). DON is also known as vomitoxin. The symptoms of ingestion of high doses include general stomach disease with loss of weight, leading to the death. DON is the most studied mycotoxin and belongs to the groups of the trichothecenes. These substances inhibit protein synthesis in eukaryotic cells (Machado et al., 2017).

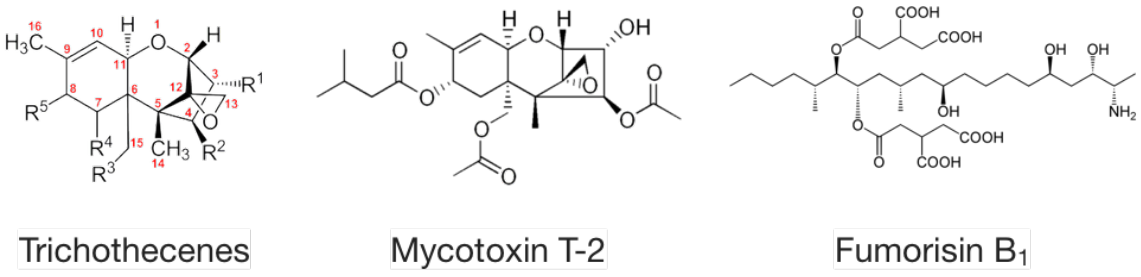


Figure 1.7 Fusarium-related mycotoxins chemical structures.

Fusarium species produce several mycotoxins. The most common is the deoxynivalenol (DON) which is a trichothecene. Other very well-known are the T2 mycotoxin and Fumorisin B_1 ("Compound Report Card," n.d.; "Fumonisin B1," 2019; "Trichothecene," 2019).

Similarly, *Fusarium* crown rot, a plant disease that produces severe problems in the basal parts of the plant, infect the stalks of cereal crops. There, DON delivered by the fungus and is translocated from the base to the top of the stems. Presence of the DON preceded the hyphal colonization and subsequently development of the disease (Beccari et al., 2018).

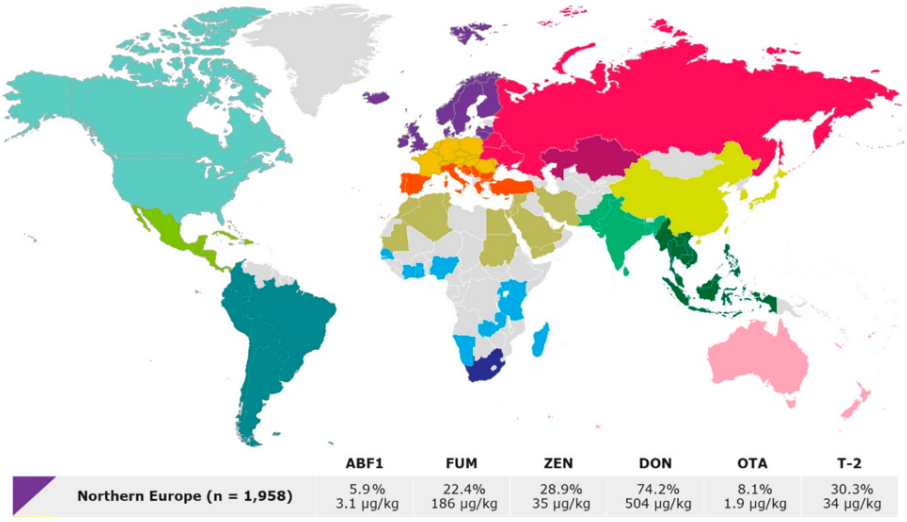


Figure 1.8 Prevalence of mycotoxins worldwide.

In a global survey made from 2008 to 2017, Northern Europe (purple) presented high prevalence of the of main mycotoxins in products used for feed and feed raw materials for livestock according to Gruber-Dorninger et al. (2019). In this study, the prevalence of the DON was 74.2% of the samples tested (n=1,958), followed by the T2 toxin (30.3%) and the zearalenone (zen) (28.9%).

In the FHB, direct infection of the heads/rachis leads to the translocation of the DON into grains. Nevertheless, the absence of symptoms of the FHB does not equal the absence of mycotoxins, which can be present at high levels (Gunupuru et al., 2017) (Figure 1.8).

1.5 Control of FHB

Control of *F. graminearum* in cereal crops then, is priority for food security. In the past, chemical control was first consideration for the plant pathogen control (Jones, 2000). Nevertheless, cultural practices and biological control agents have been introduced for some time now. Gilbert and Fernando (2004) expressed this very well, reviewing the use of cultural practices such as crop rotation along with biological control using microbial biological control agents (MBCA) in heads of wheat as well applying inoculants to the crop debris. Some others (Walkowiak et al., 2015) introduced the concept of the intraspecies control, showing how the presence of others *Fusarium sp.* could decrease the production of mycotoxins from *F. graminearum*. More recently, Dweba et al. (2017) reviewed the control strategies of FHB in wheat, noting the negative effects of the chemical control, pointing out the current widely use of biological controls and including use of resistant species as well genetic control.

1.5.1 Pesticides

The chemical treatment of the FHB has uncertain results and depends mainly on factors such as the time of application, coverage of the spikelets, climate and virulence of the fungal strain. Sometimes, chemical pesticides are affected by other conditions in the field, reducing the controlling effect of pesticides over *F. graminearum* compared with the effect over other fungi in the plot (Champeil et al.,

2004). Dweba et al. (2017), described the effectiveness of chemical pesticides in the control of FHB and included more factors, for instance: the kind and the dosage of fungicide used. Additionally, the authors addressed the matter of resistance to pesticides and exemplified it with cases as the tebuconazole in the USA and benzimidazole in China, which are both fungicides in which FHB showed resistant. It is believed that in the future, more pesticides will be obsolete to the control of this disease. In reference to this, the UK organization for pesticides registration (Health and Safety Executive -HSE-) discontinued the approval of products with carbendazim and emitted a withdrawn of the active ingredient epoxiconazole, which will enter in effect not later than October 2021. Other products recommended for use in cereal crops in EU against *F. graminearum* by the European and Mediterranean Plant Protection Organization (EPPO) and registred in UK, are listed in the Table 1.1. The table includes examples of commercial houses and their commercial products with these active ingredients.

Table 1.1 List of some chemical pesticides approved for use against *F. graminearum* in cereals by EPPO guidelines (EU) and registered in UK (Source: HSE, n.d.; OEPP/EPPO, 1997).

Active ingredient (EPPO)[1]	***Product name (UK)***	***Company***	***Target crop***
Azoxystrobin *(48)*	Amistar	Syngenta UK Limited	wheat, wheat (spring), wheat (winter)
	Affix	UPL Europe Ltd	wheat
	Azoxystar	Life Scientific Limited	wheat

	Hill-Star	Stefes GmbH	wheat
	Valiant	FMC Agro Limited	wheat
Carbendazim	-	-	-
Epoxiconazole *(78)*[2]	Adexar	BASF plc	wheat
	Cloister	Dow AgroSciences Limited	wheat
	Rapier	Ascot Pro-G Limited	wheat
	TIRO	Life Scientific Limited	wheat (spring), wheat (winter)
	Whistle	BASF plc	wheat
Tebuconazole *(96)*	Buzz Ultra DF	Arysta LifeScience Great Britain Ltd	wheat (spring), wheat (winter)
	Hi-Tebura	Hockley International Limited	wheat
	Orius	Nufarm UK Limited	wheat
	Spekfree	Rotam Agrochemical Europe Limited	wheat (spring), wheat (winter)
	Zonor	Life Scientific Limited	wheat

[1]Number in parenthesis: HSE's pesticides registration figures by December 2020.

[2]Epoxiconazole is approved until 30 April 2020 and withdrawn for sale and distribution of existing stocks of the listed products by 31 October 2020. Disposal, storage and use of existing stocks expires on 31 October 2021(HSE).

1.5.2 Resistance Genes and plant breeding

New strategies have been proposed for the control of FHB, including the developing of resistant cultivars, transgenic lines, biological control, integrated pest management (Gunupuru et al., 2017). Furthermore, Steiner et al. (2017) deepened

into the matter and presented the development of resistance-varieties as the best approach for control of FHB. Their approach is not new. The history of cloned genes for crop protection has more than 25 years. A summarised table of cloned R genes were done by Ayliffe and Lagudah (2004) (Table 1.2). The list included only a few representatives. Twenty five years later, Kourelis and Hoorn (2018) reviewed the cloned resistance R genes and proposed 9 mechanisms of the way of action of them. They presented an updated list of 30 R genes in Barley, 24 in maize, 128 in rice and 44 in wheat. The authors mentioned that many R genes confer recognition of pathogen-derived effectors and initiate effector-triggered immunity (ETI), which often involves the hypersensitive response (HR), a type of programmed cell death.

However, it is recognised that the improvement of cereal crops through new and better *R*-genes came with challenges. Most important is the overcome of the breakdown of resistance by the pathogen in gene-for-gene base resistance due to pathogen mutability. Sometimes genetic crop improvement can produce low quality of the yield (Ayliffe and Lagudah, 2004; Wulff and Moscou, 2014).

Table 1.2 Cloned cereal resistance genes (Source: Ayliffe And Lagudah, 2004)

SPECIES	Gene	Protein	Specificity	Pathogen	Disease
Barley	*mlo*	Mutant seven transmembrane	Non-race specific	*Blumeria graminis*	Powdery mildew
	Mla1	NBS-LRR	Race specific	*Blumeria graminis*	Powdery mildew
	Mla6	NBS-LRR	Race specific	*Blumeria graminis*	Powdery mildew
	Rpg1	Protein kinase	Race specific	*Puccinia graminis*	Stem rust

Maize	*Rp1-D*	NBS-LRR	Race specific	*Puccinia sorghi*	Leaf rust
	Rp3	NBS-LRR	Race specific	*Puccinia sorghi*	Leaf rust
	Hm1	HC toxin reductase	Race specific	*Cochliobolus carbonum*	Southern corn leaf blight
Rice	*Xa21*	Receptor kinase	Race specific	*Xanthomonas oryzae*	Bacterial blight
	Xa1	NBS-LRR	Race specific	*Xanthomonas oryzae*	Bacterial blight
	Xa26	Receptor kinase	Race specific	*Xanthomonas oryzae*	Bacterial blight
	Pi-b	NBS-LRR	Race specific	*Magnaporthe grisea*	Rice blast
	Pi-ta	NBS-LRR	Race specific	*Magnaporthe grisea*	Rice blast
Wheat	*Lr21*	NBS-LRR	Race specific	*Puccinia triticina*	Leaf rust
	Lr10	NBS-LRR	Race specific	*Puccinia triticina*	Leaf rust
	Pm3	NBS-LRR	Race specific	*Blumeria graminis*	Powdery mildew

Nevertheless, the technology is increasingly contributing to the aim of better cloning of genes in crops with larges genomes through new advances in genomics (Bettgenhaeuser and Krattinger, 2019).

In the other hand, research done to understand the resistance of plants could be considered for the FHB control, for instance, Hales et al. (2020) determined the genetic mechanisms in Type II susceptibility to FHB in chromosome 4D of wheat, which would lead to better crop genetic improvements in wheat.

1.5.3 Crop Rotation

Traditionally, cultural practices are recommended for any agricultural system. Crop rotation helps to decrease inoculum pressure in fields. However, the rotation of

crops and its frequency should be considered carefully. Champeil et al., (2004) described how the contamination by FHB is more severe if the preceding crop is maize, durum wheat or oats, rather than wheat or barley. A higher rotation also leads to an increase in the frequency of the head blight.

1.5.4 Detoxification

Alternative approaches have been studied to mitigate the effect of mycotoxins in animals when they feed in contaminated materials. Microorganisms are very successful converting DON to de-epoxy-DON, a harmless form of the toxin for livestock. Awad et al. (2010) reviewed this matter and highlighted the use of the strain *Eubacterium* BBSH 797, which is capable of degrading the toxin and counteract the negative effects. In another example, Saladino et al. (2016) studied the use of lactic acid bacteria (LAB) for decreasing the aflatoxin production by *Aspergillus parasiticus* CECT 2681.

In another review, Pfliegler et al. (2015) made emphasis in the use of yeast to detoxify mycotoxins. The authors explain how this approach would be taken with caution, probably applying genetic modification, with the aim of improving the response of the yeasts and their metabolites as a part of livestock feeding programs. Nevertheless, mycotoxin accumulation is a concern on public health when the toxins, and their converted forms, accumulate in products for human intake (Lee and Ryu, 2015).

1.5.5 RNA-silencing

More recent approach in the control of agriculturally important pests and pathogens include the use of target RNAs (sRNA) also known as spray-induced gene silencing (SIGS) strategies. These are small double stranded RNAs that can target plant mRNA and induce protection against pathogens. Werner et al. (2020) used this strategy against *F. graminearum* genes ARGONAUTE and DICER protecting barley leaves. Other authors (Fletcher et al., 2020) described this strategy: "RNA interference (RNAi), a eukaryotic process in which transcript expression is reduced in a sequence-specific manner", as the way to decrease chemical pesticides with very narrow effects on non-host plants or humans. The possibilities of the technique are attracting the interest of many stakeholders in the agriculture circle (Mendelsohn et al., 2020).

1.5.6 Biopesticides

Biopesticides are defined as products for plant protection, composition of which is made of biological agents ("Biopesticides Home," n.d.). The use of biopesticides is growing around 10% per year, however, remain in a small share of the total global market of pesticides, around a 5% or £2.2 billion worldwide. Biopesticides can be formulated using biological sources, like fungi, bacteria, nematodes, plant extracts, virus. Bacterial biopesticides are typically aimed for the control of insects (*Bacillus thuringiensis*) and those formulated to promote plant growth (*Bacillus subtilis*)

(Damalas and Koutroubas, 2018). The so called rhizobacterium *B. subtilis* is a model bacterium extensively used in biopesticides. Its secretome (pathways for secretion of its proteins and metabolites) has been well-studied, and the bacterial genome has been sequenced and annotated (Peng et al., 2017, 2017; Stein, 2005).

More recently, new isolates have emerged as compelling examples for fungus control: *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus velezensis*. These three strains have been shown to have genes that code for antifungals and antimicrobial compounds like fengycins, chitinases, iturins and surfactins (Chan et al., 2009; Grosu et al., 2014; Zhao et al., 2015).

1.6 *Bacillus* Spp.

Bacillus are Gram-positive, rod-shaped bacteria in the phylum firmicutes. They can be obligate aerobes or facultative anaerobes. Chemically, they test positive to catalase. Present on almost any environment, *Bacillus* includes both free-living and parasitic pathogenic species. Under stressful environmental conditions, the bacteria can produce endospores likewise, the bacteria become dormant for long periods of time. Many species of *Bacillus* can produce large amounts of enzymes and are being used in the industry, as well as in the agriculture sector -*Bacillus subtilis*, *Bacillus cereus*-. Some strains such as *Bacillus anthracis* are clinically significant (Jiang et al., 2020; Puri et al., 2016; Turnbull et al., 2007).

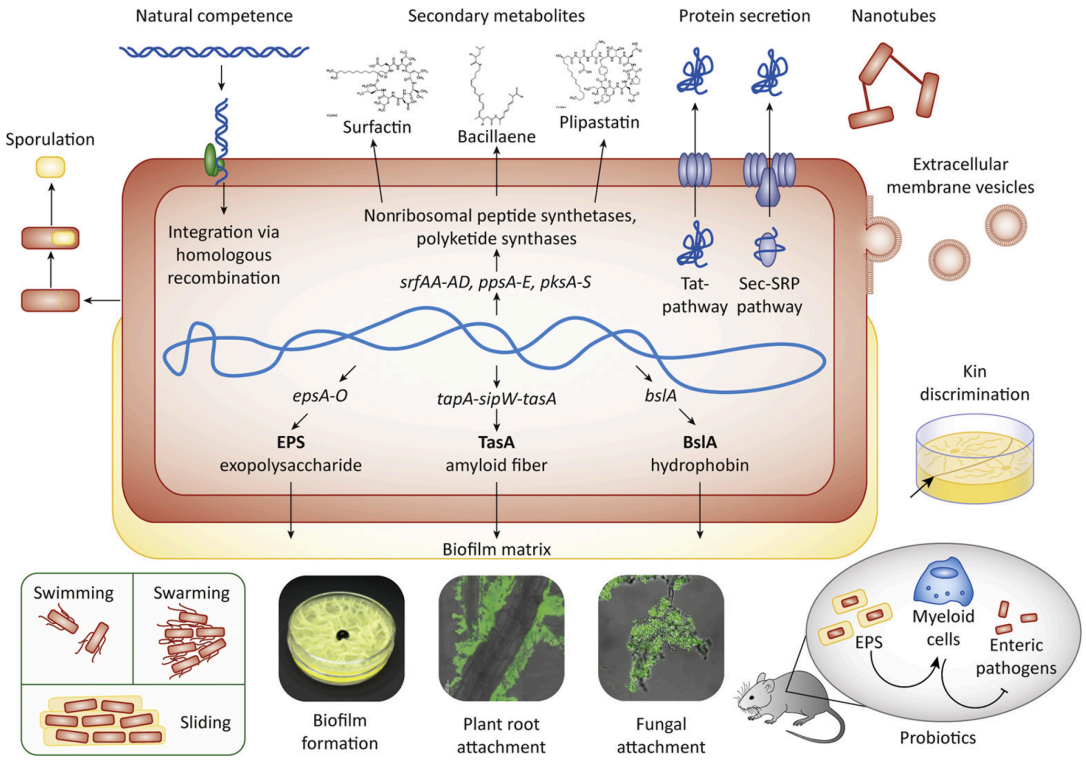


Figure 1.10 Bacillus subtilis summary of main metabolic functions.

Vibrio subtillis was first described in 1835 and in the second World War known as *Bacillus subtilis.* It was attributed the capacity to cure dysentery. It is a model in bacterial secretion (secrotome) because is able to produce antibiotics, surfactants and its characteristic biofilm. The figure shows its capability of transformation, production of secondary metabolites, protein secretion, ability of swimming, swarming and sliding, used as probiotic, ability to attach on other organisms and interact with the environment through extracellular vesicles (Kovács, 2019).

Bacillus group includes *B. subtilis, B. amyloliquefaciens, B. licheniformis,* and *B pumilus*. These bacteria have the characteristics of forming a biofilm, that is compose of the secretion of extracellular polymeric substances (EPS's) that allows the bacteria aggregate and adhere each other or to surfaces. The biofilm serves like a matrix for interchanges of micronutrients and for communication between cells (Tjalsma et al., 2000). Interestingly, these *Bacillus* species are difficult to

differentiate using only the 16S gene and all belong to the *B. subtilis* species complex. Nonetheless, in the last decades many other species have been included in this complex group and the use of whole genome sequencing (WGS) contributed to differentiate between species. In the other hand, species reported as *B. amyloliquefaciens* isolated from roots were more successful colonizing roots. By this mean, an operational group were included in the *Bacillus subtilis* complex group, called *B. amyloliquefaciens* (Fan et al., 2017).

1.6.1 PGP activity on Bacillus strains.

Plant growth promoter Rhizobacteria (PGPR) are those which can form symbiotic relations with plants protecting and facilitating their growth. Generally, there are PGPR with endophytic and epiphytic living form. The endophytic are commonly isolated from intercellular regions on plant tissues. The epiphytic are commonly isolated from the rhizoplane, rhizosphere, and plant surfaces (Ambrosini and Passaglia, 2017; Beneduzi et al., 2012; Leontidou et al., 2020).

Some are characterised for a root-colonisation capability. Rhizobacteria with PGP activity posse structures to help in the growth promoting activity such as siderophores and some produce antibiotics (Beneduzi et al., 2012; de Souza et al., 2015). Siderophores are structures that bind insoluble iron (Fe^{III}) from the environment and absorb it, making available to use by the microorganism and plant. The acquisition of iron by the microorganisms contributes with their survival and

virulence, therefore siderophores are clue metabolic compounds. Some bacteria, such as some *Pseudomonas* (Gram-negative) and some *Bacillus* (Gram-positive) are able to obtain iron with different siderophore-mediate transport systems (Ahmed and Holmström, 2014; “Iron acquisition in bacteria,” 2016; Peuckert et al., 2011). Gram-negative systems are very complex, the siderophore binds to the external layer, and is carried inside of the cell membrane from periplasma by protein systems, then by a mechanism of transport that use the ABC (ATP- binding cassette) to deliver the siderophore complex inside the cytoplasm where the Fe^{III}-siderophore is reduced to realise the Fe^{II}. On the contrary, Gram-positive bacteria has a more straightforward systems, carrying the Fe^{III}-siderophore complex from the periplasmic site to the cytoplasm by the same ABC transport mechanism. *B subtilis*, a Gram-positive bacterium, uses the second mechanism (Ahmed and Holmström, 2014). This model organism utilizes bacillibactin, nevertheless also can incorporate exogenous siderophoros (enterobactin, petrobactin, or hydroxamate-type siderophores - ferrichrome or schizokinen-) (Peuckert et al., 2011).

1.6.2 Growth-defence trade-off.

The concept of plant-defence trade off, first described in plant–insect interactions in forestry studies, was reviewed by Huot et al. (2014). This is based in the hypothesis that plants have limited resources which can be dedicated either to defence or to growth. Plant survival, reproduction and plant fitness depend on these trade-offs. As a result, studies in plant protection, will encounter limitations on their

scope. The authors noted that, plant hormone crosstalk as main part in the process (Figure 1.11).

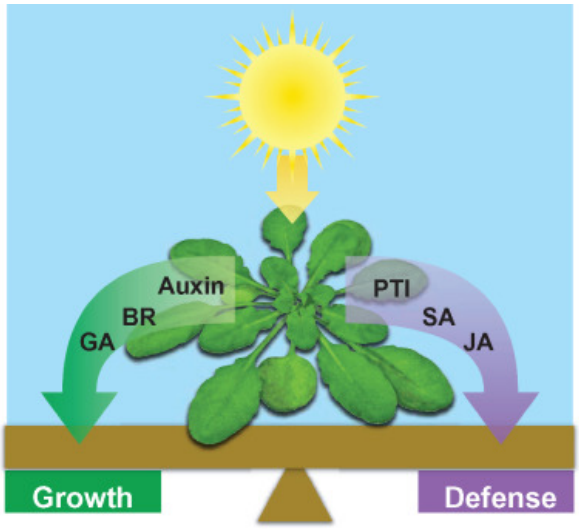


Figure 1.11 Growth defence trade-off diagram.

Plants use photosynthesis to convert light energy into chemical energy in the form of carbohydrates. These resources are then allocated towards growth or defense, depending on the presence or absence of specific stresses. This process is facilitated by hormone crosstalk and is referred to as the growth–defense trade-off. ***BR****, brassinosteroid;* ***GA****, gibberellin;* ***PTI****, pathogen-associated-molecular-pattern-triggered immunity;* ***SA****, salicylic acid;* ***JA****, jasmonates.*" (Huot et al., 2014).

Besides, Cao et al. (2017) reviewed the legume-rhizobium symbiosis, a mutualistic system. They hypothesised that this system evolved from a pathogenic relationship, then with the time, the system became of mutual benefit. It is generally accepted that rhizobia actively suppress the immune response of the host to allow the colonization and further symbiosis. The maintenance of the system is then, a delicate balance of manipulation of the host response through the lifespan of this relationship.

More recently, Neuser et al. (2019) studied the growth-related transcription factor homolog of BEE2 interacting with IBH 1 (HBI1) which regulates apoplastic reactive oxygen species (ROS) homeostasis. They proposed that HBI1 has a role in the growth–defense trade-off seen in plants. HBI1 has effects on RbohA and RbohC. Interestingly, RbohC has effects in cell size. They concluded that incompatibility between growth and defence is a result of how apoplastic ROS is modulated during both processes ("Understanding the Growth-Defense Trade-Off in Plants," 2019).

1.7 Omics approaches

1.7.1 Interaction between microorganisms

Microorganisms are ubiquitous; they shape the life in a wide range of aspects. It is believed that microbes share co-evolution with other organisms. They are able to express many characteristics that range from the fixation of nitrogen to the production of antimicrobial compounds (Beneduzi et al., 2012; Cao et al., 2017; Compant et al., 2010). They regulate their own population and the microbiota around them. They colonise and survive in a wide range of environments and made possible the life of complex organisms such as plants and animals. In a broad concept, microbes can belong to the different domains of life: archaea, bacteria (prokaryote) and eukaryote. (Lodish et al., 2000).

Additionally, whilst there is an understanding about how the microorganisms interact between themselves in the environment. Some time ago, Frey-Klett and

Garbaye (2005) explored the topic about interaction of rhizobial microorganisms, proposing a positive intervention made by the rhizosphere microflora in the mycorrhizal symbiosis (plant-mycorrhiza), emphasising the so-called 'mycorrhiza helper bacteria', which they proposed, are made of species such as: proteobacteria (*Pseudomonas*), *Burkholderia*, *Bradyrhizobium*, Firmicutes (*Bacillus* and *Paenibacillus*) and Actinomycetes (*Rhodococcus* and *Streptomyces*).

More recently, the use of hight throughput (HT) technologies have contributed to the study of these relationships in plant health (Diaz et al., 2014). In other point of view, Schmidt et al. (2016) explored the subject about volatile organic compounds (VOCs) and their role in the interaction of fungi-bacteria, concluding that bacteria can sense and response to VOCs from fungi (and vice versa) highlighting their importance as signals between microorganisms. Olsson et al. (2017) reiterated the subject of fungi-bacteria interactions, contrasting the views of antagonistic against mutualistic interactions. In their review, they concluded commenting on the novel technologies for the study of those interactions. From the use of imaging analysis such as imaging mass spectrometry (IMS) for the study of fungal-bacterial interaction at the microscopic level; to the use of omics: proteomics, genomics, transcriptomics and metabolomics. These technologies could contribute to "ping-pong" pathways rather specific, punctual metabolites. However, the authors added that, it is challenging the study of interactions of community of organisms, in which, IMS would be more helpful, for instance.

1.7.2 Genomics and transcriptomics

In the other hand, sequencing technologies have increased in availability and performance during the last two decades. Traditionally, Sanger technology was used for sequencing the whole genomes of any organism due to its reliability. It generated sequences of more than 500 base pairs (bp). Ultimately, this technology made use of lots of resources such as equipment, time and money. In the last two decades, technologies such as Illumina (Solexa) sequencing, Roche 454 sequencing, Ion Torrent and SOLiD sequencing (EMBL-EBI, 2012) became available and then new generation sequencing (NGS) data, as it is called, became the trend on the analysis of sequencing data as a high-throughput (HT) method. Because these technologies improved and changed, prices on sequencing services dropped. Additionally, NGS data works with small size but really abundant sequences of around 100 bp. Bioinformatic tools have been developed besides the new sequencing technologies and allow the analysis and comparison of many repeats with high confidence. *De novo* assembly also appeared between the new technologies for assembling whole sequences without the use of reference genomes. Altogether, powered the relative fast and efficient processing of genomic data analysis.

1.8 Epilogue

In previous studies, model bacterial strains such as the *B. subtilis* 168 or the *B. subtilis* SG6 were sequenced and characterised. In a study using the *B. subtilis* strain SG6, it was found to be successful against *F. graminearum* (*Fg*) at laboratory level (Borriss et al., 2018; Zeigler et al., 2008; Zhao et al., 2015). Additionally, in a previous proteomic analysis, the *Bacillus* strains QST713, FZB24 (commercial) and EU07 (non-commercial) were evaluated. Baysal et al. (2013) found that the EU07 strain showed differentially expressed proteins respects the other two strains. In the same study, they found that the *Bacillus* strains showed an antagonistic effect against the *F. oxysporum* in tomato.

Besides, the use of chemical pesticides has proved to be not enough to reduce the FHB. Then, discussions about alternative solutions may be offered and research should be carried out to provide highly efficient yields on fields. Biopesticides are one of the options available for the environmentally friendly control of pest diseases. Biopesticides are rising as one the favourite options by consumers and offering exciting results for farmers.

Here, this study attempts to tackle the problem of controlling FHB in cereals crops. *Bacillus* strains QST713, FZB24 and EU07 were assessed and analysed *in vitro*, *in vivo* and at genomic level to understand the mechanisms that are underlined in their potential controlling effect against the *Fg*.

Additionally, the analysis of the RNA transcripts or transcriptome, can be used to determine the virulence of eukaryote microorganisms and to find selected group of genes associated with virulence (Sibley et al., 2012). Transcriptomics were used to assess the effect of the of the interaction fungus-bacterium using the strains EU07 (*Bacillus sp.*) and K1-4 (*F. graminearum*). This will provide with knowledge which will shed light on the control effect at the genetic level.

Aim and Objectives

The overall aim of this research is to determine whether *Bacillus* strains can be effectively used to control *Fg*. Furthermore, the characterization of the molecules effective against *Fg* and identification of genes involved in the suppression of *Fg* are also important goals of this study.

To achieve these aims, specific objectives were as follows;

1. To establish *in vitro* antagonistic assays of *Bacillus* strains against *Fg*. Either testing whole bacterial cultures and cell-free bacterial broths against the *Fg*.
2. To determine if the *Bacillus* strains used have any PGP activity in *A. thaliana* and *Brachypodium distachyon* (Bd-21) plants.
3. To carry out comparative genomic study of the *Bacillus* strains to identify genes responsible for antagonistic activity.
4. To assess the interaction between Fg and EU07 using transcriptomics and to identify differentially expressed (DE) genes in the system.

Chapter 2

MATERIALS AND METHODS
Materials

2.1 Microorganisms used and culture media conditions

2.1.1 Microorganisms

F. graminearum (Fg) isolate K1-4 (*Fg*-K1-4) was obtained from Professor Paul Nicholson's laboratory and was used throughout this study. Three *Bacillus* strains, QST713, FZB24 and EU07, were used in this investigation. The strains FZB24 and EU07 were provided by Professor Baysal at Muğla Sıtkı Koçman University (Turkey). The strain EU07 was isolate from field studies and the strain FZB24 was obtained from the commercial product TAEGRO® (Novoenzymes Biologicals Inc.) (Baysal et al., 2013, 2008). The strain QST713 was isolated from the commercial product Serenade®. To do this, 100 µl of the commercial product were dissolved in 900 µl of water, vortexed thoroughly and incubated at 100°C by 10 min. Afterwards, the bacterial solution was streaked on NA medium and incubated 24h at 28°C. Bacteria with morphology similar to *Bacillus* -white appearance and non-define perimeter- were subcultured on NA medium. Additionally, a single colony was subcultured in LB broth to determine the biofilm production (Figure 2.1).

2.1.1.1 Verification of Bacillus strains by PCR and sequencing

All the isolates were subjected to PCR amplification (Thermal Cycler, Applied Biosystems) using the universal 16S primers UP 27F/1525R (5'-AGAGTTTGATC(M)TGGC- TCAG-3' and 5'-AAGGAGGTG(W)TCCA(R)- CC-3') and colony PCR technique. BioMix™ Red (Bioline) was used and the PCR regime employed was 96˚C for 5 min, denature 94˚C for 30 sec, annealing 56˚C for 30 sec and extension 72˚C for 1 min for 30 cycles followed by 10 min of at 72˚C. The PCR products were run in a 1% agarose gel to determine if the PCR worked. PCR products were then cleaned up and sent for Sanger sequencing. BLASTN analysis against the NCBI databases were performed with the sequence results obtained (Altschul et al., 1990). The isolates corresponding to *Bacillus subtilis* were kept and used in the experiments.

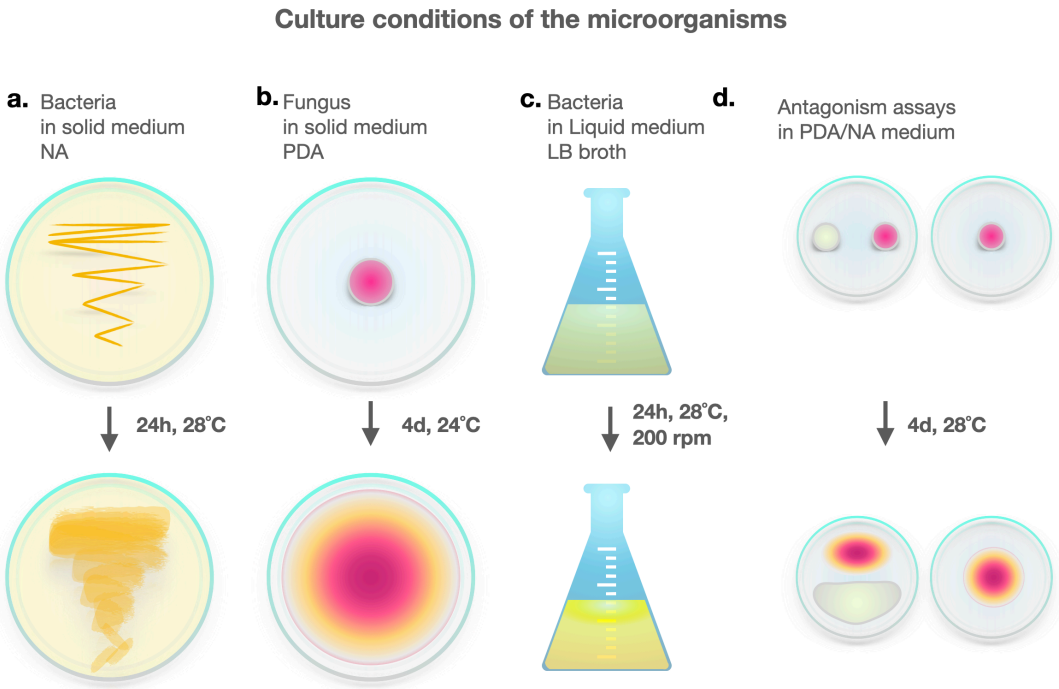


Figure 2.1 Conditions used for the cultures of microorganisms.

Conditions used to culture microorganisms. a) *Bacillus* on NA medium, b) *Fg-K1-4* on PDA medium, c) *Bacillus* in LB broth and d) *Bacillus* strains and *Fg-K1-4* on PDA/NA medium for antagonism assays.

2.1.2 Culture media conditions and storage of isolates

The fungus was maintained on PDA medium at 22 - 24˚C. Occasionally, the fungus was subcultured in SNA media or 25% of strength of PDA to reactivate the production of macroconidia (Leslie and Summerell, 2008). The *Bacillus* strains were maintained on NA medium at 28˚C. All the antagonism assays were performed in Petri dishes with medium PDA/NA (50/50). According to the experiments, bacterial broths were added before autoclaving (Figure 2.2).

For long term storage the fungus was grown on PDA media around 7d and later sterile pieces of Whatman 01 filter paper (4mm diameter) were placed over the fungus and allowed the fungus to grow over them for around 7d. Eventually, the filter papers were removed and enclosed on small sterile envelops and stored at -80˚C. Similarly, the *Bacillus* strains were grown in LB broth (72h, 28˚C, 200 rpm) and mixed half and half with 50% glycerol in cryogenic tubes. The tubes were labelled, flash frozen in liquid nitrogen and stored at -80˚C for further use.

2.2 Plant material and growth conditions

2.2.1 Plant material

In this study, *Arabidopsis thaliana* accessions Col-0, WS-*eds1*, GUS::PR, GUS::RLK, and *Brachipodium dystachyon* (*Bd*-21), were used.

2.2.1.1 Arabidopsis thaliana (At)

At plants used in this study were wild type Columbia 0 (Col-0), otherwise, Col-0 carrying *RLK::GUS* or *PR:GUS* constructs were available in the laboratory stocks as well the Ws-*eds1* (*eds1*: *enhanced disease susceptibility1*).

General conditions of cultivation were followed unless otherwise stated. Seeds of *At* were poured over saturated soil (Levington F2s Compost from BHGS Ltd) in cell trays, then covered with a lid and allowed to grow for 1 – 2 weeks (18 – 21˚C, 10 light, 14 dark) in growth room.

For PGP activity assays, ten-days old seedlings were transplanted into individual pots and soaked in water for 20 minutes. The plants were left to grow for a further 15 days with a 10h light 14h dark regime. Subsequently, plants were flooded with water (as controls) or 10% of *Bacillus* suspension, using 10ml per pot. The *Bacillus* suspensions were at 10^5 concentration of cells for QST713, FZB24 and EU07. Plants were watered or treated weekly for 9 weeks. Each treatment had six plants with 3 biological replicates. The plants were randomly arranged.

*2.2.1.2 Brachipodium dystachyon (*Bd-*21)*

The small grass *B. dystachyon* line (Bd-21) were used in roots assays, infection roots assays as well in infection head assays (IHA).

Seeds of *B. dystachyon* (diploid inbred line Bd-21) were kept a RT in a dry area. When seed germination experiments were required, seeds were placed on distilled

water, separated of the spikelet and scattered, subsequently the lemma were peeled off using straight fine point forceps and then placed over wet filter paper (90 or 60mm, Whatman) on a disposable Petri dish (90 or 60 mm), then covered with other paper, added some drops of water to make the paper wet but firm, and using the finger tips to press over the filter until the two layer of filter were sticking together with no air bubbles in between them and the seeds. The Petri dishes containing seeds were sealed with parafilm and placed on dark and cold (4˚C) for 2 -3 days to use in germination assays or for the period of 2-3 weeks to stratify them (to break the dormancy of the seed and promote germination) (Nicholson, 2017).

Methods

IN VITRO ASSAYS

2.3 Antagonism assays using *Bacillus* strains against Fg-K1-4.

2.3.1 General conditions of the antagonism assays

With the aim to demonstrate if the bacteria have the ability to suppress the fungal growth, antagonism assays were carried out using the *Bacillus* strains (QST713, FZB24 and EU07). Petri dishes with PDA/NA were used for *in vitro* antagonism assays. Sterile plug cutters (10-15mm) were used to get circular pieces of fungus and in some cases, another plug cutter was used with the *Bacillus* strains. After placing the fungal and bacterial plugs on the media in the same Petri dishes, plates were wrapped with cling film to avoid external contamination. All the assays were

performed at 28°C for 4 days in an incubator (Sanyo Biomedical Co, EU). After four days, pictures were taken using a camera in its stand. Each experiment had 4 biological replicates and each experiment was repeated at least three times. All the pictures were taken with the camera (Canon EOS-350D) set at the same high on the stand, focus and resolution by every batch of experiments to provide the uniformity, and process the images with the software ImageJ (Schneider, Rasband and Eliceiri, 2012).

2.3.2 Image processing using ImageJ

The batches of pictures were evaluated using ImageJ. The scale of the image was set using the diameter of the Petri dish (90mm) and applied globally. Subsequently, the area of the fungal growth was selected and measured. The software brought the output with the areas and then this data was imported into a spreadsheet. The data obtained was processed to obtain the percentage of inhibition for every data point of the experiments (Equation 2.1 modified from Swain and Ray, 2009) and used for the subsequent data analysis (Figure 2.2).

Equation 2.1. Percentage of inhibition of the fungal growth.

$$\%\ \text{of inhibition} = 1 - \left(\frac{\text{Sample FG}_{\text{Area}}}{\text{Control FG}_{\text{Area}}}\right) * 100$$

$$\text{FG} = \text{Fungal growth}$$

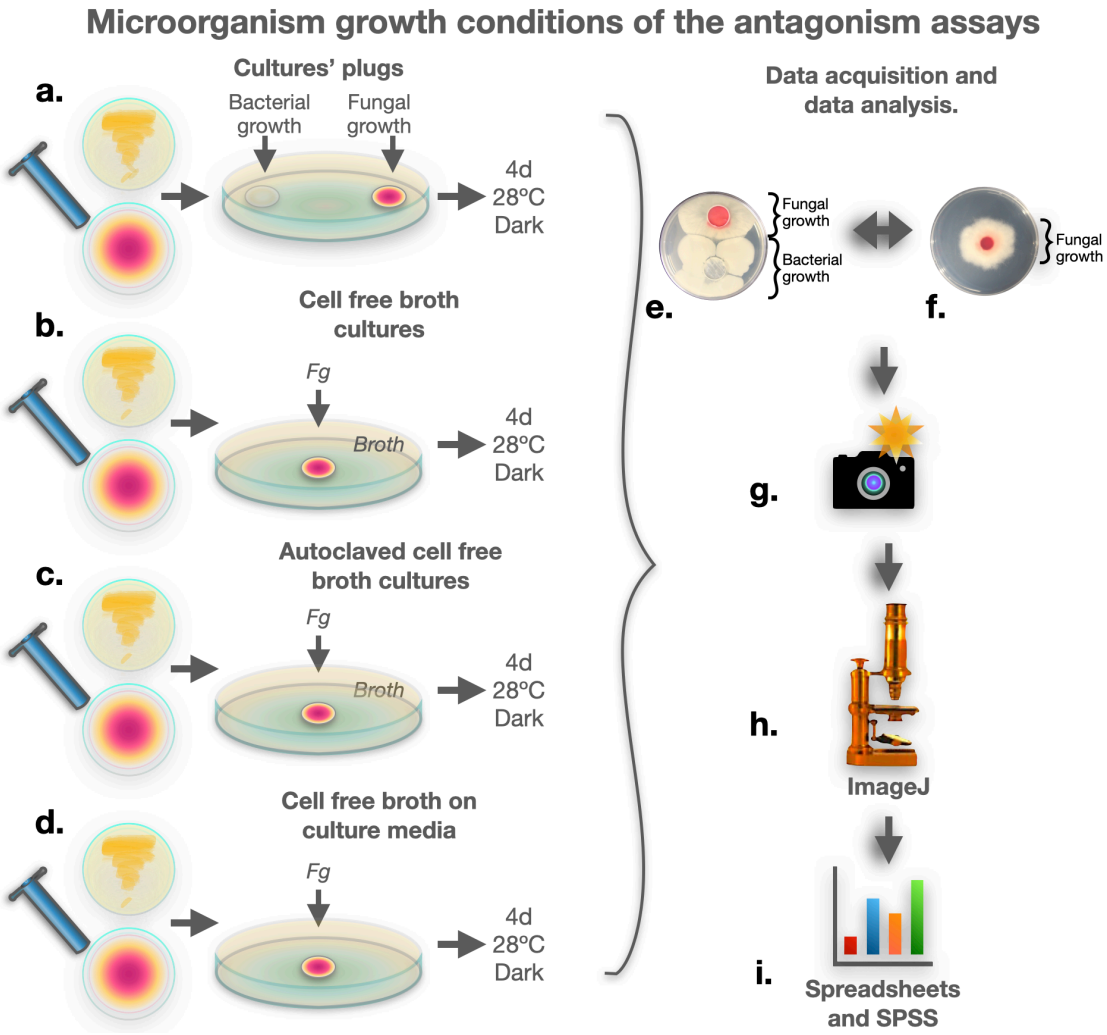


Figure 2.2 Diagram of the procedure for the antagonism assays.

Culture conditions of **a**) antagonism assay using bacterial cells **b**) antagonism assay using bacterial broths (100 µl) at 6h, 24h, 48h and 72h of culture and antagonism assay of bacterial broths (100 µg/ml $_{broth}$)at 24h, 48h and 72h of culture, **c**) antagonism assay of bacterial broths adjusted (100 µg/ml $_{broth}$ and at 24h) and autoclaved, **d**) antagonism assay of bacterial broths adjusted at 0.5, 1.0 and 2.5 µg/mL$_{medium}$ antagonism assay of bacterial broths filtrated, autoclaved and adjusted at high concentrations (10 and 20 µg/mL$_{medium}$) and antagonism assay of bacterial broths adjusted (2.5 and 5.0 µg/mL$_{medium}$), filtrated, autoclaved and proteinase K treated, **e**) final time of the experiment (4dai), identification of the bacterial/fungal growth and any contaminant, **f**) final time of the experiment (4dai), identification of the fungal growth and any contaminant, **g**) acquisition of pictures of plates at same distance, same focus and same resolution, **h**) images' import per batches into the program ImageJ and measuring the area of fungal growth and **i**) data analysis using spreadsheets and Statistical Package for the Social Sciences (SPSS).

2.3.3 Determining the amount of total protein using Bradford assay

To determine the total protein concentrations in *Bacillus* broths, a Bradford assay was performed using Bradford Reagent (B6916, Sigma Aldrich). The instructions of the manufacturer were followed for an experiment performed in a 96-wells plate. Briefly, 1µl of broth was added to the well containing 250µl of Bradford reagent for each sample. Each sample had 3 replicates. Additionally, known concentrations of Bovine Serum Albumin (BSA) (1.0, 0.8, 0.6, 0.4, 0.2 and 0.0 mg/ml) were assessed. Vials with the known concentrations of BSA were stored at -30˚C and melted on ice completely, vortexed thoroughly and used in the assays when needed (Figure 2.3f-h). The reaction was allowed over the time of minimum 10 min and the samples were read using a plate reader (Multiskan Go, Thermoscientific) at 595nm. Absorbance data for the BSA samples were obtained and transformed (net absorbance) and a plot was constructed with the data, as well the tendency line and the equation (y=mx+b). This was used as a standard curve to determine the concentration of total proteins in broths.

2.3.4 Antagonism assays using whole broths of Bacillus *strains against Fg-K1-4*

Antagonism assays of the whole bacterial broths at several dilutions (10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 in water) against *Fg-K1-4* were carried out to evaluate the interaction of the two microorganisms on PDA/NA media. Serial dilutions were subcultured in NA medium overnight and used. Two plugs were cut from the PDA/NA plates at equally distance of the edge of the Petri dish and in an opposite position of each other and taken off from the plates. One plug of the *Fg-K1-4* was placed on one

side and one plug of one of the *Bacillus* strains in the other side using sterile tweezers (Figure 2.2a). The assay was run over the duration of 4d and evaluated according to the above sections (Figure 2.2e-i).

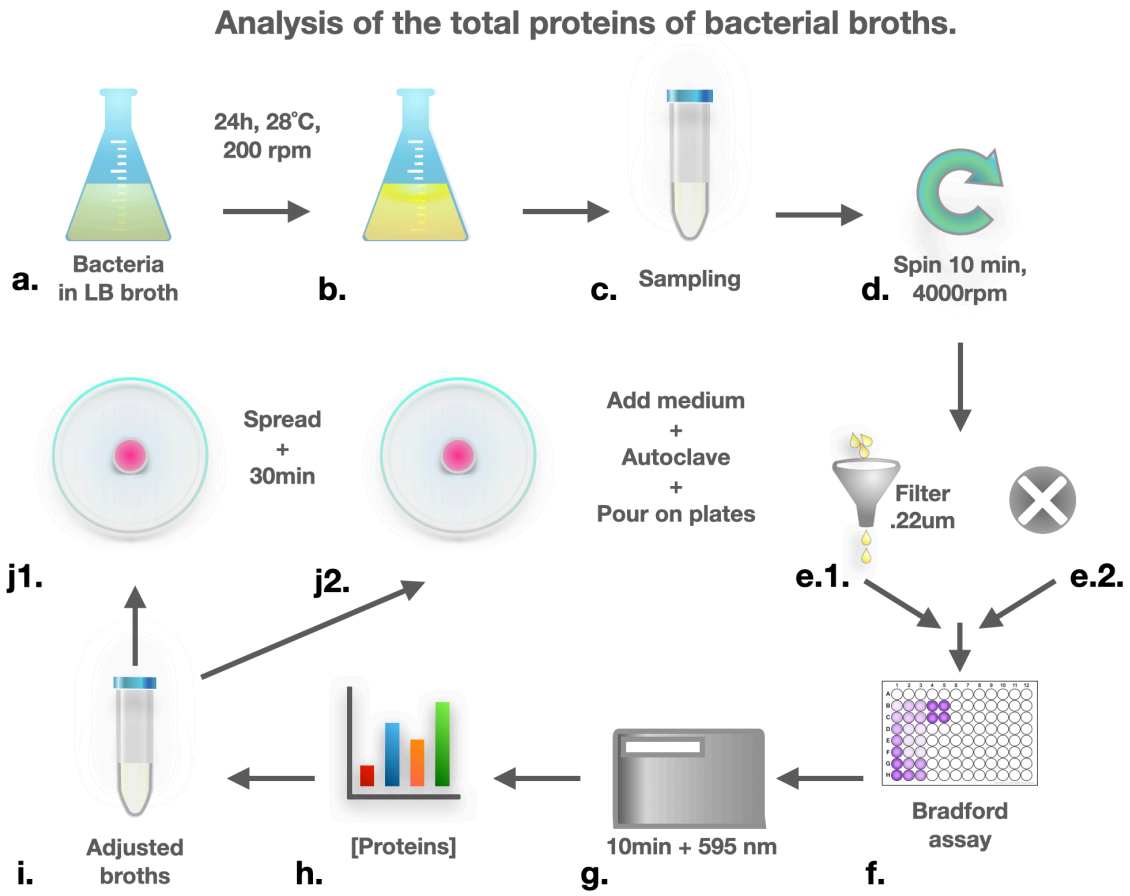


Figure 2.3 Procedure for the antagonism assays with bacterial broths using total proteins.

a) inoculation of bacteria in LB broth, **b)** bacterial broth at 24h, **c)** sampling the bacteria in centrifuge tubes, **d)**spinning for 10 min at 4000 rpm, either **e.1)** filter sterilising the broths or **e.2)** proceed to the next step, **f)** perform a Bradford assay, **g)** measure the absorbance after 10 min at 595nm for Bradford assay, **h)** acquire the data and obtain the equation to calculate the protein concentration of each sample, **i)** adjust the proteins accordingly for each experiment, **j.1)** spread the adjusted broth over PDA/NA plates and allow 30 min before inoculate with the *Fg-K1-4*, and **j.2)** add to the medium to get the working concentration on the total volume of medium and autoclave.

2.3.5 Antagonism assays using bacterial broths filtered (6, 24, 48, 72h) against Fg-K1-4

After determining that the *Bacillus–Fg-K1-4* interaction was very effective, it was decided to use only the bacterial broths in subsequent antagonism experiments. To assess the antagonism effect of broths from the *Bacillus* strains against *Fg-K1-4*, bacteria were cultured in liquid broth and at different times (6h, 24h, 48h and 72h) samples were taken, bacteria were centrifuged, and the aqueous phase was filtered using 0.22um filters (EMD Millipore Millex™). One hundred µl solution were spread over the medium (PDA/NA) using an L spreader and allowed to be absorbed by the medium for around 30 min after which a plug of the fungus was placed on the middle of the plates (Figure 2.2b). The assay was run over the duration of 4d and evaluated according to the above sections (Figure 2.2f-i). Each experiment had 4 biological replicates and was repeated at least three times.

2.3.6 Antagonism assays using adjusted filtered bacterial broths against Fg-K1-4

After determining that the bacterial broths showed an antagonistic effect against *Fg-K1-4*, an assay using adjusted proteins (total) was performed to evaluate the difference on the antagonistic effect between the *Bacillus* strains. Bacterial broths were cultured for 24h, 48h and 72h, spun, filtered (0.22 um) and assessed with a Bradford assay to determine the total protein concentration (Figure 2.3a-j1). Subsequently, the broths were adjusted and 200 µl at 100 µg/ml of protein was added to the plates, spread and allowed to be absorbed over the time of 30 minutes. The assay was run over 4d and evaluated according to the above sections

(Figure 2.3b, f-i). Each experiment had 4 biological replicates and was repeated at least three times.

2.3.7 Effect of heat treatment of bacterial broths against Fg-K1-4

Due to the antagonist effect of the adjusted-filtered *Bacillus* broth over the *Fg-K1-4*, an assay was performed to determine whether the antagonistic effect was derived from a protein component. To investigate this, *Bacillus* broths were centrifuged, filtered and autoclaved (121˚C, 1atm, 15 minutes) (Figure 2.2c). A Bradford assay was performed to normalise the protein content before and after the autoclaving process and the pre-autoclaved concentration was used to adjust the broths at 100 µg/ml total protein (Figure 2.3). Effect of autoclaved and unautoclaved broths on *Fg-K1-4* were assessed to over 4 days and evaluated according to the above sections (Figure 2.2f-i).

2.4 Heat and proteinase K treated bacterial broths

A new set of experiments were performed to determine the best concentrations of total proteins that inhibit the fungal growth.

2.4.1 Effect of bacterial broths adjusted to 0.5, 1.0 and 2.5 µg/ml against Fg-K1-4

An experiment was carried out to use low concentrations of total proteins by the total volume of the medium PDA/NA (0.5, 1.0 and 2.5 µg/ml). The same steps from autoclaved proteins were used (Figure 2.2d and Figure 2.3a-j2). Three different

concentrations of proteins per *Bacillus* strain were prepared and the experiment was carried out accordingly (Figure 2.3f-i).

2.4.2 Antagonism assay of bacterial broths adjusted to 10.0 and 20.0 µg/ml

Similarly, another experiment was carried out with high concentrations of total proteins by the total volume of the medium PDA/NA (10.0 and 20.0 µg/ml). The same steps from autoclaved proteins were used (Figure 2.2d and Figure 2.3a-j2). Two different concentrations of proteins per *Bacillus* strain were prepared and the experiment was carried out accordingly (Figure 2.2f-i).

2.4.3 Antagonism assay using bacterial broths treated with proteinase K and autoclaved

2.4.3.1 Treating Bacillus broths with Proteinase K

After adjusting the autoclaved *Bacillus* proteins in broths, bacterial broths were subjected to proteinase K treatment to assess the stability of the solution and their protein components. The average recommendations were evaluated and the conditions in Table 2.1 were used.

Bacterial broths were centrifuged, and Bradford assays were carried out, samples were treated with 100 µg/ml of proteinase K for 2h at 37˚C and used in further assays.

Later, these proteinase K treated broths were added to the PDA/NA medium at 1.0 $\mu g/mL_{medium}$ and autoclaved. An antagonism assay was performed (Figure 2.2d, f-i).

Table 2.1 Conditions used to treat *Bacillus* strains with Proteinase K.

	Proteinase K used with *Bacillus* broths	
	Recommended	Set in the assays
pH	7.5-8.0	~7.00
Tm ºC	37	37.0
Incubation time	30min - 18 h	2h
Concentration [µg/ml]	50 - 200	100

2.4.4 Antagonism assay of bacterial broths adjusted and treated with proteinase K and/or heat

2.4.5 Statistical analysis of antagonism assays of the bacterial broths against Fg-K1-4

The set of antagonism assays were performed to determine if there is any significant difference (α= 0.05) on the antagonist effect of the different treatments (*Bacillus* strains) against *Fg-K1-4*.

The experiments were established as a matrix of 3x2 or 3x3. Therefore, two-way ANOVAs were conducted using SPSS 25.0 to examine the effects of the different treatments using *Bacillus* strains (whole broth and filtered, spun, autoclaved and proteinase K treated broths) on the % of inhibition of the fungal growth (Table 2.2).

2.4.5.1 Experimental design for antagonism assays

Thus, the experiments were set as a matrix of the *Bacillus* strains (QST713, FZB24 and EU07) against the treatments (concentration of cells, proteins or applied

treatment -autoclaving or proteinase K-). The Table 2.2 shows several combinations of different treatments used.

Table 2.2 Experiment design of the antagonism assays of the *Bacillus* strains against *Fg-K1-4*.

	Treatments	6h	24h	48h	72h	10^6	10^7
	QST713	Q*6h	Q*24h	Q*48h	Q*72h	Q*10^6	Q*10^7
	FZB24	F*6h	F*24h	F*48h	F*72h	F*10^6	F*10^7
a.	EU07	E*6h	E*24h	E*48h	E*72h	E*10^6	E*10^7

	Treatments	Adjusted FAB	Autoclaved FAB&A
	QST713	Q*FAB	Q*FAB&A
	FZB24	F*FAB	F*FAB&A
d.	EU07	E*FAB	E*FAB&A

	Treatments	6h	24h	48h	72h
	QST713	Q*6h	Q*24h	Q*48h	Q*72h
	FZB24	F*6h	F*24h	F*48h	F*72h
b.	EU07	E*6h	E*24h	E*48h	E*72h

	Treatments (A&A)	0.5 ug/mL	1.0 ug/mL	2.5 ug/mL
	QST713	Q*0.5	Q*1.0	Q*2.5
	FZB24	F*0.5	F*1.0	F*2.5
e.	EU07	E*0.5	E*1.0	E*2.5

	Treatments	24h	48h	72h
	QST713	Q*24h	Q*48h	Q*72h
	FZB24	F*24h	F*48h	F*72h
c.	EU07	E*24h	E*48h	E*72h

	Treatments	A&A 10.0	A&A 20.0
	QST713	Q*10.0	Q*20.0
	FZB24	F*10.0	F*20.0
f.	EU07	E*10.0	E*20.0

	Treatments	PK+ A 2.5	PK+ A 5.0
	QST713	Q*2.5	Q*5.0
	FZB24	F*2.5	F*5.0
g.	EU07	E*2.5	E*5.0

Matrices used on the experimental design for the two-way ANOVA: a) assay section 2.2.4, b) assay section 2.2.5, c) assay section 2.2.6, d) assay section 2.2.7, e) assay section 2.3.1, f) assay section 2.3.2 and g) assay section 2.3.3.

The statistical model defines the experiment as a single factor (The Pennsylvania State University, (n.d.):

$$Y_{ij} = \mu + \tau_i + \epsilon_{ij}$$

$$\tau_i = \alpha_i + \beta_j + (\alpha\beta)_{ij}$$

where α_i is the main effect of factor A, β_j is the main effect of factor B, and $(\alpha\beta)_{ij}$ is the interaction effect. The interaction outcome would be report first and would be the failure to determine if one factor response is the same as another factor on the

different levels. The main effects, however, were interpreted directly when an interaction was not significant. Hence, the hypothesis was split in three different postulates:

The interaction of the treatments (*Bacillus* strains per treatments):

$$H_0 = \text{there is no interaction}$$

$$H_a = \text{there is interaction}$$

The main effect of the first factor (*Bacillus* strains)

$$H_0 = \mu_1 = \mu_2 = \mu_3 \ldots . \mu_i$$

$$H_a = \text{not all } \mu_i \text{ are equal}$$

The main effect of the second factor (treatments of the experiments)

$$H_0 = \mu_1 = \mu_2 = \mu_3 \ldots . \mu_j$$

$$H_a = \text{not all } \mu_j \text{ are equal}$$

The two-way ANOVA might then meet the assumptions of homogeneity, normality and non-outliners presence. To do so, residual analyses were performed to test for the assumptions of the two-way ANOVA in all the set of experiments. Outliers were assessed by inspection of a boxplot (non-presence) and histograms were including to illustrate it, normality was assessed using Shapiro-Wilk's normality test for each cell of the design on each experiment and homogeneity of variances was assessed by Levene's test.

In some cases, the data was transformed (square root) when it did not meet the above assumptions. In others, the transformation of the data (square root, log10, 1/data) did not yield better results, then it was decided to keep the original data and report the inconsistences.

The interaction effect between *Bacillus* strains and treatments was determined (interaction or non-interaction) and either an analysis of the main effect for the treatments was performed or an analysis of the simple effects at a significance of 0.05. The means (*M*) and standard deviation (*SD*) were reported as well the pairwise comparison when it was significant.

2.5 Deoxynivalenol (DON) assay

DON assay was performed to assess the viability of the *Bacillus* strains on different concentrations of the mycotoxin on plate, which could be correlated to the exposition of the bacterium to the fungus in fields (Cole et al., 2003; Madhyastha et al., 1994).

2.5.1 Preparing the DON solutions

DON (Sigma D0156) was dissolved in acetonitrile to produce a stock solution (1000mg/mL). Acetonitrile is a suitable solvent for DON due to its water miscibility and its stability at temperatures up to 25 °C for at least 24 months with a boiling point of 80°C.

Firstly, the minimal amount of inoculum was established, in 96 wells plate, 200µl of LB broth were added per well, then 1 and 5 µl of bacterial broth (24h) were added (Figure 2.8.5 (1) and incubated at 28°C in agitation (VXR basic Vibrax®) for 24h. Then the plates were inspected, and data was obtained using a plate reader adjusted to 600nm in (Multiskan Go, Thermoscientific) to determine the relative concentration of the bacteria in the different treatments.

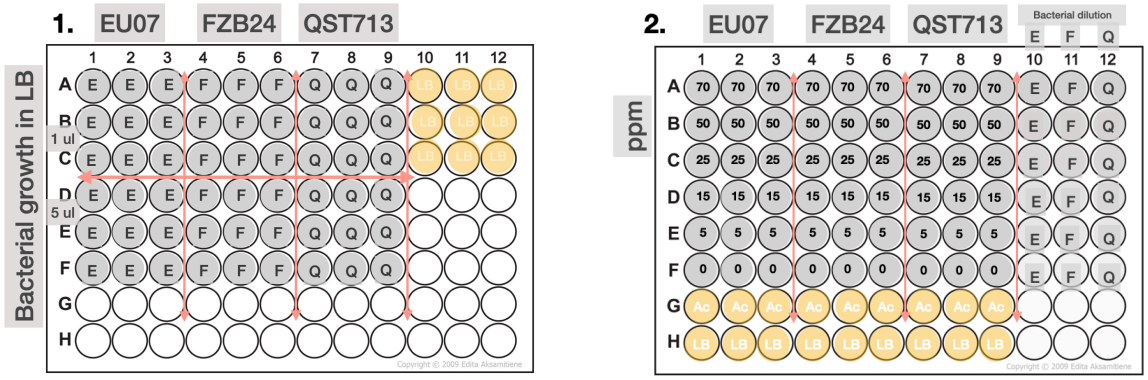


Figure 2.4 DON assay.

Bacterial broths were assessed in the minimal amount of inoculum to produce enough bacterial growth (5, 1 µl) in 200 µl of medium in 96-wells plates (1), then bacterial broths were assessed in their ability to grow in the presence of mycotoxin deoxynivalenol (2). Data was obtained using a plate reader at 600nm. Note: Ac=acetonitrile.

2.5.2 Treating Bacillus with DON solutions

A concentration of 70 ppm of DON is considered toxic for microorganisms, then from the stock solution, different amounts of DON solution were added to reach a final concentration of 70, 50, 25, 15, 5 and 0 ppm. Then it was mixed well and poured on plates. Three replicates per concentration per *Bacillus* strain, LB broth with Ac alone and *Bacillus* in LB broth untreated as controls.

The plates were left on the safety cabinet around 30 min to allow the evaporation of the acetonitrile, then incubated at 28˚C in agitation (VXR basic Vibrax®) for 24h. Three replicates were made.

IN VIVO ASSAYS

2.6 PGP activity: Effect of *Bacillus* strains on the growth of *Arabidopsis* plants.

2.6.1 Experimental design

The weight of each plant was recorded (leaves and shoots separately) and then put to dry individually in paper bags for three days at 70˚C. The dry weight was recorded. The time to develop blooms, number of primary and secondary bloom branches, height and width of shoots were recorded. Plants were examined for any natural infections by pathogens such as *Powdery mildew*.

2.6.2 Statistical analysis: Two-way ANOVA

Data obtained from the treatments of the *Arabidopsis* plants with the *Bacillus* strains were analysed using ANOVAs.

The hypothesis for each assessed factor was that the means of those factors were equal. Hence, the means of the wet weight on shoots, wet weight on leaves, dry weight on shoots, dry weight on leaves, the presence of the *PM* on each treatment and the means of the time of flowering on each treatment were equal.

$$H_0 = \mu_1 = \mu_2 = \mu_3 \ldots . \mu_k$$

Alternatively, it was hypothesized that the means were not all equal on any of those assessed factors.

$$H_a = \text{not all } \mu_k \text{ are equal}$$

A one-way ANOVA was conducted to determine if the morphological characteristics of the *Arabidopsis* plants (weigh of leaves and shoots, time of flowering, presence of pathogens) were different between the *Bacillus* strains. The plants were divided by groups: control (n = 6), QST713 (n = 6), FZB24 (n = 6) and EU07 (n = 6). The normality was assessed using histograms plots and the homogeneity of variances by the Levene's test. A post hoc test was conducted when there were significantly differences using a Tukey- HSD analysis (α=0.05)

2.7 Defence genes: treating *At* plants with bacterial broths (EU07)

Defence genes are those which express in plants due to the response to a stress inducer or a pathogen infection (Kouzai et al., 2016; Tör et al., 2003).

The marker genes for Jasmonic acid -JA- (PR1), Salicylic acid -SA- (PDF1.1), and a housekeeping gene (UBQ5-RT) were selected to evaluate the effect of the *Bacillus* suspensions in the immune-response of the *Arabidopsis* plants (Col-0). qPCR was used to quantify the relative expression level of those genes. These genes have annealing temperatures around 60ºC, they have small and similar products (~250bp) and they were used before for the by authors to investigate this responses (Moffat et al., 2012; Wang et al., 2010).

2.7.1 Experiment design

A complete randomised design was made with 5 treatments and 45-50 plants (12 days old) per treatment per replicate. Experiment was repeated at least 3 times. Treatments done were control (water only), EU07 spray 10% solution (S), EU07 spray 10% solution and infection with *Fg-K1-4* (10^6) 30 min later (*S*+*Fg*), spray with *Fg-K1-4* (10^6). The Table 2.3 shows a detailed description of the treatments. Treatments were performed through the time of 72h. All the bacterial solutions were prepared in the same way (section 2.1.1).

Table 2.3 Treatments used on *At* Col-0 plants and their sampling points to assess the immuno-response.

Treatment	*Description*	*Sampling*			
		0h	24h	48h	72h
Negative Control (A)	Spraying plants with water	S	S	S	S
Positive Control (B)	Spraying plants with *Fg* at 24h	S	S+*Fg*	S	S
Immunizing plants (C)	Spraying plants with *Bs* before treating them with *Fg*	S+*Bs*	S+*Fg*	S	S
Restoring plants (D)	Spraying plants with *Bs* after treating them with *Fg* (24h)	S	S+*Fg*	S	S
Relative control (E)	Spraying plants with *Bs* but not *Fg*	S+*Bs*	S	S+*Bs*	S

S: Spray, Bs: Bacillus EU07, Fg: F. graminearum K1-4.

All samples were carried out in the same manner, using twicers and forceps, the canopy of the plants was cut and placed in 1.5mL vials. The samples were flash frozen with liquid nitrogen and stored at -80°C till further use.

2.7.2 qPCR

qPCR was done to assess the expression of defence genes when plants were treated with the *Bacillus* (EU07).

2.7.2.1 RNA extraction from At plants

Samples were taking from the treated plants, stored (-80˚C) until until RNA extraction were carried out. TRIzol™ protocol (Themofisher, #15596026) were performed according with the manufacturer's instruction with some modification by Fantozzi and Telli, (2014) (Figure 2.7.2).

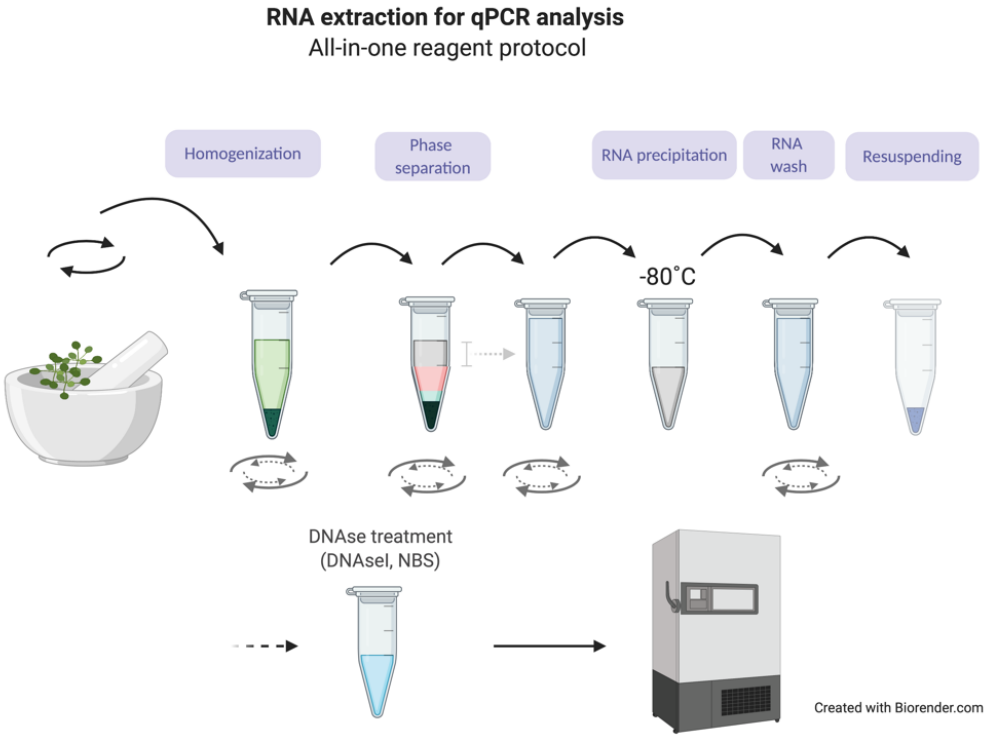


Figure 2.5 RNA extraction diagram for qPCR using plant tissue.

Trizol™ extraction were used to obtain RNA from *At* plants. Steps involved in the process include homogenization of the sample with liquid nitrogen and mix of sand, Phase separation on three layers, RNA, DNA and proteins after adding chloroform and centrifugation, RNA precipitation of the RNA (top layer) using isopropanol (1h), Wash of RNA with 75% DEPC H_2O and drying of the pellet. Finally, resuspending the pellet in 50µl of DEPC H_2O. All the RNA samples were treated with DNAse and QC checking with Nanodrop 2000, PCR (UBQ5 and ITS1/2) and gel electrophoresis. Samples were stored in -80˚C freezer.

The RNAs were treated with DNAse I (New England Biolabs; Ipswich, MA, USA; M0303) according to the manufacturer instructions to remove any residual gDNA. RNAs were then stored at -80˚C till further use. The samples were split into two tubes to avoid degradation. Quality of the samples were observed with Nanodrop2000c (Thermofisher; Wilmington, Del, USA). Results were aimed to be >1.8 in both 260/280 and 260/230 ratios, which show the purity and quality of the RNA samples, respectively.

PCR were performed with the primer UBQ5. To validate that the samples were free of DNA, a PCR was run in similar fashion as explained in Section 2.1.1.1. The primers used were ITS1/2 (5'– TCCGTAGGTGAACCTGCGG -3', 5'– GCTGCGTTCTTCATCGATGC -3') and UBQ5-RT (5'- GTAAACGTAGGTGAGTCCA -3', 5'- GACGCTTCATCTCGTCC -3').

2.7.2.2 qPCR of defence genes

To determine the relative expression of the genes *PR1* and *PDF1.1* in relation with the housekeeping gene *UBQ5*, a qPCR was performed using SensiFAST™ cDNA Synthesis Kit following the manufacturer instructions (London, UK; Bioline). The RNA samples were kept in ice, master mix were prepared over an ice block and a mix was prepared with SensiFAST™ SYBR® No-ROX One Step mix (2x) (5µl), primers 10mM (0.5µl), template (2µl), Ribo Safe RNase inhibitor (0.02µl), Reverse transcriptase (0.01µl) and DEPC-H_2O for a total volume of 10µl.

The samples placed directly in the 96-well qPCR plates, sealed and ran in LightCycler® 480 II (Roche Molecular Systems, Inc.). Three replicate per sample per gene were used, the housekeeping gene and a negative sample were included per plate. The run of the LightCycler® 480 II was programmed as follow in Table 2.4:

Table 2. 4 qPCR program in LightCycler® 480 II to analyse the defence genes *PR1*-qPCR, *PD1.1*, and *UBQ5*-RT in plants of *At* (Col-0) treated with *Bs* and *Fg-K1-4* solutions.

Step	***Description***
Pre-incubation	1 run: 2 min, 45˚C.
Amplification	40 runs: 95˚C, 10s; 60˚C, 15s; 72˚C, 15s
Melting curve	1 run: 95˚C, 60s; 1˚C, 60s; 97˚C, 15s
Cooling	1run: 40˚C and 30s.

2.7.2.3 Calculating the relative quantification using $2^{-\Delta\Delta Ct}$ method

The method $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) was used to determine the relative quantification of the defences genes mentioned above. To do so, the data of the C_p (cross points or C_q) were obtained from the data generated by the LightCycler® 480 II, using absolute quantification along the second derivative method for C_q -calling (Walch and Labaere, 2011). Then the extracted data were used to calculate the relative expression of the genes with the method $2^{-\Delta\Delta Ct}$ in Excel.

Assuming that the efficient of the amplification is same for all the samples (2), then a normalised value are calculated for the Ct, this is the change in the expression

level of the target gene minus the housekeeping (HK) gene (ΔCt). Thus, the fold change between the samples is calculated using the calibrator or the control sample normalised (ΔΔCt) (Livak and Schmittgen, 2001).

Equation 2.2. Quantification of expression with $2^{-\Delta\Delta Ct}$ method:

$$\Delta\Delta C_t = (C_{\mathrm{t,Target}} - C_{\mathrm{t,HK}})_{\Delta time} - (C_{\mathrm{t,Target}} - C_{\mathrm{t,HK}})_{time\ 0}$$

An analysis of the data was used to shed light in understanding how the bacterium affect the plant immune-system.

2.7.3 GUS assay

GUS expressing plants (PR1 and RLK), were used to widen the understanding of the PGP activity on EU07 strain on *At* plants. GUS reporter is used to detect the activity of the associated gene, in this case a PR1 (pathogen related) and a RLK (Receptor-like kinases). Both genes report the induction of the SA either by pathogen infection, stress or developmental features (Asai et al., 2000; Lincoln et al., 2018).

Seeds of *At* PR1::GUS and RLK::Gus were sown and transplanted (15 d). At 4 weeks old, they were arranged in treatments/blocks randomised and treated as described in Table 2.5. Canopy of the plants treated with~150µl of the bacterial solution or fungal solution (10^6). The plants drenched were rested on bacterial solution (10%) by 30 min and then, all the treatments were placed back in the

growth room up to 5 more days. Samples of the plants were taken at 0, 2 and 5 days after treatment and assayed with histochemical GUS staining.

Table 2.5 Treatments used on *At* PR1::GUS and RLK::GUS plants and their sampling points in order to assess the immuno-response.

		Sampling		
Treatment	Description	1d	2d	5d
Control	Spraying plants with water	✓	✓	✓
Control + physical wounds	Non-treated plants with leaves scratching with tweezers.	✓	✓	✓
Drench	EU07 (10% solution) drench	✓	✓	✓
Spray	EU07 (10% solution) spray ~150µl/plant.	✓	✓	✓
Fg	*Fg* solution sprayed over leaves	✓	✓	✓

GUS Buffer (Jefferson, 1987) were prepared as follows; 1mM of X-Gal reagent (X-GlucDIRECT; Malaga, Spain) was dissolved in ~200 µl of DMS, then 50mM Sodium phosphate buffer, pH7.0, 0.1% Triton X-100, 4mM K+ ferricynide and 100 µg/ml chloramphenicol were added. Solution was made up to a working volume and stored on freezer -30˚C in a container protected from light.

Samples were placed in GUS buffer and incubated at 37˚C for 24h. Subsequently, methanol was added to stop the solution and it was replaced by new methanol two times in order to extract the chlorophyll from leaves. To determine the GUS activity, histochemical staining in samples were compared under the microscope.

2.7.4 Plant assessment

Alongside the head infection assay (section 2.9.3), the yield of the Bd-21 plants was measured as number of spikelets per plant. Plants treated with bacterial solution as well infected or not with *Fg-K1-4* were also assessed in the number of spikelet produced by the end of the experiment.

Data was recorded and analysed with simple ANOVA to determine if there was any statistical significance on the number of heads produced.

2.8 Infection assays in Bd-21

2.8.1 Roots infection assay

2.8.1.1 Seeds vernalisation

Seeds were prepared as described above (section 2.3.1.5) for germination. After a period of 2 -3 days, seeds were taken off and allow to reach RT (~30min).

2.8.1.2 Seeds germination

Water agar (WA) media were prepared (1.5% agar in distilled water and sterilised) and poured on square plates (100mm, non-compartmentalized) allowing the plate to be filled up to 50% of capacity (~70mL of medium). Then, a row of 6-7 seeds were placed over one side of the plate. The seeds were placed parallel to the bottom and sank at least the 50% on the medium with an artist brush oriented towards the top and the germ towards the bottom (Figure 2.6.1).

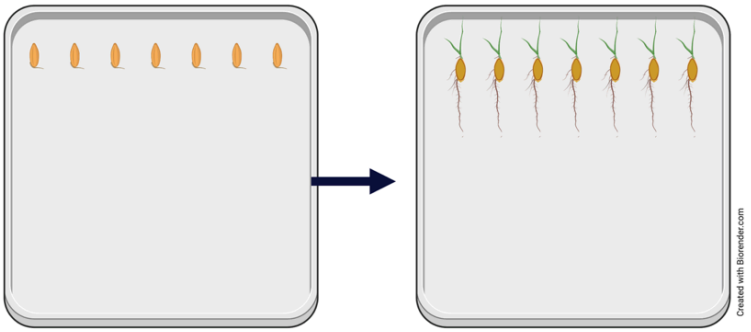


Figure 2.6 Roots assay in Bd-21.

Bd-21 seeds arrangement on square plates on WA for germination assays.

Plates were labelled, wrapped with cling film and placed on a rack with a slight angle on growth room (21˚C, 10h D /14h L) to allow the development of roots.

2.8.1.3 Seeds germination and Bacillus/ Fusarium inoculation

WA plates with seeds were allowed to develop roots until they covered the third part of the plate. Then, a drop of a suspension of the Bacillus strains or a drop of *Fusarium* suspension (10 µl) were placed through the medium near of the root, taking care of not to damage the root stem. Afterwards, pictures of the plates were taking every day for the period of 10 days to observe the growth of either microorganism.

2.8.2 Detached leaves infection assay

2.8.2.1 Plant preparation for infection assays -non-vernalised-

Seeds without lemma on wet filter paper were allowed to vernalise for 2-3 days in similar fashion to the stratification process (Section 2.3.1.5), then sown on pots (<400 ml) 8x8 cm, at 1 cm deep with saturated soil (Levington F2s Compost) in

growth room (21˚C, 10h D /14h L) for a period of around 9 weeks. Seeds with short period of vernalisation showed abundant leaves and a longer time to produce flowers.

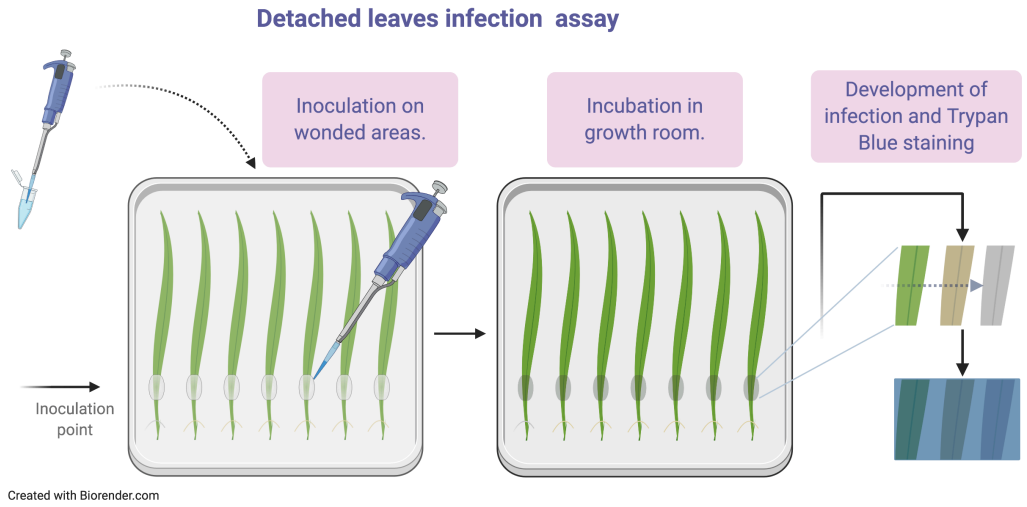


Figure 2.7 Detached leaves assay.

Detached leaves infection assays. Arrangement of the leaves non-treated (control) and treated (bacterial broth) infected (infection point) with *Fg-K1-4.* Leaves were attached to the medium sinking the collar area on the medium.

2.8.2.2 Leaf infection assay with Fg-K1-4

Leaves (<9 cm long) were cut at the collar level and sunk in distilled water to keep them turgent. To determine if the fungus could infect leaves, glass rods with round tip were sterilised individually and used to gently scratch the cuticle of the leaf blade at 5cm of distance from the collar to produce a wound on the tissue (Rana et al., 2018).

A 10µl drop of water -control - or fungal solution (10^6 spores) - treated- were deposited over the wound and allowed to dry for about 10 -20 min. Treated leaves were taken by the collar and inserted into the WA medium, 6-7 on a row, covered with the lid, labelled and sealed. Plates were stored in growth room (21˚C, 10h D /14h L) for up to 8 days to allow the leaves to develop infection.

2.8.2.3 Leaf infection assays with Fg-K1-4 and EU07 bacterial broths

In a similar way, leaves were taken and sunk on water or bacterial suspension (10%), placed on a towel and allow to dry for ~10 min. Later, mechanical damages were made on every leaf as described above (Section 2.6.2.2) and leaves were treated with spore solution, placed on WA medium and incubated on growth room for a period of up to 8 days. Pictures were taken of the development of the infection.

At the end of the experiment, the infected section was cut (one inch over and above of the infected area) and treated with trypan blue.

2.8.2.4 Trypan blue staining

To reveal hyphal structures and dead plant cells in plant tissues, trypan blue staining was used (Mauch, 2005). Sections of infected leaves of Bd-21 were cut at 2 inches long and stained with trypan blue. Phenol, glycerol, lactic acid, water (1:1:1:1) and 0.05% of trypan blue were mixed together as a stock solution and filtered. When needed, the stock solution was mixed with ethanol (>95%) 1:1. Tissues were immersed in trypan solution in a suitable container with loose lid, placed in boiling

water for one minute, put aside and rested for 24h in fume hood. Tissues were cleared of the trypan solution and distained with 40% chloral hydrate. When cleared, tissues were preserved in glycerol 50%.

2.8.3 Head infection assay

2.8.3.1 Severity of infection Index

Visual evaluation of the severity of the infection on experimental plots can be unreliable. To minimise the error due to the examiner criteria, a scale of severity of the infection were constructed (Figure 2.8.3) with a wide range of disease scoring -6 points-, to lower the impact of the error in the final statistical analysis (Bock et al., 2010; Chiang et al., 2017; Rana et al., 2018).

The scale features six score points -0 to 5 – as described in Figure 2.6.3. The scores were used to assess the head infection assay in Bd-21 (section 2.8.3.2).

2.8.3.2 Head infection assay

Head infection assay was run to determine the effectiveness of the bacterial broth treatments in grass plants (Bd-21). To prepare the plants for the assays, seeds of Bd-21 were vernalised by 2-3 weeks, then sown on pots with saturated soil as described in section 2.8.1.1. Plants were allowed to grow until spikelets (8-10 weeks) were developed and flowered.

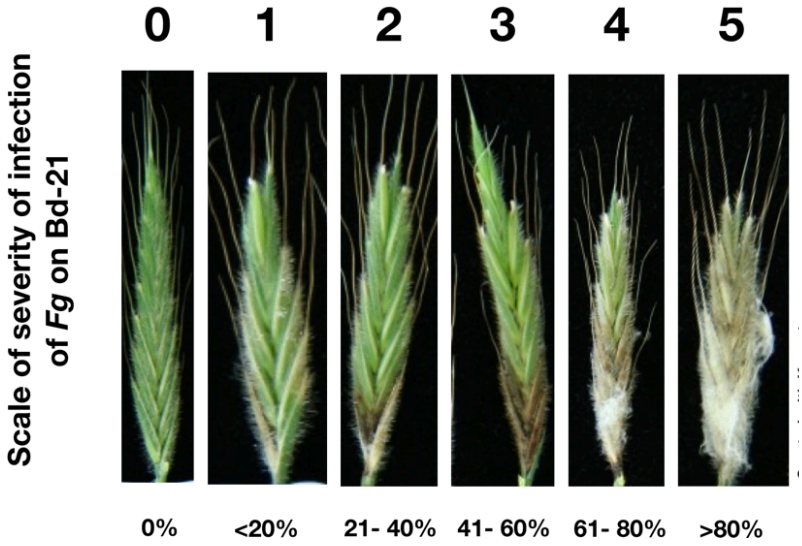


Figure 2.8. Scale of severity of the infection.

Score of 0% was assigned to a visually healthy spike **(0)**, <20% was for a spikelet showing only one floret necrotic **(1)**, 21-40% assigned to the visible infection of the basal floret and the opposite **(2)**, 41-60% was assigned to a spike with around 30% of necrotic tissue **(3)**, 61-80% were assigned to a spike with around 65% of necrotic tissue and some visible mycelia **(4)**, finally 81-100% was assigned to a spike with more than the 70% of necrotic tissue including the spikes with 100% of infection **(5)**.

Afterwards, the plants were attached to a rod to keep straight up and labelled. Then filter paper (Whatman 01) was cut in pieces (2 x 4 mm) and placed carefully between two florets per spikelet, a total of five spikelets were selected per plant. Then, a drop (10 μl) of fungal suspension (10^4) of spores were applied to the filter paper and allowed to dry ~30 min (Rana et al., 2018). Subsequently, some plants were treated with bacterial suspension (EU07, 10%) and allowed to dry in the same manner.

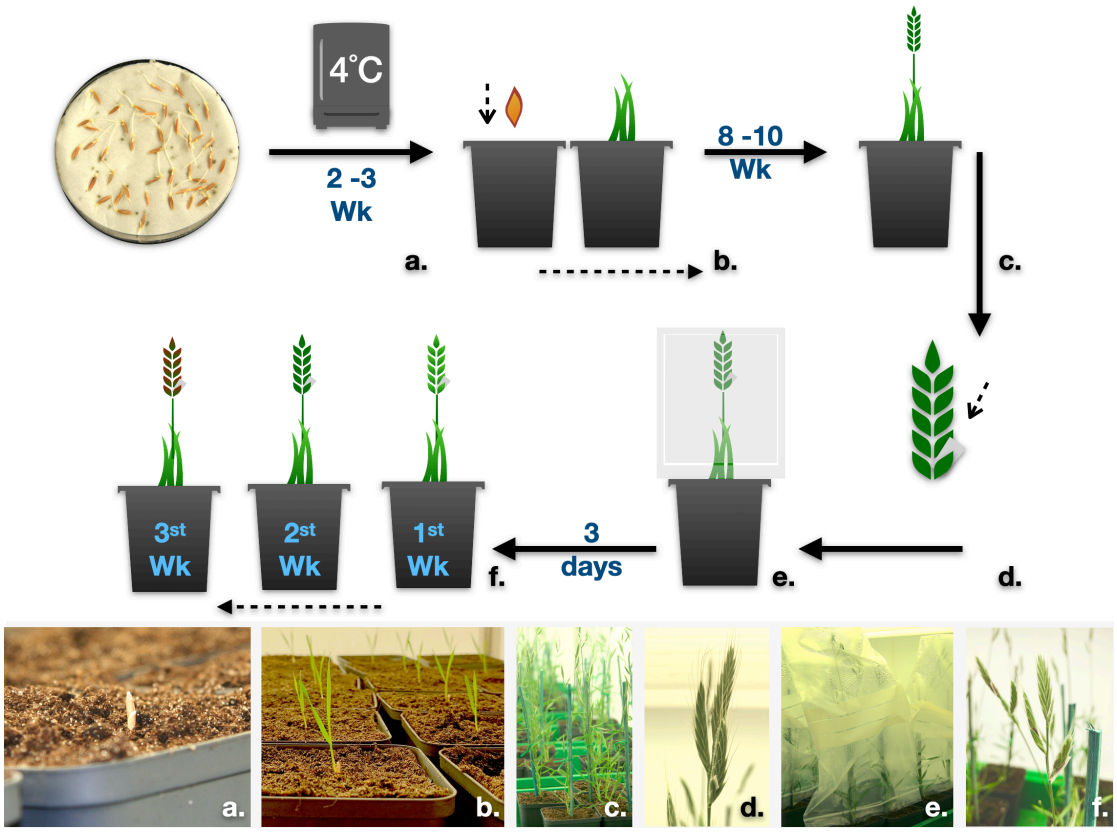


Figure 2.9. Head infection assay.

HIA were performed in 8 weeks old Bd-21 plants. Top: Seeds used were stratified for 2-3 weeks (4˚C, dark) (**a**). After this period, seeds were sown on soil and plants were allowed to grow until flowering (**b**), plants were tied straight up (**c**) and a piece of filter paper were placed in the base of the spikelet, inoculated (**d**) and covered to increase the humidity (**e**), finally the plants were assessed using the Severity of disease index (**f**). Bottom: captions during the different stages described above, accordingly.

Plants treated and untreated (control) were placed back in growth room (21˚C, 10h D /14h L) and covered individually for 3 days with polythene plain grip seal bags to allow a high humid environment, which promotes the fungal growth. After this period, covers were removed, and plants were assessed in presence or absence of disease symptoms. The scale of severity of the disease (section 2.8.3.1) were used

and values were recorded accordingly. Assessments were carried out every week for 2-4 weeks (Figure 2.8.4).

2.8.4 Statistical analysis: Two-way mixed ANOVA

Due to the necessity to understand the differences between treatments over the time, a two-way mixed ANOVA was performed. The changes on the severity of the infection (severity index) in each treatment over the time were recorded.

2.9 Infection assays with *Arabidopsis thaliana*

Infection assays were carried out with *Arabidopsis* plants using the obligate pathogen *Hyaloperonospora arabidopsidis* (*Hpa)*. Assays were done to determine if the treated plants with the *Bacillus* strains would make any difference in the sporulation of the pathogen as well the general appearance of the plants. All the plants were treated in similar fashion. Seeds of the *At* line (Col-0 or Ws-*eds1*) were sown in saturated soil, placed in germination trays, covered and allow to grow for one (Ws-*eds1*) or two weeks (Col-0). Ws-*eds1* plants tend to grow quickly compare to the Col-0 line. *Bacillus* strains were used to treat some trays immediately after seeds were sown, ~1-2mL of bacterial suspension (QST713, FZB24 or EU07) were poured over each cell on the trays using disposable Pasteur pipettes. These same trays were treated one or two more times a week.

*2.9.1 Col-0 and Ws-*eds1

2.9.1.1 Inoculation

One to two-week old Col-0 or Ws-*eds1* plants treated with bacterial broths were used to be inoculated with *Hpa* spores. From infected Ws-*eds1* plants (1week after inoculation), spores were harvested, by cutting infected seedlings and placing into a centrifuge tube (50mL) with ~6 mL of distilled water. Plants were then shaken vigorously and the solution of spores were decanted over other tube through a three layer of Miracloth® to filter the debris. Spore suspensions were assessed under a light microscope with haematocytometer. The solution of spores was adjusted when necessary.

The suspension, around 1-2 ml, was sprayed over each set of cells in a P40 module tray insert. The cells were placed back on the tray over wet tissue to keep humidity and covered with a lid, sealed and placed in growth cabinet (16˚C, 10d 14d) for one week until sporulation.

2.9.1.2 Spore counting

A week after inoculation, plants were taken from the growth cabinet and the number of spores were measured. In the same manner as the procedure to inoculate plants (section 2.9.1.1).

2.9.1.3 Data analysis

One-way ANOVA were used to analyse the data obtained. Post hoc test were carried out to determine significant differences.

OMIC'S

2.10 *Bacillus* genome sequencing

2.10.1 DNA extraction and sequencing

To carry out comparative genomics on the three *Bacillus* strains used in this project, the whole genomic DNA isolation was performed using the ISOLATE II Genomic DNA Kit (Bioline), following the instructions of the manufacturer. Fresh samples from 24h of broths of the *Bacillus* strains were used to do the extraction. To assess the quality of the extraction, 6 µl of gDNA samples were carefully mixed with DNA loading buffer (Gel Loading Dye, Purple (6X), NEB B7024) and run in a gel, 40 min, 75V. The gel was viewed in the UV transilluminator (BioSpectrum® 310 IS, UVP LLC) and a picture of the gel was taken. The quality and concentration of the gDNA was measured using Nanodrop 2000c (ThermoScientific). Genomic DNA were then sent to the Exeter sequencing services as is recommended.

2.10.2 Analysis of NGS data

Data from the Illumina® MiSeq sequencing technology were obtained from Exeter and used in the analysis. To visualize, analyse and compare the data obtained, the approach proposed by Edwards and Holt (2013) was taken. On this method, *de novo* assembly was performed on the trimmed data using SPAdes (Bankevich et al., 2012). The contigs obtained were then ordered against the reference genome with Mauve (Darling et al., 2010) and viewed in Geneious ® R10.2. Then the data was annotated (Prokka) (Seemann, 2014, Ondov et al., 2011). Finally, they were

compared with each other to uncover different genes between them using Geneious R10.2 ®. RAST 2.0 (Aziz et al., 2008) online tool was used to annotate the three different *Bacillus* strains draft genomes. Features of the whole genomes were found and classified. Manual curation was used to determine the presence of genes with PGP activity related function.

2.10.3 Comparative genomics on Bacillus strains

The whole genome of the three *Bacillus* strains were aligned with Mauve (Darling et al., 2010)l. MUMmer3.23 were used compare the genes and found those that were different between the genomes of the three *Bacillus* strains .

Table 5.7 was built and those genes with uniqueness in the strain EU07 were extracted and 15 of those were used for further analysis.

2.10.4 Confirming genes from comparative genomics: PCR and sequencing

2.10.4.1 Designing specific primers

Sequences from genes found in EU07 but not present or partially present in the other strains, found by Mummer, were selected (Table 5.7), 15 of them, and their sequences were blasted against the whole genome of the three *Bacillus* strains to confirm their presence/absence.

Once the findings were confirmed, Primer3, in Geneious ® R10.2, were used to design the primers based on an optimal product size of 200 - 250 bp, a melting

point or ~57˚C (Tm) and they were included in the target region of the gene (Untergasser et al., 2012).

PCR was run to confirm the presence or absence of those genes in the three *Bacillus* strains as described in section 2.1.1.1. Afterwards, PCR products were cleaned up and sent for sequencing using the Mix2Seq Kit (Eurofinsgenomics, Germany). Sequences from PCR products of the *Bacillus* strains were imported into Geneious® and the sequences were analysed to determine the identity of them.

2.11 Transcriptomic analysis of the *Fg-K1-4* treated with EU07 cell free culture

2.11.1 Treatment of Fungus with EU07

Firstly, the right conditions in which the effect of the bacterial broth of the EU07 over the fungus was determined. PD broths were inoculated with 100 µL of macroconidia and incubated with agitation at 150 rpm at 24˚C for two days, three replicates per treatment (total 6 flask which is the minimal number of samples to ensure a good power of the experiment) (Van den Berge et al., 2019).

After the fungus got some homogeneous growth (2d), flasks were treated with sterile H_2O_{sd} (control) or *Bacillus* pellet (EU07), 4ml per flask (Figure 2.5). Briefly, bacterial broths were grown for 24h and 4 ml (x3) were spun to obtain a pellet (4000 rpm, 10 min), the supernatant was discarded and the pellet was resuspended in 4mL of sterile H_2O_{sd}. The bacterial cells in pellet were allowed to interact with the

fungus for 6h. Afterward, 1-2ml samples from each flask were taken using a disposable Pasteur pipette.

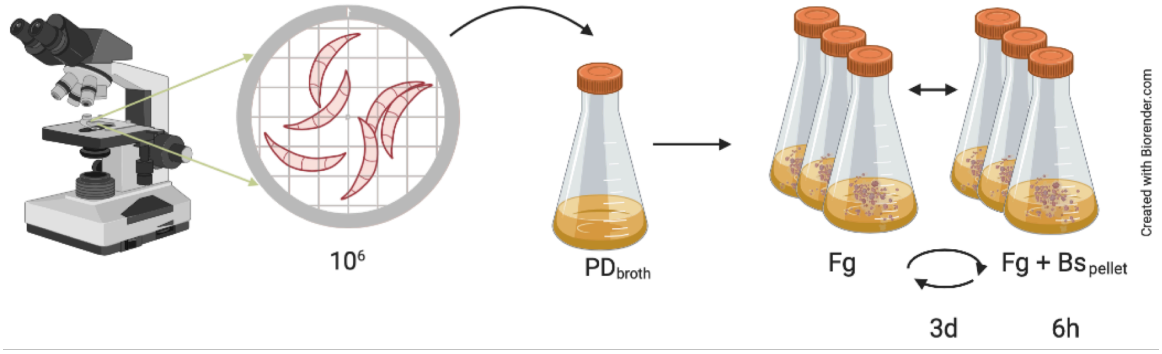


Figure 2.10 Dual culture assay for RNA sequencing.

Solutions of macroconidia (10^6) of *Fg-K1-4* were inoculated in PD broth (250:1), shaken (2-3d) and treated with the *Bacillus* broth. Total RNAs were then extracted from fungal samples, non-treated (Control/*Fg-K1-4* alone) and treated (*Fg-K1-4* + *Bacillus* pellet) and used for RNA-seq experiments.

Samples were kept in cryogen tubes, labelled and flash frozen with liquid nitrogen, then stored at -80˚C until further use.

2.11.2 Protocol of RNA extraction for NGS (RNA-seq) from fungal tissues

2.11.2.1 RNA extraction using Trizol® (suitable for use with RNA wiz reagent using a modification on precipitation step)

Eppendorf tubes were filled with 1mL of Trizol™ (or RNAwiz) and 10 µl of b-Mercaptoethanol. Frozen samples were grounded using pestle and mortar, and sterile sand (200 mesh) under liquid nitrogen. Around 8-10 scoops of the ground

tissue were placed on tubes with Trizol™, mixed very well using vortex and incubated ~5 minutes (Figure 2.8).

Chloroform (300 µl) was added to the tube, vortexed, incubated for 2-3 minutes and spun at 16,260 x g for 15 min. The supernatant was carefully recovered allowing some amount of liquid to remain in the tube and avoiding the intermediate layer. The chloroform extraction was repeated twice.

Then, in a clean tube, the supernatant was mixed with 1 ml of isopropanol (750 µl of Isopropanol and 250 H_2O DEPC when using RNA wiz reagent) and 200 µl of 7.5M ammonium acetate (1.25M), incubated at -80˚C overnight (to allow the maximum RNA precipitation) and centrifuged at 19,083 x g, 20 min to form a pellet (usually, the starting material is enough to produce a visible pellet).

The pellet was washed three times with 1ml of ethanol 75%$_{DEPC}$. Then, the pellet was resuspended using vortex, then centrifuged at 6,351 x g for 5 min. Care was taking to invert the tubes with 75% ethanol to wash all the salts presented in the tube.

After the pellet was washed, it was air-dried for 10 min and 60 µl of DEPC treated water were added. The pellets were vortexed, and the tubes were incubated at 55˚C for 5 min to dissolve the pellet completely. This yields 60 -150 ng/ µl of RNA after cleaning up.

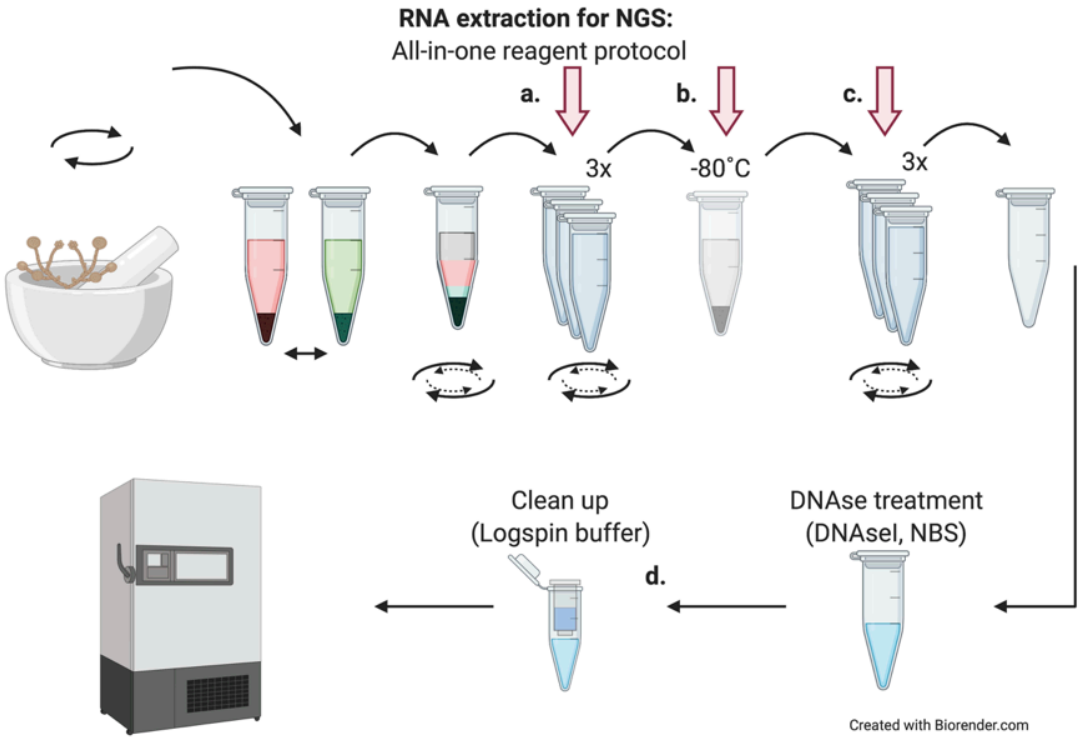


Figure 2.11 RNA extraction for new generation sequencing

The standard Trizol protocol was modified to warrant a high-quality RNA (RIN >7): (a) three chloroform extraction, (b) precipitation overnight in Isopropanol-NH_4OAc solution 3:1, (c) three ethanol 75% wash and (d) a final clean up using the LogSpin buffer in plasmid columns.

2.11.2.2 DNAse treatment

The pellet was vortexed very well to dissolve it completely to ensure the exposure of the total DNA. The concentration of the RNA was measured using a Nano drop 2000. DNAse I (NBS; London, England) was used to clean the DNA off from the samples. Briefly, 1 µl (2UI) of DNAse I and 1 µl of buffer 10X per 10 µg of RNA were used and incubated 15 min at 37˚C.

2.11.2.3 Spin columns clean up

To deactivated the DNAse I and clean up the RNA from salts, a modified LogSpin protocol was used (Yaffe et al., 2012). In short, the sample were mixed with LogSpin buffer, and ethanol and placed in a plasmid column. Centrifuged and washed with NaAC 3M then with 75% ethanol. The column was placed in a new clean tube. Finally, 100 µl of DEPC water were placed on the dry membrane. It was incubated for 1 min, then centrifuged to elute the RNA (Figure 2.12).

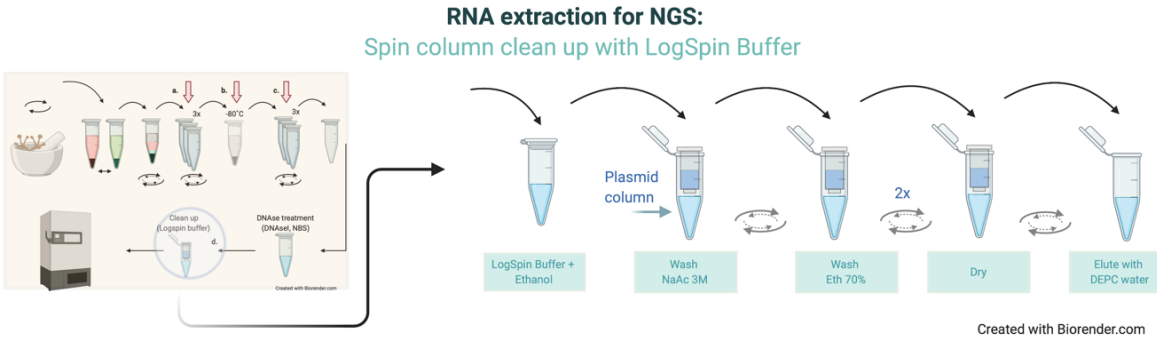


Figure 2.12 RNA clean up with spin column and LogSpin buffer.

RNA clean-up was performed using the LogSpin Buffer and plasmid columns. The steps involved mixing the RNA with the buffer and ethanol, spin, wash with sodium acetate, spin, wash with 70% ethanol, spin twice and eluted with DEPC water.

Finally, the RNA was eluted with DEPC water. All the spins were at 8000-10000 g 10s-1min.

2.11.2.4 Integrity and purity of the RNA

In house assessment of the integrity of the RNA were made with 1.5% agarose gel, run at 75V for 25minutes. Then the purity was assessed using Nanodrop 2000. Samples with ratio 260/280 >1.8 and 230/260 >1.8 were considered pure of DNA and other contaminants. Most of the samples showed ratio 260/280 >2.0 and

230/260 >2.0. Final assessment was carried out by outsourcing using a Bioanalyzer to obtain the RIN number (>7).

2.11.3 Library preparation and sequencing and quality control

Samples of *Fg-K1-4* RNA treated and non-treated with EU07 bacterial broth, were sent to Novogene (Uk) Company Limited for library construction (250~300 bp insert cDNA library (poly-A capture) and sequencing using Illumina (PE150, Q30≥80%).

Quality control (QC) was carried out by Novogen to the sample test, library preparation and sequencing steps in order to assure good quality results. Qubit 2.0 was used for tests the library concentration; Agilent 2100 was used to tests the insert size and Q-PCR was used to quantify the library effective concentration.

2.11.4 Transcriptome analysis

Analysis of the RNA sequencing were performed in the high-performance computer (HPC) or cluster computing (NBI) of the John Innes Centre (JIC). FastQC was used to assess the quality of the reads (Andrews, 2016). Adapter sequences and reads with poor quality were trimmed with Trimmomatic PE (Bolger et al., 2014). Mapping of the reads were done with Hisat2, which considers eukaryote genome configuration, when it maps to a genomic sequence contained introns allowing the gaps. Hisat2 indexes the reference genome, which is used to map the paired end reads producing .sam files (Kim et al., 2015). Samtools (v1.9) were used to sort the files and obtain the .bam file (Li et al., 2009). Sorted files can be viewed using

software like IGV (Robinson et al., 2011). However, for RNA-seq analysis it is necessary to know the expression values or the number of reads mapping to a specific gene. Stringtie 1.3.5 (Pertea et al., 2015) was used to produce the abundance table of the RNA-seq data. Stringtie take the sorted .bam files and normalised them by fragments per kilobase per million or reads per (RPKM) and by transcripts per million (TPM). This time, for the analysis of the RNA-seq data the reference genome PH-1 (Rothamsted Research, 2020) were used and reported only expression values for reference transcripts. No novel transcripts were included.

The table was then paired with the annotation genes from the tool Biomart on Ensembl ("BioMart - MartView," 2020) to obtain the name genes for the data. Finally, the final gene count table were submitted to the online tool Degust (Powell, 2015) for DE analysis."

After completing the analysis, the differentially expressed genes were obtained by comparing the expression levels using the method voom/lumma within the R tool DESeq2 (Love et al., 2014) in the online tool Degust (Powell, 2015). Samples were grouped into treatment (control and treated). Transcripts were extracted using different thresholds. The whole set of genes considered significant were in the threshold absolute log fold change (FC) of 0.585 (1.5x FC) and a false discovery rate- (FDR) adjusted P-value (q-value) <0.05. A more tighter threshold analysis was done with (FC) of 1 (2x FC) and a false discovery rate- (FDR) adjusted P-value (q-value) <0.01.

Volcano, MDS and heat maps of the data were obtained from Degust. Gene name (transcript names) were obtained from the abundance table and used in Degust.

2.11.4.1 Gene Ontology (GO) term identification, functional annotation, network and enrichment analysis

Gene ontology (GO) term identification, GO slim term, functional annotation and gene name was obtained pairing names with the database PH-1 in Ensemble. Cytoscape was used to construct the network enrichment (p=0.04) and StringDB was couple to the network to obtain protein functions (Doncheva et al., 2019; Szklarczyk et al., 2019). Additionally, StringDB online was used to construct networks, do data mining and reference mining. When needed UniprotKB was used to obtain protein functions (Consortium, 2019).

Chapter 3

In vitro assays: Antagonisms assays

Introduction

Fusarium graminearum (Fg) causes Head Blight (referred to as *Fusarium* Head Blight-FHB) in wheat and other cereal crops (Leslie and Summerell, 2006). It infects the grains in the anthesis stage, when the grains are still soft and the humidity of the environment is adequate. As a result of the infection, the grains shrink and decay with the subsequent decrease of production on fields. In addition, this fungus produces mycotoxins that can be lodged into the grains. These mycotoxins are highly stable and can persist until the grains are processed for human or livestock consumption (Beccari et al., 2018; Bottalico and Perrone, 2002; David et al., 2016). The main mycotoxins in FHB are deoxynivalenol (DON) and T-2 toxin (T-2), found alongside zearalenone (ZEN) and fumonisin B1 (FB1). These mycotoxins are toxic to human and animals and can be deadly if consumed in high concentrations (Eskola et al., 2019; Gruber-Dorninger et al., 2019; “The Global Mycotoxin Threat 2019 [Infographic],” n.d.).

Multiple efforts have been made to control this fungus and its mycotoxin production. The strategies to control it vary from crop improvement, crop management, chemical pesticides, RNA silencing, DON degradation and use of Microbial Biological Control Agents (MBCA) (Adeniji et al., 2019; Awad et al., 2010; Drakulic et al., 2017; Machado et

al., 2017; Palazzini et al., 2016; Pasquet et al., 2016; Pecoraro et al., 2018; Schweiger et al., 2016; Steiner et al., 2017). MBCAs count as excellent options, and among them, *Bacillus* strains represent a vast majority of the efforts to control this fungus (Adeniji et al., 2019; Bacon et al., 2004; Chan et al., 2009; Fan et al., 2018; Gong et al., 2015; Grosu et al., 2014; Palazzini et al., 2016; Pandin et al., 2018; Scholz et al., 2014; Zhao et al., 2014; Zhao et al., 2014; Zhao et al., 2015). *Bacillus* strains are widely used in control of plant pest/pathogen management since the beginning of the Twenty century. Specifically, *Bacillus subtilis*, which is named model bacterial organism in industry, has the capacity to produce a wide range of commercially important compounds, such as industrial enzymes. Likewise, it was first used to control dysentery during the second world war (WWII). Later, it was included in a range of bioformulations, often in plant pest control, due to its antimicrobial properties (Borriss et al., 2018; Kovács, 2019). Several studies suggest that *Bacillus* strains, such as *B. amyloliquefaciens*, *B. mojavensis* and *B. velezensis* produce antimicrobial compounds that are able to stop the growth of *Fusarium* species, given a wealth of background for future developments in commercial applications (Adeniji et al., 2019; Bacon et al., 2004; Baysal et al., 2013; Castañeda Alvarez and Sánchez, 2016; Chan et al., 2009; Liang Chen et al., 2018; Gilbert and Fernando, 2004; Gong et al., 2015; Grosu et al., 2014; Palazzini et al., 2016; Zhao et al., 2014, 2014; Zhao et al., 2015).

Some antimicrobial compounds identified from several *Bacillus* strains include fengycin (Fen D), bacillomycin (Bmy A) and Iturin (Itu C); non-peptidic antimicrobial compounds

including polyketides, aminosugars and phospholipids; signal peptides of pheromones and bacteriocins polyketides; amylocyclicin, bacillaene and difficidin (Antelmann et al., 2001; Baysal et al., 2013; Chen et al., 2007; Pan et al., 2015; Scholz et al., 2014; Tjalsma et al., 2000). These studies demonstrated that *Bacillus sp.* can control *Fusarium sp*. using different modes of action. For instance, Gong et al. (2015) determined that iturin A and plipastin A has a strong antifungal activity, contrary to surfactin. Another study showed that, *Bacillus* sp have the capability to produce volatiles, which induce deterioration of the hypha in *F. oxysporum* (Baysal et al., 2013). In a different research, Deleu et al.(2008) studied the lipopeptide fengycin, which possesses an antifungal and haemolytic activity. Apparently, compounds like fengycin and surfactin and other antimicrobial peptides make the plasma membrane of the hyphal cells more permeable, impeding their growth. However, there is still a lot of work to carry out to understand the effects of these bacteria and their metabolites in the control of plant pathogens like *Fusarium* sp.

Yet, the effectiveness of MBCA ultimately is dependent on the environmental conditions on fields. Authors such as Gilbert and Fernando (2004) concluded that, the use of bioformulations will improve the stability and long term ability of the formulations to control the FHB.

Here, three *Bacillus* strains, -two commercial (QST713, FZB24) and one non-commercial strain EU07- were investigated to reveal their ability to control *F. graminearum* at laboratory level. QST713 is commercialised by Bayer under the name Serenade® and FZB24 is commercialised as TAEGRO® (Novoenzymes Biologicals Inc.). Inversely, the

EU07 is a non-commercial strain isolated in Turkey and kept as a laboratory stock. Previous proteomic studies demonstrated the effectiveness of EU07 to control *F. oxysporum* in laboratory trials (Baysal et al., 2013; Chen et al., 2007; Merckling et al., 2009).

The aim of the work described in this chapter was to determine if these *Bacillus sp* affect the mycotoxin-producing fungus *F. graminearum* at laboratory level. To understand this, a set of antagonisms assays were carried out, in an increased complexity fashion, to determine if any of these bacteria is superior to the others in its ability to control *Fg-K1-4*. To achieve this aim, my specific objectives were as follows;

To perform dual culture assays of the fungus cocultured with the bacteria in a PDA/NA medium. To assess the potential antagonistic effect of the cell-free bacillus broths in the control of the *Fg-K1-4*. To learn the potential effect of enzymatic and heat treatment over the antagonistic effect of the *Bacillus* strains obtained in previous objectives. To learn if the mycotoxin DON has any toxic effect over the growth of the *Bacillus* strains. To perform statistical analysis using Two-way ANOVA when two different independent variables were present (e.g. bacterial treatments against different levels of dosages). To analyse the simple effect when an interaction effect is found or to analyse the mains effects when there were not interactions. Finally, to perform Tukey HSD test or post-hoc test when there is significant differences observed (Section 2.4.5)

Results

3.1 Selection and preparation of *Bacillus* strains for experiments.

3.1.1 Verification of isolates

Three different *Bacillus* strains were used, QST713, FZB24 and EU07. Stock culture of isolate EU07 were suspected to be contaminated as bacteria colonies were found to be in different colours, yellow and white. As it was not known which colonies were the right ones to carry out the experiments with, single colonies were selected, and antagonism assays were carried out with them. The results revealed that the bacteria derived from the yellow colonies did not show any antagonistic effect on the growth of the fungus. However, bacteria derived from the white colonies exhibited antagonistic effect on the fungal growth. The mixed culture was also included in the experiment and showed no antagonistic effect on the fungal growth (Figure 3.1).

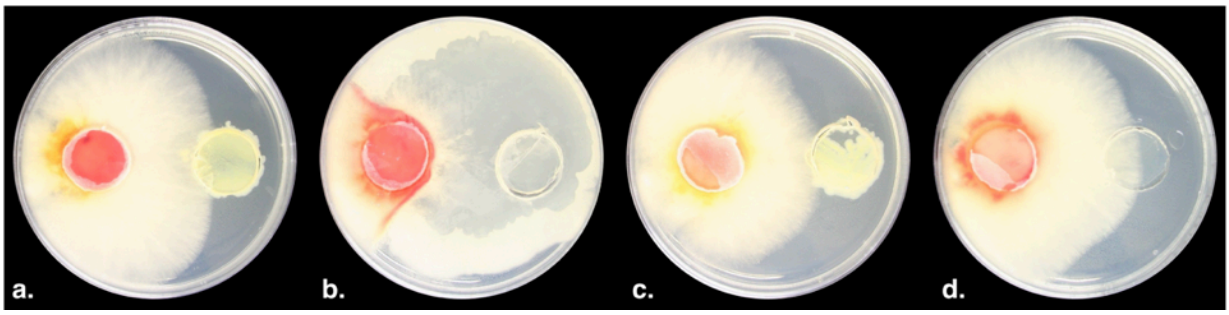


Figure 3.1 Verification of EU07 using antagonism assay.

As the bacterial culture showed mixed colony formations, single colonies were taken and antagonism assay were carried out. **(a)** The **yellow bacterium** did not show antagonism effect against the fungus, contrary, **(b)** the **white bacterium** stopped the growth of the fungus, but the **mixed culture** had no antagonistic effect **(c)**. **Control (d)** shows the growth of the fungus on the medium without any bacterium interaction.

3.1.2 Verification of Bacillus strains by sequencing

To determine whether all the bacterial strains isolated from the Serenade®, FZB24, and EU07 samples were *Bacillus* strains, colony-PCR was performed with several colonies from each strain by using universal 16S primers. PCR products were run in 1.5 % agarose gels and visualised under UV. A single band around 1500bp were obtained from each sample. Subsequently, several PCR products were cleaned, and samples were sent for Sanger sequencing (Figure 3.2).

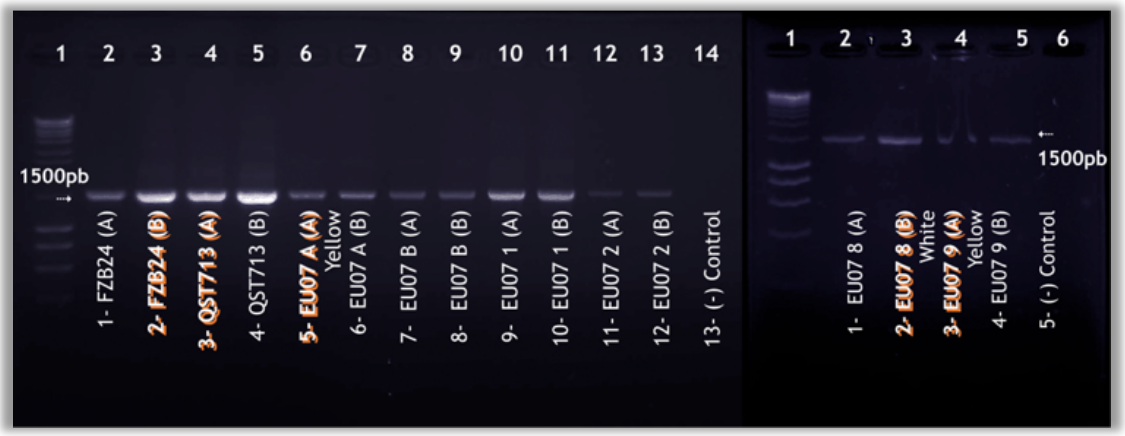


Figure 3.2 Agarose gels of the PCR products generated with the universal 16s primers.

The gel on the left shows 12 lines of putative *Bacillus* strains and the one on the right shows 4 putative *Bacillus* strains. The highlighted strains were sequenced (lanes 3, 4, 6 (left) and 3 and 4 (right).

The sequences were analysed using BLASTN and the results confirmed them to be in the *Bacillus subtilis* group. Single colonies were then selected from FZB24, QST713 and EU07, and were used throughout the subsequent experiments. Additionally, the yellow strain was identified as more closely to the endophytic Gram-positive bacterium *Microbacterium testaceum*.

3.2 Dual culture antagonism assays using *Bacillus* strains against *Fg-K1-4*

3.2.1 Antagonism assay of Bacillus strains against Fg-K1-4

The dual culture assay to determine the antagonistic effect on the *Fg-K1-4* was set up with the *Bacillus* strains QST713, FZB24 and EU07. All strains showed high inhibition of the fungal growth compared to control (Figure 3.3). The bacteria almost completely inhibited the growth of the fungus.

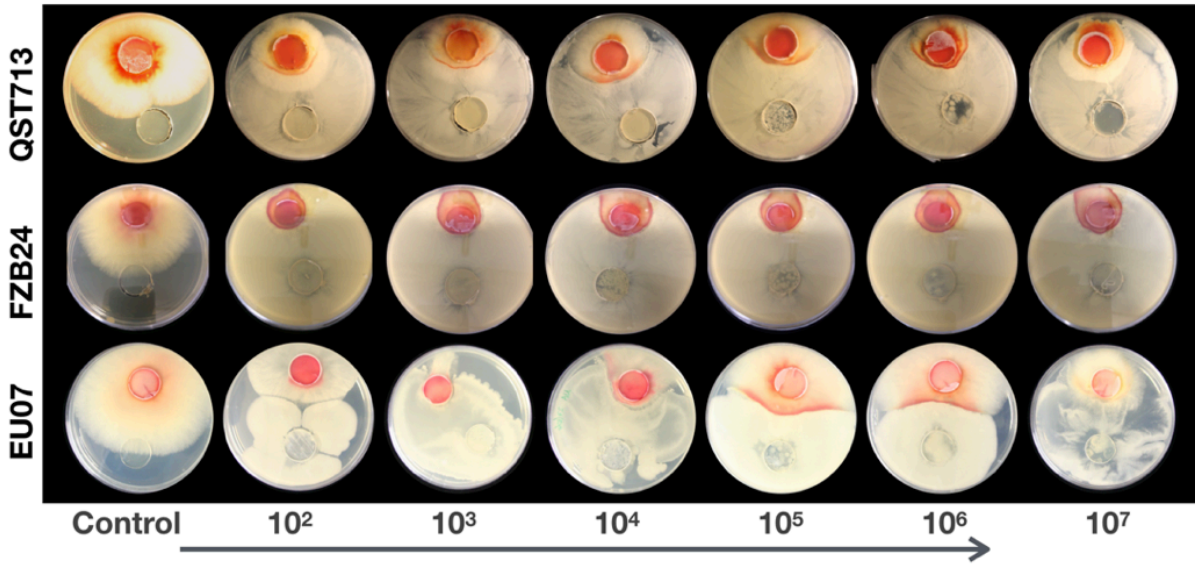


Figure 3.3 Determining effective dilution of Bacillus strains against Fg-K1-4.

From top to bottom, the rows represent the *Bacillus* strains assessed: QST713, FZB24 and EU07 (white plugs). From left to right, dilution series used: Control, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7. Dual culture experiment was set up on PDA/NA with *Fg-K1-4* (reddish plugs) and the picture was taken 4 days after incubation at 28˚C.

The percentage *Fg-K1-4* growth inhibition by the different *Bacillus* strains were in the range of 73.0 – 57.9%, 76.7 – 61.5% and 79.3 – 62.5% for QST713, FZB24 and EU07,

respectively. The highest percentages of inhibition were observed with 10^3, 10^4, 10^5 dilution series with values of 79.3 – 57.9%, 76.7 – 62.3% and 75.5 – 61.6%, respectively. A two-way ANOVA revealed that the interaction effect between *Bacillus* strains and treatments on % inhibition was not statistically significant (p = .054), but the main effects for dilutions was statistically significant (p = .018) where dilutions 10^2 and 10^4 were significantly different to 10^7. Additionally, the strains EU07 and FZB24 showed significantly higher % inhibition (p< .001) than that observed with the QST713 (Figure 3.4).

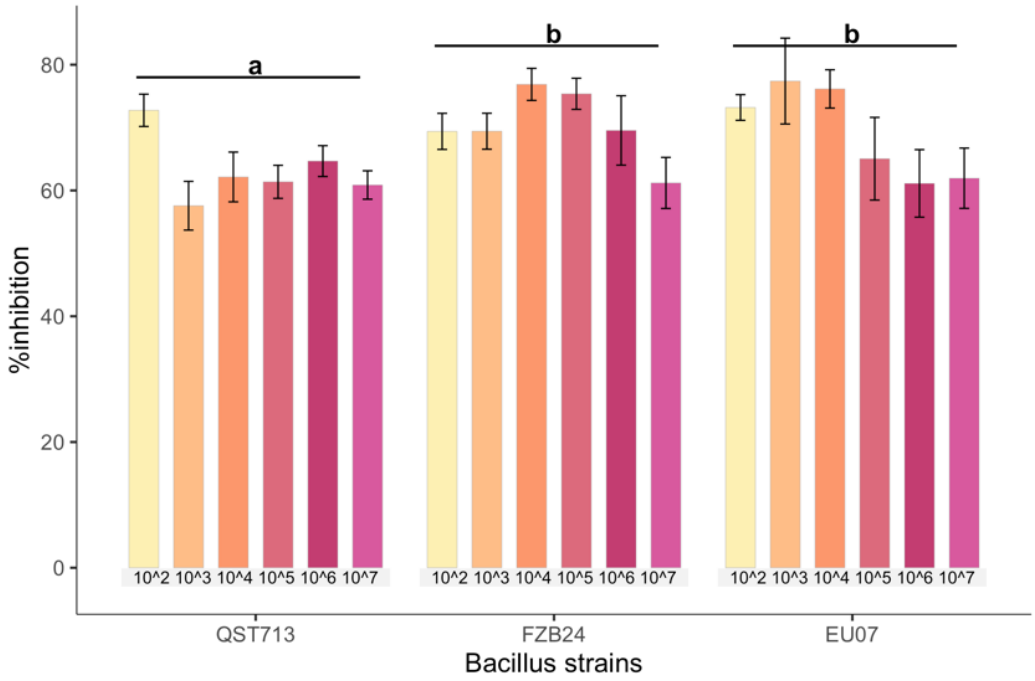


Figure 3.4 Percentage of growth inhibition of Fg-K1-4 by the Bacillus strains with different dilutions.

Bacterial broths of QST713, FZB24 and EU07 were used at different dilutions against *Fg-K1-4* showed a high inhibition with all the treatments (>57.9%). Data were from one independent experiment of four repeats and shown as the mean ± SE. Experiment was replicated at least three

times with similar results. Bars clusters with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following two-way ANOVA. n=72.

3.3 Antagonism assays using cell-free bacterial broths against Fg-K1-4

3.3.1 Determining optimal bacterial growth time for antagonistic activity

Bacterial broths were obtained at 6h, 24h, 48h and 72h after culture initiation from the *Bacillus* strains and filtered. Subsequently, the filtered broths were used to assess the inhibition effect on the *Fg-K1-4*. Results revealed that the treatment with broths from all the bacterial cultures that was tested at 6h did not have any antagonistic effect over the fungus (Figure 3.5).

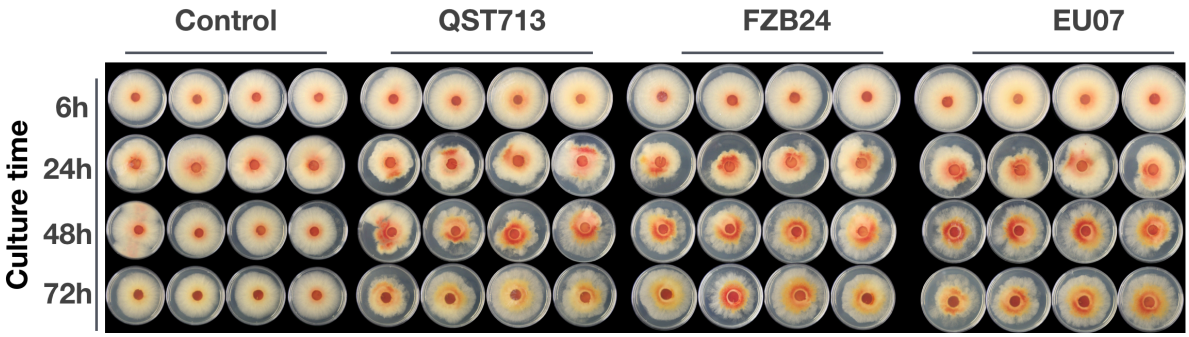


Figure 3.5 Fg-K1-4 growing on PDA/NA medium containing filtered bacterial broths.

The fungus was grown on PDA/NA medium in the presence of the 100µl filtered bacterial broths obtained from QST713, FZB24 and EU07 after 6, 24, 48 and 72h of culture.

The percentage of inhibition was calculated using equation (Equation 2.1) and data was analysed. All the treatments at 6h exhibited a very small or null antagonistic effect over the fungus (-2.7 – 0.5%). But filtrates of the bacteria at 24h and 72h had the higher levels

of inhibition with a range of 36.6 – 39.0 and 31.6 - 46.7% respectively and in average the broth from the strain FZB24 showed the highest % of inhibition (30.5%) (Figure 3.6).

The interaction effect between *Bacillus* strains and culture times on % inhibition was not statistically significant (p = .059), shown by a two-way ANOVA. Therefore, an analysis of the main effect for culture times was performed, which indicated that was statistically significant (p = .011).

The analysis of the main effect for the *Bacillus* strains showed statistically significant difference (p = .037). However, the pairwise comparison did not show significant differences between the *Bacillus* strains.

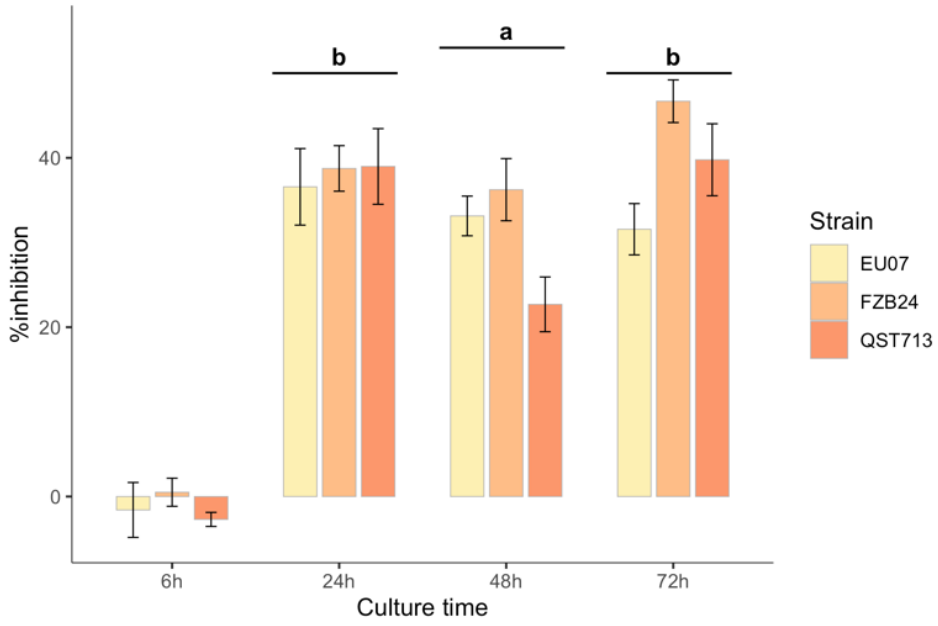


Figure 3.6 The percentage inhibition of the fungal growth by the filtered broth of Bacillus strains obtained at different times (6h, 24h, 48h and 72h).

Data were from one independent experiment of four repeats and shown as the mean ± SE. Experiment was replicated at least three times with similar results. Bars clusters with different

letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following two-way ANOVA. N=48.

Culture times displayed statistical differences, the 24h treatment was 7.4108% higher than that observed at 48h and showed statistically significant difference ($p < .046$). The 72h was 8.6525% higher than that observed at 48h ($p = .016$).

3.3.2 Antagonism assay using normalised bacterial broths

Furthermore, to determine whether the bacterial broths have a difference on their antagonistic effect against the fungus, total proteins were adjusted to 100 µg/ml. Similar to previous experiments, culture broths obtained at 24h, 48h and 72h were used (Figure 3.7).

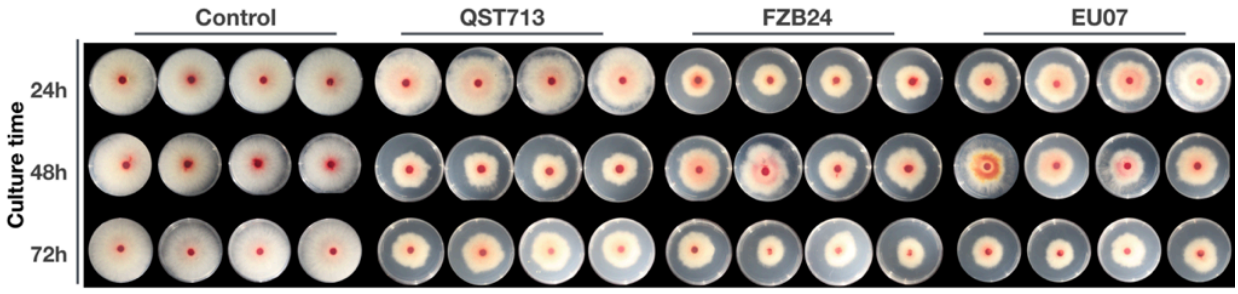


Figure 3.7 Growth inhibition of Fg-K1-4 by filtered bacterial broths that were adjusted to 100 µg/ml of total proteins.

Bacterial strains are shown at top, left to right and by culture time at left, top to bottom.

A closer inspection of the data showed that the ranges on growth inhibition of *Fg-K1-4* by culture time were 5.73 - 65.38% (24h), 58.33 - 70.07% (48h) and 69.28 - 75.60% (72h) by the strains QST713, FZB24 and EU07 respectively.

A two-way ANOVA (square root transformed) showed that there was a statistically significant interaction between *Bacillus* strains and culture times on % inhibition of the *Fg-K1-4*, ($p < .001$). There was a statistically significant difference in %inhibition of the *Fg-K1-4* by the *Bacillus* strains, with significance, for EU07 ($p < .005$), FZB24 ($p < .005$) and as for QST713 ($p < .005$). Cultures of the EU07 strain at 24h had a statistically significantly lower % inhibition of the *Fg-K1-4* than EU07 strain at 48h, 1.491 ($p < .001$), and 2.300 ($p < .001$) lower than EU07 72h (Figure 3.8).

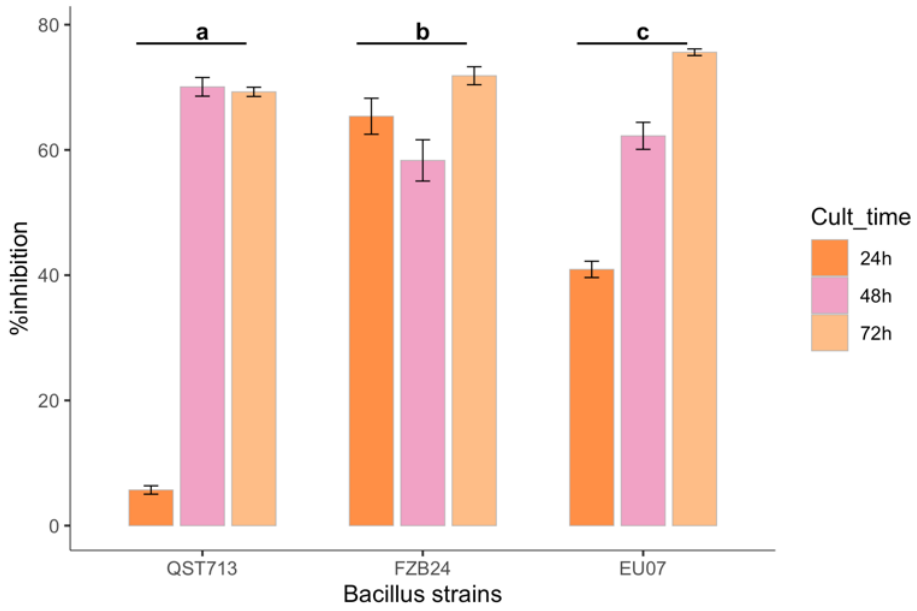


Figure 3.8 Percentage inhibition of the Fg-K1-4 by the Bacillus strains cultured at different times, filtered and adjusted.

Broths adjusted to 1.0 µg/mL of proteins from filtered broths cultured at different times (24, 48 and 72h). Data were from one independent experiment with four repeats and shown as the mean ± SE. Experiment was replicated three times with similar results. Bars clusters with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following two-way ANOVA. n=36.

3.4 Antagonism assays using adjusted and autoclaved bacterial broths

3.4.1 Antagonism assay using adjusted and autoclaved bacterial broths

After determining that the filtered-adjusted broths showed significantly statistical difference between the factors ($p < .001$) and found that the treatment 24h showed consistent fungal growth inhibition. The bacterial broths were subjected to heat treatment to understand whether the broths would be able to keep the inhibition effect. Broths were obtained from 24h bacterial cultures and adjusted to 100 µg/ml of total proteins, filtered and autoclaved. Assessments were then performed (Figure 3.9).

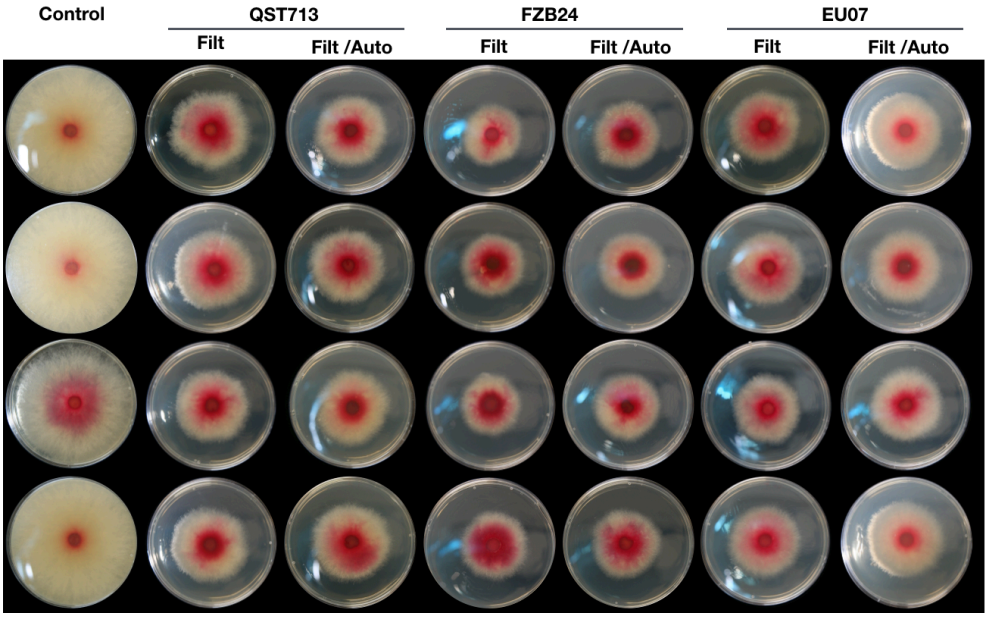


Figure 3.9 Fg-K1-4 growing on PDA/NA medium containing filtered unautoclaved or autoclaved bacterial broths.

Bacterial broths were obtained from 24h bacterial cultures, filtered, adjusted to 100 µg/ml and autoclaved. Bacterial broths are shown by bacterial treatment (top, left to right) and by type of treatment only filtered -Filt- and filtered/autoclaved -Filt/Auto- (top, left to right).

All the autoclaved *Bacillus* broths showed higher antagonistic effect in comparison to unautoclaved broths. The unautoclaved bacterial broths showed 53.9 – 73.4 %inhibition

of *Fg-K1-4* but the autoclaved bacterial broths showed an inhibition within the interval of 74.5 – 76.0 %.

A two-way ANOVA showed that there was a statistically significant difference between *Bacillus* strains and treatments on %inhibition of the *Fg-K1-4*, ($p < .001$).

There was also a statistically significant difference on %inhibition of the *Fg-K1-4* by the *Bacillus* strains either at FAB or FAB&B, for EU07 ($p < .005$) and for QST713 ($p < .005$). Additionally, there was a statistically significant difference %inhibition of the *Fg-K1-4* for FAB for all strains used, EU07, FZB24 or QST713, ($p < .0005$).

EU07 strain with FAB&A had a statistically significantly % inhibition of the Fg-K1-4 higher than EU07 with FAB, 21.935%, ($p < .001$). QST713 with FAB&A had a statistically significantly higher mean than that of QST713 with FAB, 12.785% ($p< .001$). There was no significant difference between the treatments of FAB and FAB&A for FZB24 (Figure 3.10).

3.4.2 Antagonism assays with broths adjusted to 0.5, 1.0 and 2.5 µg/ml and autoclaved

After determining that the *Bacillus* strains had an antagonistic effect (53 - 73%) against *Fg-K1-4* when heat treated, an antagonism assay were performed to evaluate different concentrations of proteins in bacterial broths (Figure 3.11).

Interestingly, the treatment with 1.0 mg/ml had a similar antagonistic effect (67.58 – 81.45%) to 2.5% mg/ml (67.79 – 75.41%) for all the *Bacillus* strains. Although, the strain QST713 showed a very low antagonistic effect at 0.5 mg/ml with 17.18% followed by

EU07 with 60.59%. Notably, a high variability of the data was observed with the treatment using 2.5% mg/ml broths.

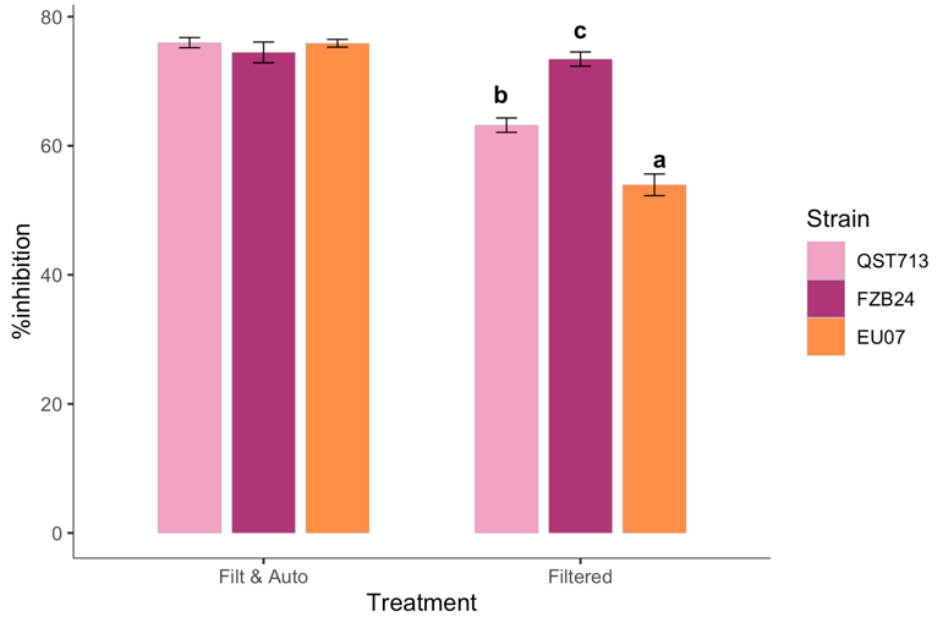


Figure 3.10 Percentage inhibition of the Fg-K1-4 by Bacillus broths that were filtered, adjusted and autoclaved.

Antagonistic effect of the *Bacillus* strains (QST713, FZB24 and EU07) against *Fg-K1-4* in % inhibition and broths adjusted (1.0 µg/mL). In general, the autoclaved treatments displayed a higher % inhibition with a little variability within *Bacillus* strains. Data were from one independent experiment of four repeats and shown as the mean ± SE. Experiments were replicated twice with similar results. Bars with different letters indicate significant difference according to Tukey's Test ($\alpha < 0.05$) following two-way ANOVA. n= 24.

The two-way ANOVA showed that there was a statistically significant difference between *Bacillus* strains on the %inhibition, ($p < .0001$). There was a statistically significant difference in %inhibition of the *Fg-K1-4* by *Bacillus* strains EU07 ($p < .0005$), as for QST713 ($p < .0005$) and for FZB24 ($p = .021$) at either 0.5, 1.0 and 2.5 mg/ml.

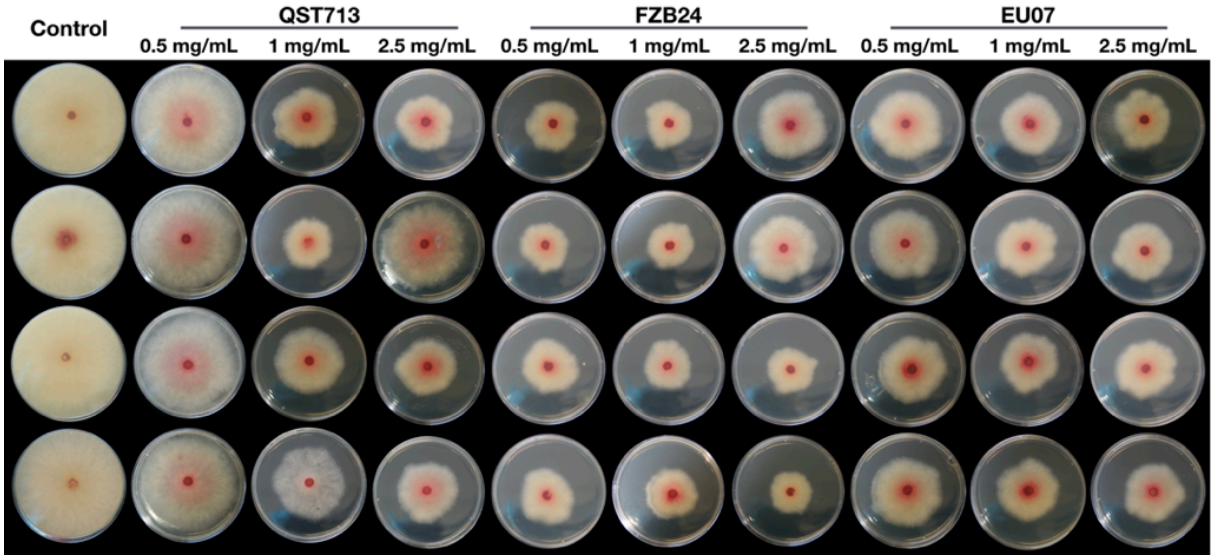


Figure 3.11 Fg-K1-4 growing on PDA/NA medium containing filtered bacterial broths adjusted to 0.5, 1.0 and 2.5 mg/ml of total proteins in medium.

Bacterial broths were obtained from 24h bacterial cultures, filtered, adjusted and autoclaved. Bacterial broths are shown by bacterial treatment (top, left to right) and by dosage (top, left to right). In general, autoclaved bacterial broths displayed low fungal growth compared with the control treatment (left column).

There was a statistically significant difference in % inhibition of the *Fg-K1-4* by dosage 0.5 mg/ml at whichever *Bacillus* strain ($p < .0005$) used and for treatments 1.0 mg/ml for any *Bacillus* strains ($p < .0005$). There was no statistically significant difference in % inhibition of the *Fg-K1-4* by treatments 2.5 mg/ml at any *Bacillus* strain ($p < .005$) (Figure 3.12).

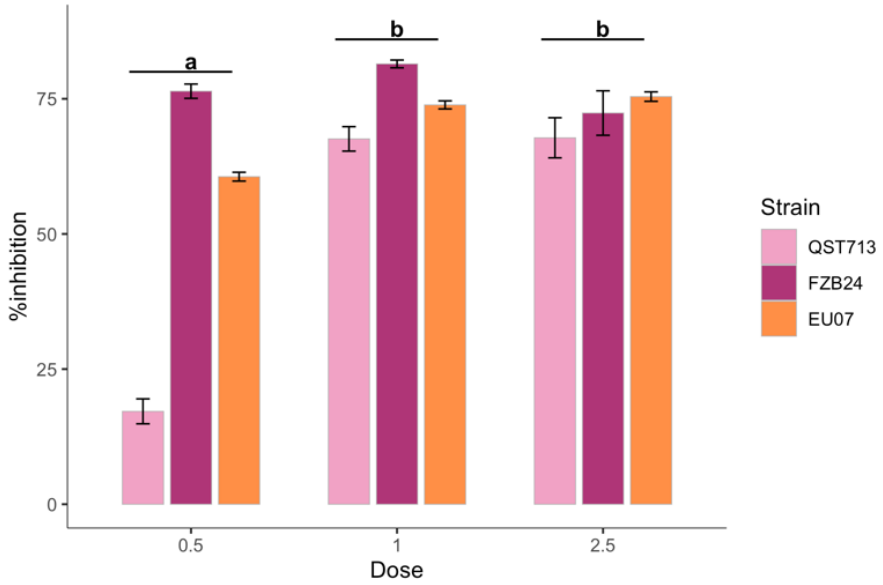


Figure 3.12 Percentage inhibition of Fg-K1-4 by the Bacillus broths, which were filtered and adjusted to 0.5, 1.0 and 2.5 mg/ml of total protein in medium.

Data were from one independent experiment of eight repeats and shown as the mean ± SE. Experiments were replicated twice, and the similar results were obtained. Bars clusters with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following two-way ANOVA. n=96.

3.4.3 Antagonism assay of bacterial broths filtrated, autoclaved and adjusted at high concentrations of total proteins (10 and 20 mg/ml) against the Fg-K1-4

Similar to above experiments, an antagonism assay was performed to evaluate if high concentrations of proteins would completely prevent the fungal growth (Figure 3.13).

The two-way ANOVA showed that there was a statistically significant difference between *Bacillus* strains and dosages (10 and 20 mg/ml) on %inhibition of the *Fg-K1-4* (p = .024).

There was a statistically significant difference in %inhibition by *Bacillus* strain EU07 either at 10 or 20 mg/ml (p

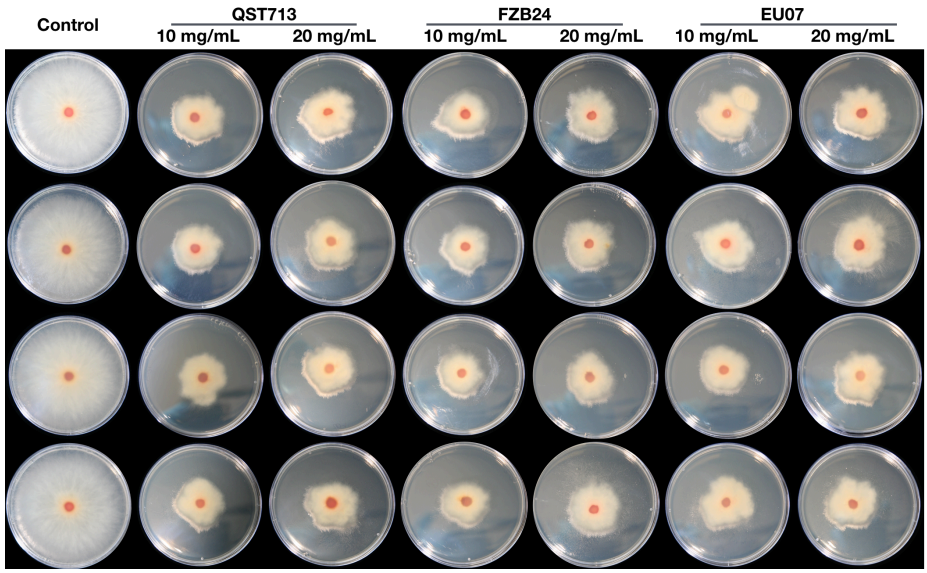


Figure 3.13 Fg-K1-4 growing on PDA/NA medium treated with bacterial broths adjusted to 10.0 and 20.0 mg/ml proteins in total medium.

Bacterial broths were obtained from 24h bacterial cultures, filtered, adjusted and autoclaved. Bacterial broths are shown by bacterial treatment (top, left to right) and by dosage (top, left to right). Hight dosages of bacterial broths displayed low fungal growth compared with the control treatment (left column).

< .009). No other statistically significant difference for the *Bacillus* strains was found.

There was a statistically significant difference in %inhibition of *Fg-K1-4* for 20 mg/ml on EU07, FZB24 or QST713 (p = .005). No other statistically significant difference in % inhibition of the *Fg-K1-4* was found on treatments.

The Tukey-HSD showed that EU07 strain with 10 mg/ml had a statistically significantly %inhibition higher than EU07 strain with 20 mg/ml, 2.457% (p < .001). FZB24 strain and QST713 strain did not showed significant differences between the treatments 10 mg/ml and 20 mg/ml.

Treatment with 20 mg/ml of FZB24 had a statistically significantly higher %inhibition than that observed with 20 mg/ml at EU07, 3.030% (p = .005). Treatment 10 mg/ml at any *Bacillus* strain did not show any significant differences in the antagonistic effect.

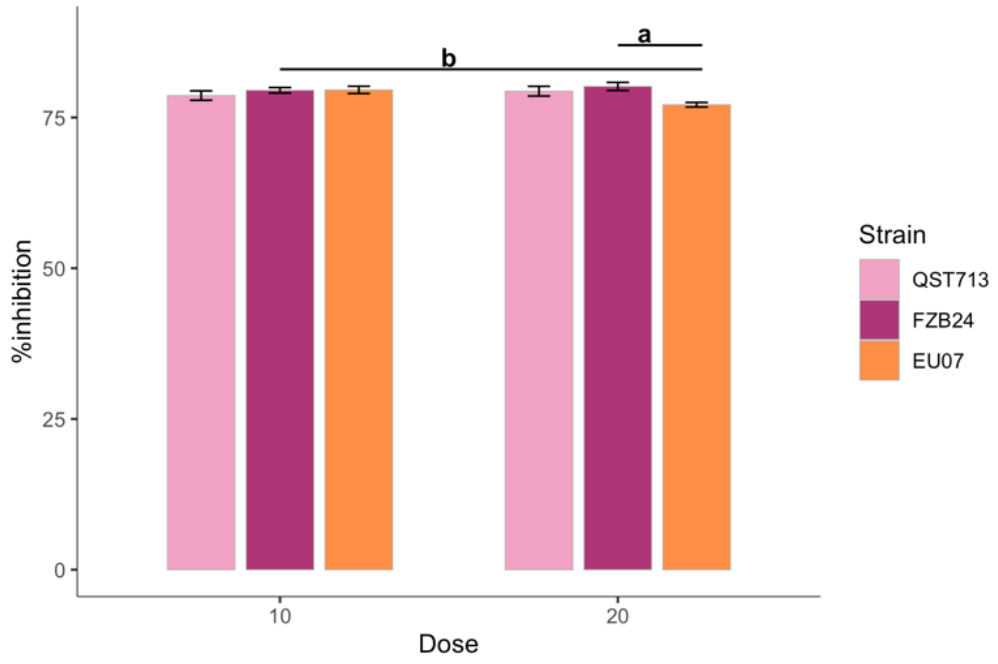


Figure 3.14 Percentage inhibition of the Fg-K1-4 by the broths of Bacillus strains (24h), adjusted to 10.0 and 20.0 mg/ml of total protein in the media.

Antagonistic effect of the *Bacillus* strains (QST713, FZB24 and EU07) against *Fg-K1-4* in % of inhibition using 10.0 and 20.0 mg/mL of total proteins from cultured broths (Filtered and autoclaved). Data were from one independent experiment of eight repeats and shown as the mean ± SE. Experiments were replicated twice, and the similar results were obtained. Bars with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following two-way ANOVA. n=48.

3.5 Antagonism assay of proteinase K treated bacterial broths against the *Fg-K1-4*

After determining that even the low concentrations of proteins have a sufficient antagonistic effect over the fungal growth (Section 3.4), it was decided to test whether

the bacterial broths kept their stability in the presence of proteinase K, which is known to degrade proteins within the given environment (Figure 3.15).

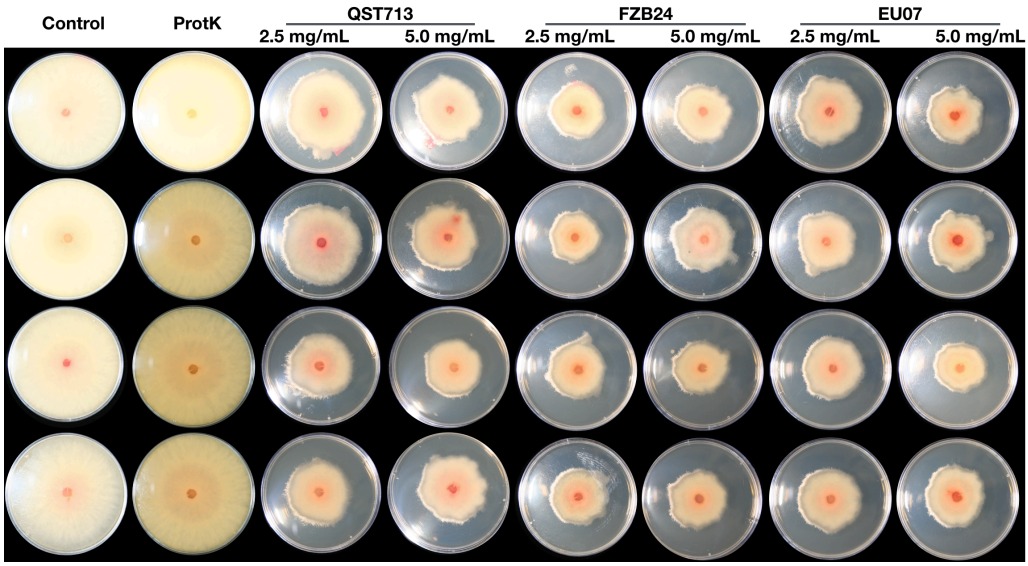


Figure 3.15 Fg-K1-4 growing on PDA/NA medium containing filtered, adjusted and proteinase K treated bacterial broths.

Bacterial broths were obtained from 24h bacterial cultures, filtered, adjusted and autoclaved. Proteinase K was used under average conditions. Bacterial broths are shown by treatment (top, left to right) and by dosage (top bottom, left to right). Control treatment (left column) and Proteinase K treatment display full fungal growth.

The assay showed that the average of the % inhibition by the treatment 2.5 mg /ml and 5.0 mg/ml was 67.89% and 71.94%, respectively. The QST713 strain showed more variability on the % inhibition for both treatments.

The interaction effect between *Bacillus* strains and dosages (2.5 mg/ml and 5.0 mg /ml of proteins) on %inhibition was not statistically significant, (p = .127). Thus, an analysis of the for the main effect, *Bacillus* strains only, was performed, which indicated that there was a statistically significant difference (p = .003).

The *Bacillus* strains EU07 treatment was statistically significant different (p = .020) than QST713 (8.357 higher %inhibition). The FZB24 treatment was higher than QST713 by 10.573 %inhibition (p = .003) (Figure 3.16).

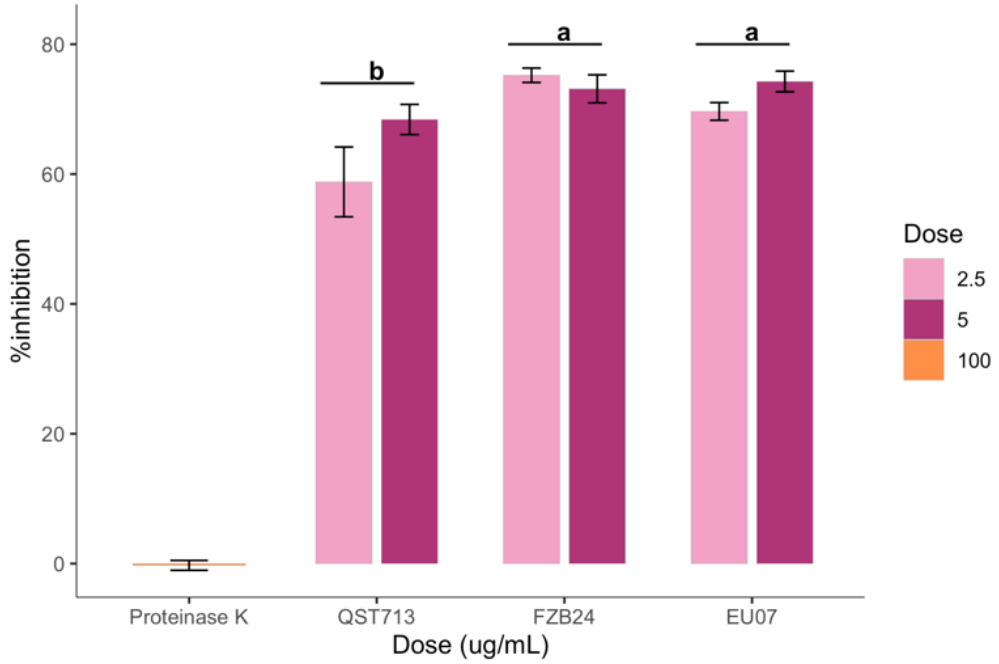


Figure 3.16 Percentage of inhibition of Fg-K1-4 by the broths of Bacillus strains (24h), adjusted to 2.5 and 5.5 µg/ml of total medium and treated with proteinase K.

Antagonistic effect of the bacterial broths of QST713, FZB24 and EU07 against *Fg-K1-4* in %inhibition, filtered, autoclaved and proteinase K treated (100 µg/mL). Data were from one independent experiment of four repeats and shown as the mean ± SE. Experiments were replicated two times and similar results were obtained. Bars clusters with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following two-way ANOVA. n=28.

3.6 Optimizing the experiment conditions for DON assays: inoculum amount and acetonitrile resistance

The mycotoxin DON or vomitoxin, is a compound that is highly soluble in acetonitrile. Because of this, an initial experiment was carried out to determine the effect of the

acetonitrile (Ac) on the growth of the *Bacillus* strains. Additionally, it was determined whether the amount of inoculum has any effect on the final concentration of bacteria.

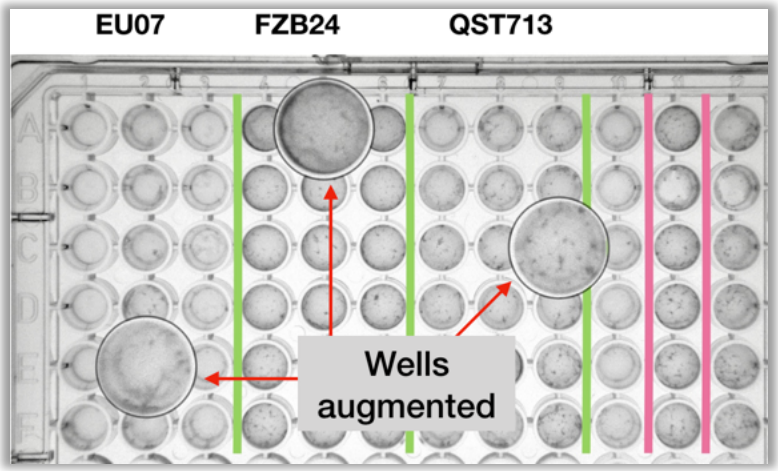


Figure 3.17 Growth of Bacillus strains in 96 wells plates after 24h incubation (28˚C, ~150rpm).

Bacillus strains (QST713, FZB24 and EU07) were grown in 96-wells plate with LB broth for the period of 24h. The three *Bacillus* strains showed biofilm formation on the bottom of the wells (augmented wells). Either, using 1 or 5 µl of inoculum produced measurable bacterial growth.

The results showed that *Bacillus* strains can grow in 96-well plates after inoculation, either, with 1 or 5 µl of bacterial broth (24h old) with a visible biofilm. Likewise, the bacteria can grow on LB medium where Ac was added in the concentrations tested in this study (Figure 3.17). However, there was a significance difference in the growth of the bacteria -as net absorbance (600nm) after 24h at 28˚C- FZB24 in comparison of the others two ($F(2,68) = 7.064$, $p = 0.002$) according the ANOVA test.

3.7 Toxicity of the DON on bacterial strains

DON assays were performed to determine the viability of the bacteria on different concentrations of this mycotoxin (0, 5, 15, 25, 50 and 70 ppm).

While the data did not meet the normality test (Shapiro p>0.05), homogeneity test (Levene's tes of equality p=0.002) and presented outliners (one outliner on EU07 and one in QST713 data), nonetheless ANOVA (two-way) is robust enough to tolerate this, therefore it was decided to carry out this analysis on regardless, because the growth of microorganisms sometimes can vary under controlled conditions.

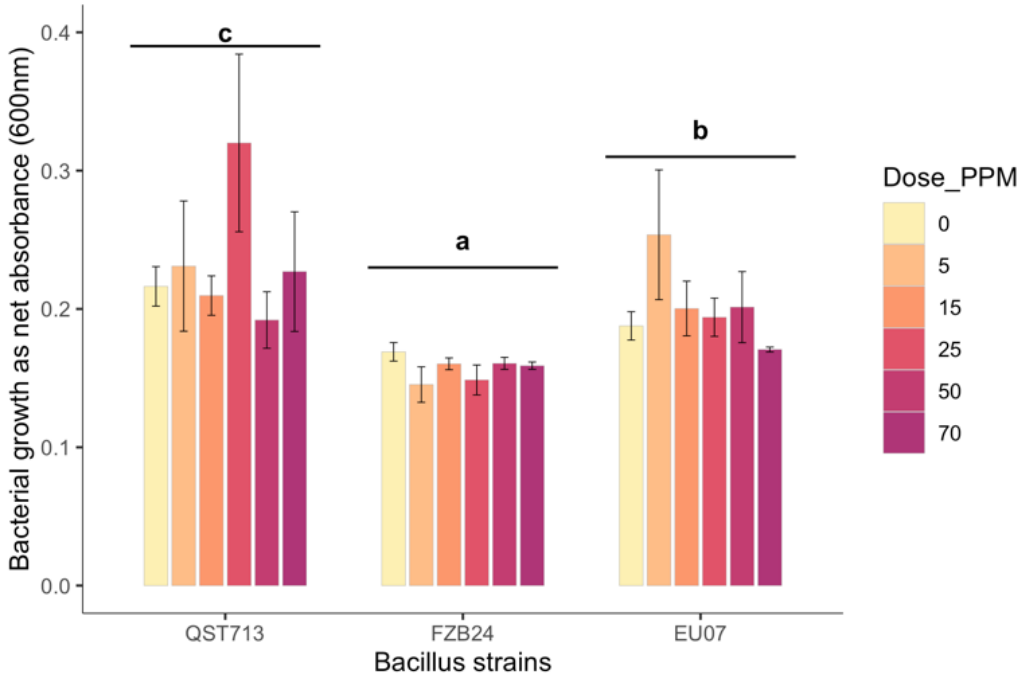


Figure 3.18 Growth of Bacillus strains in the presence of different DON concentrations.

Bacillus strains (QST713, FZB24 and EU07) were grown in the presence of DON at different concentrations (0, 5, 15, 25, 50 and 70 ppm) in LB broth, 24h, 200 rpm in 96-wells plates. Data were from one independent experiment of at least three repeats and shown as the mean ± SE. Experiments were replicated three times and similar results were obtained. Bars clusters with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following two-way ANOVA. n=72.

Firstly, the interaction analysis was made. There was no statistically significant difference between Bacillus strains and DON (dosage) over the bacterial growth (as absorbance), $F(10, 54) = 1.893$, $p = .066$, partial $\eta 2 = .260$. Therefore, the main effects were analysed, showing that there was a statistically significant difference in the bacterial growth within Bacillus strains, $F(2, 54) = 15.565$, $p < .0005$, partial $\eta 2 = .366$. Besides, there was no statistically significant difference between bacterial growth within the concentrations of DON used, $F(5, 54) = 1.115$, $p = .363$, partial $\eta 2 = .094$ (Figure 3.18).

Discussion

Antagonism assays

Antagonism assays were carried out to evaluate the effect of the *Bacillus* strains against the *Fg-K1-4*. They were assessed in their level of percentage fungal inhibition. The set of the experiments were planned to reveal the best bacterial strain under different conditions of experiments. As a result, globally, the findings support that, the three *Bacillus* strains possess antagonistic effect against *Fg-K1-4*, in *in vitro* assays. The strains EU07 and FZB24 showed the highest percentage inhibition against the *Fg-K1-4*, but EU07 was more consistent in suppression than that observed with the other two strains.

Firstly, the dual culture assay showed that, all the strains have a rapid growth on medium used (PDA/NA). After 24h, the bacteria spread over the plates and were not stopped by the presence of the fungus (Figure 3.3). Similar results were found with a *Pseudomonas* strain on Waksman's agar (Hu et al., 2010). Besides, previous studies showed that,

Bacillus strains are suitable to test on dual cultures against fungi on PDA media. Those assays were carried out for 4 or 5 days at 28˚C and the typical inhibition zone and low bacterial growth were observed (Baysal et al., 2013; Y. Zhao et al., 2014). Surprisingly, here it was found that the PDA/NA medium may provide suitable conditions for the growth of both microorganisms, thus the bacteria covered completely the surface of the medium before the mycelia of *Fg-K1-4* did.

Moreover, due to the pattern of growth of the *Bacillus* strains on PDA/NA media, it was not found the typical inhibition zone. Then, it was established a practical equation relating the growth of the fungal growth under treatment over the fungal growth of the control and ImageJ was used to accurately measure the area of the fungus growth to reflect the precise results on the assays (Swain and Ray, 2009) producing quantitative data.

Afterwards, in the dual culture assay it was decided to use several concentrations of the bacteria to look for any difference. The statistical analysis showed that, in general, at the concentration of 10^3 and 10^4, the *Bacillus* strains showed the highest percentage inhibition. Also, results showed around of 80.0% inhibition of the fungal growth. This is similar or superior to those reported in other similar studies (Castañeda-Alvarez and Sánchez, 2016; Grosu et al., 2014; Y. Zhao et al., 2014). In contrast, Zhao *et al.* 2014 found 72% inhibition at 10^4 and 88 %inhibition at 10^8 concentration of the bacterium against *Fg* with the strain *B. subtilis* SG6. For our experiment, the strains EU07 showed the best results (up to 79.3%) at 10^4, with some variability (Figure 3.4).

These results led me to assess the antagonistic effect using only the cell-free bacterial broths against the *Fg* (Baysal et al., 2013; Castañeda-Alvarez and Sánchez, 2016; Chan et al., 2009; S.-B. Li et al., 2012; Y. Zhao et al., 2014).

This assay, in general, only displayed the half of the effectiveness obtained on the assays with the whole bacterial broth. This mean that the dosage of antagonistic molecules obtained in the bacterial sample and applied to the medium, was not enough to obtain a high antagonistic effect (Figure 3.6).

Sampling the bacterial broths at 6h did not yield any antagonistic effect (Figure 3.5-6). This could be explained due to the growth rate of the *Bacillus* strains. Bernat et al., (2016), found that the growth curve of the *Bacillus subtilis* reaches the exponential phase after 18-24h or 30h (according with the strain used) in LB broth and afterwards it reached the stationary phase. Likewise, it was found that the cultures at 6h yielded a very low or almost negligible number of total proteins. Additionally, the visible production of biofilm starts in the first 18-24hrs (data not shown). In contrast, it was found that when minimal media was used, it accelerated the time in which the stationary phase and production of spores were reached (Castañeda-Alvarez and Sánchez, 2016).

The samples collected at 72h exhibited higher antagonistic effect, probably due to the consequence of the accumulation of antagonistic compounds on media, but the samples at 24h, showed consistent and similar antagonistic effect toward the treatment at 72h. In studies with extraction of iturin A, lipeptides and fengycins using High

Performance Liquid Chromatography (HPLC) method (Bernat et al., 2016; Deleu et al., 2008), it was reported that the compounds could be detected from cultures up to 72h.

Thus, the abovementioned assay prompted us to use the bacterial broths with adjusted concentration of proteins (Figure 3.7-8). Surprisingly, adjusting the protein concentration exhibited higher percentage inhibition per strain than the previous assay and similar to the dual culture assay (Figure 3.3-4).

In this case, the strain EU07 as well the FZB24 showed the best results. The strain FZB24 was superior to the EU07 in their antagonistic effect against the *Fg-K1-4* when the concentration of proteins was adjusted and sampled at 24h. The values, percentage inhibition, were very variable for the treatment 24h, and this could be due to experimental error, but the 48h and 72h were less variable and with the highest percentage inhibition, in the range of 60-70%, against the *Fg-K1-4*.

It is clear that, the antagonistic effect observed is due to molecules delivered by the *Bacillus* strains to the medium, and not only due to the rapid growth of the bacterial cells. Notably, the secretome of the *Bacillus subtilis* strain 168 has been studied extensively for its ability to produce many compounds that are beneficial for industry (Antelmann, 2001; Baysal et al., 2013; Kunst et al., 1997; Stein, 2005; Tjalsma et al., 2000). It is believed that the *Bacillus* strains used here possess a notable secretome able to produce antagonistic compounds for the control of *Fg-K1-4* similar to the *Bacillus subtilis*.

Alongside, it was found that the antagonistic compounds are thermostable and stable from proteinase K [a serine protease]. Furthermore, the treatments with heat and

proteinase K do not decrease the activity of the *Bacillus* strains but even the antagonistic effect against *Fg-K1-4*.

Firstly, to test the hypothesis that the antagonistic compounds could be proteinaceous and to determine their stability on different conditions, the broths were assessed after treating with -FAB&A- and without autoclaving -FAB- (Figure 3.10).

It must be considered that, the percentage inhibition obtained with lower concentration as low as 1.0 mg/ml were similar to those obtained with higher concentration as much as 20.0 mg/ml. This result may be due to the fact that in the assays, the whole bacterial broths were used. These broths probably contained several compounds as these were non-purified solutions. In studies where compounds were purified, the minimal inhibitory concentration were in the range of 50 to 128 µg/ml for iturin A and fengycin A (Gong et al., 2015; S.-B. Li et al., 2012; Zhao et al., 2013) . Perhaps, it is necessary higher volumes of whole bacterial broths to inhibit completely the mycelial growth on *Fg*.

Therefore, it was found that the compounds in the bacterial broths, probably need to be purified and their effect on *Fg* can be explored. Technologies such as liquid chromatography–mass spectrometry couple (LC-MC), could be used to separate and identified compounds from the treated bacterial broths (Chaimbault, 2014; Jorge et al., 2016; Sudhakar et al., 2016).

Subsequently, it was found that the antagonistic effect of the *Bacillus* strains was due to non-proteinaceous compounds, because the bacterial broths showed remarkable stability under heat conditions, which leads to thinking that the antagonistic effect is due

to bacterial metabolites and not for the action of proteins released by the bacterial cells to the medium.

Later, a further assay was made to assess the bacterial broths using proteinase K, a broad-spectrum serine protease (Jany et al., 1986). The results indicated that the antagonistic effect was high and was conserved for all the bacterial broths (Figure 3.15). *Fg-K1-4* treated only with proteinase K did not show any difference on the antagonistic effect with the control treatment. Hence, the proteinase K is not a compound capable to stop the fungal growth (Figure 3.16). The effect due to the *Bacillus* strains was significant. EU07 and FZB24 showed the best results, being better the FZB24 but not statistically different within them.

Interestingly, others (Hammami et al., 2009; Hu et al., 2010; Meng et al., 2012) have used heat treatment and proteinase treatments on *Bacillus* extracts - Bacteriocin, Protein LCI gene, LCI protein respectively- to assess their antagonistic effect. But, their efforts were negative or null, when proteinases and autoclaving conditions were used. In this project, improved antagonistic effect was obtained after treat bacterial broths with similar conditions. It is possible, that the conserved antagonistic effect is due to compounds that are degraded exposing active sites of antimicrobial molecules, which lead to the release of a more potent compound in the media after the treatments.

DON assays

All those results gave an excellent inside to determine how different or not were the phenotype of those bacteria. Nonetheless, it remained the question about the resilience

of the bacteria under the contact with the mycotoxin DON. Some suggest that this compound, secreted by *Fusarium* sp on FHB disease, has a crucial protagonist role in the development of the infection (Beccari et al., 2018). Few studies explore the toxicity of the DON on microbiota and the efforts are more directed to the detoxification of this mycotoxin using bacteria. The approaches are highly variable, ranging from detoxification of the feed, detoxification on the guts of the animals and detoxification of exposed soils. Many efforts are done to understand how detoxify food contaminated with high concentrations of DON (Awad et al., 2010; Karlovsky, 1999; Madhyastha et al., 1994; McCormick, 2013; Venkatesh and Keller, 2019).

Nonetheless here, in this study, were more of interest to determine if the three *Bacillus* strains could survive high levels of toxicity of the DON. Cultures of the *Bacillus* were made with several levels of toxins (0 to 70 ppm) and it was found that the *Bacillus* strains displayed growth in presence of the solvent of the toxin alone -acentonitrile- (Figure 3.17) and in presence of the toxin (Figure 3.18) with production of biofilm after 24h. These results are remarkable due to the consideration that Tian et al (2016) reported that the *Fg* strain 5035 produced 57 µg/g of DON and by legislation, in EU, the maximum level of DON accepted in feed products are 2 ppm (2000 µg/kg) (Gruber-Dorninger et al., 2019). Besides QST713 and EU07 strains displayed the best growth rate (net absorbance, 600nm).

Overall, investigation on antagonism assays indicated that the available *Bacillus* strains can be used to control *Fg in vitro* and gave a strong support to carry out the subsequent *in vivo* assays.

Chapter 4

In vivo assays: *Arabidopsis* and *Brachypodium*

Introduction

Dissimilarly to pathogenic bacteria, beneficial plant-associated bacteria are established in the rhizosphere, rhizoplane or roots (outside, surface or inside roots). All together, they form only 1-2% of the total bacteria present in roots of plants. These bacteria are also known as plant growth promoting rhizobacteria (PGPR).

PGP rhizobacteria produce metabolites which contribute with its activity, such as siderophores or iron sequestering compounds and bacteriocins. These compounds are effective against closely related bacteria to PGPR and usually they are antibiotics with a wider mode of action that make possible the colonization of roots by the PGPR.

Other mechanisms used by those bacteria include nitrogen fixation, auxin production, regulation of plant ethylene levels, and others (Beneduzi et al., 2012; Compant et al., 2010; Gaiero et al., 2013; Glick, 2012; Glick et al., 1999).

Recent studies determined that it is possible that rhizobacteria suppress the host immuno-response and likewise the host suppress the nutrient supply to maintain a balanced relationship (Bezrutczyk et al., 2018; Cao et al., 2017). Likewise, many

studies show that bacteria isolated from the phytosphere, such as leaves, inflorescences and other parts of the plant, contribute to the regulation of the microbiome in favor of the plant health. Hence, several *Bacillus* strains are incrementally included in studies for give full name (BCAs) prospection due to its capability of produce antibiotics and antifungal metabolites (Amin et al., 2015; Antelmann et al., 2001; Baysal et al., 2013; Berendsen et al., 2018; Borriss et al., 2018; Liang Chen et al., 2018; Das and Meena, 2018; Hu et al., 2010; Kong et al., 2018; Li et al., 2012; Luo et al., 2015; Mendis et al., 2018; Pandin et al., 2018; Perez et al., 2018; Pryor et al., 2007; Xiong et al., 2015). Remarkably, those bacteria have been reported to have PGP activity. For example the commercial strain QST713, which has been around in the market for more than 20 year, is reported to have microbial activity, induction of plant defense and growth promoting activity (Mendis et al., 2018; Pandin et al., 2018; Royalty et al., 2017).

Similarly, a wealth of knowledge has been produced to address the question about the regulation effects operated by these microorganisms inside the plants. The response of the plants to the environment and the attack of pathogens induces a cascade of events in the genetic and molecular machinery of the cells. Thought as a genetic trait, plants contain genes which encode signal for plant resistance (R) while pathogen has avirulence (*avr*) proteins. Early studies concluded that defense related genes were activated by elicitors and perceived by defense genes in a variety of ways. Some genes studied under this premise included *EDS*, *NDR1*, *PR1*.

PR1 proteins are delivered to the extracellular space of leaves in response to pathogen attack or chemical treatment. Their proteins act as the first recognition mechanism in the cell provoking a cascade of reactions to deter any pathogenic attack (Aarts et al., 1998; Dixon et al., 1991; Somssich et al., 1989; Tör et al., 2003). Additionally, plant hormones play an important role in the defense of the plant. Salicylic acid (SA), Jamonic acid (JA) and ethylene (ET) are known to be part of the plant response as resistance signaling. SA and EDS1 are related to PR proteins response, ultimately called systemic acquired resistance (SAR), which has a long term effect on plant defence (Durrant and Dong, 2004; Ferrari et al., 2003; Venugopal et al., 2009). Making use of this trait, some researchers looked into the use of similar substances to produce priming in plants for defence against pathogens. Others proposed that this defence response possesses a cost for the fitness on plants, balancing the cost of growth against the defence of the plant. Hence, a question then arises as to whether pathogens affect signaling pathways to improve their efficiency in host colonization. Studies on ABA, SA and auxin plant hormone pathways were investigated to get understanding in this relationship (Denancé et al., 2013; Eshraghi et al., 2011; Hückelhoven et al., 2013). Differently, there is the ethylene response factor (ERF), a family of 122 members, which bind the GCC box (AGCCGCC), which is found in many promoters of *R*-genes induced by JA/ethylene and found related to environmental stress response (Bacete et al., 2018). This denote what is known as the signaling pathways (JA/ET and SA), and

they seem to have an antagonistic interaction (Moffat et al., 2012). Overall, this response is consistent in vascular plants and widely found in model plants such as the tale cress plant *A. thaliana* and the well-studied *Nicotiana benthamiana*. Few studies focused in grasses, such as *Brachypodium distachyon* plant as a model organism (Hussain et al., 2018). Kouzai et al. (2016) demonstrated that apparently, *PR1* genes act similarly between *B. distachyon* and rice however, that similarity does not seem to exist between them and *A. thaliana* plants, hence many questions remain to be answered (Rodrigues et al., 2017). Equally, many other unknown questions exist about the mechanisms in which plant-beneficial bacteria can modulate the immune-response in plants, the nutrient acquisition and the growth on plants and the response of those plants to the PGP bacteria.

Resuming to the point of model plants, *Arabidopsis thaliana*, a cress plant, is the most important plant model organism for most of the genetic studies. Its invaluable research background adds a wealth of breadth to understand experiments in the context of the new research questions about plant pathogen and molecular interactions. Likewise, more recently, *Brachypodium distachyon*, a small wild grass plant, has been taken into the experimentation field when, 'cereal crops' research needs to be done. This is a quick growing plant that can be vernalised to induce floriation structures instead of leaf growth, requires less volume on growth rooms and its growth temperature is manageable (22˚C) (Hussain et al., 2018; Kouzai et al., 2016). Moreover, the Bd-21 line was sequenced and is widely used in cereal

crops studies. Finally, both, *B. distachyon* and *A. thaliana*, are susceptible to the infection of the *F. graminearum* ("Brachypodium Resources," n.d.; Bragg et al., 2015; Rana et al., 2018; Scholthof et al., 2018).

Hence, the main aim of this chapter is to evaluate the *Bacillus* strains in their effectiveness to control the effects of the *F. graminearum in vivo*. A second aim is to evaluate the immune-response of plants to get insides in the interaction between beneficial-bacteria and plants.

Arabidopsis thaliana plants were infected with the obligate oomycete *Hyaloperonospora arabidopsidis* (formerly *Peronospora parasitica*, *Hpa*) strain Emoy2, and the spore count was recorded. Firstly, Col-0 (wild type) plants -resistant to the *Hpa* but not in in young seedlings- were used. Subsequently, Ws-*eds1* *Arabidopsis* plants -were used. Their phenotype reported heavy sporulation on *Hpa* infection which is not reported in the wild type Ws-0 or in Col-0 due to susceptibility to the *Hpa* infection and the suppression of the resistance to *Hpa* has been reported (Coates and Beynon, 2010; Parker et al., 1996).

To assess the effect of bacteria on the development of the plant or PGP activity PGP assays were carried out. General fitness of the plant, development of the floral structures and other phenological characteristics were recorded. Later, similar PGP experiments were carried out using *Brachypodium* to determine if this effect was conserved between species.

To assess the infection responses, Bd-21 (*B. distachyon*) plants were used. Different parts of the plants -roots, leaves and heads- and the whole plant were used.

Finally, qPCR analysis was carried out to investigate the changes in defence genes expression when they were treated with the bacterial strain EU07 with or without infection of the *F. graminearum*.

The abovementioned experiments were done to clarify the role of the three *Bacillus* strains in the immuno-defence and growth of the selected model plants. One-way ANOVA were used to assess infection assays with *Arabidopsis* and Bd-21 plants and two-way mixed ANOVA was used to assess changes in time/treatments in infection head assays with Bd-21.

Results

Infection assays in *A. thaliana* plants

4.1 Infection assays on *Arabidopsis thaliana* plants

4.1.1 Effect of Bacillus *strains on the sporulation of Hpa on* At- *Col-0*

At-Col-0 seedlings, treated with bacterial broths at the roots level, were infected with *Hpa* (Emoy2) to determine if any inhibitory effect of *Hpa* sporulation will be observed.

Analysis of results showed some outliners in control, QST713 and EU07 treatment. Moreover, the data did not meet the normality test (Shapiro-Wilk's test ($p > .05$) or homogeneity test, still after being transformed. Then, a parametric test (Kruskal-Wallis) with a significance of 0.001, was run and it was found that the distribution of the number of spores between the treatment were not similar. Values are mean ranks unless otherwise stated. Distributions of spore counts were not similar for all groups, as assessed by visual inspection of a boxplot. The mean ranks of spore count were statistically significantly different between groups, $\chi2(3) = 17.586$, $p = .001$.

Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Adjusted p-values were presented. This post-hoc analysis revealed statistically significant differences in spore counts

between EU07 (34.07) and the other treatments, Control (59.09, p=0.015), QST713 (58.06, p=0.022) and FZB24 (66.78, p<0.001) (Figure 4.1).

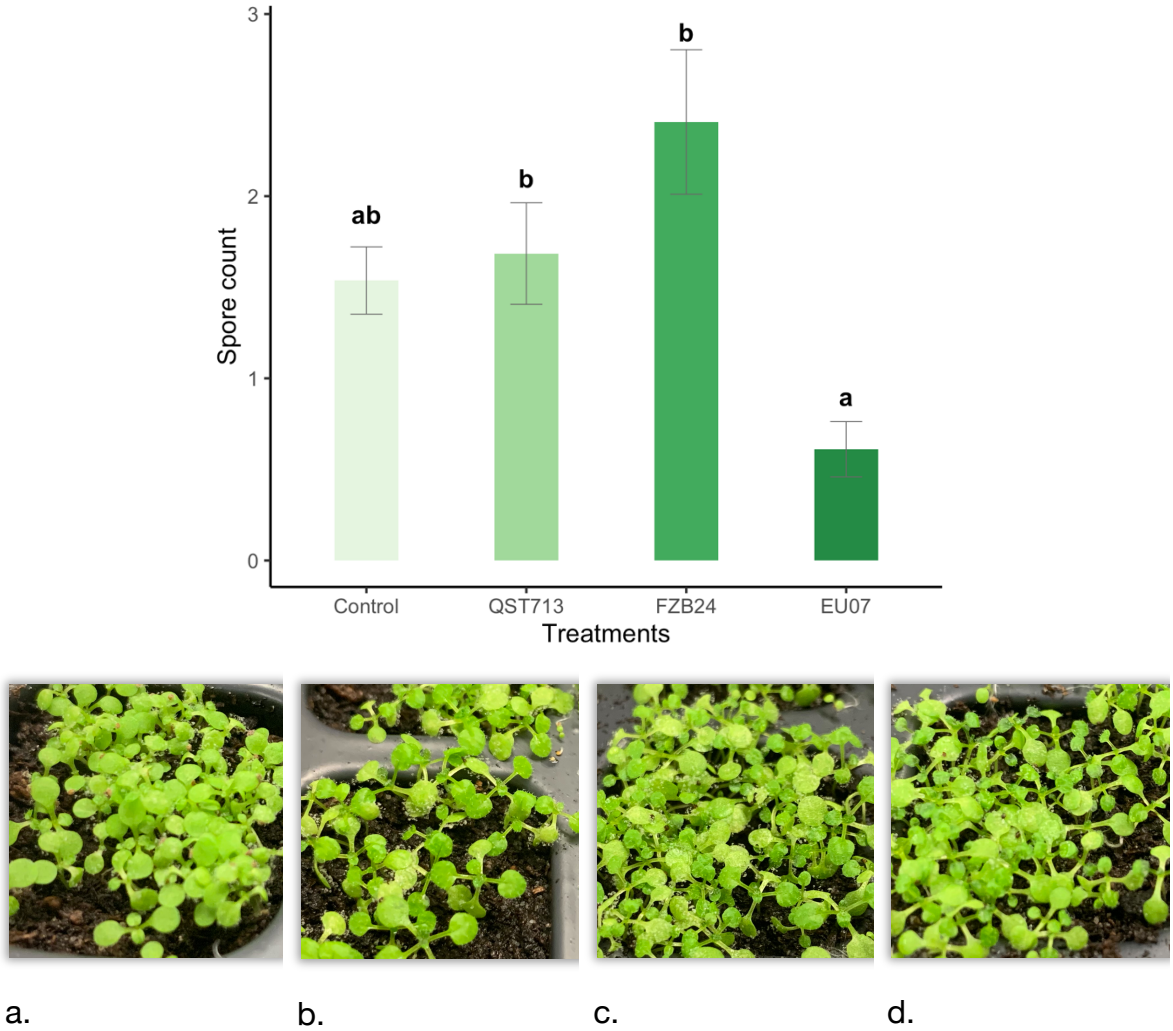


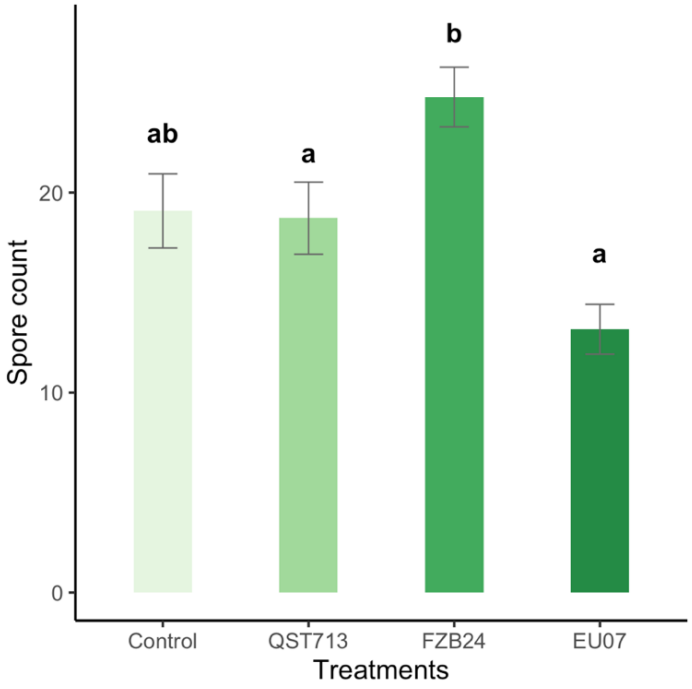
Figure 4.1 Sporulation of Hpa on Col-0 treated with* Bacillus *strains.

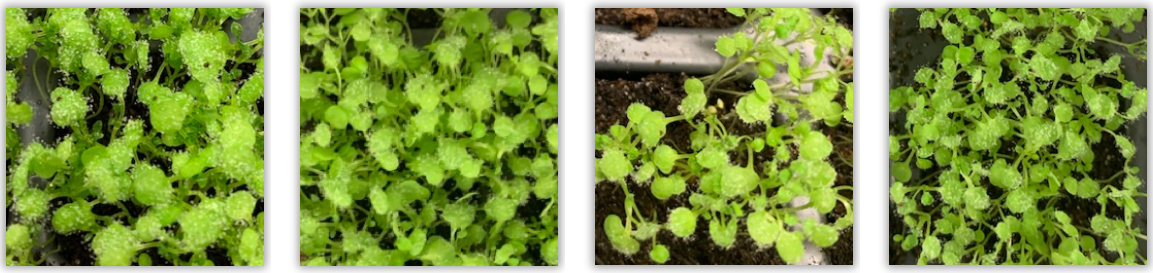
Spore count of *Hpa* on Col-0 plants treated with *Bacillus* strains (twice) (15 do) (**Top**). *At*-Col0 plants displaying sporulation of *Hpa*: **(a)** Control, **(b)** treated with QST713, **(c)** treated with FZB24 and **(d)** treated with EU07 (**bottom**). Data were from three independent experiments of nine repeats each treatment and shown as the mean ± SE. Bars with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following one-way ANOVA. n=108.

4.1.2 Effect of Bacillus *strains on the sporulation of* Hpa *on Ws-eds1 plants*

A second experiment were carried out to analyse the sporulation level on Ws-*eds1* plants infected with *Hpa*. A one-way ANOVA was conducted to determine if the ability to suppress sporulation (number of spores) was different within the bacterial treatments. The treatments were QST713 (n=36), FZB24 (n=36), EU07(n=36) and Control (n=36). There were no outliers after modified the firsts observed: QST713 (49->46), FZB24 (48->45), EU07(43->29), as assessed by boxplot; data was not normally distributed for Control ($p = .001$) and QST713 ($p = .031$) groups only, as assessed by Shapiro-Wilk test; and there was homogeneity of variances, as assessed by Levene's test of homogeneity of variances ($p = .094$).

Data is presented as mean ± standard deviation. There was a statistically significantly difference between the different treatments in spore counts, $F (3, 140) = 8.627$, $p < .0005$, $\omega 2 = 0.14$. The spore count was higher with FZB24 (24.78 ± 8.95) than that were observed on Control (19.08 ± 1.85), QST713 (18.72 ± 10.82) and EU07 (13.14 ± 1.25) treatments. Tukey post hoc analysis revealed that the spore count from FZB24 to EU07 (11.64, 95% CI (5.69 to 17.59) -Confident interval-) was statistically significant ($p < .001$), as well as spore count from FZB24 to QST713 (6.056, 95% CI (0.10 to 12.01), $p = .044$), but no other group differences were statistically significant (Figure 4.2).





a. b. c. d.

Figure 4.2 Sporulation of* Hpa *on Ws-eds1 treated with* Bacillus *strains.

Spore count on Ws-*eds1* plants treated with bacterial broths (twice) and infected with *Hpa* (15 do) (**Top**). Ws-*eds1* plants displaying sporulation of the oomycete *Hpa*: **(a)** Control, **(b)** QST713, **(c)** FZB24 and **(d)** EU07 (**bottom**). Visually, the treatment EU07 showed less signs of damage in foliage compare with the other treatments and the treatment FZB24 showed more damage due to the presence of *Hpa*. Data were from three independent experiments of twelve repeats each treatment and shown as the mean ± SE. Bars with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following one-way ANOVA. n=144.

4.2 Infection assays in roots and detached leaves of *Bd-21*

4.2.1 Roots assay with Bacillus *EU07*

Root infections assays were carried out to test if there were any *Fg-K1-4* infection occurring in the roots as well as to determine any advantage produced by the presence of the bacterium EU07.

Though, it was observed that roots were getting brown due to the infection with *Fg-K1-4*, the same effect was also observed in control roots. However, Bd-21 grew very well in WA medium, the control treatment mostly showed one main root at 11 days after sown (das). Contrary, the group treated with *Fg-K1-4*, differed in the sense that the growth of secondary roots from the crown. Remarkably, new roots managed to get same length than the first roots at the 4dpi (Figure 4.3).

In other assays, when the roots of Bd-21 were inoculated with the *Bacillus* EU07, growth of the bacterium was not observed (Data not shown). Generally, it was determined that infection assays with the *Fg-K1-4* as well as colonization assays with the bacterium assays were not pivoting points for the determination of the effectiveness of this *Bacillus* in the control of *Fusarium* diseases.

Detached leaf infection assays were shown to be promising to understand the behavior of the fungus treated with or without the bacterial broth of EU07 (Figure 4.4).

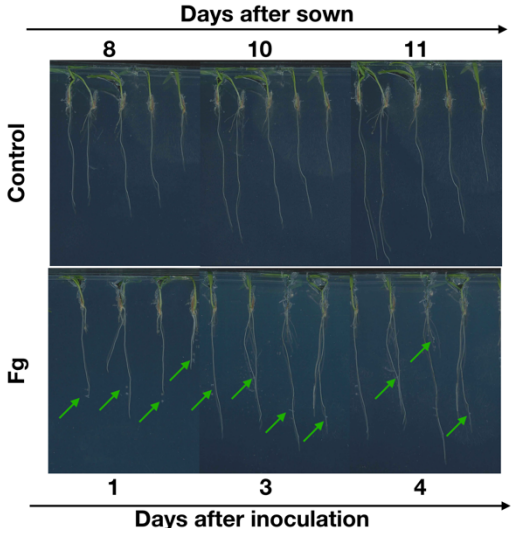


Figure 4.3 Root infection assays with n* Bd*-21.

The root phenotype of the Bd-21 after inoculation with *Fg-K1-4* displayed secondary growth of roots from the crown. Data were from one independent experiment of 6-7 replicates. Experiments were repeated twice, and similar results were obtained. n=60.

4.2.2 Detached leaves infection assay

Not surprisingly, *Fg-K1-4* treatment showed necrosis on the infection points in comparison to the controls. Likewise, the treatment of *Fg-K1-4*/EU07, in which the fungus was inoculated and later treated with EU07, showed a more virulent response (Fig 4.4).

Contrary to the *Fg*-K1-4 treatment, the presence of the necrotic areas was widely spread around the wounded area and across the width of leaves in *Fg*-K1-4/EU07. Moreover, the treatment with *Fg*-K1-4 differed from the *Fg*-K1-4/EU07 treatment because this showed visible mycelia growth around the necrotic area, which was not visible neither in Control nor in *Fg*-K1-4/EU07 treatment.

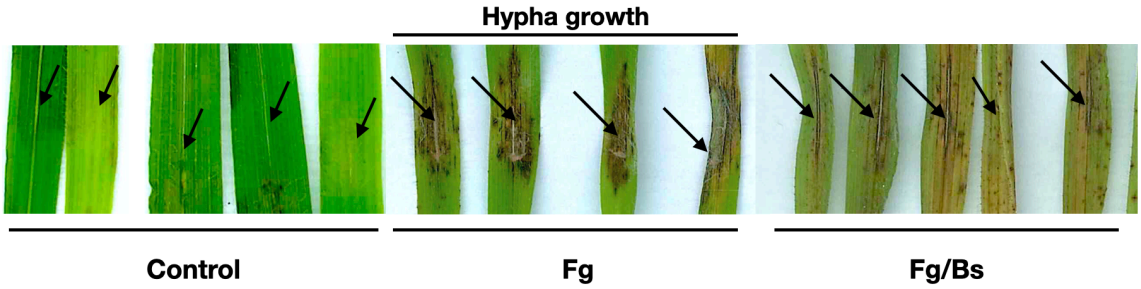


Figure 4.4 Detached leaves assay with* Bd*-21.

Bd-21 was used to assess the effect of the *Bacillus* on *Fg*-K1-4 on leaves 4 days post inoculation (dpi). Detached leaves from ~2 months old plants were wounded using a glass rod, sown in water agar (WA) and infected with Fg. **Left**: Control (treated with water only) shows the healthy leaves with some necrosis of the damaged tissues due to the laceration. **Centre**: *Fg*-K1-4 treatment shows dark necrotic areas around the infection point with conspicuous development of hypha. **Right**: *Fg*-K1-4/EU07 treatment shows less necrotic areas around the laceration point with some mycelia growing from this point. Generally, treatments of *Fg*-K1-*4*/EU07 show less dark necrotic areas and less or null hypha formation than the treatments of only *Fg*-K1-4*.*

4.2.3 Detection of mycelial growth using trypan blue stained leaves

The leaves used in the previous assay (Section 4.2.2) were stained with trypan blue and observed under microscope (Figure 4.5). Accordingly, the leaves of the control treatment did not show any infection signs, but sometimes the wound areas showed some necrosis. The *Fg-K1-4* treatment leaves showed abundant mycelia over the surface as well masses of macroconidia were observed (Fig. 4.5).

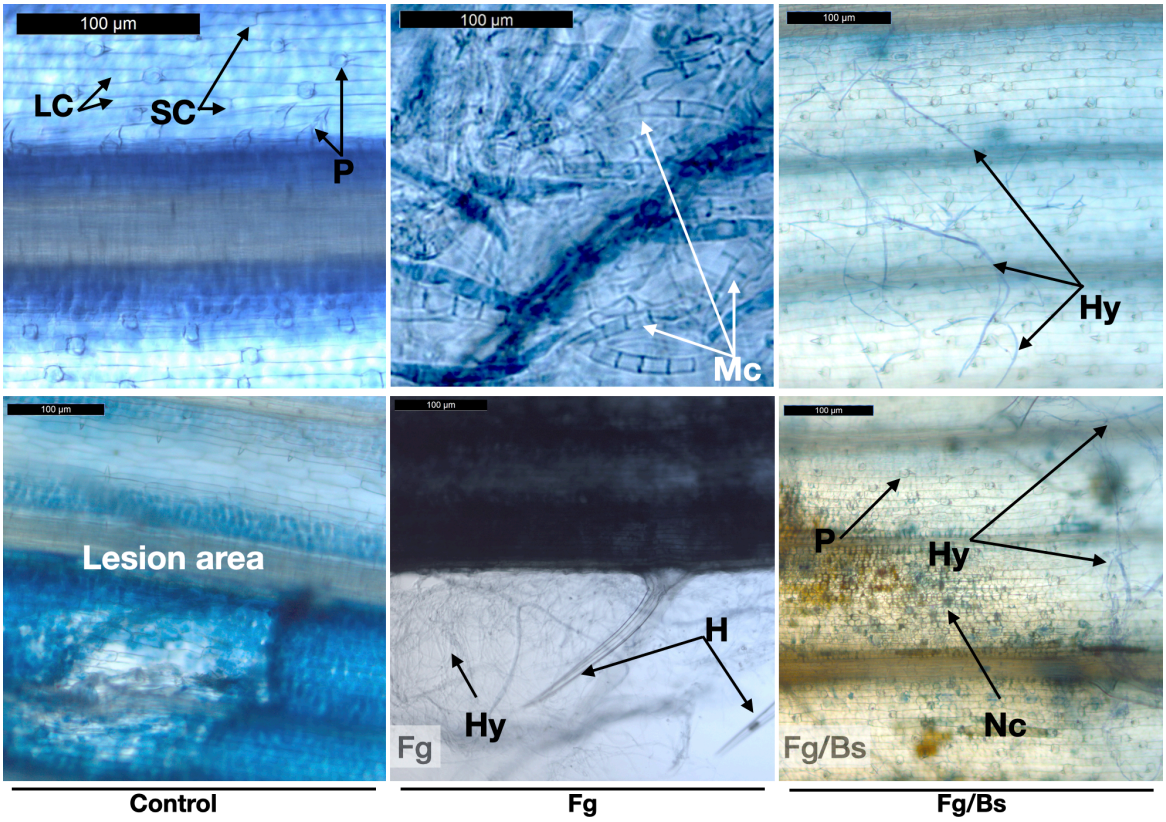


Figure 4.5 Trypan blue staining of the detached leaves of* Bd*-21.

Adaxial view of leaves of *Bd*-21 non-infected (Control), infected with *Fg*-K1-4 (Fg) and infected with *Fg*-K1-4 and treated with *Bs* (Fg/Bs) and after 4 days were stained with Trypan blue. **a)** Control treatment shows the healthy leaves with no sign or very low sign of damage. **b)** *Fg*-K1-4 treatment shows dark necrotic areas with abundant mycelia and in some cases macroconidia. **c)** *Fg*-K1-4/Bs shows no formation of macroconidia. All the treatments showed some degree of necrotic damage around the wounding area with more prevalence with the presence of *Fg*. LC: Long cells, SC: Short Cells, P: Papilla, H: Hair, Hy: Hypha, Mc: Macroconidia, Nc: Necrotic damage. Scale bars = 100 µm

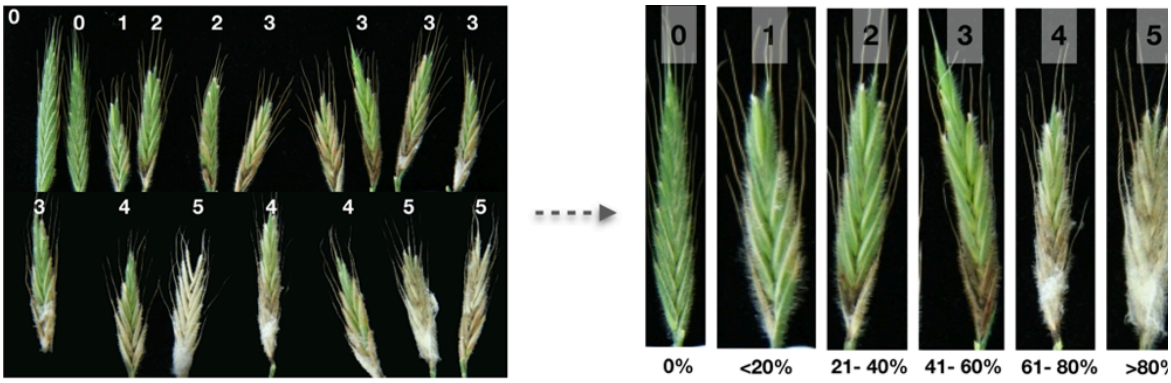
Similarly, the treatment *Fg-K1-4*/EU07 showed mycelia with less density, however no macroconidia were observed. Furthermore, trypan blue stain reveled deeper in colour, wider and concentric necrotic lesions in *Fg-K1-4* compare with the *Fg-K1-4*/EU07, according with the section above (Fig 4.5.).

4.3 Infection assays with Bd-21 heads

Infection assays were performed to understand the effectivity of the *Bacillus* EU07 to protect Bd-21 spikelets from the *Fg-K1-4*.

4.3.1 Scale of severity of head infection assay

A scale of severity was constructed to facilitate the evaluation of the infection on plants during the experiment (Figure 4.6). The scale was made with 6 assessment points clearly delimited with the aim of lower the impact of errors due to the evaluator (Bock et al., 2010; Chiang et al., 2017; Rana et al., 2018).



a. b.

Figure 4.6 Construction of the severity scale for the infection head assay (IHA).

Severity of infection scale over the observed disease progression on Bd-21 heads inoculated with *Fg-K1-4*. **a)**Two weeks after inoculation, heads were collected, and a grade was assigned accordingly. **b)** A total of six grades were made (0-5), that represents the % of progression of the disease and called Disease severity index (%DSI).

Chiang et al. (2017) propose that the more of the number of scores used to construct the scale, the less of the errors on the measurement due to the examiner.

The data was recorded with the use of the scale, then the data was transformed to a %DSI (Disease severity index) using the equation 2.3.

4.3.2 In vivo *antagonistic assay with EU07 against* Fg-K1-4 *using Bd-21*

Head Blight infection assays was performed in order to assess the effectiveness of EU07 to control the *Fg* infection (Figure 4.7) and the DSI was calculated and used as data set. The experiment was statistically analysed using two-way mixed ANOVA. There was one outlier, as assessed by boxplot. That outlier was kept as it was believed to be a normal result.

DSI was not normally distributed only for the control treatments and for *Fg-K1-4* drop treatment in T2, as assessed by Shapiro-Wilk's test ($p > .05$). However, the residuals for DSI score were normally distributed, as assessed by Normal Q-Q Plot. There was no homogeneity of variances, as assessed by Levene's test of homogeneity of variance ($p > .05$), but there was homogeneity of covariances, as assessed by Box's test of equality of covariance matrices ($p = .002$). Mauchly's test of sphericity indicated that the assumption of sphericity was violated for the two-way interaction, $\chi2(2) = .000$, p=? [control treatments effects].

There was no statistically significant interaction between the treatments and time on the %DSI score, $F (6, 59) = 1.642$, $p= .152$, partial $\eta2 = .143$. The main effect of time showed a statistically significant difference in %DSI at the different time points, $F (1, 59) = 6.182$, $p = .016$, partial $\eta2 = .095$.

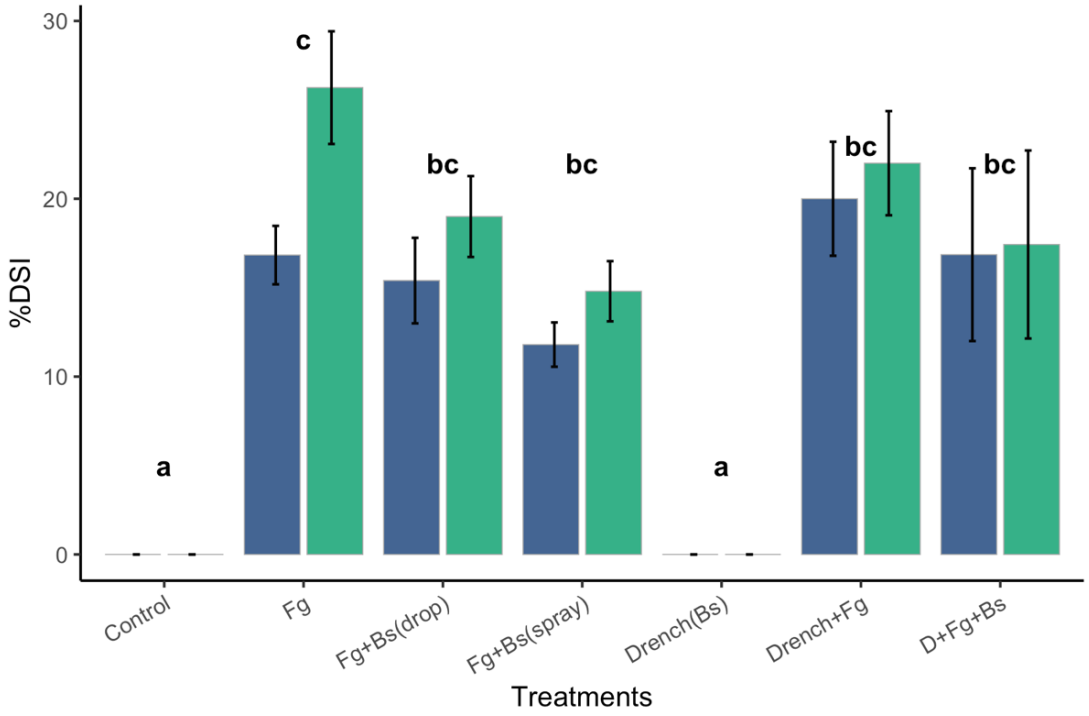


Figure 4.7 Disease severity index (%DSI) of IHA with* Bd*-21 treated with* Fg*-K1-4 and Bs at 7dpi (**Blue**) and 14dpi (**Green**).

Disease Severity Index (DSI%) on infection head assay of *Fg*-K1-4 on Bd-21 at 7dpi (**T_1 Blue**) and 14dpi (**T_2 Green**). Severity of infection of *Fg-K1-4* on Bd-21 plants was determined for the Bd-21 plants treated and non-treated with *Bacillus* EU07 (*Bs*) and infected with or without *Fg-K1-4*. Spray Treatments using *Bs* in to control *Fg-K1-4* showed significant differences with the *Fg-K1-4* treatments (drenched or not). Treatments applying drops or spraying *Bs* showed similar level of control over the *Fg*-K1-4. Data were from one independent experiment of at least seven repeats and shown as the mean ± SE. Experiments were replicated four times and similar results were obtained. Bars clusters with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following two-way mixed ANOVA. n=330. (*Fg-K1-4=F. graminearum*, D=Drench, Bs= *Bacillus* strain EU07).

The main effect of the treatments showed a statistically significant difference in %DSI at the different time points, $F(6, 59) = 23.29$, $p < .001$, partial $\eta 2 = .703$.

It is worth mentioning that during the course of the assessments it was noticed that infection by *Fg-K1-4* alone produced more presence of hypha (visible hypha and necrosis) than drenching treatments (non-visible and necrosis).

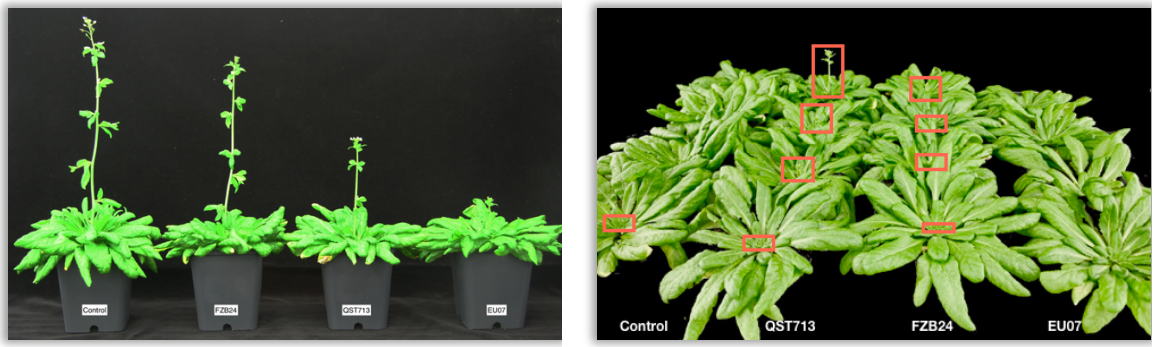
PGP activity assays in *A. thaliana* plants

4.4 Assessments of PGP activity

4.4.1 Assessing PGP activity of the Bacillus *strains in* Arabidopsis *plants*

To assess the plant growth promoter (PGP) activity of the *Bacillus* strains, *A. thaliana* (*At*) plants were flooded with 10% bacterial suspensions or water for 9 weeks, every 8 days. The plants showed different responses mainly on the pathogen response, time of flowering and weight of the leaves and shoots (Figure 4.8).

Plant characteristics recorded were: a) Presence of *Powdery mildew*, b) wet weight of shoots, c) wet weight of leaves, d) dry weight of shoots, e) dry weight of leaves, f) time for flowering, g) number of primary flowering shoots, h) number of secondary flowering shoots i) height of flowering shoots, j) width of the flowering shoots (Figure 4.9).



a. b.

Figure 4.8 Arabidopsis plants irrigated with water or solutions of bacteria for 9 weeks.

Plants *At*-Col0 were treated with bacterial solutions. Treatments done: Control, FZB24, QST713 and EU07 respectively. Solutions of bacterial broths (10%) were applied weekly by 9 weeks. **a)** treatments showed different levels of development of the floral structures (shoots). Development of floral structures were not even between bacteria Control>FZB24>QST713>EU07. **b)** Replicate of same experiment showed similar results with the strain EU07.

Following, only the a), b), c), d), e) and f) points above mentioned were statistically analysed (Figure 4.10).

4.4.1.1 Evaluating the effect of bacteria on wet weight of shoots

There was a statistically significant difference between the effects of *Bacillus* on wet weight of shoots [F (3, 20) = 8.510, p = 0.001]. A Tukey-HSD showed that the treatment FZB24 was significantly different to control (p=0.006) and to EU07 (p=.002) with 2.847 and 3.215 g more accordingly. QST713 was significantly higher than EU07 (p=.023) with 2.388 g more (Figure 4.10c).

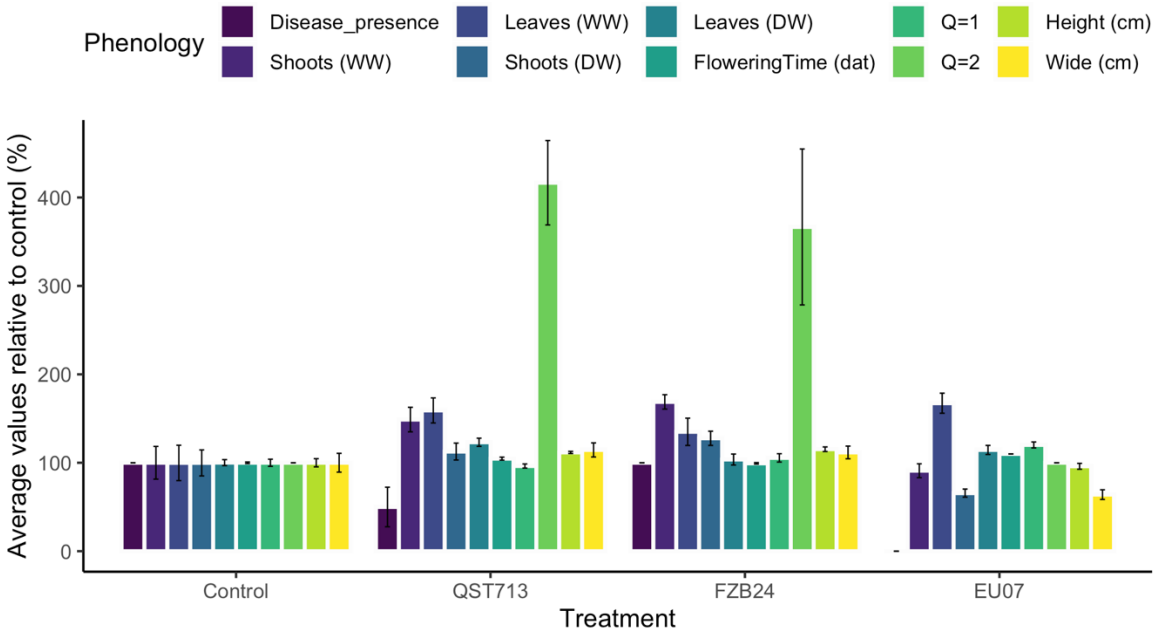


Figure 4.9 Growth characteristics of Arabidopsis plants treated with solutions of bacteria.

Growth characteristics of *Arabidopsis* plants treated with *Bacillus* solutions (QST713, FZB24 and EU07 at 10%) for 9 weeks. **(a)** Disease presence, **(b)**wet weight of shoots, **(c)** wet weight of leaves, **(d)** dry weight of shoots, **(e)** dry weight of leaves, **(f)** Flowering time, **(g)** Main shoot flower branches, **(h)** Secondary shoot, **(i)** Height of shoot, **(j)** width of shoot. Data were from one independent experiment with six repeats and shown as the mean ± SE. Experiments were replicated three times. n=24. (WW= wet weight; DW=Dry weight; cm=centimetres; Q=1 Primary shoot and Q=2 secondary shoot).

4.4.1.2 Evaluating the wet weight of leaves

There was a statistically significant difference between the effects of *Bacillus* on wet weight of leaves [F (3, 20) = 3.763, p = 0.027]. A Tukey-HSD showed that EU07 was significantly different than the control (p=0.029) with 7.347 grams more. No other significant difference was observed (Figure 4.10e).

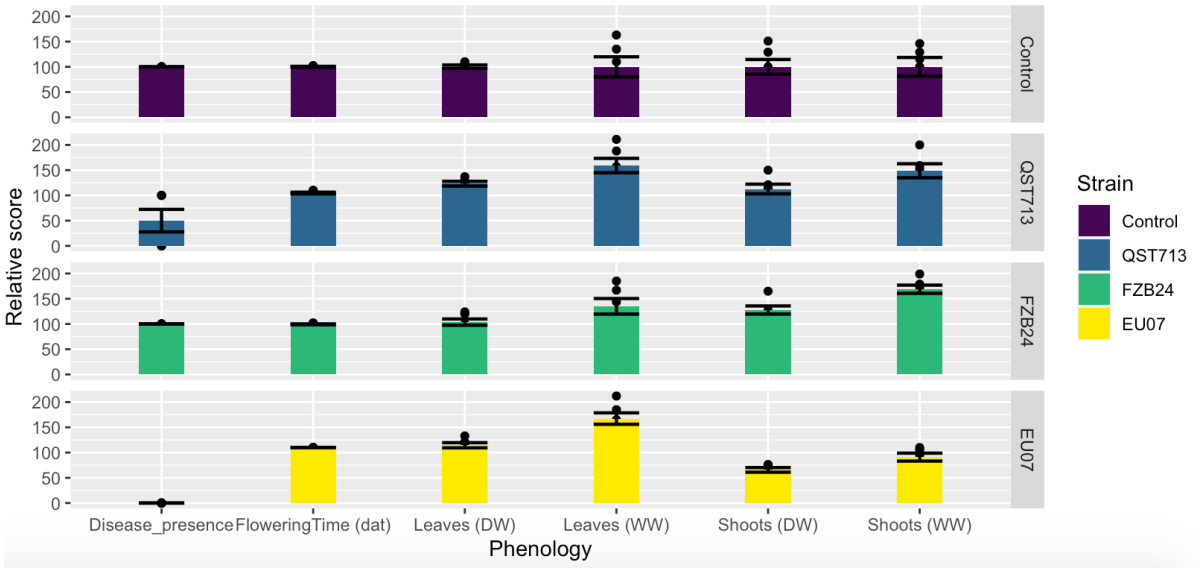


Figure 4.10 Phenotypic response of Arabidopsis plants to Bacillus strains:

Arabidopsis plants were treated with *Bacillus* suspensions (drenching 9 weeks) and phenotype characteristics were recorded. **(a)** presence of pathogens on plants, **(b)** time on which plants showed the floral shoot, **(c)** wet weight of shoots, **(d)** dry weight of shoots, **(e)** wet weight of leaves and **(f)** dry weight of leaves. Data were from one independent experiment of six repeats and shown as the mean ± SE. Experiments were replicated three times and similar results were obtained. Bars with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following one-way ANOVA. n=24.

4.4.1.3 Evaluating the dry weight of shoots

There was a statistically significant difference between the effects of *Bacillus* on dry weigh on shoots [F (4, 20) = 110.305, p <.000]. A Tukey-HSD showed that the FZB24 was significantly different than that was observed with EU07 (p=0.001) with 0.513 grams more. Moreover, the QST713 was significantly different than that was observed with EU07 (p=.015) with 0.388 grams more (Figure 4.10d).

4.4.1.4 Evaluating the dry weight of leaves

There was a statistically significant difference between the effects of *Bacillus* on dry weight of leaves [F (4, 20) = 507.643, p <.000]. A Tukey-HSD showed that QST713 was significantly different than that observed with control (p=0.015) with 0.392 grams more and to FZB24 (p=0.047) with 0.330 grams more (Figure 4.10f).

4.4.1.5 Evaluating the presence of natural pathogen infection – presence of Powdery mildew -

There was a statistically significant difference between the effects of *Bacillus* on the presence of powdery mildew (PM) [F (4, 20) = 45.000, p <.000]. A Tukey-HSD showed that the control and FZB24 both showed a significant difference to EU07 (p<.000) with 100% infected plants and to QST713 (p=0.023) with 50% infected plants on difference. QST713 was significant different to EU07 (p=023) with 50% more plants infected (Figure 3.19a). However, no PM infection was observed on plants treated with EU07 (Figure 4.10).

4.4.1.6 Evaluating the time of flowering

There was a statistically significant difference between the effects of *Bacillus* on time of flowering [F (4, 20) = 510629.69, p <.000]. A Tukey-HSD showed that EU07 was significantly different than those observed with the control (p<.000), FZB24 (p<.000) and QST713 (p=.009) with 5, 5.33 and 2.67 more days to show the floral shoot. QST713 treated plants were significantly different than the control (p=.023)

and FZB24 (p=.009) with 2.33 and 2.67 more days to show the floral shoot (Figure 4.10b).

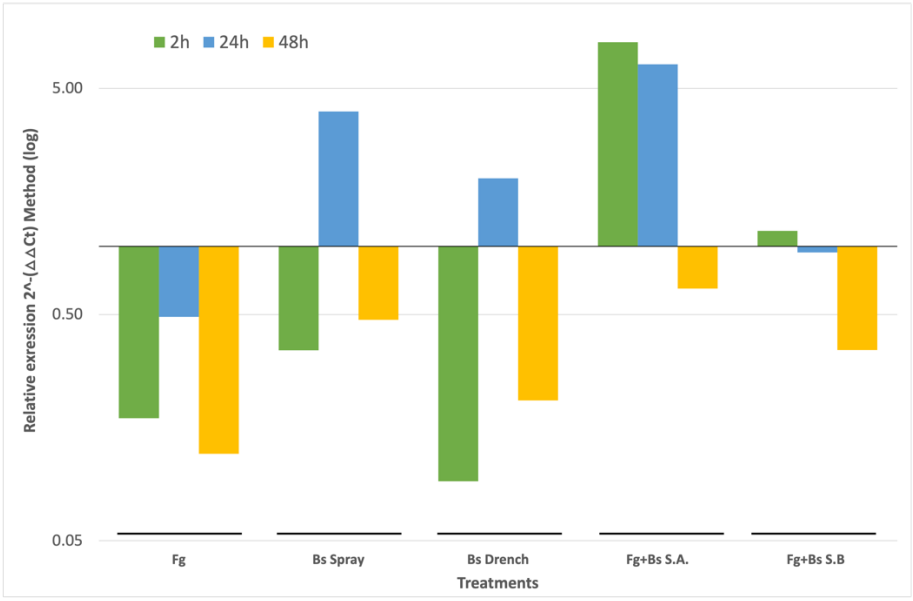
4.5 Assessing the defence genes on At-Col-0

The relative expression of the defence genes *PR1* and *PDF1.1* against *UBQ5* were quantified to determine the role of the *Bacillus* EU07 on the immune-defence of the Col-0 plants using the $2^{-\Delta\Delta Cq}$ method. The relative expression of *PR1* were observed to be downregulated (Figure 4.11 a). Conversely, the PDF1.1 gene were observed to be mostly upregulated with very high fold change (Figure 4.11 b).

The *PR1* gene showed different levels of expressions according to the different treatments. The treatment with *Fg* showed a decreased *PR1* expression level at all the times (0.174, 0.489, 0.121 folds respectively). Besides, the *Fg*+*Bs* spray, *Bs* spray and drench showed increased *PR1* expression at 24h (6.383, 3.952 and 2.00 folds, respectively). All of the other treatments showed decreased *PR1* expression levels (Figure 4.11 a).

Defence gene PDF1.1 on *At*-Col-0 treated (*Fg* and *Bs*) showed a positive tendency in levels of expression of most of the treatments. Particularly, in the treatment *Fg*+*Bs* Spray was not possible to obtain measurable expression levels at 2h and 72h, the treatment at 24h was very low (2.05E-07 folds).

a.



b.

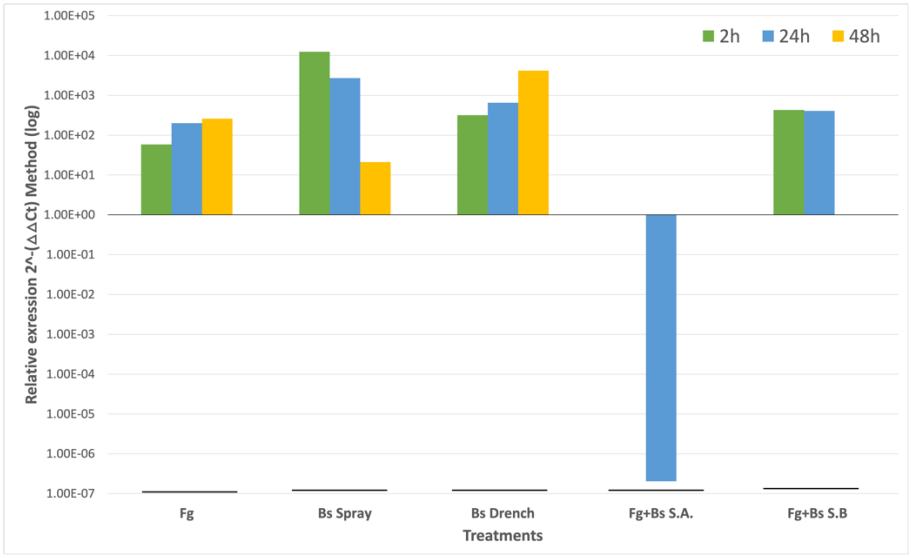


Figure 4.11 Relative expression rates of defence genes (PR1 and PDF1.1) on At Col-0 infected with Fg-K1-4 and treated with Bacillus (EU07):

The relative expression of *PR1* and *PDF1.1* genes were assessed against the house keeping (HK) gene *UBQ5* of *At*-Col-0 plants infected with *Fg-K1-4* and treated with the *Bacillus* strain EU07 for the period of up to 48H. **a)** Relative expression of the gene *PR1*/*UBQ5* ($2^{-\Delta\Delta Cp}$ method) on *A. thaliana* plants treated with Bacillus (EU07), with or without *Fg-K1-4* infection **b)** Relative expression of the gene *PDF1.1*/*UBQ5* ($2^{-\Delta\Delta Cp}$ method) on *A. thaliana* plants treated with Bacillus (EU07), with or without *Fg-K1-4* infection. (n=64). Data were from one independent experiment of 4 technical replicates. Two repeats were made, and similar results were obtained. Not data were obtained at *Fg*+*Bs* S.A. 24h, 72h and *Fg*+*Bs* S.B 72h from PDF1.1. Treatments: ***Fg***: *F. graminearum*, ***Bs*** **Spray**:EU07 *Bacillus* strain sprayed, ***Bs***

Drench: EU07 *Bacillus* strain drenched, **Fg+Bs S.A.**: *F. graminearum* + *Bacillus* strain sprayed after and **Fg+Bs S.B**: *F. graminearum* + *Bacillus* strain sprayed before.

Usually, the treatments which included bacterial treatments presented higher level of expressions (21 – 12,417 folds) than that observed with the *Fg* treatments. The treatment *Bs* Spray was the highest, at 2h (12,417) and 24h (2,712), with 212 and 14 times more expression than the *Fg* at the same time. However, the same treatment showed the decreased levels of expression, 21 folds, at 72h, 0.08x less expressed than that found with *Fg* treatment.

PGP activity assays in Bd-21 plants

4.6 PGP activity of the Bacillus strains on Bd-21

First assays with the Bd-21 treated with the *Bacillus* strain EU07 showed important differences on the production of number of heads. Observations on the assays demonstrated that plants drenched with the bacterial solution developed much more heads than those not drenched, while plants infected with *Fg-K1-4* developed smaller number of heads per plant in comparison to the control plants (Figure 4.12).

They were assessed with one-way ANOVA to determine if the number of heads in Bd-21 plants were statistically different between groups or treatments. The treatments were classified into 7 groups: Control (n = 11), *Fg* (n = 12), *Fg*+*Bs* drop (n = 10), *Fg*+*Bs* spray (n = 10), D+ Control (n = 8), D+ *Fg* (n = 8) and D+ *Fg*+spray (n = 7).

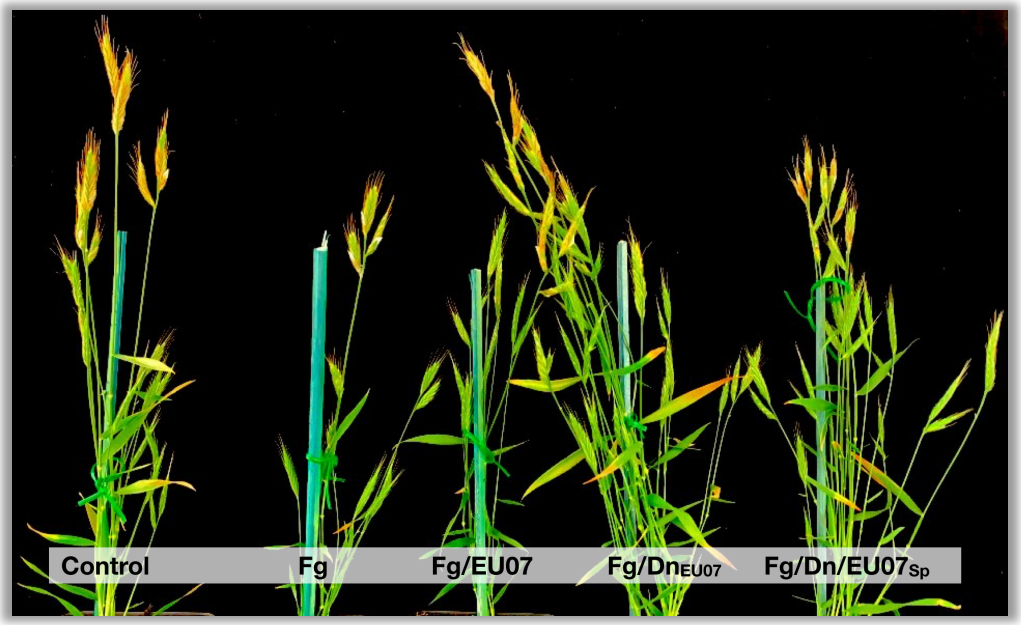


Figure 4.12* Bd*-21 plants treated with* Bacillus *EU07, effect on spike production

Bd-21 plants were infected with *Fg.* A total of 25µl drop of macroconidia at a concentration of 10^6 in the spikelet was used. The treatments were: **Control** treated with water. ***Fg*** alone. ***Fg/EU07*** infected and treated with *Bacillus* drop. ***Fg*/Dn$_{EU07}$**: Fg infected plants and drenched with EU07. ***Fg*/Dn/EU07$_{sp}$**: *Fg* infected plants, drenched with EU07 and treated with EU07 spraying the spikelets. Plants drenched (***Fg*/Dn$_{EU07}$** and ***Fg*/Dn/EU07$_{sp}$**) produced much more amount of spikelets than the control and the non-drenched plants.

There were outliers in the data (D_*Fg*_Spray[1], Fg[2], *Fg*_D_Spray[1]), as assessed by inspection of a boxplot. They were kept as they were considered normal values. Number of heads score only for *Fg_Bs* drop treatment was not normally distributed, as assessed by Shapiro-Wilk's test ($p > .05$). There was homogeneity of variances, as assessed by Levene's test for equality of variances ($p = .772$). The number of heads produced by Bd-21 in the different treatments was statistically significantly different, $F(6, 65) = 4.36$, $p = .001$.

The treatment *Fg* produced 17.00 ± 3.51 heads, a smaller number of heads compared with the control treatment (18.73 ± 3.97) and control drenched treatment (22.13 ± 4.61). In general, drenched treatments showed higher production of heads: Control Drenched produced 22.13 ± 4.61 heads, *Fg* + Drenched produced 25.22± 4.5 heads and *Fg* + Drenched + treated with spray of *Bs* (26.67 ± 5.28). Non-drenched plants infected with *Fg* and treated with *Bacillus* in spray (23.6 ± 3.98) or by drops (23.1 ± 5.9) produced more heads than *Fg* alone but less than all the drenched treatments.

Tukey post hoc analysis revealed that the drenched with *Bs* and infected with *Fg-K1-4* treatment was statistically significant in comparioson to Control (p = .019), to *Fg* (p = .002), but not to infected treatment and treated with *Bs* -Spray (p = .850) or drop (p = .738)- and the other drenched treatments -Control (p=.529) and *Fg* infected (p=.997)-. Conversely, the treatment *Fg-K1-4* was significantly different only to control treatments - drenched (p=.195) (Figure 4.13).

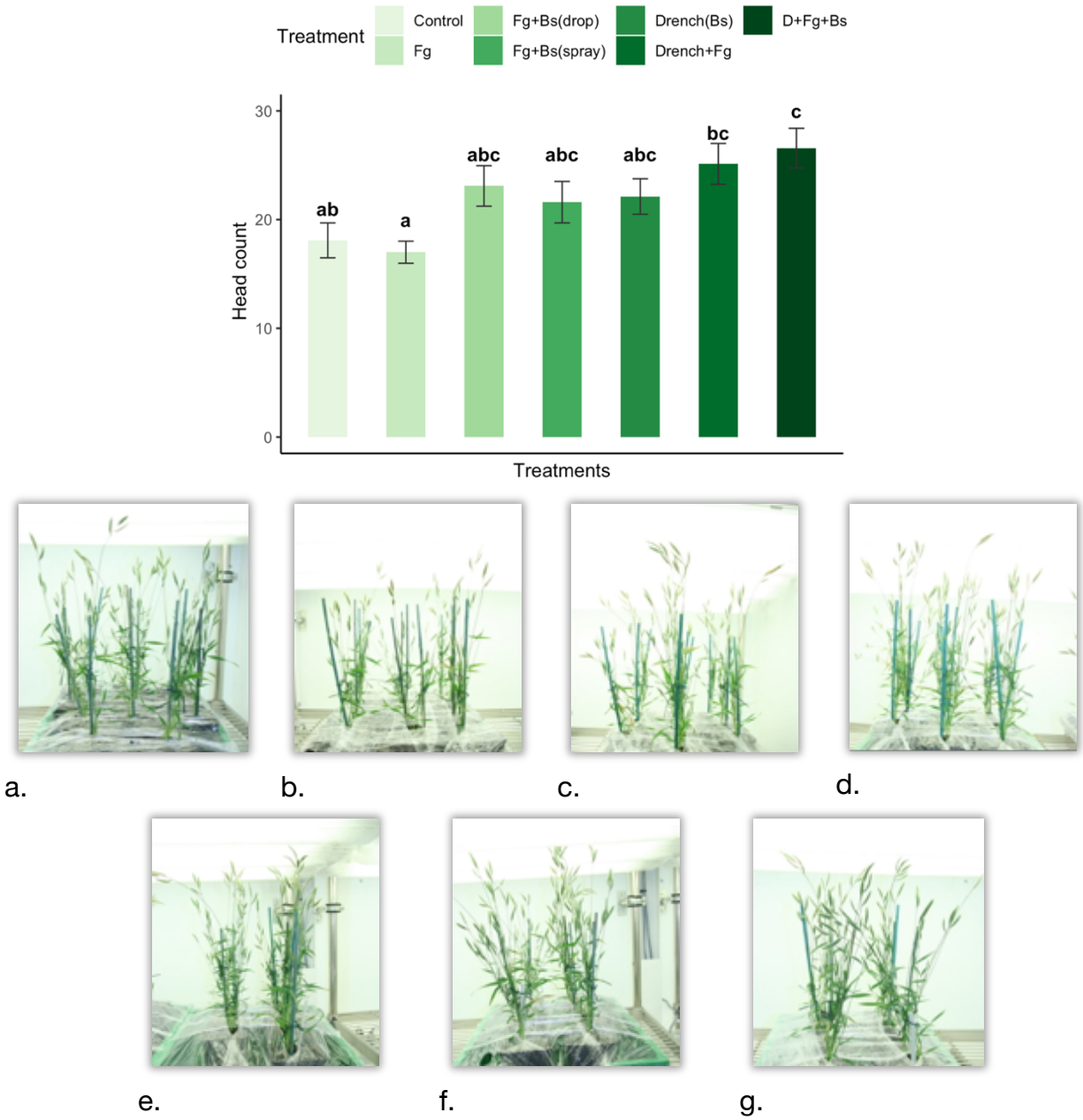


Figure 4.13 Bacillus EU07 has an effect on Bd-21 plants' spike production

Number of heads per plant of the Bd-21 treated with *Fg-K1-4* and EU07 (IHA) (**top**). Visual fitness of Bd-21 plants: **(a)** Control, **(b)** *Fg*, **(c)** *Fg*/ *Bs* -Spray, **(d)** *Fg*/ *Bs* -Drop, **(e)** Drench (Bs), **(f)** Drench/*Fg* and **(g)** Drench/*Fg*/*Bs*, (**bottom**). Data were from one independent experiment of at least seven repeats and shown as the mean ± SE. Experiments were replicated three times and similar results were obtained. Bars clusters with different letters were significantly different according to Tukey's Test ($\alpha < 0.05$) following one-way ANOVA. n=66.

Discussion

Infection assays

Infection assays have been carried out to provide a better understanding of the ability of the *Bacillus* strain in the control of the *Fg-K1-4*. The assays were focused on Bd-21, however *Arabidopsis* plants were used to complement the understanding of the outcomes due to the availability of an extensive wealth of knowledge on this plant and its well know interaction with the pathogen *Hpa* (Parker et al., 1996).

4.7.1 Arabidopsis plants

Infection assays of the *Arabidopsis* Col-0 showed that the *Bacillus* strain EU07 possessed a positive effect on the plants when they are infected with *Hpa*. The sporulation rate for this treatment was significantly better than the that observed by other two *Bacillus* strains and slightly lower than the that of control treatment. Although, the difference was not significantly different to the control, it was lower than that observed on control plants (Figure 4.1). This indicates that the EU07 can interact with the plant to decrease the sporulation of this obligate oomycete. This results also demonstrate that some other *Bacillus* strains can act in a conflicting way when they are interacting with the plant, for example exacerbating the number of spores produce by the pathogen, as it was triggering a wrong signal in the plant.

Alongside, infection assays with the *Arabidopsis* Ws-*eds1* shed light on the effect of the EU07 bacterial strain. Using the same pathogen, infection assays showed that both EU07 and the QST713 decreased the sporulation rate compare with the control treatment in contrast to that obtained with FZB24. Ws-*eds1* bear heavy sporulation of *Hpa* and retains full responsiveness to the chemical 2,6-dichloroisonicotinic acid, which is an inducer of the systemic acquired resistance (SAR) (Aarts et al., 1998; Coates and Beynon, 2010; Imran and Yun, 2020; Parker et al., 1996) . The strains EU07 and QST713 produced a similar response in Ws-

eds1 plants, however the strain EU07 appeared to have less damage in the foliage compare with the QST713 strain (Figure 4.2).

4.7.2 Brachypodium plants

On the other hand, infection assays were carried out using *Brachypodium* plants Bd-21. Infection of this plant with *Fg-K1-4* is possible and can be used to correlate the response of the cereal crops to this fungus (Pasquet et al., 2016; Rana et al., 2018). Firstly, infection in roots revealed that the *Fg-K1-4* did not distort the shape of the roots, although apparently the plant responded with the production of a secondary roots from the crown point (Figure 4.3). Some suggest that the strain *Bacillus* QST713 has genes and the capability to colonize roots, however it was not demonstrated this capability for any of the *Bacillus* strains on Bd-21 roots (data not shown) (Compant et al., 2010; Vidal-Quist et al., 2013).

Furthermore, the effect of the *Bacillus* EU07 was investigated in leaves infected with the *Fg-K1-4*. Results confirmed that the leaves infected with *Fg-K1-4* produce lesions with necrotic areas (Rana et al., 2018) and occurrence of mycelia. Nonetheless, it is more interesting that leaves infected with *Fg-K1-4* and treated with EU07 displayed much less or totally absence of the visible mycelia (Figure 4.4). Indeed, when the samples were observed under microscope (Figure 4.5), the trypan blue dye exposed the necrotic areas as well the mycelia of the fungus. Control treatment showed some necrotic areas only around the wounded infection areas (water treated), but the *Fg-K1-4* treatment showed high prevalence of mycelia and spots with macroconidia. *Fg-K1-4* can developed macroconidia as earlier as 48h (Larmour and Marchant, 1977), which may suggest that there is a suppression of macroconidia formation by the EU07.

Similarly, Zhao et al. (2014) demonstrated that the *Bacillus subtilis* strain SG6 could reduce the DON concentration in dual culture with *Fg-K1-4* in solid medium. Other authors mentioned the potential of the strain S76-3 (*B. amyloliquefaciens*) to inhibit

completely the conidium germination due to the presence of the lipopeptides iturin A (50 µg/ml) and plipastatin A (100 µg/ml), apparently with a deformation of the macroconidia and hypha. Gong et al. (2015) and Li et al. (2012) probed that the fengycin A produce deformation of hypha and chlamidiospora formation in *Fusarium solani*. Besides, fields assays of a variety of biological control agents, including *Bacillus subtilis* species, decreased the FHB severity and DON accumulation when applied in the anthesis stage (Palazzini et al., 2016).

Infection assays in floral structures (heads) were assessed against a scale of severity (Figure 4.6) to calculate the disease severity index (DSI) (Equation 2.3). Leaves infection assays (Section 4.2.2) demonstrated that during the infection, *Fg* hypha became visible over the affected lesion 8dpi (Section 2.8.2.2). In a review paper, authors mentioned how the DON mycotoxin has the role to facilitate the propagation of the fungus. The fungus uses to propagate internally by vascular bundles or above the surface by stomata. It takes only 24-32h to the fungus to reach the internal surface producing mycelial networks (Beccari et al., 2018; Gunupuru et al., 2017). Likewise, the infection head assays showed necrotic areas of the infected heads. In this experiments, high concentration of inoculum was used (10^6 spores).

Likewise, infection assays in floral structures (heads) were assessed against a scale of severity (Figure 4.6) to calculate the disease severity index (DSI) (Equation 2.3). this experiment found high %DSI in drenched plants (Figure 4.7), however the progression of the disease was lower than that observed with the treatments without drenching and much lower than the plants infected with *Fg* without any bacterial treatment. It is clear that the presence of the EU07 produces an effect in the way how the fungus can progress the infection, probably because the bacteria can decrease the DON production and lower the potential development of the

fungus. Furthermore, treatments with the EU07 showed less or null visible mycelia development supporting the control effect of the EU07 over *Fg in vivo*.

PGP activity assays

In the same fashion with the infection assays described above, PGP activity assays were carried out to get a better understanding of this endeavour in the *Bacillus* strains. A first approach included assays on *At* Col-0 plants, followed for an investigation of the relative expression of the defence genes (*PR1* and *PDF1.1*) on the same type of plants treated with the strain EU07 and at the end, this same strain was used to evaluate the production of flowering structures in Bd-21 plants.

4.7.3 PGP activity in Col-0 plants

PGP activity is an important feature of some *Bacillus* strains, for instance *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Therefore, it was decided to assess the PGP activity for the three *Bacillus* strains on *Arabidopsis* plants and it was found that results showed statistically significant differences in the effect on biomass production, immune response and flowering time by the strain EU07 compare with the other two bacterial strains.

After 9w of treatment, plants with bacterial treatments presented different characteristics in respect the control treatment. In all the cases, *Bacillus* strains increased the biomass production of the plant and stimulated the immune response of the plant (Borriss et al., 2018; Fan et al., 2018). Interestingly, the strain EU07 showed delaying in bolting and flowering time. However, other characteristics such as leaf weight and the presence of pathogens were the best compared with the other strains and the control treatment.

Some authors suggest a close relation between the salicylic acid (SA) regulators and the influence in flowering time as well the biomass production on *Arabidopsis* plants (Berrached et al., 2017; Coelho et al., 2014; Coupland, 1995; Fan et al., 2018;

Jeong et al., 2015; Salehi et al., 2005; Zhai et al., 2015). But, other studies also revealed that the Flowering Locus C (FLC), FT and GI could play significant roles in plant defence in presence of root infecting fungal pathogens (Lyons et al., 2015; Singh et al., 2013; Wang et al., 2011). Salehi et al. (2005), reported that the FLC acts as flowering repressor and they found that the leaf size and biomass were increased. They studied the FLC expressed in tobacco, finding the same responses. Coelho et al. (2014) studied this phenomenon relating the *Phosphatidylethanolamine-Binding Protein* (*PEBP*) gene family of sugarcane in *Arabidopsis* plants using *FT*/*TFL1* genes. Besides, Jeong et al. (2015) suggested a relation of the flowering time delay on *Arabidopsis* plants in an alternative pathway SnRK1/IDD8 when sugar deprivation is happening.

Here the *Bacillus* strains may be probably secreting compounds that interact with the *Arabidopsis* genes and modulate its response to induce immune response, biomass production and changes on flowering time, being more remarkable with the strain EU07.

Some authors suggest that the SA and JA/Eh pathways are antagonistic to each other (Durrant and Dong, 2004). Authors called this phenomena fitness cost, however, most recently authors have been reporting about growth defence trade offs (Cao et al., 2017; Cui et al., 2018; Neuser et al., 2019). Which means a counterproductive effect on the defence of the plant against a pathogen and the cost to growth (Eichmann and Schäfer, 2015; Huot et al., 2014). Neuser et al. (2019) explain this effect very clear and declares that the growth-related transcription factor homolog of BEE2 interacting with IBH 1 (HBI1) put in the balance the defence/growth of the plants in a system *At*/*Ps,* and detailed that this incompatibility is due to the way apoplastic ROS homeostasis is modulated in between. Huot et al. (2014) pointed out this relation with the plant hormones which either contribute to the growth (Auxin, Br, GA) or contribute to the defence of the plant (PTI, SA, JA).

On the other hand, in an early study, Pieterse et al. (1998) proposed a model for the non-pathogenic rhizobacteria mediated ISR signalling, with no discussion of the plant fitness effect. Later, Vos et al. (2013) discusses the effects on plant fitness due to the hormone-regulated inducible defence responses (Figure 4.14), where there is a reduction in growth in contraposition to any increase in defence. However, Shigenaga et al. (2017) addresses the contribution of different plant hormones to plant immunity or hormone crosstalk, indicating the promising perspective of engineer hormone crosstalk as a tool to obtain enhanced pathogen resistance and overall plant fitness.

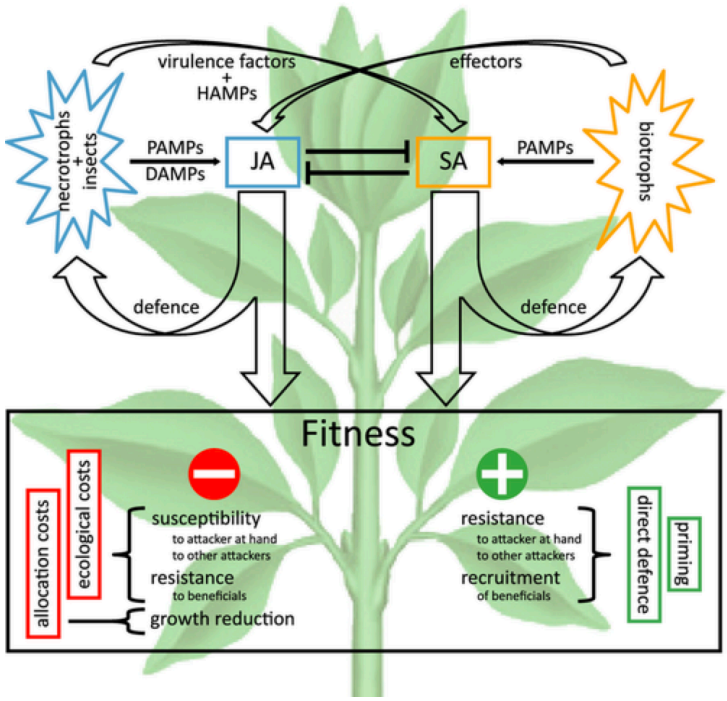


Figure 4.14 Schematic overview of hormone-regulated inducible defence responses and their effects on plant fitness.

Jasmonic acid (JA) defence response is activated after recognition of pathogen-associated molecular patterns (PAMPs) upon necrotrophic pathogens attack. Salicylic acid (SA)-dependent defence responses are activated after a (hemi) - biotrophic attack. While induced defence mechanisms have positive effects on the plant's fitness by enhancing resistance through direct activation and priming of defence, negative effects of induced plant defences

on plant fitness occur as well. The cost of priming plant's defences is considerable lower than the cost of defence activation during the pathogen attact (Source: Vos et al. (2013).

4.1.1 PGP activity in Bd-21 plants

PGP activity was assessed in Bd-21 plants by counting the number of heads produced per treatment in the head infection assays. First, experiments showed a marked difference in the number of heads produced by plants drenched with the EU07 strain (Figure 4.12) in contrast with plants sprayed with the *Bacillus* or only infected with *Fg-K1-4*. To confirm this, further assays were carried out including controls in drenching treatments (Figure 4.13). There seems to be a significant increase in the number of heads in the treatment infected with *Fg-K1-4*, drenched and sprayed with EU07 (D+Fg+Spray) compared with the control (no drenched) and with the treatment of infected plants (Fg-K1-4 only). Nevertheless, there is a relation on the number of heads on the treatments in which the plants were sprayed with the *Bacillus* strain. Results in infection head assays contrasted with PGP activity assays may suggest that EU07 could be an JA promoter and it promotes development of the plant, particularly producing more floral structures. Contrary to the effects found in *A. thaliana* Col-0, where the flowering time was delayed (this could not be demonstrated in the Bd-21 that were vernalised and stratified), the strain EU07 possesses a great advantage protecting the plant and promoting the fitness of the plant.

Globally, it is possible to conclude that these *Bacillus* strains (QST713, EU07 and FZB24) evolved in the production of compounds to facilitate the survival on the phytosphere. To do so, these strains possibly produce specific metabolites that improve the fitness of the plant or protect the plant against pathogens to keep "alive" in their environment. Most interestingly, maybe the metabolites produced by the *Bacillus* strains might be designed to affect similar traits or characteristics on

different plant species (Jeong et al., 2015; Salehi et al., 2005) as a co-response of plant-beneficial *Bacillus* against the invading pathogens.

Chapter 5

Bacterial Genomics of *Bacillus* strains

Introduction

Since the publication of the "*Experiments in plant hybridization*" in 1865 by Mendel (Mendel, 1965), scientists are dedicated to understand the genetic variability in living organisms. Genome sequencing has contributed largely to answer many questions already, nevertheless, this knowledge produced more questions which need to be addressed along the evolving of sequencing technologies such new generation sequencing (NGS).

NGS, which revolutionised the way that genome analysis is done, accelerated the time and decreased the cost of whole genomes sequencing (WGS). Traditionally, Sanger technology (first generation sequencing) was used. It was developed in 1977 by Sanger and collaborators (Sanger et al., 1977). This method was based on the termination of DNA synthesis or chain termination and proved to be tedious and time consuming (Pierce, 2006). Nonetheless, this technique was used to decode the genetic material of the simplest form, the bacteriophage ϕX174, which was the first sequenced genome, in 1977. Subsequently, in 1986, the chromosome II of *Saccharomyces cerevisiae* yeast (Goffeau et al., 1996) was sequenced, later on, in 1995 the whole genome sequence (WGS) of the *Haemophilus influenzae* bacterium was carried out using WG shotgun technique.

Soon after, the whole human genome (Human Genome Project-HGP) was sequenced, for which, bacterial artificial chromosome (BAC)-based mapping was used. After 15 years of work (from 1988), the project was completed and its results were made of public domain to show the importance of these projects for all the scientific community and humanity (Adams et al., 1991; "Human Genome Project FAQ," n.d.). Likewise, the model bacterial organism *Bacillus subtilis* (strain 168) was sequenced (Kunst et al., 1997). In this case, to illustrate the effort involved in this

project, it is necessary to mention that the whole sequencing project was done in the period of 10 years by 33 laboratories (EU, Japan, Korea) and two biotechnology companies (Kunst et al., 1997).

Later, the model plant *A. thaliana* was sequenced before of 2000, using the same technique and after spending a budget in the millions (The Arabidopsis Genome Initiative, 2000). Nowadays, 2020, the cost of the sequence of a human genome with NGS (second generation sequencing or massive parallel sequencing) is in the order of $1,000 (£1,200), decreasing the time and resources needed for similar projects (American Society for Microbiology, 2016; Cheifet, 2019; Stephens et al., 2015). Correspondingly, 20 years later, the serotype a of the *H. influenzae* was sequenced using the same technique (Fleischmann et al., 1995; Iskander et al., 2017). A summary of genetic characteristics of some species mentioned in this study is shown in the Table 5.1 with the aim to provide a better notion of the differences between genomes ("Ensembl Genomes," n.d.; "Human Genome Overview - Genome Reference Consortium," n.d.).

Table 5.1 Summary of the genetic characteristics of the major-interest species in this study.

Name	*Reference*	*Chromosomes (Ploidy)*	*Genes*	*Mbp*
Homo sapiens	NC_000001-NC_000024	23 (di)	79,441	3,272
Triticum aestivum	GCA_900519105.1	7 (3x) (poly)	146,597	14,547
Arabidopsis thaliana	GCA_000001735.1	5 (di)	55,398	135.67
Brachypodium distachyon	GCA_000005505.4	5 (di)	53,787	270.74
Fusarium graminearum	GCA_900044135.1	**4** (ha)	14,898	38.0
Bacillus subtilis-168	CP010052.1	**1** (ha)	4,424	4.2

Mbp: mega base pairs. Ploidy: haploid (ha), diploid (di), polyploid (poly).

Today, the whole genome sequencing (WGS) of a bacterium can be completed by a sequencing machine, such as Illumina Hi-seq, in three days, which gives a non-curated sequence data -raw data. Afterwards, the pipeline or the steps of cleaning and extracting the data could take less than a week to be completed. Pipelines are steps that guide the bioinformaticians in the cleaning, analysis and interpretation of the sequenced reads. This cleaned data is utilised for subsequent study of the genomic characteristics of the organism, which is called data mining. Many authors, which are in the field of bioinformatics, had published summaries of pipelines or steps to pursue a correct data analysis. This is because the information obtained from the sequencers, which is huge, depends not only in the experimental design and material isolation, but in the subsequent analysis (Brettin et al., 2015; Edwards and Holt, 2013; Huang et al., 2019; Petit and Read, 2020; Quijada et al., 2019).

Some other authors ("Biostars," 2011; Davis-Turak et al., 2017; Edwards and Holt, 2013; Bao et al., 2011) examined commercial (Geneious ®) and open software pipelines and its software. Nowadays, many universities and important research centres hold whole areas of bioinformatics, as well as bioinformatician services. A few examples of this are: a) CLIMB project (Cloud Infrastructure for Microbial Bioinformatics) -Warwick, Birmingham, Cardiff, Swansea, Bath and Leicester Universities and the QIB-; b) Technology Platforms in JIC, which uses a wide range of bioinformatic software and tools; c) GenoToul bioinformatics platform (France), they display a list of the bioinformatic tools currently in use by the service; University of Florida Research Computing in USA, which also have a webpage displaying the software currently in use ("CLIMB | Cloud Infrastructure for Microbial Bioinformatics," n.d.; "GenoToul," n.d.; "Computational techniques-JIC," n.d.; University of Florida, n.d.). Nonetheless, it is important to stress that, an important point mentioned by Davis-Turak et al. (2017) is the relation of the increase on the power of the sequencers plus the drop in the prices for sequencing, which has produced a bottleneck on analysis of high throughput (HT) data and needs adequate consistency and reproducibility by the researchers.

Returning to the point about bacterial genomes, much of the research has been carried out to understand how the phenotypes of the microorganisms compared with their genotype. In this way, microorganisms such as bacteria are very diverse and are found everywhere. Their size (~μm) and their ability to produce a great diversity of metabolites make them a subject of interest in the research and industrial field. Additionally, bacterial genomes possess some advantages compared with the eukaryotes. They are made of one chromosome with a length that vary from 0.1Mbp to less than 10 Mbp, they hold as little as few hundreds to several thousand genes only (Kuo et al., 2009) and they are haploid organisms.

WGS of bacteria can be, then, implemented as a powerful tool to determine identities of strains, to find evidence on variation between strains, to identify antimicrobial genes and new industrial's importance traits and proteins, to determine host-microorganisms interactions, modified strains and others (Borriss et al., 2018; Fan et al., 2018; "GWAS Central - Home," n.d.; McArthur et al., 2013; Scholthof et al., 2018; Tassios and Moran-Gilad, 2018; The Arabidopsis Genome Initiative, 2000).

In other words, bacterial comparative genomics, is used to determine these fine characteristics between strains, to differentiate between resistant and susceptible strains on outbreaks (Lewis et al., 2010), to investigate natural products (Seipke et al., 2012) and to determine the characteristics of bacterial-biocontrol agents (Fan et al., 2018; Pandin et al., 2018; Susič et al., 2020).

Aims and Objectives

In Chapter 3 and 4 assays demonstrated that, perhaps the three *Bacillus* strains were firstly classified as the same type and the same genus of bacteria, they are strikingly different in their response as control agent of the *F. graminearum*. Furthermore, they showed significant differences in their ability to improve the defence response of plants infected with pathogens, as well as, in their PGP activity.

The aim of the work described in this chapter was to understand the genomic differences between isolates. To achieve this, the main objectives were:

1. Isolate the bacterial genomes in home laboratory and sequence them by Exeter Sequencing Service (ESS) using short reads technology Illumina Hi-seq sequencer (paired reads) to produce NGS raw data

2. To analyse the bacterial genomes using another pipeline, which included trimming the data, assembling and comparing using Mauve and annotating using the online tool RAST. MUMmer was used to align the genomes and compared similarity of genes (dnadiff) (Aziz et al., 2008; Darling et al., 2010; Marçais et al., 2018).

4. Geneious® was used to mine data and checkpoints on data, with the aim of finding genes, generating graphic views of the genomes, designing primers and to assess the sequenced PCR products of the genes found during the comparative genome process and in previous studies (Baysal et al., 2013, 2008, *Geneious 10.4*, 2017).

Results

5.1 Genome isolation and sequencing

5.1.1 Isolation of genomic DNA from QST713, FZB24 and EU07 strains

The agarose gel of the whole genome (gDNA) of the *Bacillus* strains showed good quality and high molecular weight bands at the top of the gel. No contamination as presence of low weight bands and degradation were observed (Figure 5.1). The strain QST713 achieved a concentration of 16.7 ng/µl and a quality of 2.06, FZB24 6.9 ng/µl with 1.91 and EU07 20.2 ng/µl with of 2.04 (260/280). These samples were sent to Exeter to be sequenced.

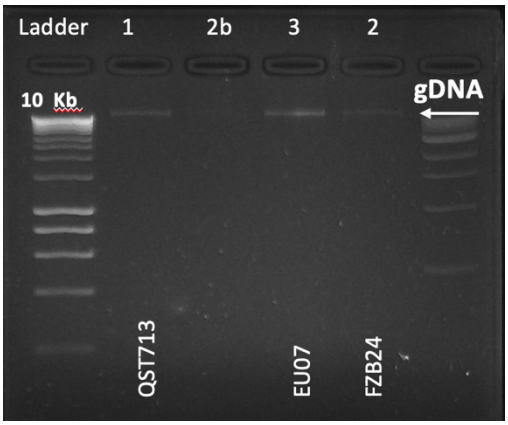


Figure 5.1 Gel of gDNA of the Bacillus strains on electrophoresis gel (1%) and Hyperladder™ 1kb ladder.

Bands of the genomes were observed at the top of the gel after 40 min (75V). For the Illumina® sequencer, the lines 1, 2 and 3 were used.

5.1.2 Analysis of NGS raw data results for the genomes of the Bacillus strains

Sequenced raw data from NGS service is delivered in the form of two fasta quality files-fastq (R1 and R2) per sample, which corresponded to the sequenced reads, forward and its complementary reverse. Raw data included the short reads, or sequenced data, and the adapters used by the sequencer to anchor them in the flow cell in which all the reactions occurred.

Inspecting the MultiQC and FastaQC quality reports, it was observed that, globally, raw data improved their quality after trimmed the adapters, going from low (Phre score <20) to medium (Phre score 20-27.5) and high quality (Phre score 27.5-40) in the reverse (R2) and forward (R1) reads, accordingly (Andrews and Andrews, 2016; Ewels et al., 2016). The Phre score is a measuring of the quality of the identification of the nucleobases generated in the sequencing. Similarly, the quality of the reads improved in the trimmed data, as observed in the Sequence Quality histograms and other FastQC reports -including per sequence quality, per sequence GC content, per base N content- which showed good quality in the trimmed data (Data not shown). These results gave confidence that, the downstream processing of the data, will give

reliable results. Additionally, FastQC reports revealed that the content of the sequences was highly pure with no contaminants from other organisms, which agrees with the Prokka assembler's report.

Therefore, in the Table 5.2, a summary of assembled data results was presented. The assemble data are the trimmed data that was compiled and ordered into contigs. Contigs are fragments of ordered and high confidence nucleotides. The colours in the table were generated by QUAST and represent the likelihood of the strains by each characteristic. Similar colours (or same) mean similar characteristics. The assembled data shows that the strain QST713 and EU07 were of similar length (4.2 Mbp) and similar N50 (~54 Kbp), contrary to the FZB24 with length is 3.9 Mbp and a N50 of 171.5 Kbp % (Andrews and Andrews, 2016; Ewels et al., 2016).

Table 5.2 *General statistics of the bacterial genomic data summarised with QUAST of the assembled data.*

Sample Name	N50 (Kbp)	N75 (Kbp)	Largest contig (Kbp)	Genome length (Mbp)
EU07	54.2bp	30.6bp	199.5bp	4.2bp
QST713	53.6bp	29.6bp	225.0bp	4.2bp
FZB24	171.5bp	98.5bp	578.3bp	3.9bp

Note: Gradient colour produced by QUAST, they reflect the similarity between samples per column description. Kbp: Kilobase pairs, Mbp: Megabase pairs.

N50: Defined as the minimum contig length needed to cover 50% of the genome, also called as a fragmentation of the assemble statistic.

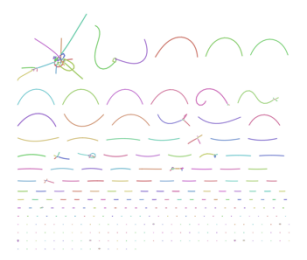
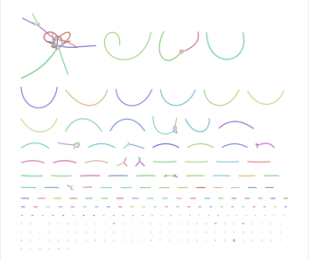
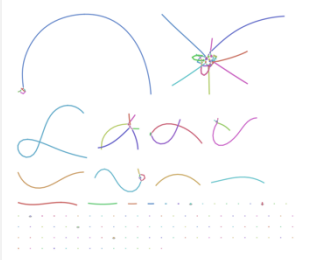
N75: Same of above, to cover the 75% of the genome.

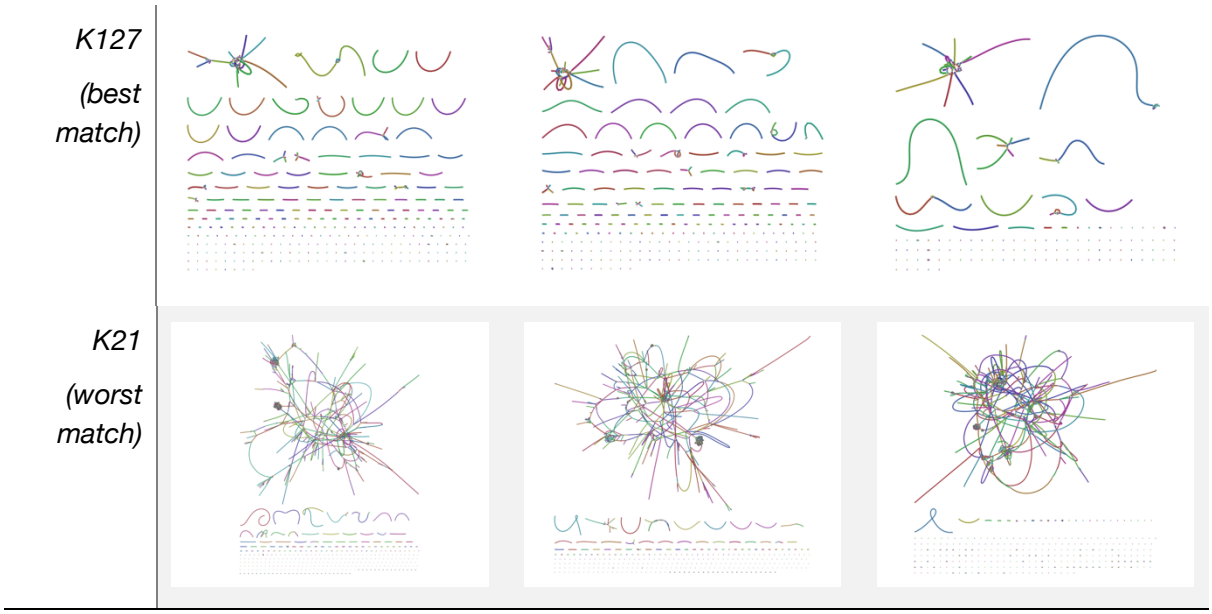
Moreover, interestingly, all the bacterial genomes shown similar %GC (46-47), similar rated of duplicate reads (21-25%) and sequences per millions (0.7 – 0.9) in the trimmed data (data not shown).

The draft genomes of the strains were assembled *de novo* with SPAdes (3.11.0), which uses K-mer number (typically 127, 99, 77, 55, 33 and 21), producing contigs and scaffolds. K-mer is the number of sequences of length K, which are possible to produce in a specific biological sequence. Spades generated assemblies of the genomes and calculates the best K-mer number, at the same time generated graphics that represent those assemblies. In the case of the three *Bacillus* strains, a K-mer of 21 produced very tangled sequence with many connections within the genomes (Table 5.3).

The assembled graphs, visualised by Bandage algorisms, are displayed in Table 5.3. The first row shows the final graph which are similar to the graphs with K-mer 127 (second row) and the last row displays the graphs with worse assembly which correspond with a K-mer of 21 (Bankevich et al., 2012; Wick et al., 2015).

Table 5.3 Bandage visualisation of assembly graphics generated by SPAdes in the assembling de novo process. Final assembly graph and by K-mer 127, as best match.

K-mer number	***QST713***	***EU07***	***FZB24***
Assembly graph			



Later, the assemble genomes were annotated with Prokka (Seemann, 2014). Annotation is the process of identification of the location of genes and coding regions on the genome. A summary of the annotation is displayed in the Table 5.4.

Table 5.4 *Summary of the statistics of features of bacterial genomes annotated with Prokka.*

	QST713	EU07	FZB24
Contigs	291	260	143
Bases	4,190,714	4,187,362	3,898,192
rRNA	13	12	11
Genes	4,161	4,161	3,817
CDS	4,063	4,064	3,721
tmRNA	1	1	1
tRNA	84	84	84
%GC	47-46	46-46	47-46

In the same manner, the strains QST713 and EU07, displayed very similar statistics and far from the strain FZB24 in their number of contigs. Other features such as RNA structures, genes and %GC (guanine-cytosine content) were remarkably similar. Usually, to do the annotation process on NGS, a reference genome is used to obtain the sequences and genes data. In the case of *Bacillus subtilis*-like bacteria, the reference genome is *Bacillus subtilis* strain 168.

Later on, the assembly was visualised using Krona. Krona shows the taxonomic classification of the bacteria (Ondov et al., 2011). Krona snapshots confirmed that the FZB24 strain belongs to a different group than the QST713 and EU07 strains. FZB24 genome is a *Bacillus* of the group *Bacillus amyloliquefaciens* (99%) with identity of *Bacillus amyloliquefaciens* (17%) and *Bacillus velezensis* (14%). QST713 genome showed identity of *Bacillus velezensis* (36%) *Bacillus amyloliquefaciens* (26%) and others *Bacillus* (Pc3 -24%-, BH072 -2%-) and EU07 is a *Bacillus* with identity of *Bacillus velezensis* (51%) *Bacillus amyloliquefaciens* (20%) and others *Bacillus* (Pc3 -14%-, BH072 -2%-) (Annexes/ data not shown).This result corresponds with the statistics in the assemble report in Table 5.2, which shows that FZB24 features are different to QST713 and EU07 and these last ones, are very similar in N50, N75, largest contig and genome length (Mbp) (Table 5.4).

The annotated .gbk files were concatenated using Geneious ®. The .gbk file is a GenBank format file that stores genome information. The linear views of the genomes were called and used for comparison purposes (Figure 5.3). Geneious ® ordered the genomes using the length of the contigs, which render a simplified view of the genome. The criteria of the ordering of the contigs differs with every specific scenario

to be analysed. Here, they were ordered by length. This way, the data complements the abovementioned results and helps to visualized differences between genomes in a graphical way.

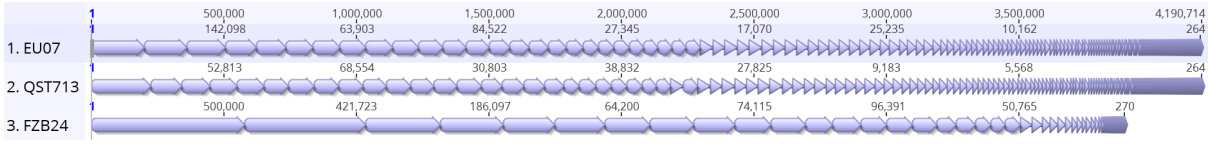


Figure 5.2 Sequence view of the three bacterial genomes using Geneious ®.

Linear genomes of the *Bacillus* strains displaying the relative length of the contigs by strain. Each segment represents a contig. Each fragmented line (lilac) corresponds to each genome, in order, QST713, FZB24 and EU07. The numbers at the top (light lilac highlighted) represent the relative length of the whole genome, the numbers over each genome represent the length on the specific contig. The bigger and the smaller contig showed in the graph are 500,000 and 270 nucleotides long respectively, in the strain FZB24.

5.2 Comparative genomics

5.2.1 Comparative genomics of the three Bacillus strains using Mummer3

In a first approach, the *Bacillus* strains sequences were compared using dnadiff from the command-line MUMmer modular package (Medema et al., 2015), the results are displayed in Table 5.5. Globally, the aligned sequences reached a high % of alignment between them. EU07 aligned 98.88% with QST713 and 89.35% with FZB24. In the same way, when FZB24 was aligned with QST713 and EU07, the % or alignment was of ~94% for both strains. finally, the alignment of QST713 to EU07 shows a 99.31% of alignment. All these alignments showed a 98.69 – 99.99% of sequence identity between them.

In the same fashion, *Bacillus* genomes were aligned using reference genomes*: B. amyloliquefaciens* ALB79, *B. velezensis* CFSAN034339, *B. velezensis* CH13, *B. velezensis* FZB42 and *B. velezensis* CC178 (Table 5.6).

It is remarkable that the strain FZB24 aligned a 99.95% with the strain *B. velezensis* FZB42 with 99.99% of sequence identity. The QST713 aligned 99.98% with *B. velezensis* CFSAN034339 as well the EU07, both with 99.99% of sequence identity.

Table 5.5 Supercontigs and data comparison obtained using dnadiff from the MUMMer package for QST713, FZB24 and EU07 strains.

Query sequence	***Aligned***	***Aligned (%)***	***Unaligned***	***Unaligned (%)***	***Reference sequence***	***Aligned***	***Aligned (%)***	***Unaligned***	***Unaligned (%)***	***Sequence identity***
EU07	4,088,690	100	95	0	EU07	4,088,690	100	95	0	100
EU07	3,649,178	94.97	193,278	5.03	FZB24	3,653,433	89.35	435,352	10.65	98.69
EU07	4,042,844	99.31	28,264	0.69	QST713	4,043,028	98.88	45,757	1.12	99.99
FZB24	3,842,360	100	96	0	FZB24	3,842,360	100	96	0	100
FZB24	3,649,116	89.63	421,992	10.37	QST713	3,645,753	94.88	196,703	5.12	98.69
FZB24	3,653,187	89.35	435,598	10.65	EU07	3,649,008	94.97	193,448	5.03	98.69
QST713	4,071,014	100	94	0	QST713	4,071,014	100	94	0	10
QST713	4,043,082	98.88	45,703	1.12	EU07	4,042,902	99.31	28,206	0.69	99.99
QST713	3,645,930	94.89	196,526	5.11	FZB24	3,649,372	89.64	421,736	10.36	98.7

Mummer reports genomes as supercontigs.fna() files.

Table 5.6 Supercontigs and data comparison obtained using dnadiff from the MUMMer package for QST713, FZB24 and EU07 strains against some reference genomes.

Query sequence	***Aligned***	***Aligned (%)***	***Unaligned***	***Unaligned (%)***	***Reference sequence***	***Aligned***	***Aligned (%)***	***Unaligned***	***Unaligned (%)***	***Sequence identity***
EU07	4,088,690	100	95	0	EU07	4,088,690	100	95	0	100
EU07	4,042,844	99.31	28,264	0.69	QST713	4,043,028	98.88	45,757	1.12	99.99
EU07	3,929,442	98.66	53,463	1.34	*B. amyloliquefaciens* ALB79	3,886,748	95.06	202,037	4.94	99.99
EU07	4,092,401	97.22	117,125	2.78	*B. velezensis* CFSAN034339	4,088,099	99.98	686	0.02	99.99
FZB24	3,842,360	100	96	0	FZB24	3,842,360	100	96	0	100
FZB24	3,852,926	99.27	28,339	0.73	*B. velezensis* CH13	3,842,352	100	104	0	99.99
FZB24	3,888,369	99.23	30,220	0.77	*B. velezensis* FZB42	3,840,646	99.95	1,810	0.05	99.99
FZB24	3,885,503	99.2	31,325	0.8	*B. velezensis* CC178	3,842,354	100	102	0	99.99
QST713	4,071,014	100	94	0	QST713	4,071,014	100	94	0	100
QST713	4,043,082	98.88	45,703	1.12	EU07	4,042,902	99.31	28,206	0.69	99.99

QST713	3,918,888	98.39	64,017	1.61	*B. amyloliquefaciens* ALB79	3,875,746	95.2	195,362	4.8	99.99
QST713	4,074,602	96.79	134,924	3.21	*B. velezensis* CFSAN034339	4,070,469	99.98	639	0.02	99.99

For reference: GCA_001709055.1 (*B. velezensis* CFSAN034339), GCA_003149795.1 (*B. amyloliquefaciens* ALB79), GCA_000015785.1 (*B. velezensis* FZB42), GCA_000494835.1 (*B. amyloliquefaciens* CC178) *B. velezensis* CC178, GCA_002002555.1 (*B. velezensis* CH13) *B. velezensis* CH13.

5.2.2 Comparative genomics of the three Bacillus *strains using Mauve*

In another approach, the *Bacillus* strains were aligned using the Mauve, which is a software for constructing multiple genome alignments in presence of large-scale evolutionary events (Darling et al., 2010). Mauve can arrange draft genomes and can reorder the contigs by a related reference genome. The Figure 5.3 is the graphical output of the three *Bacillus* strains against the reference *B. amyloliquefaciens* ALB79 (Table 5.6).

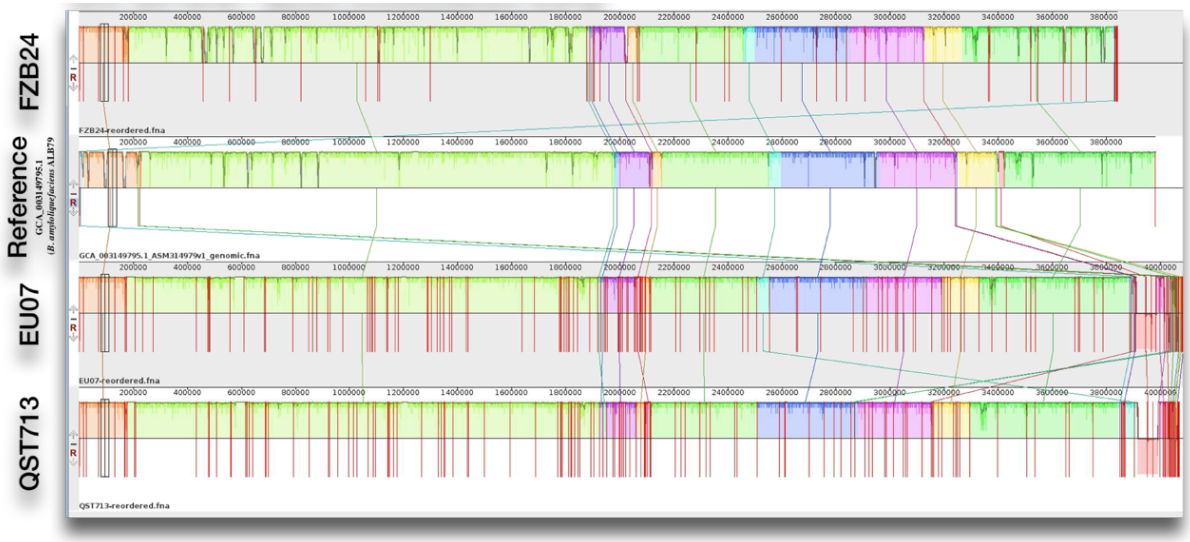


Figure 5.3 Display of the Mauve alignments of the three Bacillus genomes against a reference genome (GCA_003149795.1 (B. amyloliquefaciens ALB79).

The alignment display shows a panel for each Bacillus strain. The sequence coordinates are at scale in the top of each genome and a single black horizontal center line. Each color block represents a region of the genome, presumably homologous to others. Lines connecting colored block between strains represent homology regions. Blocks above the center line are forward direction relative to the first genome. Graphically, Mauve display shows that FZB24 is a strain totally different to EU07 and QST713 with similar arrange of homologous regions.

However, when the similarity of the strains EU07 and QST713 were examined, it is visible that many contigs/scaffolds were not aligned (smaller ones), as is observed as cluster of red lines at the end of this genomes which may represent zone of evolutionary divergency or very short Localized Co-linear Blocks (LCB).

5.2.3 Comparative genomics of the three Bacillus *strains using RAST and Seed Viewer*

Subsequently, the bacterial genomes were submitted to RAST (Aziz et al., 2008). RAST is a fully automated service for annotating prokaryote genomes on-line, it makes use of annotations across all the phylogenetic and the tree subsystems technology. As well, RAST links to the SEED viewer, a project of the Fellowship for Interpretation of Genomes (FIG) and open-source American effort. This is an integration of genomic, expression, regulatory and modelling data constructed using the SEED Project ("Home of the SEED - TheSeed," 2010).

Afterwards, a comprehensive comparison of the genomes was done using the raw data and the output gave the genes by strain and the coverage of each gene per each assembly by each set of reads. Tables with %presence, or absence, for each gene in each strain were obtained (data not shown for full set of genes) and they were sorted. Focus was done in the strain EU07, and 14 genes were selected, for those genes present in EU07 (100%) and partially present in QST713. FZB24 did not show any presence of those specific genes in the analysis. A summary of data is displayed in the Table 5.7. It displays the code of the genes (EU07_#), the percentage of coverage, the contig which the gene is located, length and the function. The last column is a prediction of the localization of the protein inside the bacterial cell using Gram Positive Cello (Yu et al., 2006) inside RAST.

In this first analysis carried out using RAST, most of the genes listed have protein products with not specified functions (hypothetical protein).

Table 5.7 Summary of the features of the top 14 genes found in bacterial comparative genomics (BCG) analysis between the strains EU07 and QST713 obtained with RAST and confirmed with Geneious ®.

Primer	*EU07**	*QST713**	*Contig*	*Start **	*Stop*	*Strand ***	*Length (bp)*	*Function*	*Location ****
EU07_01	1.00	0.00	scf_25983_10	173	3	-	171	UPF0457 protein YnzG	Cytoplasmic
EU07_02	1.00	0.00	scf_25983_44	3	230	+	228	Aspartate racemase (EC 5.1.1.13)	Memb/Cyto
EU07_03	1.00	0.11	scf_25983_132	1665	1501	-	165	hypothetical protein	Extrac/Cyto
EU07_05	1.00	0.66	scf_25983_111	4200	3880	-	321	hypothetical protein	Cytoplasmic
EU07_09	1.00	0.76	scf_25983_118	3382	2729	-	654	hypothetical protein	Cytoplasmic
EU07_18	1.00	0.87	scf_25983_137	1701	943	-	759	hypothetical protein	Cytoplasmic
EU07_20	1.00	0.88	scf_25983_91	4532	4858	+	327	hypothetical protein	Membrane
EU07_26	1.00	0.92	scf_25983_118	4006	3488	-	519	hypothetical protein	Cytoplasmic
EU07_27	1.00	0.93	scf_25983_120	881	3	-	879	Response regulator aspartate phosphatase I	Cytoplasmic
EU07_31	1.00	0.95	scf_25983_144	1953	1387	-	567	hypothetical protein	Cytoplasmic
EU07_33	1.00	0.96	scf_25983_102	4630	4124	-	507	Dihydrofolate reductase (EC 1.5.1.3)	Cyto/Extra
EU07_35	1.00	0.96	scf_25983_111	154	2	-	153	Ribonucleotide reductase of class Ib (aerobic), alpha subunit (EC 1.17.4.1)	Extracelullar
EU07_41	1.00	0.99	scf_25983_113	4904	3300	-	1605	hypothetical protein	Membrane
EU07_42	1.00	0.99	scf_25983_111	3447	3100	-	348	Phage protein	Membrane

Notes: * in %presence of the gene, **start site in the contig, *** Reverse (-) or Forward (+), ****Cello analysis based. Memb=membrane, Cyto= cytoplasmic, Extrac= extracellular.

Therefore, to determine if the protein products possess a functional known product, a new analysis was done using the tool Blast inside Geneious ® using the database Blastp UniprotKB / SwissProt (last relevant updated on April 22nd, 2020). It is a manually annotated, non-redundant protein sequence database. It combines information extracted from scientific literature and biocurator-evaluated computational analysis.

The results of the BLAST search are displayed in the Table 5.8. However, these functions occasionally were associated with a lower similarity (Table 5.8: Identical sites %) respect the reference. The information about the accession and the organism associated is included as well.

For instance, taking the first gene, EU07_01, (which is present in the EU07 -100% coverage- genome but not in the QST713 -0%-, according to RAST and Geneious® genome blast) it aligned with the top similar products O31800 and A41IS33. The first protein, aspartate racemase, is a component of the bacterial chromosome, associated with the gene ynzG in *B. subtilis* (168) and belongs to the protein family UPF0457 (uncharacterized protein families). The cytoplasmic product is associated with proteins of stress and DNA replication (genes *ynzF, ydaG, ynaB, rtp*) with extrachromosomal origin (phage-like) according with stringdb.org ("STRING: functional protein association networks," n.d.).

Table 5.8 Summary of the features of the top 15 genes found in bacterial comparative genomics using Blast with Geneious ® in the UniprotKB/SwissProt databases.

Primer EU07	***Length (bp)***	***Function (Blastp UniprotKB/SwissProt)***	***Accession***	***Organism***	***Coverage %***	***Identical sites %***
EU07_01	171	UPF0457 protein YnzG	A4IS33, O31800	*Bacillus subtilis, G. thermodenitrificans*	100.0	63.2 - 86.0
EU07_02	228	Aspartate racemase. L-aspartate/glutamate-specific racemase; Broad specificity amino-acid racemase YgeA.	A0A0H3JGH6, P03813	*Escherichia coli*	94.67 - 94.67	52.1 - 50.7
EU07_03	165	hypothetical protein **(no found Blastp)**	-	-	-	-
EU07_05	321	Single-stranded DNA-binding protein; Sister chromatid cohesion protein PDS5	O25841, Q04264	*Saccharomyces cerevisiae*	**56.60** - 27.36	33.3 - 41.4
EU07_09	654	hypothetical protein **(no found Blastp)**	-	-	-	-
EU07_18	759	hypothetical protein **(no found Blastp)**	-	-	-	-
EU07_20	327	hypothetical protein **(no found Blastp)**	-	-	-	-
EU07_26	519	Hypothetical protein. tRNA threonylcarbamoyladenosine biosynthesis protein TsaE.	Q49864, Q8WX94	*Mycobacterium leprae*	11.05	68.4
EU07_27	879	Response regulator aspartate phosphatase I.	P96649	*Bacillus subtilis*	99.32	77.3

EU07_31	567	Uncharacterized protein YobM; SPbeta prophage-derived uncharacterized protein YokH	O34377, O31999	*Bacillus subtilis*	98.40 - 98.40	81.6 - 82.2
EU07_33	507	Dihydrofolate reductase (EC 1.5.1.3)	P0ABQ4, P11045	*Escherichia coli, Bacillus subtilis*	97.02 - 100.00	97.02 - 100.00
EU07_35	153	Ribonucleotide reductase of class Ib (aerobic), alpha subunit (EC 1.17.4.1)	P50620; O31875	*Bacillus subtilis*	96.08 - 100.00	89.8 - 96.1
EU07_41	1605	Multidrug resistance ABC transporter ATP-binding/permease protein BmrA; Putative multidrug export ATP-binding/permease protein YgaD; Uncharacterized ABC transporter ATP-binding protein YfiB	O06967, P71082, P54718	*Bacillus subtilis*	93.26 - 95.51	28.6 - 27.1
EU07_42	348	Phage protein, SPbeta prophage-derived uncharacterized membrane protein YosE	O31884	*Bacillus subtilis*	100.0	92.2

Table 5.*9* Functional characteristics and similarity of proteins found in EU07, different from the strain QST713 using the database in ("STRING: functional protein association networks," n.d.)

Query Item	***Preferred Name***	***Organism***	***Bitscores* >60***	***Annotation***
ynzG	ynzG	*B. subtilis*	167.9	UPF0457 protein YnzG; Evidence 3- Function proposed based on presence of conserved amino acid motif, structural feature or limited homology; Product type h- **extrachromosomal origin**; Belongs to the UPF0457 family (83 aa)
ygeA	ygeA	*B. mojavensis*	259.2	Putative racemase YgeA; **Putative resistance proteins**; Protein involved in response to drug (230 aa)
ssb	kinC	*B. subtilis*	881.7	**Sporulation kinase** C; Phosphorylates the sporulation-regulatory protein spo0A (428 aa)
rapI	rapI	*B. subtilis*	787.3	**Response regulator** aspartate phosphatase I; Evidence 1a- Function experimentally demonstrated in the studied strain; Product type r- regulator; Belongs to the RAP family (391 aa)
yobM	yobM	*B. subtilis*	379.4	Uncharacterized protein YobM; Evidence 3- Function proposed based on presence of conserved amino acid motif, structural feature or limited homology; Product type h- **extrachromosomal** origin (185 aa)
folA	folB	*B. subtilis*	246.5	**Dihydroneopterin aldolase**; Catalyzes the conversion of 7,8-dihydroneopterin to 6-hydroxymethyl-7,8-dihydropterin (120 aa)
nrdEB	nrdEB	*B. subtilis*	2249.6	Ribonucleoside-diphosphate reductase NrdEB subunit alpha; Provides the precursors necessary for **DNA synthesis**. Catalyzes the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides (By similarity); Belongs to the ribonucleoside diphosphate reductase large chain family (1084 aa)

bmrA	bmrA	*B. subtilis*	1186.0	**Multidrug resistance** ABC transporter ATP-binding/permease protein BmrA; An efflux transporter able to transport Hoechst 33342, ethidium bromide, doxorubicin and a number of other drugs in vitro into inside out vesicles. The endogenous substrate is unknown. It has been suggested that NBD dimerization induced by ATP-binding causes a large conformational change responsible for substrate translocation. Transmembrane domains (TMD) form a pore in the inner membrane and the ATP-binding domain (NBD) is responsible for energy generation (Probable) (589 aa)
yosE	yosE	*Bacillus subtilis*	229.6	SPBc2 **prophage-derived** uncharacterized membrane protein YosE; Evidence 5- No homology to any previously reported sequences (115 aa)

The protein alignment of these accessions with the translate product of EU07_01 is illustrated in the Figure 5.3. The Geneious ® Blast showed that it seems to be present in other bacteria, such as *S. aureus* and *B. cereus*. It is a putative phage protein; defective phage region (extrachromosomal origin).

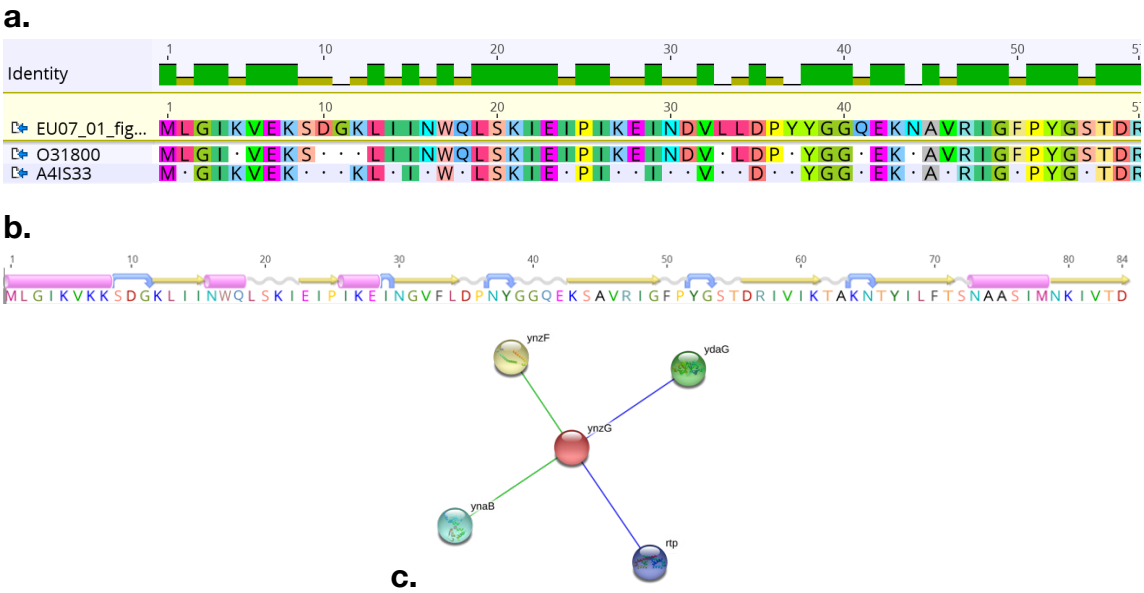


Figure 5.3 Protein product of the gene EU07_01 a putative phage protein (ynzG); defective phage region, found in EU07 but absent in QST713.

a. The protein alignment of this protein with the accession O31800 (86%) and the A4LS33 (100%) protein. The different letters correspond to different amino acids. Dots means not matches in the sequence. Top green bar represents the identity. **b.** The structure of the protein was predicted with Geneious ® (Based on the EMBOSS 6.5.7 tool Garnier). **c.** String network showing the predicted partners of the gene ynzG. Green lines mean gene on the neighbourhood and blue lines mean gene co-occurrences.

The string network as well the predicted functional of partners are displayed in Annexes for each of the genes with an associated function.

The gene EU07_02 encodes a racemases (ygeA), a protein involved in response to drugs (resistance proteins). The EU07_05 is a single-stranded DNA-binding protein (ssb), associated with ribosomal proteins (rpsF, R, D, K, rplU and others)

to recruit them to their sites of action during DNA metabolism. EU07_09, EU07_18 and EU07_20 were found to be with not specific function (hypothetical protein).

EU07_27 (rapI), which has a function in the mechanism of regulation of cellular activity. Predicted partners include phrI, immR, sacV, ydcR and others. EU07_31 is an uncharacterized protein (YobM) with characteristics of prophage-derived (YokH) related to the accessions O34377 and O31999, in *B. subtilis* (168). EU07_33 is related to the folate metabolism essential in glycine and purine synthesis (partners thyA, folC, glyA, folM and others). EU07_35 provides the precursors for DNA synthesis. Some predicted partners are yosP, nrdF, nrdIB, yosQ, for instance. The gene EU07_41 is a multidrug resistance ABC transporter ATP-binding protein BmrA, which some predicted partners: lmrA, yloB, ysaA, prsW, some of them with transport function. Lastly, EU07_42, an uncharacterised protein YosE, SPBc2 prophage-derived. It is associated with partners with similar prophage-derived function with no homology reported. Some examples are yosF, D, C, G, B and H. Finally, to summarise, these genes found in EU07 presented functions related to replication of DNA (EU07_01), precursors or repair of DNA, stress tolerance, antibiotic resistance and some are acquired as they are prophage-derived. A refined table of the genes are in the Table 5.9.

5.2.4 Comparative genomics: Confirming the Bacillus strains genes with PCR

To confirm the presence of the EU07 genes in the bacterial genomes (Table 5.7), primers were designed using Primer3 (Untergasser et al., 2012) in Geneious®. PCR products of around 200-300 bp and aligning temperatures of ~57°C were used as criteria. Those primers were used as amplicons on the three bacterial genomes. The FZB24 were found not to amplify any of the genes confirming that the FZB24 is not similar to the other two *Bacillus* strains, QST713 and EU07, as supported by the bioinformatic results (Figure 5.4).

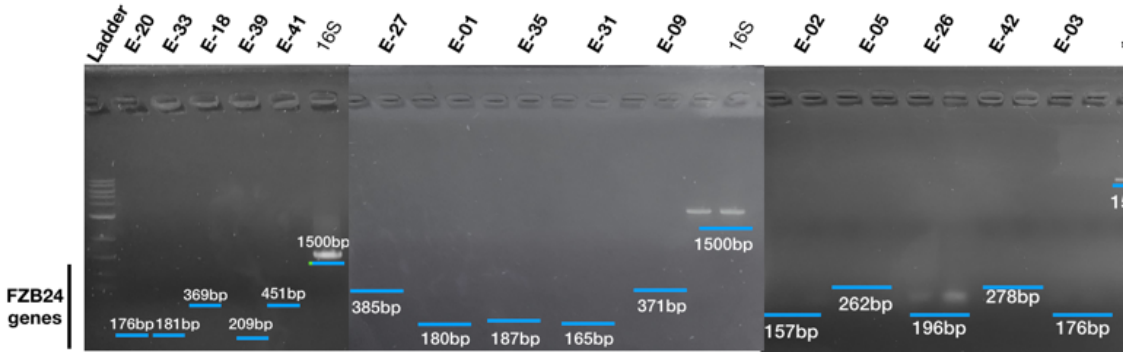


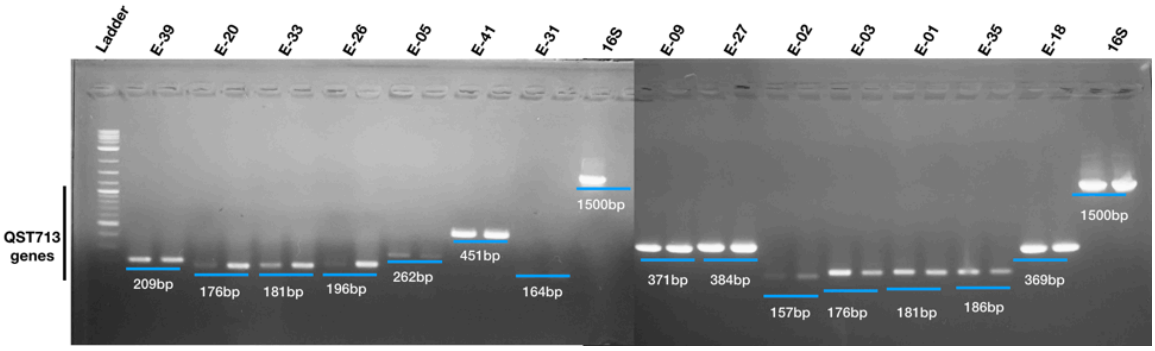
Figure 5.4 Gel of electrophoresis (1%) of the colony-PCR products from the Bacillus FZB24 (B. amyloliquefaciens).

The gel displays the amplified EU07 primers on the Bacillus FZB24. FZB24 has no amplicons with the selected primers. Only the primer 16S and E-26 showed bands (NEB 1 kb DNA Ladder). Blue lines show the duplicates of each product and the expected weight underneath.

On the other hand, when working with the strain QST713, all the primers amplified for all the genes selected (Figure 5.5 a). In this case, the amplicons of EU07_31 did not showed bands. However, the sequenced products of PCR met the expected size (bp). The sequenced products were mapped to the reference EU07, which showed match in the coordinates of the forward primer only (Figure 5.5 b), possibly because the amplified primers were there, but in a very low concentration.

Similarly, the sequenced primers of the other products of PCR from the strain QST713 were blasted against the EU07 genome in Geneious ®. Matches or partial matches were found. Similar trend was found when, the report from RAST (summary in Table 5.7) indicated genes present in EU07 assembly but not in QST713. Then a new, and final, list of selected genes was made (Table 5.8) with different levels of coverage/similarity of those genes in the strain QST713. This made evident that the fine tune results between bioinformatic tools and lab experiments at molecular biology level can differ.

a.



b.

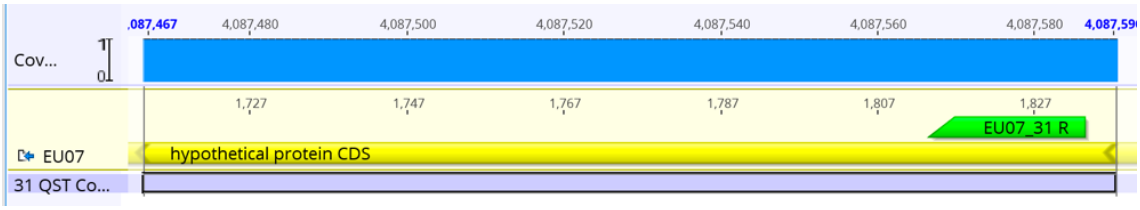


Figure 5.5 Gel of electrophoresis (1%) of the colony-PCR products from the Bacillus QST713 (B. velezensis).

a) The gel displays the amplified EU07 primers on the Bacillus QST713. QST713 amplifies for the same genes on the selected primers (NEB 1 kb DNA Ladder). Blue lines show the duplicates of each product and the expected weight underneath. b) The consensus sequence of the PCR products of the EU07-31 primer, amplified on QST713 and mapped in the EU07 genome using Geneious ®, the consensus segment –123 bp- is located between 4,087,467 – 4,087,580 coordinates, within a CDS. Green bar shows the position of the primer (reverse). Top blue band represent the coverage of the consensus sequence of the product of PCR (lilac bottom bar).

Lastly, the strain EU07 was used to test the presence of the EU07 genes with the designed primers. After running the samples on a gel, bands were observed for all the primers used (Figure 5.6). The sequenced amplicons were blasted against the EU07 genome to confirm that they belong on the specific coordinates. For example, the sequenced amplicon for EU07_33 was confirmed to be the same as the gene EU07_33. The consensus alignment (blue bar in Figure 5.6 b) was located in the middle of the CDS and roughly located by the expected product of PCR area (green blocks represent the forward and reverse primers connected by a green line).

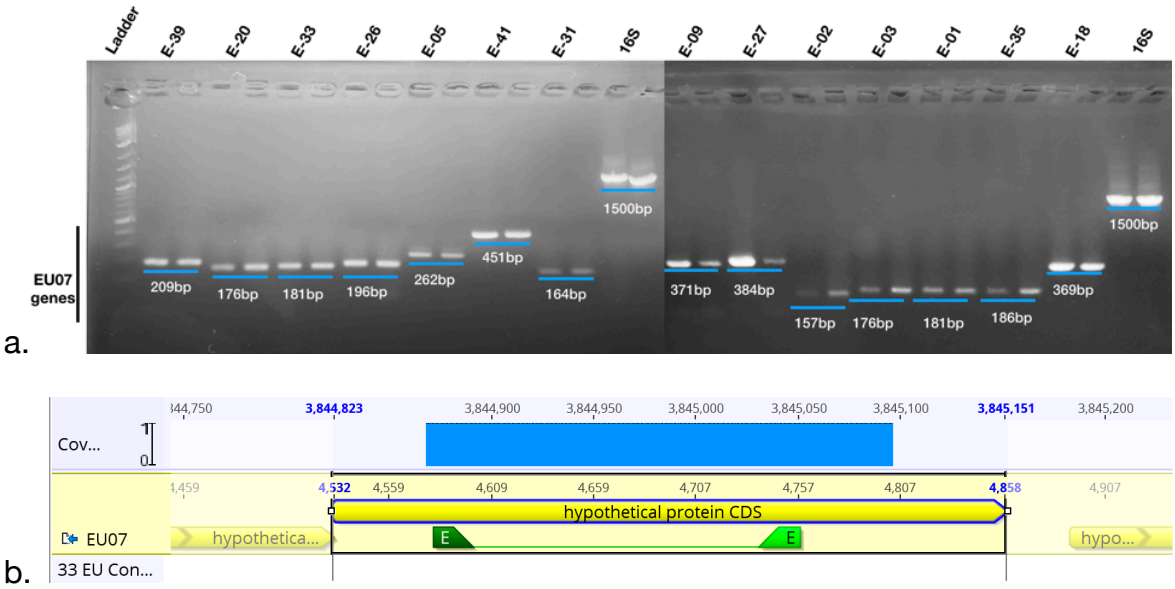


Figure 5.6 Gel of electrophoresis (1%) of the colony-PCR products from the Bacillus EU07 (B. velezensis) and view of the fragment in the EU07 genome using Geneious®.

a) The gel displays the amplified EU07 primers on the Bacillus EU07 for the selected genes (NEB 1 kb DNA Ladder). Blue lines show the duplicates of each product and the expected weight underneath. b) The consensus sequence of the PCR products of the EU07-33 gene mapped in the EU07 genome using Geneious ®, the consensus segment – 228 bp- is located between 3,844,823 – 3,845,151 coordinates, within CDS. Green bars (dark and light) show the position of the primers. Top blue band represent the coverage of the consensus sequence of the product of PCR (lilac bottom bar).

The EU07_01R, EU07_03, EU07_05, EU07_09, EU07_18, EU07_26, EU07_27, EU07_26, EU07_31R, EU07_33, EU07_41 all aligned in the expected location. The EU07_02 did not produced alignment. The EU07_35 aligned in the coordinates 3,973,622 – 3,973,833 and EU07_42 did not align in the expected location.

Globally, the results of analysis of the sequences of the PCR products to confirm that these amplicons were present on the bacterial genomes demonstrates the similarity of the strains EU07 and QST713. However, analysis of the whole genome made difference between those two strains (Table 5.7).

***Table 5.*10** *Summary of the genes associated with plasmid characteristics present in EU07 found using Geneious ® (Plasmapper, Plasmids-NEB databases).*

Sequence Name	**Name**	**Type**	**Sequence**	**Minimum**	**Maximum**	**Length**	**Direction**	**Transferred Similarity**
EU07	peroximal_target_signal1 loc	Localization	TCCAAGCTGTAG	3659747	3659758	12	forward	100.00%
EU07	araO1 reg	Regulatory Sequence	CGGCAGAAAAGT	3531936	3531947	12	forward	100.00%
EU07	araO1 reg	Regulatory Sequence	CGGCAGAAAAGT	2699634	2699645	12	forward	100.00%
EU07	araO1 reg	Regulatory Sequence	CGGCAGAAAAGT	1851342	1851353	12	forward	100.00%
EU07	EK	Gene	GATGACGACGACAAG	1737733	1737747	15	reverse	100.00%
EU07	araO1 reg	Regulatory Sequence	CGGCAGAAAAGT	1028068	1028079	12	reverse	100.00%

5.3 Phylogenetics

5.3.1 Plasmid presence in the strain EU07

Plasmids are small circular DNA chains that confer special characteristics to bacterial genome. Every plasmid has additional genes to support the survival of the bacteria in specific conditions. They are considered replicons, molecule that replicates from a single origin of replication. Although, there are specific laboratory techniques to separate the plasmids from the bacterial gDNA, for the purposes of this study, no separation was done. However, bioinformatic tools can reveal the presence of plasmid (s).

Using Geneious®, plasmid-like features were obtained doing prediction of genes with PlasMapper features (Dong et al., 2004). Plasmapper has a database of 314 features of the most common plasmids. The Table 5.10 contain the results of these annotations. Besides, blast of *B. velezensis* species reported with a plasmid were found to be 19 with lengths of 2894 – 71628 (accessions included NZ_CP022342, NZ_CP022343, NZ_CP021977, NZ_CP017776, NZ_CP030151, NZ_CP026611, NZ_CP019039 for instance). Interestingly, strains such as QST713 did not report plasmids associated.

5.3.2 Phylogenetic features of the bacterial genomes

To determine some phylogenetic features of the *Bacillus* strains, a phylogenetic tree was built using Geneious® Tree Builder. To do so, the 16S gene were extracted for the *Bacillus* strains genomes used in this study and some other selected bacterial

strains acting as references (Figure 5.7). The 16S genes were aligned and them a phylogenetic tree was called. The *Bacillus subtilis* group strains were highlighted in red. Geneious® identify this relation in the tree (Figure 5.7 a) as well in the alignment (Figure 5.7 b). A close view of the alignment supported the fact that the EU07 and QST713 are strikingly similar and they both varied from the strain 168 and FZB24 in only one nucleotide at the position 1,482.

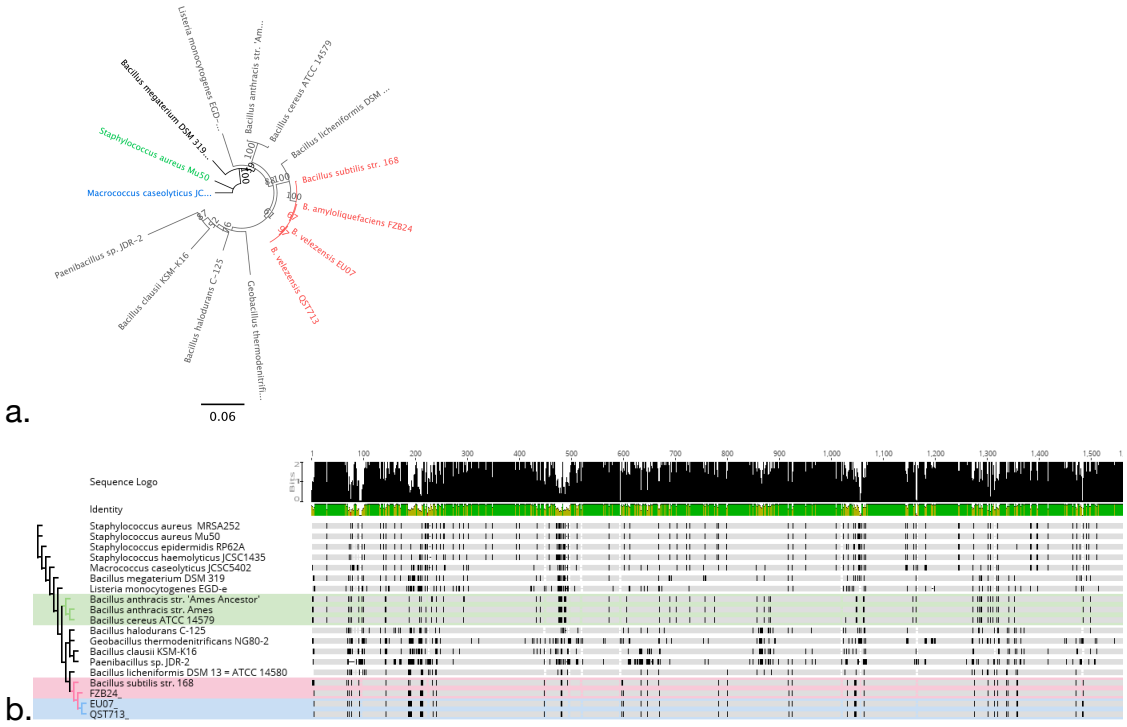


Figure 5.7 Phylogenetic tree of the three Bacillus strains and some bacteria for reference created in Geneious ® Tree builder.

a. Circular layout of the 12 bacterial genomes and the three *Bacillus* strains. In red is featuring the *Bacillus* strains and the *B. subtilis* 168, in green a relative long-distance, a coccus *S. aureus* and in blue another coccus *M. caseolyticus*. **b**. Alignment view of 16 bacterial strains, featuring *Bacillus cereus* and *B. anthracis* in green, the close related FZB24 (*B. amyloliquefaciens*) and *B. subtilis* 168, and the both *B. velesenzis* QST713 and EU07 in light-blue. The graph shows the variants between them in the 16S gene.

5.4 Metabolic and functional features

5.4.1 Important biocontrol features of the bacterial genomes

To obtain a better understanding of the special characteristics in the three *Bacillus* strains in their biocontrol capability, RAST were used to call and group the gene content of the *Bacillus* strain by features. The features represent important functional groups or subsystems. These subsystems are not a representation of the whole genome, but the genes included in the listed categories were counted. RAST provided the analysis and the data was obtained and summarised by counts in the Figure 5.8 a ("Home of the SEED - TheSeed," 2010). The three *Bacillus* strains showed similar trends in almost all the different categories. However, the categories of DNA metabolism, Phage-Plasmids and RNA metabolism differed between them (points are highlighted in orange).

In a detailed view (Figure 5.8 b), the subcategories of Phage-Prophages and RNA Processing modification presented the most relatable differences. The strain FZB24 does not have genes in the subcategory of Phage-Prophages, contrary to the strains EU07 and QST713. In the subcategory of RNA processing modification, the strain FZB24 (29) possesses more genes than EU07 and QST713 (24).

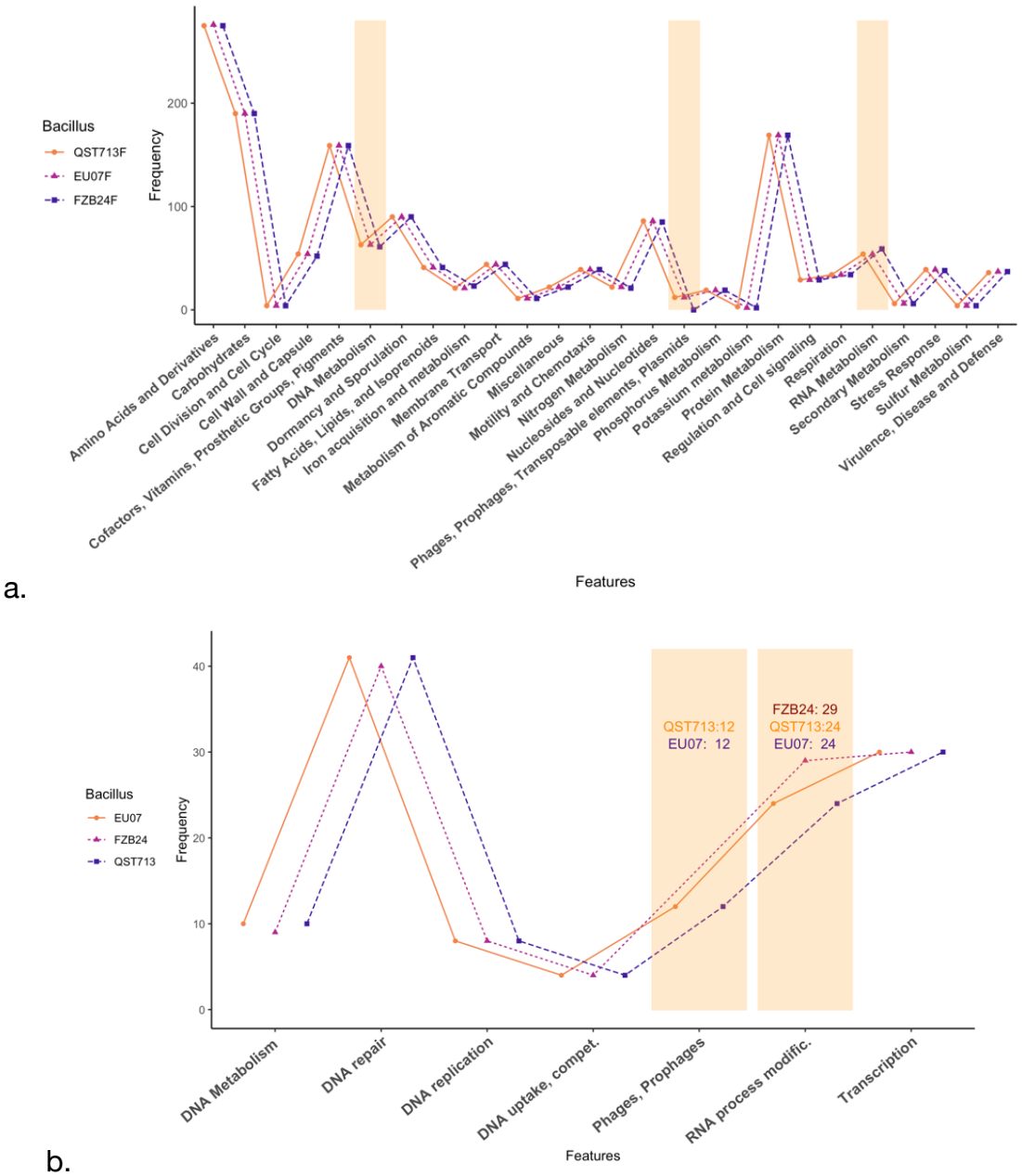


Figure 5.8 Count of the features by categories of the Bacillus strains.

a. Categories of the systems presented on the *Bacillus* strains. The highlighted area represents those categories which have differences between the strains: DNA metabolism, Phage-Prophages-Plasmids and RNA metabolism. **b.** The categories with differences were plotted by subcategories. The strain FZB24 does not have any gene in the Phage, Prophages, Plasmids category (29), but these strains have more genes in the RNA processing and modification category.

5.4.2 General metabolic features: Are these plant -growth promoting genes?

RAST was used to find out genes related to important features of the *Bacillus* strain EU07 which could be responsible of the plant protection, PGP activity and plant defence observed in the chapters 3 and 4. To do so, the SEED viewer online tool were used (Overbeek et al., 2014). The subsystem features tool was used, and the data was filtered by categories. The categories were chosen according to the importance of this study. These are plant hormones genes featuring auxin related function genes, antibacterial peptides, plant-prokaryote project and siderophores (Figure 5.9).

Surprisingly, plant hormones genes were present in EU07. Four of them were found: *trpA, trpB, trpD, trpF* (Figure 5.9a). This is an important feature, since auxin is a plant hormone which contribute in the growth of plants, a cluster in the coordinates 1,591,000 – 1,596,059 was found (Chen et al., 2007). Additionally, this set of genes are involved in other subsystems (chorismate) for the production of antibiotics. Antibacterial peptides-response (bacitracins) were present in EU07, according to RAST: bceA, graR 1, graS 1, LiaG, LiaI, liar, liaS, ydjF. A cluster of these genes were observed in the coordinates 1,725,100 – 1,729,600 (Figure 5.9b). The category of the Plant-Prokaryote project was included. This is a miscellaneous SEED category gathering sets of genes identified during plant-prokaryote studies made by the Department of Energy, USA (DoE). Some genes included in this category are responsible for iron-sulfur cluster regulation and for choline (or betaine) transport

proteins common to plants and bacteria. They were found to be distributed around the whole bacterial chromosome (Figure 5.9c).

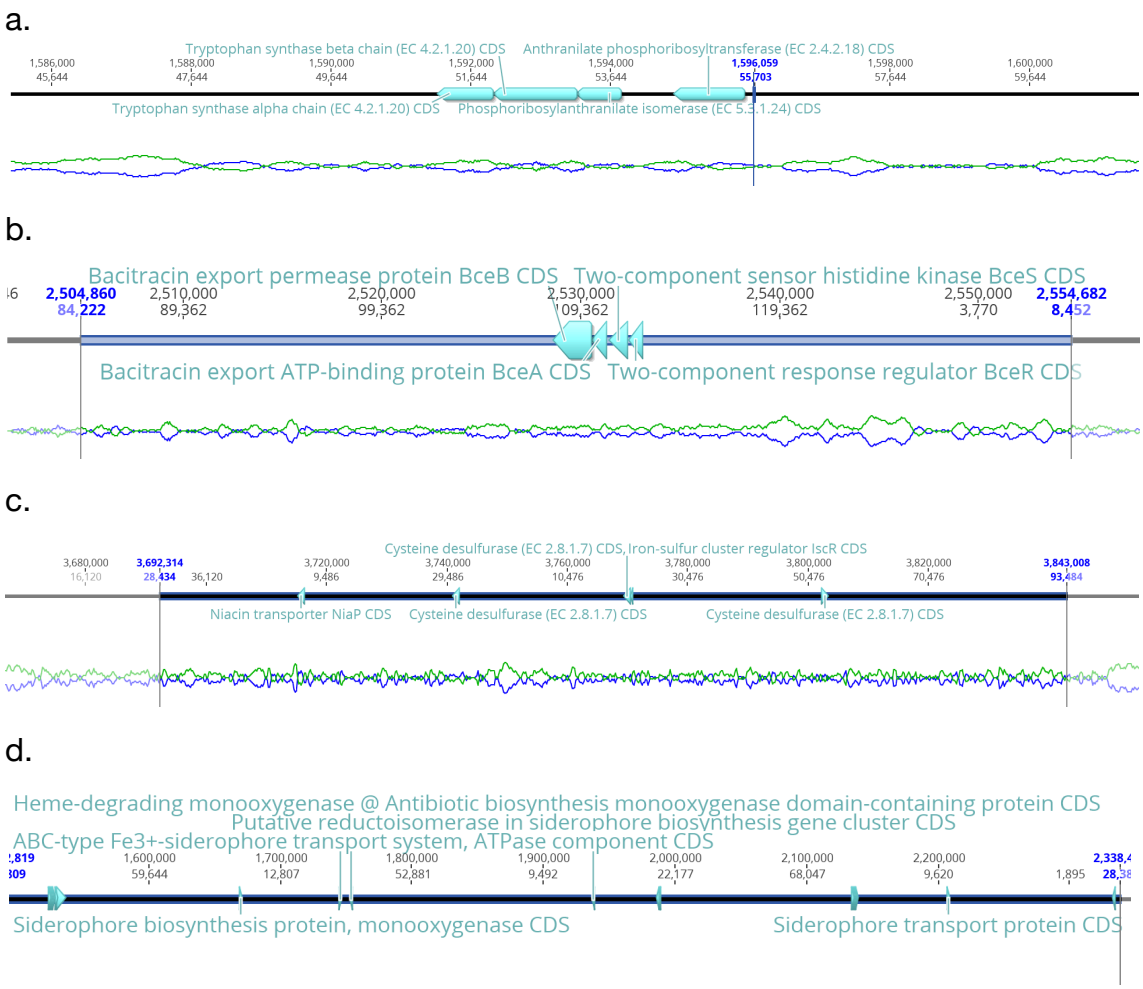


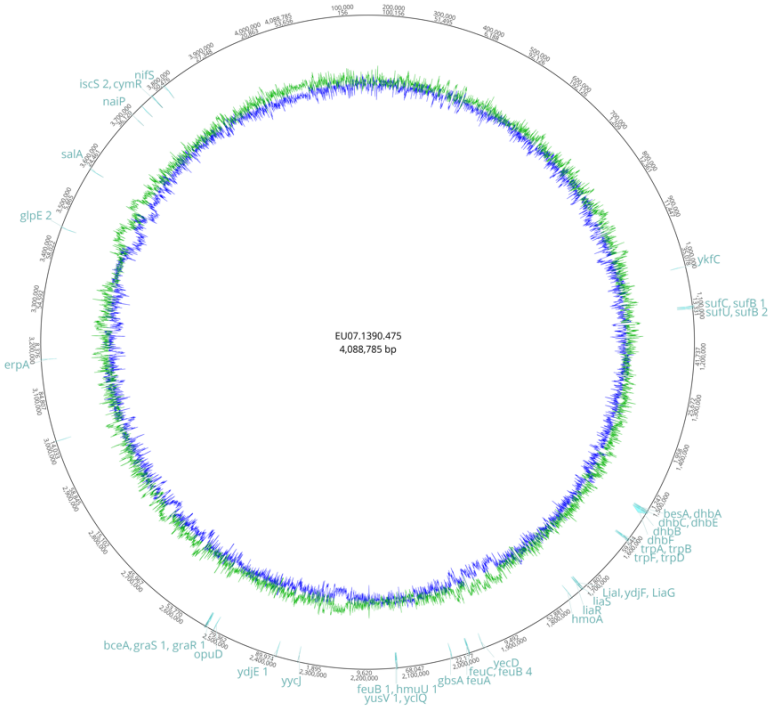
Figure 5.9 Selected subsystem protein products of the Bacillus EU07 using The Subsystems Approach to Genome Annotation -SEED- (Overbeek et al., 2014)

a. Plant hormones genes featuring auxin related function gene. **b.** Virulence and defence. **c.** Plant-Prokaryote project. **d.** Siderophores.

Finally, siderophores-related genes were included. These genes have importance as iron sequesters, increasing the availability of this element on plants ("Iron

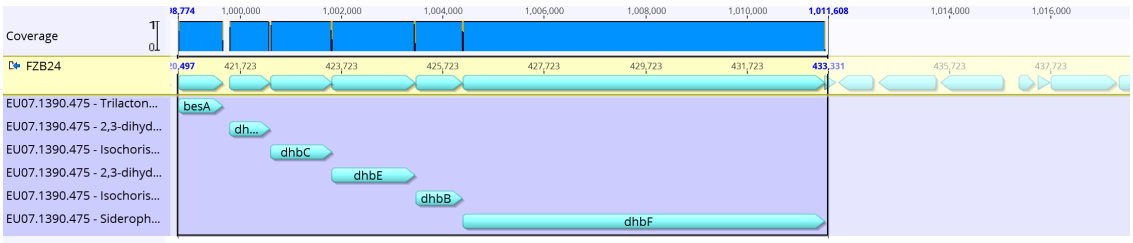
acquisition in bacteria," 2016). There was a great number of these genes in EU07: besA, dhbA, dhbB, dhbC, dhbE, dhbF, feuA, feuB 1, feuB 4, feuC, hmoA, hmuU 1, yclQ, yusV 1 and yycJ spanning through the chromosome of EU07 (Figure 5.9d).

The above genes were mapped and presented in a circular view of the bacterial chromosome, annotating them using Geneious ® (Figure 5.10), with the aim to show, graphically, the clustering of some of those genes.



a.

b.



c.

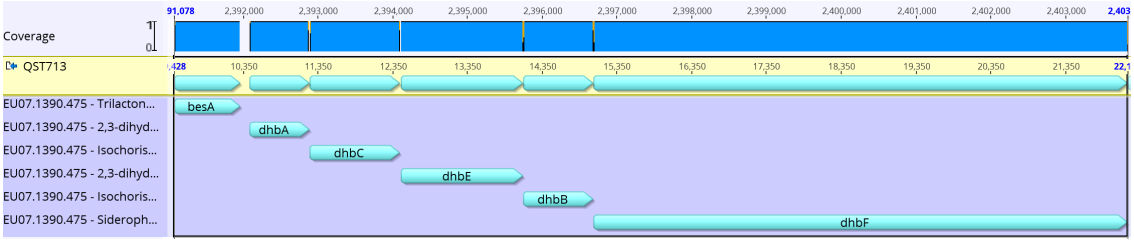


Figure 5.10 Selected subsystem protein products of the Bacillus EU07 using The Subsystems Approach to Genome Annotation -SEED- in circular genome viewed using Geneious®.

a. All the selected subsystem genes are displayed: Plant hormones -**auxin**- (trpA, trpB, trpD, trpF), **antibacterial** peptides defence -bacteriocin- (bceA, graR 1, graS 1, LiaG, LiaI, liar, liaS, ydjF), **Plant-Prokaryote** project (**trpB, trpD, trpA, trpF**, opuD, gbsA, sufB 2, ydjE 1, sufB 1, naiP, nifS, iscS 2, salA, glpE 2, ykfC, sufC, yecD, sufU, cymR, erpA) and for **siderophores** (**besA, dhbA, dhbB, dhbC, dhbE, dhbF,** feuA, feuB 1, feuB 4, feuC, hmoA, hmuU 1, yclQ, yusV 1, yycJ). The outher circle represents the nucleotides. The outside numbers represent the coordinates of the chromosome. The inside number present the position on the contig. The inside circule represent the %GC. **b)** Location of some Siderophores (highlighted in point a.) on FZB24 mapped from EU07 genome. Geneious ® showed 98.7% of pairwise identity and identical sites. **c)** Siderophores location (of highlighted in point a) on QST713 mapped from EU07 genome. Geneious ® showed 100.0% of pairwise identity and identical sites.

In the Figure 5.10b and 5.10c, a cluster of siderophores genes were extracted from the strain EU07, the corresponding genes were later mapped against FZB24 genome (5.10b) and the QST713 genome (5.10c), all the three *Bacillus* strains possess this cluster, founding similarity between these bacterial strains in this feature.

5.4.3 Antimicrobial compounds

Due to the antagonistic effect of the bacterial strains against the fungus *F. graminearum*, antimicrobial compounds were mapped to determine if they were present. Using the literature references, a list of specific bacterial antimicrobial compounds was made (table 5.11). These are frequently investigated as part of

Bacillus subtilis group bacteria. However, they do not appear under subsystems in RAST.

*Table 5.*11 *Summary of the most known antimicrobial compounds coding genes found on the different* Bacillus *strains (QST713, FZB24 and EU07)*

	EU07			***QST713***			***FZB24***		
	Name	**gene**	**Length**	**Name**	**gene**	**Length**	**Name**	**gene**	**Length**
Bacillaene (acpK)	acpK	acpK	249	acpK	acpK	249	acpK	acpK	249
Subtilisin	apr	apr	1149	apr	apr	1149	apr	apr	1149
	aprX	aprX	1329	aprX	aprX	1329	aprX	aprX	1329
Bacillaene (baeB)	baeB	baeB	693	baeB	baeB	693	baeB_1	baeB_1	693
	citB	citB	2727	citB	citB	2727	citB	citB	2727
Subtilisin	epr	epr	1746	epr	epr	1746	epr	epr	1746
Fengicyn (fenD, F, ituD)	fenF_1	fenF_1	1203	fenF_1	fenF_1	1203	fenF	fenF	1203
	fenF_2	fenF_2	1254						
	feuA	feuA	957						
	feuB_1	feuB_1	954						
	feuB_2	feuB_2	1029						
	feuB_3	feuB_3	1068						
	feuB_4	feuB_4	1008						
	feuC_1	feuC_1	1014						
	feuC_2	feuC_2	1011						
	feuC_3	feuC_3	1023						
Proteases	htrA_1	htrA_1	1353	htrA_1	htrA_1	1197	htrA_1	htrA_1	1353
	htrA_2	htrA_2	1197	htrB	htrB	1347	htrB	htrB	1362
	htrB	htrB	1347						
	leuA	leuA	1557						
	leuB	leuB	1098						
	leuC	leuC	1419						
	leuD1	leuD1	600						
	leuS	leuS	2415						
Iturin (ituB)	lgrB_1	lgrB_1	16089	lgrB_1	lgrB_1	16089			
	lgrB_2	lgrB_2	17100						
Iturin (ituC)	lgrD_1	lgrD_1	7857	lgrD_1	lgrD_1	7857	lgrD	lgrD	7860
	lgrD_2	lgrD_2	10758						
Chitinase (lytF)	lytF	lytF	1455	lytF	lytF	1455	lytF	lytF	1455
Difficidin (dfn…)	menE_1	menE_1	1464	menE_1	menE_1	1365	menE_1	menE_1	1464
Surfactins	srfAA_1	srfAA_1	10758	srfAA_1	srfAA_1	10758	srfAA	srfAA	10755
	srfAA_2	srfAA_2	7623	srfAA_2	srfAA_2	7623			
	srfAA_3	srfAA_3	2958	srfAA_3	srfAA_3	2958			
	srfAB_1	srfAB_1	10761	srfAB_1	srfAB_1	10761	srfAB	srfAB	10761
	srfAB_2	srfAB_2	6627	srfAB_2	srfAB_2	6627			
	srfAC	srfAC	3837	srfAC	srfAC	3837	srfAC	srfAC	3837
	srfAD	srfAD	732	srfAD	srfAD	732	srfAD	srfAD	732
Bacillomicyn (bmyA), Iturin (ituA)	tycC	tycC	11949	tycC	tycC	11949	tycC	tycC	11949
Cellulase (yhfE, ysdC, ytoP)	ysdC_1	ysdC_1	1074	ysdC_1	ysdC_1	1086	ysdC_1	ysdC_1	1041
	ysdC_2	ysdC_2	1041						

Those included and featured on the genomes were: bacillomycin D (bmy genes), amylocyclicin (acnA), difficidin (dfn genes), bacillaene (bae genes), chitinase like enzyme (lytF), fengycin (fen genes), cellulase like (yhf, ysd genes), protases like (germ p.e.), Iturin (itu genes), subtilisin (apr and epr genes) and surfactins (sfr genes) (Baysal et al., 2013; Bernat et al., 2016; Chan et al., 2009; Deleu et al., 2008; Gong et al., 2015; Grady et al., 2019; Luo et al., 2015; Scholz et al., 2014).

Mostly, all these genes are present on EU07 strains. FZB24 and QST713 lack of some of variants of those genes (Table 5.11). Further analysis showed that those genes absent in FZB24 were not found due to the annotation names. Blast of these genes within Geneious, demonstrated the present of analogues of these genes, with different names, highly identical. For instance, cellulases (ysdC1,2,3) were different on EU07- yto(ysdC_1), yhfE(ysdC_2), ysdC_3 -; on FZB24 - yhfE (ysdC_1), ysdC_2, yto(ysdC_3)- and on QST713 -ysdC_1, yhfE (ysdC_2), yto(ysdC_3)-.

It is remarkable that the antagonistic assays demonstrated that the overall effect of the *Bacillus* strains against the *F. graminearum* were rather similar. Then, here, the presence of antibiotic compound coding genes reported by literature are similar, correspondingly.

Discussion

5.5 Bacterial taxonomy

Bacterial whole genome sequencing popularised within last two decades due to the increased availability of the NGS (Balloux et al., 2018; Chen et al., 2007; Edwards and Holt, 2013; Pandin et al., 2018; Quijada et al., 2019; Tassios and Moran-Gilad, 2018). Here, it was sequenced the bacterial genomes of three *Bacillus* strains: QST713, FZB24 and EU07. QST713, a commercial strain, is frequently used in bacterial studies for control of plant diseases and already sequenced, accession CP025079 (Pandin et al., 2018). The bacterium FZB24, another commercial strain also known as *Bacillus amyloliquefaciens* strain SB3615, was not sequenced yet (Snyder et al., 2016), but similar strain from the same company, FZB42, was sequenced, accession CP000560.1 (Chen et al., 2007). However, for the purpose of this study and to confirm experimental results from strains used, the two above strains and EU07 were sequenced with the aim of genome comparison.

These three *Bacillus* strains have importance because they can inhibit *F. oxysporum* in tomato, according of a previous proteomic study (Baysal et al., 2013). Their approach resulted in a list of some proteins found in the cell-free supernatants and volatiles of EU07 and FZB24. They were lytic enzymes, cellulases, proteases, 1,4-b-glucanase and hydrolases. Those proteins could contribute with the degradation of pathogen cell walls. They also confirmed, using PCR, that the three *Bacillus* strains possesses antimicrobial effect genes: Fengycin (Fen D), Bacillomycin (Bmy A) and Iturin (Itu C). Finally, they found that, in the phylogenetic tree analysis of the

three *Bacillus*, FZB24 and EU07 grouped together and all the three strains were in the same group (Baysal et al., 2013). Here, using WGS, it was found that the three *Bacillus* strains are not the same. Henceforth, genotypically, the strain EU07 is more similar to QST713 than FZB24.

Results showed that the strain FZB24 is classified as a *B. amyloliquefaciens*, 99.95% similar to FZB42, but they are not the same strains. It is important to point out that some confusion might arise since these strains are commercialised with similar commercial names by several companies, through acquisitions and takeovers of companies over the time (Krebs et al., 1998; "Our crop protection products," n.d.; Snyder et al., 2016; Yao et al., 2006). Surprisingly, the strain QST713 and EU07 are the same type, *B. velezensis*, with a high similarity between them. Interestingly, our results in the antagonistic assays demonstrated that EU07 and FZB24 possess the best effect against *F. graminearum* with high confidence, similar to Baysal et al. (2013). In contrast, *in vivo* assays showed that the strains QST713 and EU07 possessed the better results overall, but EU07 is the strain with best performance.

Likewise, some authors (Dunlap et al., 2016; Fan et al., 2018, 2017) had made a distinction between the strains *B. amyloliquefaciens* and *B. velezensis*. First, it was suggested that *B. amyloliquefaciens*, *B. methylotrophicus*, *B. amyloliquefaciens* subsp. *plantarum* and *B. oryzicola* may be reclassified as *B. velezensis* (Dunlap et al., 2016). However, in a more recent publication, Fan et al. (2017) suggested that

B. amyloliquefaciens, B. velezensis, and *B. siamensis* form the Operational Group *B. amyloliquefaciens* within the *B. subtilis* Species Complex. Hence, the classification of these bacteria inside this lineage (Bacteria, Terrabacteria group, Firmicutes, Bacilli, Bacillales, Bacillaceae, *Bacillus subtilis* group). To expand on this matter, according to the taxonomic browser in NCBI, *Bacillus subtilis* group contains *B. amyloliquefaciens* group and *B. mojavensis* group between other species -*Bacillus subtilis* p.e.- ("Taxonomy browser (Bacillus)," n.d.). WGS demonstrated that the study of genotypic traits in bacterial genomes are more comprehensive than gene by gene analysis, as traditionally was made one or two decades ago, employing polymorphism and biochemical analysis of the bacterial strains.

5.6 Bacterial genomics: plant growth activity gene content

The WGS data were of high quality on this study, sufficient to perform downstream processing data and not presence of contaminants from other organisms were found.

Likewise, Pandin et al. (2018) made WG comparison of QST713 and FZB42, among others strains (Table 1) and they concentrated in identifing genes involved in biofilm formation and swarming as assets for root colonization, metabolite clusters and a biocontrol effect on the green mould disease (*T. aggressivum*) in *Agaricus bisporus* mushrooms crops. In this fashion, they found that the presence of the bacteria in

soil infected with *T. aggressivum* improved the yield compared with infected and non-treated soils, but not significant differences were observed on those treated or not with the *Bacillus* suspension without the pathogen. Interestingly, they made observations on the morphology of the pellicles (or biofilm morphology) and in the swarming (spreading of the bacteria) compared with other *Bacillus* strains (strain 168 and FZB42 p.e.). They found differences.

Here, despite these studies, the research was focused on observations made during experimentation, instead. It was noted that the swarming of the FZB24, QST713 and EU07 differed between them. The pellicle formation was also different between the strains, with more similarity between QST713 and EU07 (data not shown). Blast analysis was done to look for these genes (Pandin et al., 2018) determining that FZB24 and EU07 possess them equally.

Historically, around 15 years ago, biofilm formation was studied widely due to it was thought to be responsible for the plant protection and disease control on plants by *Bacillus* sp. First studies focused on proteomics, shedding light in the transporter of proteins in membranes, endospore formation and vegetative growth topics (Bendtsen et al., 2005; Davidson et al., 2008; Errington, 2003; Eymann et al., 2004; Hecker, 2003; Tjalsma et al., 2004). At that time, the main aim was to understand how *Bacillus subtilis* produced metabolites that were able to translocate to the outer membrane with multiple functions, mainly in antimicrobial functions and microbial physiology. Likewise, *B. subtilis* was study in their swarming and endospore

formation. For instance, spore formation is important to understand how bacteria cope in starvation and about survival strategies such as production of scavenging enzymes, genetic material and the production of antibiotics (Errington, 2003). Additionally, swarming studies took place, due to the consideration that surface migration of the cells contribute with the fixation of the bacterium aided by surfactant production and increasing in flagellated-like cells (Kearns and Losick, 2003). Moreover, the biofilm formation contributed with this trait, they found. A study by Branda et al. (2006) showed that the component of the matrix on the biofilm is made of a protein component (TasA) and a complex sugar, producing the pellicle. Davidson et al. (2008) added that transport system ATP-binding cassette has major functions in export virulence factors, viability and pathogenicity, giving as example the siderophores, which can scavenge iron from the environment from an insoluble form (Fe^{3+}), then importers mediated the intake of those nutrients and exporters secrete molecules such as lipids, proteins, peptides, etc. Some others are involved in mRNA and DNA repair. In an important work made by Beauregard et al. (2013) it was found that exist a relation between the sugars secreted by plants (*A. thaliana*) and the induction on biofilm formation by *B. subtilis*. The type of polysaccharide increased the chance of production of biofilm and is conserved between species. These sugars are signals for the kinases that controlled the master regulator Spo0A and at the same time these sugars are source for the synthesis of the biofilm matrix. Eventually, Vlamakis et al. (2013) reviewed the

subject and concluded that *Bacillus* species can lost their ability to swarm in laboratories compared with the wild types and these matrices are aided by surfactin to promote the colonization on roots. Observations of transformed bacterial cells - expressing yellow fluorescent protein (*yfp*)- observed under microscope, revealed bacteria attached to roots by biofilm which at the same time induce the systemic resistance (ISR) in plants. Because the system for biofilm production has several inputs, it is believed that many conditions could trigger its formation. Finally, Stefanic et al. (2015) found that bacteria which swarm merged in Petri dish and in roots (*At*) also showed relatedness of at least 99.9% between of bacteria species.

Here, in this study, data mining using Geneious ® contributed to learn that the *Bacillus* strains have genes that are necessary for the biofilm formation and antimicrobial compounds (Pandin et al., 2018). EU07 possesses those same genes. Our set of experiments did not determine colonization of *Bacillus* cell to roots in plants. Nonetheless, it was found that plants with soil saturated with bacterial solutions (drenched) presented different developmental rates and immuno-response to pathogens (Chapter 4). Presumably, because of the interaction *Bacillus*-roots or for induction in leave surfaces. Besides, here the *Bacillus* strains hold genes related to the auxin biosynthesis according to RAST. Furthermore, genes related to siderophores were found (21) and a cluster of 6 genes were found for Bacillibactin (Figure 11).

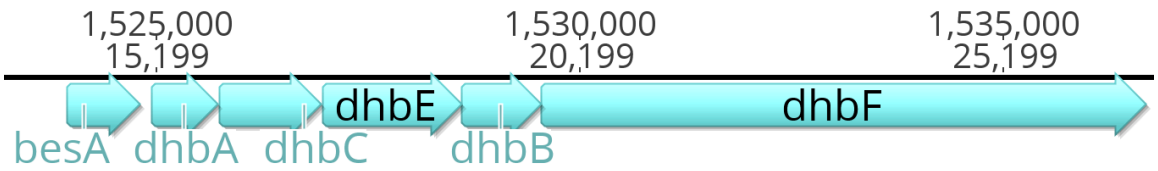


Figure 5.11 Cluster of some siderophores genes in EU07 found using Geneious®.

EU07 has around 21 genes associated to siderophores (Figure 5.6a). A cluster of those are found in the coordinates 1,523,940 – 1,536,680.

Overall, strain EU07 showed that it possesses genes necessary for plant growth promoter activity. Similar to the studied strains QT713 and FZB24, they have the potential to interact with plant, adhere to the roots and deliver mechanism for which the plants receive benefits. Special studies, with suitable-modified strains and equipment, need to be made to elucidate these mechanisms on residual presence of the bacteria on roots, number of cells, exudates secretion on roots, biofilm secretion, for instance. Nonetheless, according to the genome content, which is >99% similar between these strains, it could be expected that EU07 has equal response in plants as the QST713, hence it was demonstrated in the antagonistic and PGP activity assays that this is not the case.

5.7 Bacterial genomics: antimicrobial compounds gene content

Previously, *Bacillus* strains QST713 and FZB24 were studied in their antimicrobial genes content (Baysal et al., 2013, 2008; Fan et al., 2018; Pandin et al., 2018). Baysal et al. (2013, 2008) demonstrated that EU07 equally possesses some antimicrobial genes found in the abovementioned strains.

Many studies have looked into the function of those and other antibacterial compounds. For instance fengycin, is a lipopeptide with membrane permeabilization function on *Fusarium* sp (Patel et al., 2011). The strain JK6 possesses a surfactin lipopeptide able to suppress the tomato bacterial wilt (Xiong et al., 2015). Recently, Li et al. (2019) found that the fengycin and surfactin protect grapevine from downy mildew and activating the defence genes. Iturin A and plipastatin A were found to have *F. graminearum* spore inhibition growth (Gong et al., 2015). Additionally, other compounds like bacillomycin and bacteriocins possess antimicrobial effects against fungal pathogens. For instance, Zhang et al. (2013) found that isomers similar to bacillomycin L in *B. amyloliquefaciens* (K103) controlled *Rhizoctonia solani* Kühn. Same results, investigating bacillomycin L and surfactin, were found by Luo et al. (2015) in *B. subtilis* 916, this time controlling rice sheath blight. Other studies found that amylocyclicin, a bacteriocin in FZB42, possesses high antibacterial activity in gram-positive bacteria (Scholz et al., 2014).

Therefore, using blast in Geneious ®, it was found that the three *Bacillus* strains possess similar content in antibacterial compound genes (Table 5.10). A specific example, the circular bacteriocin amylocyclicin was found to be present in all of them. Some authors (Perez et al., 2018; Scholz et al., 2014) investigated these ribosomally synthesized peptides as bioactive compounds. Specifically, the leaderless-circular bacteriocins, they have been investigated for industrial purposes

due to their circular nature and not having an N-terminal leader peptide. (Perez et al., 2018).

In FZB42, the amylocyclicin is made of five different genes (precursor peptide, a dedicated immunity protein, ABC transporter - bacteriocin secretion but can also confer partial immunity-, membrane protein and a gene of unknown function). Interestingly, this cluster is found together in FZB24 (988,264-984,092) and QST713 (3,945,738- 3,949,910) but in EU07 only 4 genes are together (3,116,871 – 3,113,336 then 4,164,047 – 4,164,565).

Finally, can be concluded that, the antagonistic effect of the three *Bacillus* strains observed in *in vitro* assays is due to the presence of similar antibiotic genes in the three *Bacillus* strains. The genetic content observed in the NGS data confirmed this.

Hence, our scope of analysing the data based in WG comparison is justified contrary to the search for SNP's. To comment in this subject, some authors found that, SNP's in WG comparison from NGS data could be misleading, this is because of the differences, that would be found within genomes, can be due to an effect of the pipeline software used and not because of the data *per se* (Bush et al., 2019; Torkamaneh et al., 2016). Nonetheless a SNP calling done using the suggested procedure in Geneious®, showed no outcomes between QST713 and EU07 (data not shown).

Hereafter, using dnadiff, it was found that the strains EU07 and QST713 have a high similarity between them. Thus, the WG comparison to find genes differentially present between the strains became a better approach. Besides, first it was found divergency in gene content between FZB24 and the other two strains. Therefore, the work was focused to do the QST713 and EU07 genome's analysis. While, it was found that the 16S gene is totally identical between these two strains (Figure 5.7b), did not happen the same when the WG was compared using dnadiff.

Indeed, there was a difference in gene content between these two strains. Specifically, EU07 possesses some genes that are absent or partially present in QST713. The functions of those genes were related to DNA replication, repairing or precursors of DNA, stress tolerance and antibiotic resistance. Many of them are phage-derivate (tables 5.7 and 5.8). Those genes were found to be expressed in cytoplasm and membrane mainly, only two were believed to be located extracellularly, the EU07_03, which was not found in the blast search and the EU07_35, which provides precursors for DNA synthesis and is related to genes involved in ribonucleotide reductase and transport.

A summary with the protein products of these genes are in the table 5.9, which present the name of the genes according with https://string-db.org. The String database made a relation of the genes present in the organism of interest and relates them with other taxa. It was surprising to find that there was a gene, ygeA, present in EU07, which is present in *B. mojavensis*, however the *B. subtilis*, *B.*

amyloliquefaciens has only partial presence of the gene (low similarity with bitscore less than 60), and not present in other species inside of the *Bacillus subtilis* group. this gene, a putative racemase, has a function on resistance against drugs, this means, it protects the bacterial cell from toxic compounds. This demonstrates the singularity of the strain EU07.

5.8 Final remarks on previous studies with EU07

EU07 strain has been recognised as a successful strain for the control of *F. oxysporum* (Baysal et al., 2013, 2008) and its seeming role in the increasing the growth of the tomato plants. Genetic analysis showed that this strain has a good biological control capacity, according to the expression of the gene HLS. Additionally, it was found that, genes not found in QST713 (Acetoin acoA, acoB, acoR and the Acetoin utilization proteins AcuA and AcuC) previously (Baysal et al., 2013), were present in all the three *Bacillus* strains genomes.

Nevertheless, the application of genomics to the study of these three *Bacillus* strains is yet a powerful tool which contribute to determine better outcomes in the fine tune study of bacterial genomes closely related. It was found here, that the *Bacillus* strains EU07 and QST713 did not differ in these, previously found, genetic characteristics. They, actually, share a lot of similarities in the presence of genes (for the protein yrvN p.e.) for antimicrobial activity and plant growth promoter activity.

Moreover, the molecular approach used by Baysal et al. (2013, 2008) with polymorphism analysis (RAPD-PCR analysis) and biochemical test of bacteria for classification into species, now is proven to be not as accurate as it was thought. There were found differences in the results from the previous genetic analysis, for instance through the inter-simple sequence repeat (ISSR) primers (Baysal et al., 2013) which results in the Dice's matrix placed FZB24 nearer to EU07, than the QST713. Ours results placed QST713 nearer to the EU07 strain than FZB24 (Figure 5.7).

This is probably due to the advancing of tools for genetic analysis, which contributes to a better analysis of bacterial genomes, but also in the understanding of the function and regulation of genes. Overall, the knowledge surrounding the function of genes, pathways analysis, gene expression and co-expression have grown exponentially since the last two decades.

These results give value to the virtuosity of the WGS in the analysis of bacterial genomes and its features compared with traditional methods. The last two decades (2000-2020) saw an incredible input into the knowledge of the genomics of the different organisms and its relationship in the expression of genes. Nonetheless, much more work need to be done to understand completely the advantage of the EU07 over other bacterial isolates. Our study showed that small genetic variations can confer very much advantage in the effects of these isolates into the plant pathogen control environment.

Chapter 6

Transcriptomics of *Fg-K1-4*

Introduction

Microorganisms compete for a place in the same niches, displaying a range of tactics to survive. For instance, the silage industry base their success to the addition of protective bacteria to the straw and grass debris for further livestock feed (Fabiszewska et al., 2019; Hooker et al., 2019). Rhizobia microorganisms compete for nutrients and resources in the root environment or leaf surfaces as a whole system, sometimes called holobiont (Braga et al., 2016; Hassani et al., 2018; Pacheco and Segrè, 2019). The results of these interactions many times act in the benefit of the humanity (Tshikantwa et al., 2018; Zhang et al., 2018). Here is being refered as the fungal-bacteria interaction (FBI), a matter reviewed by de Boer (2017), Deveau et al. (2018) and Frey-Klett et al. (2011) and covered in this study with the interaction of *B. velesenzis* (EU07) and *F. graminearum* (K1-4), afterwards *Fg-K1-4*. In another area, there is the study of the genes involved in the development and infection of *F. graminearum*. Broadly, functional genomics is the understanding of the relationship between genotype and phenotype through the study of transcription, translation and epigenetic regulation ("What is functional genomics?," 2014). A great part of it, is the prediction of the functions of sequences, which traditionally is determined using *in situ* hybridization, DNA foot-printing,

experimental mutagenesis and transgenic knockouts (Pierce, 2006). However, advances in genome sequencing (see *Introduction, chapter 5*) lead to the use of better technologies called high-throughput (HT) technologies to decode genome transcription. European Bioinformatics Institute (EMBL-EBI) summarized and divided them in two main streams: Microarrays and HT sequencing (HTS) (Tang, 2014). Microarrays are ordered short DNAs, fixed in a solid support functioning as probes in which complementary sequences will attach (Pierce, 2006). They are used for expression-profiling, assessing thousands of genes at once and for tiling microarrays, which is employed to do mapping of transcription factor binding sites or locations of epigenetic marks. In the other hand, HTS includes RNA-sequencing and Immuno-Precipitation (ChIP). The first technique sequences cDNA in machines such as Illumina in the same fashion as the DNA. The second technique uses Chromatin ChIP sequencing with DNA sequencing to identify protein-binding sites on DNA (Tang, 2014).

Nowadays, transcriptomics is widely used and refers to the study of all set of RNA molecules transcribed from genome. Some examples of application of transcriptomics in molecular plant pathology include to: elucidate genes involve in intraspecies interaction (Walkowiak et al., 2015); explain specific mechanisms of infection and mycotoxin production (Puri et al., 2016); understand and recognize genetic mechanisms of differentially expressed (DE) genes at different stages of development (“What is functional genomics?,” 2014); recognize interaction of expressed genes and understand plant-microbe interaction of economic

importance (Kazan and Gardiner, 2018); learn about fungal defence strategy under the pressure of a biocontrol agent (Strub et al., 2019); understand virulence between isolates of same species (Huang et al., 2019) and to study plant interactions and plant genome alterations produced by plant pests (Petre et al., 2020).

RNA-sequencing (RNA-seq) has been serving the scientific community for more than 15 years. This involves the sequencing of complementary DNA (cDNA) with HT technology. The cDNA could be sourced from total, enriched mRNA or depleted rRNA. mRNA is isolated from total RNA using Poly(A) protocols.This system uses the property of polyadenylation of the RNA to separate from others (small RNA e.g.). The advantages of use Poly(A) are due to the fact that most of the previous studies were based in the same analysis, which would contribute to the results comparison and the analysis can focus on the protein-coding regions of the transcriptome (Koch et al., 2018; Stark et al., 2019). Raw data can, then, be analysed using bioinformatic tools. Demultiplexed (if needed), aligned and mapped of genes to generate a raw count table for further analysis, such as differential expression analysis.

General steps to lead a complete RNA-seq analysis have been extensively reviewed. For instance, some authors appear to agree in some steps such as experiment design, quality control, read alignment, differential gene expression (DE) analysis, functional analysis, alternative splicing, gene fusion detection and eQTL (Expression quantitative loci) mapping. Experiment design may incluide preparing the experiment (library type, depth and replicates) and planning the execution (to decrease the bias in results). RNA-seq results are greatly influenced by external

factors. Most important is the extraction protocol used and the design of the experiment in which the scientific question will be answered. After this, the number of samples used in the experiment would contribuite to decrease the technical and biological variability. In the next part, the analysis of the data may include the quality cheking (QC), transcript identification and quantification, and the differential expression analysis. Some other steps which can be carried out are visualization and analysis of the pathways in the system (Conesa et al., 2016; Koch et al., 2018; Stark et al., 2019).

The aim of this study to gain information from the interaction between *F. graminearum* (*Fg*-K1-4) and the strain of beneficial bacteria, EU07, and understand the mechanism of supression of the fungal growth using transcriptomics. The specific objectives are to design experiments: 1) to obtain three samples of the fungus as the control treatment (non-treated) and three samples of the fungus treated with the bacterial pellet; 2) isolate the total RNA using an optimised Trizol protocol to protect the integrity of the molecules of RNA; 3) obtain RNA-seq data from poly A enriched fungal mRNA; 4) analyse RNA-seq data and perform the differential expression (DE) analysis using the online tool Degust (Powell, 2015); and finally, 5) perform enrichment analysis to assess the overal interaction and pathways using the genes discovered in Objective 4.

Results

6.1 Treating Fungus *Fg*-K1-4 with EU07 for RNA-seq

6.1.1 Experiment design

The objective of this chapter is to reveal which genes of the *Fg*-K1-4 are expressed when the *Bacillus* EU07 is added to the medium. In order to investigate this, an experiment was designed with 3 replicates of the control treatment and 3 of the treated one. To do so, an exploratory experiment was made to determine the specific conditions in which the sole presence of the *Bacillus* EU07 would trigger changes in the growth of the fungus.

It was observed that all the treatments induced response, at the 2nd day of treating them, as a change on the hyphal morphology producing round structures or thickening of the hypha, which was not observed in the control treatment.

Additionally, early experiments showed that the pH of the broth changed to a more neutral level (from 5 to 6) in the treatment **c.** and **d.** All treatments were carried out at the same time point and the treatments used were: control, LB broth, whole EU07's bacterial broth, washed pellet of EU07 and only the bacterial broth (spun and filtered) (Figure 6.1).

Therefore, the treatment **d.** was chosen under the consideration that the changes on the morphology of the hypha was due to the sole presence of the bacterium.

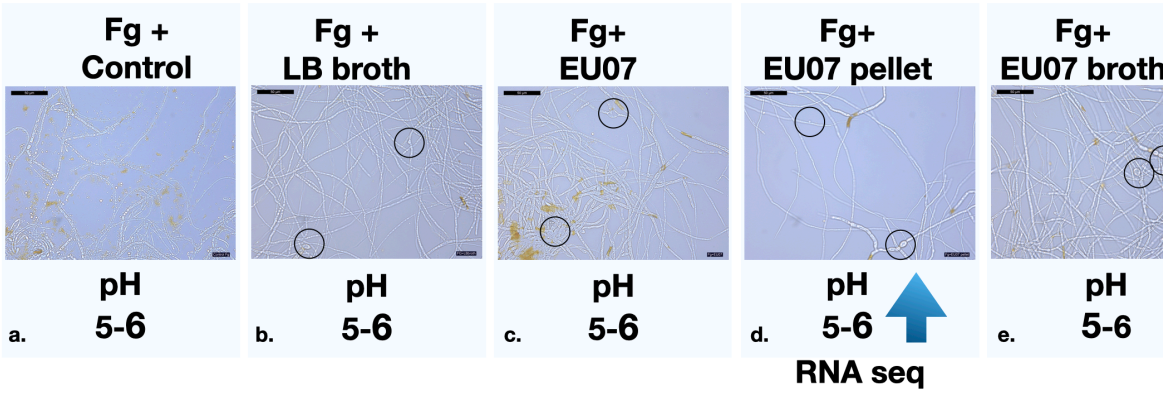


Figure 6.1 Microscopic views of the* Fg-K1-4 *growing in PD broth with and without EU07.

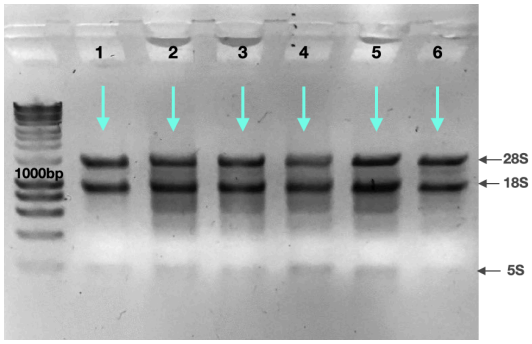
Scale bars (Top left) =. 50µm, circles surround tick-round areas of hypha. The morphology of hypha is affected after 2 days of treatment, showing thicker sections and formation of round structures compared with the control. **a.** Control treatment (5 days old and 2 days after treatment), **b.** *Fg* 5 days old with LB broth (4ml, 2 dat), **c.** *Fg* 5 days old with EU07 broth (4ml, 2 dat), **d.** *Fg* 3 days old with EU07 pellet suspended in 4mL of water (4ml, 2dat), **e.** *Fg* 3 days old with filtered EU07 broth (4ml, 2dat).

6.2 RNA sequencing of the Fg-K1-4 treated with EU07

6.2.1 RNA extraction for NGS (RNA-seq) using fungal tissues

After establishing the experimental design, the RNA extraction from the fungal mycelia was optimised. Bands of the RNA were observed in the electrophoresis gel displaying clear bands for the 28S, 18S and 5S ribosomal RNA and Nanodrop readings showed high quality ~2 in 260/280 and 260/230 absorbance ratios (Figure 6.3).

a.



Sample ID	*Concentration (ng/ µl)*	*260/280*	*260/230*	*Total µg in 62µl*	*Type sample*	*Name*
1	30.9	2.09	2.64	1.92	Eucaryote / Fungus	Fg
2	60.7	2.15	1.84	3.76	Eucaryote / Fungus	Fg
3	46.3	2.07	1.92	2.87	Eucaryote / Fungus	Fg
4	61.6	2.20	2.56	3.82	Eucaryote / Fungus	Fg/Bs
5	66.9	2.11	2.16	4.15	Eucaryote / Fungus	Fg/Bs
6	39.4	2.11	2.07	2.44	Eucaryote / Fungus	Fg/Bs

Figure 6.2 Gel electrophoresis of fungal RNA.

a) Agarose gel showing the the Hyperladder and RNA bands of fungal samples (1-3 lines) and the fungus treated with bacterial broth (4-6 lines). Bands of the ribosomal RNA subunits 28S, 18S and 5S. Samples were extracted with RNAwiz, using 1.5% of agarose, 75V and 25 min. **b)** Table of the data and characteristics of the samples sent to sequencing.

6.2.2 Quality control of fungal tissues and sequencing

6.2.2.1 Bioanalyzer results: RIN number of samples

The RNA samples were quality controlled preliminary with gel electrophoresis, sample quantification and purity with Nanodrop and sample integrity with Agilent

2100 (Figure 6.3). The concentrations of the samples were sufficient for sequencing (>38 ng/ µl) which was done by Novogene (UK) Ldt.

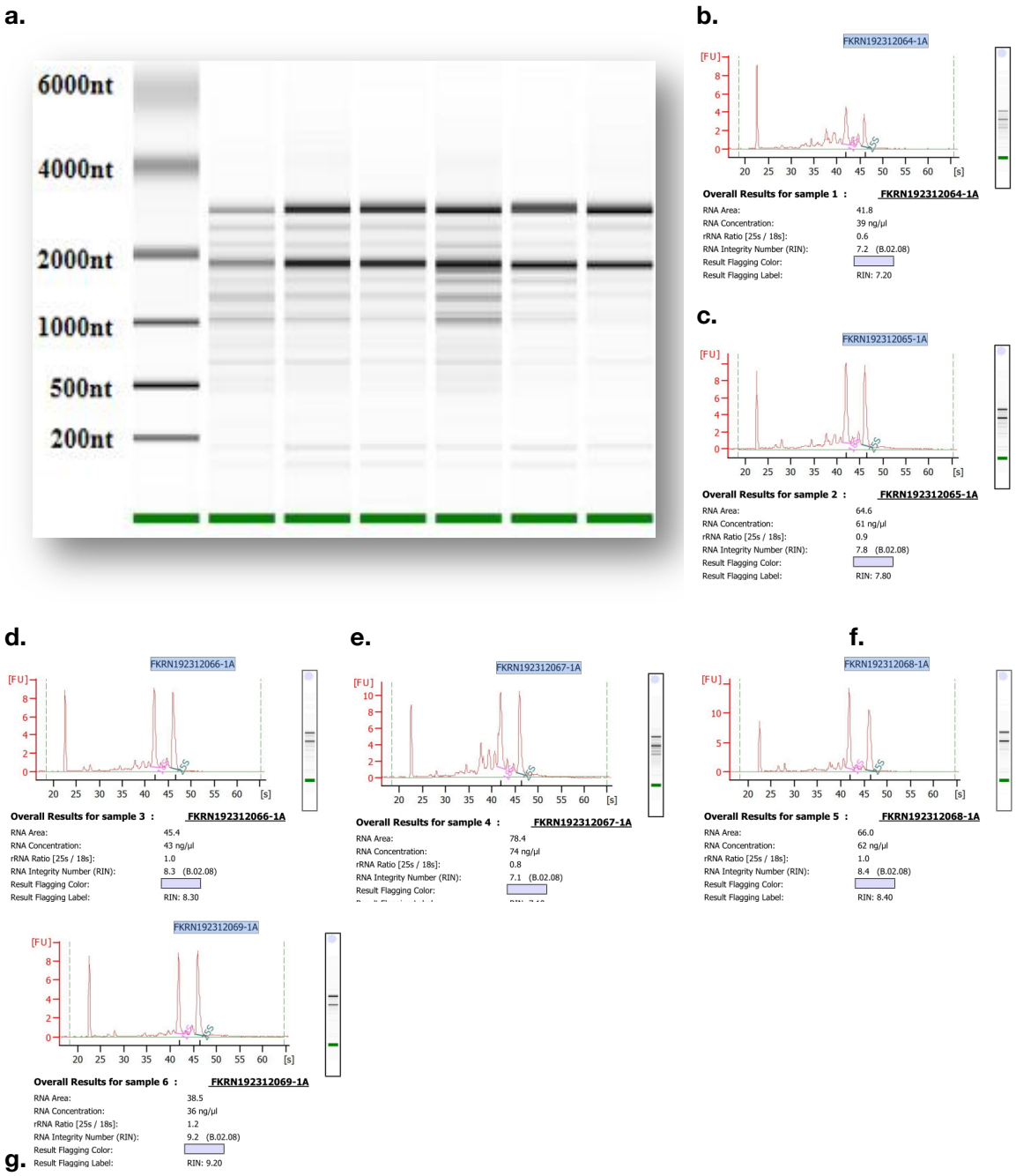


Figure 6.3 RNA sequencing data generated by Bioanalyzer.

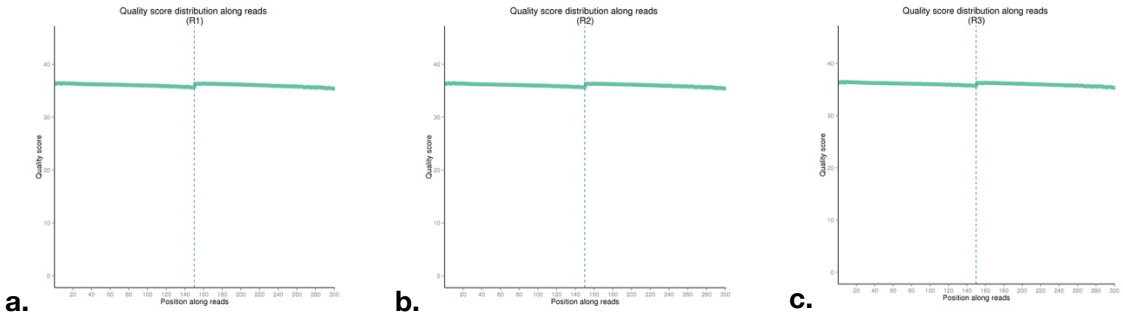
a. Electrophoresis gel: First column is the ladder, second to seventh correspond to the samples 1-6 in the order of bottom table (1-6 loaded 1µl). Electrophoresis conditions: GelConc. : 1%, Voltage : 180v, Run Time : 16min. **b-g.** Integrity test result pictures (Agilent

2100) of samples. Pictures are showing RNA area, RNA concentration, RNA integrity number and the corresponding electrophoresis column for each sample. All samples showed RIN > 6.8.

The parameters of quality control for the data such as Distribution of Sequencing Quality, Distribution of Sequencing Error Rate and Distribution of A/T/G/C Base were observed as in the highest levels.

6.2.2.2 Sequencing results: Quality score distribution of reads

The quality score distribution of the reads displays the Q-score, which is a notation of the sequence error rate and Q_{phred} and it is a relationship between sequencing error rate (e) and sequencing base quality value (Q_{phred}). Samples showed a Q-score of ~35 which mean that the error base is between $1/10^3$ and $1/10^4$, with a right base of 99.9 – 99.99%, which is within the accepted region (Figure 6.4).



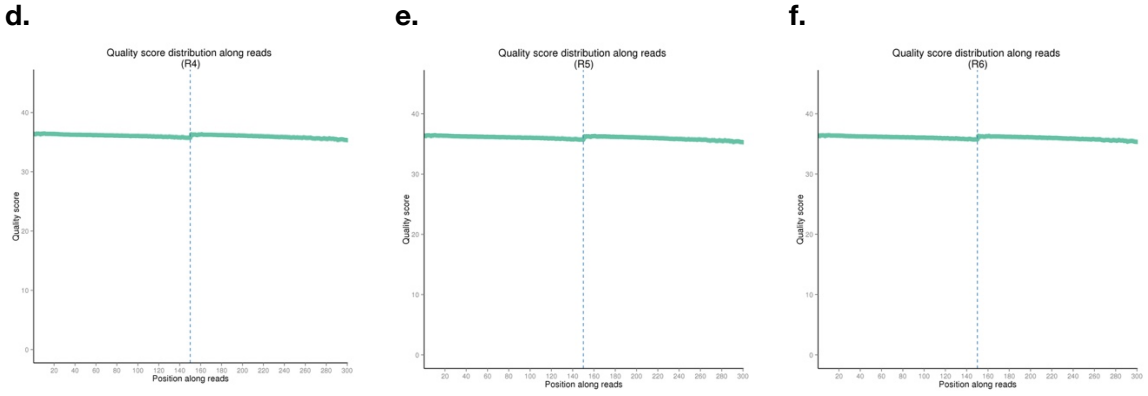
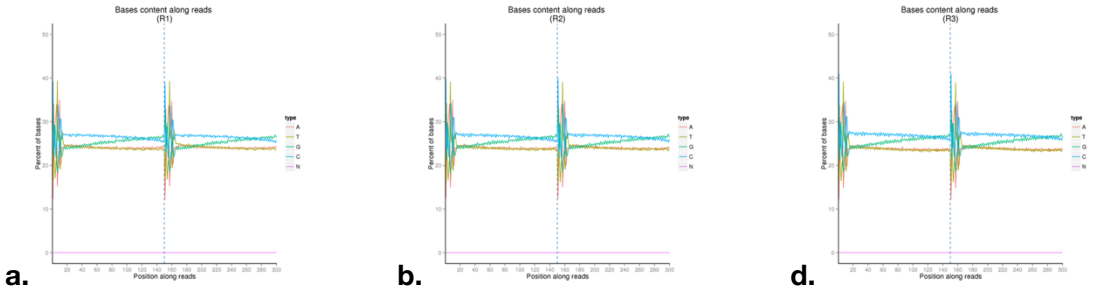


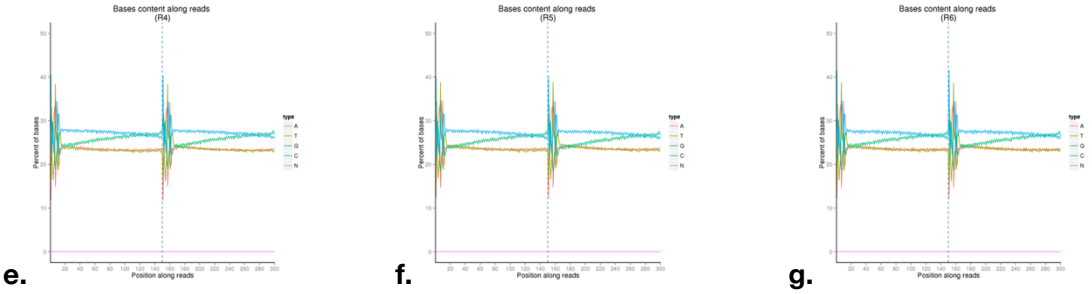
Figure 6.4 Data quality control used in the quality of RNA sequencing results: Quality score distribution of reads.

Quality score distribution along the reads' graphics of each RNA-seq sample (R1-R6). The base position is on the horizontal axis and the sequencing quality is on the vertical axis. By definition a Phred score between 30 - 40 represents an error rate of .001-0.0001 with a right base of 99.9 – 99.99%. **a-c.** Repeats of control treatment, **d-f.** repeats of bacterial treated treatment. All the scores are above of 35 (each graph presents the R1(0-150) and R2 (151-300) data).

6.2.2.3 Sequencing results: distribution of A/T/G/C Base of sequencing data.

The distribution of the bases and AT/GC content were checked. Normal picks were observed at the short (<20 read position) and medium reads (~160 read position) (Figure 6.5) indicating the sequencing worked well.





e. **f.** **g.**

Figure 6.5 Data quality control used in the quality of RNA sequencing results: distribution of A/T/G/C Base.

The distribution of the AT and GC by checking the distribution of GC content. **a-c.** Repeats of control treatment, **d-f.** repeats of bacterial treated treatment. the percentage of bases were constant between 20-30% along the reads (0-300) for all the samples. Normal peaks were observed as expected in the first 6 to 7 nucleotides (each graph presents the R1(0-150) and R2 (151-300) data).

6.2.2.4 Sequencing results: Distribution of Sequencing Error Rate and Raw Data Filtering

The distribution of the Sequencing Error Rate shows two features, the error rate which grows during the sequencing due to depletion of reagents and due to the first six bases which incompletely bind during the cDNA synthesis. The error rate for all the samples (R1 and R2) were in the ranges of 0.02-0.03%, which is good and acceptable. In the other hand, the raw data were composed only by the reads and adapters. No present of N tails (<0.00%) or low-quality reads were observed for the six samples.

Sample name	*Raw reads*	*Clean Reads*	*Raw data*	*Clean data*	*Effective (%)*	*Error (%)*	*Q30 (%)*	*GC (%)*
R1	12,694,917	12,403,487	3808,475,100	3721,046,100	97.7	0.03	94.2	51.8
R2	19,517,507	18,985,824	5855,252,100	5695,747,200	97.2	0.03	94.1	52.0

R3	17,840,027	17,568,959	5352,008,100	5270,687,700	98.4	0.03	94.1	52.4
R4	14,149,862	13,838,620	4244,958,600	4151,586,000	97.8	0.03	94.1	53.0
R5	16,994,840	16,771,164	5098,452,000	5031,349,200	98.68	0.03	94.0	52.7
R6	14,577,568	14,407,663	4373,270,400	4322,298,900	98.83	0.03	94.1	52.83

Sample: sample name. **Raw reads**: total amount of reads of raw data. For paired-end sequencing, it equals the amount of read1 and read2. **Clean reads**: total amount of reads of clean data. For paired-end sequencing, it means the amount of read1 and read2. **Raw bases**: (Raw reads) * (sequence length), calculating in GB. For paired-end sequencing like PE150, sequencing length equals 150. **Clean bases**: (Clean reads) * (sequence length), calculating in G. For paired-end sequencing like PE150, sequencing length equals 150. **Effective Rate** (%): (Clean reads/Raw reads) *100%. **Error rate**: base error rate. **Q20, Q30**: (Base count of Phred value > 20 or 30) / (Total base count). **GC content**: (G & C base count) / (Total base count).

6.2.2.5 Sequencing results: Quality control summary

The total output of data provided was a raw data of 97.55 Gb and the clean data of 28.2GB. The statistics for the quality of sequencing data are shown in Table 6.2, including raw reads, clean reads, effectiveness, error rate, Q_{pfred} score and GC content.

6.2.3 Bioinformatic analysis of the samples and preparing gene count table

Bioinformatic tools were used to clean, sort and prepare the gene count table from the raw data of RNA-seq. The downloaded .fq files were checked for errors using MD5Sum (Drepper et al., 2010), two .fq files per sample as they were paired end. Then quality checked with FastQC which produced .html files with the statistics of quality. High good quality of the raw data was observed throughout all samples. Abundance table (gene count) were obtained of the reads mapped to the reference

genome PH-1 (Rothamsted Research, 2020). A total of 14,898 transcripts were obtained.

6.2.4 Bioinformatic analysis using EU07 bacterial genome

To ensure that all the transcripts obtained during the analysis corresponded exclusively to *Fg*-K1-4, and any bacterial transcript passed the sequencing process, an analysis using the previous pipeline, was done. Using macOS terminal, the raw data was cleaned, sorted and mapped to the EU07 genome and not counts were found.

6.3 Differential expression of genes from *Fg-K1-4* treated with *Bacillus* EU07

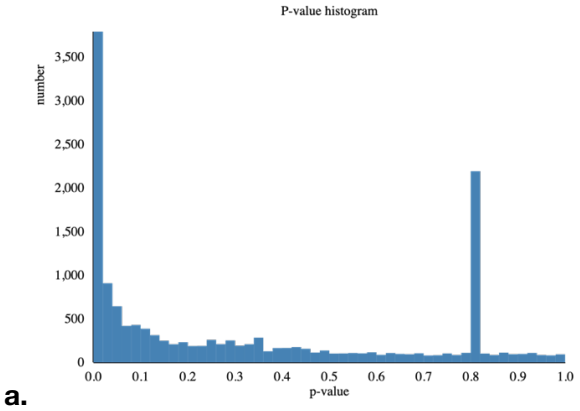
6.3.1 Degust analysis: Deferential Expression analysis

6.3.1.1 DE analysis: Quality control of gene count

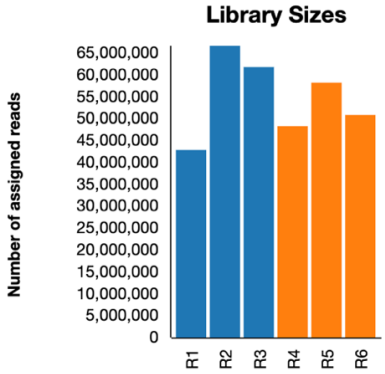
Data from gene counts obtained using bioinformatics were submitted to Degust (Powell, 2015). The tool was set to use the first three samples as non-treated and the second three samples as treated. Degust regenerated the analysis using Voom-Limma method (Law et al., 2014). For read count analysis on RNA-seq experiment, the voom method estimated “the mean-variance relationship of the log-counts, generates a precision weight for each observation and enters these into the limma empirical Bayes analysis pipeline”, which the authors suggest that are exceptional good even with data generated by microarrays.

For quality control of the data used, four graphics were generated: (a) P-value histogram, (b) library size in number of reads, (c) expression plot in log of CPM

(counts per million) and (d) relative log expression (RLE) plot (Figure 6.6), which in this experiment reflected good quality of the data. The histogram (a) shows a clear tendency with most of the reads with a low p-value (<0.1), which is a good indicator that they are reflecting a good sequencing dept in the next two graphics (b and c) library size indicate a high number of the reads, above the minimal expected for the experiment (>30,000,000) and the distribution of counts per million expressed as a log (cpm) and finally the relative log expression (RLE), samples 1-3 appear to be toward the point 0 and the samples 4-6 appear to be towards 1.



a.



b.

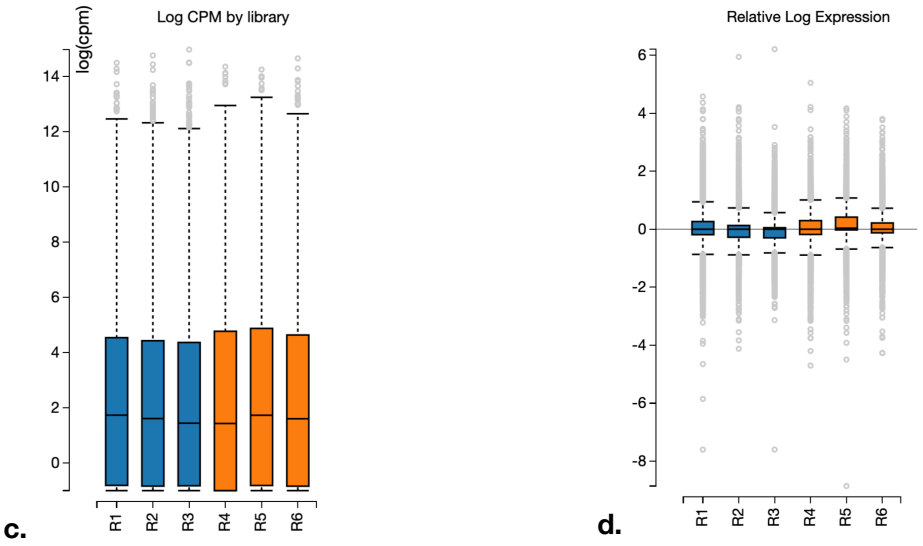


Figure 6.6 Quality control graphs of data of the Fusarium RNA-seq analysis in Degust (4.1.4).

a. P-value histogram. Samples produced a bimodal p-value distribution, the significant p-values present a pick by the 0 point. Towards 1 are those not significant, the pick observed ~0.8 are values with very low count. **b.** Library size in number of reads per sample or sequence depth. Degust takes raw count data and shows this plot as an outcome of the reads used and calculate counts per million (CPM), the least number of assigned reads was for the sample control 1, which also has a RIN=7.2, for instance. **c.** Expression plot in log of CPM (counts per million), Degust (which implement DESeq2) normalises counts per depth of the sequenced data. **d.** Relative log expression (RLE) plot, which shows the differences between the distributions of read counts across samples. Samples 4-6 (treated) appear to have a positive expression compared with samples 1-3 (non-treated).

In general, the QC plots support a good quality data for further analysis by Degust.

6.3.2 DE analysis: Data visualization with Heat map, MA, volcano and MDS plots

To visually understand the performance of the DE data, Degust uses several plots of the genes expressed. Here, it will be introduced the heat maps, multidimensional scaling (MDS) plot, log ratio(M) vs an average (A) (MA) plot and volcano plots, which display the results in a helpful manner for experiments with two different treatments (control and treated, e.g.).

Second, the MDS plot represents the variation between samples and is similar in concept to a principal component analysis (PCA) plot. A general view of the samples helps to determine how the data from a treatment (all replicates) differs from the other set of data. In Figure 6.8 control repeats are allocated far left (x axis, under -0.2) and the treated samples are allocated on far right. This gives confidence of the two treatments are different between them in the dimension 1, this is a relative magnitude of the 6 samples.

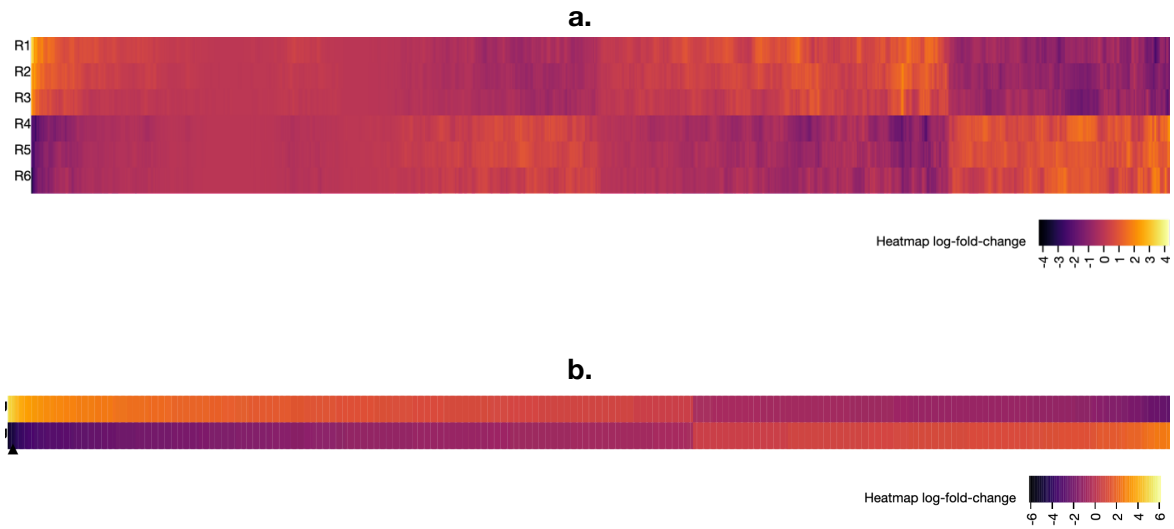


Figure 6.7 Heat map of Log Fold Change (logFC).

Log2 Fold Change Heat map. **A.** A heat map for the two different treatments (control and treated) calculated by Degust in the DE analysis using DESeq2. **B.** A heat map for the average outcome of the two different treatments. The colour scale shows a clear difference between control and treated samples. Data from Degust. Heatmap in color blind tones.

The MA plots (Figure 6.9 a-b) in DESeq2 shows the log2 fold changes due to a specific variable against the mean of normalized counts (average expression) for all the samples and contributes to visualize the gene expression data sets. The full set of genes of the gene count table was of 14,898 transcripts (Figure 6.9 a). The dots are genes and the blue ones represent those genes with no significant expression. When the false discovery rate (FCR) cut-off was changed to include those with p-value (significance) of 0.01 or less and with an absolute fold change of 1 (2x), the number of genes decreased to 709 (Figure 6.9 b). Both, increased and decreased level of expressions are observed from the treated samples comparison to the control samples.

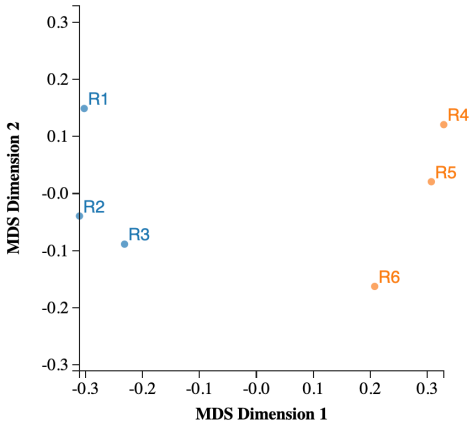


Figure 6.8 Multidimensional scaling (MDS) plot of the Fusarium RNA-seq analysis in Degust (4.1.4).

MDS (multiple dimensions plot or PCA plot) shows that the samples within the treatments are clustered together through the dimension 1. All transcripts are included (FDR cut-off 1, absolute log FC 0 relative to Control) with a total number of genes 14,898. R1, R2, R3 are the control treatment (*Fg*-K1-4 alone) and R4, R5 and R6 are the fungus treated with EU07 pellet. Dimension 1 shows a clear separation between the treatments.

Volcano plots (Figure 6.9 c-d.) are scatter plots which show statistical significance (P value) versus magnitude of change (fold change). Here, the plots are showing the DE genes between the isolates of fungal tissue treated and non-treated. Red dots are the significant fold changes, which could be the most biologically significant genes. The most upregulated genes (increase level of expression) are towards the right, the most downregulated genes (decrease level of expression) are towards the left, and the most statistically significant genes are towards the top.

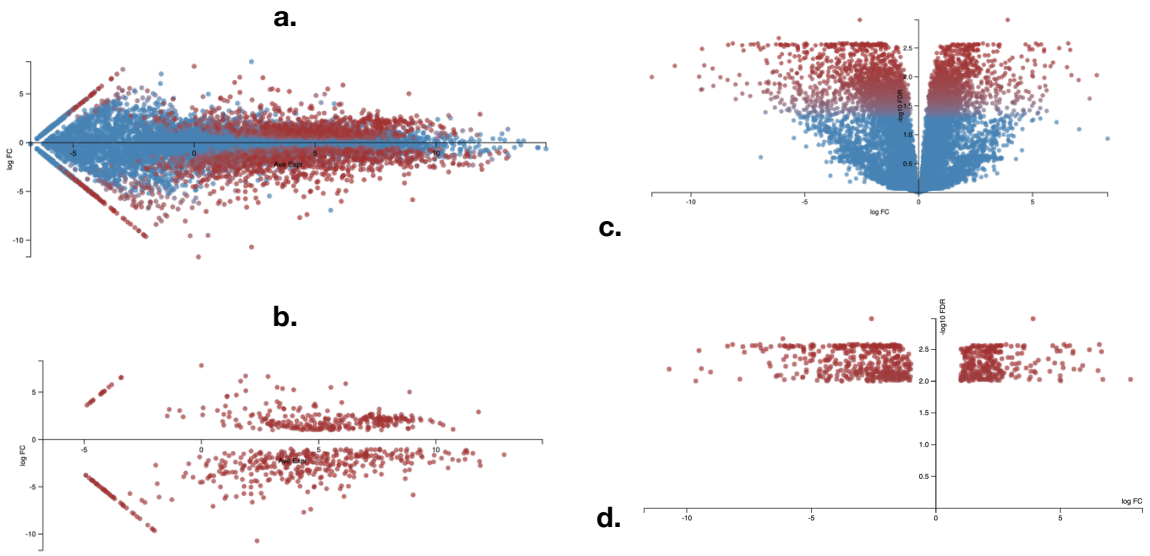


Figure 6.9 MA and volcano plots of the DE genes.

Plots of DE genes from Degust. **A.** MA plot, log ratio (M) vs an average (A), showing whole set of genes, 14,898 (positive and negative expressed). Each gene is represented by a dot. The selected parameters were FDR:1, log FC: 0 (all). **B.** MA plot with parameters of FDR:0.05, log FC:1 (2x). Here only genes with a fold change of at least the double are displayed. **C.** Volcano plot displaying all the genes DE, 14,898 in total. Threshold selected FDR:1

(confidence) and logFC:0 (all). **D.** When a threshold FDR of 0.01 and FC 1, 709 genes are found. From those, negative expressed genes were 412, and positive expressed were 297. The transcripts FGRAMPH1_01T13767 (FDR 1.03e-3; p-value 1.39e-7; FC_{EU07} -2.59) and FGRAMPH1_01T16599 (FDR 1.03e-3; p-value 1.02e-7; FC_{EU07} 3.91) are the outliner dots at the top of the graphic. Blue, genes with no significant DE; red, genes with significant DE. FDR, false discovery rate; FC, log 2-fold change.

6.3.2.1 DE analysis: Go term, Panther and UniProtKB annotation

A brief list of most significant genes can be found in the Table 6.3, classified by function according with GO term (Rothamsted Research, 2020). The genes were selected from a list found under the threshold FDR of 0.01 and FC 1. A total of 709 genes were found.

The genes were classified according to the level of expression (increased or decreased) and by the GOSlim term, which is a simplified category. From the genes with increased level of expression, the GOSlim biological process was represented by 34 genes (log FC 3.81, 1.01), the cellular component was represented by 28 genes (log FC 6.51, 1.00), the molecular function was represented by 144 genes (log FC 6.65, 1.01) and the group with not GO term found were represented by 91 genes (log FC 7.82,1.00). For those genes that were not found under the GO term, a Panther (Protein ANalysis THrough Evolutionary Relationships) code were assigned when it was available (“PANTHER,” 2020).

In the same way, the genes with decreased level of expression were 412 in the selected threshold. In the biological process group was 51 genes (log FC -1.0, -10.71), in the cellular component was 37 genes (log FC -1.1, -9.05), in the molecular

function group was 159 genes (log FC -1.0, -9.42), in the signal transduction group was 1 gene (log FC -1.3) and the group with not GO term found was 164 genes (log FC -9.6, -9.6).

Table 6.3 *Brief list of most significant genes by function according their GO term.*

Genes observed over a threshold of FDR of 0.01 and FC 1 were selected and the most increase/decrease in the express level are shown here. The Panther accession were included when it was not possible to obtain a GO term.

Transcript_id	**GO term accession**	**GO term name**	**GOSlim GOA Description**	**LogFC**	**AveExpr**	**P value**	**FDR**
FGRAMPH1_01T05373	GO:0016020	membrane	biological_process	3.8	3.35E+00	1.44E-04	5.70E-03
FGRAMPH1_01T10065	GO:1990904	ribonucleoprotein complex	biological_process	2.4	7.22E+00	1.63E-04	6.06E-03
FGRAMPH1_01T23279	GO:0042254	ribosome biogenesis	biological_process	2.3	7.58E+00	1.73E-04	6.18E-03
FGRAMPH1_01T13305	GO:0016442	RISC complex	biological_process	6.5	-3.44E+00	2.14E-04	6.79E-03
FGRAMPH1_01T21575	GO:0033177	proton-transporting two-sector ATPase complex, proton-transporting domain	biological_process	5.0	-4.21E+00	3.33E-04	8.18E-03
FGRAMPH1_01T11461	GO:0003824	catalytic activity	molecular_function	6.7	2.84E+00	4.47E-05	3.42E-03
FGRAMPH1_01T22529	GO:0016491	oxidoreductase activity	molecular_function	6.2	1.67E+00	3.51E-05	3.19E-03
FGRAMPH1_01T11459	GO:0003824	catalytic activity	molecular_function	5.5	5.52E+00	5.36E-05	3.62E-03
FGRAMPH1_01T19409	GO:0005506	iron ion binding	molecular_function	5.2	3.93E+00	1.77E-05	2.81E-03
FGRAMPH1_01T14003	GO:0003824	catalytic activity	molecular_function	5.1	-4.13E+00	1.66E-04	6.12E-03

FGRAMPH1_01T13753	GO:0005515	protein binding	molecular_function	5.1	-4.15E+00	1.18E-04	5.24E-03
FGRAMPH1_01T13305	GO:0016021	integral component of membrane	cellular_component	6.5	-3.44E+00	2.14E-04	6.79E-03
FGRAMPH1_01T21575	GO:0016020	membrane	cellular_component	5.0	-4.21E+00	3.33E-04	8.18E-03
FGRAMPH1_01T15975	GO:0005576	extracellular region	cellular_component	2.7	2.47E+00	2.66E-04	7.38E-03
FGRAMPH1_01T06069	GO:0016020	membrane	cellular_component	2.6	2.19E+00	1.29E-05	2.74E-03
FGRAMPH1_01T03539	GO:0006811	ion transport	cellular_component	2.6	5.39E-02	2.34E-04	7.04E-03
FGRAMPH1_01T15795	**255aa**	**Chromosome 2: 8,663,069-8,663,836**		**7.8**	**6.03E-05**	**4.46E-04**	**9.35E-03**
FGRAMPH1_01T27281	PTHR22950	SF8	amino acid transporter	6.7	1.88E+00	4.50E-04	9.35E-03
FGRAMPH1_01T27977	PTHR22950	SF479	amino acid transporter	6.6	-3.41E+00	3.31E-06	2.64E-03
FGRAMPH1_01T15633	PTHR39602	Family ACW-9	--	5.9	6.15E+00	1.43E-04	5.67E-03

***Transcript_id**: transcript names; **GO term accession**: Gene Ontology term accession; **GO term name**: Gene Ontology term name; **GOSlim**: Gene Ontology subsets; **GOA Description**: Gene Ontology Annotation (GOA) Description; **LogFC**: Logarithmic of Fold change Fold Change) of gene expression; **AveExpr**: Average expression of the gene; **P value**: statistic or probability; **FDR**: False Discovery Rate.*

In the case of the transcript FGRAMPH1_01T15795, coordinates Chr2: 8,663,069-8,663,836 in PH-1 strain of *F. graminearum*, a protein of 255 aa was present with not GO term or Panther code associated.

For both cases, some of the genes with no function associated in the original annotation of the PH-1 strain, could be associated with a Panther code or an UniProtKB code. For the genes negatively expressed, 164 did not show associated function, and from those, 71 genes were novel forms neither with UniProtKB nor with Panther code associated. For the genes positively expressed, 90 genes were found with not annotated function, which 56 have Panther code and only 4 have UniProtKB annotation.

6.3.2.2 DE analysis: Venn diagrams

Using the data of the number of genes from the total of DE genes obtained and within the threshold of FDR of 0.01 and FC 1, Venn diagrams were constructed to visualise the relationship of those genes within the different groups observed (Figure 6.10). Total genes found in the DE analysis are represented in the Figure 6.10 a. A total of 2,232 were selected from the threshold of FDR 0.05 and abs logFC 1 (network input). From them, the genes with increased (+ expressed) and decreased (- expressed) expression levels (FDR 0.01 and abs logFC 1) were highlighted. The Figure 6.10 b shows the relation of ratios of genes when a threshold of FDR 0.01 and abs logFC 1 (DEG -blue-) were selected.

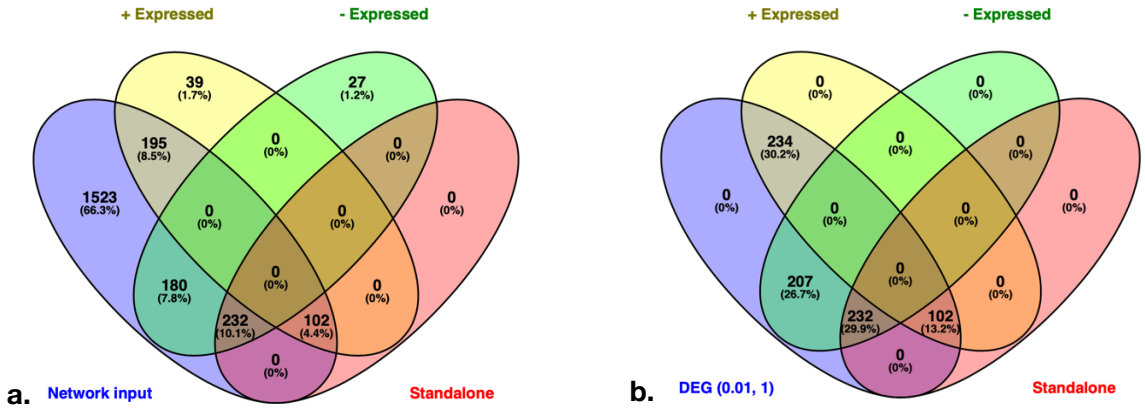


Figure 6.10 Venn diagrams of the DE genes expression relationship.

Venn plots of DE genes analysis from Degust (Oliveros, 2015). **a.** MA plot, log ratio (M) vs an average (**a**), 2,232 were selected from the threshold of FDR 0.05 and abs logFC 1 (network input) (positive and negative expressed) and (**b**) 775 genes obtained in the threshold FDR 0.01 and abs logFC 1. Each gene is represented by a dot.

6.4 Enrichment analysis

6.4.1 Cytoscape and network building

As an additional step, enrichment analysis was done using Cytoscape software (Shannon et al., 2003). Enrichment analysis helps to analyse large set of data from DE gene analysis and present that in a visually friendly manner (Merico et al., 2010). The data were pulled into the workspace using NDex and the *Fg* phibase PH-1 database were called (Liu et al., 2010). The data from the DE genes were imported into the same workspace and the StringApp (Doncheva et al., 2019) was used for the DE genes network analysis.

A total of 709 genes observed in a threshold FDR of 0.01 and FC 1 were used, and String App recognised 596 genes with interactions between them with an evidence score of 0.4 or greater (Figure 6.11).

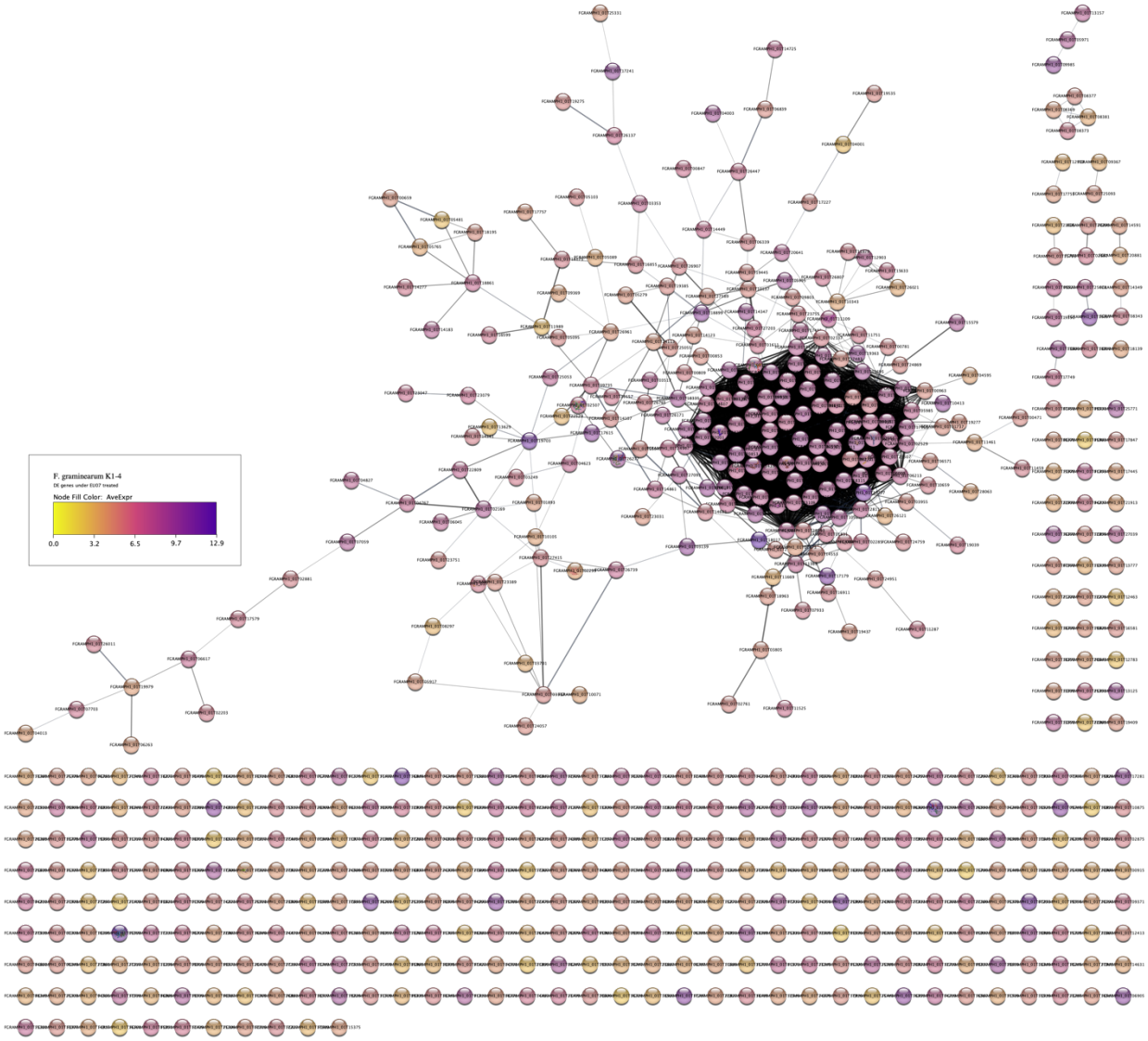


Figure 6.11 DE genes network analysis of the F. graminearum treated with EU07 RNA seq data.

STRING network analysis of proteins with significantly regulated sites detected in a transcriptomics analysis of *F. graminearum* (K1-4)treated with EU07. Log-ratios within the threshold FDR of 0.01 and FC 1 between the fungal tissue growing in PD broth and fungal tissue treated with EU07 *Bacillus* strain. A gradient of viridis plasma blind friendly were used in yellow – red – purple gradient. Proteins without any interaction partners within the network (singletons) are displayed in the bottom of the network. Scale bar represent the average expression of the observed in the DE gene analysis. Yellow (0.00) to purple

A close look to the network indicated that the DE genes grouped in the centre. At least 9 other big groups were identified. They are genes that cluster together according to experiments, co-expression data, neighbourhood, cooccurrence, databases or text-mining. Genes that clustered together in the centre are composed of 81 transcripts which are first neighbours in the system. The average expression of those genes is high (4.29 - 11.30). Go term names and string description revealed that those genes are associated to ribosome functions, which catalase the mRNA-directed protein synthesis in all organisms.

Singletons, that are genes with not associated network, were found. A total of 334 transcripts (logFC [7.82, -10.71]). From them 89 were found to be novel genes or with no associated function, other 63 were reported to have an UniProtKB code but not reported in GO terms. The rest, a total of 182 transcripts, were found to have a GO term classification. The GOSlim classification found for those were: cellular component (39), biological process (34) and molecular function (109).

An analysis of the same set of genes in the String database (Szklarczyk et al., 2019), showed that most of the genes were found to be of ribosomal function or related. A total of 21 network cluster were observed described as ribosomal or similar, one pathway was found related to KEGG database and 6 keywords, or hieratical categories, in UniprotKB (Table 6.4). The cluster CL:14560 (mixed, incl. Phosphopantetheine attachment site, and Putative esterase) and the cluster CL:10603 (mixed, incl. Amino acid permease/ SLC12A domain, and Arrestin, C-terminal) were nor related to ribosome. A further analysis of the keywords observed from the database UniprotKB shows that

there are six annotated keywords: Ribosomal protein (KW-0689), Ribonucleoprotein (KW-0687), Oxidoreductase (KW-0560), Transmembrane (KW-0812), Transmembrane helix (KW-1133) and Membrane (KW-0472). These keywords were mapped by groups in the network analysis made in Cytoscape (Figure 6.12).

Table 6.4 Functional enrichment of DE genes of Fg-K1-4/Bs treated (threshold FDR of 0.01 and FC 1.0) using String DB.

A total of 709 genes were analysed in String Network Enrichment. Total number of nodes: 674, number of edges: 3373, average node degree: 10, avg. local clustering coefficient: 0.283, expected number of edges: 1444, PPI enrichment p-value: < 1.0e-16.

#term ID	**Term description**	**OGC**	**BGC**	**Strength**	**FDR**	**Matching proteins in your network (IDs)**
						Kegg Patways
CL:687	Ribosome	71	77	1.26	5.42E-51	
CL:681	Ribosome	73	88	1.21	1.40E-50	
CL:690	Ribosome	66	72	1.26	1.06E-47	
CL:692	Ribosome	60	66	1.25	2.72E-43	
CL:693	Ribosome	56	60	1.27	8.75E-41	
CL:675	Ribosome, and Protein biosynthesis	75	187	0.9	6.17E-36	
CL:694	Ribosome	47	51	1.26	5.35E-34	
CL:695	Ribosome	37	40	1.26	1.19E-26	
CL:696	Ribosome	29	32	1.25	1.33E-20	

CL:697	Ribosomal protein, and Ribosomal S17	20	22	1.25	4.49E-14
CL:699	Ribosomal protein	15	15	1.3	7.62E-11
CL:796	mixed, incl. Ribosomal_L31e, and Ribosomal protein L1	8	8	1.3	1.53E-05
CL:715	mixed, incl. Ribosomal S3Ae family, and Ribosomal protein S7e	7	7	1.3	8.45E-05
CL:701	mixed, incl. Ribosomal protein S15P, and Ribosomal protein S19/S15	6	6	1.3	0.00047
CL:741	mixed, incl. Ribosomal L15, and Ribosomal family S4e	6	7	1.23	0.0008
CL:776	mixed, incl. Ribosomal protein S19e, and Ribosomal protein S26e	6	7	1.23	0.0008
CL:760	mixed, incl. Ribosomal L18 C-terminal region, and 60S acidic ribosomal protein P0	5	5	1.3	0.0024
CL:14560	mixed, incl. Phosphopantetheine attachment site, and Putative esterase	10	47	0.62	0.0153
CL:812	mixed, incl. Ribosomal protein L44, and Ribosomal L39 protein	4	6	1.12	0.0357
CL:10603	mixed, incl. Amino acid permease/ SLC12A domain, and Arrestin, C-terminal	10	55	0.56	0.0395

CL:10604	mixed, incl. Amino acid permease/ SLC12A domain, and Arrestin, C-terminal	9	47	0.58	0.0488
					Kegg Patways
map03010	Ribosome	72	109	1.12	1.03E-45
					Annotated Keywords in UniprotKB
KW-0689	Ribosomal protein	38	59	1.1	4.02E-23
KW-0687	Ribonucleoprotein	41	81	1.0	2.7E-22
KW-0560	Oxidoreductase	30	284	0.32	0.0127
KW-0812	Transmembrane	156	2430	0.1	0.0371
KW-1133	Transmembrane helix	155	2426	0.1	0.0376
KW-0472	Membrane	159	2518	0.1	0.0430

OGC: Observed gene count, BGC: Back-ground gene count, FDR: False discovery rate,

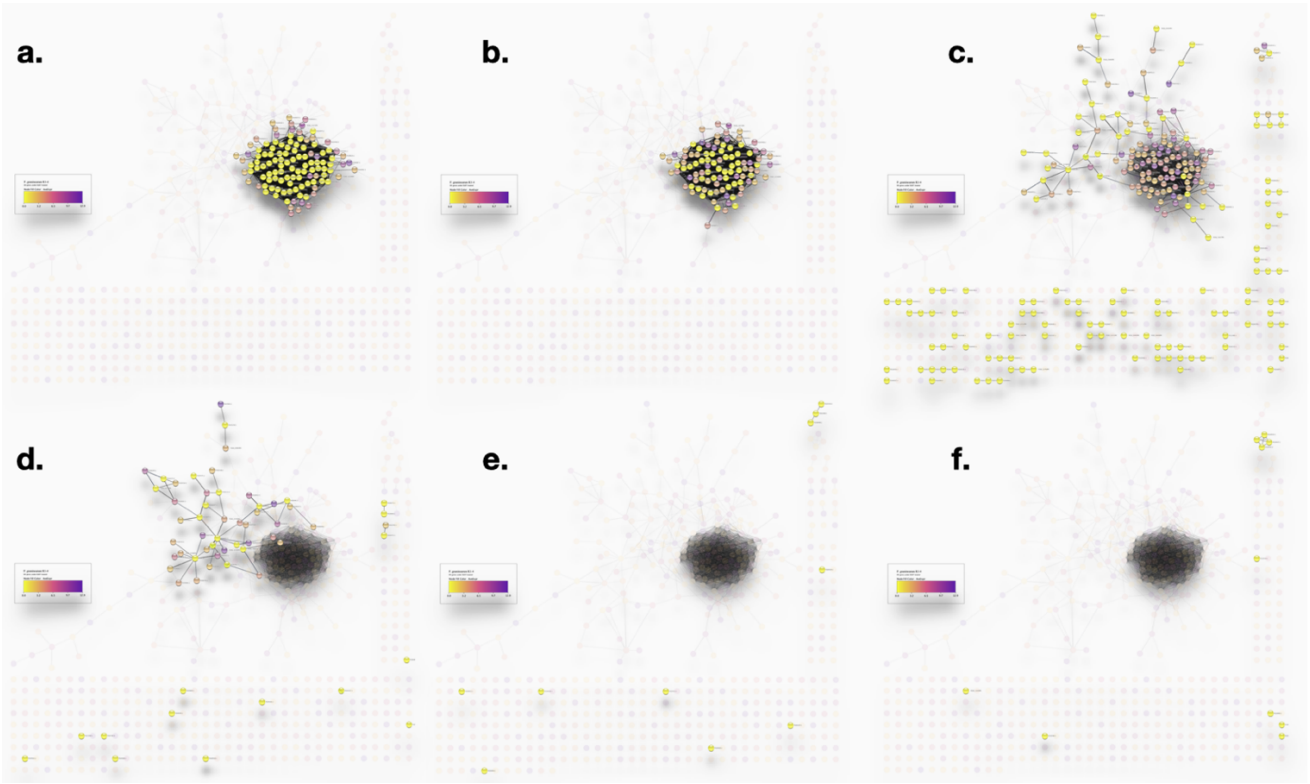


Figure 6.12 DE genes network analysis by StringDB of the Fg-K1-4 treated with EU07.

STRING network analysis of proteins with significantly regulated sites detected in a transcriptomics analysis of *F. graminearum* (*Fg-K1-4*) treated with EU07.Log-ratios within the threshold FDR of 0.01 and FC 1. **a)** Ribosome, and Protein biosynthesis represented by 75 transcripts (out of 187) FDR 6.17E-36. **b)** Ribonucleoprotein and Ribosomal protein represented by 41 transcripts (out of 81) FDR 2.70E-22. **c)** Transmembrane represented by 156 transcripts (out of 2430) FDR 0.0371. **d)** Oxidoreductase represented by 30 transcripts (out of 284) FDR 0.0127. **e)** mixed, incl. Amino acid permease/ SLC12A domain, and Arrestin, C-terminal represented by 10 transcripts (out of 55) FDR 0.0395. **f)** mixed, incl. Phosphopantetheine attachment site, and Putative esterase represented by 10 transcripts (out of 47) FDR 0.0153. Each set of transcripts were highlighted in yellow.

Discussion

6.5 Transcriptomics

6.5.1 Presence of EU07 affects the mycelium structure in Fg-K1-4 broths

In general, it was observed that the *Fg*-K1-4 mycelium was affected by the presence of the *Bacillus* strain EU07. The fungus grown in broth for 3 days and treated 48h, developed a different morphology to the control, showing swollen mycelia. As well as conglobated structures appeared at the second day of treatment. There are several studies informing of the response of mycelia due to the presence, either, of the coculture of *Bacillus* or of their metabolites (Baysal et al., 2013; Deleu et al., 2008; Gong et al., 2015; Patel et al., 2011).

Deleu et al. (2008) suggested that compounds from *Bacillus* strains such as fengycins and surfactins, possess hemolytic activity against filamentous fungus. Their results showed a concentration dependant disruption of the membranes and a higher effect produced by surfactins (40-fold) using a monolayer model of DPPC unilamellar vesicles. Patel et al. (2011) studied this effect in the commercial strain QST713, demonstrating that fengycins as its simple forms (Agrastatin1 and plipastatin A1) lead to a pore formation in fungal membranes.

Baysal et al. (2013) conducted experiments with VOC (volatile compounds) of the *Bacillus* strains used in this study. In PDA plates experiments, they found withered mycelia when a *F. oxysporum* was confronted with the strain QST713. Distorted,

swelled and disrupted mycelium were found when the strains FZB24 and EU07 were used. All the *Bacillus* strains produced mycelium growth inhibition by VOC. They suggested that the presence of the protein 3835 ID, identified in VOCs of EU07, has high similarity (99%) to SAM-dependent methyltransferases. These molecules allow methylation of small molecules required for metabolism and epigenetic regulation of macromolecules and biosynthesis of natural products (Sun et al., 2020).

Furthermore, Gong et al. (2015) used purified lipopeptides Iturin, plipastatin, and surfactin, from a *B. amyloliquefaciens* strain (S76-3) with a *Fg* (strain 5035). Microscopy observations displayed changes in the morphology of the fungus. Iturin A or plipastatin A treatments of conidia showed substantially deformed and damaged morphology and lateral expansion. Ultrastructural analysis showed damage in cell walls and plasma membranes. Similar observations were made when *Fg*-K1-4 broth were treated with EU07 (broths or pellet). Moreover, changes in pH of the fungal broth were observed. Predominantly, the pH of fungal liquid culture was low (4-5) after treatment with broths or pellets of EU07. Interestingly, addition of LB broth alone, triggered an early response and changes in the morphology of the *Fg-K1-4* mycelia. The presence of the whole bacterial broths led to an increase in the pH of the medium (6-7). The above observation prompted us to use the EU07 pellet treatment for the transcriptomic analysis, having in consideration that, this treatment would influence the fungus gene response, only

due to the presence of the bacterium. Samples were made after 6h of treatment (Gu et al., 2017; He et al., 2017; Schrey et al., 2005).

6.5.2 Optimization of RNA extraction from Fg (K1-4), a filamentous fungus, yield high quality sufficient for RNA-seq analysis

Besides this, an optimization of the RNA extraction protocol using Trizol was made. Fungal tissues are of the most difficult to process to obtain a high-quality RNA material due to the tissues content and cell structure (polysaccharides, tannins and pigments) (Dobbin et al., n.d.; Leite et al., 2012; Yaffe et al., 2012). Moreover, extractions from highly melanised structures of fungi are highly challenging when methods such as Trizol extraction are being used (Shu et al., 2014). Specifically, *Fusarium* genera is known to be great producers of secondary metabolites such as pigments, which is a taxonomic feature (Leslie and Summerell, 2006). Complementing to this, other barriers which decrease the quality of the downstream processing in RNA-seq, include the variability of the experiment, number of replicates (Kitchen et al., 2010) and quality of the samples (Kaur et al., 2014). Additionally, the presence of contaminants from the reagents used and carry through the extraction process, greatly affect the downstream process and further enzymatic reactions (Toni et al., 2018). Therefore, an RNA Trizol extraction protocol, a modification of the manufacturer, which yielded a RNA of high quality was established and used in subsequent experiments towards producing RNA-seq data.

6.5.3 DE genes analysis exhibited large data set of genes expressed in Fg-K1-4

To analyse the data, a pipeline with Hisat2 was used. The gene count table was obtained and genes were mapped using the reference genome PH-1 (Rothamsted Research, 2020), in order to investigate the transcriptional changes of *Fg*-K1-4 interacting with *B. velezensis* EU07. After the differentially expressed (DE) gene analysis was performed, a total of 14,898 genes DE were found. Further data mining was carried out to obtain insights of the experiment.

To reflect on the number of DE genes obtained in the experiment, it is necessary to compare with studies carried out on experiments related to the interaction of a *Fg* and the host (wheat) -at the time of this research, any study in *Fg*-bacterial interaction was found-. For instance, in *Fg* (strain DAOM233423) infecting wheat plants, it was found that only 8,811 DE genes were expressed during the infection (reference genome PH-1) (Pan et al., 2018), around 6,000 less compared with the *Fg*-K1-4 during the interaction with EU07.

In another study, Puri et al. (2016) found that two *Fg* isolates from different populations (3ADON-type, Asia prevalence and 15ADON-type, north America prevalence) displayed 479 up-regulated and 801 down regulated genes in a relation between the two populations in axenic cultures in mung bean agar. In these axenic cultures, a total of 14,242 and 14,564 DE genes respectively were observed. Less than 300 genes than *Fg*-K1-4. They observed pathways (KEGG) of DE analysis

included: starch and sucrose metabolism, drug metabolism (cytochrome P450) and others that were not found significantly expressed in *Fg*-K1-4. DE genes in *Fg*-K1-4, were generally ribosomal like and others related to ribonucleoprotein (p = 2.70e-22), oxidoreductase (p = 0.0127) and transmembrane (p= 0.0371). Puri et al. (2016) aimed to compare the transcriptomes of those two populations in axenic cultures purely due to the differences of those isolates (2x). While here in this study, the transcriptomics of an axenic culture of *Fg*-K1-4 was compared with the bacterium EU07 treated one. Puri *et al.* (2016) also compared the results of a transcriptomic analysis of two isolates of the fungus (3ADON and 15ADON) infecting plants. A total of 2,159 and 2,415 genes were upregulated for each isolated after 48h of infection compared with the axenic cultures. They found uniquely expressed genes in a total of 1,257 and 1,278 genes (three time point samples). They also found new KEGG pathways (pentose-phosphate pathway (p = 0.0085), RNA processing (p = 0.0002), ribosome biogenesis (p = 1.8×10−46), ribosomal proteins (p = 6.8×10−40), translation (p = 2.67×10−26), translation initiation (p = 0.0002), translation elongation (p = 0.0012), protein (p = 0.034), nucleic acid (p = 2.2×10−05) and RNA binding (p = 5.1×10−07), oxidative stress response (p = 0.0243), detoxification by modification (p = 0.0249), and genes related to nucleolus (p = 0.0437). Interestingly, this correlated well with our findings during the challenging of the *Fg*-K1-4/EU07 (DE genes of EU07 treatment relative to the axenic *Fg*-K1-4). It was observed a KEGG pathway significantly expressed (Ribosome, p=1.03E-45) and several

UniprotKB annotated keywords (Ribosomal protein, p=4.02E-23; Ribonucleoprotein, p=2.7E-22; Oxidoreductase, p=0.0127; Transmembrane, p=0.0371; Transmembrane helix, p=0.0376; Membrane, p=0.0430).

Contrary, when analysing DE genes of the *Fg*-K1-4 relative to the EU07, new UniprotKB keywords were observed (Oxidoreductase (p = 0.00011), Metal-binding (p = 0.00017), Copper (p = 0.0352) and Zinc (p = 0.0352). Only the KEGG pathway Glycine, serine and threonine metabolism was observed (p = 0.0337), which is of importance on growth and antifungal activity in fungi such as *Penicillium citrinum* W1 (Wu et al., 2015). This analysis, from the control relative to EU07 treatment, shows that *Fg*-K1-4 has a similar behaviour in axenic culture observed by Puri *et al.* (2016).

In another report using the strain PH-1, Jiang et al.(2020) studied the orphan genes (OG), which is believed, they are present in phytopathogenic fungi as a strategy to enable infection and increase virulence, also called fungal effectors. They are small-secreted proteins that are cysteine-rich but lack a common structural motif (OSP). The authors studied 50 OSPs and found three that, their absent, leads to a lost in the virulence of the strain. These effectors were found that they do not affect the vegetative growth, conidiation, sexual reproduction and virulence of *Fg* during the infection in the cultivar Xiaoyan 22. Interestingly, some of those genes were expressed in *Fg*-K1-4 when it was challenged by EU07. The gene *FGRAMPH1_01T08371* (*FGSG_13782*, p = 2.74e-3), which is a novel one, were

expressed by *Fg*-K1-4 (threshold LogFC = 1, p = 0.01) and in total 5 genes were expressed in the threshold of LogFC = 0.585, p = 0.05 (FGRAMPH1_01T17443 (FGSG_05241P0, p = 0.05), FGRAMPH1_01T09501 (FGSG_13407P0, p = 0.04), FGRAMPH1_01T28085 (FGSG_13505P0, p = 9.72e-3), FGRAMPH1_01T23171(FGSG_06780P0, p = 6.99e-3), FGRAMPH1_01T04455 (FGSG_12020P0, p = 0.01), they are novel genes as well, and some of them are present only in *Fg-K1-4*.

In another study investigating intertranscriptomic interaction between *F. verticillioides* and the *B. velezenzis* FKM10 (in a threshold of $|\log2FC| \geq 1$ and p-value < 0.05), the authors determined that a total of 792 genes in *F. verticillioides* were significantly different under co-cultivated conditions (3h), among which 535 genes showed increased expression and 257 genes displayed decreased expression levels. This demonstrate that the comparison of DE genes within species of close related *Fusarium* produce variable results. Moreover, annotated pathways of the DE genes on KEGG were found to be 243 and those significantly enriched ($p < 0.05$) were non-ribosomal peptide structures (ko01054); biosynthesis of amino acids (ko01230); citrate cycle (TCA cycle) (ko00020); 2-oxocarboxylic acid metabolism (ko01210); carbon metabolism (ko01200); valine, leucine, and isoleucine biosynthesis (ko00290); legionellosis (ko05134); sulfur metabolism (ko00920); steroid biosynthesis (ko00100); and nicotinate and nicotinamide metabolism (ko00760) (Wang et al., 2020). While the interaction of EU07 and *Fg*-

K1-4 showed two main pathways, ribosome (p=0.71) and metabolic pathways (p=0.12) when the first 2000 significant genes were analysed using StringDB. Generally, ribosomal protein genes are among the most highly expressed genes, basics for cell growth and proliferation (Petibon et al., 2020). Ghulam *et al.*, (2020) demonstrated how the ribosomal protein genes (RP) DE of duplicated ribosomal protein genes modifies ribosome composition in response to stress such as changes in growth conditions, in *Saccharomyces cerevisiae*.

Using another approach, Connolly et al. (2013) studied the effect of KMT6, a Histone H3 K27 Methyltransferase, as regulator of development and expression of secondary metabolite gene clusters in *Fg* mutants of PH-1. They distinguished several gene clusters: aurofusarin cluster (*aurO, aur1, aurC, aurJ, aurF, gip1* and *aurS*), the carotenoid cluster, deoxynivalenol (DON) synthesis, and a block of contiguous genes on chromosome 1, *FGSG_02111, FGSG_02113, FGSG_02114, FGSG_02117*, and *FGSG_02118*. In contrast, in *Fg*-K1-4, several of these genes where DE during the interaction with EU07. Most notably, from the aurofusarin cluster (*FGSG_02324P0, FGSG_02325P0, FGSG_02326P0, FGSG_02327P0, FGSG_02328P0, FGSG_02329P0*) *aurF gene FGRAMPH1_01T05599* (FGSG_02327P0, p= 0.007) and *AUR1* gene FGRAMPH1_01T05593 (FGSG_02324P0, p=0.00617) were the most significantly expressed one. Interestingly, all the genes found from those clusters decreased the level of expression during the interaction *Fg*-K1-4/EU07 (Figure 6.13 c-d).

Besides, same authors, demonstrated that a block of contiguous genes on chromosome 1, (*FGSG_02111, FGSG_02114, FGSG_02115, and FGSG_02118*), which included a TRI7 (toxin biosynthesis protein) homolog, was DE. In the case of *Fg*-K1-4, the same gene showed significantly decreased level of expression (FGRAMPH1_01T05097, FGSG_02115, p=0.0085, logFC = -2.2648) (Figure 6.13 c-d).

Additionally, other authors investigated fungal metabolites for further use in the pharmacological area (Sieber et al., 2014). They studied online data of the *Fg* (Broad Institute) and discovered that many gene clusters are regulated by secondary metabolism, specific transcription factors and global regulators as well. Some metabolites they investigated included: aurofusarin, zearalenone, orcinol/orsellinic acid, trichothecene, carotenoid, fusarin C, culmorin and fusarielin. Interestingly, the strain *Fg*-K1-4 showed that, many genes within those clusters displayed decreased level of expression, some with significant level. The cluster of aurofusarin was represented by several genes (FGRAMPH1_01T05593, p= -2.31; FGRAMPH1_01T05595, p= -1.95; FGRAMPH1_01T05597, p= -1.64; FGRAMPH1_01T05599, p= -2.22; FGRAMPH1_01T05601, p= -2.12; FGRAMPH1_01T05603, p= -2.13). As well as, from the cluster of trichothecene, several genes were DE (FGRAMPH1_01T13095, p=1.479; FGRAMPH1_01T13109, p= -4.06; FGRAMPH1_01T13115, p= -3.743; FGRAMPH1_01T13119, p= -2.013). Similar to Sieber et al. (2014), *Fg*-K1-4did not showed expression of

zearalenone (ZEN), which is found to be expressed only during infection of maize. In general, during the interaction of *Fg*-K1-4 and EU07, a decrease in the level of expression of the secondary metabolites, mycotoxin-related, were observed.

In summary, it was observed that the *Fg*-K1-4 genes were affected by the interaction with EU07 in a significant way. Firstly, the number of genes observed in the interaction was bigger when compared with that in an axenic culture. Secondly, the more important DE genes in the *Fg*-K1-4/EU07 interaction were mostly related to ribosomal pathways/ network clusters. This is important, since the ribosome and protein production are very important in the cell division and production of new cells and are energy demanding (Espinar-Marchena et al., 2017). Besides this, genes that are believed to contribute on the pathogenicity, known as fungal effectors, were almost absent in the interaction of *Fg*-K1-4/EU07. This has a great repercussion on the biological control strategy. EU07 probably has the potential not only to stop the growth of the fungus but also collaborate with plant to reduce the attack of the fungus to the plant, then the plants can defend better against the attack (Jiang et al., 2020).

More surprisingly, during the interaction of *Fg*-K1-4/EU07, genes that contribute with the infection, known as mycotoxins, are significantly decreased in the level of expression. Which mean a reduction on the overall pathogenicity of the fungus.

In conclusion, EU07 affects the level of expression of genes related to pathogenicity in *Fg*-K1-4, decreases the potential damage caused by this fungus to the plant, indicating it can be used as a plant protection agent.

Chapter 7

General discussion

Fusarium graminearum (*Fg*) is the main causal agent of the *Fusarium* head blight (FHB) in cereal crops and is of great importance in the wheat production and other cereal crops in the UK and around the world. Therefore, the general research question in this study aimed to determine whether some *Bacillus* strains have the ability to suppress the growth of the mycotoxin producing fungus *Fg*. The initial hypothesis is that these *Bacillus* strains, in fact, can control the growth of the *Fg,* and it is suspected that, this will also decrease the mycotoxin deoxynivalenol (DON) production (Baysal et al., 2013, 2008; Zhao et al., 2013; Y. Zhao et al., 2014).

Three beneficial *Bacillus* strains (QST713, FZB24 and EU07) were used to assess the control of the mycotoxin producing pathogen *Fg* (Baysal et al., 2008; "Our crop protection products," n.d.; Pandin et al., 2018). It was decided to investigate the genomic differences between these *Bacillus* strains and the effect of the EU07 on the expression of genes in the fungus. A succession of *in vitro*, *in vivo*, genomics and transcriptomics studies were carried out to determine these effects, and the genetic mechanisms behind it were evaluated.

Assays, their effects and limitations

In vitro antagonism assays

A first approach was to determine the antagonistic effect of the *Bacillus* strains against the fungus. Additionally, toxicity tests were done to learn about whether the mycotoxins would affect the bacterial growth.

Antagonistic assays demonstrated that all the *Bacillus* strains have the ability to supress the growth of the fungus *in vitro*. Remarkably, it was found that, in general, the strain FZB24 and EU07 performed better and consistent throughout the antagonistic assays. Besides, when cell-free bacterial broths of the *Bacillus* strains were used for the experiments, the antagonistic effect was conserved. Particularly, when the bacterial broths were treated with heat and proteinase K, the antagonistic effect was increased, suggesting that not some proteins but secondary metabolites were responsible for the antagonistic effects. Therefore, it was hypothesised that, the growth of *Fg* was negatively affected as a result of the presence of secreted bacterial metabolites during the *in vitro* assays (Baysal et al., 2013; Li et al., 2012; Zhao et al., 2014). It was also tested the ability of the *Bacillus* strains to grow along with the mycotoxin DON and it was found that all the *Bacillus* strains can survive and flourish in the present of the mycotoxin at high concentrations. More interestingly, QST713 and EU07 demonstrated higher growth rate than that of FZB24 (Venkatesh and Keller, 2019) in DON added LB broth.

In summary, it was found that all *Bacillus* strains have antagonistic effects against *Fg* (K1-4). Cell-free broths of the *Bacillus* strains displayed conserved antagonistic effect and when treated with enzyme or heat, bacterial broths

improved the antagonistic effect showing that FZB24 and EU07 were the best. It is possible that compounds such as iturin A, lipeptides and fengycins are responsible for the antagonistic effect (Bernat et al., 2016; Deleu et al., 2008; Gong et al., 2015)

On the other hand, DON assays demonstrated that the bacteria within the high DON concentrations could survive without any ill effect. However, it was not investigated whether the *Bacillus* strains could be able to transform DON into less toxic compounds, which also contribute to the general aim to reduce the effects of the fungi in plants and further food products (McCormick, 2013; Venkatesh and Keller, 2019).

It can be concluded that the three bacillus strains have the ability to suppress the growth of the Fg-K1-4 *in vitro* as well as be viable solutions for further investigation in DON decontaminations in food products as well as in soils with heavy levels of FHB infections in previous crops.

Limitations were found along the execution of these experiments, particularly, it was not possible to determine the identity of compounds found in cell-free bacterial broths treated with enzyme/heat. This can be achieved using technologies such as liquid chromatography-mass spectrophotometer coupled (LC-MC). On the other hand, antagonistic assays did not demonstrate the decrease of DON production from *Fg* when treated with the bacteria. Although HPLC analysis of the DON assays could increase the understanding of the

results (Chaimbault, 2014; Jorge et al., 2016), time and budget constrains made not possible to carry out on these experiments.

In vivo antagonism assays

Further assays were carried out *in vivo* to learn the effect of these *Bacillus* strains during the interaction of *Fg* and the host plant. *Arabidopsis* or Brachypodium (Bd-21) plants were used as host plants. It was clear that the presence of the bacterial suspensions, as well as free-cell bacterial broths, have an important effect in the development of the infection of the *Fg* on *A. thaliana* (*At*) and in *B. distachyon* (*Bd*-21) (Larmour and Marchant, 1977). For instance, Trypan blue staining showed less or no formation of macroconidia. This may well be due to the fact that spores of *Fg*-K1-4 were not able to germinate in the presence of EU07 and the mycelia was poorly formed (Gong et al., 2015; Li et al., 2012; Palazzini et al., 2016; Rana et al., 2018).

In regard to the PGP activity assays, conclusions can be drawn about the differences reported by the results observed in these three *Bacillus* strains. While FZB24 and EU07 were better in antagonistic assays, it was not the case with PGP activity. FZB24 showed the worst PGP performance, with non-protection of plants against pathogens. EU07 induced the defence of the plant, increased the production of floral structures in *Bd*-21, decreased the flowering time (*At* and *Bd*-21), protected the plant against opportunistic pathogens and kept biomass production in *At*. Contrary to the usual growth-defence trade-offs models, which

postulated that plants dedicate resources either for defence or for growth, it appears that EU07 contribute to the both traits (Huot et al., 2014). Moreover, the flowering time and the production of reproductive structures (flowers/heads) were influenced by EU07. Some authors mentioned that *Flowering Locus C* (FLC) and *FT*/*TFL1* genes regulate flowering time (Jeong et al., 2015; Kazan and Lyons, 2016). It is possible that these were similar results. Here, *FLC*, a flowering repressor affected by fungal infections, could be affected as well by EU07, which triggered a signal in defence genes (Figure 4.11).

Besides, assays in *At* Ws-*eds1* indicated that EU07 stimulates the ISR without loss of all the growth trade-offs. On the other hand, *At* Col-0 defence genes were found to shed light on effects at the root level, which the bacterium uses for triggering plant defence responses (SAR) (Figure 4.1 and 4.2) (Yuge Li et al., 2019; Parker et al., 1996; Vlot et al., 2021). Infection head assays (IHA) with *Bd*-21 demonstrated less percentage of disease severity index (%DSI) in heads infected with *Fg*-K1-4 when they were treated with EU07 by either spraying or drop treatments, where spread treatments performed better. Similarly, *At* plants infected with the oomycete *Hpa* showed a big drop in spore development when EU07 was used than the other two strains used, in both Col-0 and WS-*eds1* plants. Therefore, it can be put forward that *B. velezensis* EU07 possesses compounds that, in both ways, suppress the growth/development of the pathogen, as well as triggers the SA defence of the plant. Similarly, *At*-Col-0 plants treated with bacterial broths showed less infection by naturally occurring pathogen powdery mildew (PM).

The results found here are not exclusive of the strains used in this research. There is a huge amount of research in plant growth promoting bacteria and BCAs. For instance, Long Chen *et al.*, (2018) characterised *Bacillus velezensis* 157 which had antagonistic activity against a broad spectrum of pathogenic bacteria and fungi such as *E. coli*, *Streptococcus agalactiae*, *Salmonella typhimurium*, *Botrytis cinerea* and *F. oxysporum*. Mendis *et al.*, (2018) investigated the plant growth promoting bacteria using commercial strains. QST713 and *B. firmus* I-1582, which are used as active ingredients of commercially-available soil application and seed treatment products Serenade® and VOTiVO®, respectively. Susič *et al.*, (2020) further investigated *B. firmus* I-1582 and non-commercial strain for the control of nematodes. QST713 and FZB48 (*B. amyloliquefaciens*) have been used as plant growth promoting rhizobacteria for a while (Chowdhury et al., 2015). Altogether, these authors recognised the popularity of these bacteria as potential microbial biological control agents (MBCA) for crop protection. Hence, their study in the control of new or existing plant pathogens should be extended and the implementation of field trials for the necessary understanding of their effects at bigger scale could be carried out (Borriss, 2011; Borriss et al., 2018; Fan et al., 2018; Punja et al., 2019).

Genomics

Genomic sequencing of the three bacillus strains was performed and the data, particularly, belonging to the EU07, novel strain, will be deposited in public

repositories such as EBI (Knight et al., 2018). The strains QST713 and FZB24 are already in public domain (Chen et al., 2007; Pandin et al., 2018).

Subsequently, when the whole genome (WG) of the *Bacillus* strains were compared, the first question sought to determine any difference on gene content of the bacterial genomes which could support the findings obtained *in vivo* and *in vitro* assays in this study. Surprisingly, a first finding was to determine that the strain FZB24 was a different species with a genome length shorter than the other two strains. FZB24 is *Bacillus amyloliquefaciens* within the *Bacillus amyloliquefaciens* group. Another finding was that QST713 and EU07 were same *Bacillus* strain, a *Bacillus velezensis* (Table 5.2). They both have a similar genome length and similar gene content. However, analysis of the antibacterial genes content, demonstrated that both strains possess similar antimicrobial content to FZB24 (Table 5.11). This subtle similarity can be explained in the sense that these are strains classified into the same *B. subtilis* Species Complex. These bacteria produce biofilm as well (Pandin et al., 2019; Stefanic et al., 2015). Genome analysis of the strains showed that they possess genes that code for siderophores and auxin production capability. Several genes including multidrug resistance (*bmrA*), uncharacterized protein (*YobM*), extrachromosomal origin (*ynzG, nrdEB*), putative racemase (*ygeA*), sporulation kinaseC (*kinC*), were found in EU07 that differ partially or totally from QST713.

However, *in vivo* and *in vitro* assays showed that both EU07 and QST713 showed different performance. The EU07 was outstanding in the antagonistic

assays, as well as, in the *in vivo* assays. The more remarkable results were obtained in the *in vivo* assays (infection assays and in the plant-growth promoting activity). It is possible, therefore, these slight variations of these genes between QST713 and EU07 gave to the latest, the advantage to perform better in the nature. Perhaps, posttranslational mechanisms were responsible for the better performance, such as ribosomally synthesized and post-translationally modified peptides (RiPPs) (Burkhart et al., 2015; Fan et al., 2018). Yet, this postulate requires more research to increase the understanding of the superiority of the strain EU07.

Nonetheless, in recent years, the contribution of the whole bacterial genome analysis to biological control has been increased steadily. The perspectives of studies are varied, including bacterial community studies, properties of the strains, relatedness of the strains or taxonomic studies, strain of industrial and biotechnological importance, biopesticides -such as this research work-, transcription and RNA regulation, bacterial antimicrobial activity genes, ecology studies and human pathogens.

Perhaps, pangenome analysis term was first applied to the study five human pathogens (Bazinet, 2017; Kim et al., 2017; Tettelin et al., 2005). In this regards, Tettelin *et al.* (2005) aimed to study the global complexity of bacterial species in human pathogens to develop vaccines and functional characterization of genetic determinants. Taxonomic and relatedness of the bacteria have been studied using whole bacterial genome analysis which contrast with the taxonomy

analysis using the 16S rDNA gene-based (Maughan and Van der Auwera, 2011; Radnedge et al., 2003; Read et al., 2003; Zeigler et al., 2008). Evolutionary relationships and ecology studies can be constructed using genome analysis. Alcaraz *et al.* (2010) brought the matter of the advantages of the bacterial community analysis using whole genome sequencing instead of set of genes. In this regards, Um *et al.* (2013) studied a *Bacillus* spp. which inhibits antagonistic fungi in fungus-growing thermites with comparative analysis of genomes of these strains with *Bacillus subtilis*.

More recently, a great deal of research have been done to understand antibiotic resistance in genes for human pathogens using whole genome sequencing (Bengtsson-Palme et al., 2018; Forsberg et al., 2012; Fournier et al., 2014; Imran and Yun, 2020; Loman and Pallen, 2015). Yet, the studies involving whole genome sequencing to investigate the beneficial activity such as industrial use (Deng et al., 2011; Hu et al., 2010; Takami et al., 2000) antimicrobial activity (Mendis et al., 2018; Palazzini et al., 2016), plant growth promoting activity (Blake et al., 2021; Chen et al., 2019; Liang Chen et al., 2018), probiotics (Kapse et al., 2019), bioinsecticides against fungi (Li et al., 2020; Méric et al., 2018; Susič et al., 2020) or antibacterial activity and biopesticides (Mendis et al., 2018), have been exploited immensely.

Therefore, this research contributes to this trend by characterising the strain EU07 at laboratory and semi-field level and by characterising the strain itself. Its

exceptionally results will be of benefit for future applied research and possible commercial developments.

Transcriptomics

Lastly, transcriptomic studies of the *Fg* (K1-4) challenged with the *B. velezensis* EU07 were done. Analysis of the transcriptomic results showed a total of 2,232 transcripts significantly differentially expressed (DE) in the threshold FDR 0.05 and abs logFC 1. A total of 709 genes observed in a threshold FDR of 0.01 and FC 1 were used for network analysis.

Some of the most highly expressed genes were characterised to be novel genes (Table 6.3). An increase in the expression of genes related to ribosomal pathways was observed. This has importance since the ribosome and protein production are tightly related to the cell division and production of new cells and are energy demanding (Espinar-Marchena et al., 2017). This was studied by Ghulam *et al.* (2020) and Petibon *et al.*(2020), who demonstrated how stress such as changes in nutrient availability produce a response in ribosomal protein genes, which are highly expressed during growth and proliferation. It can be concluded that *Fg* (K1-4) exposed to the EU07, dedicate the energy and resources to growth and proliferate instead of produce toxins to defence against EU07.

Moreover, as was pointed out by Keller and Hohn (1997), fungi, as microorganisms with limited resources, only express appropriate catabolic pathways to survive in low growth nutrient conditions, hence the regulation of

expression of these pathways is key to direct the energy and efforts for survival. Interestingly, genes dedicated to those metabolic pathways such as the trichothecene, are clustered. Here, the analysis revealed DE genes related to mycotoxin production and pathogenesis transcripts were observed to be decreased in their level of expression, including aurofusarin and trichothecene but not zearalenone (ZEN) transcripts (Connolly et al., 2013b; Sieber et al., 2014).

Interestingly, genes that are thought to have effector activity were not significantly expressed. For example, the orphan secreted protein (OSP) gene OSP24 is important for infection which is important for growth and initial penetration and later growth in wheat rachis tissues. In our study, the level of expression of some of these OSPs during this interaction was observed to be dramatically low or absent. Fungal effectors are examples of orphan genes that have evolved for plant infection as many of them lack homologs in closely related species (Jiang et al., 2020) demonstrating the importance of this finding in our study.

Overall, the transcriptomic studies demonstrated that EU07 has the capability to trigger or suppress signals in *Fg*-K1-4 for the production mycotoxins which are known to influence the infection in plant (Beccari et al., 2018). Besides, genes that are believed to contribute on the pathogenicity, known as fungal effectors, were not significantly expressed in the interaction of Fg-K1-4/EU07. This has a great repercussion on the biological control strategy. This can suggest that the

EU07 has mechanisms of interaction with the fungus, which inhibit its capability to produce an effective infection on plants.

Therefore, it can be suggested that EU07 affects the level of expression of genes related to pathogenicity in *Fg*-K1-4, decreases the potential damage caused by this fungus to the plant, indicating that it can be used as a plant protection agent.

Lastly, there is a large body of research regarding to the use of transcriptomic studies to understand microorganism interactions. Generally speaking, the studies most commonly found about microbes' interactions using transcriptomics are focused on the effect of a pathogen to the host. Some examples include: walnut tissues and the microbes interaction (Chakraborty et al., 2016), tomato and a fungal pathogen -*V. dahlia*- (Tan et al., 2015), rice roots and *Azospirillum brasilense* (Thomas et al., 2019) between others. Some other studies focused on microbe-microbe interaction. Fungal – fungal interaction such as the beneficial fungi *Clonostachys rosea* against the pathogenic fungi *F. graminearum* studies investigated how they interact (Demissie et al., 2018), or mix of beneficial fungi interaction in specific rations with lignocellulose (Daly et al., 2017) or more complex study including beneficial fungi (*Pseudozyma flocculosa*) with pathogenic fungi (*Blumeria graminis f.sp. hordei*) and its host (*Hordeum vulgare*) (Laur et al., 2018). Finally, fungal-bacteria interactions (FBI) have been investigated as well. Gkarmiri *et al.* (2015) studied a triple RNA-seq system which involved the response of *Rhizoctonia solani* AG-3 in response to the antagonistic bacteria *Serratia proteamaculans* and S*. plymuthica*. Pierce *et*

al. (2021) studied a complex system of fungus found in cheese and collaborative bacterium analysis of bacterial mutant fitness. This study found conserved drivers of bacterial–fungal interactions. Species-specific interactions were uncovered. The implication of this work is in the understanding fungal antibiotic production, highlighting potential for broad impacts of fungi on bacterial species within microbiomes. Strub *et al.* (2019) investigated the interaction of actinobacteria *Streptomyces* sp. AV05, a BCA, against *F. verticilloides* using RNA-seq. Particularly, Wang *et al.* (2020) investigated the *B. velezensis* FKM10, which inhibits *F. verticillioides,* a pathogen of the tea crab apple (*Malus hupehensis*), and looked at the PGP activity on this pathosystem, which has a similar approach to that proposed here in this research.

Overall significance of the work

The knowledge produced here has vast repercussions for the understanding of the effect of beneficial bacteria not only in crop protection but also in environmental and climate change mitigation.

- It was demonstrated that the cell-free broths of EU07 can produce beneficial effects on plants and in control of pathogens. More significant, the plant disease control can be developed further into field assays, which do not involve the introduction of foreign strains in the field.
- It was shown that three *Bacillus* strains were viable in the presence of the mycotoxin DON.

- Besides, EU07 could be used in further research to explore its effect in the growth defence trade-off phenomena. The effects found here support the fact that this strain has positive effects in plant growth promotion and plant defence, as well as, in regulation of flowering time.
- This study also contributed with the novel EU07 genome, which will be deposited in renown databases.
- Our study, is the first to use transcriptomics analysis for the study of the interaction of *Fusarium graminearum* (K1-4) and a *Bacillus* strain (EU07). These results should be taken forward to elucidate the functions of novel transcripts found here to be DE.
- The transcriptomic data will contribute to databases such as StringDB and Ensembl Fungi where studies in the interaction of fungal-bacterial (FBI) communities are lacking.
- Finally, the whole project also gave a better understanding of the PGP activity of the bacterial strains coupled with the analysis of the genetic background of those strain, which can contribute to more informed future research.

Future directions

This study generated a lot of answers to questions raised before. However, it has also produced questions that can be answered in future work. There are several recommendations of future work to do; firstly, it is important to

investigate the effect of EU07 in a wider range of pathogens, oomycete specifically and other bacteria should be used to demonstrate the extend of the controlling effect of EU07. There is a lack of information of the effect of the *Bacillus* strains, as a seed and root protector, for example in the disease damping off protector or in the change of vigour on germination of plants or potential to increase germination and protection in seeds.

The *Bacillus* strains should be used to investigate their effects in detoxification on soils and plant surfaces with potential presence of mycotoxins. Furthermore, it is necessary to study if the strain EU07 has the same capability of growth and survive in presence of mycotoxins produced by other fungi. For instance, it is known that the climate change has produced increasing damages by fungi which migrate towards the poles due to the increasing temperatures. Damage in crops will see new species as well increase in the effect of the already established species, not to mention the spoilage in food products to the presence of these fungi. On the other hand, it is worth to explore strategies to improve crop soils, which are being degenerated by the continue application of chemical pesticides and monoculture.

Biofilm function, composition and understanding in EU07 compared with other two strains need to be studied deeply. Moreover, imaging work (fluorescent dyes/transformants with help of fluorescent/confocal microscope) would reveal movement of the bacteria/metabolites in plants. Bacteria transformation -GFP-

would contribute to improve the knowledge of the persistence of these strains as inoculum/protector in soils.

It is important to stress out some limitations detected during the execution of these experiments. For instance, it was not possible to perform PGP activity assay with the mix bacteria, to determine any level of synergism between the bacterial strains. The effects of bacteria in germination and further seed protection were not analysed as the methodologies were needed to be established. Finally, it was not possible to carry out studies on germination of *Fg* on leaves treated with these bacteria as time and equipment such as confocal microscopes and transformed variants of these strains with GFP (Pandin et al., 2019) were needed. Limited access to characteristics of variants used in plants and previous results would contribute better to the understanding of the PGP results, here only *At* Col-0, WS-EDS, PR1::GUS and RLK::GUS lines were used.

Then, the PGP activity should be explored further including the JA/SA pathway in plants such as *At*/Bd-21.

There is still the need to determine the function of genes found in EU07, which some are still not demonstrated in public databases as well as, identifying specific compounds in EU07 offer enzymatic/heat treatment and its specific effect on fungi (*Fg*). Studies around the effect of these strains in production of DON and other mycotoxins are necessary as well as to investigate effect of cell free broths on *Fg* using PD broth an imaging technique. Additionally, the effect on mycelia was not assessed, need better appreciation, to use purified specific

compounds found in EU07 broths. For instance, further investigating the structure deformation of *Fg* by *Bacillus* (all of them), with experiments that involved longer time, microscope observation and subculture of the mycelia after treated with the bacteria could help revealing further information.

Differentially expressed *Fg* genes discovered with RNA-seq analysis could be mutated or the mutant lines of Fg-K1-4 could be identified to see how that fungal line function, hence revealing the function of those genes.

Finally, EU07 could be formulated as biopesticide to use for crop protection and soil regeneration. To do this, fields assays could be done to investigate the effects of this bacteria at large scale in cereal crops.

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ORIGINAL ARTICLE

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Effect of light and dark on the growth and development of downy mildew pathogen *Hyaloperonospora arabidopsidis*

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Abstract

Disease development in plants requires a susceptible host, a virulent pathogen, and a favourable environment. Oomycete pathogens cause many important diseases and have evolved sophisticated molecular mechanisms to manipulate their hosts. Day length has been shown to impact plant–oomycete interactions but a need exists for a tractable reference system to understand the mechanistic interplay between light regulation, oomycete pathogen virulence, and plant host immunity. Here we present data demonstrating that light is a critical factor in the interaction between *Arabidopsis thaliana* and its naturally occurring downy mildew pathogen *Hyaloperonospora arabidopsidis* (Hpa). We investigated the role of light on spore germination, mycelium development, sporulation, and oospore formation of Hpa, along with defence responses in the host. We observed abundant Hpa sporulation on compatible *Arabidopsis* under day lengths ranging from 10 to 14 hr. In contrast, exposure to constant light or constant dark suppressed sporulation. Exposure to constant dark suppressed spore germination, mycelial development, and oospore formation, whereas exposure to constant light stimulated these three stages of development. A biomarker of plant immune system activation was induced under both constant light and constant dark. Altogether, these findings demonstrate that Hpa has the molecular mechanisms to perceive and respond to light and that both the host and pathogen responses are influenced by the light regime. Therefore, this pathosystem can be used for investigations to understand the molecular mechanisms through which oomycete pathogens like Hpa perceive and integrate light signals, and how light influences pathogen virulence and host immunity during their interactions.

KEYWORDS

Arabidopsis, circadian rhythm, downy mildew, light regime, oomycetes

1 | INTRODUCTION

Environmental factors such as light, temperature, and humidity play a significant role in the infection of plants by microbial pathogens and during disease development (Cheng *et al*., 2019). At the molecular level, adaptation to environmental fluctuations is influenced by circadian timing mechanisms that undergo daily adjustment and act as a seasonal timer for diverse organisms, including plants and plant-associated microbes (Johnson *et al*., 2003). Light is the one of most significant environmental signals for circadian regulation (Dunlap *et al*.,

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2004). Many organisms have circadian regulation networks that operate through similar mechanisms. For plants, light is perceived by photoreceptors and acts as a signal to regulate circadian genes (Millar, 2004; Franklin *et al*., 2005). Discrete light at different times of the day has been reported to have defined and particular effects on phase changes (Johnson *et al*., 2003). The circadian clock has also been shown to have a major effect on regulation of plant immunity (Lu *et al*., 2017; Karapetyan and Dong, 2018).

Light is known to have an effect on sporulation of several fungal and oomycete species, and the circadian clock of one fungal phytopathogen has been linked to the pathogen's virulence programme (Hevia *et al*., 2015). In contrast, there are a limited number of publications on the relation of light with development or virulence in oomycetes (Rumbolz *et al*., 2002). Early studies reported positive phototaxis of *Phytophthora cambivora* zoospores (Rumbolz *et al*., 2002) and the effect of humidity and light on discharge of sporangia of different oomycete pathogens (Fried and Stuteville, 1977; Leach *et al*., 1982; Su *et al*., 2000). Similarly, in *Plasmopara viticola*, the downy mildew pathogen of grapevine, continuous light did not have any effect on the growth of the mycelium and formation of sporangiophores, but the shape of sporangia was observed to be immature (Rumbolz *et al*., 2002). In the lettuce downy mildew pathogen *Bremia lactucae*, exposure to dark induced sporulation while light inhibited sporulation in a temperature-dependent manner: at low temperature, light was suppressive; however, with increasing temperature, the effect of suppression was decreased (Nordskog *et al*., 2007). Light also suppressed sporulation in *Peronospora belbahrii*, downy mildew of sweet basil, but light-dependent suppression of sporulation was enhanced at higher temperature. Light is also known to regulate the balance between asexual and sexual spore formation in *Phytophthora infestans*, causative agent of potato blight (Xiang and Judelson, 2014), in which exposure to constant light suppressed sporulation on plants and artificial media (Harnish, 1965). The mechanistic basis of light effects on oomycete virulence are largely unknown and likely to comprise a combination of light-regulated programmes for the host as well as the pathogen. It is also conceivable that the interacting organisms could directly influence each other's circadian programmes.

Oomycetes cause many important diseases in crops and in natural ecosystems (Kamoun *et al*., 2015). Much recent progress has been made in understanding plant–oomycete interactions through the development of reference plant–oomycete pathosystems that are amenable to genomic, genetic, and molecular approaches (Herlihy *et al*., 2019). One such pathosystem is the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Hpa) and its natural host *Arabidopsis thaliana* (Coates and Beynon, 2010). Like many oomycetes, Hpa establishes an intimate relationship with its host by forming structures called haustoria, which are used to obtain nutrients from the plant. The Hpa life cycle is completed by the formation of aerial sporangiophores, which produce asexual spores, and by sexual oospores that are formed in infected leaves (Koch and Slusarenko, 1990). Because Hpa is an obligate biotroph, it requires its host to remain alive in order to complete its life cycle (Coates and Beynon, 2010). Hpa also redirects the host's metabolism and suppresses the host defence mechanisms (Herlihy *et al*., 2019). In Hpa–*Arabidopsis* interactions, it has been established that 16 °C is the best temperature for Hpa sporulation under laboratory conditions (Dangl *et al*., 1992). However, the effect of different light/dark regimes on the sporulation of Hpa and the most productive light/dark time period for Hpa growth have not been reported. Elucidating the effect of light on the sporulation and growth of Hpa may also give some clue as to whether there is a circadian regulation of its life cycle. Here, we report the effect of different light/dark regimes on the germination, mycelial development, and sporulation of Hpa.

2 | MATERIALS AND METHODS

2.1 | Plant lines, pathogen isolates, and propagation

Hyaloperonospora arabidopsidis isolate Emoy2 was maintained on *Arabidopsis* lines Ws-*eds1* (Parker *et al*., 1996) or Col-*rpp4* (Roux *et al*., 2011). Maintenance and preparation of inoculum for experiments was performed as described previously (Tör *et al*., 2002; Woods-Tör *et al*., 2018). Transgenic *PR1-GUS* lines were obtained from Xinnian Dong (Duke University, North Carolina, USA).

2.2 | Sporulation assay

Inoculated Col-*rpp4* seedlings were exposed to three different light (L)/dark (D) periods: 12 hr L/12 hr D, 14 hr L/10 hr D, and 10 hr L/14 hr D for 7 days at 16 °C, and the amount of sporulation was assessed.

Another experiment was designed to understand the effect of extreme light regimes on Hpa sporulation. The inoculated samples were exposed to four different light regimes: 7 days constant light; 7 days constant dark; constant light for 3 days post-inoculation (dpi) and constant dark after 3 dpi; and a light/dark regime of 12 hr L/12 hr D as control. As a light source, white fluorescent bulbs (300 $mmol \cdot m^{-2} \cdot s^{-1}$, 10 HQIL 400 W lamps plus four L40/60 W fluorescent bulbs; Osram) were used. To quantify sporulation, 10 infected seedlings from each replicate were taken and placed into an Eppendorf tube containing 250 µl water. Samples were vortexed and conidiospores were counted using a haemocytometer. All experiments had a minimum of three replicas and were repeated three times. All results were evaluated and compared statistically.

2.3 | Trypan blue staining

Cotyledons of 7-day-old Col-*rpp4* were spray inoculated with Hpa-Emoy2 and were exposed to a normal 12 hr L/12 hr D cycle, constant light, or constant dark. Cotyledons were examined at 3 dpi after staining with trypan blue to highlight the mycelial growth along with sexual spore (oospores) that are produced in the interior of the leaf,

and asexual fruiting bodies (sporangia) that form on the exterior of the leaf.

Seedlings were taken from infected samples at 0 and 12 hr post-inoculation and at 1, 2, 3, 4, 5, 6, and 7 dpi. Infected leaf segments were placed in an Eppendorf tube, covered with 1 ml or a sufficient amount of trypan blue solution (10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml water, 20 mg trypan blue (Merck) in ethanol [96%; 1:2 vol/vol]) and boiled at 100 °C for 1 min. The leaf segments were then destained for 1 hr in chloral hydrate (2 mg/ml) (Sigma). All steps were carried out in a fume hood. Pathogen structures were viewed under a Axioskop 4+ microscope (Zeiss).

2.4 | GUS assay

Transgenic *PR1-GUS* lines were used. Three-week-old seedlings were exposed to constant light or dark for 1–3 days. Then, seedlings were transferred to 24-well replica plates that contained 1 ml X-Gluc histochemical staining solution (50 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt [X-Gluc] in 50 mM $NaPO_4$, pH 7) and incubated overnight at 37 °C. After staining, leaves were treated with 70% methanol for up to 4 hr. The samples were washed with ethanol, immersed in glycerol, and tissues were examined for GUS staining under a dissecting microscope.

2.5 | Germination assay using cellophane

The germination assay using cellophane on MS agar (Murashige and Skoog, 1962) was carried out as described (Bilir *et al*., 2019). Sterile pieces of cellophane were placed on the surface of MS agar in the laminar flow cabinet. Hpa spore solution was prepared and centrifuged, all spores collected, and the pellet was then resuspended in sterile water. Approximately 10 µl spore solution were dropped on each piece of cellophane. Plates was grouped and put in the three different incubators: constant light, constant dark, and 12 hr L/12 hr D regime at 16 °C for 72 hr, and examined every 12 hr under the microscope. The number of germinated Hpa spores was counted using a haemocytometer.

2.6 | Determining biomass growth using quantitative PCR

The biomass of mycelium produced by Hpa-Emoy2 up to 3 dpi was measured from samples exposed to three different light regimes by real-time quantitative PCR (qPCR). The Hpa-*Actin* gene and *At-Actin* gene were used for quantification following the protocol of Anderson and McDowell (2015). After Col-*rpp4* seedlings were inoculated with Hpa-Emoy2, samples were separated and placed under normal (D/L), constant light, and constant dark regimes as three different groups. Every 24 hr, samples were taken, and their DNA extracted and measured with qPCR as described (Livak and Schmittgen, 2001). Sequences of primers used were: Hpa-Actin/F 5′-GTTTACTACCACGGCCGAGC-3′, Hpa-Actin/R 5′-CGTACGGAAACGTTCATTGC-3′, At-Actin/F 5′-AGCATCTGGTCTGCGAGTTC-3′, and At-Actin/R 5′-ACGGATTTAATGACACAATGGC-3′.

2.7 | Statistical analysis

For statistical analysis, paired Student's *t* tests were performed on data obtained from plant infection and germination assays.

3 | RESULTS

3.1 | Optimal light regime for Hpa sporulation

We began by testing how Hpa sporulation is affected by three different light/dark periods, representing day lengths commonly encountered by the plant and pathogen in natural environments. We used a compatible interaction between the Hpa isolate Emoy2 and a mutant in the *Arabidopsis* accession Columbia (Col) that inactivates the disease resistance gene *RPP4* (*Recognition of Peronospora parasitica gene 4*, Roux *et al*., 2011). Sporulation was quantified at 4 and 7 dpi under the following light regimes: 14 hr L/10 hr D, 12 hr L/12 hr D, and 10 hr L/14 hr D. Plants grown under all three regimes supported abundant sporulation, which increased between 4 and 7 dpi (Figure 1). We observed only small, statistically insignificant

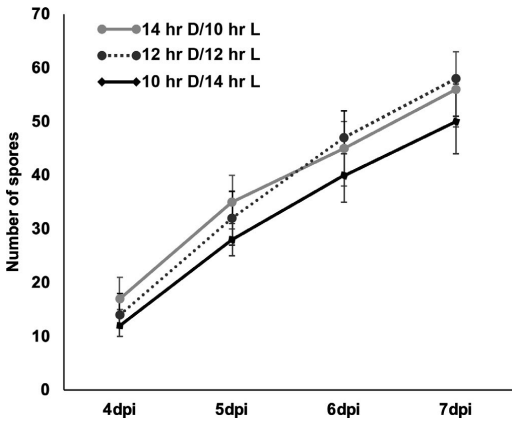

FIGURE 1 Optimization of the light (L)/dark (D) period for sporulation of *Hyaloperonospora arabidopsidis* (Hpa). Three different light/dark periods were tested to compare the amount of Hpa sporulation sporulation. These periods were 14 hr L/10 hr D, 12 hr L/12 hr D, and 10 hr L/14 hr D. Spores were harvested 4–7 days post-inoculation (dpi) and counted using a haemocytometer. Average and standard error of three replicates are shown. This experiment was repeated three times with similar results

differences in sporulation between the three regimes. We selected 12 hr L/12 hr D as the reference time period for subsequent experiments.

3.2 | Effect of constant light or dark on sporulation

The next set of experiments were designed to test how Hpa sporulation was affected by constant light or constant dark. Different light/dark conditions were compared to the 12 hr L/12 hr D reference. As expected, abundant sporulation was observed between 4 and 7 dpi on plants grown under the 12 hr L/12 hr D light regime (Figure 2a). In contrast, sporulation was dramatically reduced on seedlings exposed to constant light or dark after 3 dpi. Moreover, sporulation was almost totally suppressed on plants grown under 7 days of constant light or 7 days of constant dark regime that commenced immediately after inoculation (Figure 2b). When infected seedlings were exposed to constant light or dark after 3 dpi, there were hardly any new conidiophores and the amount of sporulation after 7 dpi was the same as at 3 dpi (Figure 2a). These experiments demonstrate that disruption of a normal light/dark regime can significantly affect the pathogen's capacity to complete the asexual phase of its life cycle.

3.3 | Recovery from suppression of sporulation by constant light

We tested whether asexual sporulation could be restored by returning plants to 12 hr L/12 hr D after treatment with constant light or dark as described above. Interestingly, seedlings that were returned to a normal 12 hr L/12 hr D regime after exposure to 7 days of constant light supported light sporulation 2 days after the shift and moderate sporulation after 4 days (Figure 2c). A similar recovery was observed in seedlings returned to the reference regime after treatment with constant light from 4 to 7 dpi (Figure 2b). In contrast, seedlings exposed to constant dark immediately after inoculation began to show a chlorotic phenotype after 4 days and the seedlings did not recover after shifting to a normal light regime, and no sporulation could be recorded (Figure 2c). Similarly, seedlings that were exposed to constant dark between 4 and 7 dpi did not survive after 7 dpi, and thus no sporulation could be recorded (Figure 2b). When seedlings were exposed to constant light or constant dark beginning immediately after inoculation for 3 days, then shifted to a normal light regime, light sporulation was recovered 7 dpi in samples exposed to constant dark. Abundant sporulation was observed 7 dpi in samples exposed to constant light, similar to plants grown under a normal light regime (Figure 2d). These experiments demonstrate that the suppression of sporulation by constant light treatment of varying durations is not a permanent effect, and that sporulation can be recovered by returning the plants to a normal regime.

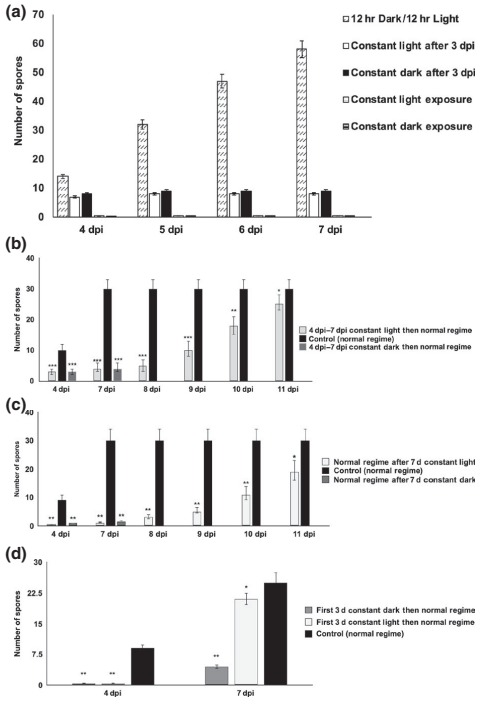

FIGURE 2 Amount of sporulation under different light regimes. (a) Five different light/dark conditions were tested. These were 12 hr light (L)/12 hr dark (D), constant light after 3 days post-inoculation (dpi), constant dark after 3 dpi, constant light exposure for 7 days, and constant dark exposure for 7 days. Spores were harvested 4–7 dpi and counted using a haemocytometer. (b) Samples were exposed to the reference light regime during the first 3 days (control regime), then were exposed to either constant light or constant dark over the subsequent 4 days (4–7 dpi). At 7 dpi, the samples were transferred to the reference light regime again and sporulation was recorded until 11 dpi. (c) Samples were exposed to constant light or constant dark for 7 days immediately after inoculation. At 7 dpi samples were transferred to the normal light regime again and sporulation was recorded until 11 dpi. (d) Samples were exposed to constant light or constant dark beginning immediately after inoculation for 3 days, then shifted to a normal light regime, with sporulation recorded at 4 and 7 dpi. All experiments were repeated three times. All results were evaluated and compared statistically. $*p < .05$, $**p < .01$, $***p < .001$, paired Student's *t* test

3.4 | Effect of different light conditions on mycelial growth of Hpa in leaves

Considering that plants grown under constant light for 7 days supported abundant Hpa sporulation after they were returned to a normal 12 hr L/12 hr D regime (Figure 2), it seemed likely that mycelium may have grown inside the leaf during exposure to constant light, but did not produce sporangia until a normal light regime was restored.

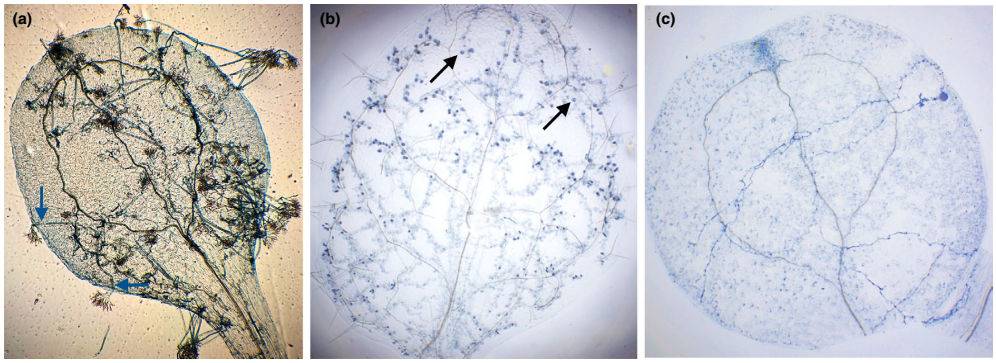

FIGURE 3 *Hyaloperonospora arabidopsidis* (Hpa) development in *Arabidopsis thaliana* leaves under different light regimes. (a) Infected plants grown under the reference 12 hr light/12 hr dark cycle; (b) infected plants grown under a constant light regime; and (c) infected plants grown under constant dark regime. Infected seedlings were stained with trypan blue 3 days post-inoculation. Blue arrows indicate conidiophores and black arrows indicates oospores [Colour figure can be viewed at wileyonlinelibrary.com]

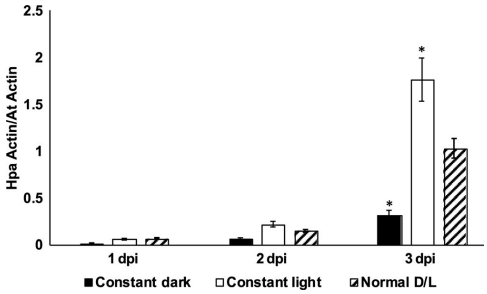

FIGURE 4 *Hyaloperonospora arabidopsidis* (Hpa) mycelial biomass production under different light regimes. Normal light, constant light, and constant dark regimes were applied. After Hpa inoculation, samples were taken every day from infected *Arabidopsis thaliana* leaves until 3 days post-inoculation (dpi) as mycelial growing phase is usually completed within the first 3 days. In all samples, mycelial growth is calculated by quantitative PCR and compared with each other. Student's *t* test, $^{*}p < .05$

To check this possibility, infected seedlings were stained with trypan blue 3 dpi.

In plants grown under the normal light cycle, mycelia had grown throughout cotyledons, sporangia had formed, and sporulation was observed over the whole surface of the cotyledon (Figure 3a). In contrast to the normal light cycle, in cotyledons exposed to constant light, there were extensive mycelia 3 dpi and abundant oospores, but no conidiophores (Figure 3b). These results indicate that vegetative growth and sexual sporulation can proceed under constant light, but asexual sporulation is suppressed.

In cotyledons exposed to constant dark, less mycelial development was observed in comparison to those that were exposed to either a normal light cycle or constant light (Figure 3c). A small number of oospores were observed, similar to that observed under the constant light experiment (Figure 3b).

To precisely assess Hpa growth in planta, we used a qPCR assay in which Hpa DNA is quantified as a proxy for pathogen biomass. During evaluation over 3 days, mycelial biomass showed an increase in all groups (Figure 4). However, the lowest biomass was observed with constant dark exposure, whilst the constant light gave the highest biomass production on every day. Constant light conditions produced a significant increase in biomass compared to that observed with normal light conditions, especially at 3 dpi. On the other hand, under constant dark conditions, biomass was significantly decreased compared to that obtained with the normal light conditions (Figure 4). Altogether, these results confirm that light is an important factor for vegetative growth and reproduction for Hpa.

3.5 | Effect of different light conditions on spore germination

Because the light and dark affect Hpa vegetative growth and sporulation, we questioned whether the light or dark affect germination of spores and whether it is necessary to have a regular light/dark regime for germination. It is challenging to accurately quantify germination on plant leaves, because trypan blue staining and clearing during the early stages of infection eliminate spores on the leaf surface. Thus, cellophane strips were used for germination assays instead of seedlings.

The germination assay was first carried out with the reference light regime (12 hr L/12 hr D). Under this regime, spores germinate after 6–8 hr and a germ tube emerges (Figure 5a). After 12 and 24 hr, germ tubes have extended on the surface of the cellophane (Figure 5b,c). After 48 hr, formation of mycelial branches was obvious and most branches were laterally oriented as they covered the surface (Figure 5d).

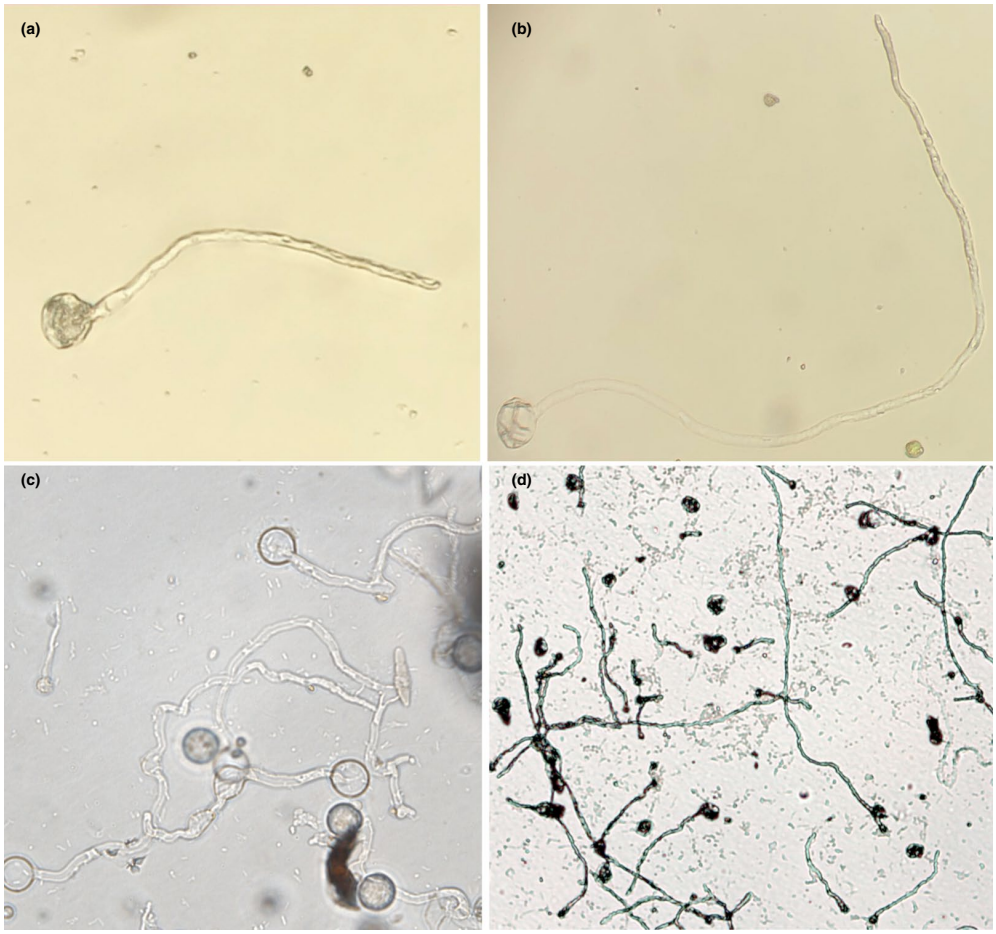

FIGURE 5 Germination of *Hyaloperonospora arabidopsidis* spores on cellophane under 12 hr light/12 hr dark regime. Spores were placed on cellophane strips and examined at regular intervals. (a) After 6 hr, spore had germinated and germ tube was produced; (b, c) after 12 and 24 hr, respectively, the germ tube became longer; (d) after 48 hr, lateral mycelial branches were obvious and hyphae began to cover the surface of the cellophane [Colour figure can be viewed at wileyonlinelibrary.com]

Germination using cellophane strips under constant light and constant dark was assessed in comparison to the reference light regime. The germination rate under the reference regime was 33% after 24 hr. The spores that were exposed to 24 hr constant dark showed a 22% germination rate, which was the lowest percentage observed within this time period. Under constant light at 1 dpi, 37% of Hpa spores were germinated on cellophane (Figure 6). At 2 dpi, the germination percentage increased for all treatments. The germination rate under constant dark was the lowest with 31%, the reference regime was 57%, and constant light was 49%. At 3 dpi, the percentage of germination under constant dark and constant light was the same as at 2 dpi. However, in the reference light regime, germination increased and reached the highest percentage. At the end of 3 days, germination seemed to be completed and spores appeared to have lost their viability. These results indicate that light is an important factor for spore germination independently of the host, and that optimal germination of spores occurs under a normal light/dark regime.

3.6 | Effect of inoculation time on Hpa mycelial biomass growth

If there is a synchronized circadian regulation of Hpa development and host defence, the inoculation time should be important for

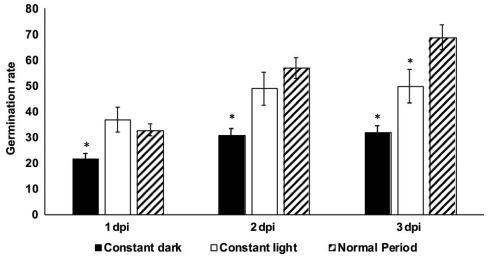

FIGURE 6 Germination rate of *Hyaloperonospora arabidopsidis* spores under different light conditions on cellophane. The spore germination on cellophane, which was exposed to constant light, constant dark, or 12 hr light/12 hr dark regimes (Normal), was determined after 1, 2, and 3 days post-inoculation (dpi). Values represent means of three experiments, and error bars correspond to the standard error of the means. Asterisks indicate statistically significant differences to the reference regime in two-tailed Student's *t* test ($p < .05$)

optimal colonization. Accordingly, previous reports have demonstrated that the time of day for inoculation can affect the degree to which Hpa can successfully colonize *Arabidopsis*, due at least in part to circadian up-regulation of host immune responses during a time period that encompasses subjective dawn. Due to these observations, the optimal infection time for Hpa development was not obvious. Therefore, biomass production between two inoculation times was compared using qPCR. Two zeitgeber time points were determined to observe the effect of day and night (or light and dark) on the development of pathogenicity. ZT0 refers to the beginning of daylight in an entrained cycle and ZT12 is the beginning of night, under experimental conditions of 12 hr L/12 hr D. One sample was inoculated at dawn (ZT0); this was followed by the beginning of the light period, and then the dark period, therefore this sample was called L/D. The other sample was set up as the opposite, with inoculation at dusk (ZT12), called D/L. To determine the dynamic range of qPCR assays, we used an infection time course of virulent Hpa-Emoy2 on Col-*rpp4* (Figure 7). All samples showed a greater biomass

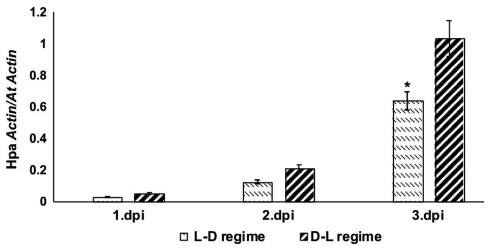

FIGURE 7 Effect of inoculation time on *Hyaloperonospora arabidopsidis* (Hpa) Emoy2 biomass on Col-*rpp4*. Col-*rpp4* seedlings were infected with Hpa at dawn (ZT = 0), labelled L-D, or dusk (ZT = 12), labelled D-L. At 1, 2, and 3 days post-inoculation (dpi), samples were taken and biomass was calculated using quantitative PCR and compared with each other. Student's *t* test, $^*p < .05$

of mycelium on the D/L cycle than L/D cycle and showed a regular increase in mycelium growth over the 3 days (Figure 7). The highest biomass was observed in the D/L cycle, where by 3 dpi, pathogen biomass in samples under the D/L cycle was approximately 56% higher than in samples inoculated under L/D cycle. The results suggest that initiating infection at dusk promotes a higher degree of virulence than initiating infection at dawn.

3.7 | Effect of continuous light/dark regimes on an immune response biomarker

Plant immune responses are characterized by the activation of a set of pathogenesis-related (*PR*) genes (Ward *et al*., 1991; Uknes *et al*., 1992). A *PR1promoter-GUS* reporter gene is considered to be a valid marker gene for activation of immunity in *A. thaliana* (Uknes *et al*., 1992), enabling transgenic *Arabidopsis PR1-GUS* plants to be employed to detect activation of immune responses. We set up an experiment to test whether the reporter gene activity can be induced by constant light and constant dark treatment with the transgenic plants containing *PR1-GUS*. In this assay, when the *GUS* reporter gene is activated by any stress factor, the plant tissues are observed to be stained blue.

In *Arabidopsis* seedlings grown under normal light regime with no pathogen infection, there were no blue-stained cells, indicating that the *GUS* gene was not induced under this condition, as expected (Figure 8a). In contrast, seedlings exposed to constant light (Figure 8b) and constant dark (Figure 8c) for 72 hr showed *GUS* activity, indicating constant light and dark regimes trigger immunity. These results indicate that induction of immunity under constant light or dark exposure could contribute to the suppression of the sporulation of Hpa.

4 | DISCUSSION

Using the Hpa–*Arabidopsis* reference system, we showed that light regimes significantly affect several stages of the Hpa disease cycle, including spore germination, mycelial development, oospore formation, and sporulation. We also obtained preliminary results suggesting that light regimes can influence the immune status of the host. These observations complement recent studies showing that the plant circadian clock system regulates the immune system in the interactions between *Arabidopsis* and Hpa (Wang *et al*., 2011; Zhang *et al*., 2013). However, the previous studies focused mainly on incompatible interactions with resistant plant hosts and did not address how light might affect Hpa in a disease-susceptible host. Therefore, this work was undertaken to investigate the effect of light on a virulent Hpa isolate.

Our initial investigations led to the selection of 12 hr L/12 hr D as a reference regime for the pathogen for ongoing experiments. Subsequent experiments showed that exposure of plants to constant light or dark regimes had a suppressive effect on sporulation (Figure 2).

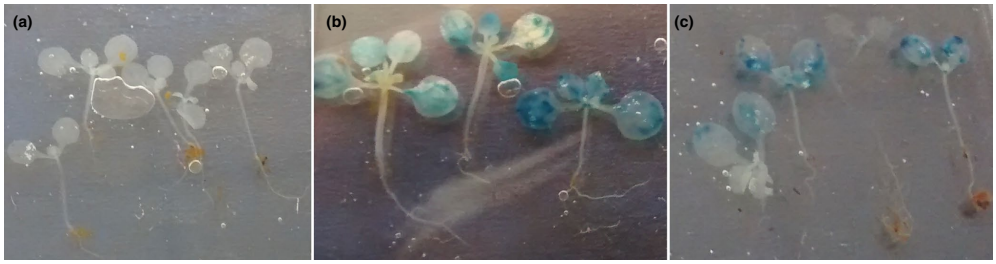

FIGURE 8 *GUS* expression in *Arabidopsis thaliana* seedlings exposed to different light regimes. (a) Seedlings grown under normal 12 hr light/12 hr dark regime; (b) seedlings exposed to constant light; and (c) seedlings exposed to constant dark. After 48 hr of exposure to these regimes, histochemical GUS assays were carried out. These experiments were repeated three times with similar results [Colour figure can be viewed at wileyonlinelibrary.com]

Similar inhibitory effects of light on sporulation of fungal and oomycete pathogens, including downy mildews, have been reported for decades (referenced in the Introduction and reviewed by Rotem *et al*., 1978). However, these studies generally have not directly addressed whether constant light inhibited vegetative (mycelial) growth in planta and/or sporulation. Our investigations showed that constant dark inhibited Hpa growth while constant light supported higher levels of Hpa biomass production than that in normal light/dark or constant dark regimes. Interestingly, constant light exposure induced abundant oospore formation. This apparent inhibition of asexual sporulation by constant light or dark was reversible; plants that were returned to the reference light regime after 4 days of constant light or dark could support abundant sporangiophore production. Similar observations have been reported for other downy mildew pathogens, for which a "recovery" period of 4 hr in the dark was sufficient to enable sporulation (reviewed by Rotem *et al*., 1978). The mechanism behind this recovery is unknown but was postulated at the time to involve enzymatic degradation of a light-induced "antisporulant". Such hypotheses can now be tested with the experimental tools of the Hpa–*Arabidopsis* pathosystem.

In this context, we tested whether constant light- or dark-treatment was sufficient to activate the plant immune system in the absence of pathogen infection. Using transgenic plants containing a fusion of *PR1* promoter to a *GUS* reporter gene, it was clear that after 24 hr, the *PR1* promoter was activated by 24 hr constant light and 48 hr constant dark (Figure 4). These results are similar to those reported in previous publications (Evrard *et al*., 2009). It has been reported that plant defence responses and hypersensitive response-associated programmed cell death triggered by pathogens is activated by light in tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), and *Arabidopsis*, and the activation of inducible resistance is dependent on phytochrome functions (Guo *et al*., 1993; Chandra-Shekara *et al*., 2006). The blue light receptor cryptochromes (*CRY*) and red/far-red light photoreceptor phytochromes (*PHY*) work together in *Arabidopsis* and they regulate many light-controlled defence responses and entrainment of the circadian clock. The photoreceptor gene *CRY1* regulates systemic acquired resistance (SAR) positively and in the *cry1* mutant, salicylic acid (SA)-induced pathogenesis-related gene *PR-1* expression is reduced, but enhanced in *CRY1-ovx* (*CRY1-overexpressor*) plants under light conditions (Wu and Yang, 2010).

We also tested whether the timing of inoculation affected Hpa's capacity to colonize the plant. A previous report demonstrated that effector-triggered immunity and basal immunity against Hpa is more efficient early in the day (Wang *et al*., 2011), and we confirmed this observation by using a different virulent isolate of Hpa. Our results demonstrate that plants inoculated at dusk supported significantly more mycelial growth than plants inoculated at dawn, even at 3 dpi. Our experiments do not point directly to an underlying mechanism, but we hypothesize that this might reflect a difference in timing of basal defence mechanisms that limit growth of virulent Hpa. Wang *et al*. (2011) noted that SA-dependent gene expression was stronger in the day than at night; accordingly, it was reported that morning and midday inoculations lead to higher SA accumulation, quicker and more intense *PR* (pathogenesis-related) gene activation and expression, and hypersensitive response, than inoculations at dusk or at night (Griebel and Zeier, 2008). These previous reports on different systems support our data and help to explain why night time inoculation is more efficient than day time inoculation.

It is important to emphasize that all experiments involving Hpa grown in planta could reflect influence of light on both the pathogen and the host. Fungal and oomycete pathogens have been shown previously to incorporate light perception into their development and virulence programmes. For example, 48 hr constant white light exposure inhibits sporulation of *P. infestans* on potato or agar plates (Xiang and Judelson, 2014). Because Hpa is an obligate pathogen that can only complete its life cycle on a compatible *Arabidopsis* host, we cannot directly assess how light influences sporulation away from the host. However, our in vitro spore germination assay indicates that light does affect the Hpa life cycle and suggests that Hpa can perceive light.

In conclusion, we have reported several lines of evidence that light is a critical factor during development of downy mildew disease on *Arabidopsis* and can influence responses in the pathogen and the host. We can now exploit this system to understand the mechanistic basis of these effects, using the well-developed tools for *Arabidopsis* in combination with a new protocol for reverse genetics in Hpa. Our future studies will focus on circadian regulation on both the host

and pathogen side. While it is well established that circadian regulation of host immunity is an important factor in immunity against Hpa and other pathogens in *Arabidopsis*, the role of circadian regulation in oomycete virulence is unexplored and therefore could be an enlightening area for future inquiries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request.

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