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Insights into the multitrophic interactions between the biocontrol agent *Bacillus subtilis* MBI 600, the pathogen *Botrytis cinerea* and their plant host

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ABSTRACT

Botrytis cinerea is a plant pathogen causing the gray mold disease in a plethora of host plants. The control of the disease is based mostly on chemical pesticides, which are responsible for environmental pollution, while they also pose risks for human health. Furthermore, *B. cinerea* resistant isolates have been identified against many fungicide groups, making the control of this disease challenging. The application of biocontrol agents can be a possible solution, but requires deep understanding of the molecular mechanisms in order to be effective. In this study, we investigated the multitrophic interactions between the biocontrol agent *Bacillus subtilis* MBI 600, a new commercialized biopesticide, the pathogen *B. cinerea* and their plant host. Our analysis showed that this biocontrol agent reduced *B. cinerea* mycelial growth *in vitro*, and was able to suppress the disease incidence on cucumber plants. Moreover, treatment with *B. subtilis* led to induction of genes involved in plant immunity. RNA-seq analysis of *B. cinerea* transcriptome upon exposure to bacterial secretome, showed that genes coding for MFS and ABC transporters were highly induced. Deletion of the *Bcmfs1* MFS transporter gene, using a CRISP/Cas9 editing method, affected its virulence and the tolerance of *B. cinerea* to bacterial secondary metabolites. These findings suggest that specific detoxification transporters are involved in these interactions, with crucial role in different aspects of *B. cinerea* physiology.

1. Introduction

Botrytis cinerea (teleomorph *Botyotinia fuckeliana* de Bary Whetz) is a filamentous ascomycete and a notorious plant pathogen, causing the gray mold disease in more than 200 host plants and belongs to the top 10 list of the most important plant pathogenic fungi (Dean et al., 2012; Dewey and Grant-Downton, 2016). The most devastated losses occur in vegetables such as tomato, lettuce and beans and in small fruits such as grape, strawberry, blackberry etc. (Droby and Lichter, 2004). Moreover, it can grow effectively during cold storage on fruits such as kiwifruit, apples and pears, causing postharvest rots (Michailides and Elmer, 2000). It is also one of the most serious fungal diseases in nurseries causing damping-off in seedlings (Capieau et al., 2004; Konstantinou et al., 2014). This pathogen forms sclerotia, which are structures able to survive for long periods under harsh environmental conditions. They play a crucial role in survival and epidemiology of the pathogen, being able to start a new disease cycle.

The control of the disease is based mostly on chemical treatments and several fungicides have been registered so far (Hahn, 2014). Among them, fungicides belonging to the groups of succinate dehydrogenase inhibitors (SDHIs), (i.e. boscalid, fluopyram, isopyrazam etc.), anilino-pyrimidines (i.e.cyprodinil), phenylpyrroles (i.e. fludioxonil), or hydroxyanilides (i.e. fenhexamid) are widely used against this pathogen. However, the high genetic variability, the short life cycle and the high rates of asexual reproduction are factors contributing to a high risk for fungicide resistance development by the pathogen (De Miccolis Angelini et al., 2016). Actually, resistant populations to several fungicide classes have emerged throughout the world (Konstantinou et al., 2015; Fillinger and Walker, 2016; Samaras et al., 2016), leading to a significant reduction of fungicide efficacy. Besides resistance, extensive use of chemical pesticides in agriculture has negative effects in the environment and human health. Presence of fungicide residues is an increasing problem, affecting the food quality and safety. Anti-*Botrytis* fungicides commonly used in vineyards have been detected in commercial wines (Economou et al., 2009; Esteve-Turrillas et al., 2016), and active ingredients of these pesticides show toxic effects in human cells, posing an increased risk for consumers health (Orton et al., 2011). This fact, in combination with the EU policy towards a drastic decrease in pesticide

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use, necessitate the development of much needed new control strategies.

Biocontrol agents are compatible with sustainable agriculture, and help to minimize the use of chemical pesticides; reducing the negative effects in environment and human health, and delaying pathogens to develop resistance to chemical pesticides. Certain fungal and bacterial species are currently used to control different plant pathogenic fungi, including *B. cinerea* (Sutton et al., 1997; Soylu et al., 2010; Pretorius et al., 2015; Karlsson et al., 2015). Species belonging to *Bacillus* genus are among the most commonly used BCAs, applied as biofertilizers or biopesticides in different crops and against a variety of soil-borne and foliar pathogens. The extensive development and registration of *Bacillus*-based bioproducts is related to some unique characteristics of this genus that include high replication rate, resistance to adverse environmental conditions, increased efficiency in plant growth promotion and broad-spectrum activity (Magno-Pérez-Bryan et al., 2015). Two main representatives of *Bacillus* species are *B. amyloliquefaciens* and *B. subtillis.,* which display strong antagonistic activity against fungi, secreting cell wall degrading enzymes, lipopeptides and other toxic compounds (Arrebola et al., 2010; Wang et al., 2016; Zhao et al., 2017; 2016; Hanif et al., 2019). Application on stored fruits and vegetables showed promising results against postharvest decays as well (Li et al., 2013; Chen et al., 2018; Calvo et al., 2019). In addition, BCAs elicit induced systemic resistance (ISR) through priming plant defense responses upon pathogen attack. More specifically, mechanisms such as cell-wall reinforcement, oxidative burst, expression of defense-related enzymes, and accumulation of secondary metabolites, have been reported in various studies (Pieterse et al., 2014; Liu et al., 2014; Nie et al., 2019). In addition to the moderate or high control efficiency of *Bacillus* spp. against *B. cinerea*, it has recently been shown that applications of similar products may eliminate fungicide resistant strains of the pathogen and thus, they may be useful in anti-resistance management schemes (Samaras et al., 2021a).

Bacillus subtilis strain MBI600 (*Bs* MBI600, from now on), is a recently commercialized biocontrol agent used for the management of various plant diseases including grey mold, but the exact mode of function against *B. cinerea* remains unknown. In addition, previous studies have showed that *Bs* MBI600 can control efficiently the soilborne fungal tomato pathogen, *Fusarium oxysporum* f.sp. *radicis lycopersici* (Samaras et al., 2018). It seems also to be effective against important tomato viruses, such as TSWV and PVY, while induces defense related genes as well (Beris et al., 2018; Dimopoulou et al., 2019). Recent studies showed also high control efficacy of *Pythium aphanidernatum* and *Fusarium oxysporum* f sp. *radicis cucumerinum*, and genome analysis of *Bs* MBI600 revealed genes involved in lipopeptides production, such as surfactin and fengycin (Samaras et al., 2021b).

Fungi have developed different strategies to cope with adverse and toxic conditions, and to be adapted in new environments. For instance, they employ transmembrane proteins, such as ATP-binding cassette (ABC) and Major Facilitator Superfamily (MFS) transporters, able to transport toxic compounds out of the cell (Coleman and Mylonakis, 2009). Many fungal species also show expansion of transporter gene families in their genomes, and increased tolerance to xenobiotic chemical compounds and fungicides (Karlsson et al., 2015; Nygren et al., 2018).

The defense mechanisms deployed by pathogens to cope with the stress caused by antagonistic organisms are still widely unknown. Thus, the main objective of this study was to investigate the multitrophic interactions between *B. cinerea*, the *Bs* MBI 600 biocontrol agent and the host plant, by using *in vitro* and *in planta* assays and RNA-seq analysis. Our results showed that this bacterial strain was antagonistic against *B. cinerea* and able to induce resistance-related genes on cucumber plants. Furthermore, *B. cinerea* transcriptomic analysis showed high induction of genes involved in detoxification process such as ABC and MFS transporters. The specific role of this MFS transporter gene further investigated using a newly established CRISP/Cas9 gene editing protocol. Our results showed that the induced MFS transporter was involved in tolerance to the bacterial chemical compounds, and fungal virulence, giving new insights towards understanding the mechanisms involved in these multitrophic interactions.

2. Material and Methods

2.1. Fungal and bacterial isolates

Bs MBI 600 strain used in the study was isolated from an experimental formulation of the product (Serifel 9.9 W P) kindly provided to us by BASF Hellas S.A. The isolation procedure was as described previously (Samaras et al., 2021b). The *Botrytis cinerea* strain B05.10 was used in all the experiments and stored as conidia suspension at −80 °C in 40 % glycerol. For conidia production culture was grown in Hydroxyapatite (HA) medium (1% (w/v) Malt extract/ 0,4% (w/v) Glucose / 0,4% (w/v) Yeast extract / 1,5% (w/v) Agar, pH 5.5) and incubated at 25 °C for 7 days.

2.2. In vitro antagonistic activity assays

Bs MBI600 was tested for its ability to inhibit the growth of *B. cinerea in vitro*. Dual cultures consisted of the bacterial isolate and the fungal species inoculated on opposite sides of the plate, at approximately 10 mm distance from the margins of the plate. The bacterial cells were streaked as a straight line, and the plates were inoculated with a 6-mm-diameter plug of mycelium taken from the colony margins of actively growing 4-day-old fungal cultures, onto 3 different media: Potato Dextrose Agar (PDA) (PDA, LabM, Hungary), Luria Broth (LB), (Tryptone 10gr, NaCl 10 gr, Yeast extract per 1ltr H_2O), and Tryptone Glucose Yeast Extract (TGY), (Casein peptone 5gr, Yeast extract 5gr, Dextrose 1gr, Bacteriological Agar 15gr per 1ltr H_2O) in 9-cm diameter petri dishes. Plates were incubated for 7 days at 25 °C and antagonistic activity was evaluated by measuring both directions of the fungal colonies and the length of the inhibition zones (mm). Relative inhibition of mycelial growth was calculated using the formula RH = (Control Diameter-treatment Diameter/control Diameter)×100. Five biological replicates were prepared per treatment and the experiment was repeated three times. Plates with only *B. cinerea* cultures were used as control.

2.3. In planta biological control assays

All the *in planta* experiments were performed on cucumber plants (cv. Bamboo). Plants were grown in plastic pots containing a 2:1 (v:v) mixture of peat and perlite, in greenhouse conditions (18−24 °C). Plants used for inoculations were at the stage of 2 fully-expanded leaves. Bacterial cultures were prepared in Tryptone Soy Broth (TSB) medium (TSB, LabM, Hungary) overnight at 37 °C. The culture centrifuged for 5 min at 4000 x g and the pellet resuspended with dd H_2O until the OD_{600} was 0.7−0.8 (approximately 2×10^{10} cfu/ml). Ten ml of the bacterial suspension were applied in each pot, by soil drenching or foliar spray, according to the type of application. Bacterial suspension was applied to cucumber leaves or roots 24 h before the inoculation. For inoculum production, *B. cinerea* was grown on HA plates and incubated for 7 days at 22 °C under continuous light to induce sporulation. Spore suspensions were prepared in Gamborg Minimal medium (3 gGamborg B5 basal salt mixtures, 1.36 g KH_2PO_4, and 9.9 g glucose per liter), collected by scraping the mycelial colony and adjusted to a concentration of 2×10^5 spores ml^{-1}. A droplet of conidia suspension (20 µl) was placed at two different points of the adaxial surface of each leaf. Control plants were sprayed or drenched with sterile tap water. Disease incidence was measured by counting the number of rotting lesions that appeared on each plant and disease severity was calculated with a disease scale as described previously (Samaras et al., 2021a).

2.4. In planta transcription analysis

For the *in-planta* transcription analysis of genes involved in ISR, cucumber leaves were treated with *B. subtilis* and inoculated with *B. cinerea* as described previously. The expression patterns of five genes, *PR1, PR10, PAL, LOX* and an *ethylene*-related gene, were chosen as they encode main representatives of defense and signaling proteins. Leaves were collected 0, 24, 48- and 72 -hs post inoculation (hpi). Four biological replicates for each time point were used. Each sample comprised three plants and four infected leaves per plant. Samples were immediately placed on liquid nitrogen and stored at −80 °C, until RNA extraction. Total RNA was extracted using the Trizol method according to manufacturer's instructions (TRItidy G™, Germany). The qRT-PCR reactions were carried out using a StepOne Plus Real-Time PCR System (Applied Biosystems) using a SYBR Green based kit (Luna® Universal One-Step RT-qPCR Kit) as previously described (Tzelepis et al., 2012). Primers for this analysis are enlisted in Table S1. Data were normalized to the cytochrome oxidase (*cox*) gene expression and relative transcripts were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Statistical analysis was conducted using the Tukey test.

2.5. Transcriptome analysis of B. cinerea upon interaction with the supernatant of Bs MBI600

To investigate the transcription patterns of *B. cinerea* during interactions with *Bs* MBI 600, RNA-seq analysis was conducted. Bacterial cells were placed on conical flasks and incubated at 37 °C for 72 hours as described above. Then, the bacterial supernatant was collected and filtered from a 0.2 silica filter. First, *B. cinerea* mycelia were grown in potato dextrose broth (PDB) for 5 days and then transferred to new flasks containing the bacterial culture filtrates. For RNA extraction, mycelia were harvested at four time points (0, 24, 48 and 72 hpi), and total RNA was isolated as described above, consisting of three biological replicates. In total, 12 RNA strand-specific libraries were generated and sequenced using the Illumina HiSeq 2500 Platform at SciLife Laboratory, Uppsala, Sweden.

2.6. RNA-seq data analysis and validation

Raw reads were subjected to a quality control using the FastQC software (Andrews, 2010). Prinseq was used to pre-process data, regarding read length, GC content, quality score and number of read duplicates. Reference genome and gene model annotation was downloaded from Ensembl Fungi (ftp://ftp.ensemblgenomes.org/pub/fungi/release-48/fasta/botrytis_cinerea/dna/). Then, filtered data were imported in Geneious Prime software (vers. 2020.0.4), as paired-end reads. The annotated genome of *B. cinerea* B05.10 strain (Accession number ASM83294v1) was used as reference. Mapping of the reads from each treatment to the reference genome was performed using Geneious RNA mapper with the following parameters: minimum mapping quality 30 bp, maximum gap per read (5%), minimum overlap identity (80 %), minimum support for intron (2 reads).

Expression levels were calculated using Geneious Prime, by counting the reads mapped to each gene. Then, the RPKM values of each gene were calculated based on the number of reads mapped to a particular gene region, which represent the number of nucleotides in a mappable region of a gene (Mortazavi et al., 2008). Differential expression analysis was performed using the DeSeq package. Gene expression levels of the isolates were compared before treatment with bacterial culture filtrates (timepoint 0 hrs) with the rest time points. The significance thresholds used for differential expression were P value < 0.05 and fold change (log2) >2. Differential expressed genes visualized as volcano plots by using Geneious tools. The heatmaps were created using the R programming language. OrthoDb software was used to find the orthologs in other phytopathogenic fungi from different taxa, according to functional annotation (Kriventseva et al., 2019). The RNA-seq data were validated with qRT-PCR as described above.

2.7. Phylogenetic analysis and transporter classification

Amino acid sequences of the ABC transporter BcatrO and homologs from different fungal species were aligned with CLUSTAL X (Thompson et al., 1997) and phylogenetic analysis was conducted using Neighbor Joining implemented in MEGA X (Kumar et al., 2018), using the JTT amino acid substitution model (Jones et al., 1992). Statistical support for branches was supported by 500 bootstraps. The MFS transporters were classified to families according to the Transporter Classification Database (Saier et al., 2016).

2.8. Construction of B. cinerea deletion strains and phenotypic analysis

Gene editing was performed using a CRISP/Cas9 approach as previously described, carrying a hygromycin resistance cassette (Leisen et al., 2020). For generation of the *Bcmfs1* deletion construct, a repair template was amplified with 60bp homology flanks with the right and left sites in the gene borders. Selection of appropriate sgRNAs was performed by the sgRNA design tool, implemented in the Geneious software. Oligonucleotides for synthesis of sgRNAs are listed in Table S1. DNA template was prepared by annealing 10 μmol each of constant sgRNA oligonucleotide (gRNArev) and protospacer specific oligonucleotide (95 °C for 5 min, from 95 °C to 85 °C at 2 °C sec^{-1}, from 85 °C to 25 °C at 0.1 °C-1), followed by fill-in with T4 DNA polymerase (New England Biolabs), and incubated for 20 min at 12 °C. sgRNA synthesis was performed using the HiScribe T7 HighYield RNA Synthesis Kit (NEB) and purified using the RNA Clean & Concentrator-25 kit (ZymoResearch, Orange, CA, USA). Cas9-NLS, containing N- and C-terminal SV40 nuclear localization signal (NLS), was purchased from New England Biolabs. For the ribonucleoprotein particle (RNP) formation, 6 μg Cas9 was incubated in cleavage buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 5% glycerol, 1 mM dithiothreitol, 0.5 mM EDTA, pH 8.0, 2 mM MgCl2) with 2 μg sgRNA for 30 min at 37 °C. (Leisen et al., 2020). Transformation of *B. cinerea* was performed using a protoplast-based protocol (Müller et al., 2018; Leisen et al., 2020). Five days later, positive colonies were transferred in HA agar plates containing the appropriate concentration of hygromycin (175 mg l^{-1}). Single spore mitotically stable transformants were subcultured for five consecutive times on selective media and validated using RT-PCR techniques.

At least three confirmed deletion strains were used for phenotypic analysis. For mycelial growth, the Δ*bcmfs1* strains were placed on Tryptone Soya Agar (TSA) plates incubated at 25 °C and mycelial diameter was measured after 5 days. For the inhibition assays, Tryptone Soya Broth (TSB) medium and bacterial culture filtrates were mixed in a 1:1 ratio and mycelial growth and relative inhibition were monitored as described above. For pathogenicity assay the Δ*Bcmfs1* isolates were placed on HA agar plates and incubated for 7 days in 25 °C until sporulation. Four-week-old tomato leaves (cv. Belladona) were inoculated with spore suspension (2×10^5 spores) and symptoms monitored as described above.

3. Results

3.1. Bs MBI 600 reduced B. cinerea growth in vitro and disease incidence in vivo

To determine the biocontrol efficiency of *Bs* MBI 600 against *B. cinerea, in vitro* assays of antagonism were performed. Dual cultures of the biocontrol agent and the pathogen were established on three different media (PDA, LB, PEG). Five days post inoculation, the bacterial strain significantly reduced the mycelial growth in all tested media. The relative inhibition of mycelial growth on PDA, LB and PEG media in the presence of *Bs* MBI 600 was 25%, 61% and 57,3%, respectively. In

addition, formation of inhibition zone was observed in LB and PEG media, with a stronger inhibition in the former medium (Fig. 1A).

To further determine the biocontrol efficiency of *Bs* MBI 600 against *B. cinerea*, *in planta* assays were conducted. Foliar and root treatments were performed on cucumber plants, following by artificial inoculation with *B. cinerea*. Our results showed that the bacterial strain significantly reduced the disease incidence in both types of application. Similarly, disease severity (lesion diameter) was significantly reduced by both treatments of *Bs* MBI 600 (Fig. 1B, C). However, root application of *Bs* MBI600 resulted in significantly smaller lesions compared to those observed on foliar-treated plants (Fig. 1B, C).

3.2. Treatment with Bs MBI 600 induced plant immunity genes

Besides the impact of *Bs* MBI 600 in control efficacy against *B. cinerea,* we also investigated whether plant treatment with this biocontrol agent could induce defense responses. Thus, the transcription patterns of five marker genes were studied at different time points. Our

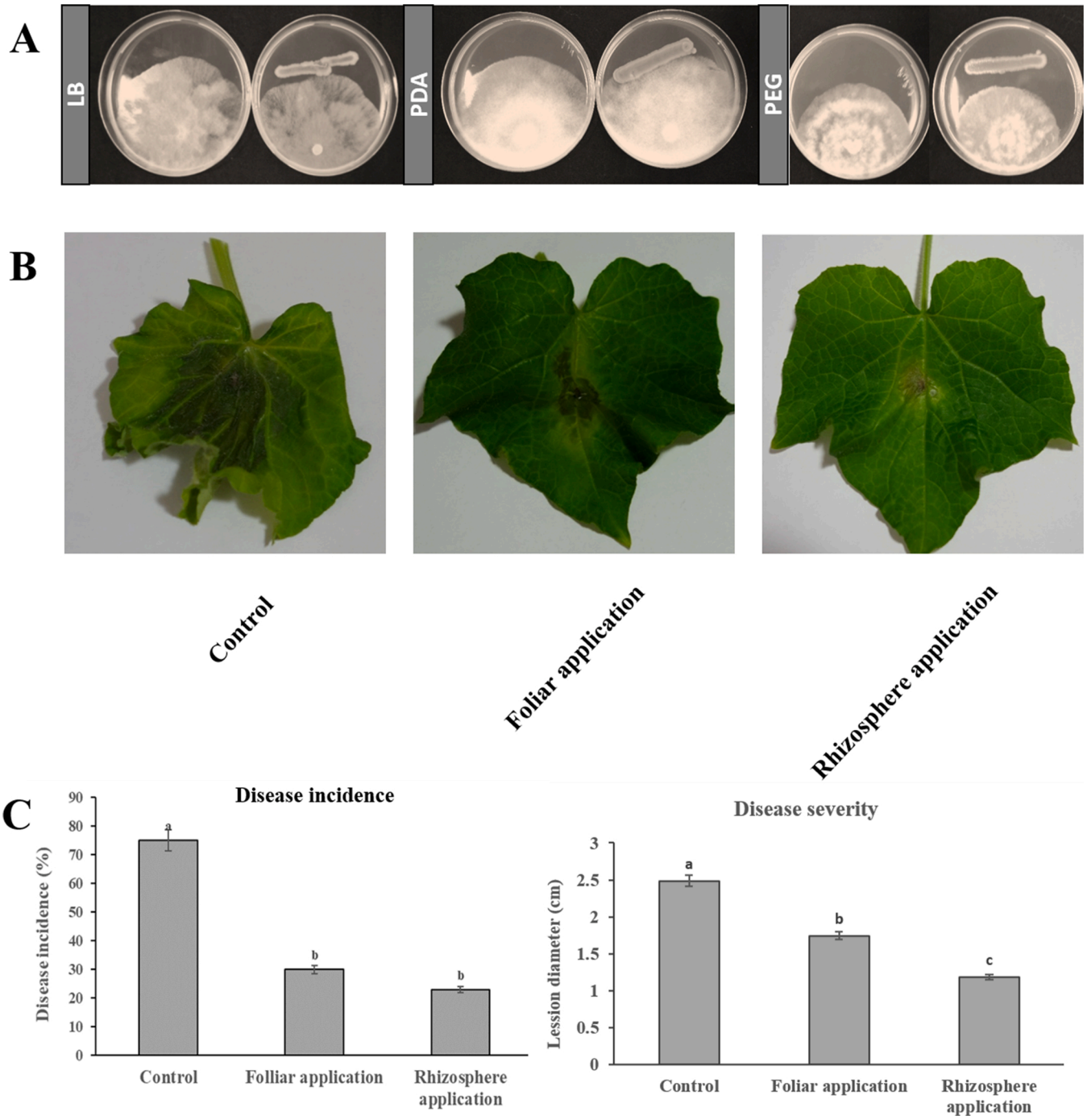

Fig. 1. Antagonistic ability assays between *Botrytis cinerea and Bacillus subtilis* MBI 600. **A.** *In vitro* interactions between *B. cinerea* and *Bs* MBI 600 cultures on three different media, PDA, PEG and LB. **B.** Gray mold symptoms on cucumber leaves from plants that received foliar, root or no (control) application with *Bs* MBI 600 and artificially inoculated with *B. cinerea*. **C.** Disease incidence (number of symptomatic leaves) and severity (lesion diameter) on cucumber plants treated with *Bs* MBI 600. Letters on the columns indicate statistically significant differences according to Fisher's LSD test ($P < 0.05$). Vertical lines on the columns indicate the standard error of the mean.

analysis showed that all tested genes were highly induced at all time points in plants treated with *Bs* MBI600 as compared to mock-inoculated ones, indicating that this bacterial strain can trigger basal immune responses (Fig. 2). The only exception in transcription patterns was in ethylene-related gene at 24 hpi between *B. cinerea* only and combination between *B. cinerea* and Bs MBI600, where no significant differences were observed. Interestingly, simultaneous inoculation of both microorganisms led to a significant increase in transcription levels of the *PR1, PR10* and *ethylene*-related genes at all tested time points as compared to individual treatments (Fig. 2).

3.3. MFS and ABC transporters were induced during interaction with Bs MBI 600 secretome

To investigate the responses that *Bs* MBI 600 potentially causes to the *B. cinerea* transcriptome, the fungus was grown on *B. subtilis* culture filtrates and mycelia were extracted at 0, 24, 48 and 72 hours post exposure. Analysis of *B. cinerea* transcriptome at the above time points in absence of the bacterial supernatant showed no significant differences (data not shown). In these samples 85 to 90% of the total reads were mapped in *B. cinerea* strain B0.5 genome. According to the annotation, 40 % of the genes were classified in relation to protein product, while 60 % were characterized as "hypothetical proteins". Data analysis revealed that gene expression patterns had been changed through the time pass. More specifically, few differentially expressed genes (DEGs) were identified in 24 h and all of them were up-regulated. However, 48 h and 72 h after exposure, significantly higher number of DEGs was observed. In detail, 53 and 58 DEGs were up-regulated in each time point, and 41 and 32 DEGs were down-regulated respectively (Fig. 3, Fig S1). Furthermore, Gene Ontology (GO) analysis on up-regulated genes showed an increased number of genes involved in metabolic process (Fig. S2). Among the annotated up-regulated genes were found the *BcatrO* gene (*XP_024546005.1)* that encodes a putative ABC transporter and the *Bcmfs1* gene (*XP_024546639.1),* that encodes a putative MFS transporter (Fig. 3A, Fig S1). Regarding the down-regulated ones, we identified genes coding for cytochromes P450 and P451 (*XP_001545401.1*, *XP_024545896.1* and *XP_024545899.1*), genes coding for putative PKS (*XP_024545898.1* and *XP_024545901.1*) and genes

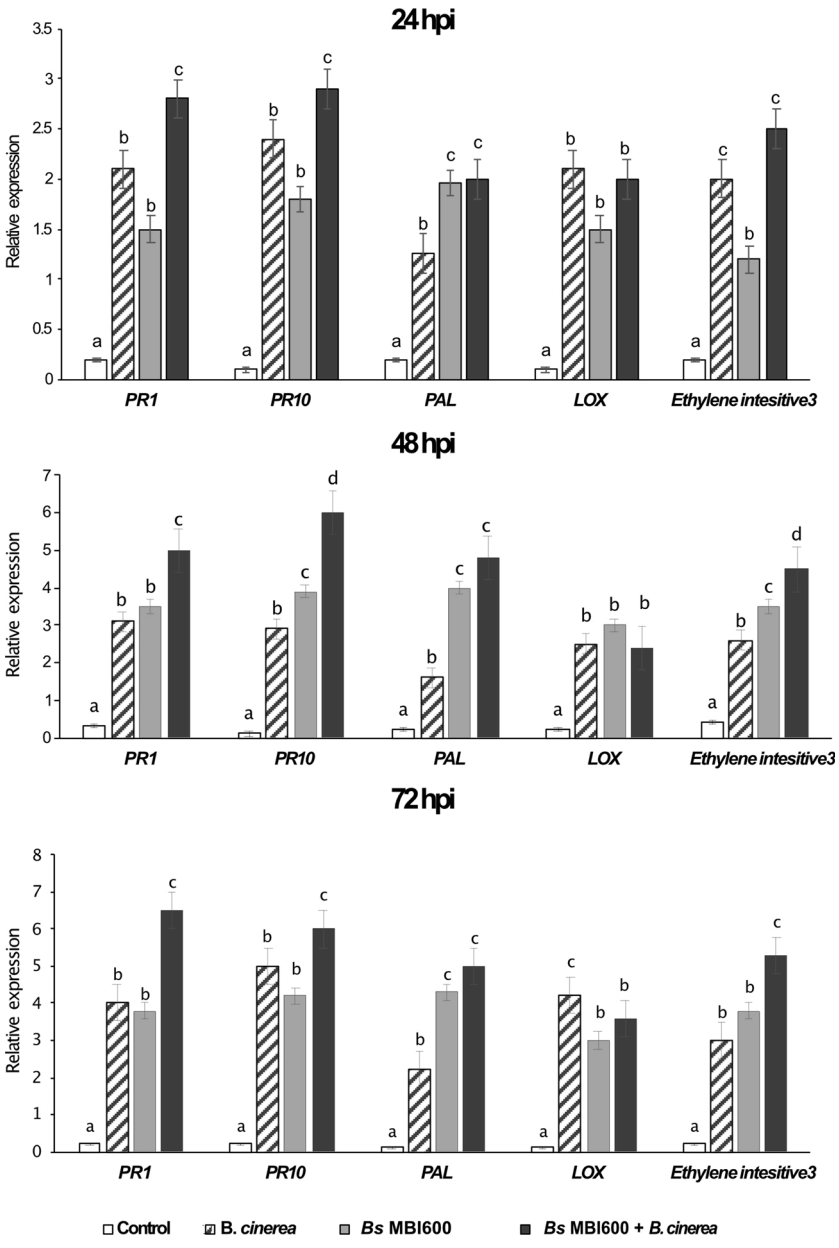

Fig. 2. Expression levels of defense-related genes (*PR1, PR10, PAL, LOX, Ethyl intensive3*) in cucumber plants after treatment with *Bacillus subtilis* MBI 600, inoculation with *Botrytis cinerea* and combined application of both. Expression levels were analyzed by qRT-PCR at 24, 48 and 72 hpi, and the cDNA samples were normalized using the endogenous cox gene. Different letters on the columns indicate statistically significant differences according to Tukey's test ($P < 0.05$). Vertical lines on the columns indicate the standard error of the mean.

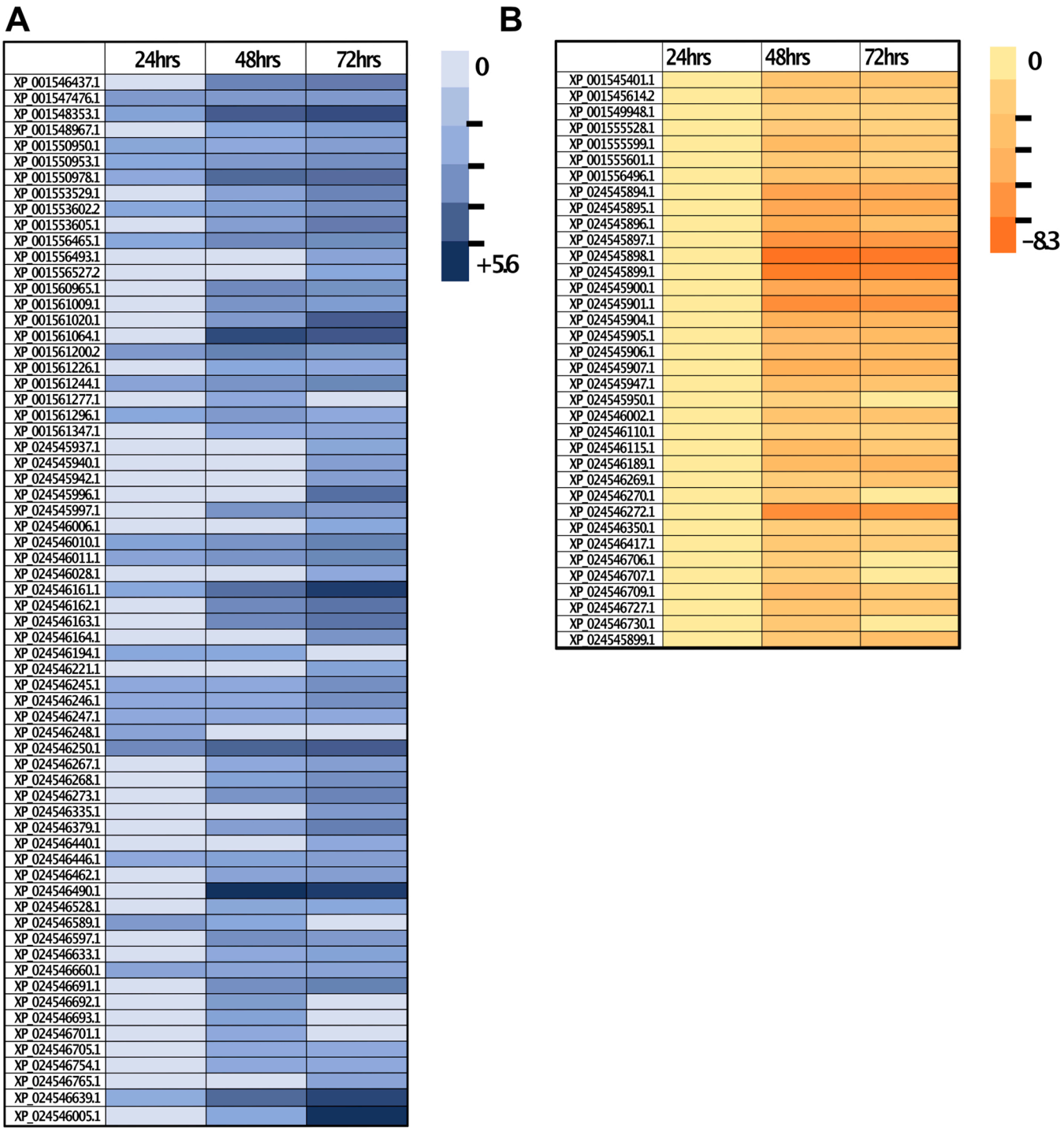

Fig. 3. Heat map showing transcription profiles of *Botrytis cinerea* during its growth in *Bacillus subtilis* MBI 600 culture filtrates at different time points (24, 48 and 74 hpi); **A**. up-regulated and **B.** downregulated genes Data were normalized with mycelia at 0 hpi (adjusted *P*-value < 0.05, absolute log2 fold change > 2). Blue and yellow colors represent up-regulated or down-regulated genes respectively.

encoding putative Major intrinsic proteins (MIP) (*XP_024545947.1* and *XP_001555528.1*) (Fig. 3B, Fig S1). Finally, RT-qPCR was conducted in a number of selected genes, confirming the RNA-seq data (Fig. S3).

3.4. The induced ABC and MFS transporters predicted to be involved in drug resistance

In order to categorize the BcatrO ABC transporter in one of the eight distinct (A–G) groups, phylogenetic analysis was conducted using homologs from a variety of ascomycete fungal species; filamentous and yeast-like ones. Neighbour Joining analysis revealed that BcatrO transporter was clustered in G group, which referred to pleiotropic drug resistance (PDR) (Fig. 4A). To further categorize this ABC transporter in subgroups (I-VII) we conducted an additional phylogenetic analysis using homologs that belong only in group G. Our analysis showed that it belongs to G–I subgroup, a subgroup including several characterized members with involvement in drug resistance (Fig. 4B) (Kovalchuk and Driessen, 2010). Finally, analysis of the Bcmfs1 MFS transporter showed that it belongs to 2.A.1.3.65 subfamily and predicted to be involved in multidrug resistance as well (Roohparvar et al., 2007; Liu et al., 2012).

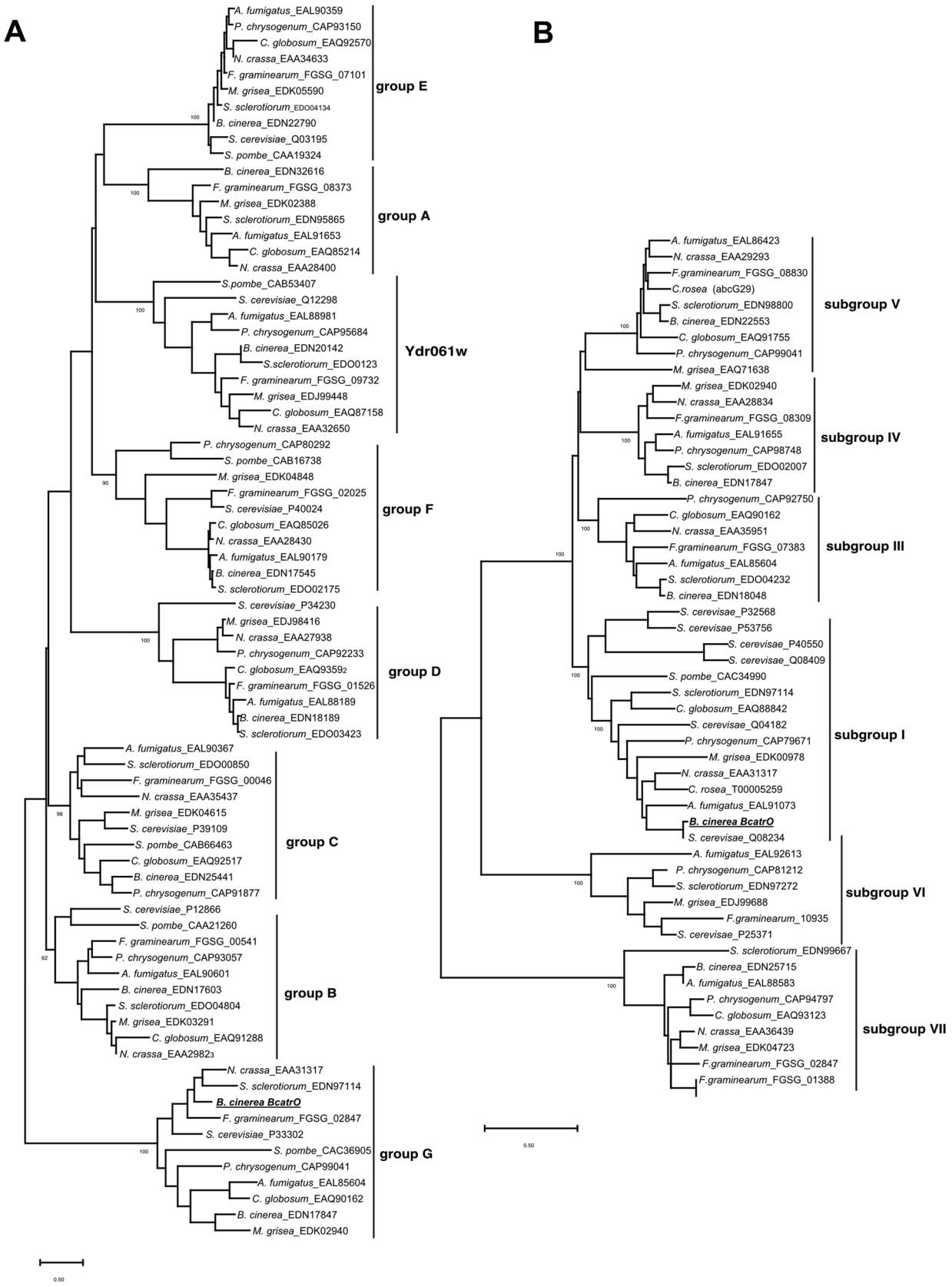

Fig. 4. Phylogeny of the BcatrO ABC transporter. **A.** Phylogenetic analysis of ABC transporters from different groups (A—G) **B.** Phylogenetic analysis of ABC transporters from different G subgroups (I-VII). Predicted amino acidic sequences were aligned using the CLUSTAL X algorithm and phylogeny was constructed in the MEGA X software using neighbour-joining method. Bootstrap support values from 500 iterations are associated with the nodes.

3.5. Deletion of Bcmfs1 reduced B. cinerea virulence and tolerance to bacterial secretome

In the next step, functional analysis of the Bcmfs1 MFS transporter was conducted. This transporter was chosen since functional data from this subgroup are scarce. To generate deletion strains, a recently published CRISP/Cas9 protocol was used (Leisen et al., 2020), conferring resistance to hygromycin and using a repair template with 60bp homology flanks with the right and left sites in the gene borders. Gene expression analysis showed no expression of the *Bcmfs1* gene in all tested mutants (Fig. S4). Further, the mycelial growth of these mutants was investigated on LB plates. Our results showed that the *Bcmfs1* mutant

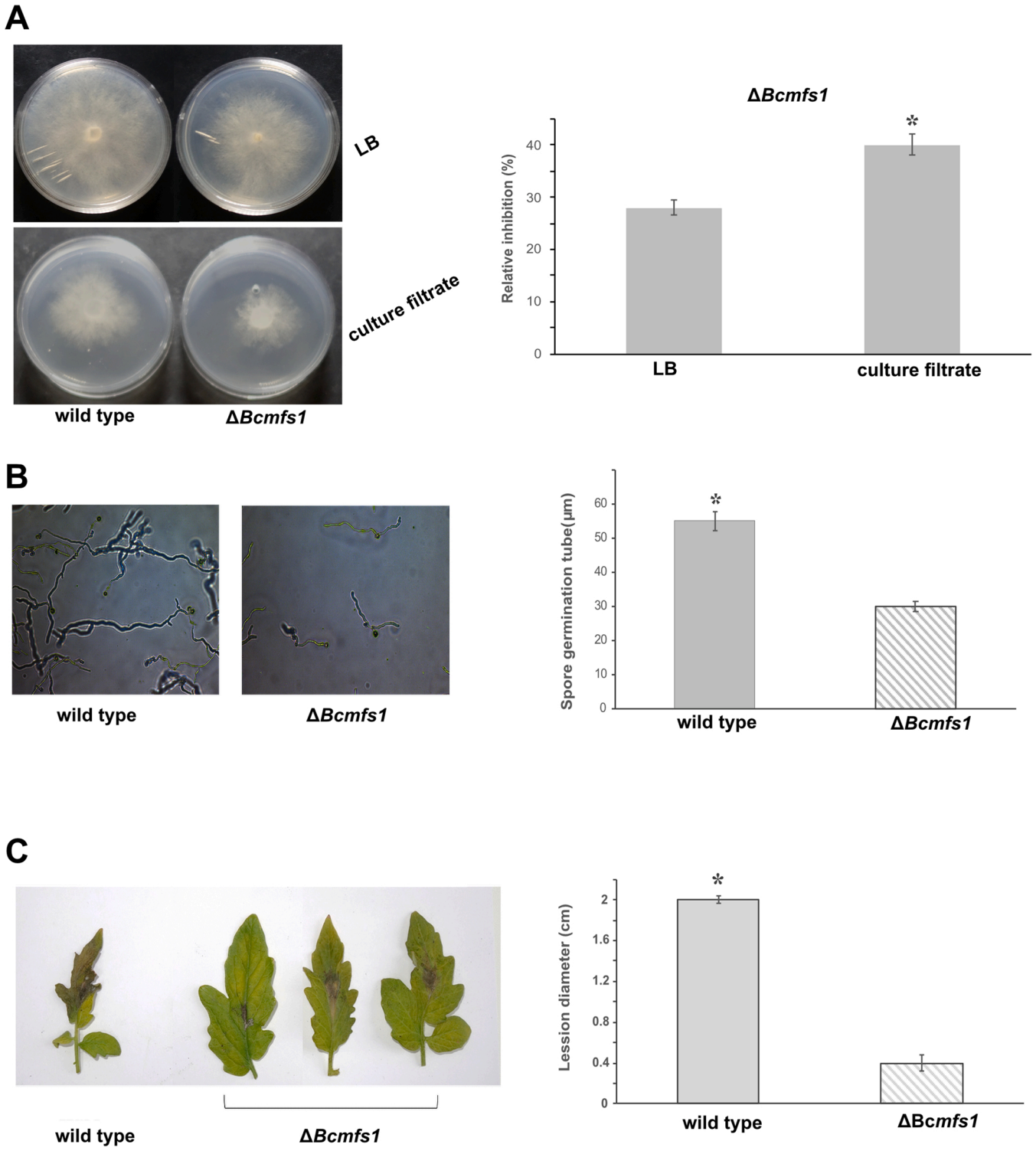

Fig. 5. Phenotypic analysis of *Bcmfs1* deletion strains. **A** Mycelial growth and relative inhibition rate on LB media in the presence or absence of *Bacillus subtilis* MBI 600 culture filtrates. **B** Conidia germination of wild type (WT) and Δ*Bcmfs1 Botrytis cinerea* strains, 18 hpi. **C.** Gray mold symptoms on tomato leaves after artificial inoculations with a wild type (WT) and Δ*Bcmfs1 B. cinerea* strains. Lesion diameter data derived as an average of 10 independent mutant strains. Vertical lines on the columns indicate the standard deviation (SD). Asterisk (*) indicate statistically significant differences according to Fisher's test ($P < 0.05$).

strains displayed a significant slower mycelial growth as compared to the wild-type strain (Fig. 5A). Moreover, the *Bcmfs1* deletion strains displayed significant higher mycelial growth inhibition rate as compared to WT, in growth media containing bacterial culture filtrates (Fig. 5A), suggesting involvement in tolerance. Microscopic observations of the conidial germination 18 hpi, revealed also reduced growth of germ tubes in the deletion strains in comparison to WT (Fig. 5B). Finally, pathogenicity assays were conducted in tomato plants to determine the virulence of these mutant strains. Interestingly, we observed that the leaves, inoculated with the Δ*Bcmfs1* strains, displayed reduced disease severity as compared to leaves inoculated with the WT strain (Fig. 5C), indicating a role of this transporter in the infection process.

4. Discussion

Botrytis cinerea is one of the most destructive plant pathogens and its control is based mostly on chemical treatments (Fillinger and Walker, 2016). Applications of biocontrol agents could be an alternative method to control this disease, but it shows many limitations and the efficacy can vary depending on climate conditions, plant cultivars etc (Nicot et al., 2016). In the current study, we investigated the biocontrol ability of *Bs* MBI 600 against gray mold on cucumber plants, which is a new, recently commercialized, biological control agent and its exact mode of action is not fully understood. In the current study, we demonstrated that *B. cinerea* deploys specific efflux membrane transporters to cope with stress caused by this biocontrol agent and additionally we showed that *Bs* MBI 600 could trigger defense mechanisms in cucumber plants.

Assessment of the antagonistic activity of this biocontrol agent against *B. cinerea in vitro* showed a clear inhibition of mycelial growth. This effect possible is attributed to the production of fungistatic compounds, since genome analysis revealed genes that are involved in production of antibiotics such as fengycin and surfactin (Samaras et al., 2021b). In agreement with the in vitro assays, the *in planta* experiments showed that *Bs* MBI 600 was able to efficiently control gray mold as has been observed previously with other *Bacillus* strains (Nie et al., 2017; Abdelkhalek et al., 2020; Toral et al., 2020), underlining that *Bs* MBI 600 strain could be an effective biocontrol agent against this pathogen.

The role of different membrane transporters in efflux process, against chemical fungicides and xenobiotics has previously been thoroughly demonstrated (Coleman and Mylonakis, 2009). Analysis of the *B. cinerea* transcriptome under growth in *Bs* MBI 600 culture filtrates, showed high induction of the *BcatrO* ABC transporter gene, which belongs to G–I subgroup. Members of this group are involved in PDR and play an important role in infection process and fungal protection from toxic compounds produced by plants (Urban et al., 1999). The fact that two G-group ABC transporter genes (*abcG8 and abcG25*), from the mycoparasite fungal species *Clonostachys rosea,* were highly induced under growth on culture filtrates derived from the bacterial species *Serratia rubidaea* but not from the *Pseudomonas chlororaphis,* indicates substrate specificity (Kamou et al., 2016). In addition, our transcriptome analysis showed induction of the *Bcmfs1* gene, coding for an MFS transporter. These transporters are also thought that they work as drug transporters, although they are more substrate-specific than the ABC ones (Dos Santos et al., 2014; Zhang et al., 2015). The Bcmfs1 transporter belongs to the 2.A.1.3.65 family which has been predicted to be involved in multidrug resistance as well (Roohparvar et al., 2007; Liu et al., 2012). Previous data from the mycoparasitic species *Trichoderma harzianum* showed that the MFS1, which is also member of this family, transports xenobiotics, such as fungicides and secondary metabolites, while transcription patterns of *C. rosea* 2.A.1.3.65 family members showed induction during contact with *Fusarium graminearum* and exposure to fungicides, fitting well with our data (Liu et al., 2012; Nygren et al., 2018).

Despite indications that MFS transporters belonging to the 2.A.1.3.65 family are involved in drug resistance; they have not been studied in details. For that reason, we decided to further investigate the role of this transporter. Deletion of this gene was conducted using a recently published CRISP/Cas9 editing protocol, which eliminates the occurrence of off-target mutations; a serious concern in conventional gene deletion techniques (Leisen et al., 2020). We found that the *Bcmfs1* mutants exhibited reduced mycelial growth. Similar data were observed in other fungal species, such as *Aspergillus carbonarius* and *Alternaria alternata*, indicating an involvement of MFS transporters in hyphal growth (Crespo-Sempere et al., 2014; Chen et al., 2017). In addition, we showed that the Bcmfs1 mutants displayed enhanced susceptibility to the *Bs* MBI 600 secretome, confirming previous studies regarding the role of this transporter in tolerance against xenobiotics (Hayashi et al., 2002). We further observed reduced virulence of the *Bcmfs1* mutants, probably because of lower fitness, since these mutants also showed reduced growth of germ tubes. In *B. cinerea*, the *mfsG* gene was also involved in virulence, while previous data from other phytopathogens, such as *Cercopsora nicotianae* and *Zymoseptoria tritici,* displayed similar results (Choquer et al., 2007; Omrane et al., 2015). In contrast, previous data of Hayashi et al., 2002 suggested that Bcmfs1 did not play any role in the pathogenicity of *B cinerea*. These differences probably are attributed to different experimental procedures used in these two studies.

Botrytis cinerea is a typical necrotrophic pathogen and plant resistance against this type of pathogens basically depends on the JA/ET signaling pathways (Pieterse et al., 2009; Houben and van de Poel, 2019). In the current study, we showed that five defense-related genes were up regulated in cucumber plants treated with this strain. Plants under priming conditions, induced defense genes 24 hpi and the induction increased significantly in 48 hpi. A previous study with *Bs* MBI600 strain, on tomato revealed that its antivirus efficiency is achieved through eliciting defense responses by activation of salicylic acid (SA)-responsive genes and a synergistic cross-talk between jasmonic acid/ ethylene (JA/ET) and SA-signaling (Dimopoulou et al., 2019). In another study, increased transcription levels of PR proteins 24 hpi were observed after treatment with *B. cereus* AR156 (Jiang et al., 2016). We also found that the *PR1*, *PR10*, *PAL*, and Ethylene intensive3 gene expression levels were higher in plants treated with both the biological control agent and the pathogen indicating a "'synergistic" action in triggering plant defense responses between these two microorganisms, as has been previously observed in other systems (Wu et al., 2018; Nie et al., 2017). It is possible that secondary metabolites produced by *Bs* MBI 600 are responsible for the induction of plant defense responses, similar to surfactin and fengycin lipopeptides, produced by variable *Bacillus* strains, and were able to act as ISR elicitors (Ongena et al., 2007).

5. Conclusions

In summary, we showed that the *Bs* MBI 600 strain can be an efficient effective biocontrol agent against gray mold on cucumber plants, working possibly through antibiosis and induction of mechanisms involved in plant immunity. Resistance in fungicides is a growing issue in *B. cinerea* management. One of resistance mechanisms is the overexpression of detoxification transporters. However, in this study we showed that this type of transporters is also involved in the interactions with *Bs* MBI 600, indicating that the efficacy of this biocontrol agent could be affected in resistant populations. In addition, our functional data provided evidence for the role of the Bcmfs1 transporter in fitness and pathogenicity, suggesting that efflux of compounds plays a crucial role in different aspects of fungal biology. Identification of the specific mechanisms, involved in interactions between biocontrol agents and pathogens, is a knowledge needed to increase the biocontrol efficacy in this field.

Authors statement

Anastasios Samaras: Conceptualization, Methodology, Software, Data curation, validation, writing original draft and reviewing.

George Karaoglanidis: supervision, writing, editing and reviewing the manuscript.

Georgios Tzelepis: Conceptualization, Methodology, supervision, writing, editing and reviewing the manuscript

Data availability

The RNA-Seq data used in this study has been deposited in the National Center for Biotechnology Information (NCBI) database under the accession number PRJNA687991.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2021.126752.

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