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Insights into the Interaction between the Biocontrol Agent *Bacillus amyloliquefaciens* QST 713, the Pathogen *Monilinia fructicola* and Peach Fruit

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Abstract: Brown rot disease caused by *Monilinia fructicola* is one of the most important peach fruit threats in the world. The use of biological control agents (BCAs), instead of synthetic fungicides, to successfully inhibit postharvest disease development is a challenge in sustainable and efficient crop management. The commercially available BCA *Bacillus amyloliquefaciens* QST 713 (formerly *Bacillus subtilis* QST713) is able to inhibit a variety of fungal pathogens and suppress several plant diseases. Our results showed that this BCA inhibited mycelial growth in vitro, and was able to suppress the disease's severity in peach fruits via delaying and reducing brown rot symptoms. A transcriptomic analysis of fruits during their pre-treatment with this biocontrol agent following *M. fructicola* challenge revealed a significant upregulation of specific differentially expressed genes (DEGs) at 48 h after inoculation (HAI). These genes are related to the activation of several transcriptional factors, such as members of the WRKY and NAC families, and receptors that are involved in pathogen recognition and signaling transduction (e.g., LRR-RLKs). Furthermore, the inhibition of *M. fructicola* by this biocontrol agent was confirmed by analyzing the expression profiles of specific fungal genes, which highlighted the direct antimicrobial impact of this bacterial strain against the fungus. Hence, these findings clearly suggest that *B. amyloliquefaciens* QST 713 is an efficient BCA against brown rot disease, which can directly inhibit *M. fructicola* and improve peach fruit tolerance.

Keywords: antimicrobial; biocontrol agent; fungal–bacterial interactions; plant immunity; transcriptomics

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1. Introduction

Monilinia fructicola (G. Winter) Honey, which causes brown rot disease in peach, is a significant necrotrophic pathogen that causes substantial losses in the production and quality of fruits [1–3]. In addition to fruit rot, several studies have shown that the disease can also cause blossom blight and twig cankers [2]. The main symptoms of the disease are observed in fruits as brown spots that rapidly develop until the whole fruit turns brown, and greyish mycelia may appear in the symptomatic areas [4,5]. Hence, the fungus can easily transmit the disease from infected harvested fruits to non-infected ones by dispersing a large number of spores that enter the host tissues through naturally occurring wounds on trees or during storage and transportation and releasing cell-wall-degrading enzymes [3,6].

The control of this disease is primarily based on conventional fungicides [7]. However, the prolonged usage of these fungicides, along with legal restrictions regarding their applications, have raised severe concerns highlighting the need to establish alternative and more sustainable disease management strategies [2]. Additionally, the pathogen seems to

harbor several mutations in the β-tubulin locus, leading to a high benzimidazole resistance level [8,9].

Biological control agents (BCAs), such as bacteria, fungi or yeasts, have been investigated as potential alternatives to ensure the efficient control of important plant diseases. Several studies have revealed that beneficial microorganisms can effectively inhibit a large number of phytopathogenic microorganisms, particularly fungi that cause pre- and postharvest fruit decays [10,11]. Thus, microorganisms belonging to the *Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp. species are considered as the most studied microorganisms regarding the biological control of plant diseases. These BCAs depict several direct or indirect modes of action at their disposal, such as the biosynthesis of antifungal volatiles, antibiotics and cell-wall-degrading enzymes, or/and the induction of plants disease-resistant mechanisms [10,12,13]. Several *Bacillus* species have posed an increasing interest as efficient BCAs of pre- and postharvest diseases against important pathogens such as *Colletotrichum* spp., *Sclerotinia sclerotiorum*, *Alternaria alternata* and *Fusarium* spp., while their suppression mechanisms have been thoroughly studied [10,12,14].

Notably, strains of the *Bacillus amyloliquefaciens* group have been extensively used as commercial plant protection products [11,15–20]. For example, *B. amyloliquefaciens* DH-4 has been reported to biosynthesize antifungal compounds as a result of its secondary metabolism, such as iturin, macrolactin, surfactin and fengycin, and its effectiveness in controlling postharvest diseases in citrus has been revealed [21]. Strains of *B. amyloliquefaciens* could induce a systemic resistance response in tomatoes and peppers under greenhouse or field conditions [13], while in banana the epiphytic bacterial strain *B. amyloliquefaciens* DGA14 has shown a high antifungal activity against crown rot, the primary postharvest disease of the certain crop [22]. Furthermore, *B. amyloliquefaciens* strains C06 and BUZ-14 significantly suppressed, by 92%, the brown rot disease incidence in harvested fruits and showed strong in vitro antagonistic activity against the causal agent. Finally, it is worth mentioning that the *B. amyloliquefaciens* strain isolated from the soil of pistachio orchards was able to reduce disease severity as well as aflatoxin production by *Aspergillus* spp. in pistachios [23].

B. amyloliquefaciens QST 713 is now commercially available as a biological plant protection product (Serenade ASO®, Bayer AgroSciences) [24] against several important foliar and soilborne plant pathogens in many crops. According to recent studies, this bio-fungicide could reduce powdery mildew disease severity in cereals by up to 60% in early applications, as well as reducing the postharvest fruit decay of mango [24], while it may additionally be employed in integrated pest management (IPM) systems to control grey mold in beans [19].

The transcriptomic responses that occur during the interaction between *M. fructicola*, the *B. amyloliquefaciens* QST 713 biocontrol agent and the peach fruits are still widely unknown. Therefore, the primary goal of this study was to identify the molecular mechanisms triggered by this bacterial strain upon *M. fructicola* challenge in peach fruits, and determine the alterations in fungal expression profiles during inoculation. We further evaluated the biocontrol activity of *B. amyloliquefaciens* QST 713 based on specific defense-related physiological indices. Our findings also demonstrated that this commercial BCA effectively suppressed fruit brown rot by directly inhibiting fungal growth.

2. Materials and Methods

2.1. Antagonistic Activity Assays In Vitro

A commercial fungicide formulation of *Bacillus amyloliquefaciens* QST 713 (Serenade ASO®, Bayer Hellas AG, Athens, Greece) was used in dual-culture fungitoxicity tests. This formulation of the biocontrol agent was dissolved in sterilized dH_2O to prepare the stock solutions (ten-fold dilution series). The sensitivity profile of *M. fructicola* single-spore strain (isolate No 2684), which was provided by Benaki Phytopathological Institute (Athens, Greece), was evaluated on potato dextrose agar (PDA), amended with the following concentrations: 0.001, 0.01, 0.1, 1, 10 and 100 mg L^{-1} a.i. of *B. amyloliquefaciens* QST 713. To prepare the stock solutions, the biocontrol agent was diluted in sterilized dH_2O, and then

1 mL of these solutions were added to 100 mL of autoclaved PDA media in 125 mL Duran glass laboratory bottles. The bottles with the amended media were gently shaken and distributed in 6 cm Petri dishes. Then, mycelial discs (4 mm in diameter) were excised from the periphery of 7-day-old *M. fructicola* culture and transferred to the BCA-amended PDA media in Petri dishes, prepared as described above. Dishes unamended with BCA served as controls. The radial growth (inhibition zone) of the fungal colony, after 7 days of incubation at 23 °C in the dark, was calculated and compared to the growth of the colony on the control plates in order to determine the minimum inhibitory concentration (MIC). Four replicate experiments were carried out for each concentration of the biocontrol agent.

2.2. Bacillus Amyloliquefaciens Treatment and Pathogen Inoculation on Fruits

In the ripening developmental stage, mature, yellow-fleshed peaches (*Prunus persica*, cultivar "Catherina") were harvested from an orchard located at the Larissa region (Greece). Before being inoculated, collected fruits were surface-sterilized as previously described [25]. Four treatments were used: fruits inoculated with *M. fructicola* (MF), fruits pre-treated with *B. amyloliquefaciens* QST 713 and then inoculated with *M. fructicola* (QST 713+MF), fruits treated only with the bacterial strain (QST 713) and untreated, mock-inoculated fruits (CT).

The fruits of all treatments were wounded (3 mm deep × 3 mm wide) at their central adaxial surface using a sterile needle. Treatments were performed as follows: (a) CT treatment: 20 µL of sterile dH_2O was injected without previous inoculation; (b) MF treatment: 20 µL of sterile dH_2O was injected into each wound, the fruits were air-dried for 12 h, and then 20 µL of 10^6 *M. fructicola* conidia/mL was inoculated into each wound; (c) QST 713+MF treatment: 20 µL of 7×10^6 bacterial spores/mL was injected into each wound, the fruits were air-dried for 12 h, and then 20 µL of 10^6 *M. fructicola* conidia/mL was also inoculated into each wound; (d) QST 713 treatment: 20 µL of 7×10^6 bacterial spores/mL was injected into each wound. To prepare the fungal inoculum, conidia from 10-day-old PDA cultures were harvested and suspended in PDB until a concentration of 10^6 conidia/mL was obtained. The fruits were kept under standard photoperiod conditions at 24 °C in sealed transparent containers with high humidity. In three independent experiments for each of the three fruit treatments (CT, MF, QST 713+MF), sections with approximately 1.5 cm diameter of peel and pulp (four mm deep) were chopped around the point of inoculation at 12, 24 and 48 h after inoculation (HAI). Pooled samples of ten fruits were used in each treatment. For subsequent usage, fruit samples were frozen in liquid nitrogen and stored at −80 °C.

2.3. Determination of Physiological Indices on Peach Fruits

For the three treatments (CT, MF, QST 713+MF), fruit tissues were used for lipid peroxidation and hydrogen peroxide assays, as well as to determine the phenolic and flavonoids content of fruits, as previously described [25].

2.4. Transcriptome Analysis and Bioinformatics

RNA-seq was performed on three treatments (CT, MF, QST 713+MF) from fruit samples collected at the three time points using three biological replicates. Total RNA from fruit samples (27 in their number) was extracted using the Monarch Total RNA Miniprep Kit (NEB, Frankfurt, Germany). RNA-seq was performed as previously described [25] using the Illumina (San Diego, CA, USA) Novaseq 6000 sequencing platform and producing 2 × 150 bp paired-end (PE) reads. The NCBI genome database was employed to obtain the reference peach genomic assembly and the cultivar Lovell [26] gene models. HISAT2 software (v2.0.5) was used for mapping the clean PE reads to the reference genomic assembly [27]. The FPKM method was utilized to calculate the levels of gene expression, and the DESeq2 R package (1.20.0) was used to assign differentially expressed genes (DEGs) [28] based on an absolute value of log2 fold change ≥ 1 by setting an adjusted p-value ≤ 0.05. Reads mapped to the *M. fructicola* transcriptome were detected from the pathogen-inoculated peach samples (MF and QST 713+MF). Thus, in order to detect fungal

transcripts, the HISAT2 software (v2.0.5) [27] was employed to map the unmapped reads against the peach genome reads to the *M. fructicola* Mfrc123 ASM869222v1 genomic assembly. The htseq-count program was utilized to assign reads at the transcript level, and the DESeq2 R software was utilized to compute the normalized transcript counts [28]. A Gene Ontology (GO) enrichment analysis was conducted using the clusterProfiler R package (3.8.1), and GO terms with a *P*-value < 0.05 were denoted as significantly enriched [29]. Finally, DEGs were assigned to KEGG orthology terms, as previously described [25].

2.5. Gene Expression Validation

In order to verify the RNA-seq data, the relative gene expression of nine peach DEGs was validated through quantitative real-time PCR (RT-qPCR) analysis, following the previously described procedure [25]. PCR reactions were performed using three replicates and the expression profiles were normalized by comparison to a housekeeping gene (LOC18789459). The $2^{-\triangle\triangle CT}$ method was used to monitor the relative quantitative expression ratios of the inoculated samples in comparison to the respective controls [30].

3. Results

3.1. Inhibitory Effect of Bacillus Amyloliquefaciens QST 713 on M. fructicola Growth In Vitro and Disease Severity on Peach Fruits

The biocontrol efficiency of *B. amyloliquefaciens* QST 713 against *M. fructicola* was in vitro assessed in antagonistic assays. Dual cultures of this bacterial strain and the pathogen were conducted on a PDA medium in six bacterial concentrations. The addition of this BCA into PDA Petri plates at different concentrations reduced the mycelial growth of *M. fructicola* after seven days of incubation in comparison to the non-amended control medium, where the fungus formed a colony with a radius ranging from 18 to 22 mm (Figure 1A). A strong inhibition zone was observed around the fungal colony, reducing the mycelial growth (Figure 1A(i–v)). The radial growth of *M. fructicola* was progressively reduced in accordance with the increased concentration of *B. amyloliquefaciens* QST 713 up to the concentration of 10 mg L^{-1} a.i., at which point the mycelium development was sparce (Figure 1B). The concentration of 100 mg L^{-1} a.i., which corresponds to 7×10^6 *B. amyloliquefaciens* spores/mL, completely inhibited the mycelial growth of *M. fructicola* (Figure 1A(vi)), and this was considered the MIC value of this BCA.

To determine the antagonistic activity of *B. amyloliquefaciens* QST 713 against *M. fructicola*, we performed antimicrobial assays on peach fruits (Figure 1). *B. amyloliquefaciens* QST 713 co-inoculation (QST 713+MF treatment) effectively inhibited the brown rot symptoms caused by *M. fructicola* compared to fruits in the MF treatment that were inoculated only with the fungus at wounded sites (Figure 1C). Notably, no lesions were observed on fruits that underwent the QST 713+MF treatment at 12 HAI, while the mean lesion diameter was 0.2 and 0.5 cm at 24 and 48 HAI, respectively (Figure 1D). No lesions were observed on either untreated, mock-inoculated peaches (CT treatment) or bacteria-inoculated fruits (QST 713 treatment) (Figure 1C).

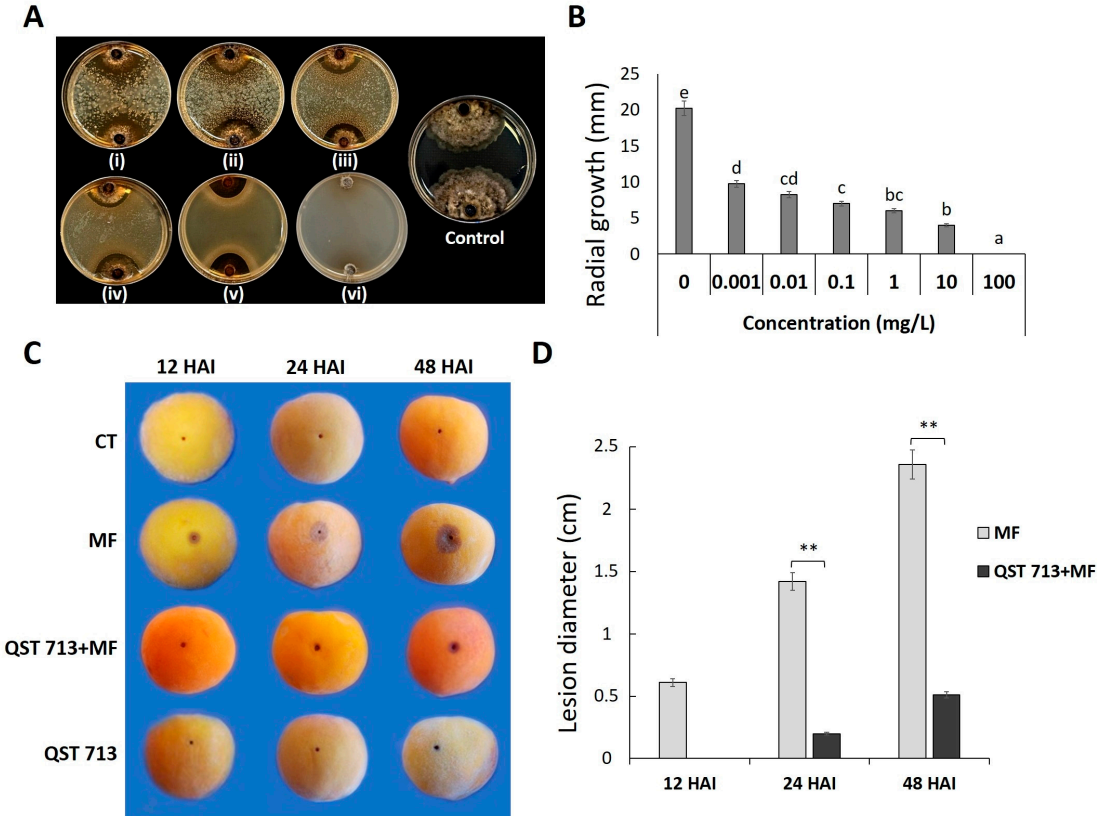

Figure 1. The biocontrol effect of *B. amyloliquefaciens* QST 713 against *M. fructicola*. (**A**) Mycelial growth of *M. fructicola* in PDA Petri plates containing PDA amended with different bacterial concentrations (i: 0.001, ii: 0.01, iii: 0.1, iv: 1.0, v: 10, vi: 100 mg/L a.i.). Plates inoculated only with the *M. fructicola* served as controls. (**B**) *M. fructicola* radial growth at different concentrations of the biocontrol agent. Different letters indicate statistical differences among different concentrations after one-way ANOVA analysis, followed by Tukey's post hoc multiple comparison test ($p < 0.05$). (**C**) Visual brown rot symptoms on fruits inoculated only with *M. fructicola* (MF treatment) or with both *B. amyloliquefaciens* QST 713 and *M. fructicola* co-inoculation (QST 713+MF treatment) at 12, 24 and 48 HAI. Untreated, mock-inoculated (CT treatment) fruits, as well as fruits pre-treated only with *B. amyloliquefaciens* QST 713 (QST 713 treatment), were used as control groups. (**D**) Mean lesion diameter on fruits upon the MF and QST 713+MF treatments. Asterisks indicate differences between treatments (** $p < 0.01$).

3.2. Physiological Changes on Fruits

There was a noticeably higher level of total flavonoids and phenolics in both the MF and QST 713+MF treatments compared to untreated peaches (CT) at all three time points (Figure 2A,B). In the MF treatment, both phenolics and flavonoids progressively increased, reaching their highest amount at 24 HAI, whereas in the QST 713+MF treatment, the highest level was observed at 48 HAI. The same pattern was also detected in H_2O_2 production (Figure 2C), where, in the MF treatment, the highest level was observed at 24 HAI, and in the QST 713+MF treatment, at 48 HAI. Finally, the lipid peroxidation level did not significantly differ between treatments (Figure 2D).

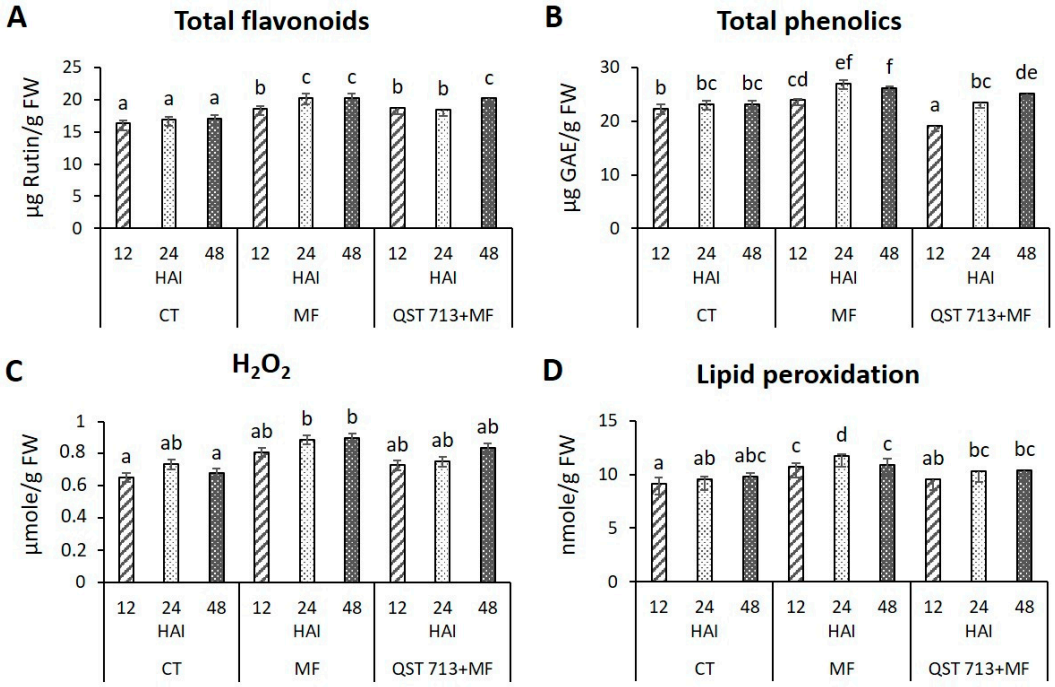

Figure 2. (**A**) Total flavonoids, (**B**) total phenolics, (**C**) hydrogen peroxide (H_2O_2) and (**D**) lipid peroxidation on fruits inoculated with *M. fructicola* (MF treatment), co-inoculated with both *B. amyloliquefaciens* QST 713 and *M. fructicola* (QST 713+MF treatment), and on untreated, mock-inoculated fruits (CT treatment). One-way ANOVA analysis was employed, followed by Tukey test for multiple comparison among treatments at all time points. The mean values of three biological replicates ± SD are displayed in bars. Means with the same letters are statistically similar ($p < 0.05$).

3.3. RNA-seq Analysis

To explore the transcriptomic changes that occurred in peach fruits in the presence of both *B. amyloliquefaciens* QST 713 and *M. fructicola*, a total RNA-seq analysis was carried out on fruit samples challenged only with the fungus (MF treatment), those co-inoculated with both the biocontrol agent and the pathogen (QST 713+MF treatment) and untreated, mock-inoculated (CT treatment) fruits. Thus, RNA-seq analysis was performed on 27 fruit samples using three biological replicates for each treatment across the three time points (12, 24 and 48 HAI). Approximately 89.79% of the 1.240.918.950 high-quality PE reads that were produced were mapped to the peach genome assembly (Table S1).

3.4. Analysis of Differentially Expressed Genes on Peach Fruits

The transcriptional responses of fruits inoculated only with the *M. fructicola*, or with both the *B. amyloliquefaciens* QST 713 and the pathogen, were determined by comparing their expression profiles against those of the untreated, mock-inoculated fruits. Thus, the DEGs of these treatments were divided into two pairwise comparison groups, namely MF-CT and QST 713+MF-CT (Table S2). The volcano plots (Figure 3A) show the correlation between the up- and downregulated DEGs for these two comparison groups, while Venn diagrams (Figure 3B) depict the common number of DEGs of each comparison group at each time point. The results showed that 10050 DEGs in the MF-CT group and 11993 in the QST 713+MF-CT group sustained a common co-expression across all time points. In the MF-CT group, 2990 DEGs (2429 up- and 561 downregulated) were identified at 12 HAI, 5317 DEGs (3956 up- and 1361 downregulated) at 24 HAI and 4941 DEGs (688 up- and 4253 downregulated) at the last time point (48 HAI). In the QST 713+MF-CT group, 3935 DEGs (1780 up- and 2155 downregulated), 1530 DEGs (927 up- and 603 downregulated) and 1178 DEGs (801 up- and 377 downregulated) were revealed at 12, 24 and 48 HAI, respectively. Our results indicate that, upon *M. fructicola* inoculation, the highest number

of upregulated DEGs was identified at 24 HAI and the highest number of downregulated DEGs was identified at 48 HAI, whereas in the QST 713+MF-CT group, the lowest number of downregulated DEGs was detected at 48 HAI.

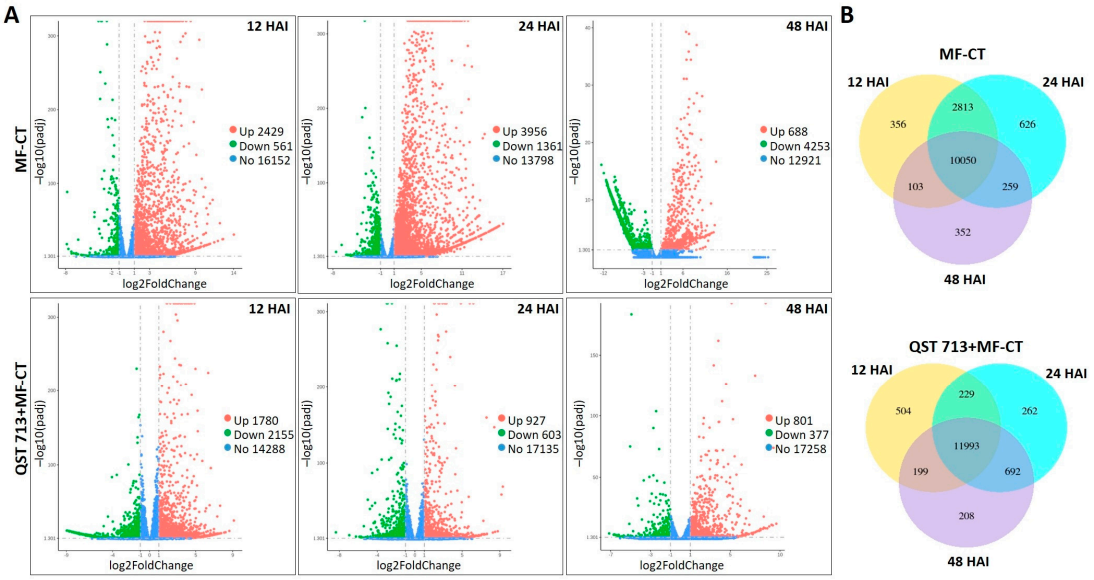

Figure 3. (**A**) Volcano plot of DEGs of both MF-CT and QST 713+MF-CT comparison groups at 12, 24 and 48 HAI. DEGs that are upregulated are represented by the red part and downregulated genes by the green. Blue represents no significantly expressed genes. (**B**) Venn diagrams illustrate the regulated DEGs for the two comparison groups (MF-CT and QST 713+MF-CT). MF: *M. fructicola* treatment; QST 713+MF: both *B. amyloliquefaciens* QST 713 and *M. fructicola* treatment; CT: untreated, mock-inoculated fruits.

3.5. Gene Ontology Categorization of DEGs

Based on the GO term enrichment analysis, DEGs were assigned to significant functional annotations (Figure S1). In the MF-CT comparison group, the most significantly enriched and constitutively highly upregulated GO molecular terms were those of 'DNA binding transcriptional factor activity' and 'transcription regulator activity', at 12 and 24 HAI. In terms of the patterns of biological processes, the 'defense response', 'response to stress' and 'response to stimulus' terms were found, at 12 HAI, to all be significantly enhanced. However, at 48 HAI, GO terms corresponding to biological process, such as 'regulation of biosynthetic processes', 'regulation of cellular metabolic processes' and 'regulation of primary metabolic process', were significantly downregulated upon *M. fructicola* infection. The GO term 'response to stimulus', corresponding to a biological process, was the most highly enriched and upregulated term in the QST 713+MF-CT group at 12 and 24 HAI, whereas the GO terms 'defense response' and 'response to stress' were constitutively enriched and mainly upregulated across all time points. In terms of the patterns of molecular functions, the GO terms 'oxidoreductase activity' and 'tetrapyrrole binding' were constitutively enriched over time, whereas the GO term 'cofactor binding' was mainly upregulated at 24 and 48 HAI.

3.6. KEGG Enrichment Analysis

In the MF-CT comparison group, the most significant highly enriched pathways were detected at the early stages of infection, at 12 and 24 HAI, according to the KEGG enrichment analysis (Figure 4). Thus, pathways related to 'plant–pathogen interaction', 'MAPK signaling pathway' and 'phenylpropanoid biosynthesis' were constitutively activated and enriched, while the KEGG pathways of 'biosynthesis of various plant secondary metabolites', and 'sesquiterpenoid and triterpenoid biosynthesis', as well as those related to linoleic metabolism, were only evident at 12 HAI. Furthermore, the 'plant hormone signal

transduction', 'ABC transporters' and 'cutin, suberine and wax biosynthesis' pathways were significantly enriched at 24 HAI. In the QST 713+MF-CT group, 'ABC transporters', 'flavonoid biosynthesis' and 'sesquiterpenoid and triterpenoid biosynthesis' were enriched at 12 HAI, whereas the 'plant–pathogen interaction' pathway was constitutively activated and highly enriched at all three times points. Furthermore, the 'MAPK signaling pathway–plant', 'biosynthesis of various plant secondary metabolites', and 'cutin, suberine and wax biosynthesis' pathways were highly enriched at 24 HAI. Finally, in the QST 713+MF-CT group, the 'MAPK signaling pathway–plant', 'sesquiterpenoid and triterpenoid biosynthesis' and 'phenylpropanoid biosynthesis' pathways were significantly enriched at the last time point.

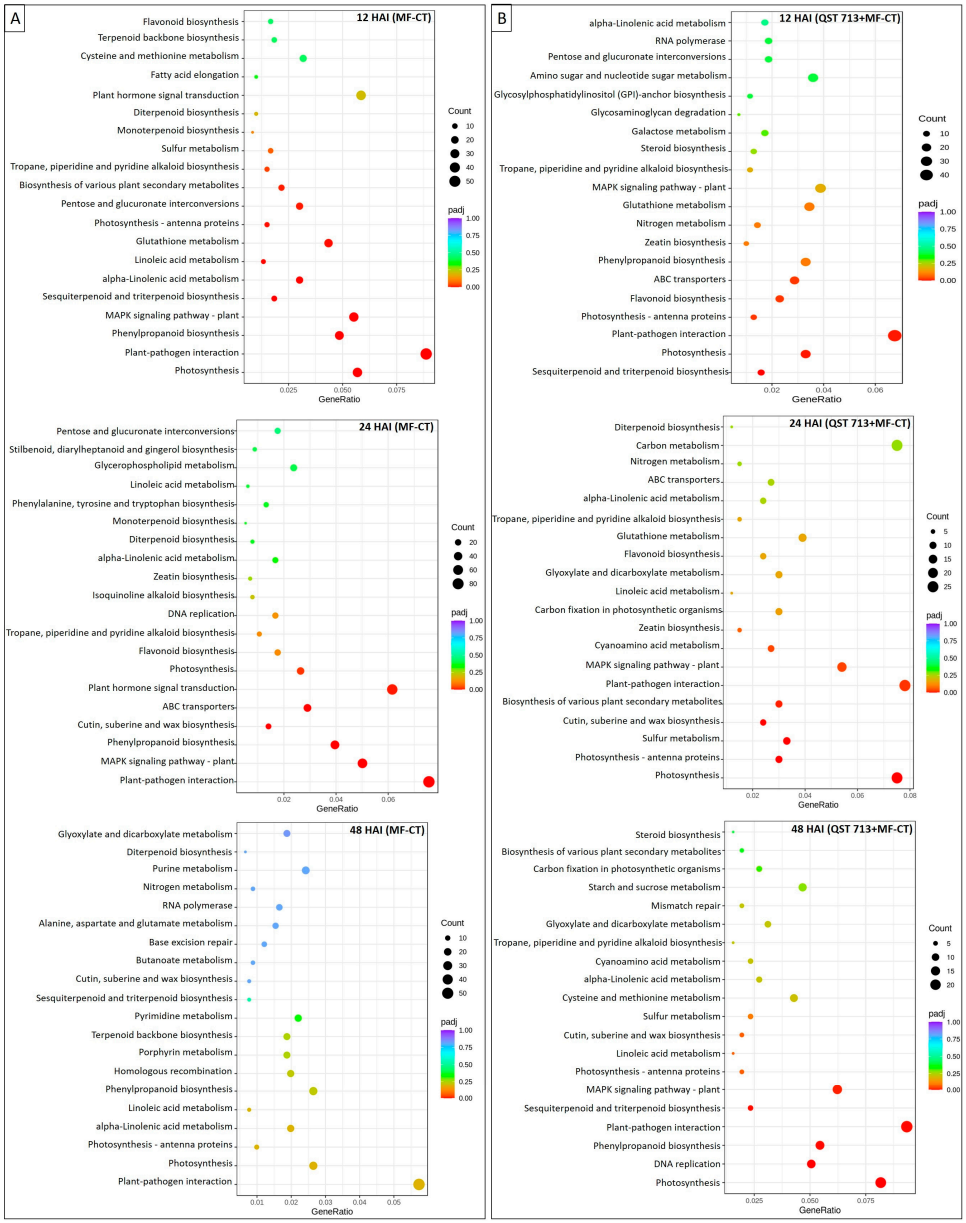

Figure 4. DEGs classification via KEGG pathway enrichment analysis in (**A**) the MF-CT comparison group and (**B**) the QST 713+MF-CT comparison group at 12, 24 and 48 HAI. The number of the DEGs that are being annotated in the relevant pathways are shown. MF: *M. fructicola*-inoculated fruits; QST 713+MF: both *B. amyloliquefaciens* QST 713- and *M. fructicola*-inoculated fruits; CT: untreated, mock-inoculated fruits.

3.7. Transcriptional Changes on Peach Fruits

In the MF-CT comparison group, DEGs related to cell-wall degradation and modification processes were more prominently upregulated at 12 and 24 HAI compared to the QST 713+MF-CT group, and were downregulated at 48 HAI, while in the QST 713+MF-CT group, most DEGs were downregulated at 12 HAI (Figure 5 and Table S3). Thus, DEGs encoding 3-ketoacyl-CoA synthase (KCS), polygalacturonase (PG) and xyloglucan endotransglucosylase/hydrolase (XTH) were the most highly induced DEGs upon *M. fructicola* inoculation, mostly at 24 HAI. Additionally, the expression patterns of DEGs encoding cellulose synthase (CesA), dirigent protein (DIR), expansin (EXP), extensin (EXT) and pectate lyase (PL) were similar to those mentioned above; they are mostly upregulated at 24 HAI and were downregulated at the latest time point. In the QST 713+MF-CT group, there was a lower number of induced DEGs compared to the MF-CT group. The majority of them were suppressed at 12 HAI, while they were mostly upregulated at 24 and 48 HAI.

Figure 5. Heat map displaying upregulated (Up) and downregulated (Down) key DEGs on fruits in the MF-CT and QST 713+MF-CT comparison groups. DEG numbers for both upregulated (blue scale) and downregulated (red scale) genes at 12, 24 and 48 HAI are presented for each group of genes. MF: *M. fructicola* treatment; QST 713+MF: both *B. amyloliquefaciens* QST 713 and *M. fructicola* treatment; CT: untreated, mock-inoculated fruits.

A high abundance of DEGs encoding several pathogen recognition receptors (PRRs) involved in pathogen perception and signaling transduction was highly upregulated in the MF-CT group at the earlier time points (12 and 24 HAI), while most of them were significantly downregulated at the latest time point (Figure 5 and Table S4). Thus, a variety of DEGs encoding several receptor-like proteins (RLPs) and receptor-like kinases (RLKs), such as those encoding members of *G*- or *L*-type lectin receptor kinases (GsSRKs, L-type LecRLKs), leucine-rich repeat-containing receptor-like serine/threonine-protein kinases (LRR-RLKs) and serine/threonine-protein kinases (STPKs), were upregulated at the early time points of infection, mostly at 24 HAI, and were downregulated at the latest time point. However, in the QST 713+MF-CT group, several DEGs encoding RLPs and RLKs were highly suppressed, mainly at 12 HAI, while most were upregulated at the latest time point (Figure 5 and Table S4).

A large number of DEGs encoding pathogenesis- and defense-related proteins were highly enriched and upregulated in the MF-CT group, particularly at 24 HAI and, to a lesser extent, at 12 HAI, whereas in the QST 713+MF-CT group, these DEGs were mainly downregulated by the first time point (12 HAI) (Figure 5 and Table S5). In the MF-CT group, a variety of DEGs encoding disease-resistance proteins belonging to various families, along with several DEGs encoding pathogenesis-related (PR) proteins, were significantly induced, reaching their highest expression level at 24 HAI. Notably, at 48 HAI, an extensive downregulation of several genes encoding disease-resistance proteins, chitinases/endochitinases and TMV resistance proteins was detected in the MF-CT comparison group. In the QST 713+MF-CT comparison group, several DEGs related to pathogenesis- and defense-related proteins were downregulated at 12 HAI. In contrast, DEGs that encode major allergen Pru ar 1- and BON1-associated proteins were highly upregulated across almost all time points.

Regarding transcription factors (TFs), a significant number of DEGs were induced in both comparison groups (Figure 5 and Table S6); however, the expression patterns of these DEGs at 12 and 24 HAI revealed that they were upregulated to a greater extent in the MF-CT group, with their peak expression levels mainly occurring at 24 HAI. However, for the group inoculated with *M. fructicola* alone (MF-CT group), a significant downregulation was observed at 48 HAI for most of the detected TFs, while in the QST 713+MF-CT group, the highest levels of suppressed and upregulated DEGs were detected at 12 and 48 HAI, respectively.

Several DEGs involved in the primary and secondary metabolism were induced in the MF-CT group at 12 and 24 HAI, while a few DEGs, such as those encoding 1-aminocyclopropane-1-carboxylate oxidase, caffeoyl shikimate esterase, S-adenosylmethionine synthase and glutamate decarboxylase, were mainly downregulated at 48 HAI. It is worth mentioning that, compared to the MF-CT group, fewer DEGs were upregulated in the QST 713+MF-CT group across the three time points, particularly at 12 and 24 HAI (Figure 5 and Table S7).

Nutrient and ion transporters were identified as highly induced DEGs in the MF-CT group, mainly at 24 HAI, including those encoding ATP-binding cassette (ABC) transporters, lysine histidine transporters and pleiotropic drug resistance (PDR) transporters. However, numerous DEGs encoding nutrient and ion transporters were suppressed at 48 HAI in this comparison group. However, in the QST 713+MF-CT group, such DEGs were induced across all time points (Figure 5, Table S8).

3.8. M. fructicola Expressed Genes

A number of genes associated with *M. fructicola* growth and pathogenicity were not detected in the QST 713+MF-CT comparison group (Table S9). Specifically, genes required for sporulation (*EYC84_003170* and *EYC84_002048*) or serving as structural constituents of the ribosome, as well as genes encoding ribosome biosynthetic proteins, such as erb1 (*EYC84_010071*), tsr1 (*EYC84_001134*), urb1 (*EYC84_000100*) and a GTP-binding ribosome biogenesis protein (*EYC84_003331*), were not detected across the three time points. Notably, the expression of genes linked to either the eicosanoid and glutathione metabolism

(*EYC84_011807*), or the Cys Met metabolism (*EYC84_005339*), as well as genes involved in cell-membrane homeostasis, were significantly disrupted in the QST 713+MF-CT group. Furthermore, genes that are associated with *M. fructicola* pathogenesis were also suppressed.

However, a number of genes encoding a variety of hydrolytic or carbohydrate-active enzymes (CAZymes) were identified in the MF-CT group, whereas, in the QST 713+MF-CT group, such genes, which are related to plant cell-wall disruption, were suppressed. Specifically, in the QST 713+MF-CT group, genes belonging to a variety of different glycoside hydrolase families (GH5, GH11, GH13, GH16, GH17, GH28, GH32, GH36, GH46, GH61 and GH76), carbohydrate esterase families (CE8, CE12 and CE16) and glycosyltransferase families (GT2, GT20, GT34 and GT39) were not expressed. Even though genes encoding polygalacturonases (*EYC84_010610*, *EYC84_010629*) were detected in the MF-CT group, these genes were significantly suppressed in the QST 713+MF-CT group. Finally, *M. fructicola* genes encoding several lytic enzymes, including class II peroxidase (*EYC84_007266*) involved in lignin degradation, pectin-methyl esterases (PMEs) (*EYC84_008212* and *EYC84_003200*) involved in pectin degradation, and three genes encoding acid (*EYC84_011533*), aspartate (*EYC84_008126*) and serine (*EYC84_008290*) proteases, were suppressed in the QST 713+MF-CT group.

3.9. Validation of DEGs by RT-qPCR

To verify the RNA-seq data, nine DEGs were selected at random. Table S10 provides a list of all gene-specific primers. A quantitative real-time PCR experiment was used to validate the log2fold change values of the RNA-seq analysis. As presented in Figure S2, the expression patterns of the selected genes were comparable to the RNA-seq results.

4. Discussion

Brown rot is a major peach fruit disease caused by *M. fructicola* and resulting in severe production losses and quality degradation [1]. The application of BCAs has lately been explored as an alternative approach to the management strategy based on the use of synthetic fungicides to control fruit decays, as antagonistic microorganisms may combine multiple modes of action [31]. Bacteria belonging to *Bacillus* species are promising BCAs due to their ability to synthesize various antimicrobial secondary metabolites, volatile compounds (VOCs) and lytic enzymes, and stimulate plants' defense mechanisms [32]. Thus, the BCAs of the *Bacillus* species have been thoroughly examined for their direct antagonistic activity against *Monilinia* spp. and biological control of brown rot disease, with *B. amyloliquefaciens* and *B. subtilis* being the most promising species [33,34]. Among them, *B. amyloliquefaciens* QST 713 (formerly *B. subtilis* QST 713) is a successful commercialized BCA product capable of suppressing the growth of numerous fungal pathogens and effectively controlling postharvest diseases, including gray mold in grapes and strawberries caused by the necrotrophic fungus *B. cinerea*, black rot in grapes caused by *Macrophoma flaccida* and brown rot in peaches caused by *M. fructicola* [19,24,33,35–37]. In our study, we investigated the effect of *B. amyloliquefaciens* QST 713 on the biological control of brown rot in peach fruits, the direct in vitro antagonistic activity of this BCA against *M. fructicola*, the defense-related physiological indices and the transcriptional responses of fruits during the confrontation of *B. amyloliquefaciens* QST 713 with *M. fructicola*. To the best of our knowledge, there is no evidence of transcriptomic changes occurring in peach fruits due to *B. amyloliquefaciens* QST 713's biocontrol activity.

Fruits pre-treated with *B. amyloliquefaciens* QST 713 (QST 713+MF treatment) significantly delayed the onset of brown rot symptoms, whereas only a downward expansion of fungal lesion at 48 HAI was evident, compared to treatment with *M. fructicola* (MF). Fruit ripening caused by pathogen infections could instantly affect plants' metabolism and cause severe oxidative damage, affecting cell membrane homeostasis [38]. As a result, following the MF treatment, a high accumulation of H_2O_2 was observed, coupled with high lipid membrane peroxidation levels, mainly at 12 HAI. Furthermore, peach fruits increased the production of total phenolics and flavonoids as a result of *M. fructicola* in-

oculation, consistent with a previous study [25]. However, upon QST 713+MF treatment, less accumulation of H_2O_2 was detected than was found in the MF treatment, leading to a lower lipid membrane peroxidation and damage to cell membranes, whereas significantly lower levels of phenolics were detected across all time points. Our results are in agreement with several studies highlighting the positive regulation of fruits' physiological metabolism by several BCAs of the *Bacillus* species [39–42].

Additionally, *B. amyloliquefaciens* QST 713 also inhibited the pathogen's mycelial growth in vitro through the formation of a clear inhibition zone, underlying the antagonistic activity of this BCA against *M. fructicola.* Our findings are in line with previous research in which QST 713 significantly suppressed *Monilinia* spp. in vitro and decreased the occurrence of peach brown rot in postharvest treatments [33,37,43]. It is well known that *B. amyloliquefaciens* QST 713 biosynthesizes and secretes a variety of secondary metabolites, such as the cyclic lipopeptides iturin A, fengycin A, fengycin B, surfactin, polyketides bacillaene and difficidin, as well as the antibiotics ericin A and ericin B [35]. These metabolites could determine the inhibitory efficacy of a competitive BCA due to their direct antimicrobial properties, their involvement in bacterial colonization on plant tissues or their stimulation of hosts' defense responses [32,44].

In parallel, the gene expression patterns of *M. fructicola* confirmed the severe damage that *B. amyloliquefaciens* QST 713 caused to fungal cell structure and pathogenicity by disrupting major metabolic pathways. Therefore, in contrast to the MF treatment, in the QST 713+MF, several fungal genes related to cell growth, ribosome biogenesis, cell-membrane homeostasis and spore germination were significantly suppressed or not expressed, mainly at the early time points, while the significant suppression of many *M. fructicola* genes related to plant cell-wall hydrolytic or carbohydrate-active enzyme (CAZymes) production was also revealed. According to the literature, iturin A and fengycin-like lipopeptides secreted by *B. amyloliquefaciens* BUZ-14 and *B. amyloliquefaciens* CPA-8, respectively, are considered the main lipopeptides responsible for the direct antifungal activity against *M. fructicola* and *M. laxa*, reducing peach brown rot symptoms in postharvest conditions [43,45]. Furthermore, the potent inhibition of *M. fructicola* growth under in vitro conditions by the purified lipopeptide surfactin biosynthesized from *B. velezensis* 1B-23 was previously reported [46], whereas the polyketides bacillaene [47] and difficidin [48], secreted by different *Bacillus* species, also showed high levels of antifungal activity against many pathogens. In addition to the above-mentioned compounds, *B. amyloliquefaciens* QST 713 also produces strong antifungal VOCs [49]. These metabolites directly affect fungal integrity and structure, causing hyphal deformity, spore perforation and the inhibition of sporulation, an irregular internal cell structure, cell membrane disruption, permeability and lysis, leading to the significant suppression of fungal growth and pathogenesis [12,50–53]. Thus, we suggest that the secretion of such a broad spectrum of antimicrobial compounds enhanced the biocontrol efficacy of QST 713 against *M. fructicola* and participated in the reduction in brown rot symptoms when formulated in postharvest conditions.

A transcriptomic analysis of peach fruits based on the two comparison groups (MF-CT and QST 713+MF-CT) that involve treatments with either only the *M. fructicola* (MF), or both the BCA and the pathogen (QST 713+MF) revealed a differential reprogramming that reflects the defense regulatory mechanisms employed in these interactions. Fruits' immune system was activated upon *M. fructicola* challenge, which is consistent with previous studies [25,54]. Notably, as a response to the early recognition of *M. fructicola* by plant recognition receptors (PRRs), the defense-related GO terms 'response to stimulus', 'response to stress' and 'defense response' were highly enriched at 12 HAI. Furthermore, based on the KEGG enrichment analysis, DEGs related to 'plant–pathogen interaction', 'MAPK signaling pathway', 'phenylpropanoid biosynthesis', 'plant hormone signal transduction', 'cutin, suberine and wax biosynthesis' and 'ABC transporters' pathways were highly induced, mostly at 24 HAI, highlighting the activation of multiple metabolic and signaling pathways upon exposure to the pathogen. Several DEGs associated with plant extracellular receptors, such as *STPK*, *CRK*, *G*- and *L*-type *LecRK*, *LRR-STK* and *WAK*, were highly induced in the

MF-CT group, which is consistent with previous findings [25]. As positive regulators of defense responses, several TFs genes encoding ZFPs, bHLHs, MYBs and WRKYs were upregulated at the early stages of *M. fructicola* infection, along with various defense- and disease-resistance related proteins. It is notable that the expression profiles of DEGs encoding the F-box protein were significantly high in the MF-CT group, mainly at 24 HAI. These proteins are related to ubiquitin-dependent catabolic processes, whereas ubiquitin, as a fundamental component of the ubiquitin pathway, determines the stress response in the activation of plant defense mechanisms [55]. Previously, high levels of F-box proteins were detected in mandarin fruits upon *Penicillium digitatum* challenge, resulting in the stimulation of stress responses in postharvest conditions [44]. However, in our study, at the latest time point in the MF-CT group, any defense-responses mainly collapsed, with the GO terms and KEGG enrichment analysis showing a weak defense response. Our results are consistent with a previous study, where peaches' responses to *M. fructicola* decreased 48 h after the infection [54]. Thus, the suppression of many DEGs related to cell-wall metabolic processes, such as those of *XTHs*, *CesAs*, *LRR-EXTs* and *DIRs* genes, may facilitate the further expansion of the pathogen at 48 HAI. Furthermore, the similar alterations in the expression profiles of many other defense-related DEGs involved in the biosynthesis of primary and secondary metabolites, pathogen perception and signaling transduction, such as those encoding TFs and PR proteins, confirmed the collapse of the peach immune system after a certain time point. Notably, a high number of fungal genes encoding for cellulose-, lignin- and pectin-degrading enzymes were highly induced in the MF-CT group, reaching the highest read counts at 48 HAI. This may further explain the weak induction of defense responses at the transcriptional level, in comparison to the early fruit responses observed shortly after *M. fructicola* inoculation.

A different expression pattern was observed on peach fruits in the QST 713+MF-CT comparison group. Based on our findings, the direct antifungal activity of *B. amyloliquefaciens* QST 713 substantially influenced the defense responses, mainly at the latest point of inoculation (48 HAI), as KEGG pathways relevant to plant defense responses were constitutively enriched, delaying pathogenesis. Furthermore, GO terms related to the activation of a defense, such as 'defense response' and 'response to stress', were constitutively enriched. Cell receptors (RLPs and RLKs) are able to recognize elicitors, such as microbe-associated molecular patterns (MAMPs) and pathogens-associated molecular patterns (PAMPs), leading to the induction of plant immunity [56,57]. Several *G*- and *L*-type *LecRK* genes were up- and downregulated, mainly at 12 HAI, indicating that PRRs constitute a complex system that is directly involved in the transduction of downstream responses and in the regulation of the immune signaling pathway [58]. It is known that MAMPs of the *Bacillus* species (peptidoglycans, lipopolysachharides, flagellins, VOCs and antimicrobial metabolites) activate the plant immune system during the postharvest suppression of pathogens, mainly by regulating host-generated hormones [32]. Based on our results, DEGs encoding members of WRKYs and MYBs were mainly upregulated at 12 HAI indicating their role in the defense-mediated role of the *B. amyloliquefaciens* QST 713 in the QST 713+MF-CT group. For example, *WRKY70*, *WRK13* and *ERF109* DEGs were significantly upregulated at 12 HAI following the BCA pre-treatment. According to the literature, both *WRKY70* and *WRKY13* regulate the induction of plants' immune systems against pathogens, suggesting their function as positive activators of defense responses, whereas *ERF109* stimulates plants' stress resistance by altering the signaling pathway [59–61]. However, *WRKY70* and *WRKY13* were only induced when *B. amyloliquefaciens* was present, which is consistent with a previous postharvest study on peach fruits, where *B. licheniformis* HG03 induced the expression of several *WRKYs* in the plant–pathogen interaction metabolic system [40]. We therefore speculate that both *M. fructicola* PAMPs, such as CWDEs, as well as *B. amyloliquefaciens* QST 713 MAMPs, contributed to the simultaneous activation of the peach immune system at 12 HAI. It is worth noting that, at the latest time point in the QST 713+MF-CT group, the upregulation of various members of TFs, along with a few DEGs encoding receptors involved in pathogen recognition and signaling transduction, occurred, in contrast to

the MF-CT group, suggesting a re-stimulation of the signaling transduction cascade and defense mechanisms following the addition of *B. amyloliquefaciens* QST 713.

5. Conclusions

In conclusion, the current research indicates that the *B. amyloliquefaciens* QST 713 biocontrol agent can effectively inhibit *M. fructicola* mycelial growth in vitro, and was able to reduce peach fruit disease symptoms, leading to high efficacy in the control of brown rot. Following the pathogen challenge, a transcriptome analysis of fruits that were pre-treated with this biocontrol agent showed a significant upregulation of particular DEGs at 48 HAI. This included genes related to the activation of different transcriptional factors, such as members of the WRKY and NAC families, and receptors involved in the recognition of pathogen and signaling cascades, including *LRR-RLKs*. The biocontrol activity of this BCA was also assessed using particular defense-related physiological indices. Furthermore, by examining the expression patterns of fungal genes during the interaction between this biocontrol agent and *M. fructicola* on peach fruits, the direct antimicrobial activity of QST 713 was confirmed. Overall, our results show that the commercial formulation of *B. amyloliquefaciens* QST 713 has a direct antagonistic impact on *M. fructicola*, and was effective in triggering, to some extent, defense-related responses in peach fruits upon fungal challenge at a late stage of infection.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14040771/s1, Figure S1: Gene Ontology-based functional enrichment and categorization of the most representative differentially expressed genes (DEGs) of the MF-CT and QST 713+MF-CT comparison groups; Figure S2: Correlation of RNA-seq and RT-qPCR data after *M. fructicola* inoculation on peach fruits at 24 HAI; Table S1: Overview of the RNA-seq data; Table S2: Differentially expressed genes (DEGs) expression profiles on peach fruits across the two comparison groups (MF–CT, QST 713+MF–CT) at 12, 24 and 48 HAI; Table S3: Differentially expressed genes (DEGs) related to cell wall degradation and modification; Table S4: Differentially expressed genes (DEGs) related to pathogen recognition and signaling; Table S5: Differentially expressed genes (DEGs) encoding transcriptional factors; Table S6: Differentially expressed genes (DEGs) associated with pathogenesis and defense-related proteins; Table S7: Differentially expressed genes (DEGs) related to metabolism (secondary and primary); Table S8: Differentially expressed genes (DEGs) related to transporters; Table S9: *M. fructicola*-expressed genes in the MF-CT and QST 713+MF-CT comparison groups; Table S10: Primers used in RT-qPCR.

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