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Role of Biofilm Formation by *Bacillus pumilus* HR10 in Biocontrol against Pine Seedling Damping-Off Disease Caused by *Rhizoctonia solani*

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Abstract: The biocontrol process mediated by plant growth-promoting rhizobacteria (PGPR) relies on multiple mechanisms. Biofilm formation plays an important role in the ability of PGPR to control plant diseases. *Bacillus pumilus* HR10, one such PGPR, promotes the growth of *Pinus thunbergii*. This study showed that the wild-type strain *B. pumilus* HR10 produces a stable and mature biofilm in vitro. Biofilm-deficient mutants of *B. pumilus* HR10 with different phenotypes were screened by mutagenesis. The contents of extracellular polysaccharides (EPS) and proteins produced by the mutant strains were significantly reduced, and the biofilms of the mutants were weakened to varying degrees. The swarming abilities of the wild-type and mutant strains were positively correlated with biofilm formation. A colonization assay demonstrated that *B. pumilus* HR10 could colonize the roots of *Pinus massoniana* seedlings in a large population and persist, while biofilm-deficient mutants showed weak colonization ability. Furthermore, a biocontrol assay showed that biocontrol efficacy of the mutants was reduced to a certain degree. We determined the inhibitory activity of *B. pumilus* HR10 and its ability to induce systemic resistance against *Rhizoctonia solani* of plants. The synthesis of lipopeptide antibiotics is probably involved in biofilm formation by *B. pumilus* HR10. These observations not only provide a reference for further research about the coordinated action between biofilm formation and the multiple biocontrol mechanisms of *B. pumilus* HR10 but also improve the understanding of the regulatory pathway of biofilm formation by *B. pumilus* HR10.

Keywords: *Bacillus pumilus* HR10; biofilm formation; root colonization; pine seedling damping-off disease; biocontrol

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) have been reported to exhibit biocontrol processes including competition, antibiosis, growth promotion, and the induction of systemic resistance in plants [1–6]. The *Bacillus* genus is one of the most frequently reported and promising PGPR with various biocontrol mechanisms. The nematicidal activity of the guanidine compound from *B. pumilus* LYMC-3 and its high potential in the biocontrol of *Bursaphelenchus xylophilus* was demonstrated [7]. *Bacillus velezensis* FZB42, a model strain for excellent plant growth promoting and biocontrol rhizobacteria, has been studied on its genes and expression involved in biocontrol process and the bacteria-plant interaction [8–10].

Due to colonizing the root of plants persistently, PGPR can effectively promote the growth of plants and inhibit the pathogens [10–12]. Persistent colonization of PGPR on roots is essential for suppressing different plant diseases, and biofilm formation is considered a key factor for efficient root

colonization [13,14]. Biofilm formation is regulated by aggregation and quorum sensing, which influences wide activities of the bacteria [15]. Therefore, exploring the distribution, expansion and colonization of bacterial biofilm in the plant rhizosphere is an important aspect to clarify the biocontrol mechanisms of PGPR.

Bacterial biofilms are formed by multicellular communities that are embedded in a self-produced matrix of extracellular substances such as extracellular polysaccharide, protein, and nucleic acids, which enhance the resistance of bacteria to adverse environments and facilitate bacterial interactions with plants [15–21]. Most cells in multilayered biofilm experience contact between cells and then aggregates formed, either in surface-attached biofilms or in flocs. The biofilm phenotypes were often observed on the solid-gas or liquid-gas surface [15,22]. Swarming motility in bacteria is a social behavior that enables rapid movement and microbial biofilm development, allowing bacteria to colonize nutrient-rich environments [4,20]. It has been reported that nonribosomally synthesized antibiotics, especially surfactin, play a significant role in suppressing diseases and are involved in regulating the process of biofilm formation [20,21]. However, the role of biofilm by *B. pumilus* involved in biocontrol efficacy against plant diseases is rarely reported.

Bacillus pumilus HR10 was isolated from the rhizosphere of *Pinus thunbergii*. The strain was found to significantly promote mycorrhizal symbionts and the growth of *P. thunbergii* [23]. In vitro testing, *B. pumilus* HR10 showed excellent inhibitory activity against *Rhizoctonia solani*, causing pine seedling damping-off disease, implying that *B. pumilus* HR10 has excellent biocontrol potential [24]. *Pinus massoniana* was chosen for the study because it is one of the most popular and native tree species in southern China. However, varieties of pathogens and insect pests' diseases occurred during the afforestation of *P. massoniana* including seedling damping-off disease. The occurrence of the disease caused the death of a large number of *P. massoniana* seedlings, which seriously threatened the sustainable development of the *P. massoniana* seedlings forestry [25,26]. Currently, the role of biofilm formation by *B. pumilus* HR10 in inhibiting pathogens is not understood, restricting further studies on the biocontrol mechanism of *B. pumilus* HR10. We propose that the biocontrol efficacy of *B. pumilus* HR10 depends on the coordinated action of inhibitory activity, induced systemic resistance and excellent colonization. Under this hypothesis, we mutated *B. pumilus* HR10 and screened biofilm-deficient mutants. Then, we compared the biofilm-related properties and gene expression, and root colonization and activity against *R. solani* of the strains.

2. Materials and Methods

2.1. Strains and Culture Conditions

Bacillus pumilus HR10 was isolated from the rhizosphere of *Pinus thunbergii*, and now it is preserved in the China Center for Type Culture Collection (CCTCC, NO: NO.M2010143). It was routinely grown at 30 °C in Luria-Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L). The pathogenic fungus of pine seedling damping-off disease, *Rhizoctonia solani* was grown in potato dextrose (PDA) medium at 25 °C.

2.2. Mutagenesis of B. pumilus HR10 and Screening of Biofilm-Deficient Mutants

The *B. pumilus* HR10 wild-type strain was treated with 0.4 mg/mL *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), and incubated in the dark for 30 min. After diluting to the appropriate concentration, the suspension was placed on an LB plate. The mutants were screened for different biofilm defects [27,28].

2.3. Colony Morphology, Growth Curve and Spore Formation

The colony morphologies of the wild-type strain and mutants were observed with a Carl Zeiss stereomicroscope (SteREO Discovery V.20, Germany). The growth curves were measured at 600 nm with a microbial growth curve tester. The suspensions (OD_{600} = 1.0) of the wild-type strain and mutants

were transferred into new media and cultured at 30 °C with shaking at 200 rpm. After cultivating for 12, 18, 24, 48, 66 and 72 h, 100 μL of culture of each strain was diluted with sterilized water to the appropriate concentration to measure the number of live bacteria. The sterile tubes containing 100 μL of each culture were incubated at 85 °C for 10 min, and the number of spores were measured. Moreover, the growth curves of cells of 4 strains were also detected by the automatic growth curve analyzer for microorganisms (Bioscreen M, Shanghai, China).

2.4. Analysis of Biofilm Formation and Swarming Motility

The biofilm formation assay of strains was carried out following a previously described method with few modifications [29]. For quantitative analysis of biofilm formation, 10 μL of liquid culture (OD_{600} = 1.0) of each strain was inoculated into a well of a 24-well microliter plate containing 1 mL of LB and was incubated without agitation at 30 °C for 96 h. The medium was carefully removed, and the wells were washed with sterilized water. Crystal violet solution (1%) was used to stain the biofilm for 15 min, and the excess stain was rinsed off with water. Pure ethanol was used to extract the dye bound to the biofilm structure, and the absorbance at 570 nm was visualized with a Multiskan Spectrum microplate reader (Thermo, Waltham, MA, USA). The biofilm formation assays were conducted 6 times.

Swarming motility assays of wild-type *B. pumilus* HR10 and biofilm mutants were performed according to Chen et al. [29,30]. One milliliter of fresh culture of each strain (OD_{600} = 0.5) was collected by centrifugation at 6000 rpm for 5 min, washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4), and resuspended in 100 μL of PBS. Subsequently, 3 μL of each culture was separately spotted onto the center of the swarming motility plates containing 0.7% agar that were dried for 20 min. The plates were placed upside down in a 30 °C incubator for 15 h. The swarming motilities of cells were observed and took pictures to record every 5 h.

2.5. Quantitative Estimation of Biofilm Polysaccharides

Standard curve of the glucose: the glucose (1 mL) concentrations were set as 0, 40, 80, 120, 160 and 200 μg/mL. Anthrone (0.2 g) was dissolved in 100 mL of sulfuric acid, and then 2 mL of liquid was slowly added into the glucose solutions. The tubes were incubated in boiling water for 10 min and then in ice-cold water for 10 min. Subsequently, the solutions were placed at room temperature for 2 min, and the absorbances were measured at 620 nm with a Multiskan Spectrum microplate reader.

Biofilm polysaccharide extraction and measurement: the cultures of *B. pumilus* HR10 wild strain and biofilm-deficient mutants were harvested at OD_{600} = 1.0. For quantitative analysis of biofilm polysaccharides, 10 μL of liquid culture of each strain was used to inoculate into the well with 1 mL LB of 24-well microliter plates and was incubated without agitation at 30 °C for 48 h. The medium was carefully removed, and the wells were washed with sterilized water. Three milliliters of ethanol were added into each well. The biofilm polysaccharides were precipitated at 4 °C overnight. Subsequently, biofilm polysaccharides were collected by centrifugation (10,000 rpm, 4 °C, 10 min). The polysaccharide pellet was washed three times with ice-cold 75% ethanol and centrifuged. Similarly, two washing steps with distilled water were performed. The biofilm polysaccharides of the wild-type strain and mutants were dissolved, and the absorbances were measured as described above [8,31].

2.6. Quantitative Estimation of Biofilm Proteins

Standard curve of bovine serum albumin (BSA): the BSA solution (1 mL) concentrations were set as 0, 20, 40, 60, 80 and 100 μg/mL. Coomassie brilliant blue G250 (0.1 g) was dissolved in 1000 mL of distilled water containing 50 mL of 95% ethanol and 100 mL of 85% phosphoric acid. The BSA solution was stained with the dye liquid (5 mL) respectively for 5 min and then the absorbance at 595 nm was measured with a Multiskan Spectrum microplate reader.

Biofilm protein extraction and measurement: the biofilms of wild-type (WT) *B. pumilus* HR10 and biofilm-deficient mutants were harvested as described above. Ammonium sulfate was added to the supernatant liquid to a final concentration of 70%. The biofilm proteins were precipitated at

4 °C overnight. Subsequently, extracellular proteins were collected by centrifugation (10,000 rpm, 4 °C, 15 min). The protein pellet was washed three times with ice-cold 50% ethanol and centrifuged. Similarly, two washing steps with distilled water were performed. The extracellular proteins obtained from WT and mutants were dissolved, and the absorbances were measured as described above.

2.7. qRT-PCR Assay of Biofilm-Related Gene Expression

The biofilms of WT and biofilm mutants were collected after 12 h, 24 h, 48 h and 72 h of incubation. The biofilms were collected by centrifugation and ground into powder with liquid nitrogen. Then, 1 mL of TRIzol (Invitrogen, Waltham, MA, USA) was added to the powder (100 mg) of each sample. The 1.5 mL tubes with powder were shaken and the samples were mixed and then incubated for 5 min at 25 °C. The solution was extracted with 200 µL of trichloromethane (99%), mixed and centrifugated. The supernatant was collected and washed 2 times with 75% ethanol. The extracted RNA was dried and dissolved with RNase-Free H_2O. The quantity and purity of isolated RNA were measured by ultraviolet absorbance NanoDrop 2000C at A260/280 (Thermo Scientific, Waltham, MA, USA) and assessed by electrophoresis on a 1% agarose gel. The first-strand cDNA was synthesized from 1 µg total RNA using the HiScript IIqRT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China), which could completely remove the residual genomic DNA in the RNA template.qRT-PCR was performed using a HiScript IIqRT reagent kit (Vazyme, Nanjing, China). The cDNA samples were diluted to 90 ng/µL. A solution of cDNA was prepared with 2 × ChamQ SYBR qPCR Master Mix. The reaction system (20 µL) included 10 µL of SYBR qPCR Master Mix (2×), 0.4 µL of forward primer (10 µM), 0.4 µL of reverse primer (10 µM), 2 µL of cDNA sample, and 7.2 µL of ddH_2O. Thermal conditions were as follows: 30 s at 95 °C for initial denaturation and 40 cycles of 10 s at 95 °C, followed by 34 s at 60 °C. The relative expression levels of *epsA* (extracellular polysaccharide-related gene), *tasA* (extracellular protein-related gene), *fliG* (flagellum motility-related gene), and *sfp* (surfactin synthesis-related gene) were measured. The *B. pumilus gyrB* gene was used as an internal reference. Experiments were performed in triplicate with three biological replicates on an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). The relative gene expression levels were analyzed using ABI Prism 7500 software and the $2^{-\Delta\Delta Ct}$ method. The primers used to amplify these genes are listed in Table 1.

Table 1. The qRT-PCR primer pairs of biofilm-related genes.

Primer [a]	Sequence (5′-3′)
epsA-qF	GCCACAAGTACAGAAAATGAGTC
epsA-qR	GATGTGAACGAATTTACGTGAGT
tasA-qF	GAATTCCTCAGCCAATTTCAG
tasA-qR	GGTGGCAACATTAATTTTACCA
fliG-qF	CAGCATGAGCATCCACAAAC
fliG-qR	GGTATAATCTTGCGTAAAGGTTG
sfp-qF	GAGAATATCACCGGAATTGAAAA
sfp-qR	GCTTTCCTTCCAGCCATAGC
gyrB-qF	GAGGGAGTCGGTAATGGTTCTT
gyrB-qR	CGAAGCTGGCTTTAAAACCG

[a] The primer pairs of *epsA*, *tasA* and *fliG* refer to the genes in the NCBI database, i.e., *epsA* (WP_007500132.1), *tasA* (WP_008342195.1), and *fliG* (WP_008344808.1) respectively.

2.8. Antifungal Activity Assay

R. solani plate was grown on PDA plates for the assay. The antifungal activity of the fermentation liquid of *B. pumilus* HR10 culture against *R. solani* was also measured. The cultures of *B. pumilus* HR10 WT and mutants were harvested to OD_{600} = 1.0 in 20 mL LB respectively. Subsequently, fermentation liquid was collected by centrifugation (10,000 rpm, 10 min) and the further removing cells with a 0.22 µm filter. One milliliter of the fermentation culture of WT *B. pumilus* HR10 and mutants

was added into a melted PDA plate respectively. The plates were incubated and observed, and the colony diameter for each treatment was recorded [6].

2.9. Cultivation of P. massoniana Seedlings

The 300 seeds of *P. massoniana* were surface-sterilized with 30% H_2O_2, after which they were placed in water agar medium with 1% agar. After growing to the cotyledon periods, 100 seedlings were grown in sterilized soil matrix for the root colonization assay. The remaining 200 seeds of *P. massoniana* were for reduced-plant-resistance assay and biocontrol assay against pine seedling damping-off disease.

2.10. Root Colonization Assay

The assay of root colonization was carried out following a described method with few modifications [32]. The overnight-grown bacterial cultures (OD_{600} = 1.0) of wild-type *B. pumilus* HR10 and biofilm mutants were collected by centrifugation at 6000 rpm for 5 min and diluted to 1×10^8 CFU/mL with PBS for use. *P. massoniana* seeds were soaked in water for 18 h. The seeds were disinfected with 30% H_2O_2, washed with sterilized water and sown on medium with 1% agar. The 10-day-old seedlings were then transplanted into sterilized bottles containing sterile soil matrix. Forty-day-old seedlings grown in sterilized bottles were inoculated with five milliliters of diluted culture. Subsequently, 1 cm of root below the base of the stem was collected at 2, 4, 6, 8, 10 and 12 dpi. The roots were gently shaken in sterilized water. Crystal violet solution (1%) was used to stain the roots for 15 min, and the excess stain was rinsed off with sterilized water. The 5 mL of pure ethanol was used to extract the dye bound to the roots, and the absorbance at 570 nm was visualized with a Multiskan Spectrum microplate reader. Simultaneously, other gently shaken roots were placed into the tube with 5 mL sterilized water. Afterwards, the tubes were prepared with shaking at 200 rpm for 20 min, and 100 µL liquid was collected, diluted, and coated on an LB plate. The number of colonies was counted two days later. Subsequently, the roots with a length of 1 cm were taken at the time with most colonies. After drying with a critical point dryer (K850, Laughton, East Sussex, UK), colonization of the HR10 WT strain and mutants on the surface of pine roots was observed under a scanning electron microscope (Quanta 200, Hillsboro, OR, USA). Each treatment contains 3 seedlings in the assays of colony counting, crystal violet staining, and SEM observation.

2.11. Assay of Plant Defense Gene Expression

P. massoniana seeds were pregerminated as described above and sown in a sterilized soil matrix. Fifteen of the fifty-day-old *P. massoniana* seedlings grown in plastic holes containing sterilized soil matrix were inoculated with two milliliters of *B. pumilus* HR10 and mutant cultures diluted to 10^8 CFU/mL with sterilized water. After 4 days, the seedlings were carefully removed, flash-frozen in liquid nitrogen separately and ground. RNA extraction was performed using the Fast Plant RNA Kit for Polysaccharides and Polyphenolics-Rlch (Zomanbio, Beijing, China). The measurement of RNA quantity, removing gDNA, synthesis of cDNA, and qRT-PCR were conducted as described above (2.7). The relative expression levels of *PR2*, *PAL* (regulation of salicylic acid-related gene), *COI* (cytochrome oxidase-related gene), and *GPX* (stress tolerance-related gene) were measured. The *P. massoniana* splicing factor *U2af* gene was used as an internal actin reference. Experiments were performed in triplicate with three biological replicates on an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). The relative gene expression levels were analyzed using ABI Prism 7500 software and the $2^{-\Delta\Delta Ct}$ method. The primers used to amplify these genes are listed in Table 2.

Table 2. The qRT-PCR primer pairs of *P. massoniana* defense resistance-related genes.

Primer [b.]	Sequence (5′-3′)
PR2-qF	CGACAACATTCGCCCCTTCT
PR2-qR	CTGCAGCGCGGTTTGAATAT
PAL-qF	CCCCTGCTCGGTTTTGCTCTG
PAL-qR	TGGCCTGCGTCATTTCTGCTG
COI-qF	GCACCTGACATGGCATTTCC
COI-qR	GATAGACCGTCCACCCAGTGC
GPX-qF	TTCACAGTCAAGGACATCAGGGGTAA
GPX-qR	CACGGGAAAGCCAGTATTTCTAAC
U2af-qF	TCGGGAGGTTGGGTCTACAT
U2af-qR	ACCAGTCCTTCAGTCCCCTT

[b] The primer pairs refer to the genes *PR2* [33], *PAL* [34], *COI* (EU701146.1), *GPX* [35] and *U2af* [36] respectively.

2.12. Biocontrol Assay

P. massoniana seedlings were cultured as described in Section 2.11 and inoculated with two milliliters of solution (10^8 CFU/mL) of *B. pumilus* HR10 and mutants dissolved with sterilized water. Four days later, each seedling was inoculated with *R. solani* in the soil at the same time of sampling in Section 2.11 [5]. One week after inoculation with the pathogen, disease severity and biocontrol efficacy were recorded and calculated according to the following formula:

$$\text{Disease incidence (\%)} = (\text{the number of damping-off diseased seedlings in each treatment/ total number of seedlings in each treatment}) \times 100.$$

$$\text{Biocontrol efficacy (\%)} = ((\text{disease incidence of untreated seedlings-disease incidence of treated seedlings})/\text{disease incidence of untreated seedlings}) \times 100.$$

Each treatment contained 16 *P. massoniana* seedlings.

2.13. Statistical Analyses

One-way analysis of variance (ANOVA) was carried out with SPSS software, and different letters indicate significant differences ($p < 0.05$).

3. Results

3.1. Colony Morphology and Growth Curve of Cells and Spores

By observing the colony morphology, three biofilm-deficient mutants of *B. pumilus* HR10 wild-type were obtained. The results are shown in Figure 1A. The three-dimensional structure of the wild-type strain was the most obvious and ductile. The structure of the MA15 mutant was relatively the simplest. The structure of the MA1330 mutant had a slight depression in the middle and poor ductility. The structure of the MA4828 mutant was simpler than that of the wild-type strain and more complicated than that of the other two mutants. These results showed that the mutants had different degrees of deficiency in colony structure.

As shown in Figure 1B, the growth in broth cultures of the mutant cell culture showed no significant changes compared with that of wild-type strain. To better understand the growth curves of *B. pumilus* HR10 and biofilm mutants, we detected and compared the quantity of cells and spores. The test results indicated that the mutations had no significant effects on the growth curves of cells and spores of mutants (Figure 1C).

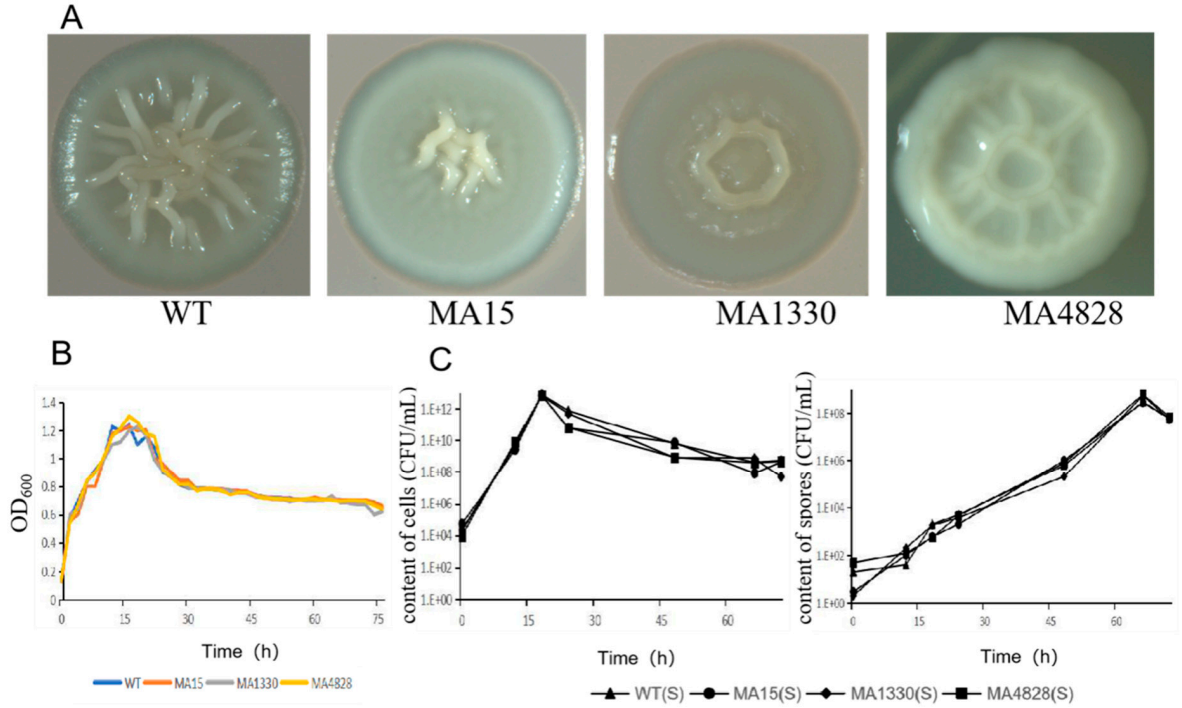

Figure 1. The colony morphology and growth curve of cells and spores. (**A**) The colony morphology of *B. pumilus* HR10 and biofilm-deficient mutants on LB agar. (**B**) The absorbances of four strains at 600 nm over 72 h. (**C**) The contents of cells (left) and spores (right) of four strains were not significantly different at each testing time.

3.2. *The Biofilm Substance Contents and Swarming Motility of the Biofilm-Deficient Mutants Declined*

After incubation for 12 h, 24 h, 48 h, and 72 h, *B. pumilus* HR10 formed wrinkly floating pellicles at the liquid–air interface of liquid cultures. In contrast, the mutants formed thinner pellicles in LB (Figure 2A). All of the strains reached peak biofilm formation at 48 h, and MA15 formed the weakest biofilm. The biofilm of MA1330 degraded at the fastest rate in the later stages. Among the mutants, MA4828 had a relatively stable ability to form biofilm, and the degradation rate of MA4828 was relatively slow (Figure 2A). After staining the biofilm formed by *B. pumilus* HR10 and mutants with crystal violet dye, the absorbances at 570 nm were quantitatively measured. These results indicate that the abilities of *B. pumilus* HR10 and the biofilm mutants to form biofilms at the liquid–gas interface are consistent with the changes in colony morphology at the solid–gas surface. The more complex the fold on the solid–gas surface is, the denser and more complete the biofilm structure on the liquid–gas surface becomes.

Biofilm polysaccharides are the key components of the three-dimensional structure of biofilms. Extracellular proteins help to maintain the hydrophobicity and surface tension of biofilms [20]. The polysaccharide contents of the mutants were significantly less than that of the wild-type strain, indicating that polysaccharides are an indispensable component for biofilm formation by *B. pumilus* HR10 (Figure 2B). The biofilm protein content of MA15 was significantly less than that of the wild-type strain, while the contents of MA1330 and MA4828 were almost the same as that of the wild-type strain.

Swarming motility has a certain effect on the social movement of bacteria and the speed of biofilm formation [15,37]. To analyze swarming motility, cell suspensions were spotted at the center of the swarming plates, and the expansion of cells was photographed (Figure 2C). As shown, the wild-type strain showed excellent swarming motility and the plate was nearly fully covered with the swarming cells. The biofilm mutants exhibited a markedly weakened swarming motility compared to the wild-type strain. Based on the above results, it is indicated that the weakened swarming motility of the mutants restricted the adhesion of bacteria and biofilm formation.

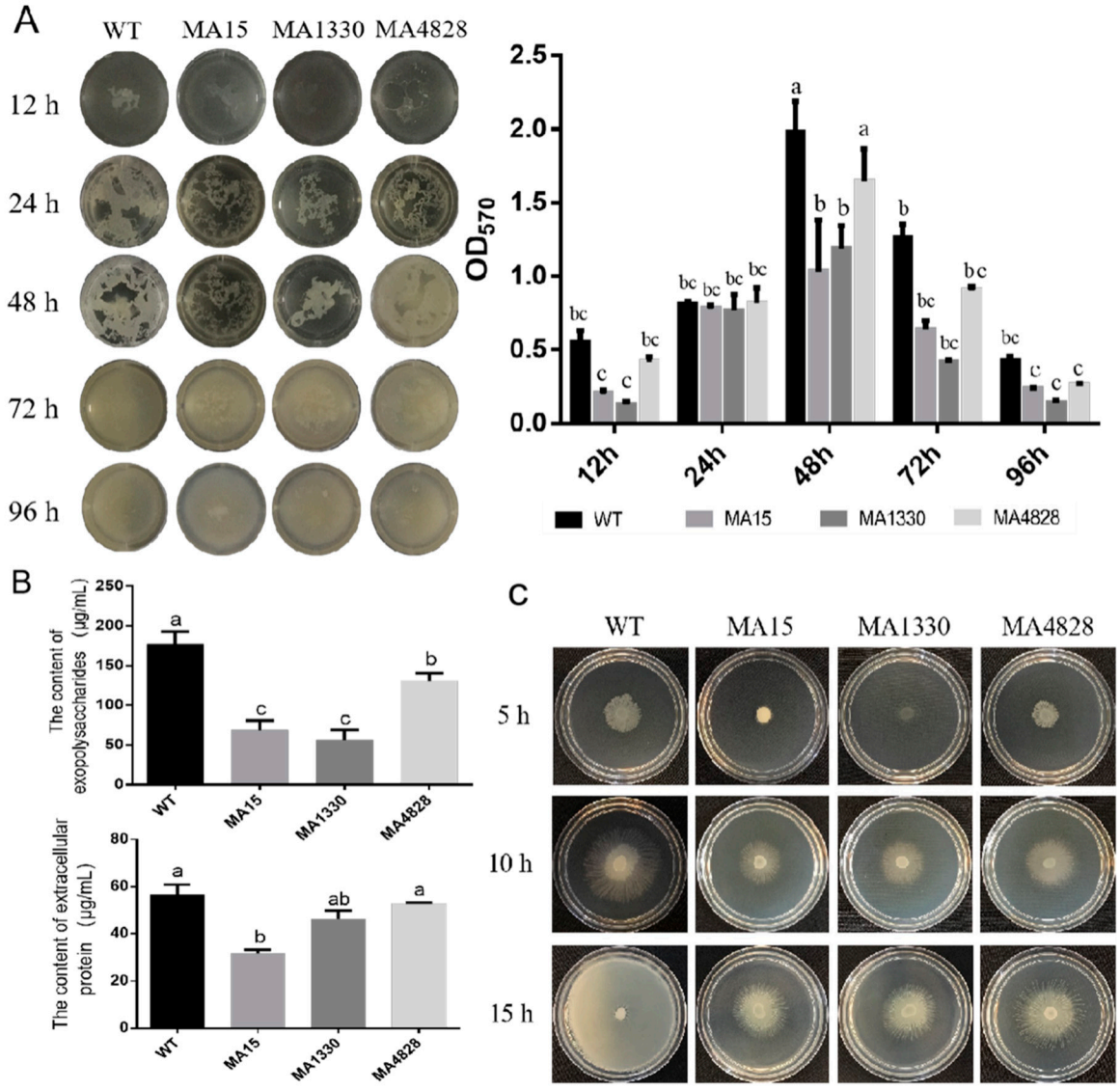

Figure 2. The biofilm-related features are significantly different between wild-type (WT) and mutants. (**A**) The biofilm architecture at the liquid–gas interface and the absorbance at 570 nm. (**B**) The contents of extracellular substances in LB medium at 48 h. (**C**) The swarming motility of cells of WT and mutants. Different letters above the bars indicate significant differences ($p < 0.05$).

3.3. Biofilm-Related Genes of HR10 Wild-Type and Mutants Showed Different Expression Levels

To further examine the effects of extracellular substances and swarming motility on biofilm formation, the transcription levels of biofilm-related genes were evaluated after incubation for 12 h, 24 h, 48 h, and 72 h. The *epsA* gene, which is a transcriptional regulator of EPS synthesis, of the four strains increased to the highest value at 48 h post-incubation (Figure 3A). The expression of the *epsA* gene in MA4828 was maintained at a high level compared to that in MA15 and MA1330. No significant changes in the *tasA* expression in the mutants were measured at different times, except for the difference in MA15 at 12 h and 24 h post-incubation (Figure 3B). The expression levels of *fliG* (movement-related gene) and *sfp* (surfactin synthesis-related gene) were maintained at the highest levels at 24 h, 48 h, and 72 h in WT, respectively, but were the lowest levels in MA15 (Figure 3C,D).

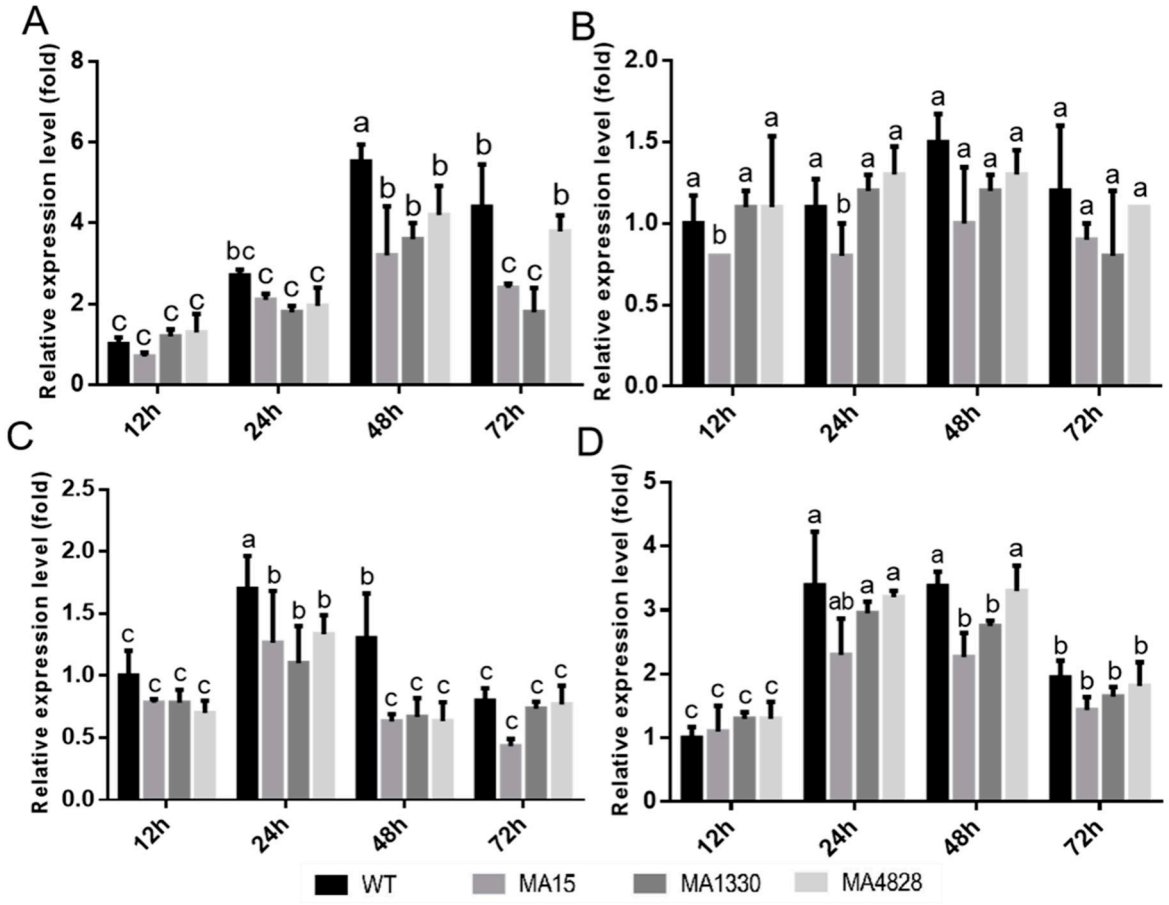

Figure 3. The biofilm-related gene expression of WT and mutants at 12 h, 24 h, 48 h, and 72 h. (**A**) The expression level of *epsA* involved in the synthesis of EPS; the main component of biofilm. (**B**) The expression level of *tasA* involved in the synthesis of extracellular protein; another vital component of biofilm. (**C**) The expression level of *fliG* involved in the movement of flagella, which contributes to swarming motility and biofilm formation. (**D**) The expression level of *sfp* involved in the synthesis of surfactin, which is related to inhibition against pathogen and biofilm formation. The expression of WT at 12 h was used as a control. Experiments were performed in triplicate with three biological replicates Different letters above the bars indicate significant differences ($p < 0.05$).

3.4. Deficiency in Biofilm Formation Reduced Root Colonization on P. massoniana Seedlings

To investigate the root colonization on *P. massoniana* seedlings of strains, the population dynamics of WT and mutants on roots were evaluated over time. The results showed that the colonization trends of strains were consistent with their biofilm formation. The population of WT was higher at each testing time point, where the highest level was reached at 10 days post inoculation (DPI). In contrast, the populations of the mutants, especially MA15 and MA1330 at 6–12 dpi, were obviously lower than that of WT. The quantitative results of crystal violet dye staining (Figure 4B,C) also showed that the absorbances of WT treatment were the highest at 4–12 dpi, while those of MA15 treatment were significantly reduced. Similar results were obtained in the SEM observations. In comparison with WT, the mutants showed different levels of reduced colonization on the roots of *P. massoniana*. In general, the deficient biofilm formation by *B. pumilus* HR10 leads to decreased colonization of the roots of *P. massoniana* seedlings.

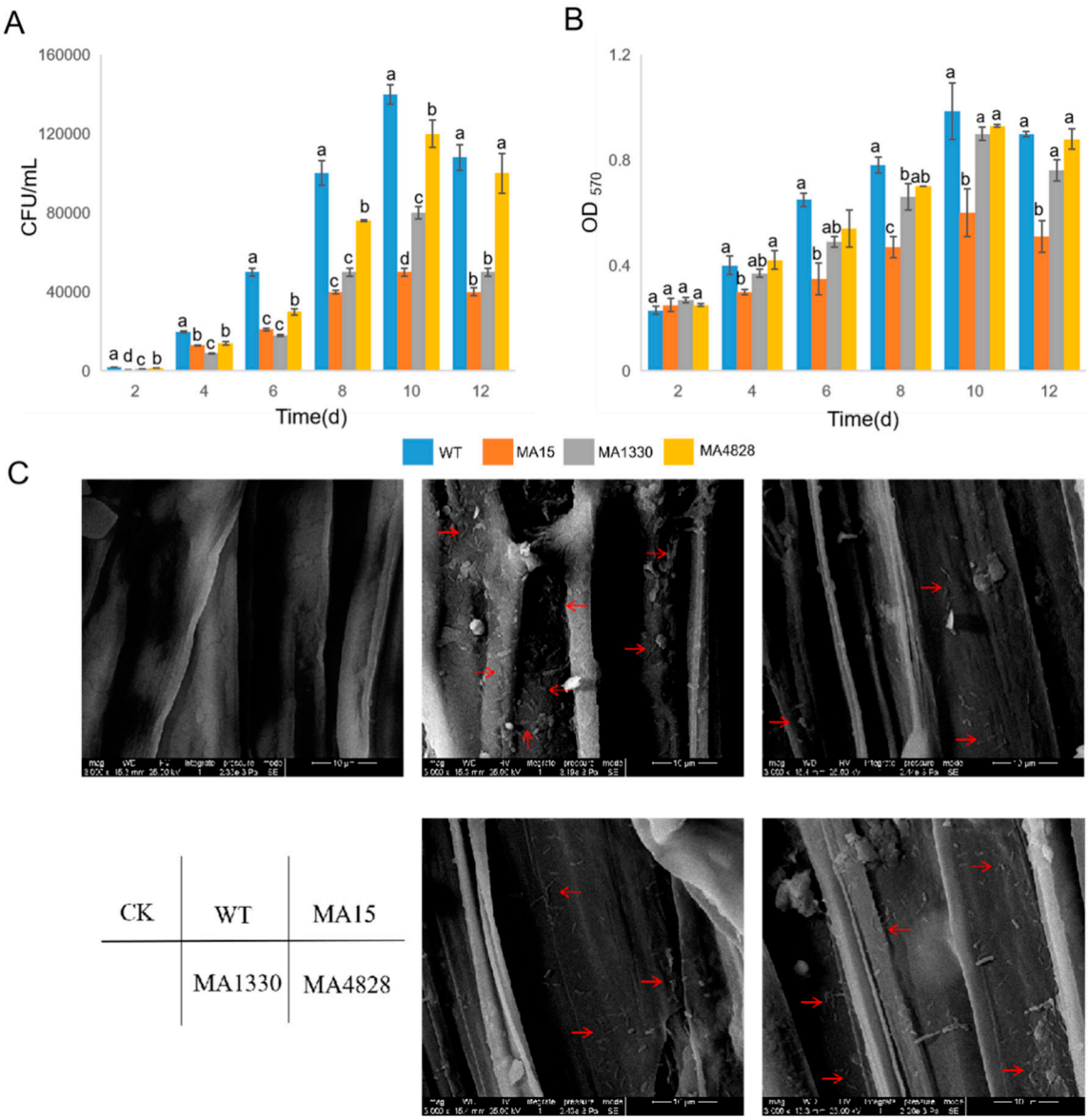

Figure 4. The colonization of *B. pumilus* HR10 WT and mutants on the roots of *P. massoniana* seedlings. (**A**) The population of cells attached to the root. (**B**) The quantitative absorbance of crystal violet staining assay of the root. (**C**) SEM micrographs of colonized seedling roots. Different letters above the bars indicate significant differences ($p < 0.05$).

3.5. Mutants Exhibited Different Inhibitory Activities against R. Solani

A test of the inhibition activity of the fermentation broth of the strains against *R. solani* was performed. The treatments with additional fermentation broth significantly inhibited the growth of *R. solani* in comparison with the control group. Moreover, the MA15 treatment exhibited weaker inhibitory activity than the other treatments (Figure 5A,B).

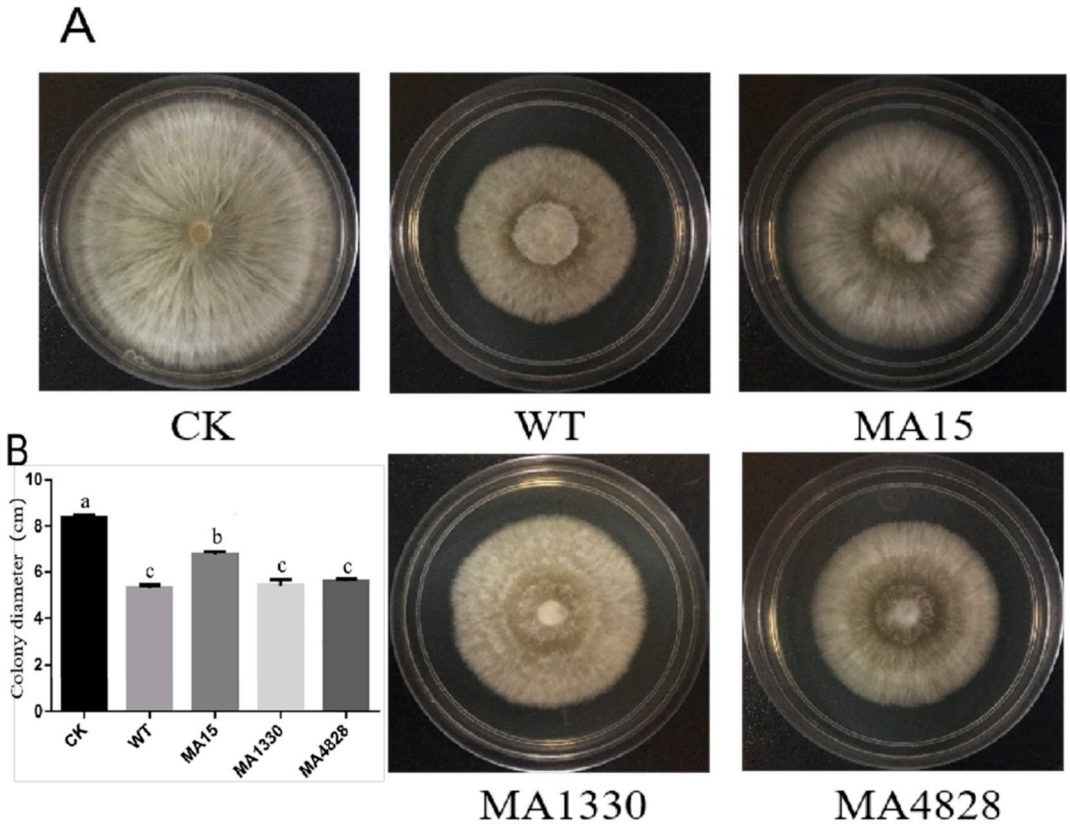

Figure 5. Inhibitory activity of fermentation liquid of WT *B. pumilus* HR10 and mutants against *R. solani*. The observation (**A**) and measurement (**B**) of the inhibition zones of fermentation liquid by 4 strains against *R. solani*. Error bars indicate the standard deviations from the results from three independent experiments. Different letters above the bars indicate significant differences ($p < 0.05$).

3.6. Biofilm Formation Contributes to Inducing the Systemic Resistance of Plants

To investigate whether the strains induce plant systemic resistance, the transcription levels of *PR2*, *PAL*, *COI*, and *GPX* were measured and evaluated. The seedlings not inoculated with bacteria served as control (CK) plants. The seedlings inoculated with WT *B. pumilus* HR10 or biofilm-deficient mutants exhibited high expression levels of *PR2* (4.2-fold, 1.8-fold, 2.5-fold and 1.9-fold compared to the expression of *PR2* in the CK, respectively), while the expression of *PAL*, *COI*, and *GPX* showed no significant differences (Figure 6). The results indicate that inoculation with WT *B. pumilus* HR10 or mutants contributes to inducing *P. massoniana* resistance to some extent.

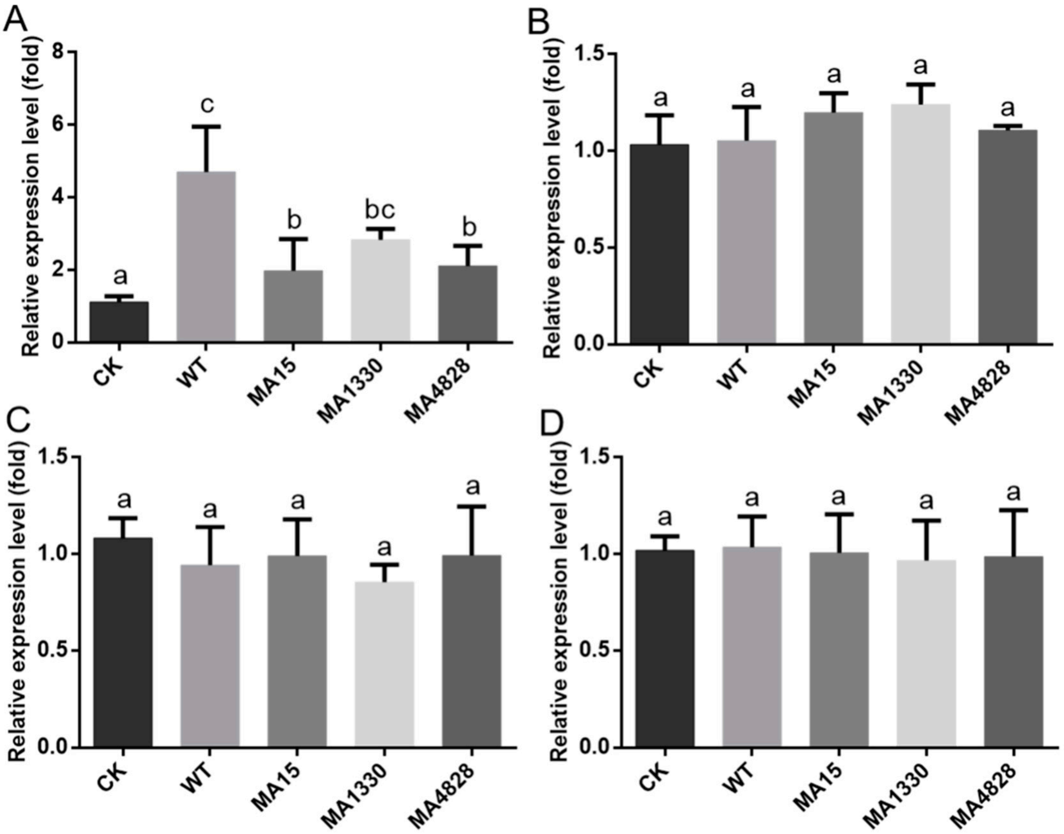

Figure 6. Transcription levels of defense-related genes in *P. massoniana* seedlings in response to WT or mutant inoculation. The related expression levels of *PR2* (**A**), *PAL* (**B**), *COI* (**C**), and *GPX* (**D**) in seedlings 4 days after WT or mutant inoculation. Experiments were performed in triplicate with three biological replicates. Different letters above the bars indicate significant differences ($p < 0.05$).

3.7. Biofilm Formation and Inhibitory Activity Are Both Required for Biocontrol against Pine Seedling Damping-Off Disease

We observed that the disease incidence of seedlings in the CK with *R. solani* reached 81.3%, and the treatment exhibited a decreasing trend. The treatment with the wild-type strain decreased disease incidence to 18.8% with excellent biocontrol efficacy. The disease incidence following MA1330 and MA4828 inoculation was 31.25%, while that of MA15 inoculation was lower (Table 3). In brief, the biofilm-deficient mutants are inefficient in the biocontrol of pine seedling damping-off disease.

Table 3. Biocontrol efficacy of WT *B. pumilus* HR10 and biofilm-deficient mutants against pine seedlings damping-off disease caused by *R. solani* under greenhouse condition. Different letters within a column indicate significant differences ($p < 0.05$). There are 16 seedlings per treatment.

Treatment	Disease Incidence (%)	Biocontrol Efficacy (%)
Blank	0	–
CK + *Rsl*	81.3 ± 1.02 a	–
WT + *Rsl*	18.8 ± 1.52 d	76.88 ± 1.42 a
MA15 + *Rsl*	43.75 ± 1.37 b	46.19 ± 1.28 c
MA1330 + *Rsl*	31.25 ± 1.46 c	61.56 ± 1.25 b
MA4828 + *Rsl*	31.25 ± 1.33 c	61.56 ± 1.21 b

4. Discussion

Bacillus spp. have been identified as biological control agents against fungal and bacterial diseases. *Bacillus subtilis* and *Bacillus amyloliquefaciens* exert growth-promoting and biocontrol functions by competition, antibiosis, and the induction of plant systemic resistance [38–40]. Successful colonization of PGPR is considered a crucial step for biocontrol [41,42]. Swarming motility and biofilm formation are major adaptive strategies for *Bacillus* in colonization [43,44]. The results of this study demonstrated that *B. pumilus* HR10 produces a mature biofilm structure, which contributes to its colonization on the roots of pine seedlings. The biocontrol efficacy of *B. pumilus* HR10 was proposed to be the result of multiple causes. This study explains the mechanisms of the biocontrol efficacy from many aspects.

The biofilm-deficient mutants of *B. pumilus* HR10 were screened by colony phenotype. The aggregate structure of three mutants, such as the middle or colony edge, exhibited different degrees of defects. The WT *B. pumilus* HR10 strain was found to be most complicated. The defects of MA15 and MA1330 were more significant than those of MA4828. The mutants formed fewer floating pellicles at the liquid air interface of liquid cultures compared to WT, consistent with their simpler colony morphology than that of WT on the solid–air interface. MA15 appeared the sparsest, and MA1330 degraded the quickest. The results suggested that the defectiveness of biofilm substances and swarming motility result in defective pellicle formation and rapid degradation.

Surfactin, a typical lipopeptide that antagonizes fungal and bacterial pathogens, is also known to be involved in swarming motility and biofilm formation [20,44]. For example, the inability of *B. subtilis* 9407 to produce surfactin led to a reduction in antagonistic activity, swarming motility and biofilm formation [4]. In accordance with qRT-PCR measurements, we found that the expression levels of *sfp* at 48 h in MA15 and MA1330 were lower than that of WT, indicating that surfactin may play an important role in the swarming motility and biofilm formation of *B.pumilus* HR10 (Figure 3).

Biofilm formation facilitates the colonization of *Bacillus* on the roots or leaves of plants [45]. Similarly, in our study, WT *B. pumilus* HR10 effectively colonized on the roots of *Pinus massoniana* seedlings, while the biofilm-deficient mutants showed significantly decreased in the colonization on the roots (Figure 4). These results demonstrated that biofilm formation by *B. pumilus* HR10 contributes to efficient colonization.

The biological control of *Bacillus* depends on its inhibitory activity against *R. solani* to a large extent [3]. The inhibitory activities of MA1330 and MA4828 showed no significant differences compared with that of WT, and MA15 exhibited the weakest activity. Surfactin is a major lipopeptide compound that suppresses pathogens [4,20]. According to the results of Figures 3D and 5, we proposed that *sfp* expression of *B. pumilus* HR10 is related to biocontrol against *R. solani*.

Previous studies have reported that biofilm formation is associated with rhizobacteria-induced systemic resistance [29,46]. However, the function of biofilm formation in the induction of systemic resistance is not very evident. In this study, we conducted qRT-PCR measurements of defense-related genes in *P. massoniana* seedlings in response to inoculation with WT or mutants. We found that the expression levels of *PR2* exhibited obvious differences, whereas the expression levels of other genes were not significant. The typical gene of the salicylic acid (SA) signaling pathway of seedlings, *PR2*, exhibited high levels when seedlings were inoculated with WT HR10 or mutants. The *PR2* transcription level increased approximately fivefold after inoculation with WT. These results demonstrated that biofilm formation by *B. pumilus* HR10 contributes its ability to induce seedling resistance by promoting the SA signaling pathway of *P. massoniana* seedlings. Deficiency in biofilm formation of the mutants significantly weakened the induced seedling resistance.

There were obvious differences in the effects of WT *B. pumilus* HR10 and mutants on the biocontrol against pine seedling damping-off disease. The biocontrol results suggested that biofilm formation plays a significant role during the biocontrol process mediated by the WT and mutants. This finding was consistent with a previous study by Luo et al., who reported that biofilm formation further strengthens the colonization of *B. subtilis* 916 and biocontrol efficacy against rice sheath blight [20]. Indeed, chemotaxis of PGPR and subsequent biofilm formation are very important for root colonization

and for providing beneficial functions for plants and PGPR [47,48]. Interestingly, the biocontrol effect of MA1330, which produced defective biofilm, was equal to that of MA4828. This may be due to MA1330 triggering higher expression of SA signaling pathway-related genes, which is necessary for the resistance of plants and biocontrol of PGPR.

Overall, the results reported here indicate that the antagonistic activity of *B. pumilus* HR10 suppresses *R. solani*, and biofilm formation also contributes to the promotion of colonization, induction of plant resistance and biocontrol against seedling damping-off disease. The results of our study may provide a theoretical basis for the field application of *B. pumilus* HR10, which is beneficial for promoting the growth of pine trees, symbiosis of mycorrhizae and biocontrol against pine diseases. A major challenge for the future will be to explore the regulatory pathway of biofilm formation of *B. pumilus* HR10 and the role of lipopeptides in the formation process. It will be interesting and necessary to determine how multiple mechanisms are synergistically involved in regulating biocontrol.

5. Conclusions

There are few studies on the relationship between biofilm formation of *Bacillus pumilus* and its biocontrol process. *B. pumilus* HR10 has high-efficiency functions such as promoting the growth of plants, assisting mycorrhizal-formation and inhibiting pathogens. In this research, biofilm related features of the HR10 strain were analyzed qualitatively or quantitatively. It was also discussed in terms of colonization on the root of *P. massoniana* seedlings and the induction of plant system resistance, which were more in line with the status of PGPR in the natural environment. The results showed that biofilm formation of *B. pumilus* HR10 plays an important role in the prevention of pine seedlings damping-off disease, and it affects biocontrol with induced resistance synergistically. It provides a basis for better exerting the application potential of *B. pumilus* HR10 in the field to promote growth and disease resistance.

Author Contributions: M.-L.Z. performed most of the experiments and data analyzation and drafted linked content of the manuscript in the manuscript. X.-Q.W. participated in planning of research work, interpretation of data and supervision of the manuscript. Y.-H.W. and Y.D. were involved in the planning and execution of the research work, analysis and interpretation of the data. All authors read and agreed to the published version of the manuscript.

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