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In Vivo Screening of Rhizobacteria Against *Phytophthora capsici* and *Bacillus subtilis* Induced Defense Gene Expression in Chili Pepper (*Capsicum annuum* L.)

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Abstract

Phytophthora capsici (Pc) affects the production of *Capsicum annuum* L., particularly in susceptible genotypes like 'California Wonder' (CW). Beneficial bacteria offer a sustainable strategy for enhancing plant defense. Five bacterial strains, *Bacillus subtilis* (BMBA), *Bacillus amyloliquefaciens* (BMBC), *Bacillus* sp. (BMBH), *Pseudomonas putida* (BMBI), and *Paenibacillus* sp. (BMBP), were tested against *P. capsici* in chili pepper. By 11 days post-inoculation (dpi), BMBH, BMBC, BMBI and BMBP showed a trend toward accelerated symptom development, reaching severity scores of 4–5, compared with control (3.66 ± 0.55), whereas BMBA delayed disease progression (2.5 ± 0.71). At 14 dpi, control plants reached maximum severity (6.0 ± 0.0), whereas BMBA-treated plants maintained lower severity (5.0 ± 0.44). Mortality was 100% in the control, compared with 33.3% in the BMBA+Pc treatment, indicating a partial and transient protective effect. Gene expression analysis in CW roots revealed strong upregulation of *PR-1*, *PR-5*, and *EAS* by *P. capsici*. In BMBA plants, *PR-1* decreased at 24 h post-inoculation (hpi), whereas *EAS* initially declined at 8 hpi but increase 18.5% at 24 hpi. These results indicate modulation of defense-related gene expression and reduced mortality, supporting the potential of BMBA as a partial biocontrol agent against *P. capsici* in chili peppers.

Keywords: *Capsicum annuum*; *Phytophthora capsici*; *Bacillus subtilis*; induced systemic resistance

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1. Introduction

Capsicum annuum L. is a chili species of major economic and social importance worldwide [1]. However, its production is severely affected by the oomycete pathogen *Phytophthora capsici* [2], which also infects a wide range of host plants, including eggplant, tomato, watermelon, squash [3], chayote [4], pumpkin, and cucumber [5]. In chili pepper, *P. capsici* induces wilting and infects all plant tissues [6], including roots, stems, leaves, and fruits [7].

In susceptible chili pepper genotypes, infection by *P. capsici* causes root, crown, stem, foliar, and fruit blight, manifested as damping off, necrosis, and fruit lesions that render pods unmarketable [8]. In contrast, resistant genotypes activate multiple defense mechanisms in response to pathogen attack, including reinforcement of cell walls through lignin deposition [9], oxidative response involving phenylalanine ammonia lyase (PAL) [10],

antioxidant enzymes such as peroxidases (POX), superoxide dismutase (SOD), polyphenol oxidase (PPO), and catalase (CAT) [11]. These responses are accompanied by the accumulation of secondary metabolites, including flavonoids [12], phenolic compounds [11], and sesquiterpene phytoalexins such as capsidiol [13]. Additionally, signaling pathways related to Ca^{2+} regulation [12], carbon fixation, and pyruvate metabolism [14] are induced during infection.

In *Capsicum annuum*, incompatible interactions with *P. capsici* activate defense-related genes, including *PR-1* (pathogenesis-related protein 1), *PR-2* (β-1,3-glucanase), *PR-5* (thaumatin-like protein), and *EAS* (5-epi-aristolochene synthase, involved in capsidiol biosynthesis). These genes serve as markers of systemic acquired resistance (SAR) and defense pathway activation [12,15–17].

Beyond intrinsic plant defenses, biological control agents such as rhizobacteria can influence pathogen establishment and disease development. Beneficial rhizobacteria enhance plant nutrition, growth, and immunity by fixing nitrogen, solubilizing nutrients, producing phytohormones, or triggering induced systemic resistance (ISR) [18–21].

Rhizobacteria produce a wide range of bioactive molecules capable of activating plant signaling pathways and promoting the synthesis of defense-related compounds [22]. For instance, inoculation of *C. annuum* with *Paenibacillus polymyxa* reduces disease severity by increasing PAL, PPO, and SOD activities, as well as the expression of *CaPR4* (*PR4*) and *CaChi2* (*PR3*) [23].

Moreover, *B. amyloliquefaciens* produces elicitors such as fengycin, bacillomycin D, surfactin, bacillaene, macrolactin, difficidin, bacilysin, 2,3-butanediol, and exopolysaccharides, which induce the transcription of defense genes in *Arabidopsis thaliana* by activating signaling pathways regulated by salicylic acid, jasmonic acid, and ethylene [24]. Similarly, inoculation of tomato with *Bacillus amyloliquefaciens* TBorg1 enhances the activity of antioxidant enzymes (PPO and POX), decreases oxidative stress markers (H_2O_2 and *MDA*), and upregulates phenylpropanoid (*C4H*, *HCT*, *CHI*) and *PR* genes (*PR-1*, *PR-5*) [25]. These findings highlight the potential of rhizobacteria to trigger systemic defense responses in plants against a broad range of pathogens.

Although *PR-1*, *PR-5*, and *EAS* are widely recognized markers of systemic acquired resistance, few studies have investigated how rhizobacteria modulate the expression of these genes in *Capsicum annuum*. To address this gap, we evaluated the effects *of Bacillus* sp. (BMBH), *Pseudomonas putida* (BMBI), *Bacillus subtilis* (BMBA), *B. amyloliquefaciens* (BMBC), and *Paenibacillus* sp. (BMBP) on plants challenged with *P. capsici*. Gene expression analyses were focused on BMBA-pretreated plants, in which mRNA levels of key defense genes (*EAS*, *PR-1*, and *PR-5*) were quantified. This approach provides insights into how beneficial rhizobacteria modulate SAR-related defense markers in the susceptible chili pepper 'California Wonder'.

2. Materials and Methods

2.1. Microbiological Material

The bacterial strains used in this study were *Bacillus* sp. (BMBH), *Pseudomonas putida* (BMBI), *Bacillus subtilis* (BMBA), *B. amyloliquefaciens* (BMBC), and *Paenibacillus* sp. (BMBP). Bacterial strains were isolated from various plant sources and rhizosphere environments in the Ciénega de Chapala region, Michoacán, Mexico: *Bacillus* sp. from *Euphorbia antisyphillitica*, *Pseudomonas putida* from *Medicago sativa*, *Bacillus subtilis* from *Solanum lycopersicum* var. *cerasiforme*, *B. amyloliquefaciens* from *Echeveria* sp., and *Paenibacillus* sp. from *Solanum ferrugineum*. All strains were identified by 16S rRNA gene sequencing (Sanger sequencing; Macrogen Inc., Rockville, MD, USA) and are part of the microbial collection of the Molecular Biology Laboratory at CIIDIR-IPN Michoacán. These strains had previously

demonstrated biocontrol potential against *P. capsici* in in vitro confrontation assays, and the present study aimed to validate their effects in planta to assess their suitability as biocontrol candidates. *B. subtilis* has been reported in previous studies from our group as a plant growth–promoting rhizobacterium (PGPR). *Phytophthora capsici* (strain 6143) was kindly provided by Dr. S. P. Fernández-Pavía of the Universidad Michoacana de San Nicolás de Hidalgo.

2.2. Inoculum Preparation

Bacterial strains were cultured on potato dextrose agar (PDA) (PDA; Difco Bacto, BD, Franklin Lakes, NJ, USA) for 24 h. The resulting colonies were suspended in sterile distilled water and adjusted to a concentration of 1×10^8 CFU mL^{-1}. Bacterial cell concentration was initially determined by plating serial dilutions and enumerating colony-forming units (CFUs) on agar plates, after which the inoculum concentration was adjusted using spectrophotometry. *P. capsici* was cultured on V8 agar medium, prepared by mixing 200 mL L^{-1} of V8 juice (V8 Vegetable Juice, Campbell Soup Company, Camden, NJ, USA), 3 g L^{-1} of calcium carbonate ($CaCO_3$; Sigma-Aldrich, St. Louis, MO, USA), and 16 g L^{-1} of bacteriological agar (Difco Bacto, BD, Franklin Lakes, NJ, USA) in distilled water.

Infective zoospores of *P. capsici* were obtained from seven-day-old mycelium. To prepare the culture, an 8 mm-diameter mycelial disk was placed in a Petri dish containing 20 mL of V8 medium. The resulting mycelium was fragmented and transferred to a 0.9% sodium chloride isotonic solution (PISA®, Industrial Química, Mexico City, Mexico) for 10 min. To promote sporangium formation, the fragments were then incubated in Petri dishes with sterile distilled water at room temperature under fluorescent light for 48 h. Zoospore release was induced by subjecting the aqueous suspension to a thermal shock of −20 °C for 15 min, followed by incubation at room temperature for 20 min. The resulting aqueous suspension was collected, and the zoospore concentration was determined using a hemocytometer (Marienfeld®, Lauda-Königshofen, Germany). The *P. capsici* inoculum was then adjusted to 10^5 zoospores mL^{-1}.

2.3. Plant Growth Conditions and Inoculation

Seeds of chili pepper 'California Wonder' (CW), a cultivar susceptible to *P. capsici* [2], were used in this study. The seeds were surface sterilized as described by [15] and germinated at 27 ± 2 °C under a 14 h photoperiod. Chili pepper seedlings were inoculated by immersing the roots in 1 mL of bacterial suspension and transplanted into pots containing 25 cm^3 of sterilized sand (one seedling per pot). The plants were maintained under the temperature and photoperiod conditions described above. Watering was performed every 24 h with sterile water (SW), and fortnightly fertilization was applied using a Nitrofoska® NPK 12–12–17 (EuroChem Agro GmbH, Frankfurt, Germany; distributed by Fertilizantes Tepeyac, Mexico City, Mexico) solution (3.15 g L^{-1} SW). When the plants had developed four to six true leaves (approximately one-month-old), they were uniformly inoculated at the base of the stem with 10^5 zoospores of *P. capsici*.

2.4. In Vivo Effect of Rhizobacteria on the Severity of P. capsici-Induced Disease

The effect of rhizobacterial inoculation on the severity of *P. capsici*-induced disease in chili pepper CW plants was evaluated under a completely randomized design (Experiment 1). Treatments consisted of plants inoculated with *P. capsici* alone (Pc) and plants co-inoculated with *P. capsici* and each bacterial strain (BMBH+Pc, BMBI+Pc, BMBA+Pc, BMBC+Pc, and BMBP+Pc). Each treatment included six biological replicates. Disease severity was recorded at 4-, 7-, 11-, and 14 days post-inoculation (dpi) with *P. capsici*. Severity was assessed according to the scale described by [26], where 0 = No visible disease symptoms; 1 = Stem necrosis without girdling; 2 = Stem necrosis with girdling; 3 = Stem

necrosis with <50% defoliation; 4 = Stem necrosis with >50% defoliation; 5 = Wilted plant; and 6 = Dead plant. Mortality (%) was calculated as the number of dead plants divided by six and multiplied by 100, with no intermediate scores assigned.

2.5. Accumulation of Defense Gene Transcripts in Chili Pepper Plants

A second experiment was established to assess the expression of defense-related genes in chili pepper (CW) plants inoculated with *P. capsici* (Pc), *Bacillus subtilis* (BMBA), or sequentially with *B. subtilis* followed by *P. capsici* (BMBA+Pc). Non-inoculated plants served as the control treatment. The experiment was conducted under a completely randomized design, with each treatment consisting of three biological replicates (three groups of three pots each). The defense-related genes analyzed included those encoding pathogenesis-related proteins (*PR-1* and *PR-5*) and 5-epi-aristolochene synthase (*EAS*). Sampling was performed at 8- and 24 h post-inoculation (hpi) with *P. capsici*. For each replicate, roots were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis.

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed as follows. For gene expression analysis, only root tissues were used. Frozen roots were ground to a fine powder in liquid nitrogen. Total RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), including an on-column DNase digestion step (Qiagen), following the manufacturer's protocol. RNA purity was assessed spectrophotometrically, and integrity was confirmed by electrophoresis on a 1% agarose gel prepared with 1× TAE buffer. First-strand cDNA was synthesized using M-MLV reverse transcriptase (Promega, Maldison, WI, USA) with oligo(dT)12-18 primers (Invitrogen, Carlsbad, CA, USA), a dNTP mix (Promega), and nuclease-free water (IDT, Integrated DNA Technologies, Coralville, IA, USA), according to the manufacturer's instructions.

Transcript accumulation of *PR-1* (Pathogenesis-related protein 1), *PR-5* (Thaumatinlike protein), and *EAS* (5-epi-aristolochene synthase) genes was quantified by real-time PCR using the CFX Connect™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with gene-specific primers (Table 1). Reactions were carried out in a final volume of 25 µL, containing 0.2 mM dNTP mix (Promega, Maldison, WI, USA), 1 mM $MgCl_2$ (Biotecmol, Mexico City, Mexico), 0.4 µM of each primer, SYBR® Green I (1:75,000) (Molecular Probes, Invitrogen), Taq polymerase (Biotecmol), 10× buffer (Biotecmol), 5 µL of cDNA, and nuclease free water (IDT, Integrated DNA Technologies, Coralville, IA, USA). Amplification was performed under the following conditions: an initial denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s, 65 °C for 35 s (annealing), and 72 °C for 35 s (extension). The absence of nonspecific products was confirmed by dissociation (melting curve) analysis. Relative gene expression levels (mRNA levels) were calculated using the $2^{-\Delta\Delta Ct}$ method [27]. Expression of target genes was normalized using the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene as the internal reference, and expression values were calibrated using Ct values obtained from control plants (non-inoculated CW).

Table 1. Primer sequences used for gene expression analysis.

Gene	Accession Number †	Sequences (5′–3′)
PR-1	XM_016683907	FW: CCCAAAATTACGCCAATCAAAG RV: ACATCTTCACGGCACCAG
PR-5	NM_001324896	FW: TGGTGGAGTCTTGCAGTGC RV: CGTGCAATGGATCGCGTG
EAS	AJ005588	FW: GCTCAAGAAATTGAACCGCCGAAG RV: TCTTCATTATAGACATCGCCCTCG
GAPDH	AJ246011	FW: GGCCTTATGACTACAGTTCACTCC RV: GATCAACCACAGAGACATCCACAG

PR-1 (Pathogenesis-related protein 1) [28], *PR-5* (Thaumatin-like protein) [29], *EAS* (5-epi-aristolochene synthase) [15], *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) [15]. † NCBI (National Center for Biotechnology Information).

2.6. Statistical Analysis

Disease severity and gene expression data in chili pepper plants were subjected to analysis of variance (ANOVA), and mean comparisons were performed using Fisher's least significant difference (LSD) test ($p < 0.05$). Statistical analyses were performed using Minitab® Statistical Software version 21 (Minitab LLC, State College, PA, USA), and all graphs were generated using GraphPad Prism version 9.5 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. In Vivo Effect of Rhizobacteria on the Severity of P. capsici Induced Disease

Initial symptoms (4 dpi) consisted of basal stem necrosis without girdling (severity rating = 1). At this early evaluation, variation in symptom severity was observed among treatments, ranging from stem necrosis without girdling to stem necrosis with girdling; however, no statistically significant differences were detected among treatments.

At 7 dpi, the BMBH+Pc treatment showed significantly higher disease severity compared with the control (Pc), with plants exhibiting stem necrosis accompanied by partial to severe defoliation (>50%) (Table 2). The remaining treatments (BMBP+Pc, BMBC+Pc, BMBA+Pc, and BMBI+Pc) did not differ significantly from the control at this time point, although numerical differences in mean severity values were observed.

Table 2. Disease severity caused by *Phytophthora capsici* in bacterized chili pepper plants.

Treatment	Severity (0–6) †			
	4	**7**	**11**	**14**
Pc	1.0 ± 0.68^{a}	1.5 ± 0.76^{bc}	3.66 ± 0.55^{ab}	6.0 ± 0.0^{a}
BMBA+Pc	1.0 ± 0.33^{a}	1.0 ± 0.68^{b}	2.5 ± 0.71^{c}	5.0 ± 0.44^{b}
BMBI+Pc	0.33 ± 0.33^{a}	0.83 ± 0.40^{b}	4.16 ± 0.70^{bc}	5.66 ± 0.21^{a}
BMBC+Pc	1.0 ± 0.44^{a}	3.0 ± 0.63^{ab}	5.66 ± 0.21^{a}	6.0 ± 0.0^{a}
BMBH+Pc	0.33 ± 0.33^{a}	4.0 ± 0.63^{a}	5.33 ± 0.21^{ab}	5.83 ± 0.16^{a}
BMBP+Pc	2.0 ± 1.0^{a}	2.5 ± 0.5^{abc}	5.83 ± 0.16^{a}	6.0 ± 0.0^{a}

Values represent the mean ± standard error (n = 6), and different letters within each column indicate significant differences according to the LSD test ($p \leq 0.05$). Evaluations were conducted at 4, 7, 11, and 14 days post-inoculation (dpi) with *P. capsici* in bacterized 'California Wonder' chili pepper plants. BMBA: *B. subtilis*. BMBP: *Paenibacillus* sp. BMBC: *B. amyloliquefaciens*. BMBH: *Bacillus* sp. BMBI: *Pseudomonas putida*. Pc: *P. capsici*. † Disease severity scale: 0 = No visible symptoms; 1 = Necrosis at the stem base without girdling; 2 = Stem necrosis with girdling; 3 = Girdling stem necrosis with less than 50% defoliation; 4 = Girdling stem necrosis with more than 50% defoliation; 5 = Wilted plant; 6 = Dead plant [26].

At 11 dpi, control plants inoculated with *P. capsici* reached a mean severity rating of 3.66 ± 0.55, corresponding to stem necrosis with less than 50% defoliation. Higher mean severity values were observed in plants co-inoculated with *P. capsici* and the bacterial strains BMBI, BMBC, BMBH, or BMBP, which were associated with severe wilting symptoms; however, these treatments did not consistently differ significantly from the control (Table 2). In contrast, co-inoculation with *B. subtilis* (BMBA+Pc) resulted in a significantly lower disease severity (2.5 ± 0.71), indicating delayed disease progression, with symptoms mainly limited to stem necrosis with girdling.

At 14 dpi, control plants inoculated with *P. capsici* (Pc) reached the maximum severity rating (6.0 ± 0.0), corresponding to plant death. Similar severity values were observed in plants co-inoculated with BMBP+Pc, BMBC+Pc, BMBH+Pc, and BMBI+Pc, none of which differed significantly from the control. In contrast, plants treated with *B. subtilis* (BMBA+Pc) exhibited a significantly lower disease severity (5.0 ± 0.44), corresponding plants that were still alive, indicating delayed disease progression rather than complete suppression (Table 2).

3.2. Mortality Caused by P. capsici

Mortality caused by *P. capsici* was evaluated at 14 days post-inoculation (dpi), showing clear differences among treatments. The control treatment (Pc) reached 100% mortality, with plants completely collapsed and exhibiting total tissue necrosis (Figure 1a). Plants treated with BMBC and BMBP also showed 100% mortality, whereas BMBH reached 90% (Figure 1b), indicating no protective effect against *P. capsici* infection.

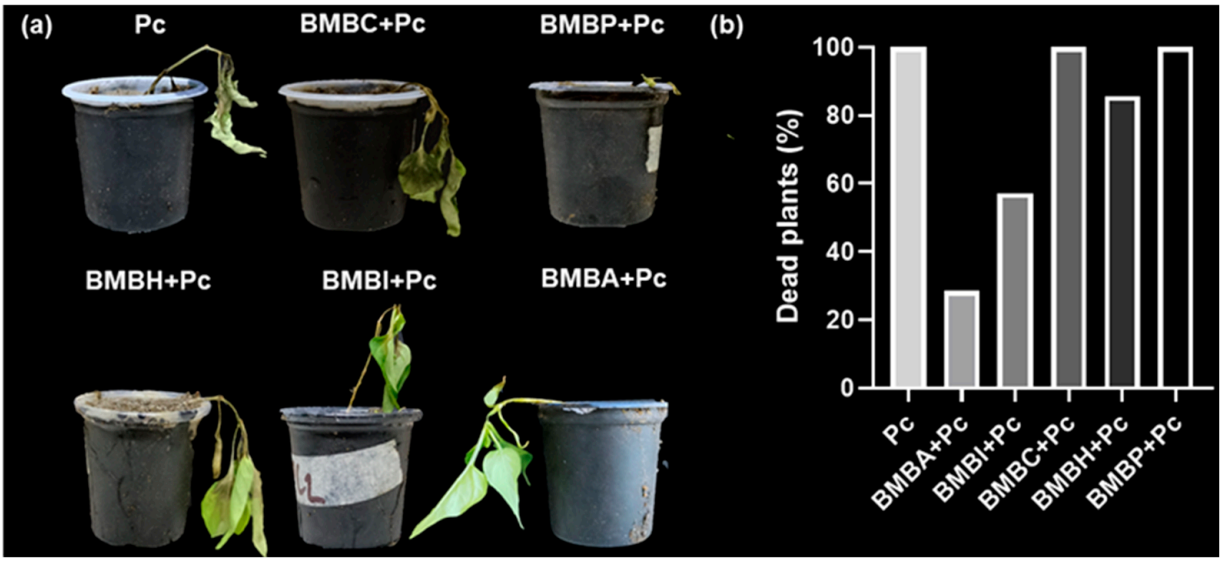

Figure 1. Mortality index at 14 days post-inoculation (dpi) with *P. capsici* in bacterized 'California Wonder' chili pepper plants. BMBA: *B. subtilis*. BMBP: *Paenibacillus* sp. BMBC: *B. amyloliquefaciens*. BMBH: *Bacillus* sp. BMBI: *Pseudomonas putida*. Pc: *P. capsici* inoculated with *P. capsici*. (**a**) Visual appearance of the symptomatic plants. (**b**) Mortality percentage recorded for each treatment.

The BMBI+Pc treatment exhibited an intermediate mortality rate of 66.7%, with some plants retaining green tissue but displaying clear wilting symptoms. In contrast, the BMBA treatment performed best, showing 33.3% mortality, with surviving plants exhibiting markedly less wilting than other treatments. These results indicate that BMBA functionsas a partial biocontrol agent against *P. capsici*, delaying disease development whereas reducing mortality in chili pepper plants.

3.3. Accumulation of Defense Related Gene Transcripts in Chili Pepper Plants Bacterized and Challenged with Phytophthora capsici

The expression of *PR-1*, *PR-5*, and *EAS* genes was evaluated at 8 and 24 h post-inoculation (hpi) with *P. capsici* in chili pepper plants (Pc), treated with *B. subtilis* (BMBA) or subjected to the tripartite interaction (Pc+BMBA) (Figure 2a–c). For the *PR-1* gene (Figure 2a), plants inoculated with *P. capsici* (Pc) showed significant upregulation at 8 hpi, reaching a maximum at 24 hpi, representing a 59.95% increase relative to 8 hpi. Plants treated with BMBA alone exhibited no significant changes in expression over time. In the tripartite treatment (BMBA+Pc), *PR-1* expression increased by 64.44% at 24 hpi. At this time point, BMBA+Pc exhibited 3.22-fold higher expression compared to BMBA alone, whereas plants inoculated only with *P. capsici* showed a 1.5-fold increase (Figure 2a).

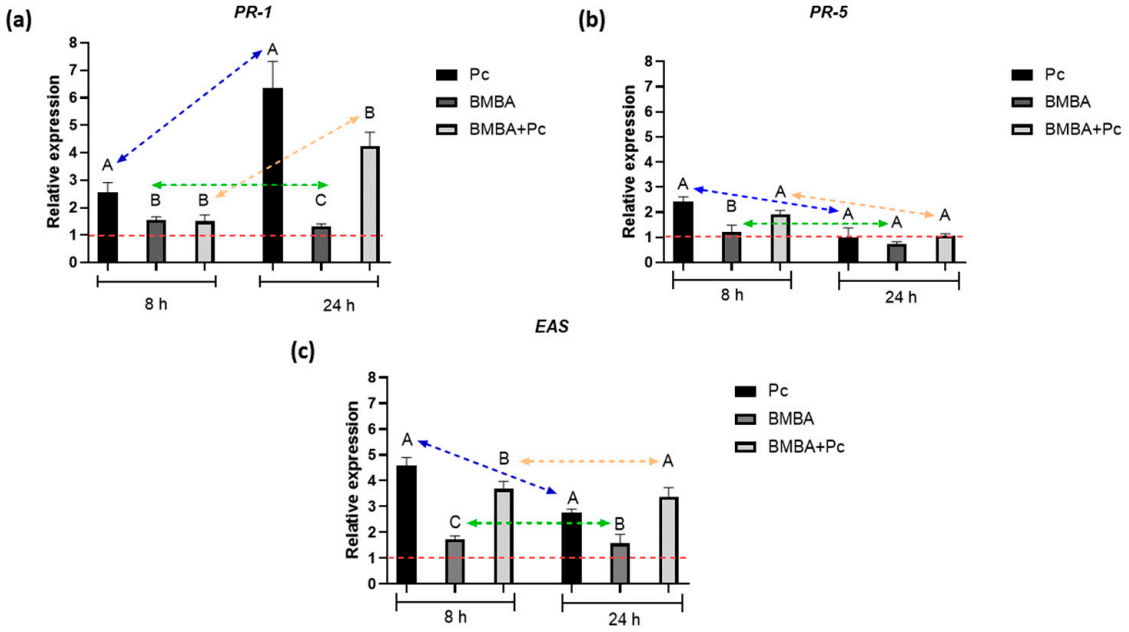

Figure 2. Relative accumulation of defense related gene transcripts in the roots of chili pepper plants (*Capsicum annuum* L. 'California Wonder') inoculated with *Bacillus subtilis* and infected with *Phytophthora capsici*. (**a**) Pathogenesis-related protein 1 (*PR-1*); (**b**) Thaumatin-like protein (*PR-5*); (**c**) 5-epi-aristolochene synthase (*EAS*). Gene expression was analyzed at 8 h and 24 h post-inoculation (hpi) with *P. capsici*. Treatments: *P. capsici* + C. Wonder (Pc); C. Wonder + *B. subtilis* (BMBA); C. Wonder + *B. subtilis* + *P. capsici* (BMBA+Pc). Each bar represents the mean of three replicates, and the line on each bar is the standard error. Different uppercase letters denote significant differences among all treatments. Data were analyzed using one-way ANOVA followed by Fisher's LSD post hoc test ($p \leq 0.05$). Arrows indicate temporal changes in gene expression. Values below the horizontal dotted (red) line indicate downregulation, and values above the line indicate upregulation; the control group was set to 1 according to the $2^{-\Delta\Delta Ct}$ (Livak) method.

For the *PR-5* gene (Figure 2b), maximum upregulation was observed in the Pc treatment at 8 hpi. In contrast, the tripartite treatment (BMBA+Pc) showed a 1.98-fold higher versus Pc treatment, but BMBA treatment was 1.27-fold lower compared to plants inoculated only with *P. capsici* (Pc).

At 24 hpi, *PR-5* expression in the Pc treatment decreased by 58.60%. A similar trend was detected in the BMBA+Pc treatment, which showed early upregulation at 8 hpi followed by a 43.26% reduction at 24 hpi. In contrast, plants treated with BMBA alone exhibited relatively stable expression levels, and the gene was even downregulated at 24 hpi (Figure 2b).

For the *EAS* gene, upregulation in plants inoculated with *P. capsici* (*Pc*) reached its maximum level at 8 hpi, showing 2.65-fold and 1.24-fold higher than BMBA and BMBA+Pc, respectively. In the tripartite interaction (BMBA+Pc), upregulation was 2.12-fold higher compared to BMBA treatment (Figure 2c).

In a similar manner, the trend of *EAS* gene expression was maintained at 24 hpi; however, expression in the Pc treatment declined by 66.15%. Plants inoculated with BMBA alone exhibited a modest but sustained increase in *EAS* expression, maintaining comparable levels at both 8 and 24 hpi. At 24 hpi, under tripartite interaction (BMBA+Pc), *EAS* expression was 2.15-fold and 1.29-fold higher than in the BMBA and Pc treatments, respectively, indicating a prolonged induction of this gene relative to the individual treatments (Figure 2c).

4. Discussion

Microorganisms can establish various types of interactions with plants, including beneficial, neutral, pathogenic, and mutualistic relationships [30]. In this context, the application of certain beneficial bacteria in agriculture plays a crucial role in enhancing plant defense against plant pathogens through both direct and indirect mechanisms [31–33]. A key mechanism involves the production of elicitors that modulate systemic signaling networks, triggering activation of defense-related pathways [34].

Our findings indicate that not all bacterial strains confer protection against *P. capsici*, as *B. amyloliquefaciens* (BMBC), *Bacillus* sp. (BMBH), and *Paenibacillus* sp. (BMBP) either failed to reduce disease severity or may facilitate pathogen colonization. This suggests that certain bacteria, despite showing antagonistic activity in vitro [35], may influence the host defense signaling or the rhizosphere environment in ways that inadvertently favor pathogen establishment, highlighting the importance of evaluating microbial effects in planta [30,36,37].

In contrast, *B. subtilis* (BMBA) demonstrated a protective effect by delaying disease progression and reducing mortality. Previous studies have reported that *B. subtilis* strains can reduce disease incidence caused by *Colletotrichum lagenarium* and *Pythium aphanidermatum* in cucumber and tomato, respectively [38]. The protective effect of *B. subtilis* is mainly attributed to the production of secondary metabolites such as acetoin and 2,3-butanediol, as well as antimicrobial bacteriocins, lipopeptides, polyketides, and hydrolytic enzymes that contribute to pathogen inhibition and the induction of plant defense responses [24].

To explore the underlying defense mechanisms, we analyzed the expression of *PR-1* and *PR-5* [39] and *EAS* [40], which are commonly associated with the salicylic acid pathway and capsidiol biosynthesis [33,40]. Following inoculation with *P. capsici*, these genes were upregulated at 8 hpi, indicating activation of the defense response during the early stages of infection. However, this transcriptional response alone was insufficient to suppress pathogen colonization, as disease progression was still observed (Table 2).

Although strains of the genus *Bacillus* have been reported to induce systemic resistance in several crops, including tomato [41], bell pepper, muskmelon, watermelon, sugar beet, and tobacco [42], such responses are often associated with induced systemic resistance (ISR) mediated mainly through jasmonic acid (JA) and ethylene (ET) signaling pathways. However, the present study evaluated only a limited set of defense-related marker genes associated with salicylic acid-dependent responses (*PR-1* and *PR-5*) and capsidiol biosynthesis (*EAS*).

In this context, during the early stage of the tripartite interaction (BMBA+Pc), the expression of *PR-1* and *PR-5* at 8 h post-inoculation (hpi) was lower than in plants inoculated only with the pathogen. These results indicate that, at this early time point, BMBA-treated plants showed a reduced early transcriptional response of these SA-dependent markers. However, only a limited set of genes and two time points were evaluated, and further studies are needed to determine whether this pattern reflects broader modulation of defense responses.

Similar patterns of defense modulation have been reported for other *Bacillus* strains. For example, *B. amyloliquefaciens* SQR9 produces extracellular compounds that activate SA, JA, and ET-mediated defenses and upregulate pathogenesis-related genes such as *PR-1*, *PR-2*, and *PR-5* [24]. Additionally, *B. subtilis* MBI600 enhances transcription of *PR-1* and *GLUA* (SA markers) as well as *CHI3*, *LOXD*, and *PAL* (JA/ET markers) in tomato, demonstrating activation of multiple defense pathways and systemic resistance [43]. In this context, although our data are limited to SA-associated markers, the observed modulation of *PR-1* and *PR-5* expression may contribute to the delayed disease progression and reduced stress observed during the early stages of *P. capsici* infection.

Notably, at 24 hpi, *EAS* expression in BMBA-treated plants increased relative to 8 hpi, whereas it declined in untreated plants. This pattern suggests that BMBA may later sustain or reactivate the phytoalexin capsidiol biosynthetic pathway, reinforcing defense responses during later stages of infection; however, additional time points need to be evaluated to confirm this trend. Similar observations have been reported, indicating that the timing and magnitude of defense gene activation are critical determinants of resistance or susceptibility to *P. capsici* and vary according to chili genotype and pathogen inoculum density [44].

The upregulation of the *EAS* gene plays an important role in plant defense against *P. capsici*. Previous studies have shown that flagellin elicitor, produced by *B. amyloliquefaciens*, can activate *EAS1* transcription in *C. annuum*, promoting the accumulation of the phytoalexin capsidiol [45]. Capsidiol exhibits antifungal activity, contributes to cell wall reinforcement, and restricts pathogen penetration, with higher phytoalexin levels correlating with reduced severity of damage caused by *P. capsici* [46,47].

In the present study, inoculation with *B. subtilis* (BMBA) induced moderate and sustained expression of *PR-1* and *EAS* in *C. annuum* plants, with similar expression levels observed at 8 and 24 h post-inoculation (hpi). This pattern suggests that bacterial inoculation, applied 30 days prior to pathogen challenge, may contribute to maintaining basal activation of specific defense-related pathways.

During the early stage of the tripartite interaction (BMBA+Pc), the expression of defense-related genes was lower than in plants inoculated with *P. capsici* alone. This observation suggests that BMBA may modulate early pathogen-induced transcriptional responses, potentially by limiting pathogen proliferation or delaying infection progression (Table 2), rather than by amplifying early defense gene expression.

At 24 hpi, bacterized plants exhibited increased *EAS* expression compared with untreated plants, which maintained relatively low transcript levels (18.54%). This indicates that, at later stages of infection, the capsidiol biosynthetic pathway remains active in bacterized plants, potentially contributing to enhanced phytoalexin accumulation and restriction of pathogen development in *B. subtilis*-treated plants.

Similar defense responses have been reported by Chávez-Díaz et al., 2017 [48], who showed that bacterial consortia composed of *Pseudomonas putida* S4 and *Pseudomonas tolaasii* A46 provided up to 90% protection against necrosis caused by *P. capsici* in chili pepper (*C. annuum* 'pasilla'). In that study, plants received three bacterial inoculations (3×10^8 CFU) at different developmental stages, resulting in stronger induction of defense-related genes, including *EAS*, *GLU* (*PR-2*, β-1,3-glucanase), *PR-1*, and *POX* (PR-9, peroxidase). Compared with those findings, the lower transcriptional induction observed in the present study suggests that increasing bacterial concentration and/or inoculation frequency could enhance the level of protection against *P. capsici* in *C. annuum* 'California Wonder'.

Overall, BMBA appears to modulate the temporal dynamics of host defense, reducing disease severity and mortality without completely preventing infection. Future studies should explore broader hormonal pathways, additional defense markers, and the effect of inoculation timing and concentration to optimize the biocontrol potential of *B. subtilis* in *C. annuum.*

Taken together, all genes were upregulated in response to the biotic stress imposed on 'California Wonder' chili pepper plants. These results indicate that *P. capsici* infection activates defense genes associated with the salicylic acid-dependent SAR (*PR-1* and *PR-5*) and capsidiol biosynthesis (*EAS*), whereas treatment with *B. subtilis* (BMBA+Pc) maintained marginal gene expression levels, with no apparent biological impact on the pathogen.

5. Conclusions

Inoculation with the screened bacteria produced differential effects on disease severity in chili pepper plants. BMBH, BMBC, and BMBP strains appeared to facilitate *P. capsici* infection, whereas treatment with *B. subtilis* (BMBA+Pc) maintained marginal gene expression levels, with no apparent biological impact on the pathogen. The transient protective effect is likely associated with the modulation of defense-related genes, including *PR-1* and *EAS*. Overall, these findings support the potential of *B. subtilis* (BMBA) as a partial biocontrol agent and highlight the importance of comprehensive assessments of plant microbe pathogen interactions prior to field application to optimize inoculation strategies for protecting susceptible *C. annuum* cultivars such as 'California Wonder'.

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