



BACILLUS-MEDIATED SUPPRESSION OF MACROPHOMINA PHASEOLINA IN STRAWBERRY VIA ANTIBIOSIS AND PAL-ASSOCIATED DEFENSE ACTIVATION

Firas K. Aljuboori¹, Rayyan Salim Mahmood Al-Heyalee², Saleh Ahmed Eesa Aljobory³, Bassam. Y. Ibrahim⁴, Alaa Hamed Mohamed⁵

^{1,2,3,4,5} Department of Plant Protection, College of Agriculture and Forestry, University of Mosul, Iraq

ABSTRACT

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Correspondence Email:

firasaljuboori@uomosul.edu.iq

Macrophomina phaseolina, the causal agent of charcoal rot, severely constrains strawberry production, and its management relies on broad-spectrum fungicides with documented environmental and public-health risks. Plant growth-promoting rhizobacteria (PGPR) therefore represents a sustainable alternative. This study evaluated *Bacillus subtilis*, *B. amyloliquefaciens*, and *B. thuringiensis* against *M. phaseolina* and their ability to induce host defense, monitored through phenylalanine ammonia-lyase (PAL) activity. Antagonism was assessed in vitro, and disease severity and PAL activity were evaluated in greenhouse experiments on two major local strawberry cultivars (Albion and Camarosa), with and without *M. phaseolina*. Non-inoculated and *Bacillus*-only controls were included. *B. subtilis* produced the highest in vitro inhibition of *M. phaseolina* (percentage inhibition of radial growth, PIRG = 38.82 ± 1.21%), significantly exceeding ($P \leq 0.05$) *B. amyloliquefaciens* and *B. thuringiensis*. In the greenhouse (cv. Albion), *B. amyloliquefaciens* provided the greatest disease control (disease severity index, DSI = 53.3 ± 1.9%; percentage disease control, PDC = 46.7%) relative to the pathogen-inoculated control (DSI = 100%), followed by *B. thuringiensis* (DSI = 66.6 ± 2.4%; PDC = 33.4%), whereas *B. subtilis* was least effective (DSI = 73.3 ± 2.1%; PDC = 26.7%). Conversely, *B. subtilis* induced the highest PAL activity (32.41 ± 1.14 nmol h⁻¹ g⁻¹ FW), a 43.0% increase over the pathogen-inoculated control (22.67 ± 0.76 nmol h⁻¹ g⁻¹ FW). All three *Bacillus* treatments significantly increased PAL activity ($P \leq 0.05$) relative to the pathogen-inoculated control, indicating activation of host defense responses. These findings support integrating *Bacillus*-based bioagents into strawberry charcoal rot management.

College of Agriculture and Forestry, University of Mosul.

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1. INTRODUCTION

Strawberries (*Fragaria* × *ananassa* Duchesne) are highly valued for their nutritional and economic importance. Their appealing flavor drives steadily rising global consumption, intensifying the need for increased production. Nonetheless, there are many problems associated with the sustainable production and farming of strawberries, the biggest being that they are prone to many soil-borne bacterial and fungal infections, especially charcoal rot caused by *Macrophomina phaseolina*. The disease has the potential to cause plant death, yield loss, and seedling mortality. The fungus is a pathogen of numerous plant hosts, with over 90 plant species having been infected (Pennerman *et al.*, 2025). In strawberry specifically, its importance has

increased since the withdrawal of methyl bromide as a pre-plant fumigant, and it is a primary cause of charcoal rot in various countries, most notably the United States, Spain, Italy, and Chile (Pedroncelli *et al.*, 2025; Lahlali *et al.*, 2022). In severe infections, *M. phaseolina* can account for more than 80% of plant mortality where inoculum pressure is high (Baggio *et al.*, 2019). Crown rot, vascular tissue discoloration, slow wilting, and seedling death are the most significant symptoms. Water deficit and elevated soil temperatures (28–35 °C) increase charcoal rot severity in strawberry under both controlled and field conditions (Pedroncelli *et al.*, 2025; Baggio *et al.*, 2019). These microscopic sclerotia play a vital role in the survival and epidemic persistence of *M. phaseolina* because the sclerotia can last for many years (Marquez *et al.*, 2021). Crop rotation and fungicide application remain useful, but their effectiveness varies with inoculum density, cropping system conditions, soil conditions, and the fungicide sensitivity of the local pathogen population. Since the general prohibition of numerous pesticides and soil modification, e.g., methyl bromide as a pre-planted fumigant, the challenge has been to find biological substitutes to control soil-borne diseases (Lahlali *et al.*, 2022). Plant growth-promoting bacteria (PGPR) have received considerable attention as a promising disease management tool and because this group has shown positive associations with the root microbiome of host plants (Yang *et al.*, 2024). *Bacillus* spp. is among the most widely commercialized PGPR biocontrol agents useful in plant pathology against a wide variety of fungal pathogens. This has been attributed to the fact that they have the capacity to produce numerous resistant spores, making them tolerant to harsh conditions in different environments, thereby increasing their survivability. Viability in the field (Li *et al.*, 2025). The success of such *Bacillus* spp. is based on the fact that they possess a wide range of biological control mechanisms, including the biosynthesis of antimicrobial lipopeptides such as Iturin A, Surfactin, and Fengycin, which target the fungal cell membrane by incorporating and disrupting phospholipid activity (Figueroa-Brambila *et al.*, 2023). They also participate in competition for nutrients and colonization sites, in addition to stimulating systemic resistance in the host plant and reshaping the root zone microbiome (Li *et al.*, 2025). Several recent studies have indicated the effectiveness of *Bacillus amyloliquefaciens* strains in inhibiting the growth of *M. phaseolina* *in vitro* and in host plants, where fungal growth was suppressed by up to 66.8% in double culture tests (Rangel-Montoya *et al.*, 2022).

Plants respond better to pathogens when systemic resistance (ISR) is induced, where plant growth-promoting bacteria (PGPR) stimulate host plant immunity as well as direct antagonistic effects (Yang *et al.*, 2024). In infected plants, the increase in phenylalanine ammonia-lyase (PAL) activity represents a clear biochemical signal indicating the activation of defense mechanisms in the phenylpropanoid pathway for lignin and phenol compound production that enhance plant immunity (Wu *et al.*,

2025). Elevated PAL activity marks phenylpropanoid pathway activation and is not by itself evidence that the ISR signaling network has been engaged

PAL catalyzes the non-oxidative deamination of L-phenylalanine to trans-cinnamic acid toward the biosynthesis of a variety of secondary metabolites by directing the carbon flux to include flavonoids, lignin precursors, and salicylic acid, which is a key signaling molecule in acquired systemic resistance (Ai *et al.*, 2025). Despite the clear role of phenylalanine ammonia-lyase (PAL) in enhancing plant immunity, to our knowledge, no study has systematically compared *Bacillus*-mediated PAL induction across strawberry cultivars with contrasting susceptibility to *M. phaseolina*, representing the gap addressed here. The novelty lies in the combined assessment of three commercially formulated species across two cultivars with *in vitro* antagonism greenhouse disease suppression and PAL activity measured on the same treatment set. Therefore, this study aimed to evaluate the potential for biological control of the fungus *M. phaseolina* on two commercial strawberry varieties, Albion and Camarosa, using three commercial strains of the bacteria *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus thuringiensis*. The study focused on *in vitro* antifungal activity and disease severity reduction under greenhouse conditions.

2. MATERIALS AND METHODS

2.1. Isolation and Morphological Identification of *Macrophomina phaseolina*

Macrophomina phaseolina was isolated from symptomatic strawberry plants (*Fragaria* × *ananassa* Duchesne) exhibiting charcoal rot symptoms at the experimental greenhouse of the College of Agriculture, University of Mosul, Iraq. Symptomatic tissue fragments underwent surface disinfection in a 4% (v/v) sodium hypochlorite solution for 3 minutes, received three rinses in sterile distilled water, and were then transferred onto Potato Dextrose Agar (PDA; Oxoid UK) supplemented with tetracycline (0.25 mg mL⁻¹). Plates were incubated at 25 ± 2°C for 5–7 days. Emerging colonies were purified by hyphal-tip transfer onto fresh PDA. Morphological identification was based on colony pigmentation, hyphal characteristics, and microsclerotia morphology according to Mihail (1992) and Smith and Carvil (1997).

2.2. Preparation of Fungal Inoculum

To prepare the fungal inoculum, a 5 mm plug excised from the advancing margin of a pure *M. phaseolina* colony was transferred to the center of a 90 mm Petri dish containing tetracycline-amended PDA (0.25 mg mL⁻¹), the antibiotic serving to suppress bacterial contamination (Mihail 1992). Plates were incubated at 25 ± 2°C for 5 days until the colony fully covered the plate surface. Following full-plate colonization, the inoculum was prepared by flooding each colonized plate with 100 mL of sterile distilled water, gently scraping the colony surface with a sterile glass rod to dislodge microsclerotia and mycelial fragments, and filtering the resulting

suspension through sterile cheesecloth to remove coarse hyphal debris (Smith and Carvil 1997). Microsclerotia density in the filtrate was quantified using a Neubauer Improved hemocytometer (Carl Zeiss, Germany) at 100× magnification and adjusted to a standard concentration of 1×10^4 microsclerotia mL^{-1} (Pastrana *et al.*, 2016). This suspension was then used directly for soil infestation as described in Sections 2.4 and 2.6.3.

2.3. Molecular Identification and Phylogenetic Analysis

2.3.1. DNA Extraction

M. phaseolina was cultivated in potato dextrose broth at $25 \pm 2^\circ\text{C}$ on a rotary shaker (150 rpm) for 5 days. Mycelial biomass was freeze-dried and ground in liquid nitrogen. Total genomic DNA was recovered with the Fungal/Yeast Genomic DNA Purification Kit (EZBioResearch USA) and its integrity was confirmed through NanoDrop, ND-1000 spectrophotometry (A260/A280 ratio of 1.8–2.0; Thermo Fisher Scientific) together with electrophoretic separation on a 1.5% agarose gel.

2.3.2. PCR Amplification of the ITS Region

ITS4 The ITS region was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) in a 25 μL reaction containing 12.5 μL of 2× DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) [0.4 μM] of each primer and [50 ng] template DNA Amplification commenced with a 5-minute denaturation at 95°C , proceeded through 35 cycles (95°C for 30 s, 52°C for 45 s, and 72°C for 60 s), and terminated with a 10-minute extension at 72°C . Expected amplicon size: ~550–600 bp. The 52°C annealing temperature follows White *et al.*, (1990) for ITS1/ITS4; specificity was confirmed by a single band of the expected size no-template controls and the resulting Sanger sequence.

2.3.3. Sequencing and Phylogenetic Tree Construction

PCR amplicons were purified (GeneJET Kit; Thermo Fisher Scientific) and bidirectionally sequenced by Sanger sequencing (Macrogen Inc., South Korea). Consensus sequences were assembled in Geneious Prime v.2023.2. BLASTU queries (identity $\geq 99\%$, coverage $\geq 98\%$) identified closest accessions. Multiple alignment was performed using MUSCLE v.5.1 (Edgar 2004). The substitution model was selected by BIC in ModelTest-NG v.1.0 (Darriba *et al.*, 2020). Maximum Likelihood phylogenetic trees were constructed in MEGA11 (Tamura *et al.*, 2021) with 1000 bootstrap replicates.

2.4. Pathogenicity Test and Koch's Postulates

Soil infestation was carried out by thoroughly mixing the contents of one fully colonized Petri dish of *M. phaseolina* (as prepared in Section 2.2) with the pot substrate prior to seedling transplanting (Pastrana *et al.*, 2016). Each colonized plate was scraped into 100 mL of sterile distilled water, and the resulting microsclerotia

suspension was poured evenly over the soil in each pot and mixed uniformly by hand to ensure homogeneous distribution of the inoculum throughout the root zone. Non-infested control pots received equivalent volumes of sterile distilled water. Healthy CV Albion seedlings at the age of 4 weeks (3–4 true leaves, approximately 8–10 cm plant height) were transplanted individually into infested and non-infested control pots with five replicate pots (three plants per pot) per treatment (Baggio *et al.*, 2019; Pedroncelli *et al.*, 2025). Plants were maintained under greenhouse conditions (22–25°C, 60–70% relative humidity, 12-hour photoperiod) and monitored daily for symptom development for 21 days post-inoculation (dpi). Koch's postulates were completed by re-isolation of the pathogen from symptomatic crown and root tissues onto PDA and molecular re-identification by ITS sequencing (Mertely *et al.*, 2005; Lahlali *et al.*, 2022). Each pot received 1×10^6 microsclerotia (100 mL at 1×10^4 mL⁻¹); the same dose was used in the greenhouse experiment.

2.5. *Bacillus* Strains and Inoculum Preparation

Three commercial biocontrol products were used: *Bacillus subtilis* strain QST 713 (Serenade® ASO; Bayer CropScience LP USA; 1.34% active ingredient with a concentration of at least 1×10^8 CFU g⁻¹), *Bacillus amyloliquefaciens* strain D747 (Double Nickel™ LC; Certis USA; 11.56% active ingredient), and *Bacillus thuringiensis* var. *kurstaki* (Dipel® WP; Valent BioSciences USA; potency 8000 IU mg⁻¹). The products were used as supplied by the manufacturer without re-isolation of the strains in order to preserve the original characteristics of the commercial formulations. Given that Dipel® WP is primarily registered as an insecticide for controlling lepidopteran larvae, it was included in the study to evaluate its potential to elicit plant defense responses and was treated as an open-ended experimental question (Palma *et al.*, 2024). Due to differences in the nature and composition of the commercial products and the methods used to express active ingredient content, the three products were not standardized based on product mass or active ingredient percentage. Instead, suspensions of each product were prepared in sterile distilled water and shaken at 150 rpm for 30 minutes. Appropriate serial dilutions were then performed, and samples were plated on Nutrient Agar (NA) and incubated at 30°C for 48 hours to assess microbial recovery and growth. Colony-forming unit (CFU mL⁻¹) counts were used to determine viable concentrations, and suspension concentrations were adjusted to 5×10^6 CFU mL⁻¹ immediately prior to each treatment.

2.6. Greenhouse Experimental Design and Treatments

2.6.1. Experimental Conditions

The trial was conducted at the College of Agriculture, University of Mosul (October 2024 - February 2025) at 22–25°C (day) / 18–20°C (night), 60–70% relative humidity, 12-hour LED photoperiod (200 µmol m⁻² s⁻¹ - 1 PPFD). Plants were irrigated every 2–3 days and fertilized weekly with half-strength NPK (20:20:20).

2.6.2. Treatments and Layout

Seedlings of cv. Albion and cv Camarosa (4 weeks old, 3-4 true leaves, 8-10 cm) were grown in 3 kg pots filled with sterilized (peat:perlite:vermiculite 2:1:1 v/v/v) substrate in a completely randomized design (CRD) with 8 treatments $\times$ 5 replicate pots (three plants per pot; disease severity and PAL activity were averaged to a single value per pot, so the experimental unit was the replicate pot, $n = 5$ pots per treatment, i.e., 15 plants per treatment), where T1: *M. phaseolina* + *B. subtilis*; T2: *M. phaseolina* + *B. amyloliquefaciens*; T3: *M. phaseolina* + *B. thuringiensis*; T4: *M. phaseolina* only (positive control); T5: healthy plants only (negative control); T6-T8: each *Bacillus* strain alone (no pathogen). Both cultivars were grown inoculated and sampled concurrently, giving a 2 (cultivar) $\times$ 8 (treatment) factorial arrangement.

2.6.3. Inoculation Timeline

The inoculation procedure followed a sequential timeline designed to allow *Bacillus* spp. to establish itself in the rhizosphere prior to pathogen challenge, consistent with protocols previously validated for PGPR biocontrol studies (Yang *et al.*, 2024; Rangel-Montoya *et al.*, 2022). Day 0 (Transplanting and First Bacterial Application): Immediately after transplanting, 50 mL of bacterial suspension (5×10^6 CFU mL⁻¹) was applied as a root-zone soil drench by pipetting the suspension slowly and uniformly around the stem base to ensure thorough infiltration into the root zone (Zhang *et al.*, 2023). Control pots received an equivalent volume (50 mL) of sterile distilled water. Day 3 (Pathogen Inoculation): *M. phaseolina* inoculum was introduced by applying 100 mL of a microsclerotia suspension (1×10^4 microsclerotia mL⁻¹ sterile distilled water) evenly around the root zone of each designated pathogen-treatment pot using a sterile graduated pipette (Pastrana *et al.*, 2016). The suspension was poured in a circular pattern at a 5 cm radius from the stem base to ensure maximum contact with the developing root system. Non-pathogen treatments received equivalent volumes of sterile distilled water. Day 10 (Second Bacterial Application): A second 50 mL dose of bacterial suspension (5×10^6 mL⁻¹ sterile distilled water) was applied. 10^6 CFU mL⁻¹) was applied as a root-zone drench to reinforce rhizosphere colonization and sustain biocontrol activity (Yang *et al.*, 2024). Controls received sterile distilled water. Disease severity and plant status were monitored every 3 days throughout the 21-day experimental period Marquez *et al.*, (2021).

2.7. In vitro Antagonism assay

Antagonistic activity was evaluated using the dual-culture assay described by Dennis and Webster (1971). A 5 mm mycelial plug taken from the advancing edge of a 7-day-old *M. phaseolina* colony was placed 20 mm from one edge of a 90 mm PDA plate, while the antagonistic bacterium (a 48-hour-old culture at a concentration of 1×10^8 CFU mL⁻¹) was streaked in a straight line 20 mm from the opposite edge, resulting in an initial separation distance of 50 mm between the two organisms. The

plates were incubated at 25 °C for 7 days in a completely randomized design. On the seventh day, the diameter of the *M. phaseolina* colony was measured in two perpendicular directions, and the mean was calculated. The percentage inhibition of radial growth (PIRG) was calculated using the following equation: $\text{PIRG (\%)} = [(CT) / C] \times 100$ where C represents the mean colony diameter in the uninoculated control treatment and T represents the mean colony diameter in the presence of the antagonistic agent. Each treatment consisted of five replicate plates, and the assay was conducted on three independent occasions.

2.8. Assessment of Disease Severity

Disease severity was evaluated at 21 dpi using the 0-4 scale of Pastrana *et al.*, (2016): 0 = no symptoms; 1 = mild chlorosis, 1-25% affected; 2 = moderate wilting, 26-50% affected; 3 = severe crown rot, 51-75% affected; 4 = plant collapse, 76-100% affected. A single disease assessment was performed at 21 dpi and all ratings were assigned by a single trained observer. The Disease Severity Index (DSI) was calculated for each replicate pot as $\text{DSI (\%)} = [\Sigma (\text{rating} \times \text{plant count}) / (\text{total plants} \times \text{maximum rating})] \times 100$ and pot values were averaged across the five replicates per treatment. The percentage disease control (PDC) was calculated as: $\text{PDC (\%)} = [(\text{DSI inoculated untreated control} - \text{DSI treatment}) / \text{DSI inoculated untreated control}] \times 100$. All DSI values are expressed as mean $\pm$ standard deviation (SD) of five pots

2.9. Extraction and Assay of PAL Activity

Leaf samples were collected to measure phenylalanine ammonia-lyase (PAL) activity at 14 days post-inoculation (14 dpi), a time point representing the sustained peak of ISR-mediated PAL induction in aerial tissues (Pieterse *et al.*, 2014; Zhang and Liu 2015). Samples were taken from the third fully expanded true leaf from the plant apex; this tissue is metabolically active and at a uniform growth stage across all plants and treatments, thereby minimizing positional variation in basal enzyme activity (Wu *et al.*, 2025; Ai *et al.*, 2025). All samples were collected between 8:00 and 10:00 AM to minimize diurnal variation in enzyme activity using five replicate pots (three plants per pot averaged to yield a single pot-level value) for each treatment. Exactly 1 g of fresh leaf tissue was collected immediately, placed on ice, and stored at -20°C until analysis. The tissue was homogenized in 3 mL of cold extraction buffer containing 50 mM sodium borate (pH 8.8), 5 mM β -mercaptoethanol, 1 mM EDTA, and 1% PVPP, followed by centrifugation at $12,000 \times g$ for 20 minutes at 4°C. PAL activity was measured spectrophotometrically according to the methods of Zucker (1965) and Zhang *et al.*, (2017) by measuring the production of trans-cinnamic acid at 290 nm after incubating the samples at 37°C for 60 minutes in a reaction mixture containing [x] mM L-phenylalanine in borate buffer (pH 8.8). Activity was expressed in nanomoles of trans-cinnamic acid per hour per

gram of fresh weight (nmol trans-cinnamic acid h⁻¹ g⁻¹ FW) using a calibration curve prepared from pure trans-cinnamic acid (Sigma-Aldrich ≥99%).

2.10. Statistical Analysis

Data were analyzed using a two-way factorial ANOVA where cultivar (Albion and Camarosa) and treatment (eight levels) were fixed factors, with the cultivar × treatment interaction included to determine whether the response of the two cultivars to the treatments differed significantly. Before performing the analysis of variance, the normality of the data distribution was checked by examining the statistical model residuals (ANOVA residuals), and the homogeneity of variances was checked using Levene's test. Means of coefficients were separated using Duncan's Multiple Range Test (DMRT) at a significance level of $P < 0.05$. All data were expressed as mean ± standard deviation (SD). All statistical analyses were performed using IBM SPSS Statistics version 26.

3. RESULTS AND DISCUSSION

3.1. Morphological and Molecular Identification of *Macrophomina phaseolina*

Fungal colonies on PDA produced flat grayish-black mycelium upon maturation. Microscopy revealed thin-walled hyaline to pale-brown septate hyphae with lateral branches at right angles constricted at origin. Microsclerotia were abundant melanized spherical to ellipsoidal (50-150 µm diameter) dark brown to black consistent with the reported morphological characteristics of *M. phaseolina* (Mihail 1992; Smith and Carvil 1997) and accord with recent pathological surveys of strawberry charcoal rot (Pedroncelli *et al.*, 2025). BLAST analysis of the ITS rDNA sequence (GenBank accession no. PX904838) yielded 99% sequence identity and ≥ 98% query coverage against verified *M. phaseolina* reference accessions (MW450822, KM979991, MT491122, MH864177, OR243962, MT491117, and OR240856) supporting the species-level identification of the isolate. Figure 1 presents the Maximum Likelihood phylogenetic tree constructed under the GTR+G substitution model selected by the Bayesian Information Criterion (BIC) in ModelTest-NG. The isolate grouped within a clade of reference accessions with 99% bootstrap support and short branch lengths indicate limited nucleotide divergence in *M. phaseolina* across several geographically diverse hosts (Gupta *et al.*, 2012).

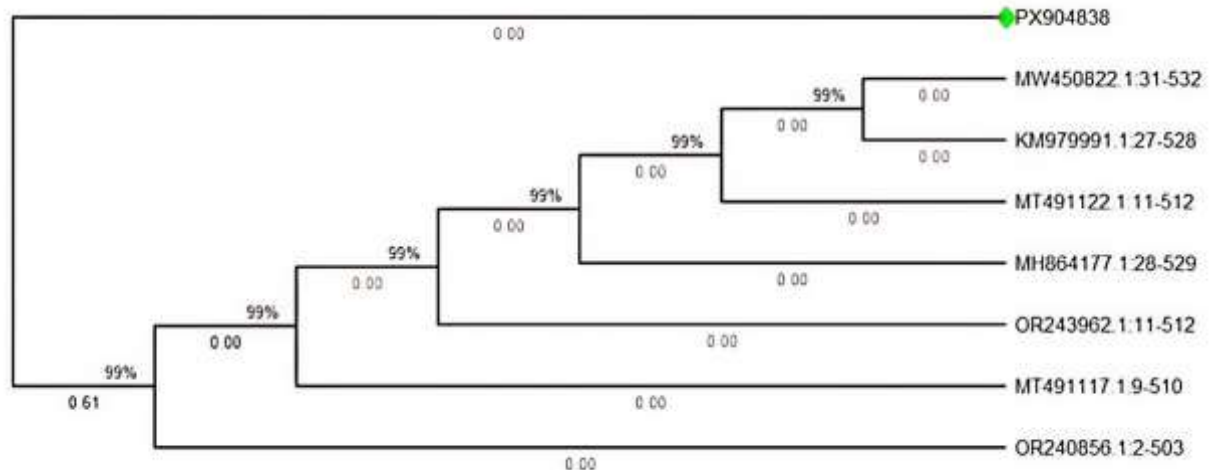


Figure 1. Maximum-likelihood phylogenetic tree showing the phylogenetic relationships of the *M. phaseolina* isolate (PX904838) with reference strains retrieved from the GenBank database. The phylogenetic tree was constructed using MEGA 12.0 software and node values represent bootstrap support (%) based on 1000 replicates.

3.2. Pathogenicity and Fulfillment of Koch's Postulates

Figure (2) indicates that the typical signs of charcoal rot, including wilting of the leaf base, vascular discoloration, and crown discoloration, first appeared at 14 dpi in some plants and developed until 21 dpi onset, therefore being asynchronous. Wilting was scored as loss of turgor beginning with the oldest leaves, crown discoloration as external browning of the crown, and vascular discoloration as brown-black staining in longitudinal sections. By the 21st day after inoculation, the infection rate was 100 percent, whereas uninoculated plants were not affected. Re-isolation from the infected crown and root tissue yielded fungal colonies morphologically similar to the original *M. phaseolina* isolate, thereby satisfying Koch's postulates. These results are consistent with the findings of Mertely *et al.*, (2005) and Lahlali *et al.*, (2022) regarding the ability of the fungus *M. phaseolina* to infect strawberries under controlled conditions



Figure 2. Characteristic symptoms of charcoal rot on strawberry plants (Albion variety) 21 days after inoculation with the fungus *M. phaseolina*. On the right, the inoculated plant shows severe rot in both the crown and roots, vascular discoloration, and general deterioration of the plant compared to the healthy, uninfected plant on the left.

3.3. *In vitro* antagonistic activity of *Bacillus* strains against *M. phaseolina*

All three *Bacillus* strains exhibited significant ($P \leq 0.05$) inhibition of *M. phaseolina* radial growth in dual-culture assays (Table 1). *B. subtilis* exhibited the highest rate of pathogen colony diameter reduction ($38.82 \pm 1.21\%$) with colony diameter decreasing to 5.20 ± 0.18 cm compared to 8.50 ± 0.14 cm in the uninoculated control sample. *B. amyloliquefaciens* and *B. thuringiensis* also showed moderate colony diameter reduction values of $30.00 \pm 1.54\%$ and $26.47 \pm 1.09\%$, respectively. All three strains were statistically distinguishable from each other and from the control sample according to Duncan's Multiple Range Test (DMRT) ($P \leq 0.05$).

Table 1. *In vitro* antifungal activity of *Bacillus* strains against *M. phaseolina* in dual culture assays on PDA.

Treatment	Colony Diameter (cm)	PIRG (%)
<i>B. subtilis</i>	5.20 ± 0.18 d	38.82 ± 1.21 a
<i>B. amyloliquefaciens</i>	5.95 ± 0.22 c	30.00 ± 1.54 b
<i>B. thuringiensis</i>	6.25 ± 0.31 b	26.47 ± 1.09 c
Control (<i>M. phaseolina</i> only)	8.50 ± 0.14 a	0 d

Different lowercase letters within a column denote significant differences according to Duncan's Multiple Range Test ($P \leq 0.05$). Data are presented as Means $\pm$ SD (n = 5 plates). PIRG = percentage inhibition of radial growth

The superior performance of *B. subtilis* may involve the reported synthesis of iturin, surfactin, and fengycin, which disrupt fungal plasma membrane integrity (Torres *et al.*, 2016). On the other hand, Rangel-Montoya *et al.* (2022) described the moderate inhibition of *B. amyloliquefaciens* by its production of chitinase and β -1,3-glucanase, which degrade fungal cell wall polysaccharides. The lower PIRG of *B. thuringiensis* is consistent with its narrower spectrum of diffusible antifungal metabolites (Palma *et al.*, 2024). None of these metabolites or enzymes were measured here, so these explanations are hypotheses drawn from the literature, and part of the inhibition may derive from formulation adjuvants. The above highlights the importance of validating biological control candidates under greenhouse conditions and realistic plant-pathogen-antagonist conditions (Yang *et al.*, 2024).



Figure 3: Dual-culture PDA plates after 7 days at $25 \pm 2^\circ\text{C}$. (A) *B. amyloliquefaciens*; (B) *B. subtilis*; (C) *B. thuringiensis* each versus *M. phaseolina*.

3.4. In Vivo Biocontrol Efficacy Against Charcoal Rot Under Greenhouse Conditions

The results presented in Table (2) indicate that the *Bacillus* treatments reduced the severity of charcoal rot disease *M. phaseolina* compared to the positive control in both the Albion and Camarosa cultivars. A clear influence of the specific isolate and cultivar on control efficacy was observed. For the Albion cultivar, the *B. amyloliquefaciens* treatment demonstrated the highest efficacy, reducing the Disease Severity Index (DSI) to 53.3% and achieving the highest Percent Disease Control (PDC) of 46.7%, followed by *B. thuringiensis* and then *B. subtilis*. Similarly, in the Camarosa cultivar, *B. amyloliquefaciens* showed the best relative performance (DSI: 66.6%; PDC: 33.4%), whereas *B. thuringiensis* was the least effective (DSI: 80.0%; PDC: 20.0%). *B. amyloliquefaciens* achieved the highest mean disease control (PDC = 40.05%) and the lowest DSI (60.0%) compared to the other treatments. Furthermore, the absence of symptoms in healthy plants (DSI = 0%) contrasted with the 100% DSI observed in the positive control, confirming the clear impact of fungal inoculation and the efficacy of the control treatments in reducing disease severity.

The differences in response between the two cultivars indicate a cultivar-treatment interaction that warrants interpretation when discussing the results.

Table 2. Effect of *Bacillus* root drench treatments on disease severity index (DSI%) and percentage of disease control (PDC%) in two strawberry cultivars (Albion and Camarosa) inoculated with *Macrophomina phaseolina* under greenhouse conditions.

Treatment	DSI%		Treatments effect	PDC%		Treatments effect
	Albion	Camarosa		Albion	Camarosa	
<i>M. phaseolina</i> + <i>B. subtilis</i>	73.3 ± 2.1b	73.3 ± 1.8 bc	73.3 ± 0.8 b	26.7± 1.41b	26.7 ± 1.8 ab	26.7 ± 0.7 b
<i>M. phaseolina</i> + <i>B.amyloliquefaciens</i>	53.3 ± 1.9 c	66.6 ± 2.3 c	60.0 ± 1.2 c	46.7 ± 1.24a	33.4 ± 1.4 a	40.05± 0.8 a
<i>M. phaseolina</i> + <i>B. thuringiensis</i>	66.6 ± 2.4 b	80.0 ± 1.6 b	73.3 ± 1.3 b	33.4 ± 1.01b	20.0 ± 1.2 b	26.7± 0.5 b
<i>M. phaseolina</i> only (+control)	100.0 a	100.0 ± 0.0 a	100 ± 0.0 a			
Healthy plants (–control)	0.0 ± 0.0 d	0.0 ± 0.0 d	0.0 ± 0.0 d			
Cultivar effect	58.64 ± 1.9 b	63.98 ± 1.4 a		35.6 ± 0.8 a	26.7 ± 0.7 b	

Different lowercase letters within each column denote significant differences (Duncan's DMRT $P \leq 0.05$). DSI (%) = $[\Sigma(\text{rating} \times \text{plant count}) / (\text{total plants} \times \text{maximum rating})] \times 100$. PDC (%) = $[(\text{DSI}_{\text{positive control}} - \text{DSI}_{\text{treatment}}) / \text{DSI}_{\text{positive control}}] \times 100$. — = not applicable. Means ± SD (n = 5 pots). DSI = disease severity index; PDC = percentage disease control.

Differences between cultivars may relate to genotype-dependent root exudate composition affecting colonization (Yang *et al.*, 2024); this was not measured and remains a hypothesis. It should be noted that the 66.8% reported by Rangel-Montoya *et al.*, (2022) represents *in vitro* radial growth inhibition, which is not directly comparable to the *in vivo* percentage disease control (PDC) of 20.0–46.7% obtained in the present greenhouse study. *In vitro* antagonism values are characteristically higher than *in planta* biocontrol efficacy due to the buffering complexity of the soil-rhizosphere environment. While direct quantification of lipopeptide bioavailability in the soil matrix was beyond the scope of this study, the observed *in vitro*-to-*in vivo* reversal is consistent with published evidence showing that soil organic matter significantly reduces the concentration of iturin A and surfactin available for antifungal activity (Rangel-Montoya *et al.*, 2022; Zhang *et al.*, 2023). Neither lipopeptide concentration nor persistence was measured here, so this interpretation remains speculative and requires direct testing. This does not hinder the possibility of obtaining similar results with the use of commercial bacterial preparations with similar conditions.

3.5. PAL-Associated Defense Activation: Phenylalanine Ammonia-Lyase (PAL) Activity

PAL enzyme activity measured 14 days post-inoculation showed differences among treatments and between the two cultivars, with response variations depending on the specific treatment and cultivar (Table 3). Regarding the effect of treatments, the *M. phaseolina* + *B. subtilis* treatment recorded the highest activity among the co-inoculations with the pathogen (30.12 nmol h⁻¹ g⁻¹ FW), followed by *M. phaseolina* + *B. amyloliquefaciens* (27.45) and *M. phaseolina* + *B. thuringiensis* (26.04), whereas plants treated with *M. phaseolina* alone exhibited the lowest activity (22.06). For individual bacterial treatments, *B. thuringiensis* recorded the highest activity (30.00), followed by *B. amyloliquefaciens* (29.07) and *B. subtilis* (28.89), while the activity in healthy, non-inoculated plants was 27.55 nmol h⁻¹ g⁻¹ FW. Regarding the cultivar effect, the mean PAL activity was 28.23 for the Albion cultivar compared to 27.06 for the Camarosa cultivar. Concerning the cultivar treatment interaction, the highest response differed between the two cultivars; the Albion cultivar exhibited the highest PAL activity when treated with *M. phaseolina* + *B. subtilis* (32.41), whereas the Camarosa cultivar showed the highest activity when treated with *B. thuringiensis* alone (32.06). Conversely, the *M. phaseolina* treatment alone resulted in the lowest PAL activity in both cultivars, recording 22.67 for Albion and 21.44 for Camarosa.

Table 3. Phenylalanine ammonia-lyase (PAL) activity (nmol trans-cinnamic acid h⁻¹g⁻¹ FW) in 14-day-old leaves of two strawberry cultivars after inoculation with various strains of *Bacillus*.

Treatments	cultivars		Treatments effect
	Albion	Camarosa	
<i>M. phaseolina</i> + <i>B. subtilis</i>	32.41 ± 1.14 a	27.83 ± 1.02 b	30.12 ± 0.54 a
<i>M. phaseolina</i> + <i>B. amyloliquefaciens</i>	28.75 ± 0.98 b	26.14 ± 0.87 b	27.45 ± 0.47 b
<i>M. phaseolina</i> + <i>B. thuringiensis</i>	26.16 ± 1.21 b	25.91 ± 0.93 b	26.04 ± 0.71b
<i>M. phaseolina</i> only	22.67 ± 0.76 c	21.44 ± 0.81 c	22.06 ± 0.85 c
<i>B. subtilis</i> only	30.18 ± 1.05 ab	27.06 ± 0.99 b	28.89 ± 0.47 ab
<i>B. amyloliquefaciens</i> only	28.44 ± 0.91 b	29.69 ± 1.11 ab	29.07 ± 0.91ab
<i>B. thuringiensis</i> only	27.93 ± 1.08 b	32.06 ± 1.24 a	30.00 ± 0.79a
Healthy plants (control)	29.32 ± 0.88 ab	25.78 ± 0.94 b	27.55 ± 0.48 b
Cultivars effects	28.23 ± 0.37 a	27.6 ± 0.48 b	

Different lowercase letters denote significant differences (Duncan's DMRT P ≤ 0.05). PAL activity expressed as nmol trans-cinnamic acid h⁻¹ g⁻¹ fresh weight (FW). Means ± SD (n = 5 pots).

These findings provide PAL-mediated biochemical evidence consistent with activation of the phenylpropanoid pathway by *Bacillus* strains. PAL is nevertheless a single indicator and cannot by itself establish ISR. This activity causes the

accumulation of lignin precursors, flavonoids, and phytoalexins. These antimicrobial agents restrain the invasion or colonization of pathogenic fungi (Zhang and Liu 2015; Li *et al.*, 2025). Jasmonic acid (JA) signaling regulates PAL and phenylpropanoid output (Joshi *et al.*, 2023; Li *et al.*, 2025), but JA was not measured here, so this remains a possible rather than a proven explanation. PAL activity in treatments with *Bacillus* alone represents readiness in metabolism, which might be related to the rapid defense mechanisms during the invasion of the pathogen. This finding provides evidence for the potential benefit of *Bacillus*-based biocontrol compared to single-mode fungicides (Yang *et al.*, 2024). It is noteworthy that the ranking of treatments by PAL activity did not fully parallel their ranking by disease control; for instance, in CV. Albion, the most effective disease-suppressing agent (*B. amyloliquefaciens*), did not record the highest PAL activity, which was instead associated with *B. subtilis*. This dissociation indicates that biocontrol efficacy in plants is a multifactorial outcome arising from the combined contributions of direct antibiosis rhizosphere competence and induced biochemical priming, rather than from PAL-associated responses alone. PAL activity should therefore be interpreted as one contributing biochemical indicator within a broader defense network, not as a sole determinant of disease outcome

4. CONCLUSION

All three preparations reduced charcoal rot severity, differing in magnitude rather than in direction. Under greenhouse conditions, *Bacillus amyloliquefaciens* and *Bacillus thuringiensis* were the most effective of the three preparations tested against charcoal rot caused by *Macrophomina phaseolina* in strawberries. Suppression was accompanied by increased PAL activity; because PAL was measured at a single time point without additional markers, the data are consistent with but do not demonstrate induced systemic resistance (ISR), and the PAL ranking did not parallel the disease-control ranking. While *B. subtilis* showed the highest *in vitro* antagonism, *B. amyloliquefaciens* consistently performed better in disease suppression under greenhouse conditions, especially in CV Albion. These results reiterate the importance of evaluating biocontrol candidates beyond preliminary *in vitro* screens. Moreover, the cultivar-dependent patterns of disease alleviation and PAL activity were associated with host genotype, suggesting genotype merits consideration when matching strains to cultivars subject to the cultivar treatment interaction (Section 3.4). These findings support further evaluation but do not yet recommend *Bacillus*-based bioagents in IPM programs; field-scale and multi-season validation is required first. Future studies should focus on *B. amyloliquefaciens*-based preparations in multi-season field efficacy combinations of multiple biocontrol agents in different soil and climatic situations, and the molecular processes that explain cultivar selective ISR to various bacterial strains.

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Conflict of Interest

The authors declare that they have no conflict of interest — whether financial, personal, professional, or scientific — with any organization or entity with a financial or non-financial interest in the subject matter or materials discussed in this manuscript.

تشبيط فطر *Macrophomina phaseolina* في الفراولة بواسطة بكتيريا *Bacillus* عبر التضاد الحيوي والتنشيط الدفاعي المرتبط بالإنزيم فينيل ألانين أمونيا لياز PAL

فراس كاظم الجبوري¹، ريان سالم محمود الحياي²، صالح احمد عيسى الجبوري³، بسام يحيى ابراهيم⁴، علاء حميد محمد⁵.
قسم وقاية النبات، كلية الزراعة والغابات، جامعة الموصل، العراق

الملخص:

يعد الفطر *Macrophomina phaseolina* المسبب لمرض تعفن الفحم عائقا رئيسيا أمام إنتاج الفراولة، وتعتمد مكافحته حاليا على مبيدات فطرية واسعة الطيف تنطوي على أخطار بيئية، لذلك تبرز بكتيريا الريزوسفير المعززة لنمو النبات كبديل مستدام. قُيِّمت هذه الدراسة فاعلية كل من *Bacillus subtilis* و *B. thuringiensis* ضد الفطر *M. phaseolina* وقدرتها على تحفيز اليات الدفاع لدى النبات المضيف، وذلك من خلال رصد نشاط إنزيم فينيل ألانين أمونيا-لاياز (PAL). جرى تقييم التضاد الحيوي مخبرياً، كما قيمت شدة المرض ونشاط الإنزيم PAL في تجارب البيت الزجاجي باستخدام صنفين من الفراولة Albion و Camarosa بوجود الفطر الممرض أو غيابه. أظهرت البكتيريا *B. subtilis* أعلى معدل تثبيط لنمو الفطر *M. phaseolina* مخبرياً إذ بلغت نسبة تثبيط النمو الشعاعي $PIRG = 38.82 \pm 1.21\%$ متفوقاً بذلك بشكل معنوي ($P \leq 0.05$) على *B. thuringiensis* و *B. amyloliquefaciens*. وفي تجارب البيت الزجاجي حققت *B. amyloliquefaciens* على الصنف Albion أفضل مستوى لمكافحة المرض حيث بلغ مؤشر شدة المرض $DSI = 53.3 \pm 1.9\%$ ونسبة مكافحة المرض $PDC = 46.7\%$ مقارنةً بمجموعة المقارنة المُلقحة بالفطر الممرض $DSI = 100\%$ ، تلتها *B. thuringiensis* ($DSI = 66.6 \pm 2.4\%$) و *B. subtilis* كانت الأقل فاعلية ($DSI = 73.3 \pm 2.1\%$ و $PDC = 26.7\%$). وفي المقابل، حفزت *B. subtilis* أعلى مستوى من نشاط الإنزيم PAL ($32.41 \pm 1.14 \text{ nmol h}^{-1} \text{ g}^{-1} \text{ FW}$) بزيادة قدرها 43.0% مقارنةً بمجموعة المقارنة المُلقحة بالفطر الممرض ($22.67 \pm 0.76 \text{ nmol h}^{-1} \text{ g}^{-1}$). وقد أدت جميع المعاملات الثلاث ببكتيريا *Bacillus* إلى زيادة معنوية ($P \leq 0.05$) في نشاط الإنزيم PAL مقارنةً بمجموعة المقارنة المُلقحة بالفطر الممرض، مما يشير إلى تنشيط استجابات الدفاع لدى النبات المضيف. وتدعم هذه النتائج دمج العوامل الحيوية القائمة على بكتيريا *Bacillus* في برامج مكافحة مرض تعفن الفحم في الفراولة.

الكلمات المفتاحية: تعفن الفحم؛ المكافحة الحيوية؛ البكتيريا المعززة لنمو النبات؛ تحفيز المقاومة الجهازية؛ *Fragaria × ananassa*.

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