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Potential Effects of *Brevibacillus* spp. in Control of Brown Spot Disease and Productivity of some Faba Bean Genotypes

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ABSTRACT

This study investigated the interaction between *Botrytis fabae*, faba bean genotypes and a promising *Brevibacillus brevis* isolate under greenhouse and field conditions in Toshka, Egypt. Several *B. fabae* isolates obtained from naturally infected foliage were compared in pathogenicity tests; isolate Bf-5 was consistently the most aggressive, causing 45% necrotic leaf area on detached leaves and was therefore used for all subsequent inoculations. Five bacterial antagonists were screened *in vitro*, and Brv-M1 showed the strongest activity, reducing radial mycelial growth of *B. fabae* by about 39% and lowering spore germination by roughly 30–35% compared with the control. Brv-M1 also produced clear halos of chitinase, β-1,3-glucanase and protease in plate assays, indicating a multi-enzymatic mode of action. GC–MS analysis of the ethyl acetate extract revealed a chemically diverse mixture of non-volatile metabolites. Under greenhouse conditions with artificial inoculation, foliar application of Brv-M1 to 17 faba bean genotypes led to moderate reductions in chocolate spot severity, generally in the range of 6–25% depending on genotype, and was accompanied by increased peroxidase, catalase and total phenolic levels in several responsive lines. In the field at Toshka, natural chocolate spot pressure remained moderate disease severity 31.7–42.5% in untreated plots, and Brv-M1 treatment lowered severity to about 22.8–39.3%, corresponding to 6–28% reduction without substantially altering disease incidence. Agronomic data across two seasons showed that a small subset of genotypes particularly G12 and G15, together with the local check Mariout 2 combined relatively low or intermediate disease levels, clear benefits from Brv-M1 treatment, and superior yield performance e.g. seed yield 51–53.5 g plant^{-1} under the hot, arid conditions of Toshka. Overall, the results indicate that Brv-M1 acts as a biologically realistic, partial suppressor of chocolate spot, mediated by a mixture of lytic enzymes and structurally diverse metabolites. The convergence of good agronomic adaptation and favourable disease response in Mariout 2, G12 and G15 highlights these genotypes as promising candidates for use in combination with Brv-M1 within integrated chocolate spot management packages. Some additional work—particularly on formulation, field performance and biosafety is still needed before Brv-M1 can be confidently integrated into faba bean integrated disease management programs.

Keywords: Faba bean, Promising genotypes, *Brevibacillus brevis*, Agronomic performance, Yield disease relationship

1. Introduction

Faba bean (*Vicia faba* L.) is a key grain legume in Mediterranean and North African agriculture, appreciated both for its rich plant-protein content and for its role in improving soil fertility and enhancing the sustainability of cropping systems. Its capacity for biological nitrogen fixation both on-farm nitrogen economy and broader environmental benefits, making the crop particularly relevant where fertilizer inputs are costly or where sustainability targets are prioritized (Raikos *et al*., 2014;

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Martineau-Côté *et al*., 2022; Jithesh *et al*., 2024). Despite its importance, faba bean productivity is frequently constrained by the combined pressure of abiotic stresses and destructive foliar diseases. In newly reclaimed desert environments in southern Egypt such as Toshka heat stress is an increasingly decisive limitation, particularly when high temperatures coincide with sensitive phenological stages and reduce pod set and seed filling. These conditions can depress yield directly and reshape host defence capacity, increasing the likelihood of more severe disease expression (Hasanuzzaman *et al*., 2013; Maalouf *et al*., 2022).

Brown spot like foliar symptoms in faba bean are often part of a spotting complex in which necrotrophic fungi can play major roles. In many production regions, chocolate spot caused primarily by *Botrytis fabae* and sometimes *B. cinerea* is widely recognized as one of the most consequential foliar diseases because lesions can expand rapidly, reduce effective leaf area, and trigger premature senescence under conducive conditions (Wakoya, and Abdissa, 2022; Webb *et al*., 2024). This disease pressure becomes more problematic when plants are simultaneously exposed to heat stress an interaction that can amplify damage and undermine yield stability. Conventional fungicide-based management can provide suppression in some contexts, yet it is frequently constrained by variable field performance, cost, residue considerations, and the longer-term risk of reduced sensitivity under repeated applications. Consequently, there is strong interest in integrating biologically based approaches that are compatible with sustainable intensification and can perform under harsh environments. Root- and plant-associated bacterial bioprotectants can mitigate disease through direct antagonism (antibiosis, lytic enzymes, competition) and/or by activating host defence pathways such as induced systemic resistance (ISR), providing multi-layered protection beyond single-target chemistry (Zhu *et al*., 2022; Egamberdieva *et al*., 2023; Collinge, 2022). Within this landscape, spore-forming bacteria are attractive candidates for field deployment because of their ecological robustness and formulation potential. The genus *Brevibacillus* including *B. brevis* has been repeatedly associated with the production of antimicrobial peptides/lipopeptides and other bioactive metabolites, and several studies have documented antagonistic activity against plant-pathogenic fungi along with traits relevant to stress (Singh *et al*., 2021; Yang *et al*., 2023; Jähne *et al.*, 2023). Such features are especially relevant for newly reclaimed lands where high temperature, low organic matter, and fluctuating moisture can reduce the reliability of less resilient biological inputs. However, two practical gaps remain critical for decision-making in newly reclaimed, heat-stressed environments. First, the efficacy of candidate antagonists must be validated under realistic conditions rather than inferred from *in vitro* inhibition alone. Second, biological control outcomes are often genotype-dependent: host genetic background can influence disease development, rhizosphere competence, and the degree to which biotic treatments translate into yield protection. Therefore, integrating genotype screening with biocontrol evaluation is essential when the objective is not only disease reduction, but also identification of production-ready materials for a specific agroecological zone.

Accordingly, this study aimed to evaluate seventeen faba bean genotypes (sixteen inbred lines and the local cultivar Mariout 2) for their response to brown spot under heat-stress conditions; identify bacterial isolates with antagonistic activity against the causal fungal pathogen associated with brown spot symptoms; quantify the impact of *Brevibacillus spp.*-based biotreatments on disease suppression and on the disease response across genotypes; and select the best-performing genotypes for Toshka conditions based on combined performance in disease tolerance/resistance, heat-stress adaptation, and productivity. This integrated framework is designed to support practical, sustainable disease management and genotype recommendation for newly reclaimed lands.

2. Materials and Methods

2.1. Plant materials and experimental site

Seventeen faba bean (*Vicia faba* L.) genotypes, including sixteen inbred lines and the local cultivar Mariout 2, were used in this study. The genotypes were evaluated for their response to brown spot disease under heat-stress conditions representative of newly reclaimed lands in southern Egypt. Field performance data were obtained from experiments conducted under Toshka conditions, characterized by high temperature regimes and typical desert soil properties.

2.2. Isolation and Pathogenicity Assessment of *Botrytis fabae*

2.2.1. Isolation and purification of the causal fungal pathogen

Naturally infected faba bean leaves exhibiting typical brown spot symptoms were collected from multiple faba bean-growing locations. Small sections from the advancing margins of necrotic lesions were surface sterilized using standard protocols and plated onto potato dextrose agar (PDA). Plates were incubated at 25 ± 1 °C, and emerging fungal colonies were subcultured to obtain pure isolates.
Fungal isolates were initially identified based on cultural and morphological characteristics following standard mycological keys. The pathogen was confirmed as *Botrytis fabae* based on colony morphology, sporulation pattern, and microscopic features consistent with previous descriptions (Williamson *et al*., 2007).

2.2.2. Pathogenicity assessment and selection of the most virulent isolate

The pathogenicity of *B. fabae* isolates was evaluated under laboratory conditions using the detached leaf assay. Healthy, fully expanded leaves were excised from faba bean plants, surface-sterilized, and placed in moist chambers. Leaves were inoculated with mycelial plugs or spore suspensions of individual isolates, while control leaves received sterile agar plugs and distilled water.
Disease severity was assessed after incubation at 20–22 °C using a visual rating scale based on lesion size and necrotic area. Reisolation of the pathogen from symptomatic tissues was performed to fulfill Koch's postulates. The isolate exhibiting the highest disease severity and consistent symptom development was selected for subsequent experiments (Brauna-Morzˇevska *et al.,* 2023).

2.3. Isolation and selection of antagonistic *Brevibacillus* spp. isolates

2.3.1. Isolation of antagonistic bacterial isolates

Bacterial isolates were recovered from plant-associated and soil samples collected from faba bean fields. Samples were serially diluted and plated on nutrient agar, followed by incubation at 30 °C. Spore-forming bacterial colonies with distinct morphological characteristics were purified and preserved for further screening.

Based on preliminary characterization, selected isolates were assigned to the genus *Brevibacillus*. Final molecular identification of the most effective isolate was performed using 16S rRNA gene sequencing according to Syed-Ab-Rahman *et al*., (2018).

2.3.2. *In vitro* antagonistic activity of *Brevibacillus* spp

The antagonistic potential of *Brevibacillus* isolates against *B. fabae* was evaluated using the dual culture assay on PDA. A mycelial disc of *B. fabae* was placed at the center of each plate, while bacterial isolates were streaked at a fixed distance from the fungal inoculum. Plates were incubated at 25 °C, and fungal growth inhibition was measured after a defined incubation period.

Percentage inhibition of radial growth was calculated relative to the control treatment. The bacterial isolate exhibiting the highest and most consistent antagonistic activity was selected for subsequent experiments (Dennis, and Webster, 1971; Köhl *et al*., 2019).

2.3.3. Cell-free filtrate assay on early fungal development

To further investigate the antifungal potential of bacterial metabolites, the effect of the cell-free culture filtrate on early mycelial development of *B. fabae* was assessed. Aliquots of the sterile bacterial filtrate were incorporated into potato dextrose agar at defined proportions before solidification. Mycelial plugs of *B. fabae* were placed at the center of each plate, while control plates contained unamended medium.

Plates were incubated at 25 ± 1 °C, and radial fungal growth was recorded at regular intervals. The inhibitory effect of the filtrate was expressed as a percentage reduction in mycelial growth relative to the control. This assay provided additional evidence for the role of diffusible antimicrobial compounds in the antagonistic activity of the *Brevibacillus* isolate (Khan *et al*. 2014; Zaidi *et al*. 2015).

2.3.4. Effect of bacterial culture filtrates on spore germination of *B. fabae.*

To investigate the antifungal potential of Brv-M1 (Brevibacillus spp.), a spore germination bioassay was conducted using its sterile culture filtrate. The bacterial isolate was grown in nutrient

broth at 28 ± 2 °C for 48 hours under continuous shaking at 150 rpm. Post incubation, cultures were centrifuged at 10,000 rpm for 10 minutes to separate bacterial cells. The supernatant was then passed through a 0.22 µm syringe filter to obtain a clear, cell-free filtrate (Jackson *et al.,* 1994).

Different concentrations of the filtrate (2.5%, 5%, 10%, 15%, and 20%) were prepared by dilution with sterile distilled water. Each concentration was mixed in equal volume with a freshly prepared *B. fabae* conidial suspension (10^5 conidia/mL). A drop from each treatment was placed on a sterile cavity slide and incubated inside a humid chamber at 25 °C for 24 hours. Spore germination was observed under a compound microscope. Conidia were considered germinated if the germ tube was longer than half the spore's length. Percent germination and inhibition were calculated relative to the untreated control. All treatments were replicated three times.

2.4. Assessment of extracellular enzyme activities (chitinase, protease, β-1,3-glucanase)

The production of extracellular lytic enzymes by the selected *Brevibacillus* isolate was evaluated to investigate potential mechanisms involved in fungal cell wall degradation.

Chitinase activity: Chitinase production was assessed using agar media supplemented with colloidal chitin as the sole carbon source. The bacterial isolate was spot-inoculated onto the medium and incubated at 30 °C. The appearance of clear halos surrounding bacterial colonies indicated chitin degradation. Enzymatic activity was estimated by measuring the diameter of the clearance zone relative to colony growth (Kumar *et al*., 2012).

β-1,3-glucanase activity was evaluated using agar media containing laminarin as a substrate. Following incubation, plates were flooded with an appropriate indicator solution to visualize zones of glucan hydrolysis. Clear zones around bacterial growth were recorded as evidence of enzyme production (Kumar *et al*., 2012).

Protease activity was determined using skim milk agar plates. The bacterial isolate was inoculated onto the medium and incubated at 30 °C. Proteolytic activity was indicated by the formation of transparent zones around colonies resulting from casein degradation. These assays were conducted to assess the enzymatic arsenal potentially contributing to the antagonistic action of *Brevibacillus* spp. against *B. fabae* (Kumar *et al*., 2012).

2.5. Gas Chromatography-Mass Spectrometry (GC–MS) Analysis of Non-Volatile Compounds

To identify the non-volatile bioactive compounds responsible for the antifungal activity of the most effective bacterial isolate (*Brevibacillus brevis*), a GC–MS analysis was conducted on the culture filtrate extract. The bacterial isolate was cultivated by inoculating two 5 mm agar plugs into 500 mL Erlenmeyer flasks containing potato dextrose broth (PDB). The cultures were incubated at 38 °C for 10 days at 100 rpm to maximize metabolite production. Post incubation, the broth was vacuum-filtered using Whatman filter paper, and the resulting filtrate was stored at 4 °C for 24 hours. Extraction of metabolites was carried out three times using ethyl acetate at a 1:1 ratio. The combined organic layers were dried over anhydrous magnesium sulfate ($MgSO_4$), filtered, and evaporated under reduced pressure at 35 °C. The dried residues were re-dissolved in 10% dimethyl sulfoxide (DMSO) and stored at −20 °C for subsequent analysis (Medeiros *et al*., 2028).

GC–MS analysis was performed using an Agilent 6890 gas chromatograph equipped with a ZB-SMS column (30 m × 0.32 mm, 0.25 µm film thickness), connected to a Jeol JMS-600H mass spectrometer operating in EI mode (70 eV). The temperature of the ion source was maintained at 50 °C. The compounds were identified by comparing their retention times and mass spectra to those available in the NIST Mass Spectral Library (NIST #: 352898).

2.6. Greenhouse Assessment of Disease Severity and Induced Biochemical Defences in Faba Bean

2.6.1. Disease incidence and severity in faba bean genotypes

A greenhouse experiment was conducted to evaluate the response of faba bean (*Vicia faba* L.) genotypes to chocolate spot disease caused by *Botrytis fabae* under controlled conditions. Seeds of the seventeen genotypes were sown in plastic pots containing sterilized growth medium and maintained

under greenhouse conditions optimized for faba bean growth. All plants were irrigated and managed uniformly throughout the experiment to reduce non-target variation among treatments.

In addition to the untreated control, a second set of plants for each genotype was assigned to a biological control treatment, in which foliage was sprayed with a suspension of the selected *Brevibacillus* isolate Brv-M1 (≈10^8 CFU mL^{-1}, adjusted by optical density at 600 nm) until run-off. Bacterial spraying was carried out 48 h before inoculation with *B. fabae* to allow sufficient time for potential induction of plant defence responses. Control plants received sprays of sterile nutrient broth or distilled water only.

Artificial inoculation was performed using the previously selected highly virulent isolate of *B. fabae* at a defined vegetative growth stage, when plants were sufficiently developed to ensure consistent and reliable symptom expression. A spore suspension was prepared from actively sporulating fungal cultures and adjusted to a standardized concentration. Inoculation was carried out by uniformly spraying the foliage until runoff. Control plants were sprayed with sterile distilled water. After inoculation, plants were maintained under conditions favorable for disease development, including high relative humidity and moderate temperatures, to promote infection and symptom expression.

Disease incidence was assessed at regular intervals following inoculation. Disease incidence and severity were assessed at three distinct time points: 21, 28, and 35 days post inoculation (DPI), in alignment with established greenhouse protocols for chocolate spot disease in faba bean (Campbell and Neher 1994), relative to the total number of plants evaluated for each genotype, using the following equation:

$$Disease\ incidence\ (\%) = \frac{Number\ of\ infected\ plants}{Total\ number\ of\ plants\ assessed}\ x\ 100$$

Disease severity was evaluated using a standardized rating scale based on the number, size, and extent of necrotic lesions on leaves. The disease severity index (DSI) for each genotype was calculated according to the following formula:

$$\text{Disease severity (\%)} = \frac{\sum(n \times v)}{N \times V} \times 100$$

where n represents the number of leaves (or plants) in each disease category, v is the numerical value of the corresponding disease rating, N is the total number of leaves (or plants) assessed, and V is the maximum disease rating value.

Based on the calculated disease severity indices, genotypes were classified into resistant, moderately resistant, or susceptible categories. This greenhouse evaluation provided a controlled and reproducible framework for comparing host responses to *B. fabae*, enabling a clear linkage between laboratory pathogenicity assays and genotype-dependent disease expression under uniform infection pressure, while minimizing environmental variability typically encountered under field conditions (Campbell and Neher 1994).

2.6.2 Enzymatic Activities of Peroxidase and Catalase

To evaluate whether bacterial treatments induced defense-related responses in faba bean plants, the activities of polyphenol oxidase (PPO) and peroxidase (POD) were determined. Leaf samples were collected from treated and untreated plants at defined time points following inoculation with *B. fabae*. Samples were immediately frozen and processed for enzyme extraction.

Enzyme activities were determined spectrophotometrically using standard assay procedures based on substrate oxidation reactions. PPO activity was estimated by monitoring the rate of catechol oxidation, while POD activity was measured using guaiacol as a substrate in the presence of hydrogen peroxide. Enzyme activities were expressed on a fresh weight basis and compared among treatments to assess induced resistance responses (Bouraoui *et al*., 2024).

2.6.3 Determination of total phenolic content

Total phenolic compounds were quantified to evaluate their potential contribution to disease resistance. Leaf tissues were homogenized and extracted using an appropriate solvent system. Total phenolic content was determined using a colorimetric assay based on the Folin Ciocalteu reagent. Absorbance was measured spectrophotometrically, and phenolic content was expressed as gallic acid equivalents per unit fresh weight. Differences among treatments were used to infer the involvement of phenolic compounds in plant defence against *B. fabae* (Lucas *et al*., 2022).

2.7. Field assessment of chocolate spot and agronomic performance in faba bean genotypes

Field trials were conducted at the Toshka Research Station, Desert Research Center, Aswan Governorate, Egypt, during two successive growing seasons (2022–2023 and 2023–2024). The experimental site represents newly reclaimed desert land characterized by sandy soil texture and exposure to high temperature regimes. A drip irrigation system was employed throughout both seasons to ensure uniform water supply under arid conditions.

Seventeen faba bean (*Vicia faba* L.) genotypes, including sixteen inbred lines and the local cultivar Mariout 2, were evaluated under field conditions to assess their performance under combined heat stress and natural disease pressure. The experiment was laid out in a randomized complete block design (RCBD) with three replications in each season. Each genotype was planted in two rows, 4 m in length, with 50 cm spacing between rows and 20 cm between hills within the row. All recommended agronomic and cultural practices for faba bean production were uniformly applied during both growing seasons.

2.7.1 Field assessment of chocolate spot disease

Chocolate spot disease caused by *Botrytis fabae* developed naturally under field conditions. Disease assessments were conducted at appropriate growth stages when symptoms were clearly expressed. Ten guarded plants were randomly selected from each plot for disease evaluation (El-Absi *et al.,* 2024).

Disease incidence was calculated as the percentage of infected plants relative to the total number of examined plants using the following equation:

$$Disease\ incidence\ (\%) = \frac{Number\ of\ infected\ plants}{Total\ number\ of\ plants\ assessed}\ x\ 100$$

Disease severity was assessed visually using a standardized rating scale based on the extent of necrotic lesions on leaves. The disease severity index (DSI) was calculated according to the following formula:

$$\text{Disease severity (\%)} = \frac{\sum(n \times v)}{N \times V} \times 100$$

where n is the number of plants falling into each disease category, v is the numerical value of the corresponding disease rating, N is the total number of evaluated plants, and V is the maximum disease rating value. Based on severity values, genotypes were classified into resistant, moderately resistant, or susceptible categories.

2.7.2 Growth and yield-related measurements

To evaluate agronomic performance under Toshka conditions, growth and yield-related traits were recorded at physiological maturity. Measurements were taken from ten guarded plants per genotype in each replication and averaged. The recorded parameters included plant height (cm), height of the first pod (cm), number of branches per plant, number of pods per plant, number of seeds per pod, seed weight per pod (g), 100-seed weight (g), biological yield per plant (g), and seed yield per plant (g).
These traits were selected to provide an integrated assessment of genotype adaptation, productivity, and stability under combined abiotic (heat stress) and biotic (chocolate spot disease) stress conditions. Yield-related parameters were further used to infer the indirect impact of disease severity on productivity and to identify genotypes combining acceptable yield potential with reduced disease expression (El-Absi *et al*., 2024).

2.8. Statistical analysis

Data from each season were subjected to separate analyses of variance (ANOVA) to evaluate genotype effects. A combined analysis across the two seasons was performed whenever homogeneity of variance was confirmed using Bartlett's test. Mean comparisons were conducted using the least significant difference (LSD) test at the 5% probability level. Statistical procedures followed standard biometrical methods for agricultural experiments (Gomez and Gomez,1984).

3. Results and Discussion

3.1. Pathogenicity assessment and selection of the most virulent *Botrytis fabae* isolate

3.1.1. Isolation and purification of the causal fungal pathogen

Faba bean leaves naturally infected with chocolate spot symptoms were collected from multiple field sites. Small sections (approximately 0.5 cm) containing both healthy and diseased tissue margins were surface-sterilized and plated onto potato dextrose agar (PDA) in 9 cm Petri dishes. Plates were incubated at 20 ± 2 °C in the dark for several days. Emerging fungal colonies with morphological features characteristic of *Botrytis* were sub-cultured to ensure purity using hyphal tip transfer or single spore isolation. For sporulation, purified isolates were grown on faba bean dextrose agar (FBDA), a semi-selective medium known to enhance condition in *Botrytis* species (Brauna-Morževska *et al*., 2023). Cultures were incubated under alternating light and dark conditions (12 h/12 h) at 20 ± 2 °C to promote sporulation. Fully sporulated cultures were preserved under refrigerated conditions for downstream applications. Fungal identification was based on morphological characteristics including colony colour, texture, conidiophore structure, and spore morphology, and confirmed as *Botrytis fabae*.

3.2. Pathogenicity assessment and selection of the most virulent isolate

Several isolates of *B. fabae* were evaluated for pathogenicity using a detached leaf assay. Healthy faba bean leaves were placed in moist chambers and inoculated with spore suspensions prepared from 7–10-day-old sporulating cultures. Disease severity was recorded based on the expansion of necrotic lesions over a period of 5–6 days post-inoculation (Koopman *et al*., 2022).

Although all tested isolates caused visible symptoms typical of chocolate spot, the level of aggressiveness varied notably. One isolate, designated Bf-5, consistently caused more extensive necrosis, with a mean disease severity of approximately 45%, as measured by necrotic leaf area. Other isolates exhibited lower pathogenic potential, with severity values generally ranging between 20–35%.

These findings align with previous reports highlighting intraspecific variability in *B. fabae* virulence, influenced by environmental adaptation, sporulation efficiency, and possibly differential toxin production (Filippetti and Ricciardi 1993). The selection of isolate Bf-5 as the most virulent strain was therefore justified for use in subsequent artificial inoculation experiments, including greenhouse screening of genotypes and biocontrol efficacy evaluation. Its consistent performance ensures uniform disease pressure across different experimental settings and supports reliable genotype differentiation.

3.3. Isolation and selection of antagonistic bacterial isolates

3.3.1. *In vitro* antagonistic activity of bacterial against *Botrytis fabae*

The dual culture assay performed on PDA medium revealed clear differences in antifungal activity among the five tested bacterial isolates against the most virulent *Botrytis fabae* strain. These included field-derived isolates and three strains sourced from the Microbial Resources Center (MIRCEN) at Ain Shams University.

Among all tested strains, the MIRCEN-derived *Brevibacillus* isolate, designated Brv-M1, consistently exhibited the strongest antagonistic performance across repeated trials. It achieved an average inhibition of 49% in radial growth of *B. fabae*, forming a broad and uniform inhibition zone around the bacterial streak. Other isolates showed moderate to weak inhibition ranging from 27% to 38%, with *Pseudomonas* strains generally demonstrating lower efficacy (Table 1).

The superior performance of Brv-M1 is likely attributable to its robust production of antifungal metabolites, including lipopeptides and cell wall–degrading enzymes, which have been well documented in other *Brevibacillus* strains with biocontrol potential (Singh *et al*., 2021; Köhl *et al*.,

2019). The isolate was therefore selected for all subsequent experiments both *in vitro* and *in vivo* including greenhouse and field assessments. This outcome confirms previous findings suggesting that even within a single bacterial genus, antagonistic efficacy can vary widely depending on strain origin and metabolic activity. The inclusion of a MIRCEN reference isolate in the screening process strengthened the comparative evaluation and enabled the identification of a more reliable biocontrol candidate (Prashanthi *et al*., 2021).

Table 1: Inhibition of *Botrytis fabae* radial growth by different bacterial isolates in dual culture assay

Bacterial isolate	Genus	Inhibition of fungal growth (%)	Notes
Brv-M1 (MIRCEN)	*Brevibacillus*	49%	Highest inhibition – selected isolate
Brv-F1	*Brevibacillus*	38%	Moderate inhibition
Ps-1	*Pseudomonas*	27%	Low inhibition
Ps-2	*Pseudomonas*	31%	Mild inhibition
Brv-F3	*Brevibacillus*	34%	Moderate inhibition
Control	—	0%	Unrestricted fungal growth

3.3.2 Suppression of early mycelial development of *Botrytis fabae* by cell-free filtrates of bacterial isolates

To further validate the antifungal capabilities of the tested bacterial isolates, their cell-free culture filtrates were evaluated for effects on the early mycelial growth of *Botrytis fabae*. The results revealed clear differences among isolates in their ability to suppress fungal colony expansion when diffusible metabolites were incorporated into PDA medium.

Among the five selected isolates, Brv-M1 (*Brevibacillus* spp.) consistently produced the strongest inhibition. Fungal colonies grown on Brv-M1-amended media were notably smaller, thinner in density, and exhibited irregular margins compared to the control and other treatments. The filtrates of the other isolates also caused visible reductions in fungal growth, though to lesser degrees.

After five days of incubation, the radial growth of *B. fabae* in the control treatment reached 72.1 mm. By contrast, growth on Brv-M1-treated plates was reduced to 43.9 mm, equivalent to a 39.1% inhibition. The next most effective isolate (*Pseudomonas* Ps-2) resulted in 45.6 mm of growth (36.7% inhibition), followed by *Brevibacillus* Brv-F3 (48.1 mm, 33.3%) and *Pseudomonas* Ps-1 (51.2 mm, 29.0%) (Table 2). These findings reinforce earlier results from spore germination and VOC inhibition assays, further suggesting that diffusible metabolites secreted by Brv-M1likely including lipopeptides, organic acids, and enzymes play a substantial role in suppressing *B. fabae* even in the absence of direct microbial contact. Similar conclusions were drawn in previous studies on the role of bacterial filtrates in disease suppression (Yuan *et al*., 2012; Chaves-López *et al*., 2015; Köhl *et al*., 2019; Mülner *et al*., 2020; dos Santos *et al*., 2023)

Table 2. Effect of cell-free culture filtrates on *B. fabae* mycelial growth (after 5 days at 25 ± 1 °C)

Bacterial isolate	Radial growth (mm)	Inhibition (%)
Brv M1 (Brevibacillus)	43.9 ± 1.5 a	39.1%
Ps 2 (Pseudomonas)	45.6 ± 1.7 ab	36.7%
Brv F3 (Brevibacillus)	48.1 ± 1.6 b	33.3%
Ps 1 (Pseudomonas)	51.2 ± 1.9 c	29.0%
Control (no bacterial filtrate)	72.1 ± 2.4 d	0%

3.3.3 Effect of bacterial culture filtrates on *Botrytis fabae* spore germination

The in vitro evaluation of cell-free culture filtrates from four bacterial isolates revealed notable differences in their ability to suppress the germination of *Botrytis fabae* conidia. All treatments resulted in significantly lower germination rates compared to the control ($p \leq 0.05$), but the degree of

inhibition varied among isolates, as shown in Fig.1, the isolate Brv-M1 (*Brevibacillus*) exhibited the strongest antifungal activity, reducing spore germination from 93.6% in the control to 44.4%, corresponding to an inhibition rate of 52.5%. Microscopic observation revealed that most conidia either failed to germinate or produced distorted, underdeveloped germ tubes.

The *Pseudomonas* isolate Ps-2 also demonstrated considerable inhibitory activity, with germination reduced to 49.0%, while Brv-F3 (a second *Brevibacillus* isolate) and Ps-1 caused moderate reductions to 55.7% and 60.0%, respectively.

Statistical analysis confirmed significant differences between treatments based on Duncan's multiple range test at the 5% level, supporting the superiority of Brv-M1 as the most effective antagonist in this assay. These results suggest that the antifungal efficacy of these isolates is likely linked to diffusible secondary metabolites, such as lipopeptides, siderophores, and extracellular enzymes, which interfere with early fungal development. Based on these laboratory screenings (Unagul *et al.,* 2005; Saha *et al*., 2008; Yetayew, 2017), Brv-M1 was selected for molecular identification and further evaluation under greenhouse and field conditions to assess its biocontrol performance in more complex environments.

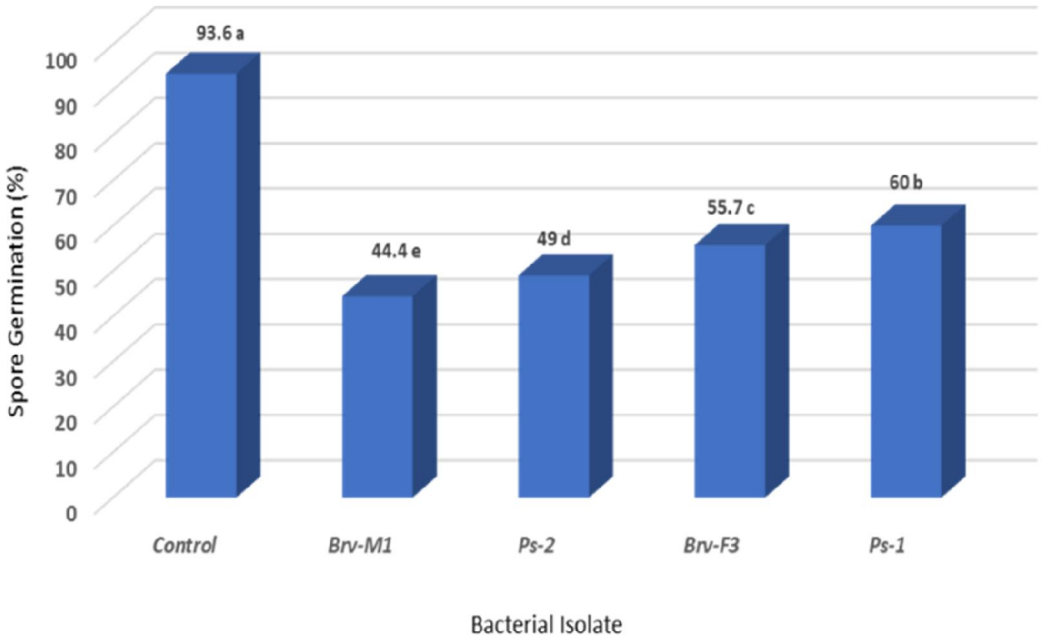

Fig. 1 Effect of different bacterial culture filtrates on *B. fabae* spore germination

3.3.4. Assessment of extracellular enzyme activities by Brevibacillus Brv-M1

To explore the enzymatic mechanisms underlying the antagonistic activity of *Brevibacillus Brv-M1*, qualitative assays were conducted to detect the secretion of hydrolytic enzymes targeting the fungal cell wall components. The bacterial isolate was cultured on media specifically designed to detect the production of chitinase, β-1,3-glucanase, and protease enzymes. Following incubation at 28 °C for 3–5 days, the appearance of clear halos around bacterial colonies served as an indicator of enzymatic activity.

The results revealed that Brv-M1 produces a diverse set of extracellular enzymes associated with fungal cell wall degradation. Notably, protease activity resulted in the formation of the widest clearance zones, averaging 15.9 mm in diameter, suggesting significant protein-degrading capacity. Chitinase and β-1,3-glucanase activities were also evident, with inhibition zones averaging 13.3 mm and 10.4 mm, respectively (Table 3). These enzymes are known to target essential structural components of fungal cell walls such as chitin and β-glucans, contributing to the breakdown of fungal integrity and impeding growth and infection processes. These findings align with previous studies that reported the involvement of such enzymes in the biocontrol arsenal of antagonistic bacteria. For instance, it has been shown that bacterial genera like *Bacillus* and *Paenibacillus* secrete hydrolytic

enzymes that compromise fungal defences and facilitate mycelial disintegration (Jackson *et al*., 2013; Ling Luo 2017).

The simultaneous production of multiple lytic enzymes by Brv-M1 points to a multi-targeted strategy of fungal suppression, where enzymatic degradation acts synergistically with other antifungal mechanisms. This enzymatic profile reinforces the potential of Brv-M1 as an effective biocontrol agent against *Botrytis fabae*, especially in integrated disease management programs.

Table 3. Lytic Enzyme Activity of *Brevibacillus Brv-M1* Against *Botrytis fabae*

Assay parameter	Brv-M1 treatment	Brv-M1 treatment	Indicators
***Chitinase* zone (mm)**	13.3 ± 0.9 a	Clear zone observed	Indicates chitin ydrolysis
β 1,3 *glucanase* zone (mm)	10.4 ± 0.8 a	Halo around colonies	Suggests glucan degradation
***Protease* zone (mm)**	15.9 ± 1.0 a	Pronounced transparent zone	High proteolytic potential

3.3.5. Effect of Brv-M1 cell-free culture filtrate on spore germination of *Botrytis fabae*

The results demonstrated that Brv-M1 culture filtrate had a dose-dependent suppressive effect on *B. fabae* spore germination. Even at the lowest concentration (2.5%), a modest reduction was observed. The strongest inhibition occurred at 20%, where spore germination dropped to 61.3%, compared to 92.9% in the control equivalent to an inhibition rate of 33.9% (Fig.2).

Microscopic examination revealed that several conidia in treated samples failed to germinate, while others exhibited stunted or malformed germ tubes, implying a disruption in early developmental processes.

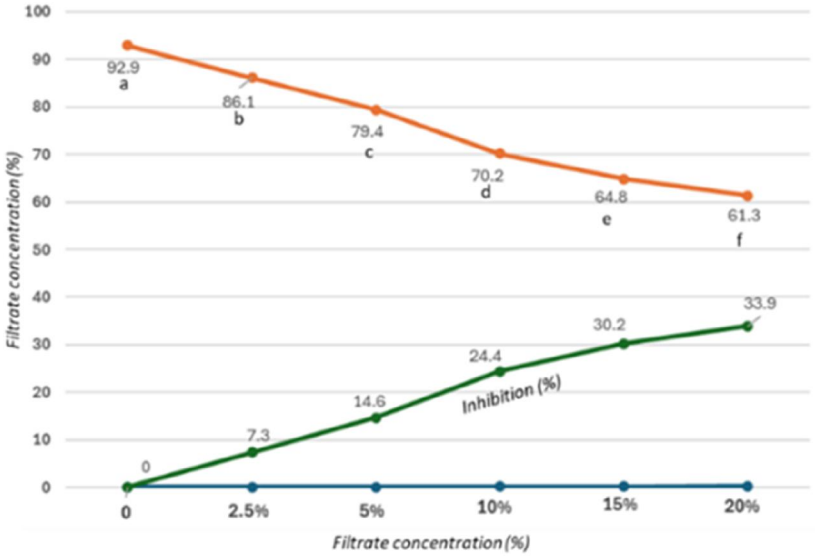

Fig 2. Effect of filtrate concentration of Brv-M1 culture filtrate on *B. fabae* spore germination

The reduction in *B. fabae* spore germination by Brv-M1 filtrate demonstrates the potential of this isolate to produce antifungal metabolites with inhibitory effects on early fungal development. The inhibition increased with concentration, suggesting a direct correlation with the quantity of bioactive compounds present in the filtrate (Oliveira *et al*., 2015; Morcillo *et al*., 2022)

These findings are consistent with previous reports indicating that cell-free filtrates from antagonistic bacteria, such as *Bacillus* and *Brevibacillus* spp., can suppress spore germination and mycelial growth through the production of lipopeptides, proteases, chitinases, and siderophores. Moreover, the microscopic deformation of germ tubes is consistent with earlier findings which attributed such effects to extracellular compounds that compromise cell wall integrity or interfere with signalling pathways essential for germ tube elongation. Despite the modest inhibition at low concentrations, the cumulative impact at higher doses suggests that Brv-M1 could serve as a viable

candidate in integrated disease management programs (Pellegrini *et al*., 2020; Baroja-Fernández *et al*., 2021; Morcillo *et al*., 2022).. Future studies should focus on identifying the exact nature of these antifungal compounds and verifying their stability and effectiveness under greenhouse and field conditions.

3.4. GC–MS identification of bioactive metabolites of *Brevibacillus brevis* Brv-M1

GC–MS profiling of the ethyl acetate extract obtained from the most effective *Brevibacillus brevis* isolate revealed a chemically diverse set of secondary metabolites. Ten major compounds were identified based on retention time, mass spectral matching, and relative peak area percentage. These compounds belong to different chemical classes, including phosphonate derivatives, long-chain alcohols, hydrocarbons, halogenated compounds, and porphyrin metal complexes (Table 4).

Among the most abundant constituents was phosphonic acid derivative (dioctadecyl ester), representing a considerable proportion of the detected extract. Long-chain alcohols such as 1-dodecanol and 1-pentadecanol were also present in measurable amounts. Additionally, cyclohexadecane was identified as one of the dominant hydrocarbon components and metal-porphyrin complexes like (2,2-dibenzyloxy-3-nitro-5,10,15,20-tetraphenyl-2,3-dihydroporphyrinato) copper (II). The diversity of detected compounds suggests that the antifungal activity observed in vitro and under greenhouse conditions is unlikely to be attributed to a single metabolite, but rather to a combination of bioactive molecules with complementary modes of action.

Table 4. Major Non-Volatile Bioactive Compounds Detected in GC–MS Analysis of *Brevibacillus brevis* Extract

No.	Compound name	Retention time (min)	Area %	Molecular formula	Molecular weight (g/mol)	Reported activity
1	(2,2Dibenzyloxy3nitro5,10,15,20tetraphenyl2,3dihydroporphyrinato) copper(II)	10.37	0.75	$C_{58}H_{40}CuN_5O_2$	901	Porphyrin–metal complex, antimicrobial
2	3Methyl1,3pentadie n5ol	19.09	3.15	$C_6H_{10}O$	98	Unsaturated alcohol, antimicrobial
3	1Dodecanol (CAS)	23.86	5.93	$C_{12}H_{26}O$	186	Long-chain alcohol, antifungal
4	Phosphonic acid, dioctadecyl ester	28.16	12.39	$C_{36}H_{75}O_3P$	586	Antimicrobial, membrane-active
5	Cyclohexadecane	32.07	10.87	$C_{16}H_{32}$	224	Hydrocarbon, antimicrobial potential
6	Silane, trichloroeicosyl	35.63	7.03	$C_{20}H_{41}Cl_3Si$	414	Surfactant, membrane-disruptive
7	1Pentadecanol	38.91	1.85	$C_{15}H_{32}O$	228	Long-chain alcohol, antimicrobial
8	1Acetyl4,4bis [4(3bromopropoxy)3,5dimethoxyphenyl] piperidine	41.56	1.22	$C_{29}H_{39}Br_2NO_7$	671	Brominated aromatic, potential biocide
9	Dimethyl2, anti4,anti9,12, anti14pentabromodecacyclo [9.9.0.0(,8).0(2,12).0(6,10). 0(11,18)0(13,1).0(16,20)] ,syn9dicarboxylate	48.23	0.79	$C_{24}H_{23}Br_5O_4$	770	Halogenated compound, antimicrobial
10	Dichloro (5,10,15,20tetraphenylporphyrinato) vanadium	54.90	1.50	$C_{44}H_{28}Cl_2N_4V$	733	Porphyrin–metal complex, bioactive

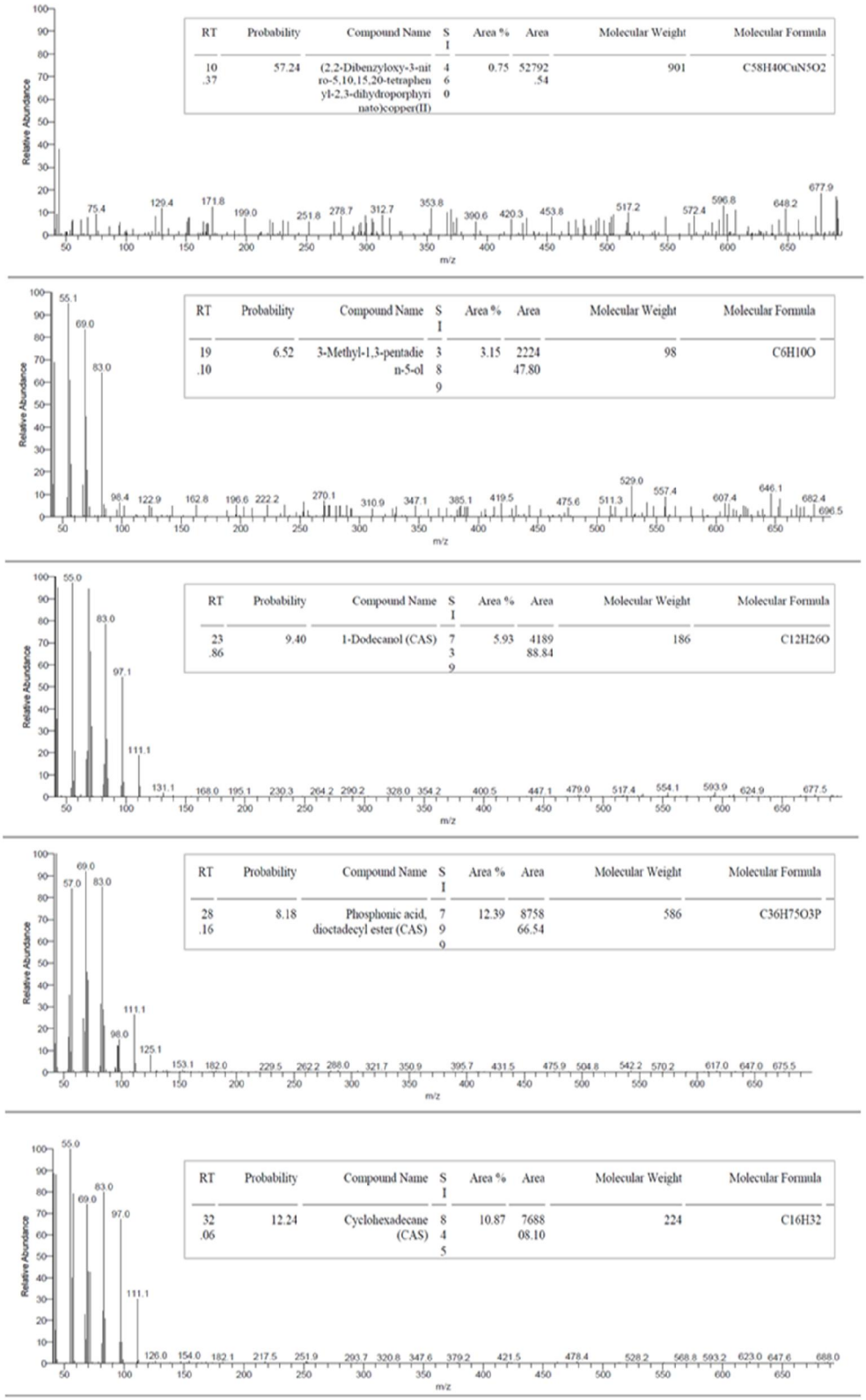

Fig. 3 a. The top ten identified non-volatile bioactive compounds in the GC–MS analysis of *Brevibacillus brevis* extract, with corresponding mass Spectra and Retention Times.

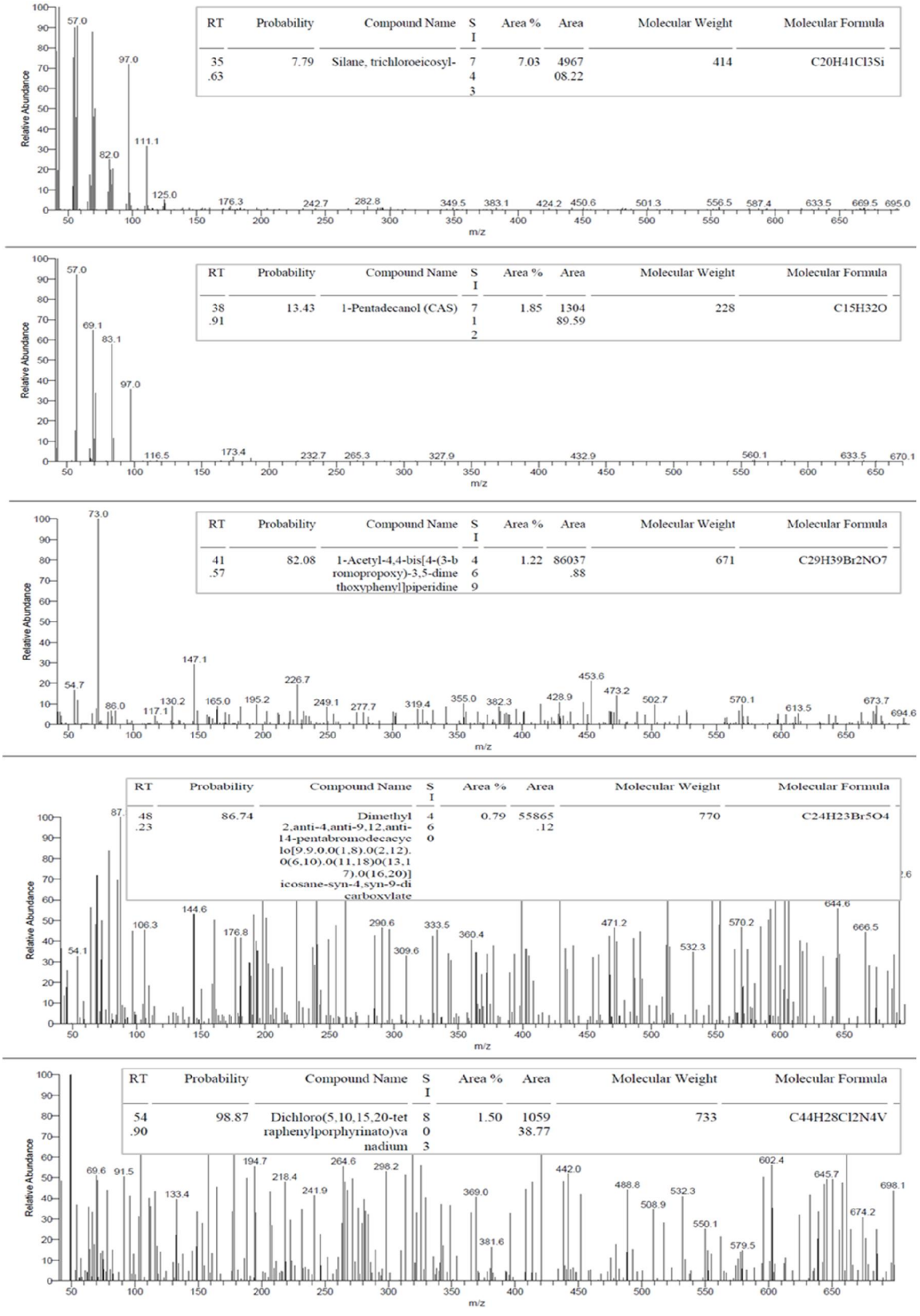

Fig. 3b: The top ten identified non-volatile bioactive compounds in the GC–MS analysis of Brevibacillus brevis extract, with corresponding mass spectra and retention Times

The identification of a phosphonic acid derivative in the extract is particularly noteworthy. Phosphonates are well known for their fungicidal properties, especially against oomycete pathogens, and are widely used in plant protection (Nowack *et al*., 2003; Sevrain *et al*., 2017). Although their regulatory status in agriculture is subject to legislative control in Europe, their biological activity as antifungal agents is well established. The presence of a phosphonate-related compound in *B. brevis* extract may partly explain the suppression of *Botrytis fabae*, potentially through interference with fungal metabolism or membrane-associated processes.

The detection of long-chain alcohols such as 1-dodecanol and 1-pentadecanol further supports the biological relevance of the extract. Long-chain fatty alcohols have been associated with antimicrobial and membrane-disruptive effects in several microbial antagonists. Their amphiphilic nature enables interaction with fungal membranes, potentially leading to permeability alterations and impaired spore germination. The role of microbial metabolites in disease suppression through competition, enzyme production, and host resistance induction has been extensively discussed by Droby *et al.* (2022) who highlighted the multifactorial mechanisms underlying microbial biocontrol.

Cyclohexadecane, although structurally simple, belongs to hydrocarbon compounds that have been reported among microbial volatile and semi-volatile fractions with antifungal properties. Bacillus-derived metabolites, including hydrocarbons and other VOC-related compounds, have been shown to suppress fungal pathogens by affecting hyphal morphology, membrane integrity, and conidial germination (Naveed *et al.,* 2023; Parveen *et al*., 2024). While the present analysis focused on non-volatile fractions, the presence of such hydrocarbons indicates that membrane-related disruption may be part of the antagonistic mechanism.

The identification of porphyrin–metal complexes, including copper-associated structures, is also of biological interest. Metal–porphyrin compounds are known to interfere with redox balance and respiratory processes in microbial cells. Their presence, even at relatively low abundance, suggests a possible contribution to oxidative stress induction in fungal tissues, which may weaken pathogen development (Sułek, *et al*., 2020; Zhang, *et al*., 2021).

Similarly, the detection of a brominated polycyclic derivative reflects the chemical complexity of the extract. Halogenated organic compounds are often associated with antimicrobial activity, and even when present at low percentages, they may exert synergistic or additive effects within a metabolite mixture.

Collectively, the GC–MS results support the earlier biological assays demonstrating inhibition of mycelial growth and spore germination of *B. fabae*. Rather than indicating a single dominant antifungal molecule, the data suggest a multi-component biochemical system, where phosphonate-like compounds, fatty alcohols, hydrocarbons, and structurally complex derivatives may act together to impair fungal establishment (Alrumman *et al*., 2019; Kang Yuan *et al*., 2023).

Importantly, the magnitude of disease reduction observed under greenhouse conditions was moderate rather than extreme, which aligns with a biological control scenario involving metabolic interference rather than acute fungicidal toxicity. Such a balanced mode of action is consistent with sustainable biocontrol strategies and reduces the risk of resistance development.

3.5. Greenhouse assessment of disease severity and induced biochemical defences in faba bean

3.5.1. Greenhouse evaluation of faba bean genotypes treated with *Brevibacillus* Brv-M1

The application of *Brevibacillus* Brv-M1 led to observable reductions in chocolate spot severity and incidence across the tested faba bean genotypes, though the extent of improvement varied notably among them. Instead of eliminating the disease, the bacterial treatment moderately suppressed its progression and, more importantly, delayed the appearance of severe symptoms particularly during the early infection stages. The reduction in disease severity following Brv-M1 treatment ranged from about 6% to 25%, depending on the genotype (Table 5). This range, although modest, is meaningful under controlled greenhouse conditions where pathogen pressure is often artificially high. Such reductions reflect the role of biocontrol agents not as standalone cures but as contributors to an integrated disease management strategy. Among all tested genotypes, G17 (Mariout 2) again emerged as the most resilient, showing the lowest infection values under both treated and untreated conditions. Its naturally higher tolerance combined with further improvement upon bacterial treatment underlines its suitability. Other genotypes such as G14, G11, and G13 showed moderate susceptibility in

untreated pots but responded relatively well to the bacterial application, suggesting partial compatibility with Brv-M1. In contrast, genotypes like G1 and G2 exhibited weaker responses. Despite bacterial treatment, the reduction in disease parameters remained limited, indicating a potential lack of responsiveness to Brv-M1 or intrinsic vulnerability to *Botrytis fabae* (Rhaïem *et al*., 2002; El-Komy, 2014; Bayoumi, *et al*., 2021; Mora-Baez, *et al*., 2024).

Table 5: Effect of *Brevibacillus* Brv-M1 Treatment on Disease Incidence and Severity of *B. fabae* under Greenhouse Conditions

Genotype code	Disease incidence (%) – control	Disease severity (%) – control	Disease incidence (%) – Brv -M1	Disease severity (%) – Brv-M1	Reduction in severity (%)
G1	88.3 ±1.2 a	65.1 ±1.5 a	74.2 ±1.3 a	51.4 ±1.7 a	21.1
G2	86.4 ±1.6 a	61.3 ±1.4 b	72.7 ±1.2 a	49.0 ±1.6 ab	20.1
G3	83.5 ±1.1 ab	59.2 ±1.6 bc	69.6 ±1.3 ab	45.7 ±1.5 b	22.8
G4	82.0 ±1.3 ab	57.0 ±1.4 bc	66.4 ±1.2 b	42.5 ±1.7 bc	25.4
G5	79.8 ±1.2 b	54.3 ±1.7 c	62.2 ±1.4 bc	39.1 ±1.3 cd	28.0
G6	77.3 ±1.5 bc	51.8 ±1.3 cd	58.4 ±1.5 cd	36.3 ±1.2 de	29.9
G7	74.7 ±1.4 cd	49.0 ±1.6 de	55.1 ±1.3 de	34.7 ±1.4 e	29.2
G8	72.3 ±1.2 cd	47.2 ±1.5 de	52.6 ±1.3 ef	32.9 ±1.6 e	30.3
G9	69.6 ±1.5 de	44.5 ±1.2 ef	49.4 ±1.1 f	30.1 ±1.5 ef	32.3
G10	67.1 ±1.3 de	42.1 ±1.4 f	47.2 ±1.3 fg	28.5 ±1.3 fg	32.3
G11	63.7 ±1.4 ef	39.4 ±1.5 fg	44.2 ±1.2 gh	25.6 ±1.4 gh	35.0
G12	61.0 ±1.1 f	37.2 ±1.3 fg	42.1 ±1.3 hi	24.0 ±1.3 hi	35.5
G13	58.6 ±1.5 fg	35.1 ±1.4 g	39.6 ±1.2 ij	22.2 ±1.4 ij	36.8
G14	56.4 ±1.2 fg	33.3 ±1.5 gh	37.2 ±1.3 jk	21.0 ±1.3 jk	36.9
G15	54.1 ±1.3 gh	31.2 ±1.4 gh	35.3 ±1.1 kl	19.7 ±1.5 kl	36.9
G16	52.7 ±1.1 gh	30.3 ±1.3 gh	34.1 ±1.4 lm	18.6 ±1.2 lm	38.6
G17 (Mariout2)	50.3 ±1.2 h	28.4 ±1.5 h	31.2 ±1.3 m	16.9 ±1.3 m	40.5

Means ± SE. Values followed by the same letter within each column are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Interestingly, while most genotypes followed the expected pattern of increasing disease severity with time, some inconsistencies were noted in the later stages (e.g., slightly lower values at 35 DPI compared to 28 DPI). This can likely be attributed to lesion aging, desiccation, or defensive responses of the plant masking visual symptoms rather than actual pathogen regression an observation that warrants further physiological studies. These outcomes mirror trends observed under field conditions, where the same genotypes showed consistent patterns in growth, yield, and disease performance. This concordance adds credibility to the greenhouse trial as a preliminary screening tool and supports the notion that bacterial biocontrol agents like *Brevibacillus* Brv-M1 and other biocontrol agents can play a valuable complementary role in managing foliar diseases of faba bean (Abay *et al*., 2017; Mengesha *et al*., 2022; Beever and Weeds, 2004; Bouhassan *et al*., 2004).

3.6. Assessment of induced resistance responses in faba bean plants

3.6.1. Enzymatic activities of peroxidase and catalase

The biochemical assays conducted to assess the defence-related responses revealed a noticeable increase in the activity of both peroxidase (PO) and catalase (CAT) enzymes in faba bean leaves after treatment with the bacterial isolates. Across the tested genotypes (G1 to G17), PO activity ranged from 0.10 to 2.50 U/ml in treated plants, compared to 0.03 to 0.79 U/ml in untreated controls. The most pronounced increase was observed in genotype G11, which exhibited the highest PO activity (2.50 U/ml) post-treatment, indicating a strong activation of the oxidative system (Table 6).

Similarly, CAT activity showed a significant enhancement after treatment, ranging from 0.33 to 6.96 U/ml, while values in the untreated controls were limited to 0.06–3.14 U/ml. Genotype G16 showed the most elevated catalase activity (6.96 U/ml), suggesting an effective detoxification of hydrogen peroxide, which is commonly accumulated during plant-pathogen interactions.

Despite the general trend of increased enzymatic activities across treatments, the magnitude of change varied significantly among genotypes, reflecting differential responsiveness to the bacterial inoculation. Genotypes G11, G12, G15, and G16 with high enzymatic responses, which may be correlated with greater induced resistance to *Botrytis fabae*.

These findings align with previous studies reporting enhanced peroxidase and catalase activity as part of the systemic acquired resistance (SAR) response in plants treated with beneficial microbes (Hassan, *et al*., 2007; El-Komy *et al*., 2015; Nowogórska and Patykowski, 2015; Tajick Ghanbary *et al*., 2020).

Table 6. Effect of bacterial treatments on enzymatic activities (U/ml) of peroxidase (PO) and catalase (CAT) Total phenolic content (mg/g FW) in leaves of faba bean genotypes.

Genotype	PO		CAT		Phenols	
	Before	After	Before	After	Before	After
G1	0.16 c	0.72 c	0.08 f	0.38 e	0.45 d	0.81 d
G2	0.03 e	0.96 c	0.09 f	2.39 b	0.38 d	1.65 b
G3	0.03 e	0.44 d	0.06 f	1.53 c	0.30 e	0.40 e
G4	0.04 e	0.23 e	0.08 f	0.57 e	0.32 e	0.55 d
G5	0.04 e	0.17 e	0.11 f	0.59 e	0.76 b	1.15 c
G6	0.20 c	0.31 e	0.72 d	2.59 b	0.65 c	1.18 c
G7	0.11 d	0.55 d	0.20 f	0.78 e	0.23 f	0.66 d
G8	0.07 d	0.50 d	0.07 f	0.75 e	0.26 f	0.74 d
G9	0.10 d	0.28 e	0.20 f	1.05 d	0.72 b	0.34 e
G10	0.22 c	0.30 e	0.39 e	0.87 d	1.30 a	1.61 b
G11	0.50 b	2.50 a	1.91 c	1.47 d	0.84 b	1.32 b
G12	0.64 b	1.96 b	1.00 c	2.49 b	0.36 d	2.59 a
G13	0.05 e	0.25 e	0.60 e	2.68 b	0.23 f	0.33 e
G14	0.11 d	0.10 e	2.79 b	3.04 b	0.76 b	1.20 c
G15	0.64 b	1.02 b	1.45 c	2.69 b	0.28 e	1.08 c
G16	0.79 a	1.75 b	3.14 a	6.96 a	1.26 a	1.76 b
G17	0.69 a	0.70 d	1.94 c	2.30 b	0.68 c	1.75 b

Means within a column followed by the same letter are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test.

3.6.2. Total phenolic content

Total phenolic content increased in most genotypes following bacterial treatment, with values ranging from 0.33 to 2.59 mg/g FW post-treatment compared to 0.23 to 1.3 mg/g FW in the untreated controls. The most marked enhancement was recorded in G12, G2, and G16, indicating a potential role for phenolic accumulation in the resistance mechanism against *B. fabae*. Phenolic compounds are well known for their antimicrobial properties and ability to reinforce cell walls, thereby limiting pathogen penetration. The increase in phenolic content in responsive genotypes suggests that bacterial inoculation not only triggers enzymatic defences but also promotes secondary metabolite production involved in plant defence (Tapia-Torres *et al*., 2014; López-Romero*, et al*., 2022; Rivas-García *et al*., 2024).

3.7. Field assessment of chocolate spot and agronomic performance in faba bean genotypes

3.7.1. Field assessment of chocolate spot disease

Under Toshka field conditions, natural infection by *Botrytis fabae* resulted in moderate chocolate spot pressure across the evaluated faba bean genotypes. In untreated plots, disease incidence ranged from about 13.1 to 23.5%, whereas disease severity varied between approximately 31.7 and 42.5% (Table 7). These values are clearly lower than those recorded under greenhouse conditions with artificial inoculation, as expected when infection develops naturally and environmental conditions fluctuate.

Despite the overall moderate disease levels, genotypes differed noticeably in their field response. Some entries (e.g. G1, G2 and G3) showed the highest severity values and can be considered more prone to chocolate spot under these conditions, whereas others such as G13–G17, including the check cultivar Mariout 2 (G17), consistently maintained the lowest severity ratings. This pattern broadly agrees with the greenhouse results, where the same group of genotypes tended to display reduced disease scores, supporting the stability of their relative ranking across environments.

Seed treatment and foliar application with *Brevibacillus brevis* Brv-M1 led to a modest but consistent reduction in disease severity. Depending on the genotype, the decrease in severity ranged from around 6% in the most susceptible lines up to roughly 28–30% in the better-performing genotypes (Table 7). By contrast, reductions in disease incidence were relatively small, typically less than 1.5 percentage points, indicating that Brv-M1 only partially affected the proportion of infected plants but was more effective in limiting lesion development and disease progression on infected foliage.

Genotypes with higher baseline severity (e.g. G1 and G2) showed only limited improvement in response to Brv-M1, and their treated severity values remained in the upper range. In contrast, several genotypes with intermediate or lower initial severity (particularly G11–G17) exhibited proportionally greater reductions, with Mariout 2 (G17) combining low severity in the control plots with one of the highest relative reductions after treatment. This suggests that the biocontrol effect of Brv-M1 is expressed more clearly when it acts in concert with inherent host tolerance, rather than compensating for high susceptibility on its own.

Compared with the greenhouse, where Brv-M1 exerted a stronger relative impact on disease development, the field response was more moderate an outcome consistent with the more complex microclimate, UV exposure, and variable leaf wetness in open field conditions, all of which can affect bacterial survival and metabolite stability on the leaf surface . Nevertheless, the observed reductions in severity, together with the better vegetative growth and yield performance of the less affected genotypes, support the potential of Brv-M1 as a useful component of integrated chocolate spot management in newly reclaimed, heat-stressed environments such as Toshka, particularly when combined with tolerant genotypes like Mariout 2.

Table 7. Effect of *Brevibacillus* Brv-M1 on field incidence and severity of chocolate spot caused by *Botrytis fabae* under Toshka conditions (combined over two seasons).

Genotype code	Disease incidence (%)		Disease severity (%)		Reduction in severity (%)
	Control	Brv-M1	Control	Brv-M1	
G1	22.8 a	21.6 a	41.8 a	39.3 a	6.0
G2	23.5 a	22.2 a	42.5 a	39.5 a	7.1
G3	21.9 b	20.8 b	40.9 b	37.2 b	9.0
G4	20.5 b	19.5 b	39.7 b	35.7 b	10.1
G5	19.7 c	18.8 c	38.5 b	33.9 b	11.9
G6	18.9 c	17.9 c	37.9 b	32.6 b	14.0
G7	20.1 b	19.3 b	39.0 b	33.9 b	13.1
G8	17.8 c	16.9 c	36.8 c	30.9 c	16.0
G9	16.5 d	15.7 d	35.2 c	28.9 c	17.9
G10	17.2 c	16.5 c	36.0 c	29.9 c	16.9
G11	15.9 d	15.1 d	34.1 c	27.3 c	19.9
G12	15.1 d	14.4 d	33.5 c	26.1 c	22.1
G13	14.3 d	13.7 d	32.2 d	24.8 c	23.0
G14	15.0 d	14.3 d	33.0 c	26.1 c	20.9
G15	13.9 d	13.3 d	31.9 d	23.6 d	26.0
G16	14.4 d	13.9 d	32.4 d	24.6 c	24.1
G17 (Mariout 2)	13.1 d	12.6 d	31.7 d	22.8 d	28.1

Means separation followed Duncan's multiple range test (group letters not shown for brevity). Reduction in severity (%) was calculated as (Control DSI − Brv-M1 DSI)/Control DSI × 100.

3.7.2. Growth and yield-related measurements

Under the hot, arid conditions of Toshka, the seventeen faba bean genotypes expressed clear and consistent differences in vegetative growth and yield over the two growing seasons. As mentioned in Table 8, Combined across years, plant height at maturity ranged from about 91.8 cm in G7 to 122.0 cm in the check cultivar Mariout 2, with G12 (120.7 cm) and G3 (118.2 cm) also maintaining tall, vigorous plants. In contrast, lines such as G7 (91.8 cm) and G8 (97.0 cm) tended to be shorter and more compact, reflecting a narrower above-ground canopy under desert conditions.

First pod height, a trait relevant for minimizing contact with soil surface and facilitating harvest also showed useful variation. Mean values spanned from roughly 22.0 cm in G9 to 35.5 cm in G3, while genotypes such as G1, G2, G10, G12 and Mariout 2 consistently placed their first pods at approximately 29–32 cm above the ground. Such combinations of adequate plant height and favourable pod placement are generally associated with better adaptation and reduced harvest losses under marginal environments.

The number of branches per plant also differentiated the genotypes. Across seasons, G14 (5.30 branches/plant), G16 (4.97), G13 (4.78), G12 and G15 (4.67) and G11 (4.03) formed the most highly branched plants, indicating a strong capacity to build a dense canopy and to provide multiple sites for pod set. By contrast, several genotypes (e.g. G1, G2, G4, G8, G10 and Mariout 2) were closer to or slightly below the overall mean (4.05 branches/plant), suggesting a more moderate branching pattern. This pattern is consistent with previous work where plant height, number of branches and pods per

plant ranked among the main contributors to grain yield in advanced faba bean genotypes across variable environments.

Seed size and pod filling further distinguished the entries. For 100-seed weight, combined means ranged from about 56.5 g in G16 and 74.0 g in G15 up to 109.1 g and 107.7 g in G2 and G8, respectively. Thus, G2, G3 and G8 clearly expressed heavier seeds than the check cultivar Mariout 2 (91.9 g).

Table 8: The genotypes mean performance for plant height, first pod height and number of branches/plant under both years (2022/23 (Y1) – 2023/24 (Y2)) as well as the combined analysis (C).

Genotypes	Plant height			First pod height			No. of branches/plant		
	Y1	Y2	C	Y1	Y2	C	Y1	Y2	C
G1	113.3	125.0	119.2	30.3	33.3	31.8	3.07	3.60	3.33
G2	108.0	118.3	113.2	29.3	33.3	31.3	3.17	3.53	3.35
G3	113.0	123.3	118.2	34.3	36.7	35.5	3.60	4.13	3.87
G4	108.3	120.0	114.2	28.0	33.0	30.5	3.13	3.60	3.37
G5	107.0	121.7	114.3	25.3	30.0	27.7	3.47	3.87	3.67
G6	106.0	113.3	109.7	25.7	27.7	26.7	3.70	4.27	3.98
G7	87.0	96.7	91.8	21.0	24.0	22.5	3.40	3.63	3.52
G8	92.3	101.7	97.0	27.0	33.0	30.0	3.03	3.63	3.33
G9	94.3	106.7	100.5	20.3	23.7	22.0	4.20	4.57	4.38
G10	91.0	96.7	93.8	27.3	34.7	31.0	3.60	3.93	3.77
G11	100.3	111.7	106.0	22.0	28.7	25.3	3.73	4.33	4.03
G12	114.7	126.7	120.7	31.0	33.3	32.2	4.43	4.90	4.67
G13	103.3	113.3	108.3	24.3	27.3	25.8	4.53	5.03	4.78
G14	100.0	110.0	105.0	20.7	25.0	22.8	4.97	5.63	5.30
G15	106.7	116.7	111.7	25.0	29.3	27.2	4.43	4.90	4.67
G16	100.7	111.7	106.2	27.0	31.0	29.0	4.73	5.20	4.97
G17 Mariout2	115.7	128.3	122.0	26.0	33.0	29.5	3.63	4.17	3.90
Means	103.6	114.2	108.9	26.2	30.4	28.3	3.81	4.29	4.05
LSD $_{0.05}$	2.9	5.0	-	1.7	3.0	-	0.14	0.22	-

The highest numbers of seeds per pod were recorded for G12 (3.49), G14 (3.53) and Mariout 2 (3.64), whereas lines such as G5, G6, G7, G15 and G16 carried fewer seeds per pod. Seed weight per pod followed a similar trend: G2, G3, G8, G12, G14 and Mariout 2 showed the largest seed weight per pod (around 3.17–3.64 g), reflecting a favourable combination of seed number and seed size within each pod. Such trait combinations are typically associated with superior sink capacity and have been highlighted as key drivers of yield in breeding populations tested under contrasting environments (Table 9).

At the whole-plant level, pods per plant, biological yield and seed yield per plant provided the clearest integrative picture. In the combined analysis, G16 (27.5 pods/plant) and G15 (24.6) had the highest pod numbers, followed by G13 (20.1) and G10 (18.4), indicating high reproductive capacity.

Biological yield per plant was greatest in Mariout 2 (123.3 g), with similarly high values in G15 (113.9 g), G12 (112.0 g), G3 (102.6 g), G13 (106.4 g) and G10 (107.0 g). These genotypes were therefore able to accumulate substantial biomass despite exposure to both heat and disease.

Seed yield per plant mirrored this pattern: G12 (53.5 g) and G15 (51.0 g) recorded the highest mean seed yields across seasons, while G13, G11, G10, G3, G9, G8, Mariout 2 and G16 all exceeded the overall mean of 40.9 g/plant. In practical terms, a relatively small subset of genotypes particularly G12, G15 and Mariout 2, and to a lesser extent G10, G11, G13, G3, G9, G8 and G16 combined vigorous vegetative growth, high reproductive capacity and stable seed production under the hot, low-humidity conditions of Toshka (Table 10). This agrees with findings from high-potential environments, where only a fraction of advanced faba bean lines achieve both high yield and acceptable stability under combined abiotic and biotic constraints (Abdalla *et al*., 2021; Abo-Hegazy, 2022; El-Abssi *et al*., 2024).

Table 10: The genotypes mean performance for number of pods/plant, biological yield/plant and seed yield/plant under both years (2022/23 (Y1) – 2023/24 (Y2)) as well as the combined analysis (C).

Genotypes	No. of pods/plant			Biological yield/plant (g)			Seed yield/plant (g)		
	Y1	Y2	C	Y1	Y2	C	Y1	Y2	C
G1	8.1	8.5	8.3	55.5	61.1	58.3	23.7	25.0	24.4
G2	11.3	11.7	11.5	92.9	98.0	95.5	39.8	41.6	40.7
G3	13.6	13.9	13.8	99.6	105.5	102.6	43.5	45.5	44.5
G4	11.3	11.7	11.5	76.6	82.4	79.5	32.8	34.7	33.8
G5	13.2	14.0	13.6	71.3	76.2	73.8	32.6	34.0	33.3
G6	13.4	14.1	13.8	79.4	83.1	81.3	35.2	36.7	36.0
G7	12.3	12.7	12.5	69.6	72.8	71.2	30.7	32.1	31.4
G8	13.0	13.7	13.3	86.2	91.6	88.9	42.3	44.0	43.1
G9	15.3	15.9	15.6	91.6	95.9	93.8	42.9	45.0	44.0
G10	18.1	18.8	18.4	105.8	108.2	107.0	44.0	46.2	45.1
G11	16.6	17.2	16.9	89.6	95.2	92.4	45.5	47.8	46.7
G12	15.5	16.5	16.0	109.0	115.0	112.0	52.5	54.5	53.5
G13	19.7	20.4	20.1	104.1	108.7	106.4	45.8	47.5	46.7
G14	10.6	11.1	10.9	78.4	82.0	80.2	31.9	33.4	32.7
G15	24.3	25.0	24.6	109.5	118.3	113.9	50.0	51.9	51.0
G16	26.9	28.0	27.5	99.6	105.4	102.5	41.3	42.9	42.1
G17 Mariout2	13.6	14.2	13.9	118.9	127.7	123.3	46.5	48.1	47.3
Means	15.1	15.7	15.4	90.4	95.7	93.1	40.1	41.8	40.9
LSD $_{0.05}$	0.4	0.3	-	1.5	2.2	-	0.9	1.1	-

3.7.3. Linking field performance with chocolate spot response and Brv-M1 treatment

Under natural infection in the field, chocolate spot pressure was moderate, which is consistent with the relatively dry conditions of Toshka compared with more humid production areas. In untreated plots, disease incidence among genotypes ranged from about 13.1% (Mariout 2) to 23.5% (G2), while

disease severity varied between 31.7% (Mariout 2) and 42.5% (G2). These values indicate that all genotypes experienced measurable infection by *Botrytis fabae*, but the epidemic did not reach the high severities that can cause severe yield loss in more conducive environments. Similar moderate field levels have been reported in chocolate spot surveys and control trials where weather conditions were only partly favourable for disease development (Mora-Báez *et al*., 2024).

Application of the most effective bacterial antagonist, *Brevibacillus* Brv-M1, led to a consistent though partial reduction in chocolate spot severity across all genotypes. In Brv-M1-treated plots, disease severity generally shifted into a range of roughly 22.8–39.3%, corresponding to reductions of about 6% (G1) up to 28.1% (Mariout 2) relative to the untreated controls. The magnitude of this effect was clearly genotype-dependent: lines with higher baseline severity tended to show modest but useful reductions, whereas several genotypes with intermediate severity exhibited more pronounced proportional responses. This pattern is in line with field reports where biological or botanical treatments against chocolate spot achieved partial but agronomically meaningful suppression typically on the order of 30–50% reduction in severity depending on product and environment (Mora-Báez *et al*., 2024).

When field disease scores are interpreted alongside the growth and yield data, a coherent picture emerges. Agronomically superior genotypes, particularly G12 and G15, along with the check Mariout 2 tended to show either lower natural severity or a stronger proportional improvement following Brv-M1 treatment. For example, Mariout 2 combined one of the lowest control severities (31.7%) with one of the highest relative reductions (to 22.8% by 28.1% reduction), while also ranking among the best genotypes for biological yield and seed yield per plant.

Similarly, G12 exhibited moderate chocolate spot severity in the untreated plots (33.5%), which decreased to about 26.1% following Brv-M1 application (22% reduction), and it was the top-yielding entry in terms of seed yield per plant. Line G15 combined a high number of pods, substantial biomass and strong seed yield with a marked reduction in severity (from 31.9% to 23.6%; 26% reduction). Other genotypes such as G13 and G16 also showed a favourable combination of acceptable disease levels, measurable Brv-M1-mediated suppression and above-average yield performance.

By contrast, genotypes that retained relatively high disease severity or responded weakly to Brv-M1 tended to fall into intermediate or lower yield classes, suggesting that greater susceptibility to chocolate spot contributed, at least in part, to reduced productivity under Toshka conditions. Negative associations between chocolate spot severity and grain yield have been repeatedly documented in field trials, supporting the use of disease scores as a complementary selection criterion alongside agronomic trait (Mora-Báez *et al*., 2024).

Overall, the field evidence indicates that combining moderate host resistance (expressed as lower natural chocolate spot severity) with biological control using Brv-M1 can help stabilize yield under desert field conditions. From a breeding and management perspective, the convergence of strong agronomic performance and favourable disease response in Mariout 2, G12 and G15, supported by promising lines such as G10, G13 and G16 identifies these genotypes as good candidates for further testing and potential deployment in integrated chocolate spot management strategies under Toshka conditions. This is fully consistent with current efforts to develop high-yielding, stress-tolerant faba bean cultivars and to embed non-chemical disease management tools within sustainable production systems.

4. Conclusion

This study demonstrated that chocolate spot in faba bean under Toshka conditions is driven by a combination of a highly aggressive *Botrytis fabae* isolate and a challenging hot, arid environment, but that both host genetics and biological control can substantially modulate disease impact. Among the tested isolates, Bf-5 was clearly the most virulent, producing markedly larger necrotic areas than other isolates in detached-leaf assays, and thus provided a robust and consistent inoculum source for greenhouse and field evaluations.

Across *in vitro* assays, the *Brevibacillus brevis* isolate Brv-M1 showed the most reliable antagonism against *B. fabae*, suppressing radial growth, reducing spore germination by roughly one-third to one-half depending on assay, and producing multiple extracellular lytic enzymes. GC–MS profiling of the Brv-M1 extract revealed a complex mixture of bioactive metabolites, including phosphonate-like derivatives, long-chain fatty alcohols, hydrocarbons and porphyrin–metal

complexes, all of which are consistent with membrane disruption and metabolic interference rather than single-target toxicity. This biochemical diversity helps explain the moderate but repeatable suppression observed across experiments.

Under greenhouse conditions with artificial inoculation, seed treatment and/or foliar application of Brv-M1 led to reductions in chocolate spot severity generally on the order of 6–25% relative to untreated controls, depending on genotype. In the field, where disease pressure and microclimate were less conducive to explosive epidemics, natural infection levels were lower, and Brv-M1 still achieved reductions in severity of roughly 6–28%, with only minor effects on disease incidence. These magnitudes are in line with realistic expectations for biological control agents and underline that Brv-M1 should be viewed as a support tool within integrated management rather than a stand-alone replacement for all other measures.

The genotypic evaluation under Toshka conditions highlighted a small set of faba bean lines that combined good agronomic performance with comparatively favorable chocolate spot responses. In particular, G12 and G15, together with the check cultivar Mariout 2, consistently expressed taller plants or robust canopies, high pod and seed yield, and relatively lower or more Brv-M1-responsive disease severity. Several additional lines (e.g. G10, G13 and G16) also showed a useful balance between yield potential and disease response, indicating a broader pool of material for breeding and deployment in reclaimed desert lands.

Overall, the results support a dual strategy for managing chocolate spot in heat-stressed environments: (i) selecting and promoting genotypes such as Mariout 2, G12 and G15 that maintain yield under moderate disease pressure, and (ii) integrating Brv-M1 as a biological partner capable of providing meaningful, though partial, suppression of *B. fabae*. However, before Brv-M1 can be recommended for routine use in integrated disease management programs, additional work is needed on formulation optimization, application strategy, and biosafety and environmental risk assessment under multi-location field conditions.

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