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Bacillus velezensis B63 and chitosan control root rot, improve growth and alter the rhizosphere microbiome of geranium

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Summary. The root rot complex of geranium plants caused by *Rhizoctonia solani* and *Macrophomina phaseolina* is a major threat, and control of these pathogens predominantly relies on chemicals. This study explored multifaceted applications of *Bacillus velezensis* (strain B63) and chitosan, assessing their biocontrol efficacy against root rot, and their subsequent effects on rhizosphere communities. Strain B63 was antagonistic to *R. solani* and *M. phaseolina*. Under field conditions, greatest efficacy was obtained with strain B63 (36% and 33% disease reductions in, respectively, two growing seasons), chitosan soaking + foliar spray 0.2% (CSF 0.2%) (33 and 27% reductions), and 0.1% chitosan soaking + foliar spray (CSF 0.1%) (33 and 26% reductions). These treatments also changed rhizosphere microbiota, as shown by numbers of colony-forming units (CFU) and 16S rRNA gene microbiome analyses. Concomitant with rhizosphere shifts, essential oil yields and composition were positively affected, as shown by gas chromatography analyses. Chitosan soaking + foliar spray 0.2% increased concentrations of citronellol (1.36-fold), geraniol (1.37-fold), citronellyl formate (1.54-fold), and geranyl formate (1.94-fold) in geranium essential oil, compared with the experimental controls. Strain B63 also increased these essential oils by 1.04- to 1.27-fold. B63 also enhanced eugenol levels by 1.35-fold. Treatments with B63 were more effective than chitosan in improving the geranium plant morphological parameters (plant height, numbers of branches, biomass). These results show that *B. velezensis* strain B63 treatments have potential for enhancing yields and product quality from geranium plant under root rot infection.

Keywords. Antagonistic bacteria, soil suppressiveness, marker genes, hydrodistillation, nitrogen fixation.

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INTRODUCTION

Plant rhizosphere microbiomes play critical roles by promoting nutrient uptake, modulating plant immunity, suppressing pathogens, and controlling diseases (Olanrewaju and Babalola, 2022). Plants communicate with soil microbial communities through the exudation of a wide variety of compounds, boosting beneficial symbioses, modifying soil chemical and physical features, and inhibiting growth of plant pathogens. Plant roots secrete 5 to 21% of all photosynthetically fixed carbon in their rhizospheres (Simon and Haichar, 2019). Pathogenic microorganisms in soil can trigger plant immunity by modulating the root metabolism to engage root microbiota to stimulate plant defense and resistance to pathogens (Hou *et al*., 2021; Lyu and Smith, 2022). This has been illustrated in research by Liu *et al.* (2017), who discovered that when cucumber roots were attacked by *Fusarium oxysporum*, tryptophan was released and functioned as a signal to attract *Bacillus amyloliquefaciens*, which then protected cucumber from the pathogen. Wang *et al.* (2019) also demonstrated positive effects of lactic and hexanoic acids in tomato root exudates on the growth of *Bacillus cereus*, and reduction of host infection by *Ralstonia solanacearum*. Carrión *et al*. (2019) demonstrated that *Rhizoctonia solani* incited proliferation of *Chitinophaga*, *Flavobacterium*, and *Pseudomonas* spp. in sugar beet rhizospheres by activating their biosynthetic gene clusters to eliminate the fungal pathogen. A new hypothetical concept (the 'cry for help') has been proposed where plants under stress recruit beneficial microbiomes recruitment, and this protects them from detrimental effects, and can provide an array of growth-promoting benefits (Ali *et al*., 2023). Changes in rhizosphere microbiome composition could predict whether plants remain healthy or become infected by pathogens (Gu *et al*., 2022).

Geranium (*Pelargonium graveolens* L'Hér.) is a widely cultivated medicinal aromatic plant, which is extensively used for its essential oil (Narnoliya *et al*., 2019). Egypt, the second-largest producer and exporter of geranium oil after China, also holds second place following Reunion Island for the quality of its oil (Narnoliya *et al*., 2019). Geranium plants grown in Egypt are susceptible to several plant pathogens, including oomycetes and fungi. These include *Fusarium semitectum*, *Rhizoctonia solani*, *Macrophomina phaseolina*, and *Pythium ultimum*, which can cause significant damage and yield losses (Dewidar *et al*., 2019). This root rot complex (Yu *et al*., 2023) causes symptoms of rotted stems and roots, wilting or yellowing leaves, browning or blackening of the xylem vessels, and plant death (Prasad *et al*., 2008). While fungi attack geranium roots, early plant symptoms are often indistinct. As infections progress these can cause severe reductions in crop yields (Coque *et al*., 2020).

Different strategies have been developed to control root rot diseases, including cultural, physical, biological, and chemical control methods (Williamson-Benavides and Dhingra, 2021). Employing microbial antagonists and natural antifungal substances to improve soil suppressiveness and enhance plant defensive priming is a promising approach to controlling soil-borne fungal pathogens (Elsayed *et al*., 2020).

The success of introducing microorganisms for plant disease control is dependent on their adaptation and survival within host rhizospheres. Choosing the best microbial strain is critical, as it has direct impact on colonization density and effectiveness. *Bacillus*, a heterotrophic saprophyte, is common in soil and aquatic habitats, particularly in soils, where it exists as latent spores at temperatures near 0°C, allowing these bacteria to resist adverse environmental conditions (Maslennikova *et al*., 2023). *Bacillus velezensis*, particularly strains GB03, QST 713, FZB42, and D747, has commercial potential for disease biocontrol and host plant growth promotion (Vallejo, 2023). These bacteria directly combat infections through competition and antagonism, and indirectly support plant defenses and growth by stimulating production of protective chemicals (Chen *et al*., 2023).

Application of natural compounds is also useful for managing plant pathogens (Azmana *et al*., 2021). The macromolecules chitin and chitosan have been used in plant disease management. Chitosan is a natural polysaccharide found in the exoskeletons of crustaceans, insect cuticles, and fungus cell walls (Piras *et al.,* 2015). Chitosan has several agricultural applications, including promoting plant growth, eliciting plant resistance to biotic and abiotic stresses, and activation of symbiotic signalling between plants and beneficial microorganisms (Li *et al.,* 2020). This compound induces defense-related enzymes, accumulation of defense-related secondary metabolites, and increases nitrogen metabolism enzymes (Román-Doval *et al.,* 2023). Chitosan also creates physical barriers around pathogen penetrations and inhibits pathogen spread in host plants (El Hadrami *et al.,* 2010).

Previous studies have indicated successful control of *R. solani* and *M. phaseolina* using *Bacillus* spp. and chitosan. However, effects of *Bacillus* and chitosan on *R. solani* and *M. phaseolina*, and on bacterial soil microbiota, and geranium physiological and biochemical responses have not been defined. The purpose of the present study was to determine the influence of *B. velezensis* B63 and chitosan on soil microbiota and on geranium morphological and physiological parameters.

MATERIALS AND METHODS

Bacillus velezensis *(B63) and culture conditions*

Bacillus velezensis B63, known for its antibacterial and antifungal activities (Elsayed *et al*., 2020; Reyad *et al.,* 2022), was obtained from Dr Tarek R. Elsayed, Microbiology Department, Faculty of Agriculture, Cairo University, Giza, Egypt. The strain was cultivated in an Erlenmeyer flask containing 50 mL of Luria-Bertani broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 1 liter of distilled water. The pH was adjusted to 7.0 with 1 N NaOH) (Bertani 1951), and was incubated in a rotary shaker at 30°C. After 24 h, the bacterial cells were centrifuged (4500 g for 10 min); the pellet was then washed three times in sterile 0.85% NaCl, and the cell density was adjusted to OD_{600} = 1.0 (approx. 10^8 CFU mL^{-1}) in 0.85%. NaCl.

Chitosan preparation

One gram of Chitosan (crab shell, Sigma Chemical) was dissolved in 40 mL of distilled water containing 9 mL of 1 M acetic acid. Sodium acetate was used to adjust the pH to 6.0. Chitosan Solutions of 0.05%, 0.1%, and 0.2% chitosan were prepared from this stock solution (Anusuya and Sathiyabama, 2014).

Bacterial in vitro *antifungal activity against* Rhizoctonia solani *and* Macrophomina phaseolina

Rhizoctonia solani and *M. phaseolina* isolated from rotted geranium roots were obtained from our previous work (Reyad *et al*., 2022).

The antagonistic effect of the bacterium (*B. velezensis* B63) on *R. solani* and *M. phaseolina* was carried out *in vitro* using dual-culture plate assays. Potato dextrose agar (PDA) plates were inoculated with a 6-mm diam. actively growing *R. solani* and *M. phaseolina* cultures that separately placed on one side of the plate. A loop of the B63 bacterial suspension (10^8 CFU mL^{-1}) was streaked at opposite sides of the plate. Control plates were cultured only with the fungal pathogens. The plates were then incubated at 25°C for *R. solani* or 30°C for *M. phaseolina*. Three replicates were used for each treatment. Upon complete mycelial growth in any treatment plate, the radial growth of the fungi in the control and treatment plates was measured and inhibition percentages were calculated as follows:

$$I\% = C\text{-}d/C \times 100$$

where: C is the mycelium radial growth of the fungus in control; d: is the mycelium radial growth of the fungus in the treatment.

Field experiment design

Field experiments were carried out in two consecutive years (2019–2020 and 2020–2021), in a field located at the Medicinal and Aromatic Plants Department Farm, Horticulture Research Institute, Agricultural Research Center, El-Qanater El-Khayreya, Qalyubia, Egypt (30° 11'36.9"N, 31°7'55.43"E). From this field, terminal cuttings (lengths = 20 cm) from healthy geranium plants were obtained from a 2-year-old geranium plant.

The experimental setup consisted of the following nine treatments (with respective abbreviations used hereafter):

1. Control plants (CK)
2. Plants treated with Topsin-M fungicide (TM)
3. Plants pre-treated with chitosan at 0.05% (CS 0.05%)
4. Plants pre-treated with chitosan at 0.1% (CS 0.1%)
5. Plants pre-treated with chitosan at 0.2% (CS 0.2%)
6. Plants pre-treated and sprayed with chitosan at 0.05% (CSF 0.05%)
7. Plants pre-treated and sprayed with chitosan at 0.1% (CSF 0.1%)
8. Plants pre-treated and sprayed with chitosan at 0.2% (CSF 0.2%)
9. Plants treated with *B. velezensis* strain B63 (10^8 CFU mL^{-1})

The plant pre-treatments were applied, respectively, using Topsin-M fungicide at the recommended dose (2 g L^{-1}), chitosan solution at 0.05%, 0.1% or 0.2%, and a suspension of *B. velezensis* strain B63 (10^8 CFU mL^{-1}), by soaking cuttings in the treatments for 30 min. The control treatment cuttings were soaked in water. The treated cuttings were then planted on 15 November in both 2019 and 2020. Chitosan spraying treatments and additional treatments with *B. velezensis* strain B63 (soil drench) (treatments from 6 to 9 above) were each applied three times: the first application was carried out 45 d after planting, the second was carried out 1 month later, and the third was applied 30 d after the second application. Strain B63 (treatment 9 above) was applied as a soil drench at the same three times.

Each experiment plot consisted of 4 rows, each of 5 m length with 60 cm spacing between the rows. The plants in the rows were at 25 cm spacings. Three replicates were used for each treatment. Chemical fertilizers (NPK) and other agricultural practices were applied as recommended for conventional geranium crop culture.

Plant morphology and disease assessment

Two cuts (the first on 15 May, the second on 15 October, in each of 2020 and 2021) were taken from the geranium plants in the trials. Plant heights, numbers of branches, and plant fresh and dry weights (g) were assessed from each plant cut.

Disease symptoms became visible only after the first cut in each trial, and disease development was assessed on 15 October in each year, as percentages of plant mortality.

Geranium essential oil yields and gas chromatography analyses

The essential oil percentage was obtained by hydrodistillation using a Clevenger-type apparatus (British Pharmacopoeia, 1963). The percentages and yields (mL) of essential oil per plant were calculated for three replicates.

For essential oil composition analysis, samples from the second-cut field harvest from the 2020–2021 trial were analyzed using Gas–liquid chromatography (GLC) in the Laboratory of the Medicinal and Aromatic Plants Research Department, Horticulture Research Institute, using Ds Chrom 6200 GC with a flame ionization detector to separate volatile oil components. The chromatograph apparatus has a capillary column composed of BPX-5.5% phenyl (equiv.) polysilphenylene-siloxane (30 m × 0.25 mm ID × 0.25 µm film). The temperature program ramp increased at a rate of 10°C min^{-1} from 70°C to 200°C. The gas flow rates were nitrogen at 1 mL min^{-1}, hydrogen at 30 mL min^{-1}, and air at 330 mL min^{-1}. Temperature of the detector was 300°C, and of the injector was and 250°C. The GC chromatogram and analysis report for each sample were used to identify the percentage of the main components of each essential oil.

Microbiological analyses

Sampling and sample preparation

At the end of each trial (October 15), the geranium plants were cut. After removing loosely attached soil, rhizosphere soil was extracted from each sample. Five grams of roots from each sample were placed in a sterile Stomacher bag and mixed with 45 mL of sterile NaCl for 60 sec at medium speed with a Stomacher 400 blender. The Stomacher mixing process was repeated three times to obtain each root cell suspension.

Estimation of numbers of viable bacteria and fungi

Tenfold serial dilutions of the microbial suspensions in sterile 0.85% NaCl obtained (as described above) were plated onto plate count agar (PCA) for estimations of total viable bacteria, or potato dextrose agar (PDA) supplemented with tetracycline (50 mg mL^{-1}) for estimations of total viable fungi. Numbers of bacterial colony forming units (CFUs) were determined after 3 d incubation at 28°C, and fungal CFUs were determined after 5 d incubation at 25°C, respectively, and were calculated per g root fresh weight.

Total community DNA extraction from geranium rhizosphere samples

To address issues related to accuracy and cost, a sampling strategy was used where different random samples were selected from each experimental treatment group.

Total community DNA (TC-DNA) was extracted from 250 mg of rhizosphere soil samples from each treatment (two replicates each). The DNeasy Power Soil kit (Qiagen) was used for the extractions according to the manufacturer's protocol. TC-DNA was diluted (1:5) using Tris-EDTA and stored at -20°C. The integrity and quality of the extracted TC-DNA were confirmed using agarose gel electrophoresis and a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

Quantification of total bacteria in TC-DNA using the 16S rRNA gene

The total numbers of bacteria with 16S rRNA gene copy numbers were estimated in TC-DNA from rhizosphere samples using universal primers (Eub338 and Eub518), according to Fierer *et al*. (2005). A real-time PCR detection system (Step One Plus Real-Time PCR System, Thermo Fisher Scientific) was used. Real-time PCR was carried out using HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne) in a final volume of 20 µL for each sample. The real-time PCR conditions used were: an initial activation step at 95°C for 12 min, followed by 40 cycles each of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and a final elongation step at 72°C for 5 min. The specific primer sequences used in this experiment are listed in Table S1.

Illumina MiSeq sequencing and analyses of 16S rRNA gene amplicons

From the sampling strategy described above, six DNA samples were analyzed using the MiSeq platform (Illumina) to target the V3–V4 region of the 16S rRNA gene (Illumina, 2013), following the manufacturer's instructions. The raw sequence reads in the FASTQ format were uploaded to the Galaxy online platform through the public server at usegalaxy.org for Bioinformatic analyses. The analyses were carried out using the default parameters within the standard operating procedure (SOP) designed for MiSeq data. The forward and reverse FASTQ files were paired with a minimum overlap length of 50 bp, maximum mismatches of 15, and a minimum quality of 30. Reads were filtered, and sequences were filtered primarily on the basis of quality. Chimera sequences were eliminated using the Chimera Search device. Operational taxonomic units (OTUs) were chosen at a cutoff level of 0.03. The representative sequences of the OTUs were selected primarily on the basis of the greatest abundance within each cluster. Taxonomic classification was determined using the Greengenes classifier. An OTU table was obtained and analyzed using STAMP software 2.1. for analysis of community composition. Sequences were deposited at the public repository Sequence Read Archive (SRA) under the accession numbers AMN40334118 to SAMN40334121, and the Bioproject accession number PRJNA1085764.

PCR-based detection of genes encoding plant growth-promoting functions

PCR was conducted on the extracted TC-DNA to amplify marker genes indicative of soil fertility and suppressiveness. These include the *nifH* gene responsible for producing dinitrogenase reductase, the *ituD* gene related to iturin A production, the *bacC* gene related to the bacitracin biosynthesis, and the *fenD* gene responsible for the fengycin antifungal antibiotic. These genes can be used to assess soil health and disease suppressiveness. Detailed primer sequences for these genes are shown in Table S1.

Field experiment design and data analyses

The experiment was complete randomized block design with three replicates for each treatment. ANOVA was used to analyze plant mortality percentages and growth parameters, and means were compared using the least significant difference test at $P \leq 0.05$. Statistical analyses were carried out using the MSTAT-C statistical package. Plant mortality percentage data were arc sin transformed before carrying out ANOVA to produce approximately constant variances. Stacked column and principal component analysis (PCA) based on MiSeq sequencing data was carried out using Origin pro-2021 version software.

RESULTS

Antifungal activity of Bacillus velezensis *B63*

In vitro, *B. velezensis* B63 inhibited ($P \leq 0.05$) *R. solani* and *M. phaseolina*. Mean mycelial radial growth diameters were 90 mm (± 0.0) for the experimental control, 51.0 mm (± 0.58) for *M. phaseolina* (by 43% reduction) and 56.0 mm (± 2.52) for *R. solani* (by 38% reduction).

Field experiments

Under field conditions, all treatments decreased ($P \leq 0.05$) disease development (percent mortality) of geranium plants, especially in 2019–2020 season. The greatest plant mortality reduction was recorded from the treatments of *B. velezensis* B63, chitosan soaking + foliar spray 0.2% (CSF 0.2%), chitosan soaking + foliar spray 0.1% (CSF 0.1%), and chitosan 0.2% soaking alone (CS 0.2%). These treatments caused 36% (*B. velezensis* B63), 33% (CSF 0.2%), 33% (CSF 0.1%), and 31% (CS 0.2%) reductions ($P \leq 0.05$) in plant mortality, compared to the control treatment in the 2019–2020 season (Table 1).

All the treatments except *B. velezensis* B63, chitosan soaking + foliar spray 0.2% (CSF 0.2%) and chitosan soaking + foliar spray 0.1% (CSF 0.1%) gave plant mortality that was similar ($P > 0.05$) to the experimental control but still gave low mortalities compared with the control. Plant mortality from *B. velezensis* B63, chitosan soaking + foliar spray 0.2% (CSF 0.2%) and chitosan soaking + foliar spray 0.1% (CSF 0.1%) reduced ($P \leq 0.05$) by, respectively, 33%, 27%, and 26% compared to the control treatment (Table 1).

The least effective treatment was chitosan 0.05% soaking alone (CS 0.05%). This treatment decreased the plant mortality percentage from 77% to 65% (by 16.12% reduction: $P \leq 0.05$) and from 80.90% to 76.01% (by 6.04% reduction) for the first and second seasons, respectively, compared to the control treatment (Table 1).

Table 1. Mean mortality proportions from root rot (at 6 months after a first cut) for geranium plants receiving different treatments in field trials carried out in 2019–2020 and 2020–2021.

Treatment [a]	2019–2020 trial		2020–2021 trial	
	Mortality (%)	Trans.*	Mortality (%)	Trans.*
CK	94.17 ± 3.33†	77.03 a	97.5 ± 0.00	80.90 a
TM	70.83 ± 8.82	57.95 bc	83.33 ± 8.30	67.71 abc
CS 0.05%	80.83 ± 6.67	64.61 b	92.92 ± 4.58	76.01 a
CS 0.1%	70.83 ± 3.33	57.39 bc	88.33 ± 4.58	71.12 ab
CS 0.2%	64.17 ± 6.67	53.44 c	83.33 ± 8.30	67.71 abc
CSF 0.05%	80.83 ± 6.67	64.61 b	88.33 ± 4.58	71.12 ab
CSF 0.1%	60.83 ± 8.82	51.52 c	74.17 ± 9.58	60.11 bc
CSF 0.2%	60.83 ± 6.67	51.35 c	73.75 ± 5.00	59.42 bc
B63	57.5 ± 2.89	49.33 c	65.83 ± 2.60	54.24 c
Prob>F	0.003	0.002	0.009	0.010

[a] CK = Control; TM = Topsin-M; CS = Chitosan soaking; CSF = Chitosan soaking and spraying; B63 = *B. velezensis*.
*Values accompanied by the same letter are not significantly different ($P > 0.05$).
± † standard error; Trans = arc sin transformed value.

Effects of treatments on geranium plant morphology

At both cuts in the two seasons, all treatments increased the geranium parameters compared with the control treatments. *Bacillus velezensis* B63 gave the greatest increases ($P \leq 0.05$) in plant height, numbers of branches, and fresh and dry weights compared with the control (Table 2).

In 2019–2020 at the first cut, plants treated with *B. velezensis* B63 had mean plant heights that were 1.35-fold greater than the controls, mean branch numbers 1.56-fold greater, and fresh weights 3.44-fold greater than the controls (Table 2). At the second cut, mean plant heights from this treatment were 1.34-fold greater than the controls, and equivalent branch numbers were increased by 1.54-fold and fresh weights by 2.74-fold (Table 2). Plant dry weights were also increased by 2.85-fold at both cuts (Table 2).

In 2020–2021, for the first cut, the *B. velezensis* B63 treatment also increased ($P \leq 0.05$) plant height by 1.84-fold, branch numbers by 2.29-fold, fresh biomass by 6.37-fold, and dry biomass by 6.63-fold (Table 2). From the second cut, these increases were 1.65-fold, 2.33-fold, 4.85-fold, and 5.04-fold (Table 2).

Treatment application methods affected the activity of chitosan for improving geranium growth. In most cases, the greatest increases in plant parameters from chitosan treatments were from chitosan soaking + foliar spray (CSF 0.2%). In 2019–2020 at the first cut, this treatment increased ($P \leq 0.05$) plant height by 1.32-fold, number of branches by 1.52-fold, fresh biomass by 2.30-fold, and dry biomass by 2.08-fold. These parameters also increased ($P \leq 0.05$) at the second cut by 1.30-fold (plant height), 1.38-fold (number of branches), 2.43-fold (fresh biomass), and 2.53-fold dry biomass) (Table 2).

The same trend was observed in 2020–2021, where the chitosan soaking + foliar spray 0.2% (CSF 0.2%) treatment increased ($P \leq 0.05$), at the first cut, means of plant height by 1.76-fold, number of branches by 2.12-fold, fresh biomass by 5.05-fold, and dry biomass by 5.26-fold. At the second cut, these four parameters increased by 1.60-fold, 2.06-fold, 3.96-fold and 4.12-fold (Table 2).

Chitosan 0.05% soaking alone (CS 0.05%) was the least effective treatment, but still gave consistent and statistically significant ($P \leq 0.05$) enhancement of geranium growth parameters across both seasons and cuts, compared to the control treatments. In 2019–2020, for the first cut, plants treated with chitosan 0.05% soaking alone (CS 0.05%) had 1.02-fold increased ($P \leq 0.05$) mean plant height, 1.20-fold increased numbers of branches, 1.36-fold increased fresh biomass, and 1.13-fold increased dry biomass. For the second cut, these parameters increased by 1.20-fold, 1.08-fold, 1.72-fold and 1.72-fold (Table 2).

The same trend was observed in 2020–2021, chitosan 0.05% soaking alone (CS 0.05%) increased ($P \leq 0.05$) plant growth parameters across both cuts (Table 2). Mean plant height increased by 1.33-fold at the first cut and by 1.21-fold at the second cut. Similarly, at the first cut, branch numbers increased by 1.53-fold, and fresh biomass by 1.50-fold, and at the second cut these parameters increased by 1.38-fold and 1.19-fold. Dry biomass also increased by 1.38-fold at the first cut and by 1.24-fold at the second, compared to the control treatment.

Geranium essential oil yields and gas chromatography analyses

Essential oil contents

All the experimental treatments affected essential oil production by the geranium plants, as shown in Table 3. During the 2019-2020 season, at the first cut, significant increases ($P \leq 0.05$) in essential oil contents (1.27-fold) and yields (4.36-fold) were recorded from the *B. velezensis* B63 treatment, compared to the controls. At the second cut elevated essential oil contents (1.29-fold) from *B. velezensis* B63 and (1.27-fold) from chitosan 0.2% (CSF 0.2%) were recorded, but essential oil yield was significantly greater (3.54-fold: $P \leq 0.05$) only for B63 compared to the control. Topsin-M fungicide and

Table 2. Mean plant heights, numbers of branches, and herbage fresh and dry weights, for two cuts of geranium plants where different treatments were applied in two field trials carried out in 2019–2020 and 2020–2021.

Treatment [a]	First cut				Second cut			
	Plant height (cm)	No. branches/ plant	Herb FW g/plant	Herb DW g/plant	Plant height (cm)	No. branches/ plant	Herb FW g/plant	Herb DW g/plant
2019–2020 trial								
CK	46.00±0.00† e	08.33±0.33 e	378.67±9.87 h	191.00±0.00 h	48.67±0.67 h	08.00±0.00 f	666.00±6.56 i	326.34±3.21 i
TM	54.00±0.58 d	10.67±0.33 cd	559.33±9.21 f	286.33±5.33 f	56.00±0.58 g	10.67±0.33 bc	976.67±5.90 h	498.10±3.01 h
CS 0.05%	47.00±0.00 e	10.00±0.58 d	516.00±3.06 g	215.00±3.06 g	58.33±0.33 f	08.67±0.33 ef	1143.00±3.79 g	560.07±1.86 g
CS 0.1%	57.00±1.15 c	12.00±0.00 b	710.67±4.06 e	353.00±4.16 d	60.67±0.33 e	09.33±0.33 de	1240.00±4.58 f	620.00±2.29 f
CS 0.2%	58.00±0.00 c	12.00±0.00 b	700.33±6.64 e	322.67±3.28 e	61.33±0.33 d	10.00±0.00 cd	1405.00±4.58 e	716.55±2.34 e
CSF 0.05%	60.33±0.88 d	11.00±0.00 c	759.33±5.21 d	368.00±4.16 c	62.00±0.00 c	10.67±0.33 bc	1497.67±1.76 d	763.81±0.90 d
CSF 0.1%	61.33±0.88 ab	12.00±0.00 b	815.00±5.69 c	387.67±3.71 b	62.67±0.33 bc	10.33±0.33 bc	1566.00±2.89 c	798.66±1.47 c
CSF 0.2%	60.67±0.33 ab	12.67±0.33 ab	869.67±7.22 b	396.67±0.88 b	63.33±0.33 b	11.00±0.00 b	1618.00±3.51 b	825.18±1.79 b
B63	62.00±0.00 a	13.00±0.00 a	1303.67±18.32 a	544.00±3.51 a	65.00±0.00 a	12.33±0.33 a	1825.33±3.38 a	930.92±1.73 a
Prob>F	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
2020–2021 trial								
CK	32.33±1.20 g	5.67±0.33 f	194.00±3.46 h	95.06±1.70 h	51.00±0.58 i	6.00±0.00 g	162.67±0.88 i	79.71±0.43 i
TM	40.33±0.33 f	9.00±0.00 e	276.00±8.19 g	135.24±4.01 g	56.67±0.33 h	7.00±0.00 f	177.33±0.67 h	88.67±0.33 h
CS 0.05%	43.00±0.00 e	8.67±0.33 e	268.67±1.45 g	131.65±0.71 g	61.67±0.33 g	9.00±0.00 e	194.33±0.67 g	99.11±0.34 g
CS 0.1%	48.33±0.88 d	10.67±0.33 d	389.00±6.08 f	198.39±3.10 f	68.67±0.33 f	10.33±0.33 d	342.00±0.58 f	174.42±0.29 f
CS 0.2%	49.67±0.33 cd	11.33±0.33 c	682.00±1.73 e	347.82±0.88 e	71.67±0.88 e	11.33±0.33 c	390.33±1.20 e	199.07±0.61 e
CSF0.05%	51.00±0.58 c	12.00±0.00 b	752.67±3.93 d	383.86±2.00 d	74.67±0.33 d	12.00±0.00 b	438.00±3.21 d	223.38±1.64 d
CSF 0.1%	55.67±0.33 b	12.00±0.00 b	822.00±5.77 c	419.22±2.94 c	79.33±0.33 c	12.00±0.00 b	599.33±0.88 c	305.66±0.45 c
CSF 0.2%	57.00±0.58 b	12.00±0.00 b	979.67±6.96 b	499.63±3.55 b	81.67±0.67 b	12.33±0.33 b	644.00±1.53 b	328.44±0.78 b
B63	59.33±0.33 a	13.00±0.00 a	1236.67±7.62 a	630.70±3.89 a	84.33±0.33 a	14.00±0.00 a	788.33±0.33 a	402.05±0.17 a
Prob>F	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] CK = Control; TM = Topsin-M; CS = Chitosan soaking; CSF = Chitosan soaking and spraying; B63 = *B. velezensis*.
Means in each row accompanied by the same letter are not significantly different ($P < 0.05$); ± † standard errors.

Table 3. Mean essential oil contents in geranium plants harvested in two cuts each in 2019–2020 or 2020–2021, after different experimental treatments were applied.

Treatment [a]	First cut		Second cut	
	Mean essential oil content (%)	Mean essential oil yield (mL plant^{-1})	Mean essential oil content (%)	Mean essential oil yield (mL plant^{-1})
2019–2020				
Control	0.200±0.000† d	0.757±0.020 h	0.310±0.000 e	2.065±0.020 h
TM	0.237±0.003 c	1.324±0.032 f	0.353±0.007 d	3.451±0.061 g
CS 0.05%	0.200±0.000 d	1.032±0.006 g	0.363±0.003 cd	4.153±0.026 f
CS 0.1%	0.237±0.003 c	1.682±0.016 e	0.380±0.000 b	4.712±0.017 e
CS 0.2%	0.247±0.003 ab	1.728±0.381 e	0.383±0.006 b	5.386±0.055 d
CSF0.05%	0.240±0.000 bc	1.822±0.013 d	0.380±0.000 b	5.691±0.007 c
CSF 0.1%	0.247±0.003 ab	2.011±0.041 c	0.370±0.006 c	5.794±0.085 c
CSF 0.2%	0.243±0.003 bc	2.116±0.034 b	0.393±0.003 a	6.364±0.040 b
B63	0.253±0.003 a	3.302±0.018 a	0.400±0.000 a	7.301±0.014 a
Prob>F	<0.0001	<0.0001	<0.0001	<0.0001
2020–2021				
Control	0.140±0.000 e	0.272±0.005 h	0.160±0.000 g	0.260±0.001 h
TM	0.233±0.003 d	0.644±0.023 g	0.247±0.003 de	0.437±0.005 g
CS 0.05%	0.243±0.003 c	0.654±0.012 g	0.237±0.003 f	0.460±0.006 g
CS 0.1%	0.267±0.003 a	1.038±0.028 f	0.240±0.000 ef	0.821±0.001 f
CS 0.2%	0.240±0.000 cd	1.637±0.004 e	0.253±0.003 cd	0.989±0.014 e
CSF0.05%	0.240±0.000 cd	1.806±0.009 d	0.250±0.000 d	1.095±0.008 d
CSF 0.1%	0.243±0.003 c	2.001±0.040 c	0.260±0.000 bc	1.558±0.002 c
CSF 0.2%	0.253±0.003 b	2.482±0.038 b	0.267±0.003 b	1.717±0.023 b
B63	0.260±0.000 ab	3.215±0.020 a	0.307±0.003 a	2.418±0.025 a
Prob>F	<0.0001	<0.0001	<0.0001	<0.0001

[a] TM = Topsin-M; CS = Chitosan soaking; CSF = Chitosan soaking and spraying; B63 = *B. velezensis*
Means in each row accompanied by the same letter are not different ($P > 0.05$)
± † standard errors.

chitosan 0.05% soaking alone (CS 0.05%) gave the least increases in oil content and yield from both cuts. During 2020-2021, the first cut showed significant ($P \leq 0.05$) increases in essential oil contents. Chitosan 0.1% soaking alone (CS 0.1%) and *B. velezensis* B63 treatments gave the greatest increases in essential oil contents by, respectively, 1.91-fold and 1.86-fold, compared to the control. Also, *B. velezensis* B63 induced an 11.82-fold increase in mean essential oil yield. At the second cut, the greatest significant increases in essential oil content (1.92-fold) and yield (9.3-fold) were recorded from the *B. velezensis* B63 treatment.

Essential oil components

Gas chromatography (GC) analyses of geranium essential oils (Figure 1) showed that the concentrations of the main components of geranium plants treated with chitosan soaking + foliar spray (CSF 0.2%) increased by 1.36-fold for citronellol, 1.37-fold for geraniol, 1.54-fold for citronellyl formate, and 1.94-fold for geranyl formate, compared to the experimental controls. *Bacillus velezensis* B63 treatment also increased these components but to lesser extents. The increases were 1.04-fold for citronellol, 1.07-fold for geraniol, 1.27-fold for citronellyl formate, and 1.17-fold for geranyl formate. In addition, the *B. velezensis* B63 treatment increased the concentration of eugenol by 1.35-fold compared to the controls.

Geranium rhizosphere bacterial communities

The dominant bacteria (more than 10% relative abundance) in the geranium rhizospheres from plants treated with *B. velezensis* B63, chitosan soaking + foliar spray 0.2% (CSF 0.2%) and untreated controls belonged to two major phyla, *Proteobacteria*, and *Actinobacteria*

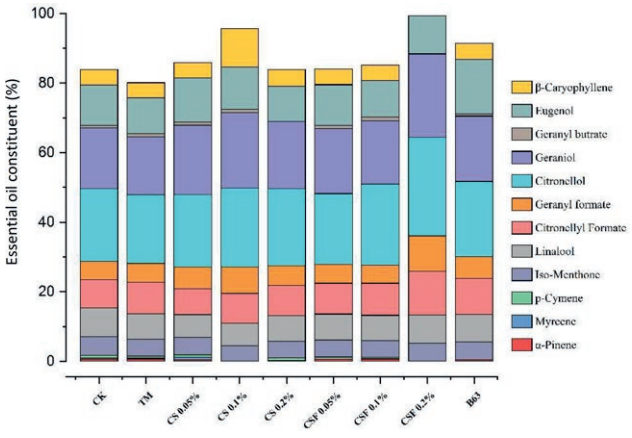

Figure 1. Mean proportions (%) of essential oils in geranium plants to which different experimental treatments were applied.

(Figure 2). *Acidobacteria* was also dominant in rhizospheres of plants treated with *B. velezensis* B63. A total of 106,061 bacterial sequences were detected from the three treatments. The greatest number of bacterial sequences (38,165; Figure S1) was obtained after chitosan treatment, while the lowest number (1,760) was detected from the *B. velezensis* B63 treatment. The control treatment gave 29,809 sequences. The bacterial sequences were affiliated with 19 phyla, 41 classes, 70 orders, 147 families, and 218 genera. The most prevalent genera (more than 3% relative abundance) detected from the control treatment were classified as *Agrobacterium*, *Kaistobacter*, and unclassified *Rhizobiales*. The most prevalent genera detected from the *B. velezensis* B63 treatment were *Kaistobacter*, *Agrobacterium*, unclassified Acidobacteria iii1-15, *Comamonadaceae* and *Rhizobiales*. Bacteria from the genera *Kaistobacter*, *Gemmata*, unclassified Acidobacteria iii1-15, and *Bacillales* were the most prevalent from the chitosan treatment (Table S2).

The chitosan soaking + foliar spray 0.2% (CSF0.2%) and *B. velezensis* B63 treatments induced increases in abundance of some minor genera of bacteria in the soil. These included *Sinorhizobium*, WD2101, unclassified *Gaiellaceae*, *Micrococcales*, *Rhodospirillaceae*, *Chthoniobacteraceae*, and *Myxococcales* compared to the control. Bacteria in *Enterobacteriaceae*, *Rhodoplanes*, *Balneimonas*, *Paracoccus*, *Gemmataceae*, and *Acidimicrobiales* increased under chitosan soaking + foliar spray 0.2% treatment (CSF 0.2%), compared to the *B. velezensis* B63 and control treatments. *Agrobacterium*, *Planctomyces*, *Sphingomonas*, *Devosia*, and several other genera decreased from the chitosan soaking + foliar spray 0.2% (CSF0.2%) treatment compared to the *B. velezensis* B63 and control. Conversely, the *B. velezensis* B63 treatment reduced the abundance of *Paracoccus*, *Planctomyces*, and several other taxa compared to the chitosan soaking + foliar spray 0.2% (CSF 0.2%) and control treatments (Table S1).

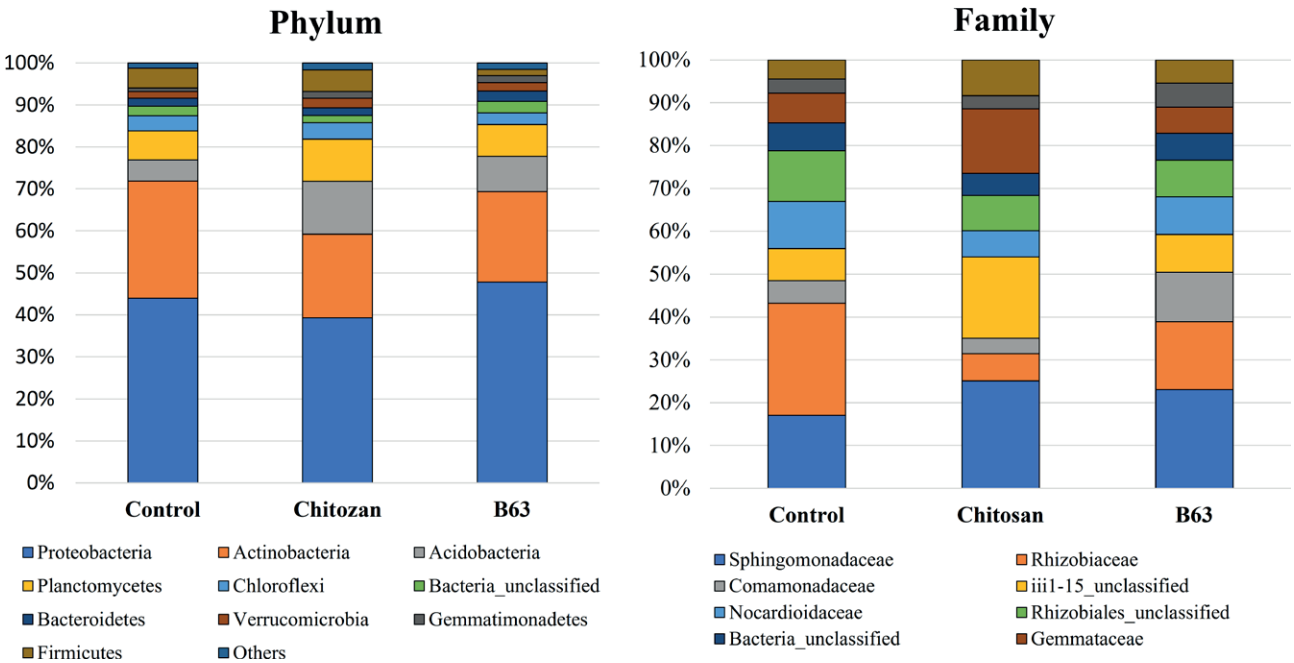

Figure 2. Microbial community compositions of geranium rhizospheres, based on MiSeq sequencing data, in response to treatment with chitosan soaking + foliar spray 0.2% (CSF 0.2%) or inoculation with *B. velezensis* B63 compared to the untreated experimental controls.

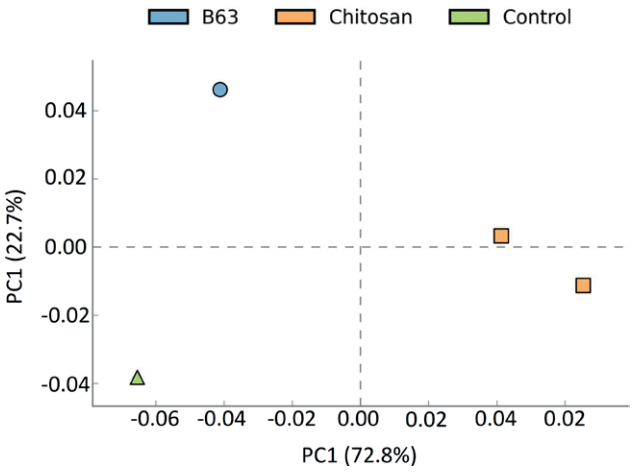

Figure 3. PCA analysis based on MiSeq sequencing data, showing separation between the microbial community compositions of the geranium rhizospheres for plants treated with chitosan soaking + foliar spray 0.2% (CSF 0.2%) or inoculation with *B. velezensis* B63 compared to experimental controls.

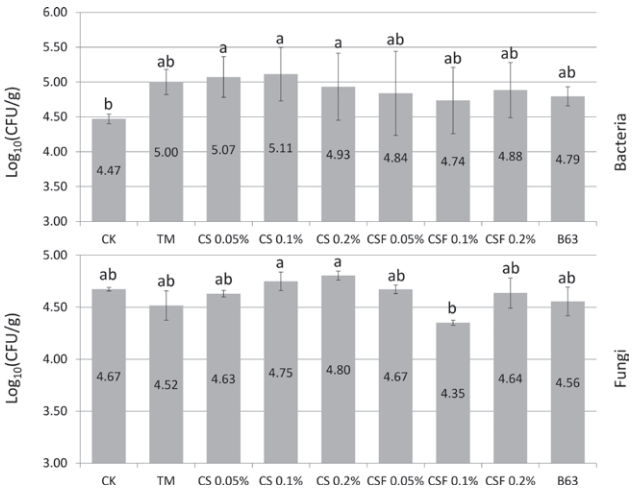

Figure 4. The total viable bacterial and fungal counts represented as Log_{10} (colony forming units/g root fresh mass). CK = Control; TM = Topsin-M; CS= Chitosan soaking; CSF = Chitosan soaking and spraying; B63 = *B. velezensis*; bars with the similar letter are not significantly different ($p \leq 0.05$).

Principle component analysis (PCA) based on the relative abundance of all bacterial OTUs obtained from geranium rhizospheres showed three distinct clusters. The first included samples from plants treated with chitosan soaking + foliar spray 0.2% (CSF 0.2%), while both *B. velezensis* B63 and control plants had diverse microbial community compositions (Figure 3).

Assessments of numbers of colony forming units (CFUs) from the geranium rhizospheres using plate count agar showed that total numbers of bacteria and fungi were not different ($P > 0.05$) between different samples (Figure 4). However, all samples from the experimental treatments tended to have greater numbers of microorganisms than the control.

The overall number of bacteria increased ($P \leq 0.05$) by 1.13-fold, 1.14-fold and 1.10-fold for Chitosan 0.05%, 0.1% and 0.2% soaking alone (CS0.05%, CS0.1% and CS0.2%) treatments, respectively, compared with the control. The other treatments did not significantly change bacterial counts compared to the control ($P > 0.05$), although they still gave greater counts than the control (Figure 4).

Figure 4 also shows that, except for the chitosan soaking + foliar spray 0.1% (CSF 0.1%) treatment, the overall number of fungi did not change ($P > 0.05$) with any of the tested treatments compared to the control. Despite this, the chitosan 0.1% and 0.2% soaking alone (CS 0.1% and CS 0.2%) treatments increased total fungal counts (1.02-fold from CS 0.1% and 1.03-fold from CS 0.2%), compared to the control, but this increase was not significantly different ($P > 0.05$). The chitosan soaking + foliar spray 0.2% (CSF 0.2%) treatment gave the lowest significant decrease in total fungal count compared to the control treatment.

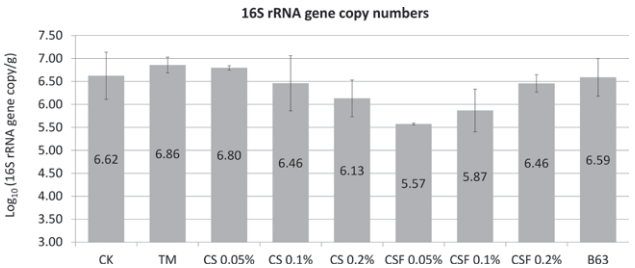

Figure 5. Real time PCR quantifications of total bacteria in TC-DNA based on 16S rRNA gene copy numbers resulting in geranium rhizosphere soil after application of different experimental treatments. CK = Control; TM = Topsin-M; CS = Chitosan soaking; CSF = Chitosan soaking and spraying; B63 = *B. velezensis*. ($P > 0.05$).

In addition to bacterial and fungal counts, 16S rRNA gene copy numbers in the geranium rhizospheres were assessed as indicators of microbial population density. However, no significant differences ($P > 0.05$) were detected between treatments. The geranium rhizosphere 16S rRNA gene copies were in the range of 5.57 to 6.86 Log_{10} copies g^{-1} of root fresh mass. Specifically, except the Topsin-M fungicide and chitosan 0.1% soaking alone (CS 0.1%) treatments, the 16S rRNA gene copies from all the treatments were less than in the control treatment (Figure 5).

Chitosan 0.05% soaking alone (CS 0.05%) gave the lowest numbers of 16S rRNA gene copies (5.57 Log_{10}

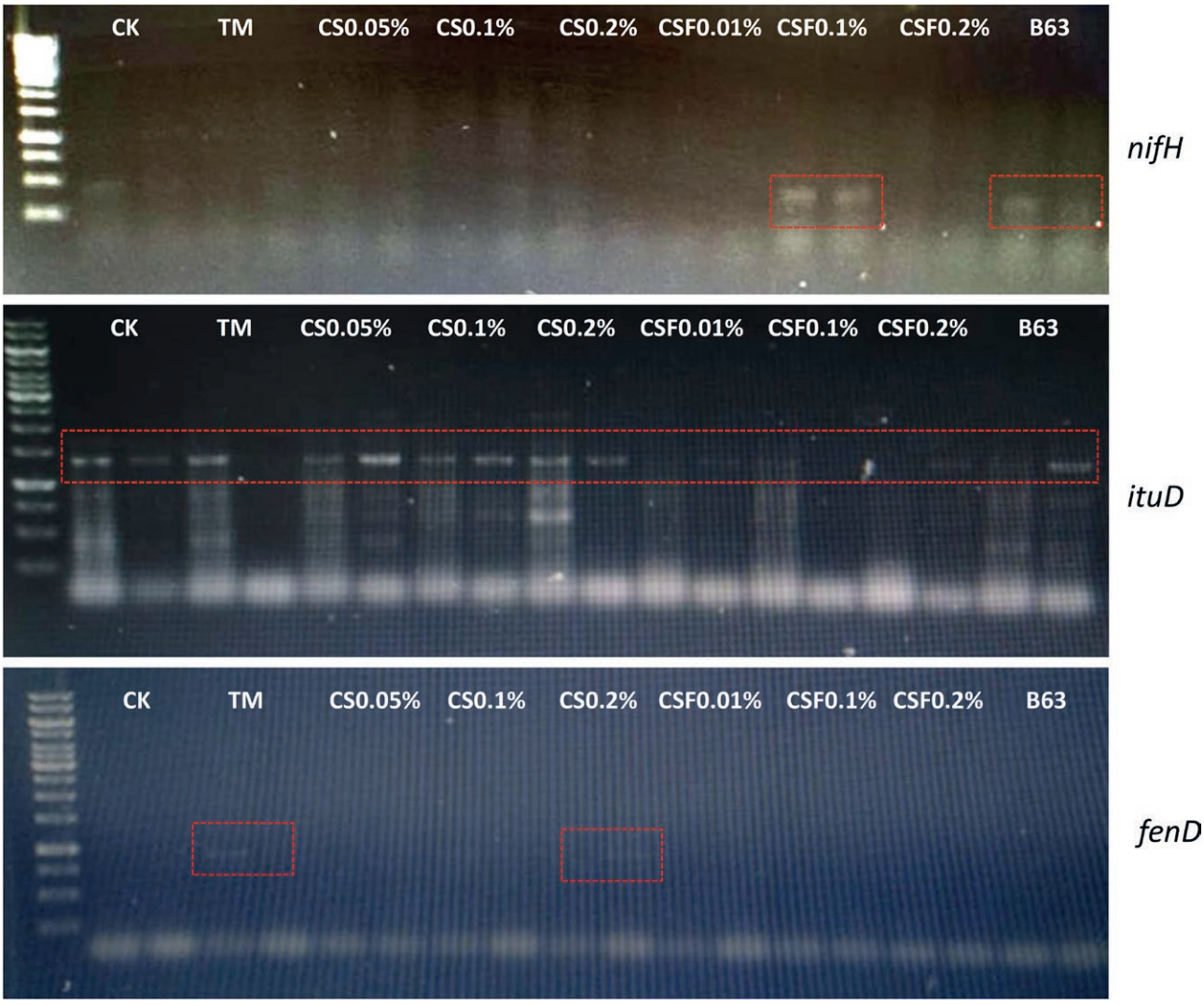

Figure 6. PCR-based detection of genes encoding plant growth promoting related functions.

copies g^{-1} of root fresh weight), followed by chitosan soaking + foliar spray (CSF 0.1%) treatment (5.87 Log_{10} copies g^{-1} of root fresh weight). Greatest numbers gene copies were recorded from the Topsin-M fungicide and chitosan 0.1% soaking alone (CS 0.1%) treatments (6.86 and 6.80 Log_{10} copies g^{-1} of root fresh weight respectively) (Figure 5).

PCR-based detection of genes encoding plant growth promoting related functions

The *nifH* gene encoding the nitrogenase reductase indicates the presence of nitrogen-fixing bacterial populations that were detected in total community DNA extracted from each rhizosphere sample. Our results showed a relatively higher band intensity for the *nifH* gene amplified from CSF 0.1 TC-DNA, followed by B63-treated plants. The *ituD* gene was detected in all treatments, at least in one of the two replicates. Only Topsin-M and SC 0.2 showed a weak *fenD* gene band (Figure 6).

DISCUSSION

A growing body of literature recognizes the importance of biological control agents and biostimulants as sustainable approaches to managing plant pathogens, particularly with increasing concern about the use of synthetic fungicides (Stukenbrock and Gurr, 2023). Bio-

control agents and biostimulants offer effective solutions for plant disease management, as they have proven efficiency for management of several diseases caused by fungi (Meena *et al.,* 2022).

The present study has shown that *B. velezensis* B63 treatment had a growth-promoting effects on geranium plants and fungicidal effects on plant pathogenic fungi. This outcome corroborates results from a previous study (Reyad *et al.,* 2022), which showed that *B. velezensis* B63 had antifungal and plant growth-promoting properties. Chitosan similarly exerted positive effects, although less pronounced than those from B63. The application of *B. velezensis* B63 and chitosan significantly influenced the soil microbial communities, reduced progression of root rot, as indicated by lower plant mortality rates, and promoted geranium growth and production of essential oils. These treatments increased geranium yields and enhanced plant quality.

Previous studies have demonstrated decreased root rot in plants treated with bioagents. For example, Gao *et al*. (2022) showed that *B. subtilis* and *B. velezensis* reduced the incidence of Fusarium root rot in *Astragalus membranaceus*. Similarly, Wang *et al*. (2023) reported that *B. velezensis* B19 was highly antagonistic to *Panax notoginseng* (Sanqi) root rot. Maslennikova *et al.* (2023) found that a mix of *B. subtilis* and *B. amyloliquefaciens* suppressed *R. solani*, and improved the physiology of potato plants. El Hadrami *et al*. (2010) and Liu *et al*. (2016) showed that chitosan played a major role in enhancing plant resistance to diseases.

In the present study, effects of *B. velezensis* B63 and chitosan on geranium root rot development were expressed as plant mortality rates across two growing seasons, and this showed that several treatments decreased plant mortality in the 2019-2020 field experiment. The most effective treatments were *B. velezensis* B63, chitosan soaking + foliar spray 0.2% or 0.1% (CSF 0.2% and 0.1%), and chitosan 0.2% soaking alone (CS 0.2%). These treatments resulted in mortality reductions of 36% for *B. velezensis* B63, 33% for CSF 0.2%, 33% for CSF 0.1%, and 31% for CS 0.2% treatment, compared to untreated experimental controls. In contrast, during the second season (2020-2021), only treatments involving *B. velezensis* B63, CSF 0.2%, and CSF 0.1% reduced plant mortality by, respectively, 33%, 27% and 26%, compared to controls. Other treatments did not differ significantly from the control. The least effective treatment over both seasons was chitosan 0.05% soaking alone (CS 0.05%), which gave a smaller reduction in geranium mortality of 16% in 2019-2020 and 6% in 2020-2021, relative to the control plants. These results indicate that chitosan efficacy was dose dependent. The observed difference in treatment efficacy, especially for chitosan treatments, may also have been due to different climatic conditions prevailing during the two growth seasons.

Bacillus velezensis B63 also strongly suppressed *in vitro* growth of *R. solani* and *M. phaseolina*. Suppression of *R. solani* and *M. phaseolina* by specific microorganisms has been associated with the formation of secondary metabolites that are toxic to the pathogens, such as siderophore bacteriocins, and cyanide (Lahlali *et al.,* 2022). The strain B63 was found to produce secondary metabolites related to biological control, including bacilysin, macrolactin, bacillaene, bacillibactin, surfactin, and fengycin, (Helal *et al*., 2022). Surfactin is important for motility, signalling, and biofilm formation as well as surface colonization (Hafeez *et al*., 2019; Helal *et al*., 2022; Reyad *et al.,* 2022).

In the present study, *B. velezensis* B63 and chitosan applications reduced geranium root rot and also improved the morphometric parameters of field-grown geranium plants during the growing seasons, including plant height, number of branches, and mass per plant. Other studies have confirmed that *Bacillus* can promote plant growth (Fan *et al*. 2018), directly or indirectly (Yánez-Mendizábal and Falconí, 2018). For example, *B. velezensis* (CE 100) effectively promoted growth of seedlings of Japanese cypress (*Chamaecyparis obtusa* Endlicher), by secreting lytic enzymes (Moon *et al*., 2021). Similarly, Zhao *et al.* (2016) demonstrated that application of *B. velezensis* (GB03) increased the growth of the traditional Chinese herbal plant *Codonopsis pilosula*. Khan *et al*. (2020), reported that *B. velezensis* Lle-9 had broad-spectrum antifungal and plant growth promotion activity. The ability of *B. velezensis* B63 to improve the geranium growth may be attributed to its plant growth-promoting traits such as nitrogen fixation and phosphate solubilization (Reyad *et al.,* 2022).

The significant influence of chitosan on agromorphological characteristics observed in the present study is comparable to previous results for sweet basil (Ghasemi Pirbalouti *et al.,* 2017), *Mentha piperita* L. (Ahmad *et al*., 2019; Da Silva *et al.,* 2021), Carla (*Momordica charantia* L.) (Sharifi-Rad *et al.,* 2020), *Amaranthus hybridus* L. (Berliana *et al.,* 2020), and Moringa (*Moringa oleifera* L.) (Zubair *et al*., 2021). These comparable results could be attributed to chitosan stimulation of production of plant hormones such as auxins and cytokinins, which promote cell elongation and division, and increase plant growth. Enhanced transportation of nitrogen in functional leaves also supports plant growth and development and enhances plant quality attributes by increasing protein synthesis and chlorophyll production (Mazrou *et al.,* 2021). In contrast to the present results, Kahromi and

Khara (2021) found that foliar application of chitosan did not affect biomass of *Dracocephalum kotschy*. A possible explanation for this could be differences in application methods, application time, and the different plant species (Kahromi and Khara, 2021).

Photosynthesis rate is important for plant productivity and is dependent on mineral nutrient uptake (Evans 2013; Li *et al.* 2023). Nutrient uptake determines chlorophyll content in leaves and photosynthetic functioning. Increased photosynthesis then increases accumulation of carbohydrates and secondary metabolites in medicinal plants (Swamy and Rao, 2009). Growing evidence also indicates that plant-microbe interactions influence the quantity and types of secondary metabolites produced by plants, ultimately affecting overall plant yields. This has created interest in the application of biostimulants to cultivated medicinal and aromatic plants using natural methods (Kıtır Sen and Duran, 2023). Concentration and composition of essential oils (Eos) in plants also have ecological significance. For example, increased EO production can be a defense mechanism against microbes (Orhan *et al*., 2012).

In the present study, applications of chitosan and *B. velezensis* B63 improved geranium essential oil yield. Specifically, *B. velezensis* strain B63 treatment gave the greatest percentage of essential oil content and greatest yield. Increased levels of essential oil in plants treated with *B. velezensis* may explain their enhanced resistance to root rot infections in the field observed in this study. This aligns with previous research demonstrating the potent antimicrobial properties of plant essential oils, making them valuable biocontrol agents against plant pathogens (Raveau *et al*., 2020). The high oil yields observed in *B. velezensis*-treated plants could be due to plant growth promotion, particularly from phosphate solubilization. Nitrogen and phosphorus are crucial elements for medicinal plants as they contribute to precursor structures of essential oils (Dehsheikh *et al*., 2020). Kıtır Sen and Duran (2023) also reported that *B. subtilis* and *B. megaterium* led to increased yields of Izmir thyme. Other reports have shown that chitosan can increase essential oil content of thyme (Emami Bistgani *et al.,* 2017), *Origanum vulgare* (Bharti *et al.,* 2013), and *Matricaria chamomilla* L. (chamomile) (Mazrou *et al*., 2021).

Chitosan soaking + foliar spray 0.2% (CSF 0.2%) and *B. velezensis* B63 also increased production of the secondary metabolites citronellol, geraniol, citronellyl formate, and geranyl formate in geranium essential oil. This bacterium strain also increased production of eugenol. These results are similar to those from previous studies, indicating that foliar chitosan applications at 0.01% promoted accumulation of artemisinin in *Artemisia annua* (Lei *et al*., 2011) and β-caryophyllene in basil (*Ocimum basilicum* L.) (Antoniazzi and Deschamps, 2006). Similarly, chitosan boosted *in vitro* saponin accumulation in *Panax ginseng* (Chamkhi *et al.,* 2021), and rosmarinic acid and quercetin in *Dracocephalum kotschyi* (Kahromi and Khara, 2021). Bacteria also release molecules that can be elicitors, inducing metabolic alterations in host plants, including accumulation of secondary metabolites. For example, *B. subtilis* uses lipoproteins such as surfactins and fengycins as elicitors to activate host plants (Chamkhi *et al*., 2021).

Rhizosphere microbiomes promote plant nutrient uptake, modulate plant immunity, suppress pathogens, and control diseases (Olanrewaju and Babalola, 2022). Ali *et al*. (2023) described the "cry for help" theory of plant responses. Plants exposed to stress actively recruit specific microorganisms to provide protection from detrimental effects. Changes in rhizosphere microbiome composition could indicate whether plants stay healthy or become infected by pathogens (Gu *et al.,* 2022). No previous research has been published on the influence of *B. velezensis* B63 and chitosan treatments on the geranium rhizosphere microbiome. The present study showed that *B. velezensis* B63 and chitosan soaking + foliar spray 0.2% (CSF 0.2%) were effective treatments for reducing geranium root rot and improving plant yields, as well as changing rhizosphere microbiome structure, including CFU and 16S rRNA gene analyses. Focus on bacterial community aims to provide insight into biological control mechanisms active in the rhizosphere. Direct quantification of pathogenic fungi can provide valuable information, but the objective was to elucidate the intricate relationships between rhizosphere bacteria and fungi, as well as host plant ability to interact with and respond to these microorganisms. By examining changes in bacterial populations, the study indirectly inferred effects on pathogenic fungi, to provide an understanding of the complex interactions involved in plant health and disease resistance.

The most obvious result from this analysis was that after treating geranium plants with *B. velezensis* B63 and chitosan soaking + foliar spray, the amounts of *Kaistobacter* showed 2-fold increases. This genus is known to have several important ecological roles, including heavy metal accumulation and bioremediation of polluted sites (Garbini *et al.,* 2022), and provision of abiotic stress tolerance and plant growth promotion (dos Santos Lopes *et al.,* 2021). Studies have also shown that *Kaistobacter* spp. can enhance plant nutrient uptake and improve resistance to pathogens (Chen *et al*., 2020). There was also a 2.3-fold and 1.4-fold increase in the amounts of bacteria in the unclassified Acidobacteria iii1–15 due to chi-

tosan treatment, and a 1.4-fold increase from *B. velezensis* B63 treatment. *Acidobacteria* are active in the rhizosphere, where they break down complex carbohydrates from plants, and have genes for extracellular peptidases, which help to release ammonium during nitrogen cycling. Additionally, these bacteria can contribute to the plant growth (Pinho *et al*., 2020). Chitosan and *B. velezensis* B63 treatments also increased the abundance of *Gemmata*, by, respectively, 2.20-fold and 1.11-fold. According to Li *et al*. (2022), *Gemmata* promote plant growth and have important roles in cucumber growth.

Chitosan and *B. velezensis* B63 treatments also induced increases in abundance of some minor bacterial genera in field soil. Various members of these groups may have crucial roles in regulating plant growth and development (Srivastava *et al*., 2022). Some of these bacteria have been identified as beneficial for plants in other pathosystems. *Rhodoplanes* and the nitrogen uptake-related *Sinorhizobium*, could provide benefits through regulation of root development, upholding hormonal balance, facilitating mobilization and acquisition of nutrients, and suppressing disease in host plants (Srivastava *et al*., 2022). Increases in these microorganisms in soil were associated with enhanced growth and development of geranium plants.

Numbers of bacterial colony-forming units (CFUs) were generally greater in all experimental treatment soil samples than in the control samples. For fungi, some changes were observed across treatments. Despite the general decrease in fungal CFU counts, application of chitosan at the tested concentrations increased fungal counts. In addition, elucidating the microbial community structure, 16S rRNA gene copy numbers indicated the density of the communities. The copy numbers were generally elevated across all treatments. Chitosan concentrations, when applied through soaking or spraying, varied markedly, indicating that the application method affected the structure of the bacterial community in geranium rhizospheres. Application of *B. velezensis* B63 resulted in considerably reduced 16S rRNA gene copy numbers, indicating an alteration in the constitution of the microbial community. Application of chitosan and inoculation with the B63 strain caused dominance of bacterial populations carrying genes associated with soil fertility (e.g., *nifH*), and disease suppression (*ituD* and *FenD*) in geranium rhizospheres. High intensity of *nifH* was recorded in plants treated with chitosan soaking + foliar spray and with *B. velezensis* B63. Biological nitrogen fixation converts nitrogen into ammonia, which is biologically usable, and is also produced through soil mineralization. The *nifH* gene is commonly used as a marker for the molecular analyses of nitrogen-fixing microorganisms. In the present study, the increase in *nifH* was consistent with the results of Liu *et al*. (2022), who reported that the copy numbers of the N_2-fixing gene *nifH* increased in watermelon rhizospheres after *Burkholderia vietnamiensis* B418 inoculation.

CONCLUSIONS

The bacterium *B. velezensis* B63 had antagonistic activity against *Rhizoctonia solani* and *Macrophomina phaseolina in vitro*. This organism also reduced damage of geranium plants during growing season more than chitosan. Both treatments led to changes in composition of the soil microbiota. Numbers of bacteria fixing nitrogen and nutrient uptake increased. This was accompanied by increases in geranium plant morphometric and physiological parameters (essential oil composition), and increases in essential oil yields. These results indicate that the chitosan and the beneficial bacterium B63 could be effective for plant disease control. The increased essential oil production and improved plant growth and yield observed in this study were likely due to the attractiveness of geranium root exudates to antimicrobial and plant growth promoting (PGP) bacterial communities (*Kaistobacter, Agrobacterium, Gemmata*, *Rhodoplanes, Sinorhizobium*, and unclassified *Acidobacteria* iii1-15) around the plant roots. Further research is required to identify the specific types of beneficial bacteria involved in this interaction, and to determine how they are influenced by root exudates under stress of pathogen infections.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.E.-H.A.R., N.A.M.E and T.R.E.; methodology, N.E.-H.A.R., N.A.M.E and T.R.E.; software, N.E.-H.A. R and T.R.E.; validation, N.E.-H.A.R.; formal analysis, N.E.-H.A.R.; investigation, N.E.-H.A.R., N.A.M.E.; resources, N.A.M.E.; data curation, N.E.-H.A.R.; funding acquisition, N.E.-H.A.R., N.A.M.E and F.A.S.; writing—original draft preparation, N.A.M.E., T.R.E. and F.A.S.; writing, review and editing, N.E.-H.A.R. All authors have read and agreed to the published version of the manuscript.

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