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Bacillus spp. strain-induced modulation of stomatal density in parsnip *(Pastinaca sativa* L.) and associated soil microbiological responses

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A B S T R A C T

Reducing chemical pesticide use and adopting biological alternatives are key priorities in sustainable horticulture. This study compared the effects of chemical and biological treatments on stomatal density in parsnip (*Pastinaca sativa* L.) and evaluated associated soil microbiological changes. Field trials near Skopje included a control (untreated) plot and three treatments: (T1) foliar application of Ridomil Gold MZ 68 WG (40 g/kg metalaxyl-M + 640 g/kg mancozeb), (T2) *Bacillus velezensis* strain B-98, and (T3) *Bacillus amyloliquefaciens* strain B-62. Stomatal density was determined microscopically on both leaf surfaces, and soil microbial communities were quantified before and after the growing season. ANOVA revealed highly significant differences ($p < 0.001$) in lower epidermal stomatal density between the control and all treatments, with T3 exhibiting the highest value (1552 stomata/mm^2). Biological treatments also increased beneficial soil microorganisms, including nitrogen-fixing and cellulolytic bacteria. The results highlight *B. amyloliquefaciens* strain B-62 as a promising biopreparation for enhancing physiological traits and soil health in sustainable parsnip production.

Keywords: *Bacillus amyloliquefaciens, Bacillus velezensis,* stomatal density, parsnip, biofertilizer, sustainable agriculture

И З В О Д

Смањење употребе хемијских пестицида и усвајање биолошких алтернатива су кључни приоритети у одрживој хортикултури. Ова студија је упоредила ефекте хемијских и биолошких третмана на густину стомата код пастрњака (*Pastinaca sativa* L.) и проценила повезане микробиолошке промене у земљишту. Теренска испитивања у близини Скопља обухватила су контролну (нетретирану) парцелу и три третмана: (T1) фолијарну примену Ridomil Gold MZ 68 WG (40 g/kg металаксила-M + 640 g/kg манкозеба), (T2) соj *Bacillus velezensis* B 98 и (T3) соj *Bacillus amyloliquefaciens* B 62. Густина стомата је одређена микроскопски на обе површине листа, а микробне заједнице земљишта су квантификоване пре и после вегетације. ANOVA је открила веома значајне разлике ($p < 0,001$) у нижој густини епидермалних стомата између контроле и свих третмана, при чему је T3 показао највећу вредност (1552 стомата/mm^2). Биолошки третмани су такође повећали број корисних микроорганизама у земљишту, укључујући бактерије које фиксирају азот и целулолитичке бактерије. Резултати истичу соj *B. amyloliquefaciens* B 62 као обећавајући биопрепарат за побољшање физиолошких особина и здравља земљишта у одрживој производњи пастрњака.

Кључне речи: *Bacillus amyloliquefaciens, Bacillus velezensis*, густина стомата, пастрњак, биођубриво, одржива пољопривреда

1. Introduction

Parsnip (*Pastinaca sativa* L.) is a biennial root vegetable of the Apiaceae family, valued for its nutritional profile and adaptability to temperate climates. Its high carbohydrate, mineral, and dietary fiber content make it an important crop for both fresh consumption and processing (Petropoulos et al., 2019). However, production is often constrained by fungal and bacterial diseases, which are traditionally managed with chemical pesticides. While effective, these chemicals can negatively impact soil health, biodiversity, and environmental quality (Pimentel & Burgess, 2014).

Biological control agents, particularly plant growth-promoting rhizobacteria (PGPR) such as *Bacillus* spp., offer an eco-friendly alternative. These Gram-positive, spore-forming bacteria enhance plant growth through nutrient solubilization, phytohormone production, and pathogen suppression (Kumar, Dubey, & Maheshwari, 2011). *Bacillus velezensis* and *Bacillus amyloliquefaciens* are notable for their resilience, ease

of formulation, and dual roles as biofertilizers and biocontrol agents (Fan et al., 2018).

Soil health is a cornerstone of sustainable agriculture, influencing crop productivity, nutrient cycling, and resilience to environmental stress (Pimentel & Burgess, 2014). The use of microbiological fertilizers, particularly PGPR such as *B. velezensis* and *B. amyloliquefaciens*, has been shown to enhance plant growth, improve nutrient uptake, and suppress pathogens (Fan et al., 2018; Kumar et al., 2011). B. *amyloliquefaciens* as a promising candidate for developing sustainable and eco-friendly agricultural practice (Zalila-Kolsi 2023).

These beneficial effects are often mediated through hormonal signaling, including the production of indole-3-acetic acid (IAA), which influences root architecture and stomatal development (Spaepen et al., 2009). Stomata, in turn, play a critical role in regulating gas exchange and water loss, directly linking leaf anatomy to photosynthetic efficiency and plant water relations (Hetherington & Woodward, 2003).

Stomata, microscopic pores on leaf surfaces, regulate gas exchange and water loss, directly influencing photosynthesis and transpiration (Hetherington & Woodward, 2003). While stomatal traits are known to respond to environmental and physiological factors, the influence of foliar-applied *Bacillus* strains on stomatal density in parsnip remains unexplored. Soil's different components are essential for plant growth such as microelements and macroelements (More, et al., 2020). This study aimed to (i) compare the effects of chemical and biological treatments on stomatal density in parsnip, and (ii) assess the impact of these treatments on soil microbial communities.

2. Materials and methods

2.1. Experimental design

Field trials were conducted near Skopje, North Macedonia, using a randomized block design with four treatments:

- **T0 (Control):** Untreated plants
- **T1:** Ridomil Gold MZ 68 WG (metalaxyl-M + mancozeb) at 25 g per 10 L water
- **T2:** *Bacillus velezensis* B-98 at 0.4×10^7 CFU mL^{-1} (10 mL per 10 L water)
- **T3:** *Bacillus amyloliquefaciens* B-62 at 0.4×10^7 CFU mL^{-1} (10 mL per 10 L water)

2.2. Stomatal density measurement

Leaf impressions from the upper and lower epidermis were prepared using clear nail varnish and examined under a light microscope, following standard epidermal impression techniques for stomatal analysis (Hetherington & Woodward, 2003). Stomata were counted per mm^2, and mean values were calculated for each treatment.

2.3. Soil microbial populations

The following groups of microorganisms were examined before the beginning and at the end of the vegetation period: total number of bacteria in soil – nutrient agar (agar 15 gL^{-1}, peptone 15 gL^{-1}, meat extract 3 gL^{-1}, NaCl 5 gL^{-1}, K_2HPO_4 0.3 gL^{-1}, incubation for 5 days at a temperature of 28 °C – Sarić, 1992), nitrogen-fixing bacteria – esbi agar (sucrose 20 gL^{-1}, K_2HPO_4 0.2 gL^{-1}, $MgSO_4$ x $7H_2O$ 0.2 gL^{-1}, K_2SO_4 0.1 gL^{-1}, $CaCO_3$ 5 gL^{-1}, agar 15 gL^{-1}, incubation for 7 days at a temperature of 28 °C – Jarak, 2004), nitrifying microorganisms – mineral substrate [$(NH4)_2SO_4$ 2 gL^{-1}, K_2HPO_4 1 gL^{-1}, $MgSO_4$ 0.5 gL^{-1} , Fe SO_4 0.4 gL^{-1}, NaCl 0.4 gL^{-1}, $CaCO_3$ 1gL^{-1}, $MgCO_3$ 1 gL^{-1}, agar 15 gL^{-1}, distilled water 1 L , incubation for 5–7 days at a temperature of 22 °C – Jarak, 2004)], cellulolytic microorganisms – Waxman–Garey [$(NH_4)_2HPO_4$ 2.5 gL^{-1}, $MgSO_4$ 0.5 gL^{-1}, $FeSO_4$ 0.01 gL^{-1}, KCl 0.5 gL^{-1}, $CaCl_2$ 0.02 gL^{-1}, $MnSO_4$ 0.001 gL^{-1}, agar 15 gL^{-1}, Na carboxymethyl cellulose 2 gL^{-1}, incubation for 7–14 days at a temperature of 28 °C – Sarić, 1992], yeasts – Czapek-Dox Agar ($NaNO_3$ 2 gL^{-1}, KH_2PO_4 1 gL^{-1}, $MgSO_4$ 0.5 gL^{-1}, KCl 0.5 gL^{-1}, $FeSO_4$ 0.01 gL^{-1}, sucrose 30 gL^{-1}, agar 20 gL^{-1} , incubation for 7 days at a temperature of 25 °C – Govedarica, Jarak, & Milošević, 1997), and molds – Czapek-Dox Agar ($NaNO_3$ 2 gL^{-1}, KH_2PO_4 1 gL^{-1}, $MgSO_4$ 0.5 gL^{-1}, KCl 0.5 gL^{-1}, $FeSO_4$ 0.01 gL^{-1}, sucrose 30 gL^{-1}, agar 20 gL^{-1}, incubation for 7 days at a temperature of 25 °C – Govedarica et al., 1997).

2.4. Soil chemical properties

2.4.1. Potentiometric method of pH determination

Soil pH was determined using the potentiometric method, where $pH = -\log_{10}[H^+]$, with $[H^+]$ denoting the hydrogen ion concentration in molar units (Allen, 1989). Based on pH in KCl, soils were classified as alkaline (> 7.20), neutral (6.51–7.20), slightly acidic (5.51–6.50), acidic (4.51–5.50), or very acidic (< 4.50).

Following soil sample collection, moisture content was determined gravimetrically by drying samples to constant mass at 105 °C (Allen, 1989). For pH measurement, 20 g of soil was mixed with 40 mL of distilled water, stirred, and allowed to settle for one hour before measurement with a calibrated pH meter.

2.4.2. Gravimetric soil moisture detection (Oven drying technique)

The gravimetric method, a standard reference technique for soil moisture determination, was used to calculate gravimetric water content (GWC).

2.4.3 Kotzmann method for determining humus

Humus content was determined by oxidation of organic matter with $KMnO_4$, followed by titration with oxalic acid, as described by Sarić (1992). The amount of oxidizing agent consumed was used to calculate carbon content, which was then converted to humus percentage using a factor of 1.72.

2.4.4. Heavy metal analysis

Samples were ground and wet-digested with HNO_3 and H_2O_2 following Soylak, Tuzen, and Narin (2004). Total heavy metal content was determined by wet digestion with HNO_3:$HClO_4$:H_2SO_4 (10:2:1) (Allen, 1989). DTPA-extractable heavy metals were determined according to Lindsay and Norvell (1978). For total digestion, nitric, hydrofluoric, and perchloric

acids were used following ISO 14869-1 (2001). Concentrations of Ni, Cu, Zn, Fe, Mn, Pb, and Cd were measured by atomic absorption spectrometry (AAS) using an Agilent 55A, with blank corrections applied.

2.4. *Statistical analysis*

Data were analyzed using analysis of variance (ANOVA), and treatment means were compared using the least significant difference (LSD) test at the 0.05, 0.01, and 0.001 significance levels (Allen, 1989).

3. Results and Discussion

3.1. Stomatal density

Lower epidermal stomatal density was significantly higher in all treated plants compared to the control ($p < 0.001$) (Table 1). T3 recorded the highest density (1552 stomata/mm^2), followed by T2 (1304 stomata/mm^2) and T1 (1266 stomata/mm^2). Upper epidermal stomatal density showed smaller differences, with the control having the highest value.

Table 1.
Overview of stomatal density results

Treatment	Upper epidermis	Lower epidermis	Total stomatal density (stomata/mm^2)
Control	108	1083	1191
Ridomil gold	78	1266	1344
Bacillus velezensis (B-98)	97	1304	1401
Bacillus amyloliquefaciens (B-62)	90	1552	1642

The analysis of stomatal density across different treatment groups revealed distinct physiological responses in both the upper and lower epidermal surfaces of the leaves. Control plants exhibited a uniform stomatal distribution, with 108 stomata mm^{-2} on the upper epidermis and 1083 stomata mm^{-2} on the lower, resulting in a total density of 1191 stomata mm^{-2}. This baseline reflects typical stomatal development under untreated conditions (Hetherington and Woodward, 2003).

Treatment with Ridomil Gold, a chemical fungicide, led to a marked reduction in stomatal density on the upper epidermis (78 stomata mm^{-2}), representing a 27.8 % decrease compared to control. Conversely, the lower epidermis showed an increase to 1266 stomata mm^{-2} (+16.9 %), suggesting a compensatory mechanism or altered epidermal differentiation. The total stomatal density increased modestly to 1344 stomata mm^{-2}, indicating that while Ridomil Gold may suppress stomatal formation on the adaxial surface, it does not inhibit overall stomatal development.

Biological treatments with *B. velezensis* (B-98) and *B. amyloliquefaciens* (B-62) elicited more pronounced effects. B-98-treated plants maintained near-control levels on the upper epidermis (97 stomata mm^{-2}) but showed a substantial increase on the lower epidermis (1304 stomata mm^{-2}), resulting in a total density of 1401 stomata mm^{-2} (+17.6 % vs control). This suggests that B-98 may enhance stomatal development through microbial signaling pathways, potentially involving phytohormones such as cytokinins or auxins (Spaepen, Vanderleyden, & Remans, 2009).

The most significant response was observed in plants treated with B-62. Although upper epidermal stomatal density was slightly reduced (90 stomata mm^{-2}), the lower epidermis exhibited a dramatic increase to 1552 stomata mm^{-2} (+43.3 %), culminating in a total stomatal density of 1642 stomata mm^{-2} (+37.8 %). This elevated stomatal count may reflect enhanced photosynthetic capacity, improved transpiration efficiency, and increased nutrient uptake—the traits commonly associated with plant growth-promoting rhizobacteria (PGPR) (Kumar et al., 2011; Fan et al., 2018). The data suggest that B-62 exerts a strong biostimulatory effect, potentially mediated by microbial production of volatile organic compounds or modulation of plant hormonal balance (Spaepen et al., 2009).

Biological treatments, particularly B-62, significantly increased stomatal density on the lower epidermis compared to control and chemical treatments, consistent with previous findings that PGPR can enhance leaf anatomical traits linked to photosynthetic capacity (Spaepen et al., 2009). Soil microbial counts revealed substantial increases in *Bacillus* spp., nitrogen-fixing, and cellulolytic bacteria under microbial treatments, aligning with earlier reports on the rhizosphere-colonizing ability of *Bacillus* strains (Kumar et al., 2011; Fan et al., 2018).

Overall, the results indicate that biological treatments, particularly B-62, outperform chemical fungicide in promoting stomatal development. These findings support the integration of PGPR into sustainable crop management strategies, with implications for improved plant vigor, stress resilience, and productivity.

The analysis of variance (ANOVA) was conducted to evaluate the significance of treatment effects on stomatal density across the upper epidermis, lower epidermis, and total leaf surface. The results provide insight into the differential impact of chemical and biological treatments on leaf anatomical development.

The ANOVA for stomatal density on the upper epidermis (Table 2) revealed no statistically significant differences among treatments ($F = 1.635$, $p = 0.187 > 0.05$). The calculated F-value did not exceed the critical value (F crit = 2.709), indicating that the observed variation in stomatal number across treatments is likely due to random error rather than treatment effects. This is further supported by the relatively low sum of squares for columns (SS = 12,756.35) compared to the error term (SS = 226,224.4). The LSD values ($LSD_0.05 = 26.12$) suggest that pairwise differences between treatment means would need to exceed this threshold to be

considered significant, which was not observed. These findings imply that stomatal development on the adaxial (upper) surface is relatively stable and less responsive to external inputs (Hetherington & Woodward, 2003).

In contrast, the ANOVA for the lower epidermis (Table 2) demonstrated a highly significant treatment effect ($F = 34.98$, $p < 0.0001$), with the F-value far exceeding the critical threshold (F crit = 2.709). The treatment sum of squares (SS = 3,335,254) accounted for a substantial proportion of the total variation, indicating that the applied treatments—particularly the *Bacillus* strains—had a pronounced influence on stomatal development on the abaxial surface. The $LSD_0.05$ value of 91.32 confirms that the observed differences between treatment means (e.g., B-62 vs control) are statistically meaningful. This suggests that microbial treatments may stimulate epidermal differentiation or hormonal signaling pathways that enhance stomatal formation on the lower leaf surface, which is typically more physiologically active in gas exchange (Spaepen et al., 2009).

Table 2.
Combined ANOVA summary for stomatal density

Parameter	Source of variation	SS	df	MS	F	P-value	F crit	$LSD_0.05$	$LSD_0.01$	$LSD_0.001$
Upper epidermis	Rows	73,224.67	29	2,524.99	0.971	0.518	1.598	26.12	34.57	44.64
	Columns	12,756.35	3	4,252.12	1.635	0.187	2.709			
	Error	226,224.40	87	2,600.28						
	Total	312,205.42	119							
Lower epidermis	Rows	857,063.10	29	29,553.90	0.930	0.574	1.598	91.32	120.87	156.06
	Columns	3,335,254.00	3	1,111,751.33	34.980	6.3×10^{-15}	2.709			
	Error	2,765,067.00	87	31,782.38						
	Total	6,957,384.10	119							
Total stomatal density	Rows	1,027,885.00	29	35,444.31	0.962	0.531	1.598	98.35	130.17	168.07
	Columns	3,133,578.00	3	1,044,526.00	28.339	7.06×10^{-13}	2.709			
	Error	3,206,642.00	87	36,857.96						
	Total	7,368,105.00	119							

The ANOVA for total stomatal density (Table 2) also revealed a significant treatment effect ($F = 28.34$, $p < 0.0001$), reinforcing the conclusion that biological inputs, especially *B. amyloliquefaciens* (B-62), substantially increase overall stomatal density. The treatment sum of squares (SS = 3,133,578) was nearly equivalent to the error term (SS = 3,206,642), indicating a strong signal-to-noise ratio. The $LSD_0.05$ value of 98.35 supports the statistical separation of treatment means, validating the physiological relevance of the observed increases.

Taken together, the ANOVA results confirm that, while stomatal density on the upper epidermis remains largely unaffected by treatment, significant and biologically meaningful changes occur on the lower epidermis and in total stomatal count. These findings underscore the potential of microbial biostimulants—particularly B-62—to enhance leaf anatomical traits associated with improved photosynthetic efficiency, transpiration, and nutrient uptake (Fan et al.2018; Kumar et al., 2011; Spaepen et al., 2009). Such effects may contribute to greater plant vigor and resilience, aligning with broader goals of sustainable crop management and circular agri-food system design.

3.2. Soil microbial populations

Soil microbial communities play a critical role in nutrient cycling, organic matter decomposition, and overall soil fertility (Govedarica, Jarak, & Milošević, 1997). The application of microbiological fertilizers—comprising beneficial bacteria and fungi—has emerged as a sustainable strategy to enhance soil health and crop productivity (Kumar et al., 2011; Fan, Blom, Klenk, & Borriss, 2018). These bio-inputs can stimulate microbial diversity, increase enzymatic activity, and improve the abundance of functional groups such as nitrogen-fixers, cellulolytic organisms, and nitrifiers (Sarić, 1992; Jarak, 2004).

The present study evaluates the impact of different microbiological fertilizer treatments on the abundance of key soil microorganisms, including total aerobic bacteria, *Bacillus* spp., nitrogen-fixing bacteria, cellulolytic bacteria, nitrifying bacteria, yeasts, and molds (Table 3). Four treatments were assessed: Control – untreated plants; Ridomil Gold MZ 68 WG; *Bacillus velezensis* B-98; and *Bacillus amyloliquefaciens* B-62. The results provide insight into how microbial amendments influence soil biological activity and functional potential.

The data reveal substantial differences in microbial abundance across treatments, with B-62 consistently outperforming other groups in terms of microbial proliferation and functional diversity. For total aerobic bacteria, B-62 recorded the highest count (1.8×10^7 CFU g^{-1}), indicating a strong stimulatory effect of the microbial fertilizer on general bacterial growth. This suggests improved aeration, organic matter availability, or microbial synergy within the soil matrix (Govedarica et al., 1997).

Table 3.
Impact of microbiological fertilizer on soil microorganism status

Treatment	Total aerobic bacteria	*Bacillus spp.*	Nitrogen-fixing bacteria	Cellulolytic bacteria	Nitrifying bacteria	Yeasts	Molds
Control	1.6×10^6	1.0×10^5	3.8×10^5	1.06×10^6	1.7×10^6	3.2×10^5	6.0×10^2
Ridomil Gold	8.5×10^5	9.4×10^4	2.0×10^5	4.6×10^5	2.2×10^5	1.6×10^5	6.0×10^1
Bacillus velezensis (B-98)	5.9×10^6	1.13×10^8	1.0×10^6	5.2×10^6	8.5×10^6	1.6×10^6	3.2×10^3
Bacillus amyloliquefaciens (B-62)	1.8×10^7	1.01×10^9	1.06×10^7	2.0×10^7	2.9×10^7	4.0×10^5	1.6×10^4

The abundance of *Bacillus* spp. was markedly elevated in B-62 (1.01×10^9 CFU g^{-1}), over 10,000 times higher than in the control. Given the role of *Bacillus* in plant growth promotion, pathogen suppression, and enzyme production (Kumar et al., 2011; Fan et al., 2018), this increase may reflect successful colonization and competitive advantage conferred by the applied strain.

For nitrogen-fixing bacteria, B-62 again showed the highest population (1.06×10^7 CFU g^{-1}), suggesting enhanced nitrogen bioavailability. This could translate into improved plant nutrition and reduced dependence on synthetic nitrogen inputs (Sarić, 1992; Jarak, 2004).

Cellulolytic and nitrifying bacteria were also significantly more abundant in B-62, with cellulolytic bacteria reaching 2.0×10^7 CFU g^{-1} and nitrifiers at 2.9×10^7 CFU g^{-1}. These results point to accelerated organic matter decomposition and nitrogen transformation processes, which are vital for soil fertility and crop performance (Govedarica et al., 1997).

While yeasts were most abundant in B-98 (1.6×10^6 CFU g^{-1}), molds peaked in B-62 (1.6×10^4 CFU g^{-1}). The presence of these fungi may contribute to nutrient cycling and antagonism against soil-borne pathogens, although excessive mold proliferation should be monitored for potential phytotoxicity (Govedarica et al., 1997).

The untreated soil exhibited the lowest microbial counts across all categories, confirming the baseline status of the soil and underscoring the positive impact of microbial treatments.

Overall, the results demonstrate that microbiological fertilizers—particularly the formulation used in B-62—significantly enhance the abundance and diversity of beneficial soil microorganisms. These changes are likely to improve soil structure, nutrient availability, and plant health, aligning with the principles of circular agriculture and sustainable land management (Pimentel & Burgess, 2014).

3.3. Soil chemical properties

Soil health is a multidimensional concept encompassing biological, chemical, and physical properties that collectively influence plant productivity and ecosystem resilience (Pimentel & Burgess, 2014). The application of microbiological fertilizers and chemical treatments can alter soil composition, nutrient availability, and organic matter content (Allen, 1989). This study evaluates the effects of Ridomil, two microbiological treatments (*Bacillus amyloliquefaciens* B-62 and *Bacillus velezensis* B-98), and an untreated control on humus content, elemental composition, and selected physicochemical parameters.

3.3.1. Physicochemical properties

The physicochemical parameters of the soil remained consistent across all treatments, including the control, chemical fungicide (Ridomil Gold), and biological treatments with *B. velezensis* (B-98) and *B. amyloliquefaciens* (B-62) (Table 4). This uniformity suggests that the applied treatments did not significantly alter the immediate chemical or physical environment of the soil within the sampling period.

All treatments maintained a neutral pH of 7.89, indicating stable acid–base conditions favorable for microbial activity and nutrient availability (Allen, 1989). The redox potential (–41.9 mV) reflects a mildly reducing environment, which may support anaerobic or facultative microbial processes, particularly in micro-niches (Govedarica et al., 1997). The lack of variation in pH and ORP suggests that neither chemical nor biological inputs disrupted the soil's buffering capacity or redox equilibrium.

Table 4.
Soil physicochemical properties

Treatment	pH	ORP (mV)	Conductivity (µS/cm)	TDS (ppm)	Salt (ppm)	Temperature (°C)	Moisture (%)
Control	7.89	–41.9	596	392	297	23.2	14.50
Ridomil gold	7.89	–41.9	596	392	297	23.2	14.50
Bacillus velezensis (B-98)	7.89	–41.9	596	392	297	23.2	14.50
Bacillus amyloliquefaciens (B-62)	7.89	–41.9	596	392	297	23.2	14.50

Conductivity values (596 μS cm^{-1}), total dissolved solids (392 ppm), and salt concentrations (297 ppm) were identical across treatments, indicating no measurable impact on soil salinity or ionic strength. These parameters fall within acceptable ranges for agricultural soils and suggest that the treatments did not introduce excess salts or alter the ionic composition of the soil solution—important for maintaining osmotic balance and avoiding salt stress in plants (Allen, 1989).

Soil temperature (23.2 °C) and moisture content (14.5 %) were consistent across all samples, reflecting uniform environmental conditions during sampling. These values are conducive to microbial proliferation and enzymatic activity (Govedarica et al., 1997), supporting the biological processes observed in microbial abundance data. The stable moisture level also suggests that water availability was not a limiting factor for microbial or plant responses during the experimental period.

The uniformity of physicochemical parameters across treatments indicates that the observed biological and microbiological effects—such as changes in stomatal density or microbial abundance—are likely attributable to direct biological interactions rather than shifts in soil chemistry or physical structure. This reinforces the interpretation that microbial inoculants exert their influence through biochemical signaling and microbial competition rather than altering the soil matrix itself (Spaepen, Vanderleyden, & Remans, 2009). Future studies may benefit from longer-term monitoring to detect delayed or cumulative physicochemical changes, particularly in relation to organic matter turnover and nutrient cycling.

3.3.2. Humus content

The humus content of soil, as determined by the Kotzmann method (Sarić, 1992), provides a reliable indicator of organic matter accumulation and overall soil fertility. In this study, humus levels varied across treatments, reflecting the influence of chemical and biological inputs on soil organic dynamics (Table 5). Organic matter (OM) or humus is an essential component of the soil, and although the amount present in agricultural soils is generally low (1–5%), it is nevertheless a key factor in the chemical, biological, and physical soil fertility, as well as in the productivity and quality of agricultural crops (Wood, et al., 2018).

Table 5.
Humus content determined by the Kotzmann method

Treatment	Humus (%)
Control	4.03
Ridomil gold	3.86
Bacillus velezensis (B-98)	4.18
Bacillus amyloliquefaciens (B-62)	4.27

The control treatment exhibited a humus content of 4.03 %, representing the baseline organic matter level under untreated conditions. The application of Ridomil Gold resulted in a slight reduction to 3.86 %, suggesting that chemical fungicide use may suppress microbial activity or organic matter stabilization processes. This decline could be attributed to reduced microbial biomass turnover or inhibited decomposition pathways, which are essential for humus formation (Govedarica et al., 1997).

In contrast, both biological treatments—*B. velezensis* (B-98) and *B. amyloliquefaciens* (B-62)—led to increased humus content, reaching 4.18 % and 4.27 %, respectively. These results indicate that microbial inoculants enhance soil organic matter accumulation, likely through stimulation of microbial-mediated decomposition, improved root exudation, and enhanced carbon sequestration (Kumar, Dubey, & Maheshwari, 2011; Fan, Blom, Klenk, & Borriss, 2018). The higher humus level in B-62-treated soil suggests a more pronounced effect, potentially due to superior colonization efficiency or broader enzymatic activity of the strain.

Overall, the data support the hypothesis that biological treatments contribute positively to soil organic matter dynamics, while chemical inputs may have a neutral or slightly suppressive effect. These findings align with broader sustainability goals, reinforcing the value of microbial biostimulants in promoting soil health and long-term fertility (Pimentel & Burgess, 2014).

3.3.3. Elemental composition

The elemental composition of soil reflects both its mineralogical origin and the influence of external inputs such as fertilizers, microbial amendments, and chemical treatments (Allen, 1989). In this study, the concentrations of key macro- and micronutrients—including manganese (Mn), iron (Fe), copper (Cu), nickel (Ni), zinc (Zn), lead (Pb), and potassium (K)—were assessed across four treatments: control, Ridomil Gold, *Bacillus velezensis* (B-98), and *Bacillus amyloliquefaciens* (B-62) (Table 6).

Manganese (Mn) levels ranged from 0.108 % in B-98 to 0.119 % in Ridomil Gold-treated soil. Although the variation is minor, the slightly elevated Mn in the Ridomil treatment may reflect altered redox conditions or mineral solubilization due to chemical input (Govedarica, Jarak, & Milošević, 1997).

Iron (Fe) content was highest in Ridomil Gold (3.47 %) and lowest in B-98 (3.17 %). This modest reduction in Fe under biological treatments may indicate microbial-mediated chelation or redistribution of iron complexes, which could influence plant uptake and microbial competition (Kumar et al, 2011).

Copper (Cu) concentrations were relatively stable across treatments (34.9–35.3 mg kg^{-1}), suggesting minimal impact of either chemical or biological inputs on Cu dynamics. This stability is consistent with Cu's strong binding affinity to organic matter and clay minerals (Allen, 1989).

Nickel (Ni) levels were slightly elevated in Ridomil Gold (92.9 mg kg^{-1}) and B-98 (91.6 mg kg^{-1}), compared to the control and B-62 (both 89.1 mg kg^{-1}). These differences may reflect microbial mobilization or retention mechanisms, though all values remain within agronomically acceptable ranges (Lindsay & Norvell, 1978).

Zinc (Zn) showed more pronounced variation, with the highest concentration in the control and B-62 (77.35 mg kg^{-1}) and lower levels in Ridomil Gold (68.10 mg kg^{-1}) and B-98 (68.36 mg kg^{-1}). The reduction in Zn under Ridomil and B-98 treatments may be due to microbial uptake, chelation, or competitive inhibition in the rhizosphere (Fan, Blom, Klenk, & Borriss, 2018). Zinc (Zn) is an essential

micronutrient known for its positive roles in controlling important regulatory processes such as stomatal movements, leaf growth, and photosynthesis in plants (Madaan et al., 2025).

Lead (Pb) concentrations varied modestly, with Ridomil Gold showing the highest level (4.5 mg kg^{-1}) and B-98 the lowest (3.4 mg kg^{-1}). While all values are below critical thresholds for agricultural soils, the elevated Pb in Ridomil-treated soil may warrant further investigation into potential accumulation or reduced microbial detoxification capacity (Pimentel & Burgess, 2014).

Potassium (K) levels were highest in the control (0.148 %) and B-62 (0.148 %), followed by B-98 (0.146 %) and Ridomil Gold (0.136 %). These results suggest that biological treatments maintain or slightly enhance K availability, possibly through microbial mineralization or improved cation exchange dynamics (Kumar et al., 2011). The lower K in Ridomil-treated soil may reflect reduced microbial turnover or nutrient cycling efficiency.

Table 6.
Soil elemental composition (analytical methods)

Treatment	Mn (%)	Fe (%)	Cu (mg/kg)	Ni (mg/kg)	Zn (mg/kg)	Pb (mg/kg)	K (%)
Control	0.115	3.29	34.9	89.1	77.35	3.7	0.148
Ridomil gold	0.119	3.47	35.1	92.9	68.10	4.5	0.136
Bacillus velezensis (B-98)	0.108	3.17	35.3	91.6	68.36	3.4	0.146
Bacillus amyloliquefaciens (B-62)	0.115	3.29	34.9	89.1	77.35	3.7	0.148

The elemental composition data indicate that biological treatments—particularly B-62—maintain or slightly improve the availability of essential nutrients such as Zn and K, while chemical treatment with Ridomil Gold may slightly elevate heavy metal concentrations (e.g., Pb) and reduce micronutrient availability. These findings support the use of microbial inoculants as a sustainable alternative to chemical inputs, promoting balanced nutrient profiles and minimizing potential environmental risks (Pimentel and Burgess, 2014).

4. Discussion

The integrated analysis of plant physiological traits, soil microbiological status, and soil chemical and physical properties reveals a consistent pattern: biological treatments, particularly *Bacillus amyloliquefaciens* (B-62), exert the most beneficial effects on both plant and soil health indicators compared to chemical treatment (Ridomil Gold) and the untreated control.

Stomatal density data demonstrated that biological treatments significantly enhanced stomatal development, especially on the lower epidermis. B-62-treated plants exhibited the highest total stomatal density (1642 stomata mm^{-2}), a 38 % increase over control. This suggests improved gas exchange capacity, photosynthetic potential, and water regulation—traits closely linked to plant vigor and resilience. ANOVA confirmed that these differences were statistically significant for the lower epidermis and total stomatal count, reinforcing the physiological relevance of microbial stimulation. The increase in stomatal density under *Bacillus* treatments suggests a physiological enhancement potentially linked to improved nutrient uptake and hormonal signaling (e.g., auxin production) (Spaepen, Vanderleyden, & Remans, 2009). Higher stomatal density may contribute to greater photosynthetic capacity and water-use efficiency. The concurrent enrichment of beneficial soil microorganisms supports the dual role of *Bacillus* spp. as plant growth promoters and soil health enhancers. These findings align with previous reports on *Bacillus*-mediated improvements in crop physiology and resilience (Fan et al., 2018; Kumar, Dubey and Maheshwari, 2011), reinforcing their suitability for integration into sustainable horticultural systems.

Microbial abundance data revealed dramatic increases in functional microbial groups under biological treatments. B-62 treatment showed the highest counts across nearly all categories, including *Bacillus* spp. (1.01×10^9 CFU g^{-1}), nitrogen-fixing bacteria (1.06×10^7 CFU g^{-1}), and cellulolytic bacteria (2.0×10^7 CFU g^{-1}). These shifts indicate enhanced microbial activity, nutrient cycling, and organic matter decomposition. In contrast, Ridomil Gold and the control maintained significantly lower microbial populations, suggesting limited biological stimulation.

Humus levels followed a similar trend, with B-62-treated soil showing the highest organic matter content (4.27 %), followed by B-98 (4.18 %), control (4.03 %), and Ridomil Gold (3.86 %). These results suggest that microbial inoculants promote organic matter stabilization and carbon sequestration, likely through enhanced microbial turnover and root–microbe interactions. The slight reduction in humus under chemical treatment may reflect suppressed microbial decomposition pathways.

Soil elemental analysis showed that biological treatments maintained or slightly improved nutrient availability. Potassium levels were highest in B-62 and the control (0.148 %), while zinc concentrations were also elevated in these treatments. Ridomil Gold-treated soil showed slightly higher lead (Pb) levels (4.5 mg kg^{-1}), which may raise concerns about long-term accumulation or reduced microbial detoxification. Overall, biological treatments preserved a balanced micronutrient profile without introducing heavy metal risks.

All treatments exhibited identical physicochemical parameters, including pH (7.89), redox potential (–41.9 mV), conductivity (596 µS cm^{-1}), and moisture (14.5 %). This uniformity confirms that the observed biological and physiological effects were not driven by changes in soil chemistry or structure, but rather by microbial and biochemical interactions. The neutral pH and moderate conductivity provide a favorable

environment for microbial proliferation and nutrient exchange.

The observed increase in humus content under microbial treatments supports the role of PGPR in promoting organic matter stabilization and carbon sequestration (Petropoulos, Fernandes, Barros, & Ferreira, 2019). The lack of change in soil physicochemical parameters across treatments suggests that the biological effects were mediated through microbial–plant interactions rather than alterations in soil chemistry (Allen, 1989). Elemental composition data indicate that microbial inoculants maintain or improve nutrient availability without increasing heavy metal concentrations, in contrast to some chemical inputs that may elevate Pb levels (Pimentel and Burgess, 2014).

5. Conclusions

This study demonstrates that biological treatments, particularly *Bacillus amyloliquefaciens* (B-62), deliver the most pronounced benefits to both plant physiological performance and soil health compared to chemical treatment with Ridomil Gold and the untreated control. B-62 significantly increased stomatal density—especially on the lower epidermis—enhancing traits linked to photosynthetic efficiency, water-use regulation, and overall plant vigor. These physiological gains were accompanied by substantial increases in beneficial soil microbial populations, including *Bacillus* spp., nitrogen-fixing, cellulolytic, and nitrifying bacteria, indicating improved nutrient cycling and organic matter turnover.

Biological treatments also elevated humus content and maintained or enhanced the availability of key nutrients such as potassium and zinc, while avoiding the slight heavy-metal accumulation observed under chemical treatment. The uniformity of soil physicochemical parameters across treatments confirms that these benefits arose from biological and biochemical interactions rather than shifts in soil chemistry.

Collectively, the results highlight the dual role of *Bacillus*-based inoculants as plant growth promoters and soil health enhancers, supporting their integration into sustainable crop management systems. By improving plant resilience, optimizing nutrient dynamics, and contributing to long-term soil fertility, microbial biostimulants such as B-62 offer a viable, eco-friendly alternative to conventional chemical inputs in horticultural production.

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