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Original article

Controlling root rot disease in colored peppers utilizing mixtures of bio-agents or antioxidants with regard to yield qualities

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Abstract

Root rot caused by soil-borne fungi severely limits the productivity of colored sweet pepper in Saudi Arabia, challenging the sustainability of intensive greenhouse systems. This study assessed whether mixtures of microbial antagonists, with or without an antioxidant formulation, provided superior suppression of root rot and improvement of fruit quality compared to single applications. Greenhouse and commercial greenhouse experiments evaluated single strains of *Trichoderma harzianum*, *Bacillus subtilis*, and *Pseudomonas fluorescens*, their pairwise mixtures, and their combinations with a potassium tartrate-based antioxidant over two consecutive growing seasons. All biocontrol combinations significantly reduced disease incidence relative to the untreated control and increased marketable yield. The *T. harzianum* + *B. subtilis* mixture provided the greatest disease suppression and highest fruit yield, followed by *T. harzianum* + *P. fluorescens*. When *B. subtilis* was combined with an antioxidant, it produced the strongest reductions in root rot incidence. Treatments most effectively suppressing disease also enhanced total phenolic content, total soluble solids (TSS), and vitamin C (V.C.) concentration in fruits, which correlated positively with improved disease resistance and yield. These findings demonstrated that integrating compatible biocontrol agents, particularly *Bacillus subtilis,* with antioxidants can offer a promising and environmentally sound strategy for managing pepper root rot while simultaneously improving fruit nutritional quality in greenhouse production systems in Saudi Arabia.

Keywords: antioxidant, biological control, *B. subtilis, P. fluorescens,* pepper, *T. harzianum*

Introduction

Sweet pepper (*Capsicum annuum*), particularly the colored varieties, represents a horticultural crop of strategic importance in Saudi Arabia. In 2023, the Kingdom imported approximately 8,020 t of fresh bell peppers (0.28% of global imports, ranking 33rd). It exported approximately 913.5 t (0.03% of global exports, ranking 48th), with Kuwait, Bahrain, and the United Arab Emirates as the primary destinations (Ministry of Environment, Water, and Agriculture, 2024). Domestic production reached 128,920 t, reflecting a 7.6% increase compared to 2022 (FAO, 2021). Although data on harvested area specifically for colored peppers are scarce, the protected-vegetable sector—which includes greenhouse production—covers approximately 6,096 hectares nationwide.

Despite its growing economic value, sweet pepper production faces significant constraints from soil-borne diseases, which are among the most destructive factors limiting crop productivity worldwide (Alegbejo *et al.* 2006; Babay-Ahari *et al.* 2009; Ali 2021). Pathogens such as *Pythium* spp., *Rhizoctonia solani*, *Fusarium solani*, *Verticillium* spp., *Phytophthora* spp., and *Sclerotinia sclerotiorum* are frequently implicated. Among these, *Phytophthora capsici* is a particularly aggressive causal agent of pepper root rot, capable of infecting plants at all stages of growth. It induces damping-off in seedlings within a few days of infestation, in addition to crown rot, leaf and fruit blight, and stem discoloration (Akgül & Mirik 2008) Moreover, foliar diseases represent an additional constraint in sweet pepper production, and recent image-analysis tools have enabled objective quantification and identification under field conditions (Rajamanickam *et al.* 2025).

Chemical fungicides have traditionally been used to manage such pathogens; however, their extensive use poses risks to human health and the environment. Cultural practices and chemical control remain common strategies for managing *R. solani* and similar pathogens (Ahmed *et al.* 2019; El-Feky *et al.* 2019; Jalal *et al.* 2024); however, increasing attention has shifted toward biological control as a safer and more sustainable alternative (Gebily *et al.* 2021; Mitter *et al*. 2021). Effective biocontrol agents employ multiple mechanisms of action, enhancing their persistence and reducing the likelihood of pathogen resistance, while leaving no harmful residues in food systems (Ahmed 2008; Choudhary & Johri 2009; Ahmed *et al.* 2019; Niu *et al*. 2020).

Among the most extensively studied antagonists, *T. harzianum* is considered a model biocontrol agent due to its ease of isolation, rapid growth, and versatile mechanisms of action, including mycoparasitism, antifungal metabolite production, and secretion of hydrolytic enzymes (Mukesh *et al.* 2016; Ali 2021; Andersson and Hughes 2023). Moreover, volatile compounds produced by *Trichoderma* spp. can induce systemic resistance in plants (Jash & Sitansu 2007; Bansal *et al.*

2021). Several bacterial species, particularly within the *Bacillus* and *Pseudomonas* genera, also demonstrate strong potential against soil-borne pathogens (Niu *et al.* 2020; Poveda & Eugui 2022; Mohdly *et al.* 2024). For instance, *B. subtilis* effectively suppresses *R. solani* through antibiosis, production of volatile metabolites, secretion of lytic enzymes, and resource competition (Nakkeeran *et al.* 2002; Michelsen & Stougaard 2011; Simamora *et al.* 2024). Similarly, *Pseudomonas fluorescens* synthesizes siderophores and cell-wall-degrading enzymes, which contribute to its broad antifungal activity and potential use in novel fungicidal formulations (El-Gamal *et al.* 2016; Latha *et al.* 2009; Poveda & Eugui 2022; Mohdly *et al.* 2024).

Combining multiple antagonists may further enhance disease suppression, as synergistic interactions can increase efficacy and stability (Abd El Moity 1985; Yobo *et al.* 2011; Ali 2021; Mohdly *et al.* 2024). Building on this premise, the present study aimed to (i) assess the pathogenicity of soil-borne fungi causing damping-off and root rot in pepper, (ii) evaluate the antifungal activity of *T. harzianum*, *B. subtilis*, and *P. fluorescens*, individually and in combination, against these pathogens, and (iii) determine whether integrating bio-agents with antioxidants can enhance disease control and improve the growth and yield of sweet pepper.

Materials and Methods

Soil-borne diseases cause considerable yield losses in pepper plants, primarily due to the continuous cultivation of crops in the same soil, which facilitates the persistence and buildup of pathogenic inocula. To investigate the causal pathogens associated with root rot and wilt symptoms, infected pepper plants were collected from commercial fields in Abha, in Saudi Arabia. The isolated fungi were purified and identified based on their morphological and microscopic characteristics, representing the most common soil-borne pathogens affecting pepper production.

Pathogenicity assay (*in vivo*)

Preparation of pathogenic fungal inocula

Identification of the causal pathogens was carried out through visual assessment of disease symptoms and microscopic examination. The isolated fungi were purified and identified based on their morphological and microscopic characteristics, following the standard descriptions for *Rhizoctonia solani* (Sneh *et al.* 1992), *Fusarium* spp. (Booth 1971), and *Phytophthora capsici* (Akter *et al.* 2007). For subsequent pathogenicity and greenhouse experiments, inocula of *R. solani*,

F. solani, and *P. capsici* were prepared by growing each pathogen on a maize–sand meal medium composed of 90 g riverbed sand, 10 g maize meal, and 20 mL distilled water supplemented with 0.2% peptone in 250-mL Erlenmeyer flasks, following the method described by Nawar and Mostafa (1982). This procedure ensured standardized inoculum production, suitable for reliable assessments of soil infestation and disease development.

Soil infestation procedure

Inocula of *R. solani, F. solani*, and *Phytophthora capsici* isolates (each gm containing $8x10^5$ CFU) were added to soil at a rate of 10 gm per kg of soil. Pots containing contaminated soil were planted with two separate pepper varieties (Olympus and Shanghai). Pots with non-infested soil and the same amount of autoclaved sand and maize meal were used as controls. Each treatment was divided into 10 pots (each pot contained two seeds). Each treatment consisted of three replicates. Each pathogen (*R. solani*, *M. phaseolina*, and *F. solani*) was inoculated in a separate experiment; no combined inoculation with multiple pathogens was performed.

Commercial greenhouse experiments

The study was conducted at Saleh Obaid Al-Habashi's farm in Abha, Saudi Arabia (18°02'54.5"N 42°48'45.5"E). Abha lies on the Red Sea coastal plain and has an arid coastal desert climate, with an average annual temperature of 25–26°C and low, irregular rainfall of 50–80 mm, mainly between November and March (Fick & Hijmans 2017; FAO 2021). The sandy-loam soil, low inorganic matter that was moderately saline, provided a suitable environment for soil-borne pathogens. Experiments were conducted during the 2022/2023 and 2023/2024 seasons under plastic greenhouses using naturally contaminated soil. Pepper (*Capsicum annuum* L.) cv. Shanghai seedlings were grown in plots of 40 plants per treatment with three replicates, arranged in a completely randomized design, and supplied through drip irrigation and fertigation systems to assess the efficacy of bio-agent and antioxidant mixtures against root rot.

Isolation, purification, and identification of biocontrol agents

Three different biocontrol agents were employed in this study: *Trichoderma* sp. (THSC), *Bacillus subtilis*, and *Pseudomonas fluorescens*. The *Trichoderma* isolate (ITS GenBank accession no. MT110634) supports assignment to the *T. harzianum* species complex (THSC); species-level confirmation would require additional loci such as *tef1α* and *rpb2*. *B. subtilis* (GenBank accession

no. MT110640). It was kindly obtained from the Central Laboratory for Organic Agriculture, Giza, Egypt. *P. fluorescens* (biotype A, 77K isolate) was kindly provided by Dr. A. F. Abd El-Rahman, Bacterial Diseases Research Department, Agricultural Research Centre, Giza, Egypt. *T. harzianum* was cultured on Brain and Hemming's (1945) liquid gliotoxin fermentation medium (GFM) for 11 days at 25°C in total darkness to stimulate toxin synthesis (Abd El Moity 1981). *B. subtilis* was grown for two days at 25°C on Dowson's (1957) nutrient glucose medium (NGM), whereas *P. fluorescens* was cultivated for five days at 25°C on King's medium (King *et al.* 1954). The resulting bio-agent cultures were adjusted to a concentration of 5×10^7 CFU mL^{-1} and formulated with 5% Arabic gum and 0.5% potassium soap to enhance adhesion and uniform distribution on plant surfaces (Ali 2021). Pepper (*Capsicum annuum* L.) seedlings were immersed in the diluted (1:30) bio-agent suspensions for 30 minutes prior to transplanting to ensure effective colonization and early protection against root-rot pathogens.

Application of antioxidant

The antioxidant used consisted of: ammonium tartrate, 2 g/L; dipotassium hydrogen orthophosphate, 2 g/L; glucose, 25 g/L; ferrous sulfate, 1 mg/L; and agar, 20 g/L dissolved in distilled water. Tested compounds were potassium tartrate (A), 300 mg/L, ascorbic acid (A.A) 100 mg/L, folic acid (FA) 20 mg/L, a mixture of micronutrients (MN) containing Zn sulfate 500 mg/L, Mn sulfate 500 mg/L, Cu sulfate 100 mg/L, boric acid 100 mg/L and selenium 1.0 ppm, thiamine (TH) 100 ppm (Yousef *et al.* 2017) An antioxidant solution was produced by dissolving 5 g/L of the compound in water. Pepper seedlings were immersed separately in this solution for 30 minutes.

Preparation of a mixture of bio-agents and an antioxidant mixture

To enhance the effectiveness of individual bio-agents in disease control, all three possible combinations of the bio-agents were prepared and tested. Preliminary studies are necessary to further explore these combinations and determine the most suitable mixing ratios. Each mixture was prepared by combining two antagonists at a 1:1 ratio. The three specific combinations were: *T. harzianum* with *B. subtilis*, *T. harzianum* with *P. fluorescens*, and *B. subtilis* with *P. fluorescens*. Each bio-agent was also mixed separately with antioxidants at a 1:1 (v:v) ratio to evaluate the effectiveness of these mixtures compared to individual bio-agents or antioxidants, and to assess their potential synergistic effects. Pepper seedlings treated differently were transplanted into soil

inoculated with the plant pathogens, as previously mentioned. Pepper seedlings that were not treated acted as the control group. Pepper plants treated with different bio-agents or antioxidants were treated similarly in terms of irrigation and fertilization. Forty-five days after transplantation, each treatment was evaluated, and the percentage of disease incidence and synergistic effect was calculated as follows:

$$\text{Disease incidence \%} = \frac{Number\ of\ dead\ plants\ X\ 100}{Total\ number\ of\ plants}$$

The synergistic effect was calculated using the following formula :

$$\text{Synergism (x)} = \frac{100 - \text{C X } 100}{(\text{A} + \text{B})/\ 2}$$

X = Percent synergism

C = Disease incidence (%) observed in plants treated with the mixture (A + B)

(A + B) = Mean disease incidence (%) observed in plants treated separately with A and B

Assessment of TSS, V.C, and total phenol content

TSS, V.C., and total phenol content were assessed in healthy plant samples obtained 30 days after transplanting from various treatments. Healthy plants were harvested, and TSS, V.C., and phenols were measured to see if the various treatments had any influence on plant chemical composition. Pepper juice was extracted using an electric juicer and then filtered through four layers of cotton cloth until no juice remained. Homogeneous suspensions were analyzed to assess any reduction in total soluble solids (TSS). The TSS was measured at an ambient temperature of 25 ± 1°C using an automatic refractometer (A610, Hanon Equipment Co., Jinan, China). The ascorbic acid (vitamin C) content (mg/100 g fresh weight) was determined using the method outlined by A.O.A.C. (2000). Total phenol content was determined using the Folin–Ciocalteu reagent according to Ainsworth and Gillespie (2007). Correlations between chemical changes, disease incidence, and yield were examined. The data were organized and statistically analyzed using standard procedures, including the general linear model (GLM) in SAS (1996). Duncan's multiple range test was employed to identify means with significant differences.

A robust statistical design underpinned the credibility of the presented results by explicitly characterizing uncertainty and applying post hoc procedures to limit inflation of Type I error. Differences were analyzed in the synergism index between treatments using one-way analysis of variance (ANOVA), followed by Tukey's HSD test to delineate statistically homogeneous groups in line with contemporary recommendations. The effects of sizes and confidence intervals were reported rather than relying solely on p-values (Wasserstein, Schirm, & Lazar 2019; Lenth 2020). This inferential framework was complemented by multivariate visualization. Heatmaps were used to condense the high-dimensional response matrix, enabling the detection of correlation blocks and major response gradients. Principal component analysis (PCA) provided a reduced-dimensional representation that clarified the latent structure and relationships among variables, thereby supporting more focused hypothesis refinement and the development of robust explanatory and predictive models. PCA was conducted on treatment means for disease incidence (DI%), disease reduction (%), synergism index, fruit yield (kg m^{-2}), total phenols (mg g^{-1} FW), total soluble solids (TSS, °Brix), and vitamin C (g 100 g^{-1} FW). All variables were standardized to a zero mean and unit variance, and the analysis was based on the correlation matrix. The first two principal components (PC1 and PC2), accounting for 80.6% and 9.7% of the total variance, respectively, were subsequently used to generate a biplot illustrating the joint configuration of treatments and traits (Hair *et al.* 2019; Luecken & Theis 2019; Atia *et al*. 2021; Omar *et al*. 2021). Taken together, the combination of formal inferential testing with multivariate graphical tools promoted a more cautious, reproducible, and generalizable causal interpretation of the findings.

Results

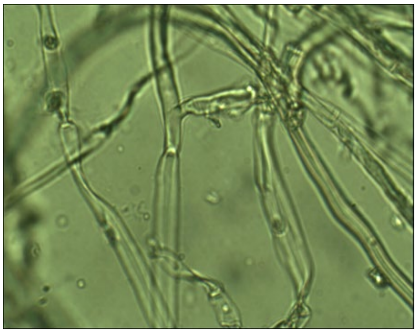
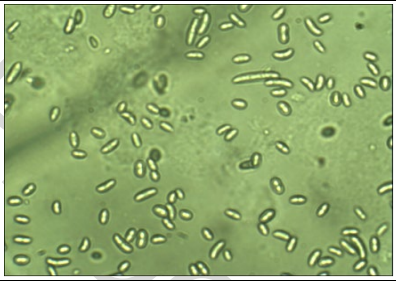
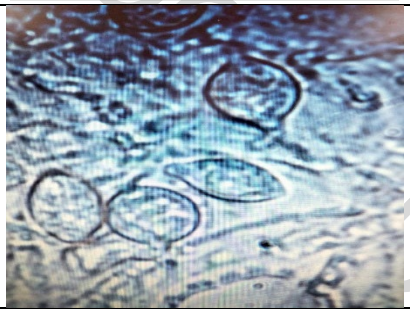
A greenhouse experiment was conducted to determine the sensitivity of two pepper cultivars to the three pathogens under investigation. Table 1 displays the obtained data. The presented data suggest that the Shanghai variety was more sensitive to many infections than the Olympus variety. Olympus showed resistance against different pathogens compared to the other variety. Regarding *R. solani*, no significant differences were found between the percentages of disease incidence recorded in different varieties, and the percentage of damping off ranged from 25% in the Olympus variety to 35% in Shanghai. On the other hand, *Phytophthora capsici* was the most aggressive pathogen, followed by *R. solani*, whereas *F. solani* was the least pathogenic fungus (**Fig.1**).

Table 1. Susceptibility of two colored pepper varieties to three root rot pathogens under greenhouse conditions

pathogens	% disease incidence	
	Olympus	Shanghai
F. solani	5 c	15 c
R. solani	25 b	35 b
Phytophthora capsici	30 a	45 a
Control	0 d	0 d

Values with the same letter are not significantly different

Fig. 1. sensitivity of pepper plants (variety Shanghai) against *Rhizoctonia solani*, *Fusarium solani*

Isolated *Rhizoctonia solani* emerging seedlings of sweet pepper		
Isolated *Fusarium solani* emerging seedlings of sweet pepper		
Isolated *Phytophthora* spp.		
control	----------	

and *Phytophthora capsici* compared to the control treatment under greenhouse conditions

Commercial greenhouse experiments

The experiment was carried out on a highly sensitive pepper cultivar (Shanghai) to determine which treatment was most successful for suppressing root rot pathogens. Table 2 shows that all treatments, whether single bio-agent or combined, had significant effects on disease incidence and yield when compared to the control treatment. Data also show that *B. subtilis* was the most effective when

compared to the other two bio-agents, *T. harzianum* and *P. fluorescens*. *T. harzianum* ranked second only to *B. subtilis* when it came to disease reduction. Data also show that mixing two different bio-agents at a 1:1 ratio resulted in a synergistic impact in all combinations. These synergistic effects were achieved by comparing the effect of the mixture to the mean of the results obtained when the two involved isolated bio-agents were applied. The data indicate that the most favorable results were achieved with a mixture of *T. harzianum* and *B. subtilis*, followed by *T. harzianum* and *P. fluorescens*. In contrast, the combination of *B. subtilis* and *P. fluorescens* ranked last. When *T. harzianum* was combined with *B. subtilis*, a synergistic impact was observed, with results ranging from 28.5 to 50% in 2022 and 2023, respectively. In contrast, when *B. subtilis* was combined with *P. fluorescens*, only a slight synergistic effect (3.3%) was observed in 2022. The best pathogen suppression treatments resulted in increased yields, with the best mixture (B. subtilis and *T. harzianum*) yielding the highest yields (12.3 and 13.6 kg/m²) across both seasons. When *P. fluorescens* was combined with *B. subtilis* or *T. harzianum*, the treatment's efficacy was significantly increased.

The effect of applying single bio-agents or mixtures on the root rot reduction of colored pepper in relation to the chemical component

To explore the mechanism of these treatments and their effects on disease incidence and yield, three chemical analyses were conducted on treated plants for comparison. Phenol contents, vitamin C, and TSS levels were compared to those in the control treatment. The obtained data show that when disease incidence decreased, phenol content increased in all treatments when compared to the control or other single treatments.

Data also show that the correlation between disease incidence reduction and an increase in TSS and VC contents was pronounced when the most efficient treatment (*T. harzianum* and *B. subtilis*) in the two examined seasons was compared to the control. The data show that phenol content was higher in all treatments than in the control treatment. A similar result was obtained when TSS or VC in treated plants was compared to that in control plants.

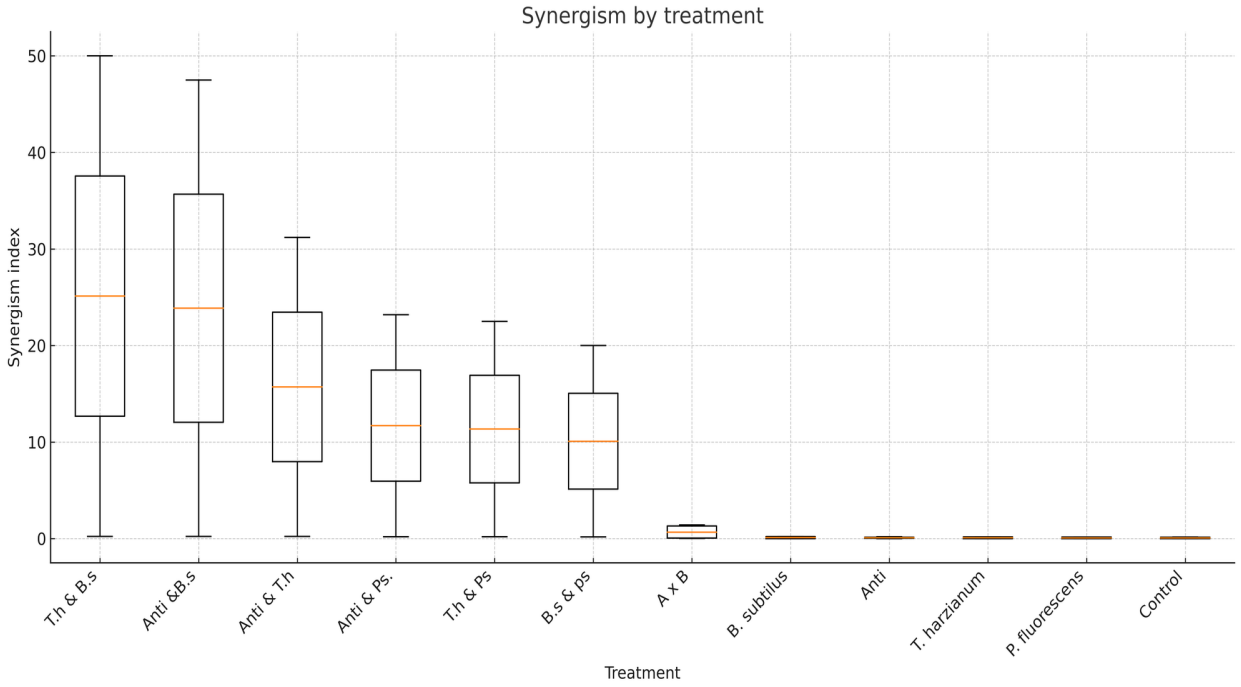
Table 2. Effect of applying a single bio-agent or mixtures on pepper root rot disease incidence in relation to treated plant fruit yield over two seasons, 2022/23 and 2023/24

Traits	DI %			Reduction %			Synergistic			Yield kg/m^2			Phenol mg/g			TSS			VC		
Years / Treatments	2022/2023	20232024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean
***T. harzianum* (*T.h*)**	26.70	23.30	25.00	46.60	56.80	51.70	0.00	0.00	0.00	10.30	11.00	10.65	18.10	19.50	18.80	7.30	7.40	7.35	0.16	0.18	0.17
***B. subtilis* (*B.s*)**	20.00	16.70	18.35	60.00	69.10	64.55	0.00	0.00	0.00	11.00	12.20	11.60	20.00	20.70	20.35	7.40	7.40	7.40	0.20	0.21	0.21
***P. fluorescens* (*P.s*)**	30.00	28.30	29.15	40.00	47.60	43.80	0.00	0.00	0.00	9.60	9.10	9.35	15.30	17.10	16.20	7.10	7.30	7.20	0.14	0.16	0.15
T.h & B.s	16.70	10.00	13.35	66.60	81.50	74.05	28.50	50.00	39.25	12.30	13.60	12.95	20.90	23.60	22.25	7.70	7.50	7.60	0.22	0.24	0.23
T.h & P.s	22.00	18.00	20.00	56.00	66.70	61.35	12.00	22.50	17.25	9.20	11.70	10.45	20.40	23.20	21.80	7.40	7.30	7.35	0.18	0.20	0.19
B.s & P.s	25.00	20.00	22.50	50.00	63.00	56.50	3.30	20.00	11.65	11.00	9.50	10.25	18.20	19.10	18.65	7.40	7.40	7.40	0.17	0.18	0.18
Control	50.00	45.00	47.50	0.00	0.00	0.00	0.00	0.00	0.00	6.10	6.30	6.20	11.00	12.00	11.50	6.50	6.50	6.50	0.14	0.14	0.14
Grand mean	27.20	23.04	25.12	45.60	54.96	50.28	6.26	13.21	9.74	9.93	10.49	10.21	17.70	19.31	18.51	7.26	7.26	7.26	0.17	0.19	0.18
LSD at	0.05	0.01		0.05	0.01		0.05	0.01		0.05	0.01		0.05	0.01		0.05	0.01		0.05	0.01	
Treatments (A)	1.069	1.437		1.058	1.432		0.676	0.905		1.266	1.709		1.02	1.398		1.104	1.498		0.012	0.015	
Years (B)	0.561	0.769		0.569	0.76		0.354	0.485		0.678	0.917		0.55	0.744		0.593	0.799		0.005	0.008	
A x B	1.502	2.039		1.494	2.02		0.944	1.28		1.788	2.407		1.452	1.969		1.569	2.112		0.013	0.023	

Table 3. Effect of a combination of antioxidant and bioagents on root rot in pepper in relation to fruit yield of treated plants in two successive seasons, 2022/23 and 2023/24

Traits	DI %			Reduction %			Synergistic			Yield kg/m^2			Phenol mg/g			TSS			VC		
Years / Treatments	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean	2022/2023	2023/2024	Mean
T. harzianum* (*T.h*)	26.70	23.30	25.00	46.60	56.80	51.70	0.00	0.00	0.00	10.30	11.00	10.65	18.10	19.50	18.80	7.30	7.40	7.35	0.16	0.18	0.17
***B. subtilis* (*B.s*)**	20.00	16.70	18.35	60.00	69.10	64.55	0.00	0.00	0.00	11.00	12.20	11.60	20.00	20.70	20.35	7.40	7.40	7.40	0.20	0.21	0.21
***P. fluorescens* (*P.s*)**	30.00	28.30	29.15	40.00	47.60	43.80	0.00	0.00	0.00	9.60	9.10	9.35	15.30	17.10	16.20	7.10	7.30	7.20	0.14	0.16	0.15
Antioxidant (Anti)	36.70	34.00	35.35	26.60	37.00	31.80	0.00	0.00	0.00	9.80	10.00	9.90	21.30	21.40	21.35	7.40	7.20	7.30	0.18	0.19	0.19
Anti & T.h	21.00	18.00	19.50	58.00	64.00	61.00	33.80	31.20	32.50	12.00	13.50	12.75	23.70	25.20	24.45	7.80	7.60	7.70	0.22	0.23	0.23
Anti &B.s	18.00	12.00	15.00	64.00	76.00	70.00	36.50	47.50	42.00	12. 1	14.50	14.50	26.50	27.00	26.75	7.60	7.70	7.65	0.21	0.23	0.22
Anti & P.s	26.00	22.00	24.00	48.00	59.30	53.65	22.00	23.20	22.60	9.80	9.50	9.65	20.70	21.00	20.85	6.90	6.80	6.85	0.18	0.20	0.19
control	50.00	54.00	52.00	0.00	0.00	0.00	0.00	0.00	0.00	6.10	6.30	6.20	12.00	12.00	12.00	6. 5	6.70	6.70	0.14	0.17	0.16
Grand mean	25.49	22.04	23.76	49.03	58.54	53.79	13.19	14.56	13.87	10.42	11.40	11.20	20.80	21.70	21.25	7.36	7.34	7.35	0.18	0.20	0.19
LSD at	0.05	0.01		0.05	0.01		0.05	0.01		0.05	0.01		0.05	0.01		0.05	0.01		0.05	0.01	
Treatments (A)	1.055	1.421		1.034	1.393		0.745	1.004		1.036	1.395		1.15	1.549		0.845	1.138		0.03	0.042	
Years (B)	0.528	0.711		0.517	0.696		0.373	0.502		0.518	0.698		0.575	0.774		0.423	0.569		0.016	0.021	
A x B	1.492	2.01		1.463	1.97		1.054	1.42		1.465	1.973		1.627	2.19		1.195	1.61		0.044	0.06	

*Th, *T. harzianum*, Bs, *B. subtilis*; Ps, *P. fluorescens*; Anti, antioxidant; A×B, interaction between treatments (A) and seasons (B); Control, inoculated untreated control.



Th, *T. harzianum*, Bs, *B. subtilis*; Ps, *P. fluorescens*; Anti, antioxidant; A×B, interaction between treatments (A) and seasons (B); Control, inoculated untreated control.

Fig. 2. Boxplots of fruit yield by treatment

As shown in Figure 2, the panel displays distributional summaries of the synergism index (y-axis, "Synergistic 1") across treatments (x-axis). Each box displays the median (horizontal line), interquartile range (IQR), and whiskers extending to 1.5×IQR. Values near zero indicate additive/no interaction, while larger positive values indicate more substantial synergistic effects. Consortia exhibit the highest central tendencies and widest dispersion, consistent with biologically meaningful synergy. In particular, *T. harzianum* × *B. subtilis* and Anti × *B. subtilis* showed the largest medians (≈20–25 units) and upper whiskers reaching ~45–50, followed by Anti × *T. harzianum*, *T. harzianum* × Ps, and *B. subtilis* × *P. fluorescens* (medians ≈10–16). By contrast, single-agent treatments (*T. harzianum*, *B. subtilis*, *P. fluorescens*) and controls cluster at or near zero, indicating negligible or no synergism. The very low spread for "A × B" and the near-zero values for "Anti" also support this pattern. These distributional contrasts align with the multivariate findings: the PCA biplot places consortia opposite to the DI% vectors and in the direction of reduction, and the correlation matrix shows negative associations between DI and synergism/reduction indices. Together, the boxplots substantiate the study's primary claim that

combinations of biocontrol agents yield superior interaction effects relative to single strains and untreated controls.

Table 4. Analysis of variance (mean squares) for pepper root rot and damping-off as affected by antioxidant–bioagent treatments across two consecutive seasons, with associated biochemical traits (phenols, TSS, vitamin C)

Mean squares								
Single bio-agent or mixtures on pepper root rot disease during two growing seasons								
SOV	**d.f**	**DI**	**Reduction**	**Synergistic**	**Yield**	**Phenol**	**TSS**	**VC**
Replications	**2**	2.645	2.049	0.126	0.065	1.893	0.188	0.001
Treatments	**13**	353.639 **	1689.384 **	704.266 **	13.752 *	40.775 **	0.362 **	0.009 **
Treatments (A)	**6**	324.736 **	1468.423 **	1014.216 **	15.17 **	44.621 **	0.271 **	0.005 **
Years (B)	**1**	0.175	12.055 **	164.812 **	0.809	1.561	0.028	0.001
A X B	**6**	441.43 **	2189.9 **	484.227 **	14.487 **	43.452 **	0.505 **	0.007 **
Error	**26**	**0.809**	**0.796**	**0.312**	**1.128**	**0.754**	**0.867**	**0.001**
Combinations of antioxidants and bioagents on damping off in pepper during two growing seasons								
Replications	**2**	0.614	2.446	0.346	4.841	3.571	2.753	0.001
Treatments	**15**	323.709 **	1490.311 **	876.225 **	50.108 **	188.065 **	19.131 **	0.015 **
Treatments (A)	**7**	566.173 **	2775.322 **	1434.997 **	96.442 **	376.153 **	40.702 **	0.030 **
Years (B)	**1**	0.001	2.210	28.985 **	0.521	1.367	0.003	0.000
A X B	**7**	127.489 **	417.884 **	438.487 **	10.858 **	26.647 **	0.292	0.002
Error	**30**	**0.801**	**0.770**	**0.400**	**0.772**	**0.952**	**0.514**	**0.001**

SOV = source of variation; d.f. = degrees of freedom; A = Treatments; B = Year; A×B = interaction; DI = disease incidence (%); TSS = total soluble solids; VC = vitamin C; ** and * denote significance at $p \leq 0.01$ and $p \leq 0.05$, respectively. Clarification: In this table, "Treatments" refers to the overall treatment structure (all regimes considered together), while "Treatments (A)" denotes the main effect of factor A (treatment regiments) in the two-way ANOVA across two seasons. "Years (B)" denotes the seasonal effect and "A×B" denotes the interaction between treatments and seasons.

In Table 3 it can be seen that a mixture of *B. subtilis* and antioxidant showed approximately twice as much reduction in disease incidence as antioxidant treatment (64 and 76% in the two seasons) and had a synergistic effect (36.5 and 47.5% in the two seasons, respectively). The mixture of *T. harzianum* with an antioxidant ranked second after *B. subtilis* with an antioxidant, with a disease incidence reduction of 58.64%. A synergistic effect was observed, and the percentage of disease reduction increased by 64% compared to the mean effect of the antioxidant and *B. subtilis* alone. When *P. fluorescens* was mixed with the antioxidant, an apparent increase in the efficacy of

the treatment was noticed in the two seasons, and 22 and 23.2 synergistic effect of *P. fluorescens* was observed. Yield was increased when the different bio-agents were combined with the antioxidant. The highest yield was recorded when *B. subtilis* was combined with the antioxidant, yielding 12.1 and 14.5 kg/m² in the two seasons, followed by *T. harzianum* with the antioxidant.

Based on ANOVA mean squares, Table 4 shows that treatments and the A × B interaction significantly affected disease metrics and biochemical traits (DI, reduction, synergistic, yield, phenols, TSS, VC), whereas the year effect was limited. These patterns substantiate the efficacy and seasonal robustness of antioxidant–bioagent combinations.

The effects of combining antioxidant and bio-agents on the reduction of disease incidence in peppers in relation to the chemical components of treated plants

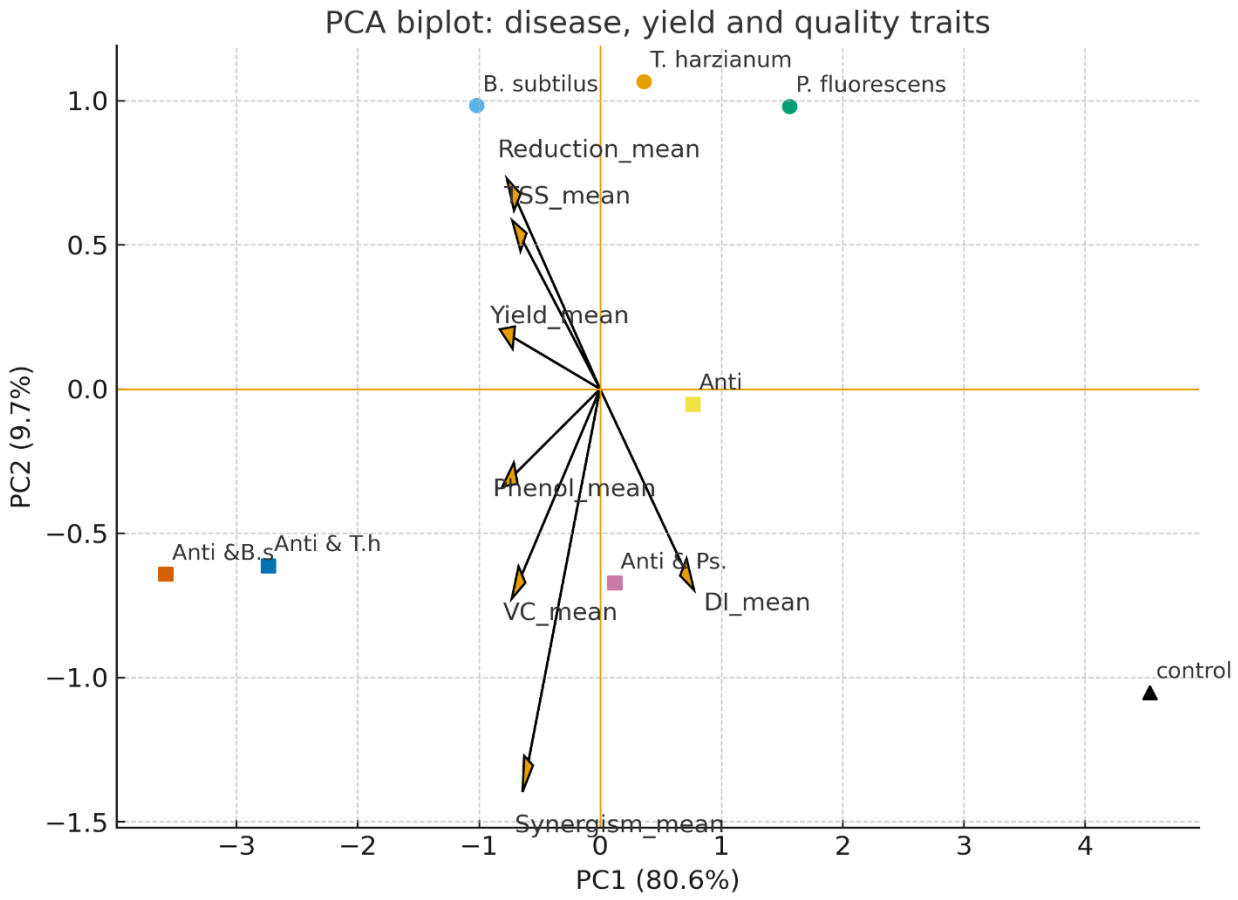


Fig. 3. | PCA biplot showing treatment separation and variable loadings

The PCA biplot (Fig. 3) clearly separated the untreated control from all treatments along PC1, mainly driven by high DI_mean and low reduction_mean, yield_mean and quality traits. Bioagent-only treatments (*T. harzianum*, *B. subtilis*, *P. fluorescens*) were located on the opposite side of PC1, associated with higher disease reduction and yield. Antioxidant–bioagent combinations, particularly anti & Bs and anti &Th, clustered in the quadrant characterized by high phenolic content, TSS, and vitamin C, as well as improved yield. Synergism_mean loaded negatively on PC1 and PC2, indicating that increasing synergistic interactions was associated with lower DI and higher yield and quality traits.

Table 4 presents the efficacy of various treatments, either single or combined with bio-agents and antioxidants, in reducing disease incidence, as well as specific chemical analyses (phenols, VC, and TSS) of treated plants. The purpose of this association is to better understand the mechanism and way of action of various treatments on plant physiology, and consequently, disease control. The data obtained demonstrated that phenol levels in all treated plants were higher than in control plants. The same result was seen when TSS or VC in treated plants were compared to plants from the control treatment. This explains why different treatments have different levels of efficacy.

When *B. subtilis* and antioxidants were applied during the two seasons studied, the highest level of disease control was observed, with a 64% reduction in disease incidence compared to the control treatment. This combination also resulted in the highest phenol concentration (26.5 and 27 µg/g in the two seasons, respectively) when compared to other treatments. Throughout the experiment's two seasons, the best treatments were linked to an increase in fruit quality features such as vitamin C and TSS.

The correlation structure revealed a coherent organization of the metrics into biologically meaningful blocks: disease-incidence measures (DI%, DI%_1, DI%_2) form a tight, internally consistent cluster, whereas reduction and synergism indices co-cluster with strong positive associations; critically, DI is negatively correlated with both reduction and synergism, indicating that treatments achieving larger interaction effects also register lower disease. Replicate variables (suffixes "_1", "_2") show near-unity correlations within constructs, reinforcing measurement reliability across assessments. These relationships presented in Fig. 4. mirror the multivariate geometry observed in the PCA biplot, where DI loadings oppose the reduction/synergism direction

along PC1, thereby situating consortia in the more efficacious quadrant and controls in the adverse one. Beyond corroborating the study's central claim that bioagent combinations outperform single strains, the block-diagonal pattern provided a principled rationale for composite endpoints and dimension-reduction strategies (e.g., PCA/PLS) that summarize treatment performance while mitigating multicollinearity and overfitting, ultimately improving inferential stability and the translational interpretability of ranking decisions for greenhouse and field deployment.

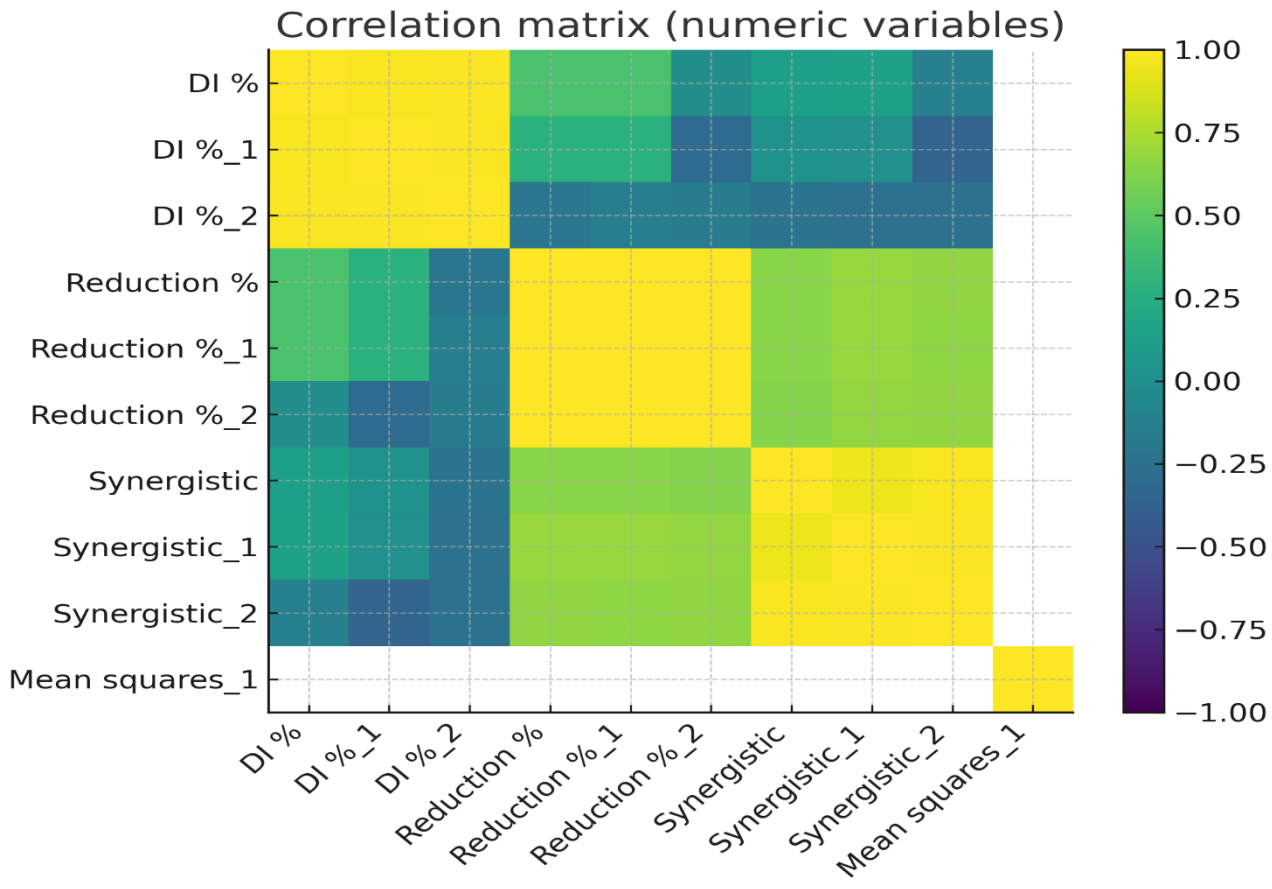


Fig. 4. | Correlation matrix among numeric variables

Discussion

This study has advanced the current understanding of root-rot pathology in pepper by combining classical pathogenicity assessments with a comparative evaluation of single and combined biological control agents (bioagents), and by integrating univariate and multivariate statistical evidence to interpret treatment performance. Consistent with prior reports, pathogenic isolates associated with damping-off and root rot—such as *Rhizoctonia solani*, *Fusarium solani*, and *Phytophthora capsici*—were able to induce pre- and post-emergence losses as well as root lesions across cultivars (Sid Ahmed *et al.* 2003; Akgül & Mirik 2008; Yobo *et al.* 2011; El-Feky *et al.* 2019). Against this backdrop, the presented data show that antagonistic fungi and bacteria significantly reduced disease metrics relative to inoculated controls. Notably, while single strains (e.g., *T. harzianum*, *B. subtilis*, *P. fluorescens*) provided measurable suppression, combinations of agents and agent + antioxidant treatments delivered superior performance, indicating that factorial strategies harnessed complementary mechanisms to produce supra-additive (synergistic) outcomes. This pattern is not merely descriptive: it aligns with the growing consensus that multi-strain consortia can reconfigure rhizosphere interactions to stabilize disease suppression more effectively than single inoculants (Köhl *et al.* 2019; Trivedi *et al.* 2020).

The comparative efficacy across agents is congruent with earlier work but also refines it in two ways. First, in the relative ranking it was observed that *B. subtilis* generally outperforms *T. harzianum, with P. fluorescens* often trailing echoing patterns noted in pepper pathosystems (Rini & Sulochana 2007), while emphasizing that the effect of sizes remains context-dependent (i.e., soil, pathogen pressure, and host genotype). Second, these findings quantify the additional benefit of pairing agents that differ in their primary modes of action. *Trichoderma* spp. are frequently dominant contributors to commercial biocontrol portfolios, with recognized mechanisms including mycoparasitism, enzyme secretion, competition for space and nutrients, and induced systemic resistance (Pandya & Saraf 2010; Huang *et al.* 2011; Ali 2021). In parallel, *Bacillus subtilis* and *Pseudomonas fluorescens* can suppress pathogens through multiple mechanisms, including the production of antibiotics and siderophores, interference with pathogen signaling, and modulation of plant immunity (Chilosi *et al*. 2020; Yuliar *et al.* 2025).

When these agents are co-applied, their distinct biochemical arsenals and ecological niches plausibly combine to produce stronger, more robust suppression than any single mechanism alone

(Sreedevi *et al*. 2011; Muhanna *et al*. 2016). The present results corroborate this multi-mechanistic interpretation, as the discussed treatments achieved clear reductions in disease incidence relative to the inoculated control. For example, single-agent applications reduced disease incidence by 51.70% with *T. harzianum* and 64.55% with *B. subtilis*, while the combined antioxidant–*B. subtilis* treatment achieved 70.00% reduction. At the same time, inter-study heterogeneity reported for *Trichoderma* (Mannai *et al*. 2018) underscores that local microbiomes, abiotic conditions, and inoculation strategies modulate realized efficacy, an issue which will be returned to below when discussing translational implications.

A central contribution of this study was the triangulation of evidence across statistical lenses. A one-way ANOVA framework—augmented by Tukey's HSD for multiple comparisons—showed that consortia generally achieved significantly higher synergism indices than single strains. These inferential results were consistent with the distributional profiles in the boxplots, where medians for consortia (e.g., *T. harzianum* × *B. subtilis*; *Anti* × *B. subtilis*) were substantially above zero, with upper whiskers extending into higher-effect regions, while single-agent treatments clustered at or near zero, indicating additive or negligible interaction. Rather than interpreting dispersion as unreliability, it was considered to be an ecological signature of context dependence: synergy emerges when strains occupy complementary niches and exchange metabolites, and its magnitude depends on microenvironmental contingencies (Köhl *et al.* 2019). In practical terms, this motivates the factorial optimization of dose ratios, order of arrival, and carrier formulations to stabilize synergy across seasons and sites—steps that are rarely explored in single-factor screening but are essential for productizable biocontrol.

The multivariate analyses provide independent, mechanistically interpretable corroboration. The correlation matrix displayed a block-diagonal structure in which disease incidence (DI) measures clustered tightly and were negatively correlated with both reduction and synergism indices. This geometry implies that the same underlying processes driving higher "reduction" and "synergism" also correspond to lower disease incidence—an interpretation consistent with cooperative, complementary functions in consortia (Carrión *et al*. 2019). High within-construct correlations across repeated assessments support measurement reliability and justify the use of dimension reduction and composite endpoints, particularly when traits covary as coordinated suites rather than acting independently. The PCA biplot integrated these relationships:

PC1 captured ≈80% of the variance and separated the DI vectors from the reduction/synergism direction, placing consortia on the low-DI/high-reduction side and aligning control/antioxidant-only conditions with high DI. This is precisely the signal expected if multi-strain inocula reprogram rhizosphere interactions toward disease-suppressive states (Trivedi *et al.* 2020). From an applied perspective, projecting treatments onto PC1 yields a parsimonious composite index for ranking candidates; used alongside confidence intervals from ANOVA/Tukey, it creates a transparent decision framework for down-selection before more resource-intensive field trials.

An additional novel element is the augmentation of antioxidants. The results of the presented research indicated that adding an antioxidant to *Trichoderma* or *B. subtilis* enhanced disease suppression and, in some cases, increased total phenolics and vitamin C (V.C.), with concomitant effects on TSS and yield-related traits. The synergy between antioxidants and bioagents is biologically plausible. Antioxidants can modulate plant redox homeostasis, interact with signaling pathways (e.g., salicylic acid, jasmonate/ethylene), and thereby amplify induced resistance, while also shaping the biochemical milieu that pathogens encounter in planta. Concomitantly, bioagents supply growth regulators and elicit defense priming (Karlidag *et al.* 2012; Constantinescu *et al*. 2009; Kashyap *et al*. 2022), suggesting a dual pathway, direct antagonism of pathogens and host-mediated fortification, that converges on lower DI and improved plant vigor. Although earlier reports described such interactions (Ali 2013; Singh *et al*. 2008; Latha *et al.* 2009), the present study extends their scope by integrating synergism metrics, correlation structure, and PCA to show that antioxidant + bioagent treatments do not merely "add" effects but reposition the treatment centroid in multivariate space toward the desirable quadrant. This multilevel coherence (univariate, multivariate, and physiological co-benefits) strengthens confidence in the interpretation and its translational relevance.

The study's strengths were threefold. First, it benchmarked multiple pathogen taxa and bioagents within a single experimental frame, allowing more direct contrasts than cross-study comparisons can provide. Second, it coupled standard inferential tests (ANOVA with multiplicity control) with effect-size-aware visualization (boxplots with Tukey letters) and multivariate synthesis (correlation/PCA), thereby limiting overinterpretation of single p-values and guarding against spurious univariate conclusions. Third, it interrogated both direct disease outcomes and related quality/physiology indicators (phenolics, V.C., TSS, yield), thereby connecting pathogen

suppression to agronomic value. These design features collectively provided a robust, decision-oriented view of biocontrol performance that is often lacking in narrowly scoped trials.

Nevertheless, several limitations warrant consideration and chart directions for future work. First, while greenhouse or controlled environments reduce noise and clarify mechanisms, they may not fully capture the complexity of the field (microclimate heterogeneity, native microbiome competition, and fluctuating pathogen pressure). Multisite, multiseason validations are therefore essential to quantify robustness. Second, the realized synergy of consortia can be sensitive to inoculum ratios, timing, and carrier matrices; future designs should explicitly optimize these factors and test their interaction with soil physicochemical properties. Third, although the antioxidant augmentation used here produced compelling gains, the generality and mechanistic underpinnings (e.g., redox signaling vs. direct antimicrobial contribution) merit targeted follow-up using transcriptomics/metabolomics to link phenotype to pathway-level change. Finally, while PCA and correlation analyses clarified structure, additional multivariate or integrative approaches (e.g., sparse PLS for prediction, structural equation models for causal hypotheses) could further disentangle direct and indirect effects among disease, physiology, and yield.

Within the existing literature, the present findings largely concur with reports that *Trichoderma*, *Bacillus*, and *Pseudomonas* can individually suppress soil-borne pathogens; however, they highlight two important distinctions. First, it was demonstrated, with convergent statistical evidence, that carefully composed consortia outperform single agents not only in terms of mean disease metrics but also in multivariate positioning, consistent with theory on cooperative disease suppression (Köhl *et al*. 2019; Trivedi *et al.* 2020; Carrión *et al*. 2019). Second, it was demonstrated that targeted biochemical augmentation (antioxidant) can alter both plant defense chemistry (phenolics, V.C.) and performance endpoints (TSS, yield), supporting a host-centric complement to microbial antagonism. While others have hinted at such dual-track strategies (Ali 2013; Chilosi *et al.* 2020; Kashyap *et al.* 2022), the present integration across statistical layers and outcome axes provides a more holistic template for screening and optimization.

Two broader implications follow. Theoretically, the data support a systems view of biocontrol in which pathogen suppression emerges from distributed, partially redundant functions—antibiosis, competitive exclusion, enzymatic degradation, and induced resistance—operating across microbial members and the host. This functional diversity may not only enhance

mean efficacy but also reduce the evolutionary potential of pathogens to adapt, by imposing multifactorial, shifting constraints rather than a single, fixed selective pressure. Practically, the results advocate for formulation pipelines that (i) co-develop complementary strains, (ii) calibrate inoculum ratios and timing to the target pathosystem, and (iii) consider biochemical adjuvants that amplify host defenses without compromising microbial viability. The use of composite multivariate scores (e.g., PC1 projections) alongside classical inferential tests offers a pragmatic, transparent way to prioritize candidates for field-scale testing, while minimizing the risk of overfitting to any single endpoint.

In conclusion, this study demonstrated that bioagent consortia—especially combinations of *T. harzianum* and *B. subtilis*—achieved greater and more durable suppression of pepper root rot than single-strain treatments, and that antioxidant augmentation could further magnify these benefits by enhancing host defense chemistry and, ultimately, yield. These conclusions are supported by consistent signals across univariate (ANOVA/Tukey), distributional (boxplots), and multivariate (correlation/PCA) analyses, indicating that the observed advantages are structural features of the data rather than artefacts of any single analytic lens. Future research should (a) validate these findings across seasons and locations; (b) optimize consortia composition, dosing, and delivery; and (c) pair efficacy trials with microbiome, transcriptome, and metabolome profiling to resolve mechanisms and improve transferability. By integrating complementary modes of action with host-centered biochemical support and robust, multilayer statistics, the work contributed to a clear, generalizable framework for designing next-generation biocontrol strategies against soil-borne pathogens in pepper and, by extension, other horticultural crops.

Conclusion

The presented findings indicated that deploying consortia of bio-control isolates with complementary modes of action could substantially improve disease suppression relative to single-strain applications. Multi-strain formulations were more likely to realize true synergistic interactions—combining antibiosis, niche competition, and induced systemic resistance—to shift the rhizosphere toward disease-suppressive states and sustain low disease pressure. This biological advantage translates agronomically: by protecting and invigorating the root system, consortia enhance nutrient uptake and transport, supporting greater assimilate allocation to fruit set and ultimately resulting in higher yields. Practically, these results support co-formulation strategies that

optimize strain compatibility, dose ratios, and timing to stabilize synergy under variable greenhouse and field conditions. Future work should validate the durability of these effects across seasons and environments, and pair efficacy trials with microbiome and metabolomic profiling to elucidate the mechanisms that underpin robust performance and guide data-driven design of next-generation biocontrol products.

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