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Research Article

Integrated Biological Control of Alternaria Leaf Spot in Spinach through *Trichoderma harzianum* and *Bacillus subtilis* Mediated Disease Suppression and Growth Promotion

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ABSTRACT

Spinach (*Spinacia oleracea* L.) is a cool-season annual plant native to countries in the southwestern and central regions of Asia. *Alternaria alternata*, the causal agent of Alternaria leaf spot, is a major constraint to spinach production worldwide. The objective of this study was to determine the relative efficacy of biological control agents (BCAs) under lab and field conditions. In vitro testing showed that Bacillus subtilis provided the greatest early inhibition (50.0% inhibition at 8 days). *Trichoderma harzianum* showed the most consistent antagonistic activity (29.67% inhibition at 12 days) when compared to the other BCAs. Based on consistent *in vitro* antagonistic performance, *T. harzianum* and *B. subtilis* were selected for evaluation under field conditions using a Randomized Complete Block Design (RCBD). The field trial showed that the treatment involving BCAs (*T. harzianum* + *B. subtilis*) resulted in significantly lower severity scores of 12.0%, representing a 65.7% reduction compared to the untreated control (35.2%). The *T. harzianum* + *B. subtilis* treatment also provided the greatest vigour in plant growth with higher fresh weight (27.30 g) and total dry weight (2.25 g). These improvements are likely associated with the known mycoparasitic activity of *T. harzianum* and the induction of plant defence responses by *B. subtilis*. The data support the idea that a combination of BCAs could be a viable, eco-friendly option for integrated management of Alternaria leaf spot in spinach production.

Keywords: *Alternaria alternata*, *Bacillus subtilis*, Biological control, Disease management, Spinach, *Trichoderma harzianum*, Sustainable agriculture

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INTRODUCTION

Spinach (*Spinacia oleracea* L.) is a cool season annual plant native to countries in the southwest and central regions of Asia. It is one of the most widely grown vegetables in the world (Gong *et al*., 2021). Fungi have caused enormous production losses in plants across the world. *A. alternata* is a genus of fungus that infects a wide variety of crops and causes economic losses. Among them, Alternaria leaf spot caused by *A. alternata* is one of the most severe diseases (Nowicki *et al*., 2015). *Alternaria* is a well-known plant pathogen causing at least 20% crop loss and it can cause up to 80% loss under favorable conditions (Bhargava & Singh, 2020). *Alternaria* produces host-specific toxins that cause necrotic lesions on spinach leaves that can result in yield losses of up to 60% under favorable environmental conditions (Meena *et al*., 2017). Excessive reliance on chemical fungicides has raised serious concerns due to soil contamination, risks to human health and the emergence of fungicide-resistant strains of *A. alternata*, prompting increased interest in environmentally safe disease management strategies (Liu *et al*., 2020;

Khan *et al*., 2021) reported these and this has resulted in a third concern: seeking eco-friendly selections for plant disease control. One of the eco-friendly approaches for plant disease control is biocontrol agents. Some of the well-known biocontrol agents include *Trichoderma harzianum* and *Bacillus subtilis*. Both biocontrol agents demonstrated tremendous ability in suppressing fungal pathogens. *T. harzianum* is the most predominantly used soil fungus in disease management of plants through antagonism by competition for nutrition with pathogens, direct parasitic action against fungi and the production of antifungal metabolites by which the fungus inhibits the pathogen's growth (Keswani *et al*., 2020; Singh *et al*., 2022). Similarly, *B. subtilis* possesses plant growth-promoting rhizobacteria (PGPR) traits that enhance plant health and resilience. It synthesizes several antifungal lipopeptides, such as surfactin, iturin and fengycin. These lipopeptides do not only hinder the development of pathogenic fungi but also help the plant through enhancing the plant's own defence mechanisms (Fan *et al*., 2017). It also affects plant hormones like auxins and cytokinin’s, which leads to stronger root growth and hence helps the plant to deal with disease and stress in a better way (Calvo *et al*., 2019). Based on these considerations, the present study was designed to evaluate the comparative efficacy of selected biocontrol agents against *A. alternata* under laboratory and field conditions in spinach.

MATERIALS AND METHODS

Pathogen Isolation, purification and identification

Diseased spinach leaves having typical symptoms were collected from the vegetable field of the University of Agriculture Faisalabad. The leaves were washed in tap water and air dried. Leaves were cut into 5 × 5 mm pieces, surface sterilized with 1% sodium hypochlorite (NaOCl) for 60 seconds, rinsed twice in sterile distilled water and dried on sterile filter paper. Potato Dextrose Agar (PDA) was prepared (Potato starch=20g, Dextrose=20g and Agar=20g) and used to isolate the pathogen. Media was sterilized at 121^{o}C for 15 min on 103Kpa. On lukewarm conditions media was poured into petri dishes (90mm). On solidification, the leaves pieces were place on the surface of the media plates and incubated at 25 ± 2°C for 2 days. On appearance of fungal colonies, they were placed into fresh PDA plates for sub-culturing to obtain pure cultures.

Biocontrol Agents and Preparation of Inoculum

Cultures of *T. harzianum, B. subtilis, Metarhizium robertsii and Metarhizium anisopliae* were collected from Plant Bacteriology Section, Oilseed Research Institute, AARI, Faisalabad. *T. harzianum*, *M. robertsii* and *M. anisopliae* were multiplied on Potato Dextrose Media at 28°C and spores’ concentration of 1×10^{7} mL^{-1} was used. *B. subtilis* was multiplied in Nutrient Broth at 30°C and 1×10^{8} cfu mL^{-1} was used to assess the antifungal efficacy against *A. alternata*.

In vitro Antagonistic Efficacy

Antagonistic activity was assessed for four biological control agents: *B. subtilis*, *T. harzianum*, *M. robertsii* and *M. anisopliae*, using a dual culture technique on PDA. A 5 mm mycelial disc of *A. alternata* was placed on one edge of a PDA plate and 60 mm on the opposite side of the plate was a similar-sized disc of *T. harzianum*, *M. robertsii* and *M. anisopliae*. *B. subtilis* was evaluated by mixing it in the media and placing the *A. alternata* culture bit in the center like poisoned food technique. Plates were incubated at 25 ± 2°C, with the radial growth of the pathogen measured after days 4, 8 and 12. Percent growth inhibition (PGI) was calculated by using the following formula:

$$\mathrm{PGI}\ (\%) = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{C}} \times 100$$

Where C represents radial growth of the pathogen in the control and T represents radial growth in the treated plates. Treatments were arranged in a completely randomized design with three replicates.

In planta Experiment

A field experiment was performed by using a randomized complete block design (RCBD) with four treatments (Table 1) and ten replications. Each field plot consisted of a 1 m × 1 m area, with a 0.5 m barrier between each treatment and replication plot. A spore suspension of *A. alternata* (1×10^{6} conidia mL^{-1}) was uniformly sprayed on the plants when they were at the 3–4 leaf stage of growth with hand sprayer. BCAs were applied at sowing.

Disease Assessment

While 45 days after sowing, disease severity was assessed using a modified 0–5 disease rating scale (Table 2). Disease incidence and disease severity were calculated using standard procedures.

Growth and Physiological Measurement

From each field plot, ten plants were randomly selected to measure the height (cm), leave length and width (cm), chlorophyll content (SPAD units) with SPAD-502-meter, fresh biomass (shoot and root) and total dry weight (oven dried at 70°C for 72 hours).

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Table 1. Treatments of BCAs for field evaluation.

Treatment	Agents
T1	*T. harzianum*
T2	*B. subtilis*
T3	*T. harzianum* + *B. Subtilis*
T4	*A. alternata* only

Table 2. Disease Rating Scale according to Bhat *et al*. (2013).

Disease ratings	Leaf area infected (%)	Response
0	Disease free	Immune
1	0.1-10.0	Highly Resistant
2	10.1-25.0	Resistant
3	25.1-50.0	Moderately Resistant
4	50.1-75.0	Susceptible
5	>75	Highly Susceptible

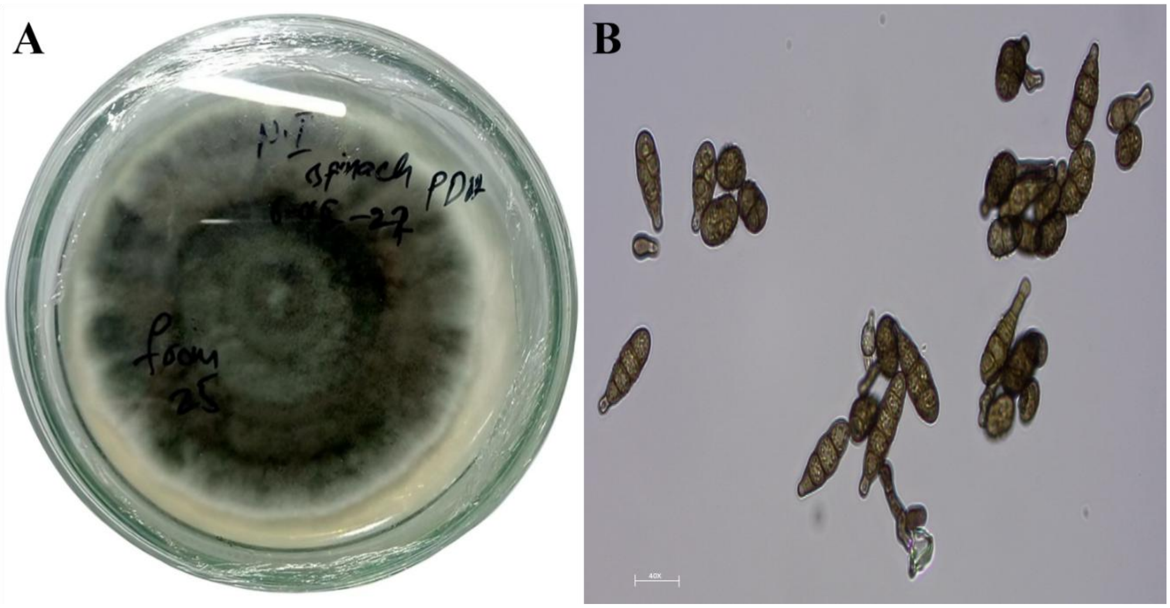

Figure 1. Morphological characterization of *Alternaria* pathogen associated with leaf spots of spinach. A) Pure culture of *A. alternata* isolated from spinach B) Spores of *A. alternata* under microscope at the magnification of 40X.

Statistical Analysis

The data were analyzed with analysis of variance (ANOVA). Treatment means were compared using the least significant difference (LSD) test at P ≤ 0.05 in Statistix version 8.1.

RESULTS

Pathogen Identification on Morphological Basis and *In vitro* Antagonistic Efficacy

The pathogen associated with the symptoms of leaf spot in spinach was isolated on the basis of colony morphology and spore shape and colour. The pathogen was identified based on colony morphology and microscopic characteristics (Figure 1) following standard taxonomic keys; however, molecular confirmation was not conducted and is acknowledged as a limitation of this study. The dual culture assay demonstrated that all tested Biological Control Agents (BCAs) significantly inhibited the radial growth of *A. alternata* at all observation intervals shown in Table 3. However, the magnitude and consistency of inhibition varied among treatments. *B. subtilis* exhibited the strongest early antagonistic response, restricting pathogen growth by 40.00% at day 4. Inhibition peaked at nearly 50% by day 8. The rapid early antagonism observed with *B. subtilis* may be associated with its ability to colonize the substrate and produce antifungal metabolites during initial incubation. However, the decline in activity after day 12 suggests a possible reduction in persistence or competitive activity over prolonged incubation.

Table 3. Mean percent growth inhibition (PGI) of *A. alternata* under *in vitro* experiment.

Treatments	PGI Mean ± SE (%)		
	4 Days	8 Days	12 Days
B. subtilis	40.00 ± 2.88 a	49.97 ± 4.18 a	22.54 ± 5.64 ab
T. harzianum	33.33 ± 3.33 ab	40.47 ± 4.76 b	29.67 ± 3.70 a
M. robertsii	28.33 ± 1.66 c	39.52 ± 2.07 b	11.57 ± 0.46 b
M. anisopliae	23.33 ± 1.66 b	24.03 ± 2.63 c	14.21 ± 2.68 b
Control	6.66 ± 1.66 d	11.33 ± 0.88 d	9.33 ± 0.66 c

Means followed by the same letter in a column are not statistically different (LSD test, P ≤ 0.05).

Table 4. ANOVA summary for field traits

Trait	Source of Variation	DF	F-value	Significance
Plant Height (cm)	Treatment	3	75.49	P < 0.001***
Leaf Length (cm)	Treatment	3	44.95	P < 0.001***
Leaf Width (cm)	Treatment	3	71.86	P < 0.001***
Fresh Weight (g)	Treatment	3	12.07	P < 0.001***
Chlorophyll (SPAD)	Treatment	3	72.34	P < 0.001***
Disease Severity (%)	Treatment	3	95.42	P < 0.001***
Total Dry Weight (g)	Treatment	3	146.90	P < 0.001***

*** indicates significance at P ≤ 0.001.

Table 5. Field evaluation of biocontrol treatments in spinach.

Treatment	PH (cm)	LL (cm)	LW (cm)	CC (SPAD)	FW (g)	TDW (g)	DS (%)
T1	12.50 b	12.98 b	5.46 b	41.98 b	25.10 b	2.02 b	18.30 c
T2	11.91 c	12.28 c	5.13 c	40.40 c	22.61 c	1.76 c	21.90 b
T3	13.35 a	13.95 a	5.91 a	44.20 a	27.30 a	2.25 a	12.00 d
T4	9.06 d	10.18 d	4.03 d	35.30 d	15.23 d	1.20 d	35.20 a

PH=Plant Height, LL=Leaf Length, LW=Leaf Width, CC=Chlorophyll Content, FW=Fresh Weight, TDW=Total Dry Weight and DS=Disease Severity. Means followed by the same letter in a column are not statistically different (LSD test, P ≤ 0.05).

In contrast, *T. harzianum*, while having lower initial levels of inhibition than *B. subtilis*, exhibited consistent performance throughout. *T. harzianum* produced an inhibition of mycelial growth of *A. alternata* of 33.33% on day 4 and 40.47% on day 8 and continued to have some inhibitory effect (29.67%) on day 12. *T. harzianum* sustained inhibition, constant competition, mycoparasitism and enzyme production, are typical characteristics of *Trichoderma* spp. *M. robertsii* and *M. anisopliae* also produced some inhibition in the growth of *A. alternata*, but both were less effective and stable than *T. harzianum*, especially by day 12, when both fungi were no longer able to effectively compete against the *A. alternata*. The control plate proves that *A. alternata* was virulent and confirms the effects of the treatments.

Field Experiment

The analysis of variance results show strong F values associated with each trait that suggest that the combined effect of biocontrol agents resulted in a significant reduction of pathogen damage and an increase in the rate of plant growth. The results of the field trial confirmed that each of the growth (physiological) parameters and disease parameters evaluated were significantly affected (P < 0.001) by all biocontrol treatments mentioned in Table 4. This provides evidence that combining BCAs has an evident impact on the overall performance of the plant under normal environmental conditions. Generally speaking, the pattern for the order of treatment (T3 > T1 > T2 > T4) for almost all traits is consistent. When using *T. harzianum* in combination with *B. subtilis* (T3) resulted in the greatest positive effect on *A. alternata* and appeared to have a synergistic effect on enhancing both suppression of Alternaria leaf spot and increased vigour of the plant. The untreated control showed the least growth with the highest level of disease severity.

Effect of BCAs on Growth, Physiology and Disease Severity

The most favorable results in plant growth and biomass are attributable to the combination (T3) of *T. harzianum* and *B. subtilis*, with T3 exhibiting Plant Height, Leaf Area, Leaf Area Index and Total Chlorophyll levels greater than all of the other treatments. In addition, T3 also reduced plant disease severity (12%) by 65.7% compared to the untreated control, which demonstrates that *T. harzianum* and *B. subtilis* provide a very high level of protection as a combination of two BCAs. *T. harzianum*, when applied alone, did produce favorable results in regard to plant growth, but to a minor degree than the combination of *B. subtilis* and *T. harzianum*. *B. subtilis* produced moderate effects with respect to both plant growth promotion and systemic resistance to disease. For the untreated control, plant vigour was the lowest and disease severity was the highest for *A. alternata* for spinach as a result of the lack of biological control.

Overall, the combined use of the two microbial BCAs, *T. harzianum* and *B. subtilis*, provides the best suppression of Alternaria leaf spotting and increases plant health and productivity simultaneously.

DISCUSSION

An eco-friendly and efficient way to manage Alternaria leaf spot in spinach plants can be found in the results of this study that support the integrated biocontrol approach through the use of *T. harzianum* and *B. subtilis*. The integrated biocontrol treatment (T3) had the least amount of disease severity (12%) and improved all major growth and yield characteristics. This shows that these BCAs act in a dual capacity as both protective and growth-promoting in real world field situations. They therefore represent promising candidates for incorporation into sustainable spinach production systems.

While laboratory assays demonstrate short-term antagonism, they do not necessarily reflect long-term ecological stability under field conditions. Laboratory experiments provide evidence of how effectively BCAs work on fungal and bacterial targets during a relatively short period, while environmental variation and other microbial competitors limit the effectiveness of many BCAs and may not produce equal or superior results in field conditions. The *T. harzianum* antagonist continues to provide strong protection against Alternaria leaf spot throughout the study, indicating that *T. harzianum* has adapted well to rhizosphere colonization and has developed the ability to withstand environmental stresses such as high temperatures and drought. Previous studies have shown that many *Trichoderma* spp. will remain viable in the field for long periods of time and will continue to effectively control harmful microorganisms (Dutta *et al*., 2022; Mukherjee & Ghosh, 2023). These findings further support that ecological stability is often the best predictor for success in the field, as opposed to laboratory inhibition.

The increased effectiveness of the third treatment over the first two treatments is mainly attributed to the mechanisms of the biocontrol agents on their target pathogens. *T. harzianum* has several modes of action against target organisms, for example, it kills target organisms by a process called "mycoparasitism" and the coiling of hyphae around the target organism and produces cell wall degrading enzymes (Guzmán-Guzmán, 2023). In addition, *T. harzianum* activates the host plant's natural defence mechanisms against pathogens (Poveda, 2022). *B. subtilis* produces bioactive compounds to create a systemic resistance against pathogens and improve plant immunity, whereas *T. harzianum* promotes plant immunity and reduces the growth of many types of fungi by using surfactin and iturin as the key bioactive compounds to protect the plant. The results from this research confirm earlier reports that both *B. subtilis* and *T. harzianum* acted on the pathogens in complementary, but parallel manners (Maketon *et al*., 2008; Poveda, 2022).

The T3 application provided us with further evidence to confirm that applying *B. subtilis* and *T. harzianum* together is effective because in addition to confirming the mutual relationship between fungi and bacteria, we also noted that plants with combined applications of both fungi and bacteria produced greater amounts of chlorophyll and biomass and exhibited increased rates of root growth compared to their separate applications alone. The results suggest that *B. subtilis* supports greater establishment in the rhizosphere for better nutrient and mineral uptake (enhancing, therefore, overall performance) while *T. harzianum* has the ability to support better development of the root system and enhanced overall performance through increased soil–plant interactions. There are numerous publications that document the benefits of co-application of *Trichoderma* and *Bacillus* to increase crop performance and reduce soil-borne plant pathogens at the same time (Attia *et al*., 2025; Firdu *et al*., 2022; Petcu *et al*., 2023; Zaim *et al*., 2018; Singh *et al*., 2021). Similar trend has been reported in earlier study on potato common scab, where plant growth–promoting rhizobacteria significantly reduced disease severity under both laboratory and field conditions, confirming the effectiveness of microbial-based disease management across different host–pathogen systems (Usman *et al*., 2025).

These results are also consistent with earlier studies comparing the use of biological and chemical methods for managing *Alternaria* diseases. Studies completed in the past document that the application of chemical fungicides (mancozeb and propiconazole) to combat *Alternaria* can reduce the incidence of *Alternaria* by 25–30% *in vitro*, however, *Trichoderma* species alone can provide inhibition levels equal to some synthetic fungicides (Pun *et al*., 2020; Glory *et al*., 2022). In addition, similar results have been reported on the use of *T. harzianum* and *B. subtilis* to improve the growth of mustard blight and cabbage diseases and to inhibit the growth of *Alternaria* spp. at levels comparable to synthetic fungicides (Gupta *et al*., 2020). The present results therefore confirm that the combination of *T. harzianum* and *B. subtilis* offers a promising residue-free alternative under the conditions evaluated in this study for managing Alternaria leaf spot disease.

This observation is supported by other research showing that the combination of *T. harzianum* and *B. subtilis* improves the overall health of plants and decreases the incidence of diseases from many different types of crops and pathogens. Evidence includes all of the studies previously referenced which documented large reductions in fusarium wilt in bananas, chickpeas and other crop species using both organisms together (Kavino *et al*. 2007; Karthikeyan *et al*. 2005; Saravanakumar *et al*. 2016). Additionally, the antagonist and nematode suppressor characteristics of *T. harzianum* have been documented in peppers (Ramakrishnan *et al*., 2019a; Ramakrishnan *et al*., 2019b). The findings from this experiment confirm earlier studies regarding the combined effect of using both organisms together to improve biological control and plant growth. Altogether, the dual approach used in this experiment represents an effective, sustainable method of managing plant diseases in more modern Integrated Disease Management (IDM) practices.

CONCLUSION

The study concludes that the combined application of *T. harzianum* and *B. subtilis* provides a highly effective, sustainable and environmentally safe strategy for the management of Alternaria leaf spot in spinach, significantly reducing disease severity while improving plant growth, physiological performance and yield. The synergy between fungal mycoparasitism and bacterial-induced systemic resistance ensures superior and stable biocontrol compared with individual treatments or chemical fungicides. These findings support the integration of both BCAs as a practical component of sustainable crop protection programs, particularly in residue-sensitive leafy vegetables. Nevertheless, the findings are based on a single-season field trial and future studies should focus on multi-season validation, formulation development and large-scale application.

AUTHOR CONTRIBUTIONS

Numaad Ihsan: Experimental design and conduction, Methodology, Writing – original draft. Muhammad Usman: Data recording, Writing – review and editing. Muhammad Ehetisham-ul-Haq: Statistical analysis, Writing – original draft, Writing – review and editing. Huma Abbas and Amjad Abbas: Conceptualization, Supervision, Project administration, Resources, Validation.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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