Fortification of Rice Seedlings with Growth-Promoting Microorganisms for Tolerance to Brown Spot Disease and Enhancement of Yield.

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Abstract
Production of rice, a critical staple food crop worldwide, particularly in Africa, is significantly constrained by fungal diseases, including Alternaria brown spot, caused by *Alternaria alternata*. The use of conventional chemical fungicides, though effective, poses significant environmental and health concerns, necessitating the search for sustainable alternatives. Due to this, a field experiment was conducted at the Teaching and Research Farms, Federal University of Technology, Akure, to assess the effects of biocontrol agents *Trichoderma harzianum* and *Bacillus subtilis* on the growth, disease incidence, severity, yield, and proximate content of rice. The trial consisted of 2 x 6 factorial combinations of two priming durations (24 and 48 hrs.) with six bioagents: priming with *T. harzianum* (10^5/s/ml) for 24 hrs., inoculation with *A. alternat*a; priming with *T. harzianum* (10^5/s/ml) for 48 hrs., inoculation with *A. alternata*; priming with *B. subtilis* (10^4/cfu/ml) for 24 hrs., inoculation with *A. alternata;* priming with *B. subtilis* (10^4/cfu/ml) for 48 hrs., inoculation with *A. alternata;* priming with *B. subtilis* + *T. harzianum* for 24 hrs., inoculation with *A. alternata;* priming with *B. subtilis* + *T. harzianum* for 48 hrs., inoculation with *A. alternata*; Standard check, seed dressing with Seed Plus, inoculation with *Alternaria alternata*, Negative control, priming with water, inoculation with *Alternaria alternata, and* Positive control, priming with water only. The experiment was arraigned in a completely randomized design (CRD) with three replications. The results indicated that *Trichoderma harzianum* and *Bacillus subtilis*, individually and in combination, significantly reduced disease incidence and severity while enhancing plant growth and yield. The study concludes that the integration of these biocontrol agents can serve as an effective, eco-friendly strategy for managing Alternaria brown spot in rice production. This conclusion highlights the potential of using biocontrol agents as a sustainable alternative to chemical pesticides.

Keywords: Rice, *Trichoderma harzianum*, *Bacillus subtilis*, brown spot disease, *Alternaria alternata*. biological control

Introduction

Rice (*Oryza sativa*) is a vital staple food crop essential for global food security, particularly in Africa, where it serves as a primary dietary staple. As a member of the Poaceae family, rice is among the most significant cereal crops in Nigeria, supporting the diet of more than half of the world's population (IRRI, 2013). Despite its importance, rice production is constrained by various factors, including biotic and abiotic stresses that significantly impact yield and quality. Among the biotic challenges, diseases caused by fungal pathogens, including *Alternaria alternata*, are particularly detrimental to rice production. Alternaria brown spot, caused by *A. alternata*, is a seed-borne disease that causes seed discoloration, seedling blight, and necrotic lesions on leaves, stems, and panicles, ultimately leading to substantial yield losses (Chiangsin *et al.*, 2018). The management of this disease has traditionally relied on chemical fungicides, which, while effective, pose environmental concerns such as soil and water contamination. Consequently, the search for sustainable and eco-friendly disease management strategies has gained momentum in recent years.

Biological control agents (BCAs) such as *Trichoderma harzianum* and *Bacillus subtilis* have emerged as promising alternatives to synthetic fungicides. *Trichoderma harzianum* is a widely studied fungal biocontrol agent known for its antagonistic properties, including mycoparasitism, antibiosis, and competition for nutrients and space (Manzar *et al.*, 2022). Similarly, *Bacillus subtilis*, a beneficial rhizobacterium, has demonstrated efficacy in suppressing plant pathogens through mechanisms such as the production of antibiotics, induction of systemic resistance, and nutrient solubilization (Patel *et al.*, 2023). These biological agents not only mitigate plant diseases but also enhance plant growth and yield by improving nutrient uptake and promoting root development.

Materials and Methods

Experimental Site Description

The study was carried out at the Federal University of Technology's Department of Crop, Soil, and Pest Management Laboratory in Akure, Ondo State, Nigeria (7°16'N and 5°12'E). Akure is located in Nigeria's rain forest region, which experiences an average 1300–1600 mm of rainfall annually and 27°C temperatures. The relative humidity is less than 60 % during the dry season and between 85 and 100 % during the wet season. The elevation of Akure is roughly 351 meters above sea level. The rainfall pattern is bimodal, peaking in June and July and September, with a brief dry spell in August in between. The soil type is predominantly sandy clay loam.

Experimental Procedure and Treatments Application

The certified seeds of the early-maturing, high-yield, and stress-tolerant FARO 44 rice cultivar were acquired from the International Institute of Tropical Agriculture (IITA). The floating method was used to evaluate the seeds for germination. The Department of Crop, Soil, and Pest Management at the Federal University of Technology, Akure, provided the *Bacillus subtilis* and *Trichoderma harzianum*. After being isolated from contaminated rice seeds, the pathogen *Alternaria alternata* was cultivated on potato dextrose agar (PDA). Rice seeds were inoculated with *Alternaria* alternata spore suspensions and subjected to the treatments involving the sole and combination of *T. harzianum* and *B. subtilis*. Treated seeds were allowed to germinate and grow for three (3) weeks in *A. alternata*-infested medium in the laboratory prior to the field evaluation. The field trial consisted of 2 x 6 factorial combinations of two priming durations (24 and 48 hrs.) with six bioagents: priming with *T. harzianum* (10^5/s/ml) for 24 hrs., inoculation with *A. alternata*; priming with *T. harzianum* (10^5/s/ml) for 48 hrs., inoculation with *A. alternata*; priming with *B. subtilis* (10^4/cfu/ml) for 24 hrs., inoculation with *A. alternata;* priming with *B. subtilis* (10^4/cfu/ml) for 48 hrs., inoculation with *A. alternata;* priming with *B. subtilis* + *T. harzianum* for 24 hrs., inoculation with *A. alternata;* priming with *B. subtilis* + *T. harzianum* for 48 hrs., inoculation with *A. alternata*; standard check; seed dressing with Seed Plus; and inoculation with *Alternaria alternata.* Negative control, priming with water, inoculation with *Alternaria alternata,* positive control, priming with water only in a completely randomized design (CRD) with three replications. The seedlings from the laboratory were transplanted to the plastic pots, having 10 kg of soil. The soil in each pot was infested with spore suspension of *A. alternata* at 10^7 s/ml. The rice plants were allowed to grow till maturity. Standard agronomic practices were uniformly followed to maintain plots free from biotic stresses.

Data Collection and Analysis

The study employed two-way ANOVA to assess for significance among treatments and gathered data on the number of leaves, plant height (cm), and number of tillers. Disease incidence and severity were assessed using standard disease rating scales. Panicle number, weight, and grain yield were also evaluated. Number of infected panicles, weight of panicles, weight of 100 grains, shoot dry weight (g), moisture content, ash content, fat content, crude fibre, protein content, and carbohydrate content were determined at harvest.

Determination of disease incidence (DI, %) **and severity** (DS, %)

Disease incidence was determined using the formula

$$\text{Disease incidence} = \frac{\text{No. of infected leaves}}{\text{Total number of leaves}} \times 100$$

The disease severity was assessed by using a 0 to 5 rating scale (Shahzad and Bhat, 2005). Five disease categories were made on the basis of percent leaf area infected.

Disease severity (DS) was calculated by using the formula

$$\text{Disease Severity} = \frac{\Sigma (n \times v)}{N \times G} \times 100$$

Where Σ = Summation; n = Number of leaves in each category; v = Numerical value of each category; N = Total number of leaves examined; G = Maximum numerical value.

The disease severity (DS, %) rating is based on these five scales: no infection (1), poorly infected (2), slightly infected (3), strongly infected (4), and severely infected (5).

Results

Effect of *Trichoderma harzianum*, Bacillus *subtilis,* and priming duration on plant height (cm), number of leaves, and number of tillers produced by rice plants

Table 1 displays the results on the effect of *Trichoderma harzianum*, Bacillus *subtilis,* and duration of seed priming on plant height (cm), number of leaves, and number of tillers produced by rice plants. Rice plant height, number of leaves, and tillers were significantly improved by all the bioagents relative to the negative control (NC). BS+TH had the highest values of plant height and number of leaves (33.74 cm and 19.63, respectively). Plant height obtained for BS + TH was not significantly different compared with TH only but showed a significant increase in comparison with BS only, PC, and SC. The number of leaves of rice (19.63, 19.37, and 17.75) recorded for BS + TH, SC, and BS only, respectively, were not significantly different from one another, while BS had the highest number of tillers of rice plants but showed no significant differences compared with other treatments. The duration of priming on plant height, number of leaves, and tillers of rice plants revealed that 48 hours had the highest values but showed no significant difference when compared together, while there was no significant difference considering the interaction between the bioagents and the priming duration.

Table 1: Effect of *Trichoderma harzianum*, Bacillus *subtilis* and priming duration on plant height (cm), number of leaves and number of tillers produced by rice plant

Treatments	Plant Height (cm)	Number of Leaves	Number of Tillers
Bioagents (B)			
TH Only	32.22ab	16.17b	21.08ab
BS Only	29.11bc	17.75ab	25.17a
BS + TH	33.74a	19.63a	21.07ab
SC	27.81cd	19.37a	23.33ab
PC	28.47bcd	13.33c	19.67b
NC	25.21d	15.77bc	21.00ab
Duration (D) of seed priming			
24 Hrs.	29.34a	16.65a	21.19a
48 Hrs.	29.51a	17.35a	22.58a
B x D	ns	ns	ns

Values followed by similar letters under the same column are not significantly different at p = 0.05; * = significant at 5% level of probability; ns = not significant

Effect of *Trichoderma harzianum*, Bacillus *subtilis,* and priming duration on disease incidence, severity, and number of panicles of rice

The results on the effect of bioagents and priming duration on disease incidence, severity, and number of panicles of rice are shown in Table 2. The highest values of disease incidence and severity were obtained for the negative control (NC), and they were significantly higher than other treatments. There was no significant difference between the priming durations (24 and 48 hours) on disease incidence and severity, while the 48-hour priming duration had a significantly higher value for the number of panicles compared with 24 hours. The results on the interaction between the treatments (bioagents and priming duration) showed that priming duration had a lower value (7.12 %) of disease incidence, and it was significantly lower than the value recorded for bioagents, while there was no significant difference in the values obtained for priming duration and bioagents on disease severity and number of panicles.

Table 2: Effect of *Trichoderma harzianum*, Bacillus *subtilis* and priming duration on disease incidence, disease severity and number of panicles of rice on the field

Treatments	Disease Incidence (%)	Disease Severity (%)	Number of Panicles
Bioagents (B)			
TH Only	1.51b	0.00b	48.00ab
BS Only	6.69b	1.00b	48.75a
BS + TH	3.81b	1.00b	45.75ab
SC	7.03b	1.00b	39.00c
PC	1.19b	1.00b	43.50bc
NC	32.49a	2.00a	45.50ab
Duration (D) of seed priming			
24 Hrs.	6.86a	1.17a	42.58b
48 Hrs.	7.38a	1.17a	47.58a
B x D	*	ns	ns

Values followed by similar letters under the same column are not significantly different at p = 0.05; * = significant at 5% level of probability; ns = not significant

Effect of *Trichoderma harzianum*, Bacillus *subtilis,* and priming duration on the number of infected panicles, weight of panicles, weight of 100 grains, and shoot dry weight of rice at harvest.

The data presented in Table 3 are the results on the effects of *Trichoderma harzianum*, Bacillus *subtilis,* and priming duration on the number of infected panicles, the weight of panicles, the weight of 100 grains, and the shoot dry weight of rice at harvest. The number of infected panicles was highest in the negative control compared with other bioagents. BS + TH had the highest weight of panicles, weight of 100 grains, and shoot dry weight of rice but showed no significant difference compared with BS and TH only. The least weight of panicles, weight of 100 grains, and shoot dry weight of rice were obtained for the negative control (NC). 48 hours of priming duration showed significantly higher values for the weight of panicles, the weight of 100 grains, and the shoot dry weight of rice compared with 24 hours of priming duration. The results further showed that the interaction between the bioagents and the inoculation time were not significantly different on the number of infected panicles, the weight of panicles, or the weight of 100 grains, while the value recorded for the priming period was significantly higher than the bioagents on shoot dry weight.

Table 3: Effect of *Trichoderma harzianum*, Bacillus *subtilis* and priming duration on number of infected panicles, weight of panicles, weight of 100 grains and shoot dry weight of rice at harvest.

Treatments	NIP	WOP (g)	W100 (g)	SDW (g)
Bioagents (B)				
TH Only	13.00a	99.38abc	2.20ab	88.48ab
BS Only	8.00b	119.21ab	2.25ab	105.75a
BS + TH	5.67b	128.50a	2.38a	108.30a
SC	14.00a	77.28cd	2.15b	51.43b
PC	9.00b	86.80bc	2.07b	60.45b
NC	14.50a	45.45d	1.83c	33.67c
Duration (D) of priming				
24 Hrs.	13.00a	103.25a	2.08b	95.05b
48 Hrs.	8.39b	82.31b	2.22a	101.65a
B x D	ns	ns	ns	*

Values followed by similar letters under the same column are not significantly different at p = 0.05; * = significant at 5% level of probability; ns = not significant; TH = *Trichoderma harzianum,* BS= *Bacillus subtillis,* SC= Standard check, PC= Positive control, NC= Negative control, NIP= Number of Infected Panicles, WOP= Weight of Panicles, W100= Weight of 100 grains, SDW= Shoot Dry Weight.

Effect of *Trichoderma harzianum*, Bacillus *subtilis,* and priming duration on the proximate content of rice grains at harvest

Table 4 presents the results on the effect of *Trichoderma harzianum,* Bacillus *subtilis,* and priming duration on the proximate content of rice grains at harvest. Both the bioagents and the priming durations improved moisture content, ash content, fat content, crude fibre, protein content, and carbohydrate content of rice at harvest. The results on the rice's crude fibre, protein, and carbohydrate content were not significantly different comparing the bioagents with one another except for the standard and positive controls for crude fibre and protein content. The negative control had the least ash content among the treatments, while the fat content obtained for TH and BS only were not significantly different from each other but significantly low compared with the positive control (PC). The highest moisture content was obtained for TH only, although it showed no significant difference compared with BS only and PC. The effect of priming duration revealed that there were no significant differences in the values of ash content, fat content, crude fibre, protein content, and carbohydrate between 24 and 48 hours. However, 48 hours' duration was significantly higher than 24 hours considering the rice grain moisture content. There were no significant differences in the interaction between the bioagents and priming duration for moisture, ash, fat, crude fibre, protein, and carbohydrate content in rice grains.

Table 4: Effect of *Trichoderma harzianum*, Bacillus *subtilis* and priming duration on the Proximate content of rice grains at harvest.

Treatments	M/C	ASH	FAT	C/F	PRO	CHO
Bioagents (B)						
TH Only	11.19a	1.59abc	3.86ab	4.88ab	16.35ab	63.28a
BS Only	10.48ab	1.69abc	3.47b	3.39b	17.69a	63.72a
BS + TH	9.52c	1.74ab	3.53b	4.85a	15.70ab	64.74a
SC	10.08bc	1.96a	4.33ab	3.75b	17.49a	62.38a
PC	10.36abc	1.55bc	4.71a	4.67a	14.77b	63.81a
NC	10.11bc	1.30c	4.14ab	4.16ab	16.76ab	62.35a
Duration (D) of seed priming						
24 Hrs.	10.49a	1.71a	3.85a	4.25a	16.69a	63.31a
48 Hrs.	10.08b	1.57a	4.16a	4.32a	16.23a	63.46a
B x D	ns	ns	ns	ns	ns	ns

Values followed by similar letters under the same column are not significantly different at p = 0.05; * = significant at 5% level of probability; ns = not significant; TH= *Trichoderma harzianum* BS= *Bacillus subtillis*, SC= Standard check, PC= Positive control, NC= Negative control, M/C= Moisture content, ASH= Ash content, FAT= Fat content, C/F= Crude fibre, PRO= Protein content, CHO= Carbohydrate content.

Discussion

This study focuses on the biological agents *Trichoderma harzianum* and *Bacillus subtilis*' capacity to prevent rice brown spot disease by inducing a plant defence response. It has been observed that *Trichoderma spp*. are an environmentally friendly biological control agent for plant diseases, allowing for a reduction in the usage of chemical fungicides (Puyam, 2016). According to Devi *et al*. (2022), Bacillus is a bacterial genus that is commonly found and has been utilized as a biocontrol agent due to its ability to promote plant growth and enhance resistance against various pathogens. This dual role makes Bacillus subtilis a valuable ally in sustainable agriculture practices. Bacillus species are suitable candidates for biocontrol agents because of their ability to create endospores, which makes them resistant to harsh environmental conditions. Bacillus may produce extracellular metabolites and siderophores as a result of its antagonistic activity (Miljaković *et al*., 2020). The field experiment, when comparing treatment BS+TH to other untreated plots, showed a significant increase in all growth parameters (plant height, number of leaves, and number of tillers). This could be that *Trichoderma species* function as bio-fertilizers, promoting improvement in nutrient solubilization and uptake by plants (Vinale *et al.*, 2006). The results obtained from the combination of Trichoderma with Bacillus resulted in a significant reduction in disease incidence and disease severity compared to other treatments. This could be due to the production of several antimicrobial agents and complex compounds that are important in the antagonistic biocontrol activities, which played a significant role in the stimulation or inhibition of spore formation. The current study aligns with the earlier research conducted by Abdel-Fattah *et al*. (2007), which found that spraying a *T. harzianum* spore suspension at 10^8 spores/mL significantly reduced disease severity (DS) and disease incidence (DI) on plant leaves and also significantly increased grain yield, total grain, carbohydrate, and protein. This indicates that the application of *T. harzianum* not only helps manage plant diseases but also enhances the overall productivity and nutritional quality of the crops. Such findings underscore the potential benefits of utilizing biocontrol agents in agricultural practices. Additionally, it was noted that the treatment with the bioagents, especially BS+TH treatments, gave the highest yield in terms of the number of panicles, weight of panicles, and weight of 100 grains. This is as a result of a decrease in the incidence and severity of brown spot disease on the rice plant. This outcome is consistent with the findings of Benitez *et al*. (2004), which showed that *T. harzianum* application is critical to the formation of yield, enhancement of yield quality, optimal growth component, quantity of seed, and number of filled seed. This indicates that the use of *T. harzianum* not only mitigates disease impact but also contributes positively to various yield-related factors. Consequently, this reinforces the importance of biological control methods in sustainable agriculture practices. According to Yadev *et al*. (2013), *Trichoderma spp*. also stimulate plant growth and yield through secondary metabolite production, producing phytohormones that optimize plant growth. Crucially, *Trichoderma spp*. also regulate pathogenic fungi in the soil and promote the plant's revitalization phase. In addition, it has been reported that *Bacillus spp*., in conjunction with mycorrhizal fungi, produce siderophores that improve nutrient acquisition in wheat grain and root tissues (Yadav *et a*l., 2021). One important factor that determines rice's nutritional value is its proximate composition, which includes its moisture, protein, fat, ash, fibre, and carbohydrate content. Well-known biocontrol agents like *Trichoderma harzianum* and *Bacillus subtilis* improve plant development and health, which may have an effect on rice grains' nutritional value. It is well known that *Trichoderma harzianum* and *Bacillus subtilis* can increase plant growth and provide systemic resistance to pathogens. They can also irritate plant pathogens and produce compounds that encourage growth, which may enhance nutrient intake and metabolism. It has been reported by Sharma *et al*. (2020) that *B. subtilis* and *T. harzianum* can both raise the protein content of rice grains. This is probably because these biocontrol agents enable better uptake and utilization of nitrogen. Singh *et al*. (2017) reported that higher ash content has been linked to treatments with *T. harzianum* and *B. subtilis*, suggesting that rice grains are absorbing and accumulating minerals more readily. According to Yadav *et al*. (2021), after rice has been treated with these biocontrol agents, rice grains' fibre content has somewhat increased, increasing the dietary fibre content and overall nutritional quality. In contrast, *B. subtilis* typically has a more noticeable impact on the amount of protein and ash, most likely because of its potent antagonistic qualities and capacity to increase nutrient absorption. However, *T. harzianum* is more successful in raising fibre levels; this may be because of its contribution to enhancing overall plant resilience.

Conclusion

The use of bioagents is a good substitute for chemical fungicides. *Trichoderma harzianum* demonstrated potent antagonistic activity against *Alternaria alternata*, most likely by means of nutritional competition, antifungal chemical synthesis, and systemic resistance induction in rice plants. In comparison to the standard check and control, this biocontrol agent significantly decreased the incidence and severity of disease while also improving the general health and resilience of rice plants. *Bacillus subtilis,* also known for its broad-spectrum antimicrobial properties and ability to promote plant growth, reduced disease incidence and severity. This disease control strategy is economical and environmentally friendly; it encourages plant growth and reduces the toxicity that comes with using synthetic chemicals. It is recommended to employ *Trichoderma harzianum* and *Bacillus subtilis* as biocontrol agents to control rice brown disease. These biocontrol agents not only help manage disease but also enhance the overall vitality of the plants, making them more robust against future threats. By utilizing natural methods like these, farmers can foster sustainable agriculture while minimizing environmental impact.

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Competing Interests
Authors have declared that no competing interests exist.

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