MULTIVARIATE EVALUATION OF WHEAT GENOTYPES AND MICROBIAL INOCULATION FOR ENHANCED PERFORMANCE UNDER WHEAT LEAF RUST PRESSURE

A. H. Aslam[1], R. Irshad[1], M. F. Khan[2], A. Ali[1], F.T. Zahra[1], H. Abbas[1], A. Abbas[1], M. A. Ali[1*]

[1]Department of Plant Pathology, University of Agriculture, Faisalabad, 38040, Pakistan
[2]National Seed Research and Training Center, University of Agriculture, Faisalabad, Pakistan.
*Corresponding author's email: amjad.ali@uaf.edu.pk

ABSTRACT

Wheat leaf rust (WLR) caused by *Puccinia triticina* remains an important constraint to wheat productivity and microbial seed treatment may complement host resistance by reducing disease development while sustaining plant growth and yield. However, genotype-dependent responses to individual and combined microbial treatments under field WLR pressure remain insufficiently characterized. Therefore, the present study was carried out to investigate the efficacy of beneficial microbes, *Trichoderma harzianum* and *Bacillus* sp. MN54, in managing WLR and enhancing growth and yield of wheat under field conditions. Twenty-five wheat genotypes were initially screened for disease response, and three representative genotypes, namely Galaxy-13 (moderately susceptible), Subhani-21 (moderately resistant), and Arooj-22 (resistant), were selected based on their contrasting responses to WLR severity. These genotypes were further evaluated using seed priming with *T. harzianum*, *Bacillus* sp. MN54, and their combination in a randomized complete block design (RCBD). The combined application of *T. harzianum* + *Bacillus* sp. MN54 significantly improved morpho-physiological and yield traits, resulting in increases of up to 13.0% in plant height, 17.9% in root length, 26.1% in fresh root weight, 50.0% in dry root weight, 35.1% in spike length, 61.1% in spike weight, 22.7% in grains per spike, 28.5% in 1000-grain weight, and 45.5% in grain yield per plant compared to the control. Microbial treatments significantly reduced disease severity, with the lowest values in Arooj-22 (12.0%) and Subhani-21 (17.3%) after seed priming with *T. harzianum*. Compared with the respective untreated controls, disease severity was reduced by approximately 37.8% in Arooj-22 and 45.4% in Subhani-21. Significant genotypic variation was observed, with Arooj-22 exhibiting superior resistance and responsiveness to microbial treatments. Correlation analysis revealed strong positive relationships among growth and yield traits, while WLR severity showed significant negative associations with yield components ($r = -0.80$ to -0.82). Principal component analysis (PCA) confirmed that yield traits and disease severity were the primary drivers of variation. These findings demonstrate that microbial seed treatments, particularly in combination, can effectively suppress WLR, enhance host growth, and improve grain yield and grain biomass in wheat, providing a sustainable approach for integrated disease management.

Keywords: Wheat leaf rust, *Bacillus* sp. MN54, *Puccinia triticina*, seed priming, *Triticum aestivum*; *Trichoderma harzianum*.

Received May 05, 2026 Published Online October 19, 2026 https://doi.org/10.36899/JAPS.2027.1.0003

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a globally important cereal crop, contributing nearly 20% of the caloric and protein intake for the human population (Mottaleb *et al*., 2023). In Pakistan and other South Asian regions, wheat is a staple crop essential for food security and economic stability (Shahzad *et al*., 2022). However, wheat productivity is severely constrained by biotic stresses, among which WLR caused by *Puccinia triticina* is one of the most prevalent and destructive diseases worldwide (Ali *et al*., 2018). Under conducive environmental conditions, WLR epidemics can lead to significant yield penalties ranging from 10-40%, and in highly susceptible cultivars, losses may exceed 50%, primarily due to reductions in photosynthetic efficiency, premature leaf senescence, and impaired assimilate translocation (El-Orabey and Elkot, 2020).

Puccinia triticina is an obligate biotrophic fungus with a macrocyclic and heteroecious life cycle, producing five spore stages that facilitate survival, genetic recombination, and rapid dissemination (Chiuraise, 2022). The uredinial stage plays a crucial epidemiological role, enabling repeated infection cycles and rapid disease spread within a season (Aslam *et al*., 2026; Peterson *et al*., 1948). The pathogen exhibits high genetic variability driven by mutation, recombination, and long-distance dispersal, leading to the continuous emergence of new virulent races capable of

overcoming host resistance (Figueroa *et al*., 2022). Host genetic resistance remains the most economical and environmentally sustainable approach for disease management. Major resistance genes (e.g., *Lr9*, *Lr24*) provide race-specific protection but are often short-lived due to pathogen evolution, whereas adult plant resistance genes such as *Lr34* and *Lr46* confer partial yet durable resistance by reducing disease development and pathogen reproduction (Kolmer, 2015; Hussien *et al*., 2024). Therefore, continuous screening of wheat germplasm under field conditions is essential for identifying stable resistance sources and supporting breeding strategies based on gene pyramiding (Hussien *et al*., 2024; Singh *et al*., 2014). Fungicides such as triazoles and strobilurins are effective in managing WLR, but their repeated and excessive use has raised serious concerns, including environmental contamination, potential risks to human health, and the emergence of fungicide-resistant pathogen populations (Balba, 2007; Islam *et al*., 2024). Consequently, there is a growing interest in sustainable and eco-friendly alternatives, particularly biological control approaches.

Plant growth-promoting microorganisms such as *Trichoderma harzianum* and *Bacillus* sp. MN54 enhance plant development through multiple mechanisms, including improved nutrient acquisition, phytohormone production, and induction of plant defense responses (Ali *et al*., 2020; Zohaib *et al*., 2024; Irshad *et al*., 2026). These microbes can activate induced systemic resistance, enhance antioxidant activity, and suppress pathogen development through antibiosis and competitive interactions (Razzaq *et al*., 2024). Moreover, microbial consortia often provide greater benefits than individual inoculants because of their complementary modes of action in the rhizosphere (Sharma *et al*., 2020). Such synergistic interactions can improve plant vigor while simultaneously reducing disease pressure. Nonetheless, the compatibility of fungal and bacterial inoculants in combined applications is not always guaranteed, as differences in colonization dynamics, resource competition, and rhizosphere niche preferences may influence their establishment and functional efficiency. Understanding these ecological interactions under field conditions remains a key challenge in developing effective microbial consortia. However, the combined effects of beneficial microbial inoculants on wheat performance under rust stress remain insufficiently explored under field conditions, particularly in the context of locally cultivated wheat varieties. It was hypothesized that the combined application of *Trichoderma* and *Bacillus* would exhibit synergistic effects, resulting in greater suppression of WLR and enhanced growth and yield of wheat compared to individual inoculations. Therefore, the present study aimed to evaluate wheat genotypes for resistance against *P. triticina* under field conditions and to assess the efficacy of microbial seed priming using *T. harzianum*, *Bacillus* sp. MN54, and their combination in managing WLR and improving wheat growth and yield.

MATERIALS AND METHODS

The experimental site was located in the research area of the Department of Plant Pathology, University of Agriculture, Faisalabad (31.4370723 N, 73.0591642 E), characterized by a semi-arid subtropical climatic zone with mild winters and moderate relative humidity. The experiments were conducted during the 2025-2026 wheat-growing season under prevailing field conditions. The soil of the experimental field was classified as sandy loam with uniform fertility status. All the recommended agronomic practices for wheat cultivation in Punjab were uniformly adopted to minimize confounding environmental effects. The present investigation comprised two independent and sequential experiments. The first experiment involved screening 25 wheat germplasm lines to evaluate their response to *P. triticina*, the causal agent of WLR. Based on the observed disease responses, three contrasting genotypes were selected for the subsequent microbial evaluation. The second experiment was conducted separately to evaluate microbial seed priming with *T. harzianum* and *Bacillus* sp. MN54, applied individually and in combination, for managing WLR and growth promotion under field conditions.

Plant Material and Experimental Design for Genotype Screening: Twenty-five bread wheat *T. aestivum* genotypes were obtained from Ayub Agricultural Research Institute, Faisalabad. These genotypes represented diverse genetic backgrounds commonly cultivated in Pakistan, including Galaxy-13, Subhani-21, Arooj-22, Mexi Pak-65, Koshistan-97, Punjab-11, T-9, Ujala-16, C-271, Pothowar-93, C-5, Anaj-11, Inqalab-91, Pasban-90, Dilkash-20, Millat-11, Sehar-06, Lasani-08, Chakwal-06, C-273, AS-2002, Akbar-19, Kohinoor-83, Shafaq-06, and ARRI-11. The screening experiment was laid out in a RCBD with three replications to account for field variability. Each genotype was sown in 1 m-long rows, maintaining 30 cm inter-row spacing and 15 cm intra-row spacing. Each replication consisted of 25 entries, excluding spreader rows. The universally susceptible wheat cultivar 'Morocco' was planted along plot borders and after every tenth test entry to ensure uniform disease pressure, in accordance with standard rust epidemiological practices (Peterson *et al*., 1948).

Culturing of Biocontrol agents in the Lab: Based on differential host responses observed during the resistance screening trial, three representative wheat genotypes (Galaxy-13, Subhani-21, and Arooj-22) were selected for the management study. The fungal antagonist *T. harzianum* was cultured on potato dextrose broth prepared using 200–300 g potato extract and 20 g dextrose per liter of distilled water. The medium was sterilized at 121°C and 15 kPa for 20 min.

Inoculated plates were incubated at 25 ± 2°C for 7-10 days to allow profuse sporulation. Conidia were harvested by flooding plates with sterile distilled water containing 0.01% Tween-20 and gently scraping the colony surface. The suspension was filtered through sterile muslin cloth, and spore concentration was adjusted to 1×10^7 conidia mL^{-1} using a hemocytometer, following standard mycological quantification procedures (Harman *et al.*, 2004). The bacterial antagonist *Bacillus* sp. MN54 was cultured in nutrient broth containing tryptone (10 g L^{-1}), sodium chloride (10 g L^{-1}), and yeast extract (5 g L^{-1}). Cultures were incubated at 28–30°C for 24–48 h on a rotary shaker (150–180 rpm). Bacterial population density was standardized to 1×10^8 CFU mL^{-1} using serial dilution and plate counting techniques (Tripathi *et al.*, 2025).

Seed priming was conducted under aseptic conditions in a laminar airflow cabinet. A total of twelve sterile Petri dishes were prepared and systematically assigned to four treatments, with three independent replicates per treatment. The first three Petri dishes were assigned to treatment T_1, the next three to T_2, the subsequent three to T_3, and the final three to T_4 (control). The structure and composition of the treatments are summarized in Table 1. Seeds of each genotype were immersed in the respective treatment suspensions for 6-12 h to promote uniform microbial adhesion and early rhizosphere establishment. Following priming, seeds were shade-dried under sterile conditions to restore their original moisture content before further use.

Table 1. Description of microbial seed priming treatments

Treatment Code	Treatment Description
T_1	Seed priming with *T. harzianum* (1×10^7 conidia mL^{-1})
T_2	Seed priming with *Bacillus* sp. MN54 (1×10^8 CFU mL^{-1})
T_3	Combined seed priming with *T. harzianum* (1×10^7 conidia mL^{-1}) + *Bacillus* sp. MN54 (1×10^8 CFU mL^{-1})
T_4	Sterile distilled water (control)

Cultivation Layout of Seed-Treated Genotypes: Genotypes of wheat were cultivated in the experimental area of the Department of Plant Pathology, University of Agriculture, Faisalabad (UAF). The experiment was conducted using a RCBD with three replications. Four treatments, as described in Table 1, were evaluated on three wheat genotypes under field conditions. The genotypes were grown at temperatures ranging from 20 to 30°C, with row-to-row and plant-to-plant spacing of 30 and 15 cm, respectively. Each block consisted of twelve rows, with ten plants maintained per row. The rust-susceptible cultivar (Morocco) was planted along the borders of each block and after every fourth row to facilitate uniform disease development and ensure adequate disease pressure throughout the experimental area.

Obtaining Rust Inoculum: In order to serve as the primary inoculum for the spread of wheat rust, Morocco (a rust-susceptible variety) was used. For ensuring uniform distribution of inoculum, Morocco (Spreader) was planted on the boundaries of blocks and interplanted between rows of the experimental plot.

Readings of Morpho-Physiological Characters: Morpho-physiological, yield, and disease parameters were recorded to evaluate plant performance under WLR stress. Plant height (PH, cm) was measured at physiological maturity from the soil surface to the spike tip (excluding awns) using a meter scale. Root length was determined after careful uprooting and washing of roots. Productive tillers per plant were manually counted at maturity. Spike length was measured from spike base to apex, and spike weight was recorded using a digital balance. The number of grains per spike was counted after manual threshing. Thousand-grain weight was determined by weighing 1000 randomly selected grains using an electronic balance. Grain yield per plant was calculated by weighing total grains harvested from representative plants. Fresh shoot weight was recorded immediately after harvest while dry shoot weight was measured after oven-drying samples at 70°C to constant weight.

Determination of Severity: Disease severity was measured by following the Modified Cobb scale as described by Peterson *et al.* (1948). Host response was assessed separately from disease severity and classified as R, MR, MR-MS, MS, and S according to uredinia size, sporulation intensity, and associated chlorotic or necrotic tissue. Intermediate responses were denoted by a hyphen between adjacent categories.

Statistical Analysis: The collected data were subjected to statistical analysis using Statistix 8.1 under a RCBD. Treatment effects were first evaluated through analysis of variance (ANOVA), and differences among treatment means were subsequently separated using the Least Significant Difference (LSD) test. For the microbial experiment, the multivariate dataset comprised 36 experimental units (3 genotypes × 4 microbial treatments × 3 replications). PCA and Pearson correlation were conducted on these 36 observations using the measured growth, yield and WLR severity

variables. Data were mean-centered and scaled to unit variance before PCA. Because the multivariate analyses were intended primarily for exploratory characterization of trait relationships and treatment or genotype patterns rather than formal population-level inference, the resulting PCA structure and correlations were interpreted descriptively. All PCA analyses and visualizations were conducted in RStudio (version 4.4.1), and the final graphs were exported in PNG format for presentation of the results.

RESULTS

Field-Based Assessment of Wheat Genotypes for WLR Response: The evaluation of 25 wheat genotypes for their response to WLR under field conditions revealed considerable variation in disease severity (Table 2). Based on severity values, the genotypes were categorized into four distinct host response classes, namely resistant (R), moderately resistant (MR), moderately resistant to moderately susceptible (MR-MS), and susceptible (S). Arooj-22 exhibited the strongest resistance, recording the lowest disease severity (9.2%) and was classified as resistant (R). Genotypes such as Subhani-21 (15.3%), Millat-11 (14.9%), Dilkash-20 (14.0%), Akbar-19 (18.5%), Ujala-16 (19.0%), and Shafaq-06 (20.3%) also maintained comparatively low severity levels and were grouped under the moderately resistant (MR) category, indicating effective suppression of disease development under field conditions. In contrast to the above low severity group, a larger proportion of genotypes fell within the MR-MS category, including Anaj-11, ARRI-11, C-271, C-273, C-5, Chakwal-06, Kohistan-97, Mexi Pak-65, Punjab-11, Sehar-06, and T-9, with severity values generally ranging between 30-40%. These genotypes displayed intermediate responses, suggesting partial resistance with moderate disease progression.

A significant increase in disease severity was observed among the genotypes classified as MS to S. The highest severity was recorded in Kohinoor-83 (79.3%), followed by Inqalab-91 (73.9%), Lasani-08 (71.5%), Pothowar-93 (70.9%), AS-2002 (67.0%) and Pasban-90 (67.0%).

Table 2: Host response of 25 diverse wheat genotypes to WLR disease of wheat under field conditions

Sr. No.	Variety	Disease Severity (%)	Host Response
1	Akbar-19	18.5^{jk}	MR
2	Anaj-11	34.0^{fgh}	MR-MS
3	Arooj-22	9.2^{m}	R
4	ARRI-11	39.9^{d}	MR-MS
5	AS-2002	67.0^{c}	S
6	C-271	37.2^{def}	MR-MS
7	C-273	35.6^{efg}	MR-MS
8	C-5	30.0^{h}	MR-MS
9	Chakwal-06	35.6^{efg}	MR-MS
10	Dilkash-20	14.0^{l}	MR
11	Galaxy-13	24.8^{i}	MR
12	Inqalab-91	73.9^{b}	S
13	Kohinoor-83	79.3^{a}	S
14	Kohistan-97	39.0^{de}	MR-MS
15	Lasani-08	71.5^{b}	S
16	Mexi Pak-65	34.2^{fg}	MR-MS
17	Millat-11	14.9^{kl}	MR
18	Pasban-90	67.0^{c}	S
19	Pothowar-93	70.9^{bc}	S
20	Punjab-11	32.2^{gh}	MR-MS
21	Sehar-06	35.6^{efg}	MR-MS
22	Shafaq-06	20.3^{j}	MR
23	Subhani-21	15.3^{kl}	MR
24	T-9	37.8^{def}	MR-MS
25	Ujala-16	19.0^{jk}	MR

Whereas R = Resistant, MR = Moderately Resistant, MR-MS = Moderately Resistant-Moderately Susceptible, MS = Moderately Susceptible, S = Susceptible, and S = Susceptible. Disease severity (%) was estimated using the Modified Cobb scale (Peterson *et al*., 1948). Values followed by different letters differ significantly at $P \leq 0.05$.

Multivariate Delineation of Wheat Genotypic Response to WLR: A rigorous multivariate framework was employed to characterize the response of 25 diverse wheat genotypes to WLR under field conditions (Figure 1A-D). The frequency distribution of severity values revealed a distinctly heterogeneous pattern, with clear separation between low- and high-disease classes, indicating significant phenotypic variability within the tested germplasm (Figure 1A). Stratification of genotypes based on host response categories demonstrated a well-defined progression in disease intensity (Fig. 1B). Resistant (R) and moderately resistant (MR) groups consistently exhibited lower severity values, whereas moderately susceptible (MR-MS) and susceptible (S) categories showed progressively higher disease expression. The limited overlap among these groups reflects strong concordance between qualitative host response classification and quantitative disease severity. This non-uniform distribution suggests the presence of discrete resistance levels rather than a continuous gradient. Genotypes such as Kohinoor-83, Inqalab-91, Lasani-08, Pasban-90, and Pothowar-93 were positioned on the positive side of PC1, corresponding to higher disease levels. In contrast, Arooj-22, Subhani-21, Akbar-19, Shafaq-06, and Ujala-16 were located on the negative axis, reflecting comparatively lower disease expression. Hierarchical clustering using Ward's method partitioned the genotypes into three distinct clusters (Figure 1C). The first cluster comprised highly susceptible genotypes characterized by elevated incidence and severity values. The second cluster included moderately susceptible lines with intermediate disease levels. The third cluster consisted primarily of resistant to moderately resistant genotypes with consistently low disease metrics.

Silhouette analysis supported the three-cluster classification (Figure 1D), with most genotypes exhibiting silhouette width values between approximately 0.75 and 0.90. Minimal negative values were observed, confirming strong intra-cluster similarity and clear separation among clusters. This statistically coherent stratification offers a robust foundation for resistance classification and strategic deployment of superior genotypes in breeding programs targeting durable rust resistance.

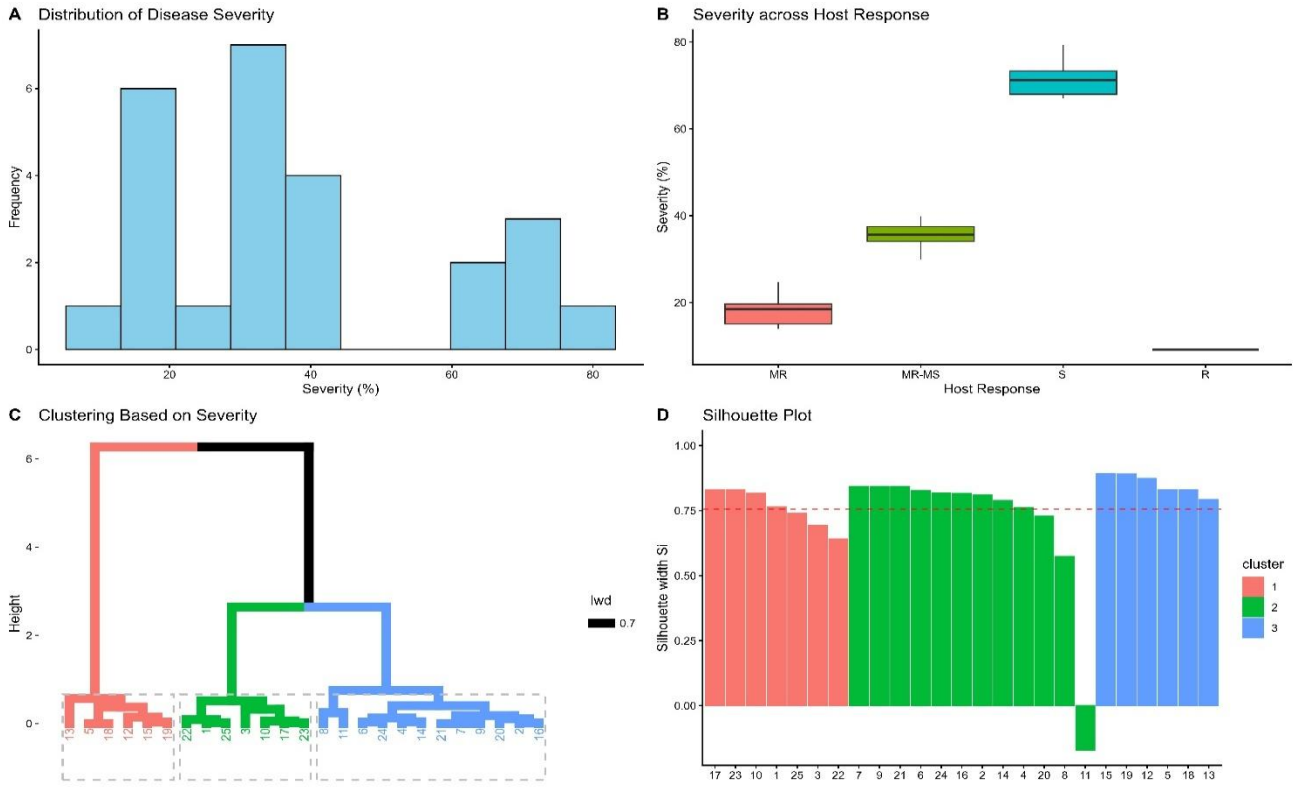

Figure 1. Multivariate classification of 25 wheat genotypes for WLR under field conditions: (A) Distribution of disease severity (%). (B) Boxplot comparing severity across host responses (R, MR, MR-MS, S), showing a clear increase in severity from resistant to susceptible categories. (C) Dendrogram from hierarchical clustering (Ward's method) grouping genotypes into three clusters based on similarity in disease severity. (D) Silhouette plot illustrating the quality of the three-cluster classification.

Bio-Inoculant Effects on Wheat Growth under WLR Stress: The results demonstrated highly significant ($P \leq 0.05$) effects of bio-inoculation on all measured growth traits, as presented in Table 3. The combined application of *T. harzianum* and *Bacillus* sp. MN54 consistently produced superior performance across varieties.

Plant height varied among genotypes and treatments, with values ranging from 79.0 cm in Galaxy-13 under control to 97.7 cm in Subhani-21 under combined inoculation of *T. harzianum* and *Bacillus* sp. MN54, while Arooj-22 gave comparatively higher values (89 cm under control) and reached 94.3 cm under combined treatment (Table 3). Combined inoculation significantly increased plant height by 13.0% in Galaxy-13, 11.0% in Subhani-21, and 6.1% in Arooj-22 compared with control ($P \leq 0.05$). Individual application of *T. harzianum* also improved plant stature, reaching 93.3 cm in Subhani-21 and 93.7 cm in Arooj-22, whereas *Bacillus* sp. MN54 alone produced comparatively moderate increases across varieties. Root length showed significant treatment and varietal differences, ranging from 11.7 cm in Subhani-21 under control to 17.7 cm in Arooj-22 under the combined treatment. Relative to the control, *T. harzianum* alone increased root length by 12.1% in Galaxy-13, 12.8% in Subhani-21, and 4.9% in Arooj-22, while the combined treatment enhanced root length by 15.2%, 17.9%, and 7.9%, respectively ($P \leq 0.05$). Fresh root weight ranged from 2.0 g in Subhani-21 under control to 3.7 g in Arooj-22 under *T. harzianum* + *Bacillus* sp. MN54. The consortium treatment increased fresh biomass by 26.1% in Galaxy-13, 25.0% in Subhani-21, and 12.1% in Arooj-22 compared with untreated plants, as given in Table 3. A similar pattern was observed for dry root weight, which varied between 1.0 g in Subhani-21 under control and 2.6 g in Arooj-22 under combined inoculation. Dual application enhanced dry biomass by 42.9% in Galaxy-13, 50.0% in Subhani-21, and 23.8% in Arooj-22 relative to the control. Tiller number ranged from 3.7 in Galaxy-13 under control to 7.0 in Arooj-22 under both *T. harzianum* alone and the combined treatment. The consortium application increased tiller production by 70.3% in Galaxy-13, 50.0% in Subhani-21, and 40.0% in Arooj-22 compared with control ($p \leq 0.05$). The combined application of *T. harzianum* and *Bacillus* sp. MN54 consistently produced the most pronounced improvements across all measured traits, followed by *T. harzianum* applied alone, then *Bacillus* sp. MN54, while the untreated control recorded the lowest performance throughout. Across varieties, Arooj-22 exhibited superior vegetative performance, followed by Subhani-21 and Galaxy-13.

Table 3. Effect of *T. harzianum*, *Bacillus* sp. MN54, and their combined application on growth attributes of three wheat varieties under field conditions

Variety	Treatment	PH	RL	FRW	DRW	Tillers
Galaxy-13	*T. harzianum*	86.3^{fg}	14.8^{cd}	2.8^{cd}	1.6^{d}	5.3^{abcde}
	Bacillus sp. MN54	84.7^{g}	14^{de}	2.6^{de}	1.4^{de}	4.3^{cde}
	T. harzianum + *Bacillus* sp. MN54	89.3^{de}	15.2^{c}	2.9^{c}	2^{c}	6.3^{ab}
	Control	79^{h}	13.2^{ef}	2.3^{ef}	1.4^{def}	3.7^{e}
Subhani-21	*T. harzianum*	93.3^{bc}	13.2^{ef}	2.3^{ef}	1.4^{de}	5^{bcde}
	Bacillus sp. MN54	93^{bc}	12.5^{fg}	2.1^{fg}	1.2^{ef}	4^{de}
	T. harzianum + *Bacillus* sp. MN54	97.7^{a}	13.8^{e}	2.5^{e}	1.5^{de}	6^{abc}
	Control	88^{ef}	11.7^{g}	2^{g}	1^{f}	4^{de}
Arooj-22	*T. harzianum*	93.7^{bc}	17.2^{ab}	3.5^{ab}	2.5^{ab}	7^{a}
	Bacillus sp. MN54	91.3^{cd}	16.3^{b}	3.2^{b}	2.2^{abc}	5.7^{abcd}
	T. harzianum + *Bacillus* sp. MN54	94.3^{b}	17.7^{a}	3.7^{a}	2.6^{a}	7^{a}
	Control	89^{def}	16.4^{b}	3.3^{b}	2.1^{bc}	5^{bcde}

Data represent plant height (PH), root length (RL), fresh root weight (FRW), dry root weight (DRW), and number of tillers per plant (Tillers). Values are means of replicated observations. Different superscript letters within each column indicate significant differences among treatments at $P \leq 0.05$.

Integrated Effect of Microbial Inoculants on Wheat Productivity under WLR Stress: Yield-related traits and disease severity were significantly influenced by bio-inoculant treatments with clear varietal differences ($P \leq 0.05$) (Table 4). Spike length (SL) ranged from 7.7 cm in Subhani-21 and Arooj-22 under control to 12.1 cm in Galaxy-13 under *T. harzianum* + *Bacillus* sp. MN54. The combined inoculation increased spike length by 26.0% in Galaxy-13, 26.0% in Subhani-21, and 35.1% in Arooj-22 compared with their respective controls. Individual application of *T. harzianum* also improved spike elongation, particularly in Galaxy-13 (10.9 cm), whereas Control plants consistently recorded the shortest spikes. Spike weight (SW) showed a similar positive trend, varying from 1.2 g (Galaxy-13, control) to 2.9 g (Arooj-22, *T. harzianum* + *Bacillus* sp. MN54). The consortium treatment enhanced spike weight by 50.0% in Galaxy-13, 41.2% in Subhani-21, and 61.1% in Arooj-22 relative to the control, indicating substantial improvement in reproductive biomass. The number of grains per spike ranged from 44 (Subhani-21, control) to 59 (Arooj-22, combined treatment). Dual inoculation increased grain number by 13.9% in Galaxy-13, 22.7% in Subhani-21, and 14.1% in Arooj-

22 compared with untreated plants. Likewise, 1000-grain weight was significantly enhanced, reaching a maximum of 48 g in Arooj-22 under combined inoculation, representing increases of 22.2% (Galaxy-13), 28.5% (Subhani-21), and 24.0% (Arooj-22) over the control (Table 4). Grain yield followed the same superior trend under microbial synergy. The highest yield (4.8 g) per plant was recorded in Arooj-22 treated with *T. harzianum* + *Bacillus* sp. MN54, reflecting a 45.5% increase over the control. In Galaxy-13 and Subhani-21, yield improvements of 42.9% and 29.0%, respectively, were observed under combined treatment.

In contrast, disease severity was highest in control plots, reaching 38.7% in Galaxy-13 and 31.7% in Subhani-21, whereas bio-inoculated treatments significantly reduced disease severity. The lowest disease severity (12.0%) was recorded in Arooj-22 treated with *T. harzianum* alone, followed by the combined treatment of *T. harzianum* and *Bacillus* sp. MN54 (13.3%). The results have collectively revealed that *T. harzianum* + *Bacillus* sp. MN54 and *T. harzianum* alone effectively suppressed disease severity while enhancing yield attributes.

Table 4. Effect of *T. harzianum*, *Bacillus* sp. MN54, and their combined application on yield and yield-related traits and WLR severity of three wheat varieties under field conditions

Variety	Treatment	SL	SW	NOGPS	TGW	YPP	WLRS
Galaxy-13	*T. harzianum*	10.9^{b}	1.5^{de}	52^{cd}	38^{e}	3.5^{efg}	17.7^{fg}
	Bacillus sp. MN54	10.3^{c}	1.3^{ef}	49^{f}	35^{f}	3.2^{gh}	25.7^{c}
	T. harzianum+ *Bacillus* sp. MN54	12.1^{a}	1.8^{c}	55^{b}	40.7^{cd}	4^{bc}	23.7^{cd}
	Control	9.6^{de}	1.2^{f}	48.3^{f}	33.3^{f}	2.8^{I}	38.7^{a}
Subhani-21	*T. harzianum*	9.2^{e}	2.2^{b}	49.3^{ef}	41^{c}	3.7^{cde}	17.3^{fg}
	Bacillus sp. MN54	8.5^{f}	1.9^{c}	50.7^{def}	38.3^{de}	3.6^{def}	23.7^{cde}
	T. harzianum+ *Bacillus* sp. MN54	9.7^{d}	2.4^{b}	54^{bc}	43.7^{b}	4^{bc}	18.3^{ef}
	Control	7.7^{g}	1.7^{cd}	44^{g}	34^{f}	3.1^{hI}	31.7^{b}
Arooj-22	*T. harzianum*	9.3^{e}	2.7^{ab}	57.7^{a}	44.3^{b}	4.3^{b}	12^{h}
	Bacillus sp. MN54	8.4^{f}	2.4^{b}	53.3^{bc}	41^{c}	3.9^{cd}	16^{fg}
	T. harzianum+ *Bacillus* sp. MN54	10.4^{c}	2.9^{a}	59^{a}	48^{a}	4.8^{a}	13.3^{gh}
	Control	7.7^{g}	1.8^{d}	51.7^{cde}	38.7^{cde}	3.3^{fgh}	19.3^{def}

Data represent spike length (SL), spike weight (SW), number of grains per spike (NOGPS), thousand-grain weight (TGW), yield per plant (YPP), and wheat leaf rust severity (WLRS). Different superscript letters within each column indicate significant differences among treatments at $P \leq 0.05$.

Multivariate Evaluation of Wheat Performance under WLR Pressure: To elucidate the underlying relationships among growth, yield, and disease-related parameters, PCA was performed (Figure 2). PCA revealed that PC1 explained 72.24% of the total variance, while PC2 accounted for 14.35%, together capturing 86.59% of overall variability (Figure 2A). The correlation circle indicated strong positive associations among plant height, root traits (root length, fresh root weight, dry root weight), number of grains per spike, tillers, yield per plant, and thousand-grain weight, whereas WLR severity was negatively oriented relative to most growth and yield traits (Figure 2B). The PCA distribution of varieties (Figure 2C) showed clear differentiation patterns. Arooj-22 clustered toward higher growth and yield attributes, while Subhani-21 was separated along PC2 with moderate trait expression, and Galaxy-13 occupied an intermediate to comparatively lower performance position. Treatment-wise distribution (Figure 2D) showed that the combined application of *T. harzianum* + *Bacillus* sp. MN54 grouped with superior trait performance, followed by *T. harzianum* whereas the control and *Bacillus* sp. MN54 treatments were associated with comparatively lower growth and yield responses.

Correlation of Growth and Yield Traits with WLR Severity: The Pearson correlation matrices presented in Fig. 3 illustrate the relationships among the traits studied. The strength and direction of associations among these traits are expressed as Pearson correlation coefficients (r values), ranging from −1 to +1, with positive values indicating direct relationships and negative values indicating inverse relationships. Scatterplots displayed below the diagonal represent the distribution and linear trends between trait pairs, while histograms along the diagonal show the frequency distribution of individual traits.

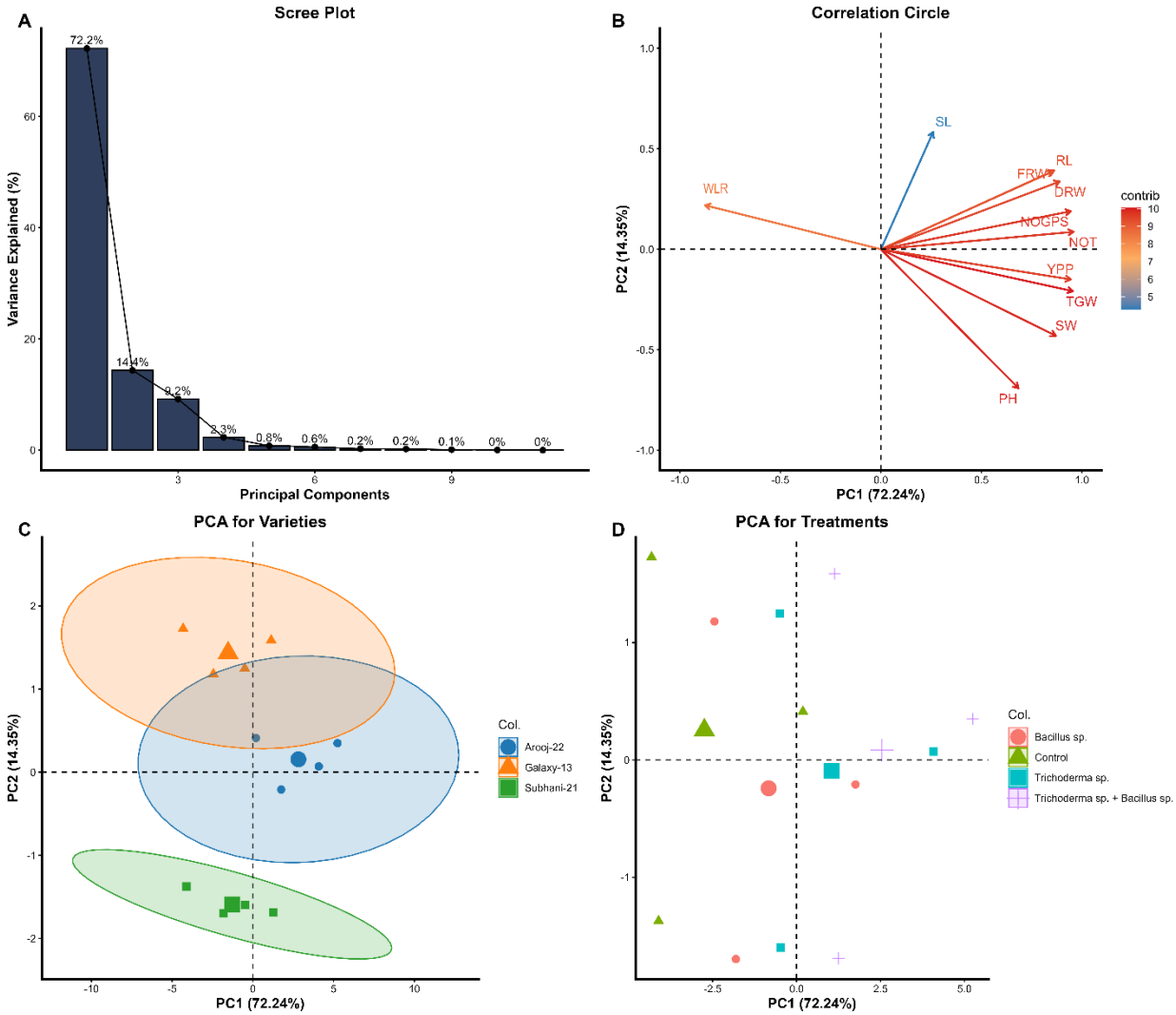

Figure 2. PCA of all measured traits in the studies under different bio-inoculant treatments. (A) scree plot showing variance explained by principal components (PC1 = 72.24%, PC2 = 14.35%); (B) correlation circle illustrating relationships among growth, yield traits, and WLR severity; (C) PCA distribution of wheat varieties showing distinct grouping patterns; and (D) PCA distribution of treatments indicating separation according to performance levels.

The highly positive correlation was observed between yield per plant and 1000-grain weight (r = 0.97, P < 0.001), followed by number of tillers (r = 0.91, P < 0.001), spike weight (r = 0.90, P < 0.001), and number of grains per spike (r = 0.89, P < 0.001). These results indicated a close association between yield per plant and major sink-related traits. Yield per plant also showed significant positive correlations with fresh root weight (r = 0.68, P = 0.015) and dry root weight (r = 0.72, P = 0.008). Strong and significant interrelationships were also observed among yield component traits. FRW and DRW were almost perfectly correlated (r = 0.99, P < 0.001). Similarly, number of tillers exhibited highly significant positive correlations with number of grains per spike (r = 0.92, P < 0.001), spike weight (r = 0.80, P = 0.002), and 1000-grain weight (r = 0.91, P < 0.001). Spike weight was also significantly correlated with 1000-grain weight (r = 0.93, P < 0.001). Plant height showed significant positive correlations with spike weight (r = 0.86, P < 0.001). In contrast, spike length and root length did not show significant correlations with yield per plant. WLR exhibited significant negative correlations with several important growth and yield traits. WLR showed a strong negative correlation with yield per plant (r = −0.80, P = 0.002). Significant negative correlations were also observed between WLR and 1000-grain weight (r = −0.82, P = 0.001), spike weight (r = −0.77, P = 0.003), number of grains per spike (r = −0.71, P = 0.009), and number of tillers (r = −0.77, P = 0.003). These negative associations indicate that increased WLR severity was associated with reductions in biomass accumulation and yield components.

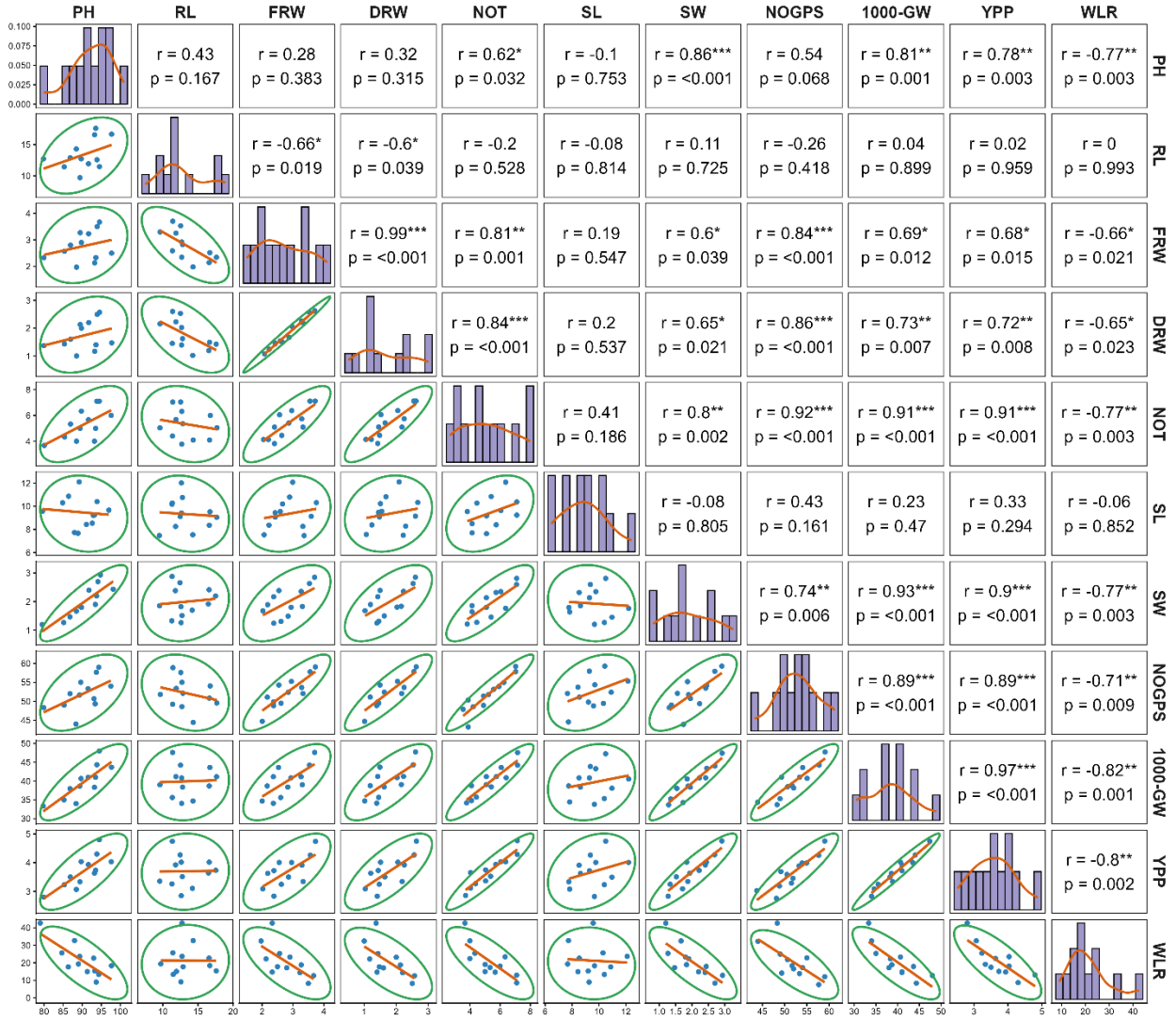

Figure 3. Correlation matrix of measured parameters of wheat samples. Diagonal histograms show trait distributions. The upper triangle presents Pearson correlation coefficients (*r*) with significance levels (P* < 0.05, ***P* < 0.01, ****P* < 0.001). The lower triangle shows scatter plots with trend lines and confidence ellipses. Trait abbreviations: PH (Plant Height), RL (Root Length), FRW (Fresh Root Weight), DRW (Dry Root Weight), 1000-GW (1000-Grain Weight), SW (Spike Weight), NOGPS (No. of Grains per Spike), NOT (No. of Tillers), SL (Spike Length), WLR (Wheat Leaf Rust).**

DISCUSSION

The present study demonstrated substantial genetic variability among the evaluated wheat genotypes in response to WLR under field conditions. The clear stratification of genotypes into resistant, moderately resistant, moderately susceptible, and susceptible categories confirms the availability of exploitable resistance within the tested germplasm. Similar levels of field-based variability have been documented by earlier workers (Jabran *et al.*, 2021; Srinivas *et al.*, 2024), who emphasized that natural screening under epidemic conditions provides a realistic assessment of host-pathogen interactions. The superior performance of Arooj-22, characterized by minimal disease incidence and severity, suggests the presence of effective resistance mechanisms, potentially associated with adult plant resistance genes known for durability and quantitative expression (Kolmer, 2015; Herrera-Foessel *et al.*, 2011).

The exceptionally strong positive association between disease incidence and severity (r = 0.98) reflects the synchronized progression of infection frequency and lesion development. Comparable relationships have been reported in wheat rust epidemiology (Aatif *et al.*, 2024; Riaz *et al.*, 2016), which observed that increases in infection units are

directly translated into enhanced tissue colonization and sporulation intensity. Hierarchical clustering effectively separated genotypes into biologically meaningful groups corresponding to resistance levels. The stability of clusters, supported by silhouette analysis, aligns with findings of Poudel *et al*. (2017) and Mecha *et al*. (2017), who reported that multivariate clustering enhances discrimination among wheat genotypes beyond conventional disease scoring. Such classification approaches are increasingly recommended for resistance breeding programs because they integrate multiple disease parameters simultaneously (Shah *et al*., 2018). Beyond genetic resistance, microbial inoculation significantly improved vegetative growth and grain yield attributes under rust pressure. The combined application of *T. harzianum* and *Bacillus* sp. MN54 consistently enhanced plant height, root biomass, tiller production, and yield components while reducing disease severity. These results corroborate earlier findings that *Trichoderma* species promote root proliferation, nutrient uptake, and induce systemic resistance (Irshad *et al*., 2026). Likewise, *Bacillus* species have been widely reported to control diseases and enhance plant vigor through phytohormone production, phosphate solubilization, and ISR activation (Choudhary and Johri, 2009; Sharf *et al*., 2021). Similar synergistic effects of microbial consortia in suppressing foliar diseases while improving productivity have been documented by Chandra and Bhatt (2022). However, the greater performance observed under combined inoculation reflects an enhanced effect of *T. harzianum* and *Bacillus* sp. MN54, rather than providing statistical evidence of true microbial synergy. Several studies have documented that *T. harzianum* enhances rhizosphere competence through aggressive root colonization, secretion of hydrolytic enzymes, and elicitor-mediated activation of host defenses, whereas *Bacillus* species contribute via phytohormone biosynthesis (indole-3-acetic acid), siderophore production, and antibiosis through lipopeptides (Harman *et al*., 2004; Choudhary and Johri, 2009). Co-inoculation may provide complementary modes of action, whereby the activities of *Trichoderma* and *Bacillus* collectively support root development and plant defense. Physiologically, these microbial interactions may influence defense-associated signaling, including SA and JA mediated pathways, thereby contributing to induced resistance and improved stress tolerance (Sharf *et al*., 2021; Razzaq *et al*., 2024). With this, improved root architecture associated with dual inoculation also helps in increasing nutrient acquisition and assimilation, which could contribute to the observed increases in biomass accumulation and yield under WLR pressure. Therefore, the superior performance of the combined inoculation indicates an enhanced combined effect of *T. harzianum* and *Bacillus* sp. MN54, with the greater response under co-inoculation suggesting complementary contributions of the two microbial agents. However, this response should not be interpreted as statistical synergism in the absence of a significant interaction effect between the individual microbial treatments.

The strong negative correlations between WLR severity and major yield components indicate that increased disease intensity directly compromised assimilate partitioning and grain filling. Comparable negative associations between rust severity and yield have been consistently reported by El-Orabey and Elkot (2020) and Jabran *et al*. (2021), highlighting that reductions in green leaf area due to rust infection limit photosynthetic efficiency and carbohydrate translocation. The PCA integrating growth, yield, and disease parameters further confirmed that agronomic performance and rust resistance were closely interconnected, consistent with observations by Kumar *et al*. (2024) and Yadav *et al*. (2025). In this regard, the findings of the present study emphasize that fungal-bacterial consortia could be ecologically compatible in natural ecosystems. Unlike controlled environments, field systems involve complex interactions among soil microbiota, host plants, and environmental factors. The consistent performance of dual inoculation across genotypes suggests that such consortia can establish stable functional interactions in the rhizosphere, providing a reliable and sustainable approach for integrated disease management. These findings demonstrate that both inherent genetic resistance and microbial inoculation contribute significantly to mitigate the impact of WLR while sustaining productivity. The integration of multivariate statistical tools with field-based disease assessment provides a robust framework for identifying superior genotypes and effective management strategies. Such integrated approaches are increasingly advocated for sustainable wheat production under evolving rust epidemics.

Conclusion: This study revealed significant genetic variability among wheat genotypes for WLR resistance under field conditions, enabling clear identification of resistant and susceptible groups. Arooj-22 exhibited stable resistance with minimal disease severity, while several cultivars showed moderate to high susceptibility. Strong associations between disease incidence and severity, supported by multivariate analysis, confirmed reliable differentiation of resistance. Bio-inoculation significantly improved growth and yield traits under WLR pressure. The combined application of *T. harzianum* and *Bacillus* sp. MN54 consistently enhanced biomass, yield components, and grain yield while reducing disease severity. The negative correlation between WLR severity and yield traits highlighted the direct impact of disease on productivity.

Acknowledgements: The authors are thankful to the Department of Prof. Dr. Muhammad Naveed, Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad for providing bacterial strain for this study. The authors gratefully acknowledge financial support from Higher Education Commission (HEC) of Pakistan through Quality Seed

Project (No. PSDP 1011 and FD-0220) at National Seed Research and Training Center, University of Agriculture, Faisalabad.

REFERENCES

Aatif, H.M., S. Saeed, Y. Ali, S. Iqbal, C.M.S. Hanif, S. Ahmad, A. Raza, R.S. Lucas, H. Ali, A.F.F. Alrefaei and T.M.M. Sheikh (2024). Genotypes, epidemiological variables and fungicide application associated with wheat leaf rust development and grain yield. Heliyon 10: e32564. https://doi.org/10.1016/j.heliyon.2024.e32564

Ali, A., A. Javaid, A. Shoaib and I.H. Khan (2020). Effect of soil amendment with *Chenopodium album* dry biomass and two *Trichoderma* species on growth of chickpea var. Noor 2009 in *Sclerotium rolfsii* contaminated soil. Egypt. J. Biol. Pest Control 30: 102. https://doi.org/10.1186/s41938-020-00305-1

Ali, M.A., A. Abbas, M. Atiq, N.A. Rajput, K. Naveed and N. Javed (2018). Plant diseases of major crops and the way forward for their management. In: Developing Sustainable Agriculture in Pakistan. CRC Press; Boca Raton. https://doi.org/10.1201/9781351208239-20

Aslam, A.H., Z. Ali, K. Saleem, R. Maqbool, A.A. Dababat, F. Özdemir, R. Lahlali, A. Nurbekov, M. El Jarroudi, S. Bhavani and M.A. Ali (2026). Escalating threat of wheat stripe rust under climate change: pathogen evolution, resistance durability, and future management. Plants 15: 1073. https://doi.org/10.3390/plants15071073

Balba, H. (2007). Review of strobilurin fungicide chemicals. J. Environ. Sci. Health B 42: 441–451. https://doi.org/10.1080/03601230701316465

Chandra, D. and P. Bhatt (2022). Unravelling plant-microbe synergy. Academic Press; London. https://shop.elsevier.com/books/unravelling-plant-microbe-synergy/chandra/978-0-323-99896-3

Chiuraise, N. (2022). Pathogen variation and genetic control of *Puccinia triticina* in Zimbabwe. University of the Free State; Bloemfontein. http://hdl.handle.net/11660/12308

Choudhary, D.K. and B.N. Johri (2009). Interactions of *Bacillus* spp. and plants with special reference to induced systemic resistance. Microbiol. Res. 164: 493–513. https://doi.org/10.1016/j.micres.2008.08.007

El-Orabey, W.M. and A.F. Elkot (2020). Prediction of leaf rust severity and yield loss in wheat based on environmental factors. J. Plant Dis. Prot. 127: 507–519. https://doi.org/10.1007/s41348-020-00304-2

Figueroa, M., P.N. Dodds, E.C. Henningsen and J. Sperschneider (2022). Global landscape of rust epidemics by *Puccinia* species: current and future perspectives. In: Plant Relationships: Fungal-Plant Interactions. pp. 391–423. https://doi.org/10.1007/978-3-031-16503-0_17

Harman, G.E., C.R. Howell, A. Viterbo, I. Chet and M. Lorito (2004). *Trichoderma* species—opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. 2: 43–56. https://doi.org/10.1038/nrmicro797

Herrera-Foessel, S.A., E.S. Lagudah, J. Huerta-Espino, M.J. Hayden, H.S. Bariana, D. Singh and R.P. Singh (2011). New slow-rusting leaf rust and stripe rust resistance genes Lr67 and Yr46 in wheat are pleiotropic or closely linked. Theor. Appl. Genet. 122: 239–249. https://doi.org/10.1007/s00122-010-1439-x

Hussien, A.M., M. Moussa, M.A. El-Maghraby, A.M. Elframawy and H.M. Shakam (2024). Molecular marker detection of the leaf rust resistance genes Lr34, Lr74, Lr75 and Lr80 and their importance for partial resistance in bread wheat genotypes. Egypt. J. Bot. 64: 197–216. https://doi.org/10.21608/ejbo.2024.266747.2687

Irshad, R., A.H. Aslam, M. Tariq, I. Shafique, M. Usman, M. Naveed and A. Abbas (2026). Synergetic effect of seed-primed biocontrol agents and biochar soil amendment on wheat productivity and leaf rust suppression. Integr. Plant Biotechnol. 4: 73–83. https://doi.org/10.55627/pbiotech.004.01.1779

Islam, T., Danishuddin, N.T. Tamanna, M.N. Matin, H.R. Barai and M.A. Haque (2024). Resistance mechanisms of plant pathogenic fungi to fungicide, environmental impacts of fungicides and sustainable solutions. Plants 13: 2737. https://doi.org/10.3390/plants13192737

Jabran, M., U. Arshad, H.M.U. Aslam, A. Abbas, A. Haseeb, A. Hussain, S. Hussain, W. Sabir, A. Jabbar and M.A. Ali (2021). Multivariate analysis of morpho-physiological and grain yield traits in advance lines of bread wheat under different leaf rust disease regimes. Pak. J. Agric. Sci. 58. https://doi.org/10.21162/PAKJAS/21.1445

Kolmer, J. (2015). First report of a wheat leaf rust (*Puccinia triticina*) phenotype with high virulence to durum wheat in the Great Plains region of the United States. Plant Dis. 99: 156. https://doi.org/10.1094/PDIS-06-14-0667-PDN

Kumar, A., V.K. Yadav, C. Maurya, S.V. Singh, J. Kumar and B.S.D. Naik (2024). Estimate the genetic diversity with PCA in bread wheat (*Triticum aestivum* L.). J. Sci. Res. Rep. 30: 207–217. https://doi.org/10.9734/jsrr/2024/v30i51935

Mecha, B., S. Alamerew, A. Assefa, E. Assefa and D. Dutamo (2017). Genetic diversity based on multivariate analysis for yield and its contributing characters in bread wheat (*Triticum aestivum* L.) genotypes. Agri. Res. Tech. 8: 555748. https://doi.org/10.19080/ARTOAJ.2017.08.555748

Mottaleb, K.A., G. Kruseman, A. Frija, K. Sonder and S. Lopez-Ridaura (2023). Projecting wheat demand in China and India for 2030 and 2050: implications for food security. Front. Nutr. 9:1077443. https://doi.org/10.3389/fnut.2022.1077443

Peterson, R.F., A. Campbell and A. Hannah (1948). A diagrammatic scale for estimating rust intensity on leaves and stems of cereals. Can. J. Res. 26: 496–500. https://doi.org/10.1139/cjr48c-033

Poudel, A., D.B. Thapa and M. Sapkota (2017). Assessment of genetic diversity of bread wheat (*Triticum aestivum* L.) genotypes through cluster and principal component analysis. Int. J. Exp. Res. Rev. 11: 1–9. DOI:10.13140/RG.2.2.17911.11681

Razzaq, M.U., I. Shafique, E. Naz, S. Jamil, M. Anas, A. Javed, U. Murtaza and A. Abbas (2024). Seed priming with bacterial and fungal biocontrol agents alter physio-chemical parameters to suppress leaf rust disease in bread wheat. Integr. Plant Biotechnol. 2: 51–62. https://doi.org/10.55627/pbiotech.002.01.01110

Riaz, A., S. Periyannan, E. Aitken and L. Hickey (2016). A rapid phenotyping method for adult plant resistance to leaf rust in wheat. Plant Methods 12: 17. https://doi.org/10.1186/s13007-016-0117-7

Shah, I.A., I. Khan, S.A. Mir, M. Pukhta, Z.A. Dar and A. Lone (2018). Genetic diversity by multivariate analysis using R software. Int. J. Pure Appl. Biosci. 6: 181–190. https://doi.org/10.18782/2320-7051.6596

Shahzad, A., A. Hamid, A. Hussain, M. Rashid, M. Khan and Z. Hussain (2022). Current situation and future prospects of wheat production in Pakistan. J. Life Soc. Sci. 2022: 5. https://doi.org/10.64013/bbasrjlifess.v2022i1.5

Sharf, W., A. Javaid, A. Shoaib and I.H. Khan (2021). Induction of resistance in chili against *Sclerotium rolfsii* by plant growth promoting rhizobacteria and *Anagallis arvensis*. Egypt. J. Biol. Pest Control 31: 16. https://doi.org/10.1186/s41938-021-00364-y

Sharma, P., P. Jambhulkar, M. Raja, S. Sain and S. Javeria (2020). *Trichoderma* spp. in consortium and their rhizospheric interactions. In: *Trichoderma*: Host Pathogen Interactions and Applications. pp. 267–292. https://doi.org/10.1007/978-981-15-3321-1

Singh, A., R. Knox, R. Depauw, A. Singh, R. Cuthbert, H. Campbell, S. Shorter and S. Bhavani (2014). Stripe rust and leaf rust resistance QTL mapping, epistatic interactions and co-localization with stem rust resistance loci in spring wheat evaluated over three continents. Theor. Appl. Genet. 127: 2465–2477. https://doi.org/10.1007/s00122-014-2390-z

Srinivas, K., V.K. Singh, B. Srinivas, K.K. Sameriya, L. Prasad and G. Singh (2024). Determining the impact of stripe rust and leaf rust on grain yield and yield components losses in Indian wheat cultivars. Cereal Res. Commun. 52: 733–746. https://doi.org/10.1007/s42976-023-00435-w

Tripathi, K., Y. Srivastava and N. Kumar (2025). Enumeration of soil microbiota: calculation of colony forming units for microbial quantification. In: Biotechnology Lab Techniques: Culture Media, Microscopy and Microbial Analysis. pp. 89–91. https://doi.org/10.70593/978-93-49307-52-0_16

Yadav, J.K., S. Sinha, H. Shukla, A. Singh, T.K. Sahu, S.K. Jha, J. Kumari, M. Verma, S. Kumar and R. Singh (2025). Genetic dissection of leaf rust resistance in a diversity panel of tetraploid wheat (*Triticum turgidum*). BMC Plant Biol. 25: 406. https://doi.org/10.1186/s12870-025-06330-2

Zohaib, K.A., U. Bashir, I.H. Khan, A. Javaid and W. Anwar (2024). Synergistic effect of plant growth promoting rhizobacteria and *Cirsium arvense* against black scurf disease of potato. Sains Malays. 53: 3071–3083. https://doi.org/10.17576/jsm-2024-5309-13