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EXPLORING THE BIOCONTROL POTENTIAL OF HALOTOLERANT RHIZOBACTERIA AGAINST *FUSARIUM* WILT IN RICE

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ABSTRACT

Fusarium wilt caused by soil fungus like *Fusarium oxysporum* poses a significant threat to rice production around the world, including Pakistan and The Gambia. Conventional methods for managing such diseases are not sustainable. Therefore, the present study was carried out to evaluate the biocontrol potential of halotolerant plant growth-promoting rhizobacteria (PGPRs) against *Fusarium oxysporum* PF5. Out of 510 rhizobacteria isolates, 24 halotolerant isolates (11 from Pakistan and 13 from The Gambia) were selected to evaluate their potential against the growth of selected fungus. Dual cultures *in vitro* showed that isolate 6 and 11 from Pakistan and isolate B and G from The Gambia, inhibited the growth of fungi by 70%. These isolates also showed the production of hydrogen cyanide (HCN) (52 µg/mL) and glucanase (4 µg/mL). In addition, intense production of hydrolytic enzymes protease (70 µg/mL) and chitinase (4 µg/mL) was recorded by isolates 4 and 9 respectively. Scanning electron microscopy graphs evidenced a significant ultrastructural damaged and a severe hyphal breakdown by the bacterial isolates (6, 11 and B, G) in *Fusarium* co-cultures as compared to the control fungal culture. The GC-MS metabolomics highlighted the presence of key bioactive compounds such as oleic acid, imidazol [1,2-a] pyridine. Therefore, it can be concluded that the selected isolates can be considered as a promising candidate as a biocontrol agent against *Fusarium* wilt in rice on the basis of strong antifungal activity, hydrolytic enzymes and bioactive metabolites production as well as structural damage to *Fusarium* hyphae. The future work will focus on controlled pot experiment and then field application for practical implication of the selected isolates.

Keywords: *Oryza sativa*, *Fusarium* wilt, Antagonistic activity, Secondary metabolites, Chitinase

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INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food crop supplying more than 20% of global calorie consumption and up to 80% of dietary needs in many parts of Asia (Asati and Yadav, 2023; Mohapatra *et al*., 2025). Despite its significance, its production remained threaten due to biotic and abiotic stresses. Among biotic stresses, *Fusarium oxysporum*, a soil borne fungus is one of the most destructive phytopathogens. It can live in the soil for an extended period (5-10 years) due to formation of thick-walled chlamydospores and causes vascular wilt and root rot diseases in more than 100 crops, including rice (Stoddard *et al*., 2010). In severely impacted areas, this pathogen causes yield losses of 30 to 70% because it thrives in saline, wet and nutrient-deficient soils (Mustapha *et al*., 2022). This fungus has detrimental effects on both the quality and quantity of the crops and are difficult to control due to their aggressive pathogenic behavior. *Fusarium* infects the plant tissues by producing mycotoxins making the grains of the plant unsafe for human and animal usage (Agha *et al.,* 2023). Similarly, Kamble *et al.* (2024) reported that *F. oxysporum* triggers the secretion of protein effectors and toxins in plant tissue leading to the vascular dysfunction, hampering the water and nutrients transport to the plant and eventually causing plant death. Husna *et al.* (2025) reported that seed borne pathogen of *Fusarium* specie resulted in chlorosis and leaf elongation of rice plant leading to Bakanae Disease.

Salt stress is another constraint that severely affect rice growth and productivity by inducing osmotic stress in the plant through metabolic disruption and overproduction of reactive oxygen species (Wang *et al.,* 2025). Increased salt concentration weakens the plant and

makes it more susceptible for the pathogen attack and resulted in increased disease severity. Salinity stress modified the biochemical and enzymatic defense system by interrupting the signaling pathway leading to oxidative damage that increased the risk of disease incidence in plant. *Fusarium* infestations and yield reductions highlight the need for sustainable mitigation strategies in Pakistan (PAK) and The Gambia (GMB). Traditional methods of treating fungal disease like fungicides are effective but only provide short-term respite and worsen the environment (Mishra *et al*., 2016). Due to environmental concerns, these traditional methods need to be replaced with sustainable environmental friendly measures. Halotolerant plant growth-promoting rhizobacteria (PGPR) have become a viable eco-friendly substitute due to their capacity to increase plant resilience and suppress the pathogens (Chandran *et al*., 2025). These halotolerant are already adopted to withstand with stress conditions, therefore, they can tolerate the biotic stress in a way that is more efficient and can perform dual role (Hidri *et al.,* 2022). These bacteria use a variety of mechanism to induce systemic resistance in the plants against the disease. These mechanisms include but not limited to the production of antibiotics such as pyolutrin, 2,4-diacetylphloroglucinol, siderophores, ammonia and hydrogen cyanide (Kenawy *et al.* (2019). Release of enzymes such as chitinases, glucanases and secondary metabolites containing bioactive compounds make them potential biocontrol agent (Hong *et al.,* 2016). Similarly, mechanism of plant growth and development by the PGPR include nitrogen fixation, phytohormone production and nutrient solubilization (Liu *et al.,* 2025).

To best of our knowledge, studies are reported on the effect of PGPR on rice under single stress, however, literature is scanty on the effect of PGPR isolated from different agro-climatic regions of rice under combined stresses such as salinity and *F. oxysporum*. Moreover, no literature is found about the role of bacterial isolates from The Gambia in *Fusarium* and salt stress mitigation. Therefore, this study was aimed to isolate and characterize salt-tolerant bacteria from different agroecological regions of Pakistan and The Gambia to evaluate their potential against both stresses in rice.

MATERIALS AND METHODS

The rhizosphere soil samples were collected from salt-affected rice fields in Pakistan and The Gambia to isolate salt-tolerant bacteria in three replicates from each site. The sampling areas in Pakistan were Ali Pur (29° 23' 4.70" N, 70° 54' 41.65" E), Murad Pur Janubi (29°22'30.4"N 70°40'52.3"E), Gujranwala (32° 9' 58.8636" N, 74° 11' 45.2400" E), Dera Ismail Khan (31° 49' 53" N, 70° 54' 07" E), Mardan (34°20'8N 71°54'10E), Waraich (31° 22′ 16″N, 74° 18′ 45"E), Qambar (27°35'09.3"N 68°00'21.7"E), Larkana (27° 22' 29" N, 68° 05' 50" E), Waggan (27°29′40″N, 67°57′49″E), Padidan (26°46'29.1"N, 68°18'7.09"E), Tando Muhamad Khan (25° 00' 60.00" N, 68° 28' 59.99" E), Matli (25.0428° N, 68.6588° E), Gulachi (27° 42′ 55″N, 68° 10′ 57″E) and Khorwah (24° 45' 11.74" N, 68° 24' 54.58" E), and in The Gambia were Kotu (13º27´37″N, 16º42´22″W), Sifoe (13º11´15″N, 16º41´40″W), Mandinari (13º22´16″N, 16º36´05″W), Pirang (13º16´16″N, 16º32´07″W), Faraba (13º15´35″N, 16º31´09″W), Salikenni (13º29´12″N, 15º58´03″W), Kerewan (13º29´36″N, 16º05´26″W), Saba (13º31´11″N, 16º03´00″W), Darsilameh (13º31´55″N, 16º08´13″W), Tambana (13º30´29″N, 16º10´31″W), Banni (13º31´20″N, 16º00´21″W), Jahaur Mandinka (13º42´53″N, 15º16´39″W), Memmeh (13.5209° N, 16.2294° W). The phytopathogen, *F. oxysporum* strain PF5 (Accession number MF522223.1), was obtained from the stock of the Applied Microbiology and Biotechnology Laboratory (AMBL) of the department of Biosciences, COMSATS University Islamabad, Pakistan.

Sampled soil physical and chemical properties: The soil sample type in Pakistan was alkaline-sodic, with a pH range of 8.0 to 10.0 and a high clay content. On the other hand, The Gambia's soil was mainly saline-sodic sandy-loam with a slightly lower pH (7.5-8). The level of soil in Pakistan is described as chronic with an ECe greater than 8 dS m^{-1}. At the same time, The Gambia is exposed to fluctuating soil salinity due to tidal cycles from the Atlantic Ocean and the River Gambia.

Soil sampling and bacteria isolation: Rhizosphere soil samples were collected using the randomized composite soil sampling technique, following the methodologies described by Lauber *et al.* (2008). The samples were kept in sterile plastic zipper bags and placed in ice cooler and stored at 4 °C for further processing. Rhizobacteria were isolated using serial dilution and plating techniques following the methods established by Hussain *et al*. (2022). Briefly, a ten-fold serial dilution was conducted, followed by plating of 100 µL of each soil solution on nutrient agar (NA) containing 3% sodium chloride (NaCl). Isolated colonies with clear morphological traits were purified on NA and were stored individually in slants at -20 °C.

The pure colonies were screened for their maximum salt-tolerant capacity on different NaCl concentrations (5%, 10%, 15%, and 20%) to evaluate halotolerance ability. The 24 bacterial isolates out of 510 isolates screened for salt tolerance, growth rate, and colony vigor were evaluated for biocontrol trait assays. The isolates were assessed for phytopathogen control potential including hydrogen cyanide (HCN), glucanase, protease and chitinase production potential. The selected isolates were cultured in nutrient broth and incubated at 28 ± 2 °C for subsequent characterization. All the

experiments related to the current study were performed in completely randomized design (CRD) with three replicates.

Antagonistic activity against *F. oxysporum* PF5: The antagonistic activity of the selected isolates against the fungal phytopathogen *F. oxysporum* PF5 (MF522223.1) were determined using the dual culture (point inoculation) method, as described by Ali *et al.* (2020) with modifications. A 5-mm fungal plug disc was placed on a potato dextrose agar (PDA) plate and 3 µL of bacterial broth culture was point inoculated 3 cm away from the fungal disc plug at the center of a petri plate. The control plate contained only the fungal pathogen. The plates were incubated for ten days at 27 ± 2 ℃, and fungal radial growth was measured using a calibrated ruler from day 3 to day 10. The percentage inhibition (%) of each isolate was determined using the relationship given by Abdelmoteleb *et al*. (2017):

$$\mathbf{I\%} = \frac{\mathbf{R} - \mathbf{r}}{\mathbf{R}} \times \mathbf{100}$$

Where R: fungal control colony diameter
r: fungal treatment/dual culture colony diameter
The experiments for all the isolates were performed in triplicate (n = 3) under CRD for statistical reliability.

Biochemical assays for antifungal traits: The selected isolates were screened for their potential to produce key antifungal hydrolytic enzymes such as glucanase, protease and chitinase including HCN according to reported standard protocols. Each experiment was performed in CRD with three replicates.

The isolates ability to produce HCN was assayed using the modified picric acid-based method (Raj *et al.,* 2024). For the assay, bacterial cultures were spread on nutrient agar. Three impregnated filter strips were affixed to the petri plate lid and the inoculated agar was inverted over them, sealed with parafilm and incubated at 37 °C for 72 h. The HCN production was indicated by a colour shift from yellow to orange-brown/red, reddish-brown, or brown, corresponding to increasing HCN levels. No color change suggests a negative results. For quantitative analysis, filter strips were eluted in 5 mL of 50% ethanol for 30 min, vortexed and absorbance was measured at 510 nm. A standard curve was generated using serially diluted KCN (10 mg/mL). An uninoculated medium + catalase served as a negative control. The HCN concentrations were determined by extrapolating from the standard curve.

The Glucanase activity of bacterial isolates was evaluated using a modified 3,5-dinitrosalicylic acid (DNSA) reducing sugar assay (Jain et al., 2020). For enzyme production, 100 µL of 24 hr bacterial culture was inoculated into 5 mL of mineral salt medium (MSM) and incubated at 37 °C with shaking (125 *rpm*) for 72 hr. The bacterial culture was centrifuged at 10,000 × g, the cell-free supernatant was collected for glucanase enzyme assays. Glucanase activity was determined by mixing 750 µL of supernatant with an equal volume of DNSA reagent followed by heating at 90-100 °C for 5 min. The absorbance of the resulting solution was measured at 540 nm. A standard curve was prepared using serial dilutions of D-glucose (10 mg/mL) for quantification. Enzyme activity was expressed as µg of reducing sugars released per mL per minute (µg/mL/min) under the assay conditions. Uninoculated medium treated with pectinase served as the negative control.

Protease production was quantified according to the modified caseinolytic method described by Tsuchida *et al*. (1986). Bacterial cultures grown on glucose-yeast-peptone (GYP) medium was centrifuged at 12,000 × g to obtain cell-free supernatants. Protease activity was determined using a 1375 µL reaction mixture consisted of 250 µL supernatant and 250 µL 2% (w/v) casein solution in 50 mM phosphate buffer. The reaction mixture was incubated at 37 °C for 30 minutes and terminated by adding 250 µL of 0.4 M trichloroacetic acid (TCA). After centrifugation, 250 µL of supernatant was mixed with 312.5 µL 0.4 M Na_2CO_3 and 62.5 µL diluted Folin-Ciocalteu's reagent. Samples were incubated for 30 min in the dark to allow color to develop and absorbance was measured at 660 nm. Uninoculated medium treated identically served as the negative control. A standard curve was generated using L-tyrosine (0-100 µg/mL). One unit (U) of protease activity was defined as the amount of enzyme releasing 1 µg tyrosine equivalents per minute under assay conditions.

The chitinolytic activity was determined using the 3,5-dinitrosalicylic acid (DNSA) method using colloid chitin as a substrate according to the method described by Sadfi *et al.* (2001). Bacterial isolates were grown in an autoclaved colloidal chitin medium for 24-hour. A 100 µL inoculum from a bacterial culture was added to 5 mL of medium and incubated at 37 °C for 96 hours with shaking at 125 *rpm*. After incubation, the culture was centrifuged at 10,000 *rpm* for 5 minutes to obtain a cell-free supernatant containing chitinase. The reaction mixture was incubated at 50 ℃ for 30 min and the reaction was quenched by adding 750 µL DNSA reagent in warm distilled water.

For the assay, 750 µL of culture supernatant was mixed with 750 µL DNSA reagent, heated in a water bath at 90–100 °C for 5 minutes and the resulting colour change (yellow to orange-red) was measured spectrophotometrically at 540 nm. A standard curve was generated using serial dilutions of D-glucose (0–10 mg/mL) and enzyme activity was calculated based on reducing sugar release. An uninoculated medium treated with chitinase served as a negative control.

The scanning electron microscope (SEM) was used to assess the antagonistic effects of the isolates on the *Fusarium oxysporum* PF5 following the method described by Oberai and Khanna (2019). The *Fusarium*

oxysporum and the selected bacterial strains (6, 11, B, and G) were co-inoculated and cultured in 45 mL of autoclaved potato dextrose broth (PDB) medium for 7 days for scanning electron microscopy analysis. The culture was centrifuged at 6000 *rpm* in 15 minutes. The residue (*Fusarium* mycelia-bacterial pellet cake) of the co-culture was air-dried at ambient temperature in a laminar flow hood for 7 days. The dried residue cake was collected for SEM analysis.

A piece of *Fusarium* mycelia-bacterial pellet cake, about 5 mm in size was placed onto the 5 mm square piece of glass slide and flushed with a 0.1 M phosphate buffer (pH 7.0). Then it was fixed in 3% glutaraldehyde at 4 °C for ten minutes, submerged in 0.1 M phosphate buffer (pH 7.0) for thirty minutes and in one to two drops of 2% $KMnO_4$ solution for ten minutes. Then washed with 50% and 100% absolute ethanol and dried in laminar flow hood until completely dried. The samples were prepared for SEM examination by mounting them onto 5 mm² glass slide pieces. The samples were operated at an accelerating voltage of 5 kV.

The crude antibiotics and antifungal compounds from the four selected rhizohalophiles isolates from both region were extracted using the liquid-liquid extraction (LLE) modified method outlined by Kamal *et al*. (2022) for gas chromatography mass spectroscopy (GC-MS) analysis. A loopful of 24-hour-old bacterial isolates were inoculated into 45 mL of sterilized nutrient broth medium and incubated at 120 *rpm* on a rotary shaker for 96 hours at room temperature (Kaaria *et al*., 2012). Following incubation, the culture broth was centrifuged for 10 minutes. The 20 mL of cell-free supernatant was collected into two new falcons and mixed with 20 mL of chloroform, (for non-polar metabolites) and ethanol, (for polar metabolites) separately in a ratio of 1:1. The chloroform-added supernatant formed two layers; using a pipette, 2 mL of the solvent layer pipetted into a labeled 2 mL Eppendorf. The ethanol-added culture supernatant was used as an aqueous extract. The both extracts were filtered using nylon syringe filters into new Eppendorf tubes and were immediately stored at -20 °C for GC-MS analysis.

The mass spectrum interpretation of GC-MS was conducted using the National Institute of Standards and Technology (NIST) database, which contains over 62,235 spectral patterns. The mass spectra of the unidentified compounds were compared with those of the known compounds archived in the NIST library. The identity, molecular weight and structure of the test material components were verified and recorded.

Statistical analysis: All the experiment related to the current study were performed in a completely randomized design with three replications of each treatment. Data collected from the experiment were analyzed using one-way Analysis of Variance (ANOVA) with Statistix version 8.1 to evaluate the biocontrol potential of rhizobacterial isolates. Comparison of means were made using Least Significant Difference (LSD) at a significance level of $p \leq 0.05$ (Steel *et al.,* 1997).

RESULTS

Antagonistic activity against *F. oxysporum* PF5: The results in Fig. 1 indicated that isolate 6 from Pakistan showed the highest inhibition by day 10 (D10) with approximately 70% significant inhibition of *Fusarium* mycelial growth followed by isolate 11 with 69% significant inhibition as compared to the control at $p \leq 0.05$. Isolates 9 and 10 showed moderate significant inhibition of 60% and 55% respectively (Fig. 1C) compared to the control. On the other hand, isolate B and G from The Gambia showed the significant percentage inhibition of about 70%, while isolates D followed closely with a percentage inhibition range of 65% respectively at $p \leq 0.0001$ (Fig. 1C). Isolate M and D showed the least percentage inhibition of the phytopathogen compared to the control. Overall, isolates 6 and 11 from Pakistan, and isolate B from The Gambia showed the highest percentage inhibition of *F. oxysporum* PF5.

Hydrogen cyanide (HCN) and Glucanase production: A number of the rhizosphere isolates from Pakistan and The Gambia were screened for producing hydrogen cyanide (HCN) and glucanase (Fig. 2A). Results indicated that the Pakistani isolates showed more HCN production compared to their Gambian counterparts. The isolates 8 showed maximum significant production of HCN as compared to other isolates producing approximately 51 µg/mL followed by isolate 5 with 50 µg/mL non-significant increase as compared to isolates 8. Similarly, isolate 3 also showed good production of HCN (47 µg/mL at $p \leq 0.0001$. On the other hand, isolate L from The Gambia showed maximum significant (27 µg/mL) production of HCN followed by M and E (20 µg/mL) that are non-significant to each other.

In case of glucanase, isolates from the Gambia were better glucanase producers compared to the Pakistani isolates (Fig. 2B). The highest glucanase producer among the Gambia isolates was M (4 µg/mL) that showed significant increase over other isolates at $p \leq 0.0001$ followed by J and K ranging from 2 to 3 µg/mL glucanase production. Pakistani isolates 9 showed the highest amount of glucanase (4 µg/mL) production followed by 5 and 7 isolates with non-significant difference among each other. The rest of the isolates from both regions were poor producers of HCN and glucanase.

Production of Protease and Chitinase: In this study, all the selected isolates demonstrated a strong ability to produce both hydrolytic enzymes, however, isolates from Pakistan showed the highest potential for producing both

enzymes as compared to the isolates from The Gambia. The best protease producer among the Pakistan isolates were 3 and 4 with significant amount of 69-70 µg/mL respectively followed by isolate 6 and 11 in the range of 61-65 µg/mL at $p \leq 0.0001$ (Fig. 2C). On the other hand, the best protease producers among The Gambia isolates were F and H with non-significant increase of 67 and 65 µg/mL respectively followed by C and M isolates (60 and 59 µg/mL) respectively.

The results for the chitinase showed that the isolates of both regions showed good production of these enzymes however, maximum significant production was recorded by isolate 9 of Pakistan in the range of 4 µg/mL as compared to all other isolates $p \leq 0.05$ (Fig. 2D). Isolate 7 and 5 also showed good production of chitinase in the range of 3.3-3.7 µg/mL respectively. Similarly, the highest producer of chitinase among the Gambia isolates was M (4 µg/mL) that showed significant increase over other isolates followed by J and K in the range of 2-3 µg/mL at $p \leq 0.0001$.

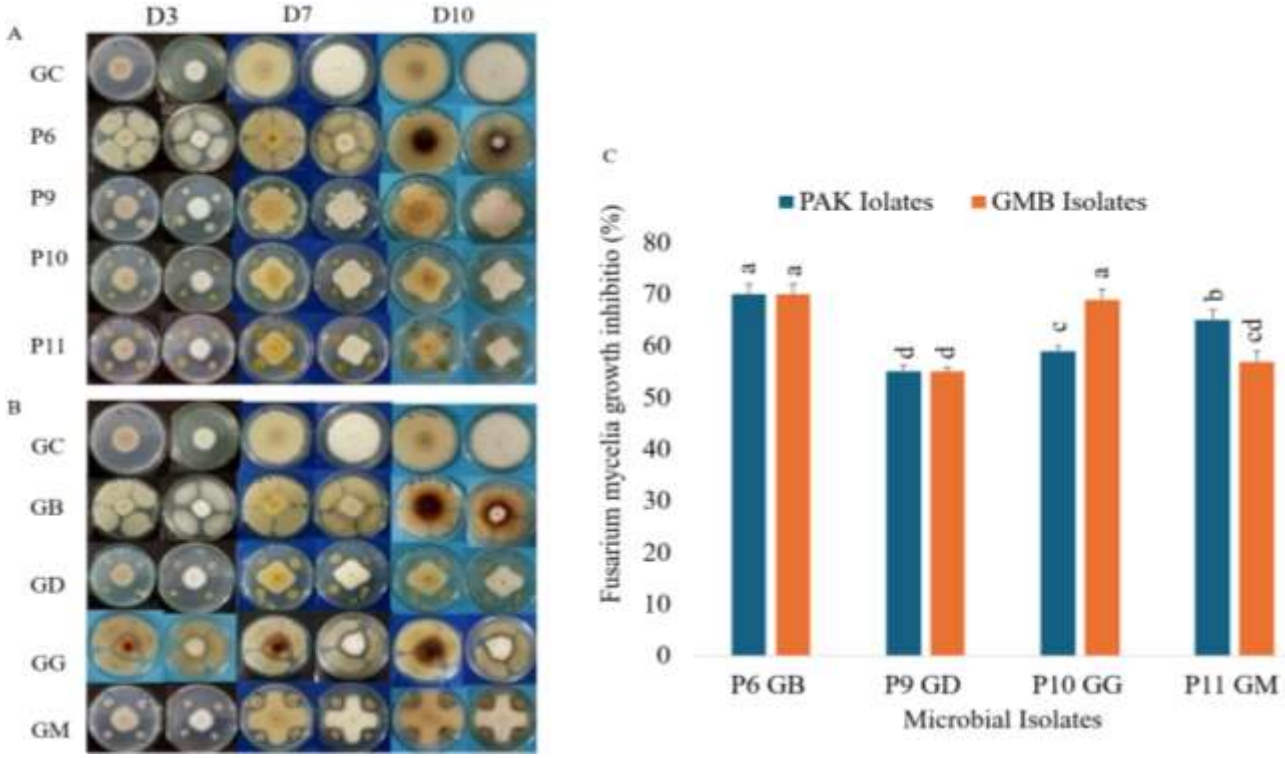

Fig. 1: In vitro antagonism assay images (A and B) showed visual representation and graphs (C) showed percentage inhibition of *Fusarium* by microbial isolates from Pakistan (6, 9, 10, 11) and The Gambia (B, D, G, M) compared to the control. Isolates labelled in Arabic numerals depicts Pakistan isolates, while the Isolates from The Gambia are shown in alphabet letters. The shared LSD letter(s) above each error bar indicates non-significant difference, otherwise, significant difference among the isolates.
Symbols used, D3: Days 3, D7: Days 7, D10: Days 10.

Scanning Electron Microscope (SEM) of bacteria against *F. oxysporum:* The results of SEM micrographs revealed that scaffold architecture of fungal hyphae remained well-organized, intact and smooth with a continuous hyphal network in the control groups (Fig 3A and B). Moreover, control group showed clear and visible pores and unaltered mycelial surfaces with no surface disruption or presence of external deposits were observed. On the other hand, the *Fusarium* mycelia co-cultured with each bacterial isolate, were ruptured, degraded and lost their integrity and morphology. The bacterial isolates brought significant morphological changes in the fungal hyphae. The treated fungal hyphae showed extensive distortion, surface roughening and collapse of the mycelial network. Large clusters of granular or aggregated material were observed coating the fungal surface specifically by isolate 6, 11 and B. These deposits masked the natural hyphal architecture and indicating strong physical interaction between fungal mycelia and the bacterial isolates specifically 6 and 11. The mycelial structure appeared fragmented or degraded at several regions by 6 and 11 isolates more prominently. The isolates 6, 11 and B significantly altered the pore size and structure of the hyphal network. These results

demonstrate the efficacy of the four selected bacterial strains, thereby justifying their selection for further investigation as potential biocontrol agents against the phytopathogen *Fusarium oxysporum*, the causative agent of rice wilt and root rot diseases.

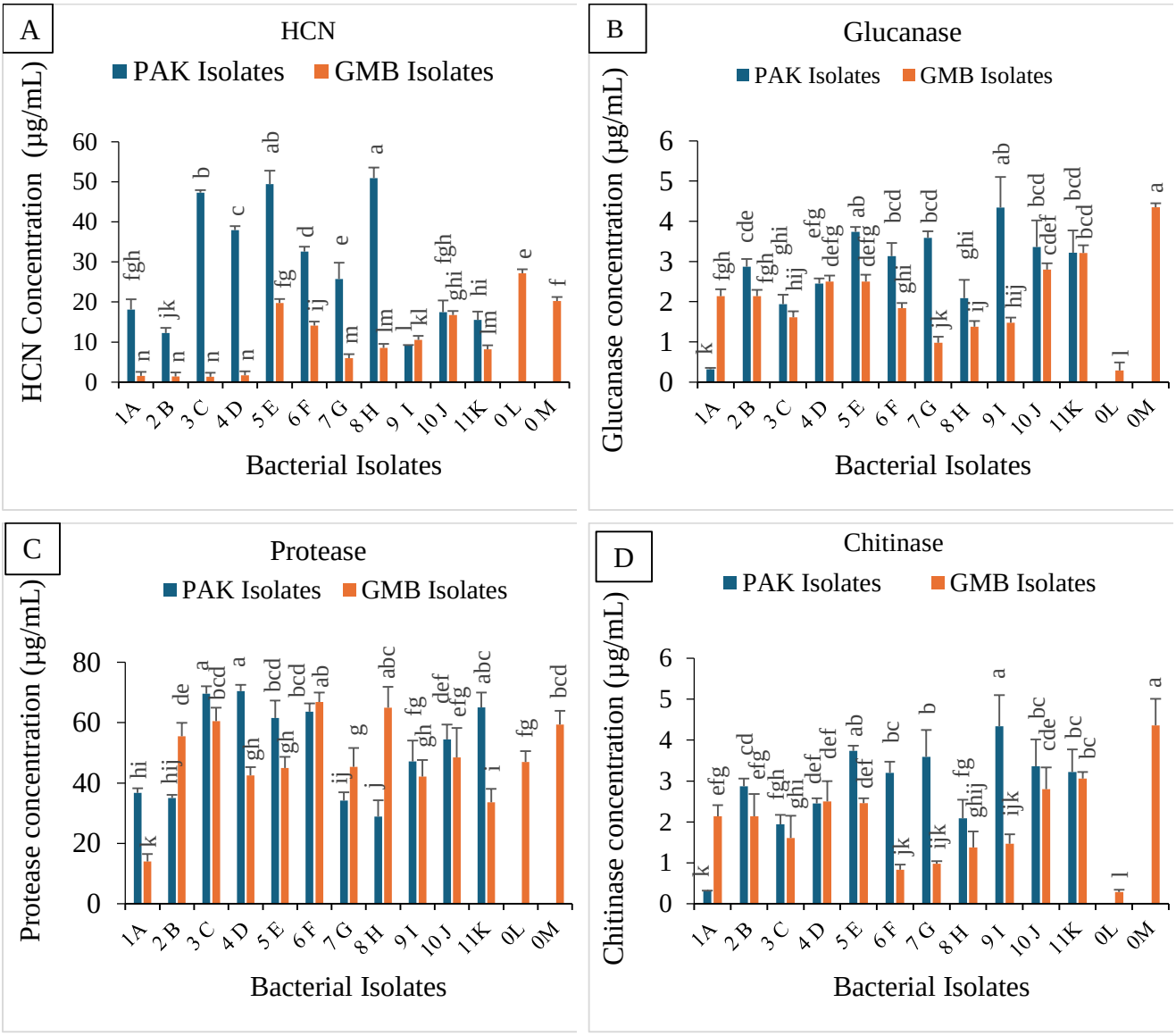

Fig. 2: Biocontrol traits of isolates from Pakistan and The Gambia: A) HCN production, B) Glucanase production, C) Protease production and D) Chitinase production. Isolates labelled in Arabic numerals depicts Pakistan isolates, while the Isolates from The Gambia are shown in alphabet letters. The shared LSD letters showed non-significant difference, otherwise, significant difference among the isolates.

Bacterial metabolites profile analysis through GC-MS: The GC-MS analysis of chloroform and ethanol extracts from isolates 6, 11, B and G showed that isolate B produced highest number of secondary metabolites above 40 compounds as compared to other isolates. Bacterial isolates from both regions (specifically isolate B) produced more non-polar secondary metabolites in trichloromethane (TCM), commonly called chloroform extracts compared to the polar extract in ethanol (EtOH). Isolates 11 and B from Pakistan and The Gambia, respectively, produced more reported plant growth promoting and biocontrol secondary metabolites in both TCM and EtOH compared to counterparts isolates 6. The GC-MS analysis revealed diverse secondary metabolites with putative antifungal activity (Tables 1–3). In Pakistan isolates, 2-(2-phenoxythiinyl) imidazo[1, 2a]pyridine

(18.72% peak area) and oleic acid (24.04%) dominated the chloroform and ethanol extracts of isolate 6, respectively, whereas 1,4-benzenedicarboxylic acid bis(2-ethylhexyl) ester (2.08%) and n-hexadecanoic acid (1.05%) were minor constituents. Similarly, isolate 11 yielded p-xylene (9.27%) and oleic acid (40.8%) as primary metabolites, with 2-pentanol (2.94%) and n-hexadecanoic acid (2.44%) at lower abundances. The Gambia isolates showed similar analogous trends; isolate B produced 2-(2-penoxythiinyl) imidazo[1,2a] pyridine (18.02%) and oleic acid (41.15%) as predominant compounds, while 2-pentanol (0.88%) and Methanone (1.05%) were least abundant. It is interesting to note that oleic acid consistently showed high abundance across extracts, indicating that it may have an antifungal function.

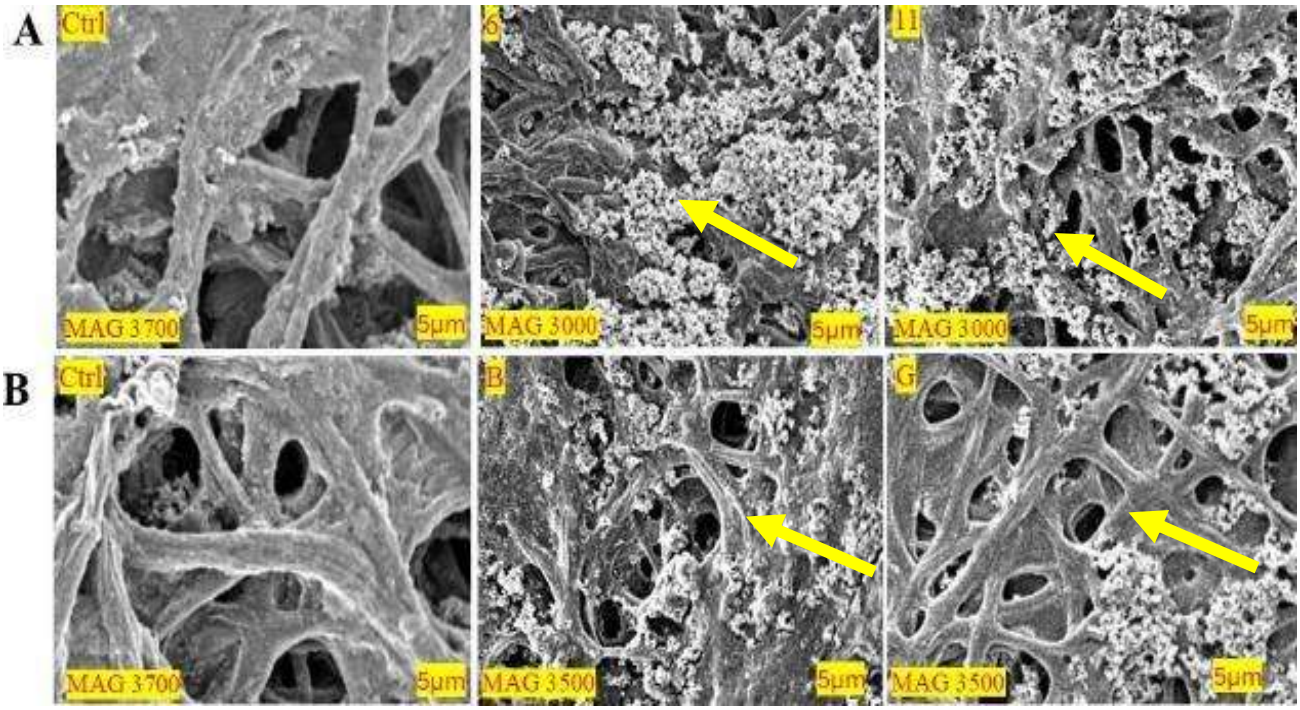

Fig. 3: SEM micrographs of antagonistic effects of selected bacteria PGPRs on *F. oxysporum* mycelial growth. Panel A: shows the fungal control (Ctrl) and co-culture of *F. oxysporum* with isolates 6 and 11 from Pakistan; Panel B: shows the fungal control (Ctrl) and co-culture of *F. oxysporum* with isolates B and G from The Gambia.

Table 1: Secondary metabolites present in isolate 6 as identified in chloroform and ethanol extracts

S/N	Peak Number	Retention time (Min)	Compound	Molecular formula	Molecular Weight	Peak Area (%)
			Isolate 6 in chloroform Extract			
1	1	2.361	2-Pentanol	$C_5H_{12}O$	88.15	2.16
2	3	2.668	p-Xylene and o-Xylene	C_8H_{10}	106.18	7.81
3	4	2.737	Cyclohexanone	$C_6H_{10}O$	98.15	4.69
4	14	23.814	1,4-Benzendicarboxylic acid, bis(2-ethylhexyl) ester	$C_8H_6O_4$	166.13	2.08
5	15	27.328	2-(2-Phenoxythiinyl) imidazo[1,2-a] pyridine	$C_{19}H_{12}N_2OS$	316.38	18.72
			Isolate 6 in ethanol Extract			
6	1	17.334	n-hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	1.05
7	2	18.951	Oleic acid	$C_{18}H_{34}O_2$	282.47	24.03
8	3	19.188	trans-13-octadecanoic acid	$C_{18}H_{34}O_2$	282.47	3.36
9	4	19.233	9-octadecanoic acid E)-	$C_{18}H_{34}O_2$	282.47	1.33
10	5	19.386	[1,2,4] Oxadiazole, 5-(2-fluorophenyl)-3-(4-pyrrol-1-ylphenyl)-	$C_{16}H_{11}FN_4O$	294.29	5.12
11	9	20.672	cis-11-Eicosenoic acid	$C_{20}H_{38}O_2$	310.51	2.14

Table 2: Secondary metabolites present in isolate 11 as identified in chloroform and ethanol extracts

S/N	Peak Number	Retention time (Min)	Compound	Molecular formula	Molecular Weight	Peak Area (%)
Isolate 11 in chloroform Extract						
1	2	2.366	2-Pentanol	$C_7H_{14}O_2$	130.19	2.94
2	4	2.673	p-xylene	C_8H_{10}	106.18	9.27
3	5	2.741	Cyclohexanone	$C_6H_{10}O$	98.15	5.72
4	13	12.474	Sulfurous acid, hexyl pentyl ester	$C_{11}H_{24}O_3S$	236.37	3.34
5	17	23.816	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	390.56	1.71
Isolate 11 in ethanol Extract						
6	3	17.338	n-hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	2.44
7	4	18.957	Oleic acid	$C_{18}H_{34}O_2$	282.47	40.8
8	5	19.186	9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$	282.47	8.25
9	6	20.676	cis-13-Eicosenoic acid	$C_{20}H_{38}O_2$	310.51	7.57
10	7	21.65	Cyclopropaneoctanal, 2-octyl-	$C_{19}H_{36}O$	280.5	7.32
11	11	23.216	cis-11-Eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$	324.5	9.95

Table 3: Secondary metabolites present in isolate B as identified in chloroform and ethanol extracts

S/N	Peak Number	Retention time (Min)	Compound	Molecular formula	Molecular Weight	Peak Area (%)
Isolate B in chloroform Extract						
1	1	2.189	2-Pentanol, acetate	$C_7H_{14}O_2$	130.19	0.88
2	3	2.364	2-Pentanol, acetate	$C_7H_{14}O_2$	130.19	5.1
3	5	2.671	o-Xylene and p-Xylene	C_8H_{10}	106.18	18.02
4	25	17.855	Octadecanal	$C_{18}H_{36}O$	268.48	1.88
5	26	18.954	9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$	282.47	2.29
Isolate B in ethanol Extract						
6	1	9.575	Pentanethioic acid, O-methyl ester	$C_6H_{12}OS$	132.22	3.39
7	3	14.302	Methanone, (1-hydroxycyclohexyl) phenyl-	$C_{13}H_{16}O_2$	204.27	1.05
8	4	17.336	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	2.61
9	5	18.953	Oleic Acid	$C_{18}H_{34}O_2$	282.47	41.15
10	8	19.26	Oleic Acid	$C_{18}H_{34}O_2$	282.47	1.6
11	9	20.676	cis-13-Eicosenoic acid	$C_{20}H_{38}O_2$	310.51	6.23

DISCUSSION

In the current study, an in vitro assessment of antagonistic activity and inhibition through a dual culture assay demonstrated a significant mycelial growth inhibition of *F. oxysporum* PF5 by various bacterial isolates. Isolates 6 and 11, along with isolates B and G, displayed the highest inhibition percentages of approximately 70% by day 10. This notable suppression could indicate a strong, direct antagonistic interaction, possibly involving competition, antibiosis or release of lytic enzymes (Torekhanova, 2025). A similar comprehensive approach utilizing in vitro antifungal screening, biochemical traits analysis, scanning electron microscopy (SEM) and GC-MS metabolite profiling was conducted to identify effective biocontrol strains against *F. oxysporum* PF5 (Mohanan *et al*., 2025). Akhtar *et al.* (2023) reported that *Lactobacillus sp*. showed 82% inhibition of mycelial growth of the *Rhizoctonia solani* and improved the growth and disease resistance potential of rice crop. Similarly, a previous study showed that inoculation with *Bacillus amyloliguefaciens* improved the disease resistance and antioxidant status of the rice seedlings under *R. solani* stress (Srivastava *et al.,* 2016). The consistent inhibition observed at various time points highlights the stability and robustness of these interactions under in vitro conditions. Recent research shows that soil salinity not only disrupts soil nutrient balance, photosynthetic parameters and weakens the plants leading to poor growth and development. This disruption compromises the plant systemic immunity makes plant vulnerable to fungal pathogen attack (Shafi *et al*., 2023). The ability of isolates 6, 11 and B to survive beyond 15% NaCl and remain metabolically active suggest they can mitigate biotic stress through direct

engagement, diffusible compounds, osmotic adjustment and compatible solute accumulation.

The assessment of key biocontrol traits, including hydrogen cyanide (HCN), glucanase, protease and chitinase production was conducted to elucidate the mechanisms that drive fungal antagonism. The isolates from Pakistan primarily showed superior HCN production, while those from The Gambia exhibited enhanced glucanase activity. The HCN production is an important biocontrol trait that is produced through antibiosis mechanism. The HCN inhibit the cytochrome oxidase enzyme and other metalloenzymes, thus interferes with the respiratory chain of the fungus (Nandi *et al.,* 2017). Isolates 6 and 11 along with B and G exhibited significant protease activity, essential for the degradation of fungal cell walls. Growth of fungal pathogen is also inhibited by breaking the glycoproteins of the fungal cell wall with the help of proteases (Banani *et al.,* 2014). These both traits produced by our bacterial isolates provide strong evidence for biocontrol mechanism. Similar results have previously been reported by other researchers (Fatima *et al.,* 2022). Our isolates reported chitinase and glucanase production that are very important enzyme with respect to fungal disease inhibition. These enzymes have capability to degrade various structural components of the pathogen cell wall such as chitin, glucan and glycoprotein (Berini *et al.,* 2018) and inhibit its growth. The essential nutrients become less soluble and unavailable to be taken by the plant and the microbe in the saline soil with high pH. Therefore, the PGPR stimulate the production of HCN and siderophores along with production of other organic compounds and enzymes in order to obtain these nutrients. The reason behind the increased production of HCN by the Pakistani isolate might be due to the higher level of salinity with high pH for nutrient acquisition (Rijavec and Lapanje, 2016). Similarly, the differences in production of hydrolytic enzymes such as chitinase and protease by the isolates can be an adaptive to their environment of origin with high level of salinity that make them resilient to mitigate with the stress through variety of mechanisms (Essghaier *et al.,* 2021).

The SEM image of co-cultured fungal mycelia displayed significant structural disruption, a loss of hyphal integrity; lysis and deformation caused by the selected bacterial isolates further justified the current findings. The results corroborated the biochemical data and clearly indicated the direct inhibitory effect of the chosen PGPRs on the fungal pathogen. Similar results were reported by Sahgal *et al.* (2024) who stated that co-culturing of fungal pathogens and *Pseudomonas* strains AS19 and AS21 resulted in significant degradation of the mycelium that might be due to the production of hydrolytic enzymes. These results support the findings related to the biochemical traits and the SEM results, the production of hydrolytic enzymes such as chitinase are responsible for the degradation of cell wall of the fungi that is evident in the form of mycelial surface deformation. Similarly, Akocaka et al. (2015), who stated that due to the influence of enzymes, diffusible volatiles organic compounds (VOCs) and the metabolite produced by the bacterial isolates, are the causal agent of these changes, have reported similar morphological changes in the fungal mycelium.

The solvent extraction method, commonly referred to as liquid-liquid extraction, was utilized to isolate crude polar and nonpolar secondary metabolites by the use of chloroform (trichloromethane (TCM)) and ethanol respectively (Krug *et al*., 2025). These solvents demonstrate efficacy in extracting bacterial metabolites that possesses antimicrobial activity (Fanoro and Oluwafemi, 2025). In the GC-MS analysis of cell-free culture extracts, a diverse array of bioactive polar and non-polar secondary metabolites of known or putative antifungal properties were identified in the current isolates. Notably, oleic acid, consistently present in high abundance in the ethanol extracts of all the potent strains, had been shown to interfere with fungal membrane structure and hyphal growth and development. Fatty acid molecules reduced the mycelial growth of different fungi in a previous study. Among those, oleic acid at 100 µM reduced the mycelial growth of *P. ultimum* and at 1000 µM reduced that of *C. perniciosa* (Walters *et al.,* 2004). Oleic acid, which is an unsaturated fatty acid, penetrates into the lipid bilayer of fungal cell membrane and disrupts the structure and membrane organization. These changes ultimately lead to increase in fluidity of cell membrane. The hydrophobic interactions that maintain the membrane integrity, becomes weak and results in enhanced permeability of the membrane. Consequently, vital intracellular components such as proteins, electrolytes and nucleic acids leak out of the cell, disturb cellular homeostasis and ultimately leads to fungal cell death (Qui *et al.,* 2021). Compounds like (2-Phenoxythiinyl) imidazo [1,2-a] pyridine, p-xylene, cis-13-eicosenoic acid, and 9,17-octadecadienal (Z) were detected from isolates 6, 11, B and G and have been reported to possess antimicrobial properties. For example, imidazo-thiinyl derivatives have been associated with antifungal activity through the induction of oxidative stress by disrupting cell membrane integrity. In addition, it is reported that long-chain aldehydes and unsaturated fatty acids interfere with fungal metabolites or signaling pathways. More importantly, plasticizer-type compounds, like bi(2-ethylhexyl)phthalate and terephthalate esters, were found in trace quantities, consistent with earlier reports of such molecules released by microbial endophytes or isolated from extracts of bioactive plants (Sun *et al*., 2024). Although some of these are considered as environmental contaminants, their frequent association with antifungal effects in microbial extracts cannot be overlooked and warrants further functional validation.

Together, these results suggest a multifarious mode of action of selected PGPR strains. The isolates not only demonstrated promising antifungal activity but also produced strong biochemical and hydrolytic enzymes as well as bioactive compound that make them a strong candidate for development of biocontrol inoculants.

Conclusions and future recommendations: Results of this study demonstrated that the PGPR isolated from salt-affected rice-growing soils from Pakistan and The Gambia have a great potential for biological control of *F. oxysporum*. *In vitro* antifungal assay demonstrated that isolates 6, 11, B and G were able to suppress *Fusarium* more effectively as compared to other isolates. Isolate 8 showed highest HCN production while M showed maximum gluconase production. Maximum significant increase in protease was recorded by isolate 3 and 4 while isolate M and 9 showed maximum chitinase production. Moreover, the SEM results of isolate 6, 11 and B showed best antagonistic activity. Antifungal secondary metabolite (oleic acid, imidazo[1,2-a]pyridine derivatives) production also confirmed the biocontrol potential of these isolates. Taken together, all the findings indicates that selected isolates have the ability to inhibit fungal pathogens structurally and chemically, confirming their potential as sustainable biocontrol agent. However, further investigation in the form of pot and field experiment are needed to assess effects of these isolates in controlled and natural conditions.

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Data Availability: The four newly sequenced datasets were uploaded to the NCBI database, including these four strains with the following accession numbers: PQ555678, PQ555679, PQ555683, and PQ555692. All other relevant data regarding this manuscript will be made available on request.

Credit authorship contribution statement: PAC and AN designed the study, PAC performed the research experiment and wrote draft manuscript, AN devised ideas and supervised the whole experiment, HY and RN helped in data analysis, reviewing and editing the manuscript. MS and MNH provided the critical revision and final approval of the article. AZ helped in analysis and resources.

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