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RAPID COMMUNICATION

New report of *Rhizoctonia solani* anastomosis group AG-7 associated with root rot disease of black gram in Pakistan

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Abstract

Black gram [*Vigna mungo* (L.) Hepper] is an important and nutritionally rich pulse crop mainly grown in tropical and subtropical environments. From July to August 2023–2024, black gram plants in Faisalabad, Pakistan, were observed with severe wilting and root rot disease complex symptoms. Following morphological and molecular characterization, the causal pathogen was identified as *Rhizoctonia solani* anastomosis group AG-7. Based on the present findings and a review of the literature, this is the first report of *R. solani* as the causal agent of root rot disease in black gram both in Pakistan and worldwide.

Keywords: black gram, characterization, pathogenicity, *Rhizoctonia solani*, root rot

Introduction

Black gram [*Vigna mungo* (L.) Hepper], a member of the Fabaceae family, is an important short duration, nutrient-rich legume crop widely grown on the Indian subcontinent as well as in Southeast Asia (Vishalakshi *et al.* 2017). Because of its many advantages for soil and human health, it was domesticated from its ancestor, *Vigna mungo* var. *silvestris*, in northern South Asia (Lukoki *et al.* 1980). The cultivation and consumption of black gram has been extended to other regions of Asia, such as Pakistan, Bangladesh, Sri Lanka, Nepal, and Myanmar. With a nutritional composition of 25% protein, 60% carbs, 1.3% fat, and vital vitamins and minerals, black gram serves as a vital component of a healthy vegan diet (Qayyum *et al.* 2019; Dey *et al.* 2022). It is good for humans due to its high and easily digestible protein content (20.8–30.5%) and carbohydrate (56.5–63.7%) on dry weight basis (Qayyum *et al.* 2019). In Pakistan in 2022, black gram was grown over an area of 7,000 hectares with a total production of 4,200 tons (Anonymous 2023). However, despite its significance, the yield of black gram in Pakistan is far less than in other countries. Legumes including black gram are globally facing increasing challenges due to biotic stresses, such as diseases (yellow mosaic, leaf crinkle, powdery mildew) and abiotic stresses, including climate change. This changing climate is disrupting an ecosystem, thereby increasing the potential risk of new plant pathogens and diseases to crops which are emerging (Priyadi and Upadhyay 2021; Das *et al.* 2023). Hence, accurate detection of plant pathogens and analysis of their pathogenic variabilities within the population is critical to developing proper control strategies and breeding schemes (Ozer *et al.* 2020).

In July and August 2023–2024, during the regular inspection of a black gram crop for diseases in the breeders' experimental fields at the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan, plants of different genotypes were observed with severe wilting and root rot disease complex (WRRDC) symptoms. Infected plants showed a range of disease

symptoms, including vascular browning, premature leaf drop, chlorosis, necrosis, and plant death (Fig. 1A). Therefore, this study was done to isolate, identify (using morphological and molecular techniques) and to confirm the pathogenicity of the pathogens associated with WRRDC in black gram.

Materials and Methods

Sampling and pathogen isolation

Root samples from 50 WRRDC infected plants were collected and cut into small pieces of about 5 mm in length after washing with tap water. The fragments were then surface disinfected using a 3% sodium hypochlorite (NaOCl) solution for 5 minutes. After drying in a laminar air flow cabinet, these tissue fragments were transferred onto potato dextrose agar (PDA) medium in Petri plates supplemented with streptomycin (100 mg · l^{-1}) and chloramphenicol (49.9 mg · l^{-1}) and incubated at 25°C for 3–4 days (Ozer *et al*. 2019). The growing hyphal tips were then further transferred onto Petri plates containing PDA for purification, morphology-based identification of *R. solani* and future work.

Morphological and molecular characterization

To confirm the morphology-based identification of *R. solani* isolates following molecular characterization, whole genomic DNA was obtained from 14 isolates of 7-day-old cultures using a modified CTAB method (Doyle and Doyle 1987). The DNA isolated from fungal cultures was PCR amplified using universal fungal ITS primers (ITS1/ITS4) (White *et al.* 1990) and *R. solani* specific primers (Rhsp1/ITS4B) (Salazar *et al.* 2000). The PCR product of two representative highly pathogenic isolates "FF3 and FF4" was custom-sequenced (Eurofins Genomics, USA). The BioEdit program was used to analyze the obtained sequences and a consensus sequence was submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/). For phylogenetic reconstruction, reference (rDNA-ITS) region sequences of several fungal species were obtained from GenBank and aligned using MUSCLE (Edgar 2004). The phylogenetic tree was reconstructed using IQ-Tree (http://iqtree.cibiv.univie.ac.at/) with 1,000 ultrafast-bootstrap replicates and visualized in FigTree v1.4.4 (https://tree.bio.ed.ac.uk/software/figtree/).

Pathogenicity tests

Pathogenicity tests of the 14 *R. solani* isolates were conducted in a laboratory using the agar plate method (Sajjad *et al.* 2024). For this purpose, the fungus *R. solani* was cultured on PDA in Petri plates for 7–10 days. Seeds of black gram accession "38272" (showing maximum recovery of *R. solani* isolates under field conditions) were surface sterilized in 3% NaOCl for 10 minutes and washed in autoclaved distilled water three times and then air dried properly. Five seeds per Petri plate were then transferred to PDA medium containing the *R. solani* cultures. There were three replicates for each treatment. The plates were then incubated at 25 ± 2°C for 12 hours of alternating cycles of day/night under fluorescent light in an incubator. An identical set having the same number of surface sterilized seeds on un-inoculated PDA was also included as a negative control. Seeds were examined daily, and the final data was collected as germination percentage, seedling mortality percentage, infection percentage, infection type range, disease severity index and disease response 15 days post inoculation. The seedling mortality percentage was calculated as the percentage of viable seed germination (produced by both plumule and radical) but not produced by a healthy plant following the rating system described by Sajjad *et al*. (2024).

To determine the ability of *R. solani* to cause root rot in black gram plants, a pathogenicity test was also performed on the accession "38272" in earthen pots under glasshouse conditions following the method described by Abd-Elsalam *et al.* (2010). For this purpose, the culture of two *R. solani* isolates viz; FF3 and FF4 (showing the highest rate of disease severity under the agar plate method) was multiplied in 250 ml flasks, each having 25 g of autoclaved sorghum grains and 20 ml of tap-water for 3 weeks. Earthen pots (28 cm diameter and 35.6 cm high) were filled with sterilized soil and inoculated with mass culture of the *R. solani* at a rate of 15 g per kg of soil. After seven days surface disinfected seeds of black gram accession "38272" were sown at the rate of 12 seeds per pot in three replications (2 pots/replicate). An identical set having sterilized un-inoculated soil was included as a healthy control. The experimental unit was maintained in a glasshouse at a temperature range of 25–28°C with a 12-hour light/dark cycle. Conventional agronomic practices were followed to keep the crop in good condition. The experimental unit was observed daily for germination percentage and to determine the percentage of infected plants. To fulfil the Koch's postulate *R. solani* was re-isolated from inoculated plants and the same experiment was repeated.

Results and Discussion

Of the purified fungal colonies from the roots of infected black gram plants, 28% were tentatively identified

as *Rhizoctonia solani based on their morphological and cultural characteristics*. Initially the *R. solani* colonies were light white and after seven days of incubation, these colonies became dark brown and fluffy (Fig. 1B). Microscopic examination showed that the hyphae were brown, tended to branch at right angles to the parent hyphae, and had a septum near the base of the branches. The hyphal portion near the septum was also found to be slightly constricted (Fig. 1C). The hyphal cells at the advancing edge of the colony were 5–12 µm wide and up to 250 µm long. In addition to normal vegetative hyphae, simple or branched chains of short, broad cells, hyaline or brown, barrel-shaped, pyriform, irregular, or lobate known as monilioid cells were also observed (Fig. 1D–E). These morphological and cultural characteristics of the isolated *R. solani* isolates were consistent with earlier descriptions of *R. solani* by Ogoshi (1975), Sneh *et al.* (1991) and Palacıoglu *et al.* (2024).

Following molecular analysis, all 14 isolates morphologically identified as *R. solani* amplified a single band of the expected PCR product size of approximately 715 bp and 700 bp with ITS and Rhsp1 primers pairs, respectively (Salazar *et al.* 2000). The purified PCR product of the ITS region from two representative, highly pathogenic isolates, FF3 and FF4, were sanger sequenced and the analyzed sequences were deposited in GenBank under accession numbers PV790576 and PV790577. The phylogenetic tree showed that both isolates clustered within a well-defined clade with other *R. solani* isolates from AG-7 (Accession No. AB000003, FJ35104, AF153791, OP784261, OP784258) (Fig. 2). The characterized culture of *R. solani* isolates, FF3 (GenBank accession number PV790576) and FF4 (GenBank accession number PV790577) were deposited (FMB–CC–UAF 252) with the fungal molecular biology lab-culture collection (FMB–CC) at the University of Agriculture, Faisalabad.

The pathogenicity test performed using the agar plate method showed that the disinfected seeds of accession "38272" showed 83.3% germination when directly placed on PDA containing 7-days-old culture of *R. solani*. However, seedling length was 23.6 mm to 46.0 mm; percent decrease in seedling length over healthy control was 60.8% to 23.6%. The infection type range due to seedling necrosis was 1–4; disease severity index (calculated based on the necrosis score) was 3.1 to 3.4, and the disease response was moderately susceptible (Fig. 3A–B).

To determine the ability of *R. solani* to cause root rot on black gram plants, a pathogenicity test was also conducted in earthen pots using two of the most virulent isolates, FF3 and FF4, which displayed the highest disease severity in the agar plate test. Observation

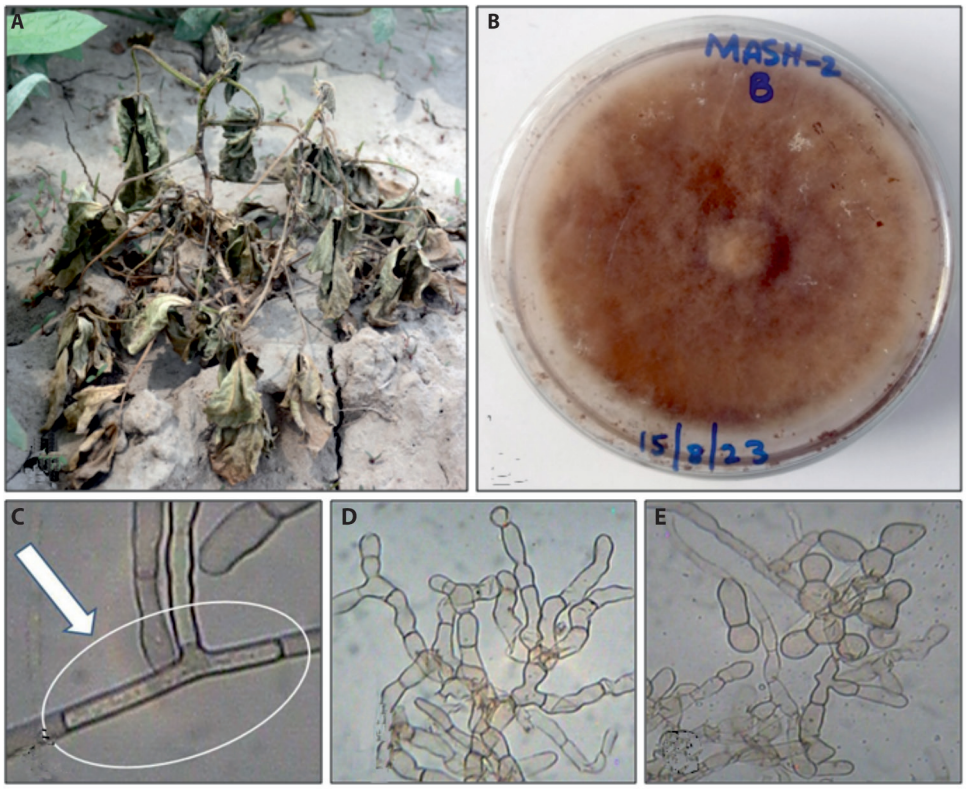

Fig. 1. Symptoms, morphology, and microscopic features of *Rhizoctonia solani*. A – wilt symptoms on aerial parts in black gram under field conditions; B – *R. solani* colony on PDA after 7 days; C – right-angle branching of septate hyphae; D-E – Barrel-shaped monilioid cells of sclerotia

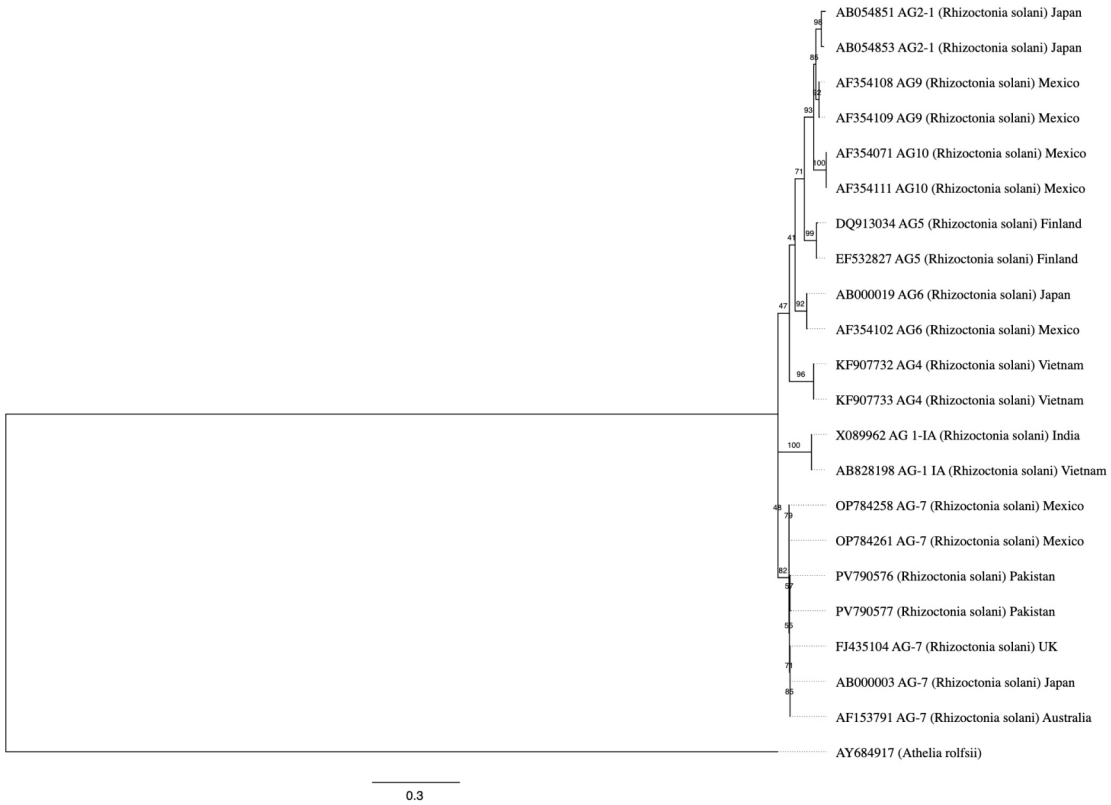

Fig. 2. Phylogenetic relationship among the *R. solani* isolates (PV790576 and PV790577) from black gram with reference *R. solani* isolates belonging to different anastomosis groups (AGs). The phylogenetic tree was reconstructed using IQ-Tree with 1,000 ultra-fast bootstrap replicates and visualized in FigTree v1.4.4

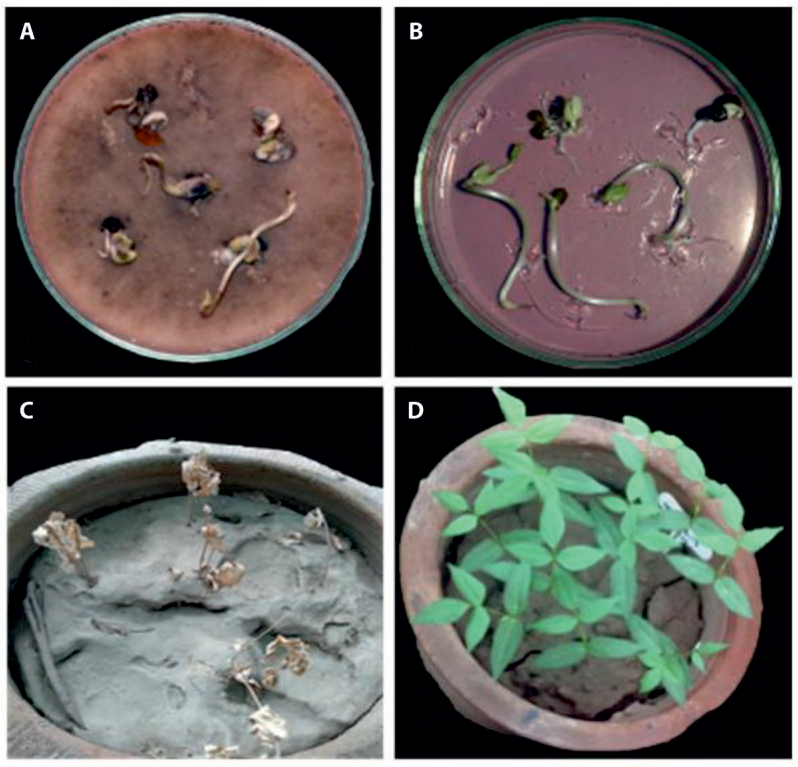

Fig. 3. Pathogenicity test of *Rhizoctonia solani*. A – seed germination and seedling growth on *R. solani* culture on PDA; B – seed germination and seedling growth on un-inoculated PDA; C – black gram plants in inoculated pots; and D – black gram plants in un-inoculated pots (healthy control)

showed that the germination percentage and survival of the plants was 100% in the case of un-inoculated pots while germination was 78% to 80% and infection was 100% in the case of inoculated pots, confirming the pathogenicity of isolated *R. solani* (Fig. 3C–D). To fulfil Koch's postulate, *R. solani* was re-isolated from these plants and the same experiment was repeated. Severe symptoms similar to those observed in the field and pot experiments were recorded. Conversely, the negative controls (un-inoculated) remained symptomless or free from disease.

Rhizoctonia solani is a common soil-inhabiting fungal pathogen that inflicts huge losses to several commercially significant crops including black gram and can cause severe diseases such as hypocotyl, crown, root, stem collar, bud and fruit rots, as well as web blights, wire stem and damping-off in legumes (Ajayi-Oyetunde and Bradley 2018; Palacıoglu *et al.* 2024; Bhuiyan *et al.* 2025). These diseases result in significant yield losses and pose challenges to effective crop management. The economic ramifications of the impact of *R. solani* on agriculture is well-documented (Biswal and Das 2024). According to estimates, *R. solani* causes an average yield loss of 20% in legumes. Under extreme cases, the losses may be as high as 30–60% or even total loss of the legume crops (Ajayi-Oyetunde and Bradley 2018; Akber *et al.* 2023). Black gram has been cultivated globally for a long time; however, there are no prior reports or literature documenting *R. solani* as a cause of root rot in black gram (Kumar *et al.* 2018). To the best of our knowledge, this is the first report of the *R. solani* AG-7 anastomosis group causing root rot on black gram. The emergence of *R. solani* in black gram could potentially be an outcome of climate change. Climate plays an important role in the aggravation of diseases. Increasing temperatures and altered precipitation patterns can especially exacerbate the emergence and severity of *R. solani* in bean crops (Biswal and Das 2024; Lahlali *et al.* 2024). Rising temperatures and extreme weather events like droughts or heavy rainfall can create favorable conditions for *R. solani* outbreaks in black gam. The widespread presence of *R. solani* in diverse environments, coupled with its ability to cause severe damage to a wide range of crops, demonstrates its remarkable versatility and capacity to adapt to different conditions, making it a significant agricultural pathogen with a wide distribution. Recent studies suggest that environmental changes impact pathogen distribution, allowing them to thrive in previously unsuitable areas and infect a wider range of host plants. This adaptability is evident in the pathogen's ability to infect both traditional crops and newer, high-value horticultural species. Furthermore, climate forecasts also signify more challenges in combating *R. solani* global expansion in food crops (Biswal and Das 2024). In the future it can be a potential risk to crops including black gram cultivation. Newly emerging plant pathogens and diseases can become epidemics if not timely identified and controlled, as changing climatic conditions may create more favorable environments for pathogens to spread and establish themselves in new and non-native areas (Priyadi and Upadhyay 2021). The present research may be helpful in the future to devise disease management measures to minimize the spread of *R. solani* in black gram sowing regions. The control of *R. solani* is difficult due to its high variability, wide host range, and ability for long term survival in the soil (Erper *et al.* 2021; Palacıoglu *et al.* 2024). Therefore, future research is needed to develop integrated management strategies against *R. solani* by combining chemical and biological control methods, implementing crop rotation practices, and breeding for resistant crop varieties to effectively manage this pathogen across different agricultural systems.

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