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Multifaceted benefits of *Bacillus amyloliquefaciens* strain FBZ24 in the management of wilt disease in tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*

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ABSTRACT

Our study endeavors to evaluate the efficacy of endophytic *Bacillus* against *Fusarium* wilt disease and understand the resistance mechanism in tomato plants. A total of 21 endophytic bacterial strains were characterized at both biochemical and molecular levels and were screened against *F. oxysporum* f. sp. *lycopersici* by dual culture. Strain FZB24 recorded the maximum percent inhibition (48.3%) of *F. oxysporum* f. sp. *lycopersici* under *in vitro* conditions. Bacterial strains were evaluated for growth promoting characters and the presence of antibiotic genes through PCR analysis. FZB24 and TEB3 strains increased plant growth in the tomato plants *in vitro*. Strains FZB24, TEB3, TEB5, TEB6 and TEB9 had all the evaluated antibiotic biosynthetic genes *viz.,* Iturin A, Iturin C, Surfactin, Bacillomycin A and Bacillomycin D. Corn starch-based formulation prepared with *B. amyloliquefaciens* (FZB24) was evaluated both under glass house and field conditions for *Fusarium* wilt disease management. *B. amyloliquefaciens* (FZB24) treatment induced defense enzymatic activities such as PO, PPO, PAL, CAT and SOD in the treated tomato plants. FZB 24 was found to be effective when applied as a combination of seed treatment @ 4 g/kg + seedling dip @ 4 g/l + soil application @ 500 g/ha + foliar spray @ 500 g/ha in reducing disease incidence. This treatment also recorded higher defense enzyme activities, maximum plant growth promotion and higher yield.

1. Introduction

Tomato (*Solanum lycopersicum* L.), belonging to the family Solanaceae, is one of the most important commercial vegetable crops cultivated worldwide for culinary purpose as well as processing. Total area under tomato cultivation is increasing day by day due to its nutrient value, demand and high yield [1]. Tomato crops are affected by various fungal, bacterial and viral diseases [2] causing huge crop loss. Among them, *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* Sacc. is a highly destructive soil borne disease affecting both green house and field grown tomatoes. In India, it was estimated to cause crop loss of up to 45% [3,4]. Chemical pesticides are widely adopted by farmers to manage the disease [5]. However, continuous use of pesticides for disease management will lead to the development of resistant strains of the pathogen, environmental pollution and human health hazards [6]. To overcome this predicament, the focus has been shifted to the use of biocontrol agents to curb diseases, as a safe and promising alternative to synthetic pesticides [1].

Endophytic bacteria are those which colonize the plant internally without causing any substantial harm [7]. Recent studies determined the potential role of endophytic bacteria in plant growth promotion and phytopathogen control [8]. Among endophytic bacteria, *Bacillus* species are the most commonly recognized microorganisms found to colonize plants [9]. In addition to plant growth promotion through the biosynthesis of phytohormones, their endophytic ability plays a major role in the biocontrol of soil-borne plant pathogens [10]. It is apparent that *Bacillus* sp. produces antimicrobial lipopetides and antifungal compounds like surfactin, iturin, bacillomycin and fengycin [11] which suppresses the pathogenic microorganisms. Induced systemic resistance (ISR) is a process of active resistance dependent on the host plant's physical or chemical barriers activated by biotic or abiotic agents. The involvement of ISR in disease suppression has been studied in a wide

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range of biocontrol microorganisms. It is justified by the ability of *Bacillus* sp. to induce systemic resistance in plants through the production of plant defense enzymes like polyphenol oxidase (PPO), peroxidase (PO) and phenylalanine ammonia lyase (PAL) [12]. Based on this background, a study was carried out with the objective to identify the efficacy of endophytic bacterial strains and their mechanisms implicated in the effective management of *Fusarium* wilt of tomato under both glasshouse and field conditions.

2. Materials and methods

2.1. Isolation and identification of Fusarium oxysporum f. sp. lycopersici

Infected tomato samples were collected from Thondamuthur village of Coimbatore district (Tamil Nadu, India) and the pathogen was isolated on Potato dextrose agar (PDA) medium adapting tissue segment method, which was purified and used for further studies. The pathogen was identified by its morphological characters as was described by Booth [13] and further confirmed using PCR amplification of 18S-28S rRNA region using universal primer pair ITS 1 and ITS 4 [14]. To determine its pathogenic nature, the isolated pathogen was inoculated on susceptible tomato cv. Deepti 22 day old seedlings by standard root dip method [15,16].

2.2. Isolation of endophytic bacterial strains

Endophytic bacterial strains were isolated from the healthy root, stem and leaf portions of tomato plants. The samples were cut into 2–3 cm size and surface sterilized with 1% sodium hypochlorite (NaOCl) for 10 min. They were rinsed four times with 0.02 M sterile potassium phosphate buffer (pH 7.0) and 0.1 ml aliquot from the final buffer was added to 9.9 ml Nutrient Broth (NB) for sterility check. If there was any bacterial growth after an incubation period of 48 h during sterility check, then those samples were discarded. Triturated samples (0.5 g) were used to make serial dilutions up to (10^{10}) in phosphate buffer with a sterile mortar and pestle in 9.5 ml of the final buffer wash. Dilution of each sample was plated (0.1 ml) on Nutrient Agar (NA) medium and incubated at 28 ± 2 °C for 48–72 h. One representative of each bacterium, as evident from their colony type and morphology was transferred to fresh NA plates to establish pure cultures. FZB24 strain of *Bacillus amyloliquefaciens* was obtained from Novozymes South Asia Pvt. Ltd Bangalore.

2.3. In vitro efficacy of endophytic bacterial strains against F. oxysporum f. sp. lycopersici

Endophytic bacterial strains were tested for their antagonistic activity against mycelial growth of *F. oxysporum* f. sp. *lycopersici* following the dual culture technique [17]. A mycelial disc of 8 mm diameter from 7 days old culture of *F. oxysporum* f. sp. *lycopersici* was placed at one side of the (10 mm away from periphery). Petri plate containing PDA medium and bacterial cultures were streaked onto the medium exactly opposite to the mycelial disc. The plates were incubated at room temperature (30 ± 2 °C) for 10 days. Percent inhibition (PI) of mycelial growth was calculated using the formula proposed by Dennis and Webster [18].

2.4. Plant-growth promoting activity of endophytic bacterial strains on tomato seedlings

Endophytic bacteria were inoculated into NB and incubated at 150 rpm with shaking for 48 h at room temperature (28 ± 2 °C). The bacterial suspension was then centrifuged at 12,000 ×*g* for 15 min and the cells were resuspended in Phosphate Buffer (PB) (0.01 M, pH 7.0). The concentration was adjusted to approximately 10^8 cfu ml^{-1} (OD_{595} = 0.3) [19]. Tomato seeds (1 g) which were surface-sterilized with 2% sodium hypochlorite for 30 s, rinsed in sterile distilled water and dried overnight under a sterile air stream were soaked in 10 ml of the bacterial suspension (containing 3×10^8 cfu/ml) for 2 h and dried overnight under shade. Based on the standard roll towel method, the seedling vigour index was determined in order to assess the plant-growth promoting activity of the bacterial strains [20]. 25 bacterized seeds were kept over the presoaked germination paper and incubated in the growth chamber for 15 days at 3 replications for each bacterial strain. Root length and shoot length of individual seedlings were measured along with their germination percentage. The vigour index was calculated using the formula of Abdul Baki and Anderson [21]:

Vigour Index = (shoot length + root length) × % germination

2.5. Biochemical and molecular characterization of endophytic bacterial strains

Isolated strains were characterized based on the various biochemical tests *viz.*, Gram's staining, KOH test, catalase test, starch hydrolysis, gelatin hydrolysis, growth at 7 per cent NaCl citrate utilization test and methyl red test [22]. DNA was isolated by the modified Cetyl trimethyl ammonium bromide (CTAB) method reported by Melody [23] and confirmed with PCR analysis using 16S rRNA intervening sequence specific primer pairs BCF1 (5′CGGGAGGCAGCAGTAGGGAAT3′) and BCR2(5′CTCCCCAGGCGGAGTGCTTAAT3′) as proposed by Cano et al. [24]. The reaction was performed with a volume of 20 µl containing ~50 ng of total DNA, 5 mM each of dNTPs (Fermentas, USA), 20 pmol each of the forward and reverse primers and 0.5 U of Taq DNA polymerase (Bangalore Genei, India). The PCR products were resolved on 1.2% agarose at 50 V, stained with ethidium bromide (0.5 µg/ml) and documented using gel documentation system (Alpha Innotech Corporation, San Leandro, California). Amplified PCR products were purified and sequenced. Sequences were queried for similarity within the NCBI GenBank database using the BLAST tool. Sequence homology was established with the existing reference sequences in NCBI database and submitted to NCBI Genbank. Phylogeny studies were conducted based on their 16S rRNA region through Neighbor Joining method using MEGA 6.0 tool with 1000 bootstrap replicates and condensed with a cut-off value of 80%.

2.6. Detection of antimicrobial peptide (AMP) biosynthetic genes

PCR assay was performed to investigate the presence of AMP biosynthetic genes such as iturin A, iturin C, surfactin, bacillomycin A and bacillomycin D using their specific primers [25,26].

2.7. Evaluation of B. amyloliquefaciens (FZB24) under glass house conditions against F. oxysporum f. sp. lycopersici

Cornstarch-based formulation of *B. amyloliquefaciens* (FZB24) with a minimum population of 1×10^{10} cfu g^{-1} was delivered through seed treatment (4 g/kg), seedling dip (4 g/l), soil application (500 g/ha) and foliar spray (500 g/ha). This formulation contained 13% *B. amyloliquefaciens* (FZB24) and 87% cornstarch, obtained from M/S. Novozymes South Asia Pvt. Ltd, Bangalore, India. Dry seed treatment was given to tomato seeds by mixing the FZB24 formulation @ 4 g/kg of seeds. For seedling dip, 25 days old seedlings were uprooted and their roots were dipped in water containing FZB24 formulation for 30 min. The treated seedlings were transplanted into plastic pots (45 × 60 cm) containing sterilized pot mixture (red earth:sand:FYM 1:1:1 w/w/w). Isolated *F. oxysporum* f. sp. *lycopersici* was mass multiplied in a sand–maize medium and inoculated into the sterilized pot mixture at 5% (w/w). Three seedlings were maintained per pot. Soil application of FZB24 formulation was given at the time of transplanting. The corn starch based FZB24 formulation was dispersed in