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RESEARCH ARTICLE

Metabolite profiling of PGPR *Bacillus subtilis* BGKMR1: A potential strategy for managing *Fusarium equiseti* causing wilt in bitter gourd (*Momordica charantia* L.)

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Abstract

Wilt caused by *Fusarium equiseti* is one of the most destructive diseases that leads to substantial yield loss in bitter gourd. The *F. equiseti* strain CBEFE1((PQ111513.1) which was identified morphologically with falcate shaped three septate macroconidia and oval shaped microconidia and molecularly confirmed through amplification of the ITS region at 560bp was used for this study. Plant growth promoting rhizobacteria (PGPR) act as a sustainable biocontrol agent against major plant pathogens through multiple mode of actions. PGPR were isolated from native rhizopshere region in bitter gourd. The isolated PGPR *Bacillus subtilis* BGKMR 1 shows maximum mycelial inhibition of 68.73 % against *F. equiseti* in dual plate assay. The presence of metabolites produced by *B. subtilis* and *F. equiseti* along with its interaction were identified through the GCMS profiling. Primarily, pathogenic compound squalene (8.88 %) was identified in *F. equiseti* and antifungal compound 1,4-benzenedicarboxylic acid (6.1 %) was identified in *B. subtilis*. Further, *B. subtilis* BGKMR 1 during its interaction shows propanoic acid (14.69 %) which was found to have effectiveness against *F. equiseti*. KEGG analysis revealed pyrimidine metabolism in *F. equiseti* and nitrogen, alanine**,** aspartate and glutamate metabolism in *B. subtilis* and propanoate metabolism pathways were detected during its interaction. *B. subtilis* BGKMR 1 promote the seed germination rate upto 96 % and vigor index to 1919.04 when compared to control. The seed treatment with soil application of *B. subtilis* BGKMR1 reduced disease incidence upto 25.95 % under glass house condition. Moreover, *B. subtilis* BGKMR1 treated plants shows enhanced defense enzymes activity such as peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase indicating induction of systemic resistance. Therefore, *B. subtilis* BGKMR 1 contains novel antifungal metabolites which were identified via GC-MS and induced defense enzyme activity highlights its practical application as a sustainable and eco-friendly solution for managing wilt disease in bitter gourd.

Keywords

B. subtilis; F. equiseti; KEGG; metaboanalyst; rhizosphere

Introduction

Bitter gourd (*Momordica charantia* L.) a member of the cucurbitaceae family, is a widely cultivated vegetable known for its medicinal and nutritional properties. It is extensively grown in Asia, with more than 340000 hectares cultivated annually (1). By tradition, it has been used to treat diabetes with its bitter taste attributed to the presence of momordicin and the fruit contain high amountof vitamin A, E and

C. It also has excessive amount of potassium, iron, calcium, magnesium, phosphorus and zinc (2).

However, bitter gourd cultivation is often hindered by soil-borne pathogens such as *Phytopthora sp.*, *Pythium sp.*, *Verticillium* wilt and *Fusarium sp.* which causes significant yield losses. Among these, *Fusarium* spp. pose a major challenge due to their saprophytic nature, formation of chlamydospores and ability to persist in the soil for extended periods, making conventional management strategies like crop rotation and soil sterilization less effective. *Fusarium* spp. can cause systemic vascular wilting, affecting the plant throughout its lifecycle and leading to yield losses of upto 40 % (3).

F. equiseti has been reported to cause severe leaf spot in bitter gourd with 37 % disease incidence in Pakistan (4). Moreover, *F. equiseti* has been identified as causal agent inciting wilt in watermelon with 10% disease incidence and in cauliflower with 45 % disease incidence (5, 6). It also leads to considerable yield reduction in tomato, causing up to a 60 % loss in Northern Algeria (7). Thus, this pathogen plays a significantly role in reducing vegetable yield.

Unlike chemical methods, which may lead to resistance development, environmental contamination and harm to non-target organisms, biological control agents are safer and more sustainable. Biological control involves the use of beneficial microorganisms, such as plant growth-promoting rhizobacteria (PGPR) (8).

Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and promote plant growth either directly, by facilitating nutrient uptake or producing phytohormones or indirectly by suppressing phytopathogens through mechanisms like competition, production of antifungal compounds and induction of systemic resistance (9). PGPRs like *B. subtilis*, *P. flouroscences, Agrobacterium radiobacter* 84, *Chaetomium globusum*, *Sterptomyces sp.*, are widely recognized as effective biocontrol agents which are available in market (10). In addition to pathogen suppression, PGPRs can also alleviate abiotic stresses such as soil salinity. For instance, certain strains of *B. subtilis* produce ACC deaminase, which reduces ethylene levels and reactive oxygen species (ROS) induced by salinity stress, thereby promoting plant growth and yield (11). *B. subtilis* produce various bioactive secondary metabolites such as fengycin, surfactin, iturin which shows strong antifungal activity against plant pathogens. This bacterium also promotes plant growth through mechanisms such as biofilm formation, chemotaxis, improved nutrient availability, and modulation of phytohormone homeostasis. Moreover, its root colonization enhances plant defense mechanisms, offering dual benefits of disease suppression and growth promotion (12).

This research focuses on the native rhizospheric PGPR isolate *B. subtilis* BGKMR1, a potent biocontrol agent. The study aims to investigate its antifungal activity against *F. equiseti* and to explore metabolite interactions between *B. subtilis* and the pathogen and evaluate its plant growth-promoting activity. The practical application of *B. subtilis* BGKMR1 in mitigating wilt disease in bitter gourd will also be assessed under greenhouse conditions, providing insights into sustainable and effective disease management strategies.

Materials and Methods

Collection of pathogenic strain

The *F. equiseti* strain CBEFE1 causing wilt in bitter gourd used in this study was previously reported and identified. The strain was stored in the laboratory and subsequently employed for experiments. The culture was further purified using the pure culture technique to ensure consistency in studying the pathogen's traits and its role in disease development.

Isolation of antagonists from rhizospheric region in bitter gourd

Rhizospheric organisms were collected from bitter gourd field from Kurumbalur, Vadakarai, Annamangalam, in Perambalur district and Kavilipalayam, Kodangipalayam in Coimbatore district of Tamil Nadu. The bacteria were isolated using the serial dilution method, with dilutions ranging from 1×10^{-1} to 1×10^{-8} in Nutrient Agar (NA) medium. The isolated bacteria were further analyzed for colony morphology, colony character and tested for their antagonistic and PGPR activities (14) (Table 1).

Table 1. Collection of antagonists from rhizospheric region in bitter gourd

Isolate code	Place of collection	GPS COORDINATE	Region
BGKMR1	Kurumbalur	N 11.211874	rhizosphere
BGVKI1	Vadakarai	N 11.394517 E78.844312	rhizosphere
BGANM1	Annamangalam	N 11.334418	rhizosphere
BGKPM1	Kavilipalayam	N10.706	rhizosphere
BGKDM1	Kodangipalayam	N 11.013599	rhizosphere

Effect of rhizospheric antagonists against *F. equiseti under in-vitro*

The antagonistic effect of rhizospheric bacteria against *F. equiseti* was assessed using the dual culture method. A 9-mm mycelial disc of the pathogen, taken from a 10-days-old cultures, was cut using a sterile cork borer and placed on one side of the petri dish containing solidified potato dextrose agar (PDA) medium. The actively growing bacteria were selected and streaked on the opposite side of the pathogen. The pathogen without antagonists was kept as control. The *in vitro* assay was carried out with four replications for each treatment, along with appropriate controls. The plates were incubated at 28 ± 2°C for seven days, after which the diameter of mycelial growth of the pathogen was measured. The percentage inhibition of mycelial growth compared to the control was calculated using Vincent's formula (15).

$$I = \frac{100\ (C - T)}{C}$$

where I= Percent inhibition over control, C=Pathogen growth in control, T=Pathogen growth in treatment.

Molecular characterization of effective rhizospheric antagonist against *F. equiseti*

Isolation of genomic DNA

The phenol-chloroform method was employed to isolate genomic DNA from effective rhizosphere antagonists, as confirmed by the dual plate method. The effective bacterial isolate was cultured overnight in a 2 mL centrifuge tube at -4°C.

The culture was centrifuged at 10000 rpm for 10 min at room temperature. The supernatant was discarded and the pellet was resuspended in 800 µL of TE buffer with 5 µL of lysozyme, 1 µL of proteinase. And then 45 µL of freshly prepared 10% SDS were added and incubated for 1 hour at 65°C followed by centrifugation at 10000 rpm for 5 min and kept at room temperature. The resultant supernatant was taken and added with an equal volume of phenol-chloroform-isoamyl alcohol (24:1:1) and kept at - 4 ° c for overnight. The incubated sample was then centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and 500 µL of 70 % ethanol was added. After centrifuging at 12000 rpm, the supernatant was removed and the pellet was air-dried. The DNA was dissolved in 50 µL of TE buffer and stored at -20°C. The purity of the genomic DNA was evaluated using agarose gel electrophoresis.

Polymerase chain reaction (PCR)

The extracted DNA sample was prepared using 25 µL of master mix, 19 µL of double-distilled water and 2 µL each of the forward primer (27F: 5' AGAGTTTGATCTGGCTCAG 3') and reverse primer (1492R: 5' AGGGTTGCGCTCGTTG 3'). The PCR reaction was performed using an Eppendorf PCR machine under the following conditions: initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72° C for 2 min. The amplified PCR product was analyzed using 1% agarose gel electrophoresis and visualized with a gel documentation system. The PCR product was sequenced using the Sanger sequencing method. The sequence was analyzed using the NCBI BLAST tool and subsequently submitted to the NCBI database (16).

Metabolic profiling of *B. subtilis* against *F. equiseti* and its interaction

The *B. subtilis* isolate that showed maximum mycelial inhibition against *F. equiseti* under *in vitro* conditions was employed for GCMS to identify the metabolites involved in the suppression of pathogen. Cultures of *B. subtilis* BGKMR1, *F. equiseti* and co-culture of *B. subtilis* and *F. equiseti* were grown in PDA broth for 3 days. The cultures were centrifuged and metabolites was extracted using methanol method. The metabolites were detected through GCMS analysis using a Shimadzu Gas Chromatograph (QP 2020) equipped with an Rxi-5 MS column and a Turbo Mass Gold mass detector. The column employed was an Elite-1 (100 % Dimethyl Polysiloxane), 30 m × 0.25 mm ID. Helium was used as the carrier gas at a flow rate of 1 mL/min. The software used for GC-MS was Turbo Mass 5.1 and it was conducted at the Department of Agricultural Microbiology, Agricultural College and Research Institute, Coimbatore. The selected metabolites were used for pathway analysis to construct metabolic map reflective of the experimental conditions using metaboanalyst 6.0. KEGG metabolic pathways were performed to reveal differences in metabolite enrichment between pathogen and the biocontrol agent (17).

Evaluation of plant growth promotion activity of *B. subtilis* under *invitro* conditions

The plant growth-promoting potential of *B. subtilis* BGKMR1 was assessed on bitter gourd using the roll towel method. Seeds were surface-sterilized with 0.1 % sodium hypochlorite solution and subsequently treated with a suspension of *B. subtilis* (10^{-8} CFU/mL) for 30 min. Control seeds were soaked in sterile distilled water under identical conditions. Both treated and control seeds were placed on sterile blotter papers in the roll towel setup, moistened with sterile distilled water and incubated at 25°C under a 12-hr light/dark cycle. Plant growth parameters including germination percentage, root length, shoot length and vigor index were measured to assess the growth-promoting effect of the bacterial treatment (18).

Efficacy of *B. subtilis* against *F. equiseti* under glasshouse condition

The efficacy of effective *B. subtilis* (BGKMR1) was evaluated under pot culture conditions. Earthern pots were filled with 5 kg of sterilized red soil and sand (1:1) and used for seed sowing. The experiment consisted of six treatments withs four replications (5 seeds per replication) and was conducted in a complete randomized block design. The treatments were as follows: T1 - seed treatment with *B. subtilis* (BGKMR1) at a rate of 10 g/kg of talc-based formulation, T2 - seed treatment with carbendazim (0.1 %) at 1 g/kg of seed. T3- T1 + soil application of *B. subtilis* (0.1 %) at 15-day intervals for the 2nd, 3rd, 4th and 5th spray, T4 -T1 combined with soil application of carbendazim (0.1 %) at 15-day intervals for the 2nd, 3rd, 4th and 5th spray, T5 - pathogen inoculated control, T6- uninoculated control. The pots were maintained with relative humidity at 80 % and temperature range of 26-28 °C. Percent disease severity and disease incidence were recorded for analysis (19) (Table 2).

Table 2. Details of treatment for testing the efficacy of *B. subtilis* against *F. equiseti*

Treatment no	Treatment details
T1	Seed treatment with *B. subtilis* (BGKMR1) (10 gm / kg talc based)
T2	Seed treatment with carbendazim (0.1%) (1gm /kg of seed)
T3	T1 + Soil application with *B. subtilis* (0.1%) @ 15 days interval 2nd,3rd,4th,5th spray
T4	T1 + Soil Application with Carbendazim (0.1%) @ 15 days interval 2nd,3rd,4th,5th spray
T5	Inoculated Control
T6	Uninoculated Control

$$\text{Percent diseases severity} = \frac{\text{Number of leaf} \times \text{grade obtained}}{\text{Total number of leaves} \times \text{maximum grade}} \times 100$$

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected plant}}{\text{Total number of plant observed}} \times 100$$

Defense enzyme analysis

Sample preparation

Leaf material (0.5 g) was blended with 2 mL of a 0.1 M sodium phosphate buffer at a pH of 6.5 by using pestle and mortar and maintained at a temperature of 4 °C. After homogenization, the resulting mixture was centrifuged at 10000 rpm for 2 min. The supernatant devoid of solid debris was collected and utilized as the enzyme source for estimating plant defense enzymes including Phenylalanine ammonia lyase (PAL), Peroxidase (PO) and Polyphenol oxidase (PPO).

Peroxidase Activity (PO)

The PO activity was assessed following the procedure (20). A 100 µL aliquot of enzyme extract was combined with 1.5 mL of 0.05 M pyrogallol (Sigma Aldrich, USA) and 0.5 mL of 1 %

hydrogen peroxide. The absorbance change was measured at 420 nm every 30 seconds for three min at room temperature (28 ± 2 °C). A heat-treated enzyme sample served as the control. The enzyme activity was determined by calculating the change in absorbance of the reaction mixture per minute per gram of fresh tissue.

Polyphenol Oxidase Activity (PPO)

The reaction mixture included 100 Σµl of enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5), with the reaction initiated by adding 200 µl of 0.01 M catechol. The activity change was tracked by measuring the absorbance at 495 nm every 30 seconds for three minutes. The enzyme activity was expressed as the change in absorbance per min per g of fresh tissue (21).

Phenylalanine Ammonia Lyase Activity (PAL)

To measure PAL activity, 200 µL of enzyme extract was mixed with 1.3 mL of water and 0.5 mL of borate buffer. The reaction was started by adding 1 mL of 12 mM L-phenylalanine and incubated at 32 ± 2 ℃ for one hr. The reaction was stopped by adding 0.5 mL of 2 N HCl and a blank was used as a control. Absorbance was read at 290 nm and the enzyme activity was expressed as µmol of cinnamic acid produced per minute per gram of fresh tissue, according to a standard protocol (22).

Statistical Analysis

The visual representations including graphs and charts were generated using R software 4.3.1. A completely randomized design (CRD) was implemented for the *in vitro* studies, while a randomized block design (RBD) was utilized for the *in vivo* experiments.

Results and Discussion

Collection of wilt infected pathogen in bitter gourd

The *F. equiseti* strain CBEFE1 used in this study was identified and documented (13). The strain was obtained and maintained as pure culture in the laboratory. The colony morphology of *F. equiseti* strain CBEFE1 was characterized by pink-colored colonies with fluffy growth and orange pigmentation. Microscopic observations revealed oval-shaped microconidia and three-septate, sickle-shaped macroconidia. Pathogenicity testing confirmed its virulence as bitter gourd plants inoculated with the pathogenic strain exhibited characteristic symptoms, including yellowing of leaves and wilting. Molecular confirmation using universal primers (ITS 1 and ITS 4) further validated the identity of the strain as *F. equiseti*. The sequence was submitted in NCBI (PQ111513.1) The strain's characteristic colony morphology, including orange pigmentation and sickle-shaped macroconidia, matches earlier descriptions of this species (23).

Isolation and antifungal activity of rhizospheric bacteria against *F. equiseti*

Five distinct bacterial isolates were obtained from the rhizospheric region using the serial dilution method at dilutions of 10^{-5} and 10^{-6}. After isolation, these bacteria were subjected to purification through the pure culture technique, ensuring that each bacterial colony was free of contamination and suitable for further testing. The isolated rhizosphere bacteria showed rough surfaces and slimy margins. All five bacterial isolates were screened against *F. equiseti* using the dual plate method. Among the bacterial isolates, *B. subtilis* (BGKMR1) was found to be more effective recording 73.83 % mycelial inhibition over control followed by BGKDM1 with a 62.57 % inhibition and BGANM1 45.58 % showed least mycelial inhibition. The genomic DNA of the BGKMR1 bacteria was extracted and a single band of intact genomic DNA was visualized on the agarose gel. Using the 27f and 1492r primers, the 16s rDNA region approximately 1200 bp in size, was successfully amplified from the genomic DNA. The amplified PCR product of the BGKMR1 isolate was sequenced and submitted in NCBI nucleotide database under the accession number PP930618 and BLAST sequence analysis was performed. The results revealed a sequence match with *B. subtilis* upto 99 % similarity. Phylogenetic analysis indicated that PP930618 shares a close evolutionary relationship with PP702077 and CP103769. Reports are there on the isolation of rhizobacterial strain such as *B. subtilis, Burkholderia sp.*, *B. aryabhatta* and have tested their antifungal efficacy against *F. equiseti* under *in vitro* assay and the result revealed a percent mycelial inhibition of 75 %, 71.25 %, 70.25 % and submitted to GenBank under accession numbers of AB366333, KT265082, KR045599.1 respectively (24) (Table 3) (Fig. 1, 2).

Metabolite profiling of *B. subtilis* against *F. equiseti* and its interaction

Metabolite Profiling

GC-MS analysis of *F.equiseti* revealed the presence of unique metabolites such as squalene (8.88 %), cyclohexanol (7.37 %), bis (2-ethylhexyl) phthalate (6.87 %), n-hexadecanoic acid (6.99 %) and fumaric acid (0.52 %). Squalene, a precursor in sterol biosynthesis, plays a critical role in maintaining fungal membrane integrity, aiding survival and enhancing virulence during host infection. Similar bioactive compounds, including coumarin, nonadecane and hexadecanoic acid have been identified in other *F. equiseti* isolates (25). The major metabolites detected in *B. subtilis* were 1,4-benzenedicarboxylic acid (6.1%), bis (2-ethylhexyl) phthalate (6.85%), propanoic acid (4.94 %), dodecanoic acid (3.36 %) and pentadecanoic acid. The finding was compared with previous report which showed compounds such as 1,4-benzenedicarboxylic acid and bis (2-ethylhexyl) phthalate exhibiting antimicrobial properties potentially

Table 3. Antifungal activity of rhizospheric bacteria against *F. equiseti*

T.no	Isolate code	Percent mycelial growth	Percent mycelial inhibition
1	BGKMR1	28.27^{e}	68.73^{a}
2	BGVKI1	45.62^{d}	49.31^{b}
3	BGANM1	59.65^{c}	33.72^{c}
4	BGKPM1	67.23^{b}	25.3^{d}
5	BGKDM1	73.35^{a}	18.5^{e}
	Control	90	90
	CD (p = 0.05)	1.38	0.79

* Values of mean of four replicates

* Values in parentheses are arcsine transformed values

* In a column mean followed by a common letter are not significantly different at the 5% level by Duncan multiple range test.

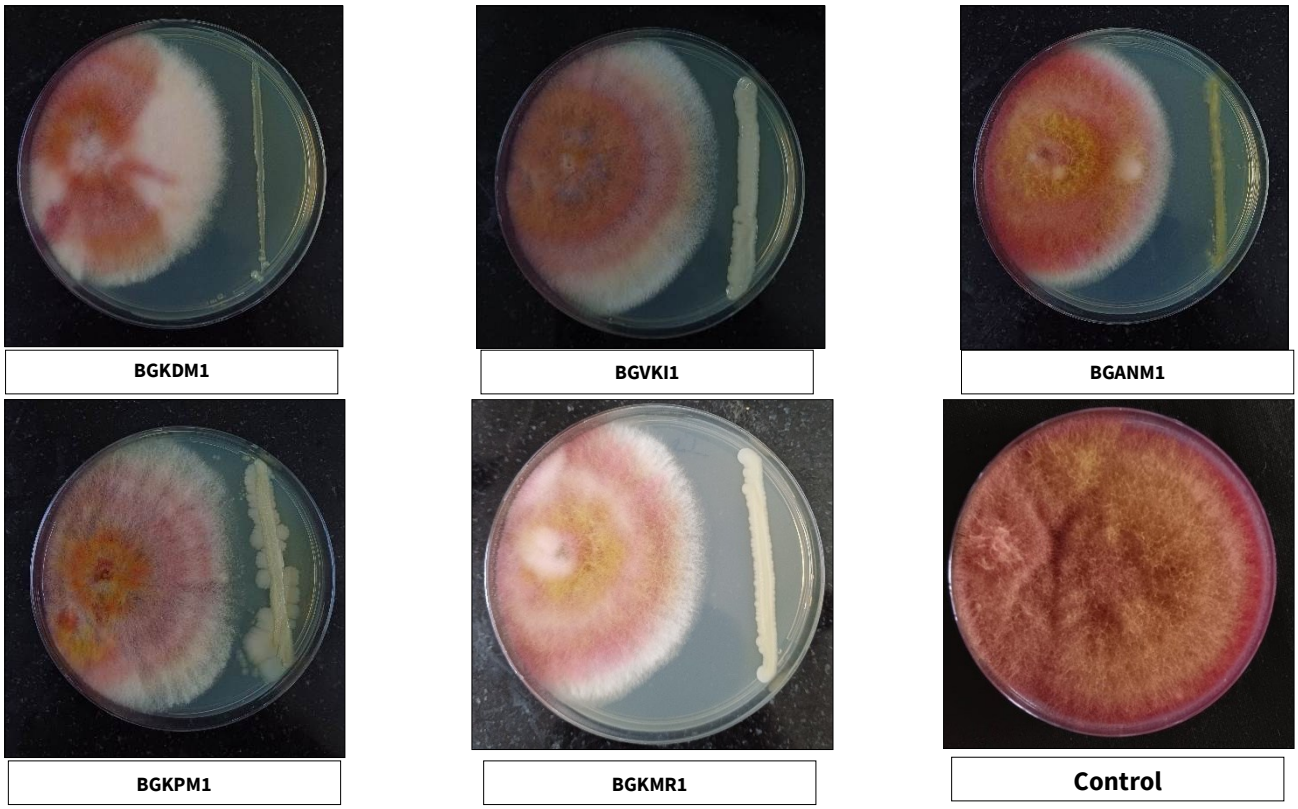

Fig. 1. Antifungal activity of isolated rhizospheric bacteria against *F. equiseti* (Dual-plate assay).

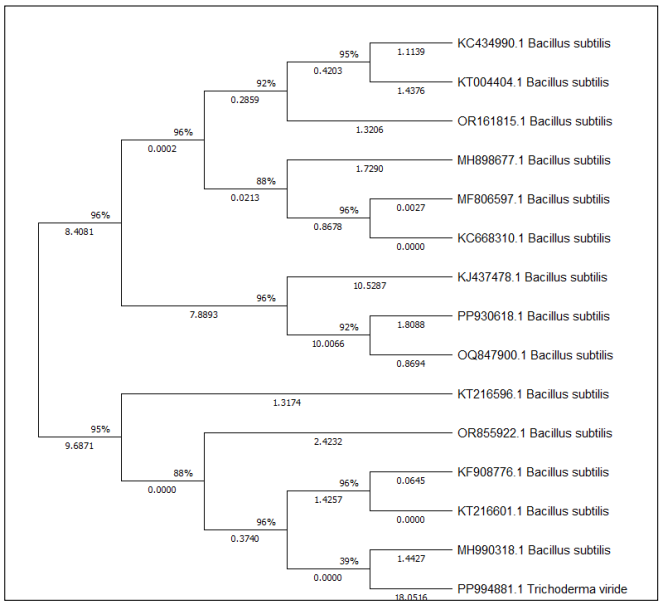

Fig. 2. A phylogenetic tree illustrating the evolutionary relationships of *B. subtilis* PP930618.1, constructed using the maximum likelihood method based on the 16S rDNA region. Bootstrap values (in percentages) are indicated at the nodes, reflecting the confidence levels of the clustering. The scale bar represents the evolutionary distance in terms of substitutions per site.

disrupting fungal enzyme systems or metabolic pathways essential for fungal growth (26). During the interaction between *B. subtilis* and *F. equiseti*, additional metabolites were detected including propanoic acid (14.69 %), dimethyl (S)-(-)-malate (8.18 %), 2-butanone (7.65 %) and 2-thiazolamine (7.76 %). Propanoic acid, a short-chain fatty acid (SCFA) is known to lower local pH, creating an inhospitable environment for fungal pathogens while disrupting their metabolic processes. These findings align with previous studies, where propionic acid was characterized as SCFAs and their derivatives as critical contributors to the antifungal activity of *B. subtilis* (27) (Fig. 3) (Table S1, S2, S3).

Pathway Analysis

The metabolic pathways of *F. equiseti* identified ten major pathways, including nitrogen, phenylalanine and propanoate metabolism. In particular, phenylalanine metabolism plays a role in synthesizing secondary metabolites such as phenylacetic acid and polyketides, which suppress plant defenses and contribute to fungal virulence. Similarly, studies on *F. equiseti* in soybean identified enriched vitamin B6 metabolism pathways, which are essential for fungal infection processes (28). In contrast, pathway analysis of *B. subtilis* highlighted nitrogen metabolism, butanoate metabolism and alanine, aspartate and glutamate metabolism. Nitrogen metabolism regulates the production of antifungal peptides like surfactins and iturins, which disrupt fungal membranes and inhibit spore germination. Previous reports identified metabolic pathways in *B. subtilis* related to valine, leucine, and isoleucine biosynthesis (29). The metabolic pathways involved in the interaction between *F. equiseti* and *B. subtilis* were identified including propanoate metabolism, phenylalanine metabolism, butanoate metabolism, alanine, aspartate and glutamate metabolism, glutathione metabolism, glyoxylate and dicarboxylate metabolism, glycine, serine and threonine metabolism, cysteine and methionine metabolism, drug metabolism and amino sugar and nucleotide sugar metabolism.The propanoate metabolism pathway interfere with fungal carbohydrate or amino acid metabolism, reducing energy production and growth. A similar finding was earlier observed where a comparative analysis of nontargeted metabolites of *B. subtilis* against *F. oxysporum* identified 79 different metabolites (30). KEGG analysis indicated that these metabolites were involved in 10 metabolic pathways, with three pathways related to amino acid metabolism, specifically alanine, aspartate and glutamate metabolism (Fig. 4).

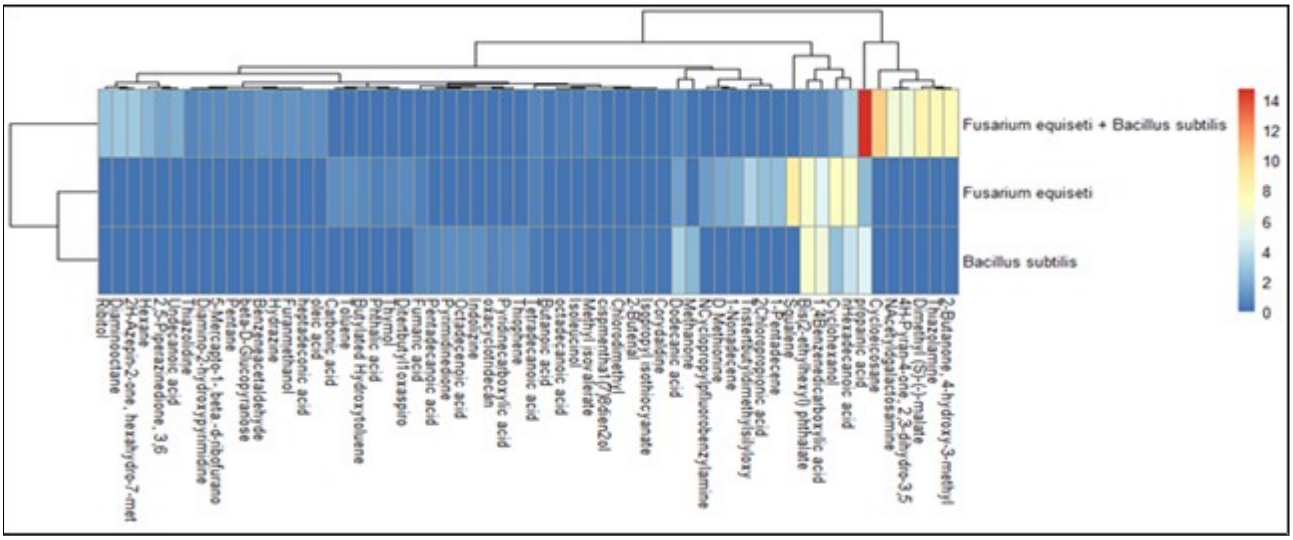

Fig. 3. Heatmap showing the bipartite interaction between *F. equiseti* and *B. subtilis* BGKMR1. The heatmap illustrates metabolite ratios associated with their interaction, with significant metabolites (p < 0.01) highlighted. Red indicates a high metabolite ratio, blue represents a moderate ratio and white indicates a low ratio. Propionic acid is marked in red, indicating its high abundance. Squalene and 1,4-benzene dicarboxylic acid are represented in blue, indicating a moderate ratio. Ribitol and diaminooctane are shown in white, indicating a low ratio.

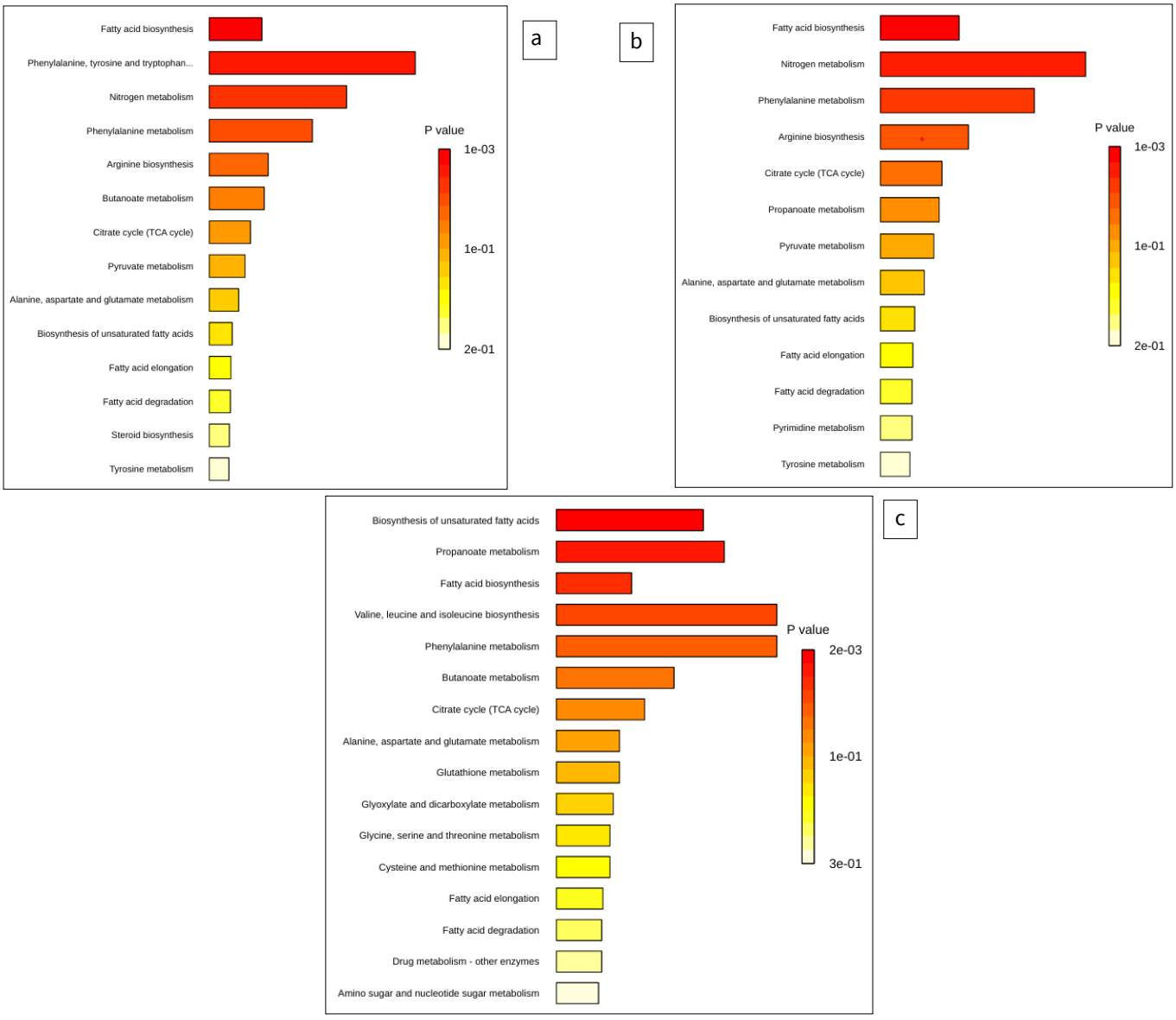

Fig. 4: KEGG pathway enrichment analysis showing significant pathways (log p < 0.05) for a) *F. equiseti*, b) *B. subtilis* BGKMR1 c) their interaction. In *F. equiseti*, red bars indicate high enrichment for phenylalanine, pyruvate and nitrogen metabolism, while white bars represent low enrichment for tyrosine metabolism. In *B. subtilis* BGKMR1, red bars highlight nitrogen metabolism and white bars show low enrichment for tyrosine metabolism. The interaction analysis reveals high enrichment for propanoate metabolism (red) and low enrichment for amino sugar and nucleotide sugar metabolism (white).

Evaluation of plant growth promotion activity of *B. subtilis* BGKMR 1 under invitro

Seed treatment with talc-based *B. subtilis* formulation resulted in significant improvements in plant growth promotion, achieving a germination of 96 % and a vigor index of 1919.04. A similar outcome was observed in the study, where seeds treated with *B. subtilis* culture broth after being immersed in water at 60 °C for 10 min showed an increase in germination percentage from 65 % to 83.3 % (31) (Table 4) (Fig. 5).

Table 4. Evaluation of pant growth promotion activity of *B. subtilis* BGKMR 1 under *in vitro* by roll towel method

Treatment	Germination Rate (%)	Vigour index
B.subtilis (1×10^8 CFU)	96%	1919.04
Control	49%	614.46

a. Control b. *B. subtilis* (1× 10^8 treated)

Fig. 5. Evaluation of plant growth promotion activity of *B. subtilis BGKMR 1* (Roll-towel method).

Efficacy of *B. subtilis* BGKMR1 against *F. equiseti* under glasshouse condition

The study assessed the effectiveness of *B. subtilis* BGKMR 1 against *F. equiseti* under glasshouse condition. The result of the study indicates that seed treatment with *B. subtilis* (BGKMR1) at a rate of 10 g/kg shows disease incidence upto 38.66% and 47.98% reduction compared to the control followed by seed treatment with carbendazim (0.1 %) which showed lower disease incidence of 33.22 % and 55.30 % reduction over control. The combination of seed treatment and soil application of *B. subtilis* (10 mL/kg of seed) further reduced the disease incidence to 25.95 %, achieving a 65.08 % reduction over the control followed by carbendazim (0.1 %) seed treatment with soil application 0.1 %. These results indicate that both seed treatment with soil application of *B. subtilis* BGKMR 1 have the greatest efficacy against *F. equiseti* under glass house condition. Similarly, applying *B. subtilis* through seed treatment and soil applications under pot conditions led to disease reductions of up to 29.7 % (32) (Table 5) (Fig. 6).

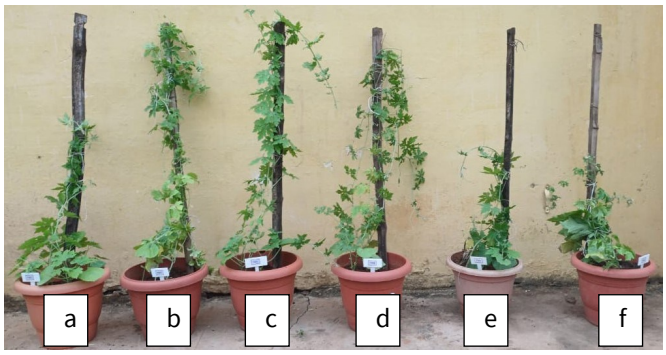

Fig. 6. Efficacy of *B. subtilis* (BGKMR1) against *F. equiseti* under glasshouse condition. a. Seed Treatment with *B. subtilis* b. Seed treatment with carbendazim c. Seed treatment and soil application of *B. subtilis* d. Seed treatment and soil application of carbendazim e. Inoculated control f. uninoculated control.

Defense enzyme assay

Plants treated with *B. subtilis* BGKMR 1 showed enhanced enzymatic activity across all assays compared to the controls, demonstrating a robust defense response. In the peroxidase (POD) assay, activity peaked at 4.10 OD/min/gm on day 75. Increased POD activity is associated with the strengthening of cell walls through lignification, which hinders pathogen penetration. Similarly, polyphenol oxidase (PPO) activity reached a maximum of 3.50 OD/min/gm on day 75, indicating an enhanced biochemical defense mechanism that produces toxic quinones, creating an unfavourable environment for the pathogen. Phenylalanine ammonia-lyase (PAL) activity peaked at 18.75 OD/min/gm on day 75, reflecting heightened production of phenolic compounds, which play a crucial role in plant defense. PAL also facilitates the synthesis of salicylic acid precursors, a key signal in systemic acquired resistance (SAR). Compared to the uninoculated and inoculated controls, the treatment with *B. subtilis* significantly increased the activity of POD, PPO and PAL, suggesting its potential as a biocontrol agent against *F. equiseti*. These findings align with previous studies, as the work on *F. oxysporum* in bitter gourd, where treatment with *Trichoderma asperellum* T-66 significantly increased enzymatic activities, strengthening plant defences (33) (Fig. 7).

Table 5. Efficacy of *B. subtilis* BGKMR1 against *F. equiseti* under glasshouse condition

Treatment detail	Diseases incidence (%)	Percent reduction over control (%)
Seed Treatment with *B. subtilis* (BGKMR1)(10 gm / kg talc based)	38.66^{d}	47.98^{a}
Seed treatment with Carbendazim (0.1%) (1gm/kg of seed)	33.22^{c}	55.3^{b}
T1 + Soil Application with *B. subtilis* (0.1%) @ 15 days interval 2nd, 3rd, 4th, 5th spray	25.95^{b}	65.08^{c}
T1 + Soil Application with Carbendazim (0.1%) @ 15 days interval 2nd, 3rd, 4th, 5th spray	20.65^{a}	72.12^{d}
Inoculated Control	98.15	98.15
uninoculated control	74.33	74.33
C.D(p=0.05)	0.86	1.6

* Values of mean of four replicates
* Values in parentheses are arcsine transformed values
* In a column mean followed by a common letter are not significantly different at the 5% level by Duncan multiple range test

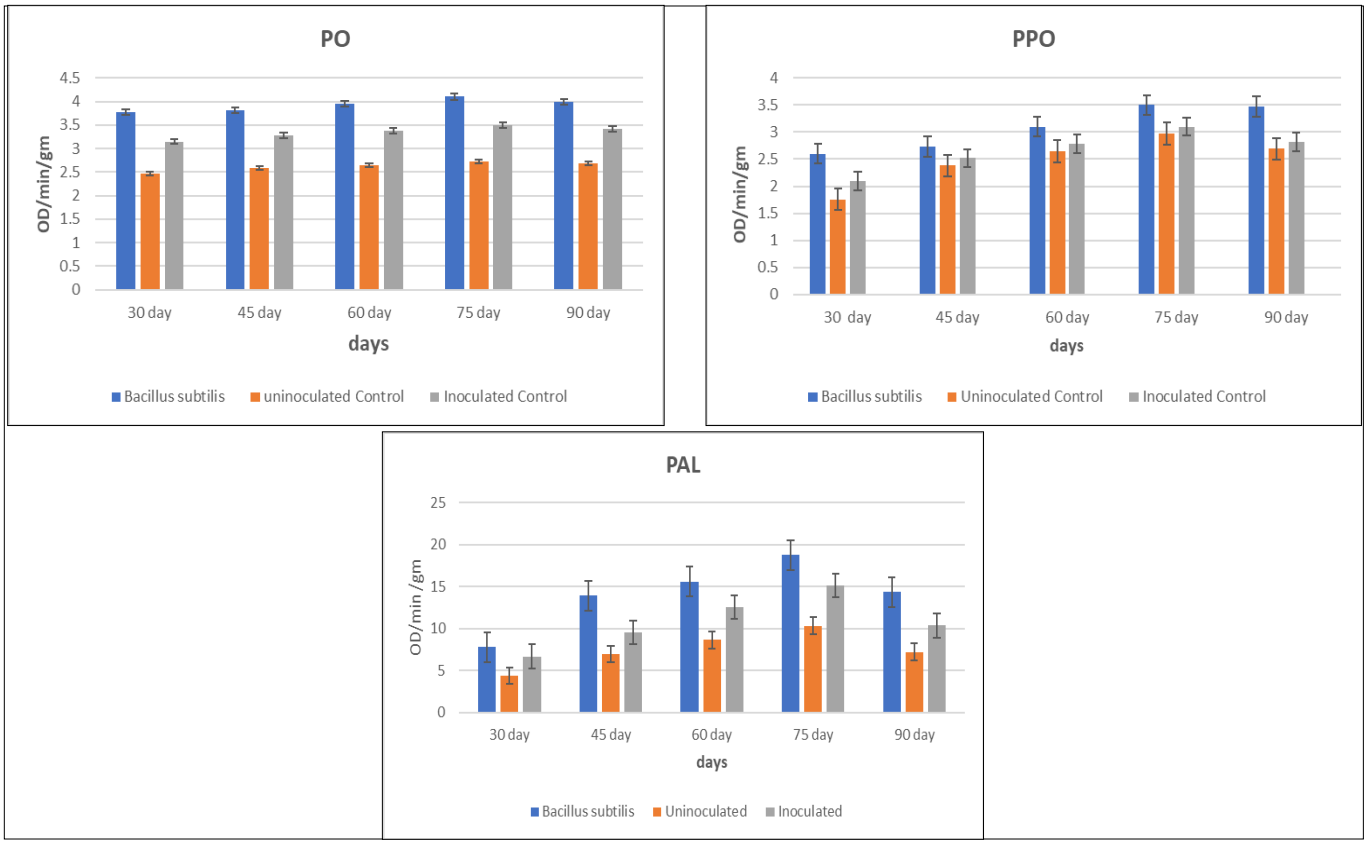

Fig. 7. Defense enzyme assay (a..PO, b.PPO, c.PAL) of *B. subtilis* (BGKMR1) against *F. equiseti* in bitter gourd plants.

Conclusion

This study demonstrates that *B. subtilis* BGKMR1 is an effective biocontrol agent against *F. equiseti*, the wilt pathogen in bitter gourd. It significantly reduced disease incidence, improved seed germination and enhanced plant defence enzyme activity, promoting systemic resistance. Key antifungal metabolites and beneficial metabolic pathways were identified, highlighting its ability to suppress fungal growth and promote plant health. By delving deeper into the metabolic interactions of *B. subtilis*, researchers can uncover the specific metabolites and biochemical pathways involved in these processes. This knowledge will enhance the use of *B. subtilis* in sustainable agriculture, leading to healthier plants and reduced reliance on chemical pesticides.

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Authors' contributions

RS carried out experiment and data recorded, VS formulating the research concepts and approved the final manuscript, HS research ideas and reviewing the articles, JI helped in laboratory techniques, IVP summarizing and revising the manuscript, SA contribute for defence enzymes related studies.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical issues: None

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