Bacillus thuringiensis - a potent PGPR showing *in vitro* antagonism against *Fusarium equiseti* and *in vivo* suppression of yellow leaf disease of *Brassica juncea*

SHAMBHU SWARNAKAR AND ARKA PRATIM CHAKRABORTY*
Department of Botany, Raiganj University, Raiganj, Uttar Dinajpur-733134, West Bengal

Yellows symptoms in leaves of *Brassica juncea* was noticed during January and February 2022 form the area of Khowaspur (Lat 25.7726710; Long 88.0328480), Karandighi, Uttar Dinajpur District, West Bengal. The initial signs of the disease were irregularly shaped dull yellow regions along the leaf margins. Lesions could be sparse and dispersed throughout the leaves or densely packed over vast areas of leaves.On the basis of morphology of the fungal mycelia, various shapes and sizes of conidia by compound, scanning electron microscopy and molecular methods the fungus was identified as *Fusarium equiseti* (ON783721) was   and was submitted to NCBI GenBank. *Bacillus thuringiensis* (OQ415951), isolated from rhizospheric soil of *Brassica juncea* showed positive response in IAA production, phosphate solubilization, catalase production, starch solubilization. *B.thuringiensis* showed *in vitro* antagonism against *Fusarium equiseti* (ON783721). *B.thuringiensis* also inhibited the growth of the tested pathogen markedly. The scanning electron microscopic image of the transition zone between mycelia of *F. equiseti* and cell of *B. thuringiensis* during *in vitro* study was analyzed. GC MS analysis showed several antifungal compounds produced by *B. thuringiensis*. GC MS analysis result revealed the presence of major components including Tris esters, 1,2 bis benzene thymol, N-methyl-1- adamantaneacetamide etc. Field experiments confirmed significant improvement in plant growth parameters and reduction in disease severity in *B. thuringiensis* treated plants. The present study has highlighted the role of potent PGPR- *Bacillus thuringiensis* as plant growth promoter and biocontrol agent against the fungal pathogen infecting mustard plants.

Keywords : *Bacillus thuringiensis*, *Fusarium equiseti*, GC MS analysis, *In vivo* disease suppression, *In vitro* antagonism, Yellow leaf disease

INTRODUCTION

Mustard (*Brassica juncea* L.) is a vital oilseed crop that holds a prominent position in global agriculture, particularly within India, which is among the largest producers and consumers of mustard seed and oil (Ahmad *et al*. 2008).

The crop contributes significantly to livestock feed, human nutrition, and various agro-industrial applications, making it an essential component of sustainable agriculture and rural livelihoods. The economic importance of mustard is underscored by its role in providing edible oil and protein-rich seeds, which are vital for improving food security and nutritional security in many regions (Singh *et al*. 2015).

Despite its economic significance, mustard cultivation faces numerous challenges, with biotic stresses being the most pressing constraints affecting yield stability and crop quality. Among these, fungal pathogens are particularly problematic, causing a range of diseases that impair photosynthesis, reduce overall productivity, and deteriorate oil quality. The most common symptoms include yellowing of the leaves, which is often associated with pathogen attack, leading to decreased photosynthetic capacity and ultimately yield loss (Chakraborty and Chakraborty,2015; Nandi *et al*. 2022). Fungal diseases such as leaf blights, wilt, root rot, and foliar spots are widespread and pose a significant threat to sustainable production systems.

The genus *Fusarium* comprises a diverse group of soil-borne fungi notorious for their pathogenicity

*Correspondence :arka.botanyrgu@gmail.com

across a broad spectrum of crops worldwide. Species within this genus, such as *Fusarium oxysporum* and *Fusarium solani*, are well-documented for their ability to cause wilt diseases, root rot, and leaf blight, resulting in substantial economic losses. Among these, *Fusarium equiseti* has emerged as an increasingly prevalent pathogen affecting several crop species, including mustard. This pathogen is recognized for its capacity to survive in soil for extended periods, produce potent mycotoxins, and infect multiple host plants, thereby complicating management strategies (Gajera *et al.* 2016; Manasa *et al.* 2017).

Traditional control methods primarily involve the application of chemical fungicides, which, while effective to some extent, are associated with various drawbacks. These include increasing production costs, environmental contamination, and potential health hazards to farmers and consumers (Sant *et al.* 2011; Chakraborty *et al.* 2013). The growing concerns over pesticide residues and the development of fungicide-resistant pathogen populations necessitate the exploration of eco-friendly and sustainable disease management alternatives.

In recent years, the focus has shifted toward biological control agents, particularly plant growth-promoting rhizobacteria (PGPR), as viable, environmentally sustainable options. PGPR are beneficial microbes that colonize plant roots and enhance plant growth through multiple mechanisms. These include synthesis of phytohormones such as indole-3-acetic acid (IAA), solubilization of essential nutrients like phosphates, nitrogen fixation, and production of bioactive antifungal metabolites that suppress phytopathogens (Lugtenberg and Kamilova, 2009; Glick, 2012). The use of PGPR not only promotes plant growth but also improves resistance against pathogen attack, reducing dependence on chemical inputs.

Bacillus thuringiensis (Bt), predominantly recognized for its insecticidal properties, has garnered attention for its antagonistic potential against fungal pathogens. While its role in biocontrol of insect pests is well-established, recent studies indicate that certain strains of Bt can produce antifungal compounds, inhibit the growth of phytopathogenic fungi, and enhance plant health (Akram et al., 2013; Kaur et al., 2020; Sharma et al., 2018). The dual activity of Bt as an insecticide and a biocontrol agent presents an integrated approach to crop management.

This study aims to investigate several interconnected aspects of disease management in mustard cultivation which will involve pathogen and antagonist isolation and their testing both in vitro and in vivo. The disease to be studies is leaf yellowing of *B. juncea* in the Khowaspur region of Uttar Dinajpur, West Bengal.

MATERIALS AND METHODS

Study site and sample collection

Field surveys were conducted during January–February 2022 in Khowaspur (Lat 25.7726710; Long 88.0328480), Karandighi block, Uttar Dinajpur district, West Bengal. Mustard plants showing distinct yellow leaf symptoms were selected for pathogen isolation. Leaf samples with lesions were collected in sterile polythene bags, transported to the laboratory, and processed immediately. Rhizospheric soil samples were also collected from the same plants for bacterial isolation (Swarnakr *et al.* 2022).

Isolation and identification of fungal pathogen

Symptomatic leaf pieces were surface sterilized in 0.1% sodium hypochlorite for 1 min, rinsed thrice in sterile distilled water, and plated on potato dextrose agar (PDA). Fungal colonies were purified by hyphal tip method (Barnett *et al.* 1998). Morphological characteristics of mycelia and conidia were examined under compound microscopy and scanning electron microscopy (SEM). Genomic DNA was extracted using a fungal DNA kit. The internal transcribed spacer (ITS) region was amplified by PCR and sequenced. BLAST analysis confirmed the pathogen as *Fusarium equiseti*, and the sequence was deposited in GenBank (Accession No. ON783721) (Leslie *et al.* 2006; Gupta *et al.* 2014).

Isolation and biochemical characterization of bacteria

Rhizospheric soil samples were serially diluted and plated on nutrient agar. Colonies with distinct morphology were isolated and purified. The most promising isolate was subjected to biochemical assays including IAA production (Salkowski reagent method followed by HPLC quantification) (Pikovskaya *et al.* 1948), phosphate solubilization on Pikovskaya's agar (Borriss*et al.* 2011), catalase activity, and starch hydrolysis test.

Antagonistic assay

Dual culture assay was performed by co-culturing *B. thuringiensis* with *F. equiseti* on PDA plates (Akram *et al.* 2013). The inhibition percentage was calculated using the formula :

$$\text{Inhibition (\%)} = \frac{R1 - R2}{R1} \times 100$$

where R1 = radial growth of the pathogen in control, and R2 = radial growth in the presence of the bacterium.

Scanning electron microscopy of interaction zone

Mycelial discs of *F. equiseti* were co-cultured with bacterial cells, and the transition zone was fixed in 2.5% glutaraldehyde, dehydrated in ethanol series, gold-coated, and examined under SEM to study bacterial–fungal interactions (Vinale *et al.* 2008).

GC–MS analysis of bacterial metabolites

Culture filtrates of *B. thuringiensis* were extracted with ethyl acetate and concentrated under vacuum. The crude extract was analyzed by GC–MS (Agilent system), and compounds were identified by comparing mass spectra with NIST library database (Raaijmakers *et al.* 2006).

Transmission Electron Microscopy (TEM) Sample Preparation

To compare internal leaf structures of healthy and *Fusarium equiseti* –infected Mustard plants, samples (1–2 mm) were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) following standard TEM protocols (Graham*et al.* 2007; Swarnakar *et al.* 2022). Fixation was performed at room temperature, followed by post-fixation at 4°C. Samples were washed with phosphate buffer and dehydrated through a graded ethanol series (30–100%). Resin infiltration was carried out with LR White resin, followed by polymerization at 56°C. Ultrathin sections were cut using a Leica EM UC7 ultramicrotome, mounted on grids, and examined under a CRYO-TEM (TALOS S, Thermo Scientific) at AIIMS, New Delhi.

Field experiments

Field experiments were conducted to evaluate the effect of plant growth–promoting rhizobacteria (PGPR)- *B. thuringiensis* and pathogen- *F. equiseti* inoculation on mustard (*Brassica* sp.) varieties B-9, B-54, and NRCYS-05-02 under field conditions. The experiment was laid out in a randomized complete block design (RCBD) to minimize variability due to soil and environmental heterogeneity.

Four treatments were included: untreated control, application of *B. pumilus* alone, *F. equiseti* alone, *F. equiseti* combined with *B. thuringiensis*. A factorial arrangement of three varieties and four treatments (3 × 4) was followed. Each treatment was replicated three times and all treatment–variety combinations were randomly allocated within each block. Individual plots measured 3 × 3 m or 4 × 3 m, with a buffer space of 0.5–1.0 m maintained between plots to prevent cross-contamination. Mustard seeds were sown at a row spacing of 30–45 cm and plant spacing of 10–15 cm following recommended agronomic practices. For PGPR treatments, seeds were coated separately with *B. thuringiensis* (10^8 cfu) using 1–2% adhesive (Tween 20) prior to sowing. Pathogen inoculation was carried out in designated plots, while combined treatments received both pathogen and respective PGPR strains (Yang *et al.* 2024).

RESULTS AND DISCUSSION

Pathogen and rhizospheric bacterial isolation and identification

Yellowing symptoms in *Brassica juncea* leaves initially appeared as irregular patches along the leaf margins, later progressively enlarging to cover extensive areas of the leaf surface. These lesions

were either sparsely dispersed or densely aggregated, often coalescing to form larger necrotic zones leading to significant tissue deterioration. Morphological examination of the pathogenic isolates revealed septate mycelia and spindle-shaped conidia, which are characteristic features of *Fusarium* spp. Based on these morphological features, coupled with molecular characterization, the pathogen was definitively identified as *Fusarium equiseti* (Gen Bank Accession: ON783721). The molecular identification was confirmed through PCR amplification and sequence analysis, aligning closely with existing sequences in the NCBI database, and consistent with prior reports of *F. equiseti* infecting crop plants in diverse agro-ecosystems (Manasa et al., 2017; Gajera et al., 2016; Gupta et al., 2014; Swarnakar et al., 2023). Parallel to pathogen identification, a bacterial isolate from the rhizosphere was characterized for plant growth-promoting traits. The isolate, identified as *Bacillus thuringiensis* (GenBank Accession: OQ415951).

In vitro traits of the bacterium

This bacterium demonstrated multiple beneficial activities, including production of indole-3-acetic acid (IAA), phosphate solubilization, catalase activity, and starch hydrolysis. (Table 1, Fig. 1). Quantitative estimation of IAA via high-performance liquid chromatography (HPLC) showed production levels of 139.271 ng/il, markedly higher than the control sample supplemented with tryptophan (121.512 ng/il). The bacterium produced significant levels of indole-3-acetic acid (IAA), which is consistent with earlier reports where PGPR enhanced plant growth through hormone modulation (Leslie *et al.* 2006; Chakraborty et al. 2022). The IAA concentration detected in this study was higher than the control, indicating its ability to utilize tryptophan precursors efficiently. Similar findings were reported by Manasa *et al.* (2017), where PGPR-mediated IAA enhanced root elongation and nutrient uptake in oilseed crops. This indicates a robust IAA biosynthesis capacity, crucial for promoting root elongation and plant growth under biotic and abiotic stresses (Fig. 2) (Swarnakar & Chakraborty, 2025).

In vitro antagonism

The antagonistic potential of *B. thuringiensis* against *F. equiseti* was evaluated through dual culture assays. Results demonstrated notable fungal growth suppression, with an inhibition rate of approximately 83%. A clear zone of inhibition emerged between bacterial colonies and fungal hyphae. Under microscopic observation, the fungal hyphae exhibited distorted and deformed structures, indicating direct antagonism and cellular disruption. Scanning electron microscopy (SEM) provided further insights, showing colonization of *B. thuringiensis* cells around hyphal surfaces, which correlated with physical disintegration of the hyphal structure and collapse of the mycelial network (Fig. 3). Such morphological distortions suggest that bacterial metabolites or cell wall-degrading enzymes produced during interaction exert potent antifungal effects. In this study, *Bacillus thuringiensis* isolated from rhizospheric soil exhibited multiple plant growth-promoting attributes. Apart from growth promotion, *B. thuringiensis* demonstrated remarkable antagonism (83%) against *F. equiseti*. Dual culture assays have been extensively used to evaluate microbial antagonism, and Bacillus species are consistently among the most effective biocontrol agents (Gajera *et al.* 2016). For instance, *B. subtilis* and *B. amyloliquefaciens* have been shown to inhibit *Fusarium* spp. through competition, antibiosis, and mycoparasitism (Sant *et al.* 2011). Our SEM observations further confirmed that *B. thuringiensis* colonized the pathogen's hyphae and disrupted their integrity, which correlates with similar microscopic evidence reported in *Bacillus-Fusarium* interactions (Lugtenberg *et al.* 2009).

GC- MS analysis of bacterial culture filtrate

Further chemical analysis of *B. thuringiensis* culture filtrates using gas chromatography–mass spectrometry (GC-MS) identified various bioactive compounds with known antifungal activity. Prominent among these were tris esters, 1,2-bis benzene thymol, and N-methyl-1-adamanta-neacetamide, compounds recognized for their antimicrobial properties. These metabolites likely contribute to the biocontrol efficacy observed *in vitro* and may play a role in plant pathogen suppression *in vivo* (Fig. 4; Table 2) (Raaijmakers et al., 2006). The GC–MS analysis of *B. thuringiensis* culture filtrate revealed antifungal

Table 1 : *In vitro* Plant growth–promoting traits of *Bacillus thuringiensis*

PGPR isolate	IAA production	Phosphate solubilization	Siderophore production	Catalase activity	Starch solubilization
Bacillus thuringiensis	++	++	++	++	++

(+ indicates positive reaction- activity present)

Table 2 : Some major compounds found in the culture filtrate of *Bacillus thuringiensis* in solvent ethylacetate
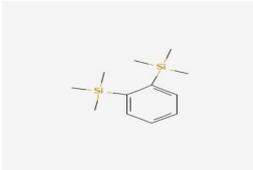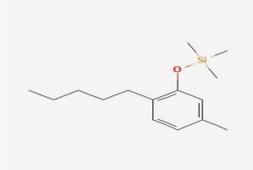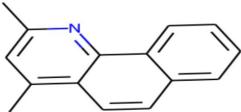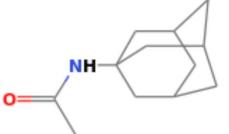
Compound name	Molecular formula	Mol.Weight (g/mol)	RT	Area %	Molecular Structure	Bioactivity
1,2-Bis(Trimethylsilyl)Benzene	$C_{12}H_{22}Si_2$	222.47	17.301	95.61		Antimicrobial Activity
Trimethyl-(5-methyl-2-propan-2-ylphenoxy)silane	$C_{16}H_{26}OSi$	278.51	17.301	95.61		Antimicrobial Activity
Arsenous acid, Tris(trimethylsilyl) ester	$C_9H_{27}AsO_3Si_3$	342.49	17.301	95.61		Antimicrobial antioxidant activity
Benzo(h)quinoline, 2, 4-Dimethyl-	$C_{15}H_{13}N$	207.27	17.591	4.39		Antimicrobial Activity
Methyltris(trimethylsiloxy) silane	$C_{10}H_{30}O_3Si_4$	310.7	17.591	4.39		Antimicrobial antioxidant activity
N-Methyl-1-adamantaneacetamide	C13H21NO	207.31	17.591	4.39		Antimicrobial Activity, Antimutagen Activity, Anti-inflammatory Activity

Table 3 : Effect of *B. thuringiensis* on growth parameters of mustard in terms of % increase in plant height and root length

Treatment	% increase in height after			% increase in root length after		
	15 days	30 days	45 days	15 days	30 days	45 days
Untreated healthy	22±1.15	30±1.23	42±1.15	09±0.577	14±0.69	19±0.115
Treated with *B. thuringiensis*	39±0.115	54±0.35	64±0.115	13±1.154	19±0.577	24±1.23

Mean of 10 replicate plants / treatment; Differences between control and treatments significant at P=0.05

Table 4 : Evaluation of Yellow Leaf disease development of mustard following application of *B. thuringiensis*

	Leaf Disease Index*	
Plant Mustard	Untreated Healthy inoculated with *F. equiseti*	Treated with *B. thuringiensis* and inoculated with *F. equiseti*
% of leaf area affected	80.3	8.7
Category	Highly Susceptible (HS)	Resistant (R)

*Average of 10 plants /treatment – 45 days after inoculation with *F. equiseti* Disease score: 0-5 0=0%lesion area; disease reaction= Highly Resistant (HR)
1=1-10%lesion area; disease reaction= Resistant (R); 2=11-30%lesion area; disease reaction= Moderately Resistant (MR); 3=31-50%lesion area; disease reaction= Moderately Susceptible (MS); 4=51-75%lesion area; disease reaction= Susceptible (S); 5=76-100%lesion area; disease reaction= Highly Susceptible (HS)

compounds such as thymol derivatives, Tris esters, and N-methyl-1-adamantaneacetamide. Thymol and its derivatives are well-known for their broad-spectrum antimicrobial activities, including disruption of fungal membranes (Glick *et al.* 2012). Likewise, adamantane derivatives have been reported to interfere with sterol biosynthesis in fungi, thereby inhibiting mycelial growth (Arora *et al.* 2017). These findings corroborate the hypothesis that secondary metabolites play a crucial role in the antagonistic activity of PGPR. Our results extend the functional role of *B. thuringiensis*, which is primarily recognized as an insecticidal bacterium producing Cry and Vip proteins (Tilak *et al.* 2006). Recent studies, however, have suggested that B. thuringiensis also synthesizes bioactive metabolites with antifungal properties (Akram *et al.* 2013), making it a dual-purpose bioagent. The dual activity—plant growth promotion and pathogen suppression—makes *B. thuringiensis* an attractive candidate for integrated disease management strategies.

Ultrstructural studies in situ

The present investigation identified *Fusarium equiseti* as the causal organism of yellowing symptoms in *Brassica juncea* in Uttar Dinajpur, West Bengal. Transmission electron microscopy (TEM) analysis of infected leaves revealed significant ultrastructural alterations. Disruptions were observed in chloroplast organization, vacuole integrity, and the presence of balloon-shaped aberrations—potential indicators of cellular stress and pathogen-induced damage. These structural changes compromise photosynthesis and overall cellular function, ultimately leading to symptom development and plant decline (Fig. 5). The TEM data provided valuable insights into the cellular-level interactions between the pathogen, host, and biocontrol bacteria, highlighting the potential for microbial antagonists to mitigate disease progression effectively. Although *Fusarium oxysporum* and *F. solani* are more frequently reported in association with wilt and root rot of mustard and other oilseed crops (Ahmad *et al.* 2008; Nandi *et al.* 2022), our findings add evidence that *F. equiseti* can also infect mustard foliage. Previous studies have indicated that *F. equiseti* is a cosmopolitan pathogen capable of infecting cereals, legumes, and oilseeds, often resulting in chlorosis, necrosis, and yield losses (Goswami *et al.* 2004).

In vivo test

In keeping with the strong antagonism and metabolite production observed under controlled conditions, plants treated with *B. thuringiensis* exhibited significantly improved growth

Fig. 1: *In vitro* Plant growth–promoting traits of *Bacillus thuringiensis* : IAA Production (A) ; Catalase test (B); Phosphate solubilisation (C); Starch solubilisation (D); Siderophore production (E); Antibiotic sensitivity test (F).

Fig 2 : (A) IAA production, (B) Analysis of tryptophan and IAA content of culture filtrate of *B. thuringiensis strain showed increased* production of tryptophan- 121.5 ng/micro liter and IAA- 139.27ng/micro liter as compared to control through HPLC analysis.

Fig 3 : (A) SEM image of the isolate- MUSRH-SL-02, (B & C) *In vitro* antagonism of *Bacillus thuringiensis* against *Fusarium equiseti,* (C) Interaction between cell of bacterium *Bacillus thuringiensis* and mycelium of *Fusarium equiseti*

Fig 4: GC-MS analysis of the culture filtrate of *Bacillus thuringiensis* showed some presence of antifungal compounds that may be effective to control *Fusarium equiseti*.

Fig 5: (A) Healthy *Brassica* leaves under transmission electron microscopy, (B) Infected *Brassica* leaves under transmission electron microscopy

Fig 6: Evaluation of *in vivo* growth promoting activities of *B. thuringiensis* in terms of changes in plant height and root length of mustard plants.

Fig 7: Untreated healthy mustard (A) and plants artificially inoculated with *F. equiseti* (B); Plant growth following application of *B. thuringiensis* and challenge inoculation with *F. equiseti* (C & D).

performance as compared to pathogen-inoculated plants and control (Figs. 6 and 7; Table 3). Plant growth in terms of increase in plant height and root length following application of *B. thuringiensis* has been presented in Table 2. Untreated plants artificially inoculated with *F. equiseti* showed yellow leaf symptoms (Fig. 6B). Plant growth of different stages following application of *B. thuringiensis* and challenge inoculation with *F. equiseti* have been documented in Fig 7 C, D. Yellow leaf symptom was also reduced to some extent as evident with disease score and per cent leaf area affected (Table 4) after application of *B. thuringiensis*.

CONCLUSION

The present investigation confirmed *Fusarium equiseti* as the causal agent of yellow leaf symptoms in *Brassica juncea* in the Khowaspur region of Uttar Dinajpur, West Bengal. Alongside this, a rhizospheric isolate of *Bacillus thuringiensis* demonstrated multiple plant growth-promoting traits, including indole-3-acetic acid production, phosphate solubilization, catalase activity, and starch hydrolysis. Importantly, the isolate exhibited strong antagonism against *F. equiseti*, achieving up to 83% inhibition *in vitro.* Scanning electron microscopy revealed direct bacterial–fungal interactions, while GC–MS analysis of bacterial metabolites indicated the presence of several antifungal compounds, such as thymol derivatives and adamantane acetamide. These findings highlight the dual functionality of *B. thuringiensis* as both a plant growth-promoting rhizobacterium and a biocontrol agent against a pathogenic *Fusarium* species. Its ability to produce phytohormones and antifungal metabolites suggests that this bacterium has significant potential for inclusion in eco-friendly disease management strategies for mustard cultivation.

FUTURE PROSPECTS

◆   Formulation development – For practical applications, suitable carrier-based formulations of *B. thuringiensis* need to be developed to ensure stability, viability, and ease of application for farmers.

◆   Mode of action studies – Further work is needed to elucidate the precise mechanisms of antifungal activity, including transcriptomic and metabolomic analyses of both the bacterium and the pathogen.Integration with crop management – The potential of *B. thuringiensis* can be maximized when integrated with other PGPR and biological agents as part of an Integrated Pest and Disease Management (IPDM) approach.

◆   Commercial potential – Considering the dual role of *B. thuringiensis* in insect and fungal suppression, this bacterium could serve as a model for developing next-generation bioinoculants with wide-spectrum benefits in sustainable agriculture.In conclusion, *B. thuringiensis* represents a promising bioresource for mustard disease management, offering an environmentally friendly alternative to chemical fungicides and contributing to sustainable crop production systems.

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DECLARATION

Conflict of Interest. Authors declare no conflict of interest.

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