Antiviral efficacy of *Bacillus* sp. against *Groundnut bud necrosis orthotospovirus* in cowpea

Abstract

Tomato spotted wilt, a disease caused by *Groundnut bud necrosis virus* (GBNV), is a major disease affecting tomatoes causing great yield loss to the farming community.In this study, the GBNV-TNAU 1 virus isolate was obtained from infected plants and maintainedthrough mechanical transmission on cowpea cv. CO7, which induced chlorotic and necrotic local lesions by the fourth-day post-inoculation (dpi). The pathogenicity of GBNV on tomato cv. PKM1 was confirmed through sap transmission. *In vitro* screening of *Bacillus sp. viz., B. amyloliquefaciens* (Ka1), *B. subtilis* (Bbv 57), *and B. subtilis* (BST8) demonstrated effective reduction of GBNV-induced lesions in cowpea. *B. amyloliquefaciens* (Ka1) and *B. subtilis* (BST8) displayed a maximum germination percentage of 100%, while *B. amyloliquefaciens* (MM12) exhibited 83.33%.Futhermore, maximum vigour index of 1743.8,1699.6 & 1436.5 was noticedin *B. amyloliquefaciens* (Ka1),*B. subtilis* (BST8)&*B. subtilis* (Bbv 57) treated plants. These findings provide valuable insights on sustainable management strategies against the disease, benefiting farmers by enhancing tomato cultivation productivity and profitability.

Key words: Tomato, GBNV,*Bacillus* sp., cowpea, SEM, vigour index.

Introduction:

Tomato (*Solanum lycopersicum* L.), a member of the Solanaceous family, is widely recognized as “Red gold” because of its luscious importance for culinary and nutritional purposes. Hundred grams of tomato provide 3.9% carbohydrates, 0.9% proteins, 2.6% sugar, 1.2% fibre, 0.2% fat, 18.9 mg of vitamin C, and 12.58 mg of lycopene (Hanif *et al*., 2006). Due to its extensive cultivation and unique nutritional qualities, the tomato has emerged as the second most grown vegetable crop worldwide, after potatoes. Tomatoes are grown in India over a vast area of 0.84 million hectares, producing a yield of 24.2 tonnes per hectare, with a total yield of 203.314 lakh tonnes (Indiastat, 2021-22). However, tomato cultivation worldwide is hindered by biotic and abiotic stresses (Chaudhry *et al*., 2022). Among the biotic stresses posed to tomatoes, damping off, Fusarium wilt, bacterial wilt, and viral infections are particularly detrimental,

resulting in significant losses in crop yield. (Brahimi *et al*.2017). Tospoviruses are a significant challenge to the economic cultivation of tomatoes, with over 20 different types found globally (Zhu *et al.*, 2019). *Peanut bud necrosis virus* (PBNV), also known as *Groundnut bud necrosis orthotospovirus* (GBNV), is particularly harmful to tomato plants and can cause catastrophic crop losses.

Groundnut bud necrosis virus belonging to the genus orthotospovirus, family Tospoviridae and order Bunyavirales was renamed as *Groundnut bud necrosis orthotospovirus* (ICTV, 2021 release).GBNV is transmitted through thrips in a circulative and propagative manner. The virus is characterized by an enveloped isometric virus particle with a diameter of 80-120 nm. Its genome includes L RNA (8.9 kb) encoding virus replicase protein of 337 kDa, M RNA (4.8 kb) encoding glycoproteins (34 kDa) and movement protein (127 kDa), and S RNA (3.05 kb) encoding non-structural small protein (34 kDa) and nucleocapsid protein (28 KDa) (Mandal *et al*.2012).Tomato plants that have been infected by the GBNV exhibit necrotic and chlorotic spots on their young leaves, stem, and petioles(Basavaraj *et al.*, 2017). As the infection progresses, the young bud dries out and the growth becomes stunted, resulting in the yellowing of leaves and ultimately leading to death. Infected plants produce fruits with concentric chlorotic rings with decreased size.In India, GBNV has caused yield losses of more than 80% according to Dasgupta, Malathi, and Mukherjee (2003).

Management of GBNV in tomato is primarily achieved by removing infected plants and using systemic insecticides to manage the insect vector. Nevertheless, no tomato varieties have been identified as resistant to GBNV thus far. Genetic engineering via RNAi is a time-consuming approach and is accompanied by biosafety concerns(Ahmad *et al.*,2021). An alternative approach is to induce host immune responses by employing beneficial microorganisms such as Plant Growth Promoting Rhizobacteria (PGPR).PGPR are recognized for their ability to colonize plant roots, thereby increasing plant growth, nutrient absorption, and the production of growth factors and vitamins(Prasad *et al*.,2015). Additionally, PGPR can stimulate systemic resistance or function antagonistically to several biotic stress factors. PGPR are commercially used to combat fungal and bacterial infections, and an increasing number of studies highlight their potential efficacy against viruses. Sporulating Gram-positive bacteria, particularly Bacillus spp., have been successfully employed to control plant diseases (Kloepper *et al*., 2004).

Bacillus spp. are known for their effectiveness in controlling plant diseases, especially those caused by bacteria and fungi. However, there are few studies that have explored the potential of Bacillus spp. in managing virus diseases (Harish *et al*., 2008; Vinodkumar *et al*., 2018). Previous studies have shown that the application of plant growth promoting rhizobacteria (PGPR), such as *P. fluorescens* strains (CoP-1/CoT-1/CHAO), significantly reduced the incidence of *Tomato spotted wilt virus* (TSWV) and promoted growth in both glasshouse and field conditions (Kandan *et al*., 2005). In addition, *Bacillus amyloliquefaciens* (VB7) and *Bacillus licheniformis* (CoEH6) were found to effectively suppress *Tobacco Streak Virus* (TSV) symptoms in cowpea and reduce TSV incidence in tomato under field conditions (Vinodkumar *et al.,* 2018). *Bacillus amyloliquefaciens* strain MBI600 was also effective in reducing the incidence of TSWV under different environmental conditions (Beris *et al*., 2018). PGPR have also been reported to control other virus diseases in various crops, including *Cucumber mosaic virus* (CMV) in pepper and tomato, cotton leaf curl, *Tomato mottle virus* in tomato, and *Tobacco mosaic virus* in tobacco (Lee & Ryu, 2016; Ramzan *et al*., 2016; Zehnder *et al*., 2000; Murphy *et al*., 2000; Wang *et al.,* 2009). Although the exact mechanisms by which *Bacillus* spp. induce systemic resistance against virus diseases are not yet fully understood, previous studies suggest that MAMP molecules such as flagellin and elongation factor can induce systemic resistance in plants by activating their corresponding PRRs and triggering transcriptional changes, leading to MAMP-triggered immunity (MTI) (Vanthana *et al*., 2019). Therefore, this research aims to evaluate the effectiveness of *Bacillus* spp. in inhibiting viral activity within cowpea plants, specifically focusing on the local lesions caused by the virus.

Materials and methods:

Virus isolation and characterization

GBNV-infected plants collected from the Thondamuthur area (11°00' 52.2" N 76°48' 23.7" E) of the Coimbatore district, Tamil Nadu, India were inoculated in cowpea (*Vignaungiculata*cv.CO7). The cowpea plant was selected since it is known to cause local lesion symptoms three to four days after inoculation. These plants were grown in the PL480 glasshouse at the Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu, India under insect-free conditions. Sap inoculation was performed using infected tomato leaves extracted with sodium Phosphate buffer (0.01 M) in a pre-chilled pestle and mortar.Leaves of seven-day-old cowpea plants were dusted with 600 mesh carborundum and the sap was inoculated to the plants by

gently rubbing the surface. Two minutes after inoculation, the plants were washed with sterile water and observed for symptom expression. Following the manifestation of symptoms, the virus inoculum was amplified and subsequently re-inoculated into 25-day-old tomato plants (*Solanum lycopercicum* cv. PKM1) to confirm the pathogenic nature of the virus in tomato plants.

Molecular detection of GBNV

Total RNA was isolated from the infected plantsusing the Trizol method (Chomczynski and Sacchi, 1987) and quantifiedin Nanodrop (BIODROP).First-strand cDNA synthesis was performed using a kit from Thermo Scientific (RevertAid first strand cDNA synthesis kit, USA). The reaction mixture consists of Reaction buffer (4 µl), dNTPs (2 µl), random primer (1 µl), reverse transcriptase (1 µl), RNase inhibitor (1 µl), and total RNA (3 µl, 1800 ng), which was made upto to 20 µl with DEPC-treated water. The mixture was incubated for 60 minutes at 45 °C, followed by a 5 minutes incubation at 70 °C.

Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify the genomic component of GBNV using movement protein gene-specific primers.PCR reaction was carried out in a 20 µl Master mix, with 4 µl forward and reverse primers (5 M each), 8 µl of nuclease-free water, and 4 µl of cDNA.The PCR was carried out in a thermal cycler (Biorad) under the following PCR conditions: initial denaturation of 94°C for 5 min; 35 cycles of denaturation for 94°C for 1 min; annealing for 52°C for 1 min; extension for 72°C for 1 min; and a final extension of 72°C for 10 min. The RT-PCR product was run in 1.2% agarose gel, stained with ethidium bromide, and examined under a gel documentation unit. The amplified GBNV products was sequenced at Biokart pvt ltd., submitted to the NCBI Genbank database, and the accession number was obtained.

Preparations of bacterial suspensions

In this study, *Bacillus* sp. from the culture collection centre of the Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu, India was used. The strains include, *B. endophyticus* (COEH7), *B. subtilis* (BST 18), *B. firmus* (TNAU 1), *B. subtilis* (BST 8), *B. pumilus* (TEB 10), *B.subtilis* (Bbv 57), *B. amyloliquefaciens* (MM12), *B.amyloliquefaciens* (Ka1) and*B. subtilis* (EBPBS 4).Two hundred ml of bacterial cell suspensions were prepared, placed on a shaker operating at 120 rpm and maintained at a temperature of 28 ± 2 °C for a duration of 24 hours. After incubation, the bacterial cells were harvested by centrifugation at

6000 rpm for 5 minutes. The resulting bacterial pellet was then resuspended in distilled water and adjusted to achieve a concentration of 2.5 × 10^10 colony-forming units (CFU) per millilitre.

Bacterial suspension- seed treatment for growth promotion test

One gram of tomato seeds (PKM 1)were surface sterilized using a 1% (v/v) sodium hypochlorite solution for 3 minutes. The sterilized seeds were then rinsed twice with distilled water and shade dried on a blotter sheet. The bacterial suspensions supplemented with 0.2% sterilized carboxymethyl cellulose (CMC) were used for surface bacterization of the sterilized tomato seeds. The bacterial suspensions along with seeds were incubated in a shaker at 26 °C for 6 hours to allow the bacterial cells to adhere to the seed coat. After the incubation period, the seeds were driedin a shade area.

Efficacy of *Bacillus* sp. on seed germination and seedling vigour of tomato under *in vitro*

A total of 25 tomato seeds were evenly distributed on a paper towel and covered with another pre-soaked paper towel. To prevent drying, the paper towels were rolled together with polythene packaging.The rolled paper towels were then placed in an incubation chamber maintained at a constant temperature of 24 ± 1 ℃. After the incubation period, the paper towels were unrolled, and the number of germinated seeds was recorded(ISTA, 2003). The seedling vigour index was calculated after ten days of incubation (Abdul Baki and Anderson, 1973). To determine the vigour index, the average lengths of the roots and shoots were measured for each inoculation variant. The vigour index (VI) was computed using the formula VI = (mean root length + mean shoot length) × germination percent.

Efficacy of*Bacillus* sp. against GBNV inoculumunder glasshouse condition

To test the antiviral efficacy of *Bacillus* sp., the bacterial cell suspensions mixed with 1% Tween 20 (10 ml), 1% glycerol (10 ml), and 1% polyvinylpyrrolidone (10 g) were used.Seven days old cowpea plants (two leaf stage) were treated separately with a 1% bacterial suspension of each isolate as a foliar spray following a standard protocol (Vinodkumar*et al*.,2017). After 24 h, the plants were challenge inoculated with the freshly prepared GBNV inoculum and incubated at 28±2 °C. The experiment was repeated five times with five plants per replication. Inoculated control and a healthy control were also maintained. The number of lesions per plant was recorded to assess the antiviral activity of *Bacillus* sp.

Scanning Electron Microscopy (SEM) of *Bacillus* on cowpea phylloplane

Twenty-four hours after treatment, cowpea leaves (1x1 cm) were exercised from plants and immersed in a solution containing 4 g/L of glutaraldehyde in 0.1 mol/L cacodylate buffer and kept at a temperature of 4°C overnight for fixation. Subsequently, the samples were rinsed twice using 0.1 mol/L cacodylate buffer (pH 7.3) and then subjected to post-fixation in a solution containing 1 g/L of osmium tetroxide (OsO4) in 0.1 mol/L cacodylate buffer for 1 hour. The samples were washed twice using sterile deionized water and subsequently subjected to freeze-drying. The resulting dried samples were mounted on specimen stubs, coated with a layer of gold using a sputter coating technique, and examined using a Quanta 200 Model SEM(Olmez, H., & Temur, S. D., 2010).

Statistical analysis

The data were statistically analysed using IBM SPSS Statistics software version 28.0.0.0. Data were subjected to analysis of variance (ANOVA) at significant levels ($P < 0.05$) and means were compared by Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1984)

Results and discussion:

Symptomatology

From the field survey, it was observed that, GBNV initially produced chlorotic ring spots which later turned to necrotic ring spots on the leaves. The disease occurred in all the stages of the crop from young stage to flowering stage. Severe infection on young shoots lead to bud blight necrosis. On stem and petioles, GBNV caused necrotic streaks. GBNV infection on early crop stage caused wilting and stunting of the whole plant(Fig.1). Similar results were found in the studies of Suganyadevi *et al.,* 2018, who reported that GBNV on tomato plants produced chlorotic and necrotic ringspots on leaves followed by necrotic streaks on stems, petioles and chlorotic rings on infected fruits.

Mechanical transmission of GBNV and pathogenicity test

Cowpea plants at two leaf stage (7 days old) was used for inoculation. The same was done in tomato plants @ 30 DAS for proving pathogenicity of GBNV. On cowpea, the virus produced chlorotic spots at 4 days after inoculation and necrotic ring spots at 8 days after inoculation. Systemic infection of crinkling of top leaves in virus-inoculated cowpea plants was also observed. On tomato, the virus produced chlorotic spots on leaves on 6 days after sap inoculation and necrotic ring spots 10 days post inoculation. Eventually, systemic infections of bud necrosis and necrotic streaks on the stem on 21 days after inoculation, were produced thus proving the Koch postulates.Similar symptoms were observed by Vanthana *et al.* (2019) upon sap inoculationof GBNV on cowpea plants and tomato plants.In cowpea plants (CO7), GBNV inoculation exhibited chlorotic ring spots which later turn to necrotic spots within 4-5 dpi and in tomato plants necrotic rings on leaves and necrotic streaks were observed (Fig.2a and 2b).

Molecular detection of GBNV in infected plants

The isolate, GBNV-TNAU 1 was amplified with the product size of~ 903 bp (Fig.3) and the amplified product was partially sequenced. Likewise, Rahul *et al.*, in 2022 have characterized the GBNV isolate with movement protein gene with an amplification size of 903 bp.After analysis and submission to the NCBI Genbank database, the accession number (OQ871573) was generated.

Efficacy of *Bacillus* sp. on seed germination and seedling vigour of tomato under *in vitro*

Bacillus is indeed a commonly occurring genus of bacteria that plays a significant role in biocontrol and plant growth promotion activities (Nandhini *et al*., 2012). The *Bacillus* sp. used in our study has increased the germination percentage of tomato seeds treated with PGPR strains, which ranged from 100 to 83.3 %. Among PGPR treatments, *B. amyloliquefaciens* (Ka1) and *B. subtilis* (BST 8) showed maximum germination percent (100 %), followed by *B. subtilis* (Bbv 57) (90%), *B. endophyticus* (COEH7), *B. subtilis* (BST 18) &*B. pumilus* (TEB 10) (87.5 %), *B. subtilis* (EBPBS 4) (84%) and *B.amyloliquefaciens* (MM12) (83.33%) as compared to control (76%).In all the treated plants, there was an increase in root length ranging from 11.47 to 6.27 cm and shoot length ranging from 6.56 to 4.47. The highest root length of 11.47 cm observed in the plants treated with *B. amyloliquefaciens* (Ka1) followed by 10.43 cm in *B. subtilis* (BST 8) and 9.81 cm in *B. subtilis* (Bbv 57), whereas the least root length was observed in *B. endophyticus* (COEH7) 6.28 cm compared to control the root length was only 6.27 cm. The

highest shoot length of 6.56 cm observed in the plants treated with *B. subtilis* (BST 8) followed by 6.28 cm in *B. endophyticus* (COEH7) and 6.14 cm in *B. subtilis* (Bbv 57), whereas the least shoot length of 4.633 cmwas observed in *B. pumilus* (COEH7)compared to control (4.46 cm). The highest vigour index was 1743.8 in*B. amyloliquefaciens* (Ka1) and lowest was 932.5 in *B. firmus* (TNAU 1)treated plants(Fig 4; table.1).Similarly, from the studies of Devi *et al.*,(2020) it is concluded that *Bacillus* sp. enhanced the seedling germination that ranged from 60-95%.Also, the *Bacillus velezensis* ERBS51 treated seeds had the highest germination per cent (95%) and vigour index of 1073.50 and 1472.5at 7th and 14th day. The studies conducted by Sundaramoorthy &Balabaskar (2012) and Agarwal & Agarwal (2013) provided evidence for the positive effects of certain *Bacillus* isolates on plant growth. These isolates significantly improved seed germination, vigour index, shoot length, and root length in tomato plants compared to those without bacterial inoculation. The findings from these studies support the concept that employing plant growth-promoting bacteria like *Bacillus* can be a highly promising approach to enhancing crop productivity in sustainable agriculture.

Efficacy of *Bacillus* sp.against GBNVunder glasshouse condition

The management of plant diseases using microorganisms for biological control is receiving a lot of interest globally (Mehetre *et al.*, 2021).In the present study, screening of the nine isolates of *Bacillus* spp. against GBNV revealed that, *B. amyloliquefaciens* Ka 1 effectively reduced the number of lesions from 9.44 lesions per leaf in the virus-inoculated control to 0.575 lesions per leaf. This was followed by *B. subtilis* -Bbv57 and *B. subtilis*- BST 8, which were effective in reducing the number of lesions to 1.39 and 2.07 lesions per leaf, respectively(Fig.5). The number of lesions in the other *Bacillus* sp. treated cowpea plants ranged from 2.49 to 5.25 lesions per leaf. The results revealed that *B. amyloliquefaciens(Ka1), B. subtilis (Bbv 57) and B. subtilis (BST8)* were effective in reducing the lesions compared to other treatments (Table.2).The findings of our current study align with previous research conducted by Jonathan *et al.* (2005), who investigated the effects of *Bacillus subtilis* on germination and seedling vigour using different native isolates.In the study by Vinodkumar *et al*., (2018), the inoculation of *B. amyloliquefaciens* VB7 suppressed TSV symptoms in cowpea plants, indicating a beneficial effect in reducing their severity. Similarly, Senthilraja (2018) reported that the inoculation of *B.licheniformis* effectively suppressed TSWV symptoms in cowpea plants, highlighting a positive impact in reducing symptom severity.The mechanisms behind these improvements

involve the production of phytohormones, increased nutrient availability, antibiotic production, ethylene reduction, induced systemic resistance, and competition for resources (Ambreetha *et al*.,2018).

Colonization dynamics of *Bacillus*sp. on cowpea phylloplane

Colonization of *Bacillus* sp. on the cowpea phylloplane was observed 24 hours after treatment.The scanning electron microscopy (SEM) examination demonstrated a strong attachment of *Bacillus* sp. to the phylloplane, with the bacteria firmly adhering to the leaf surfaces and forming cohesive clusters(Fig.6).SEM results revealed that the cells were straight and rod-shaped with round ends, organized in chains, and motile.Colonization of *Bacillus*enhances its persistence over the leaf surface and acts as a physical barrier against pathogens, competes for resources, exhibits antimicrobial activity, and induces systemic resistance (Junges*et al.*,2013).

Conclusion

In summary, this study aimed to investigate the growth-promoting and resistance-inducing abilities of various *Bacillus* species against GBNV. Nine different *Bacillus* sp. were evaluated using a roll towel assay to assess their impact on growth promotion. The results demonstrated that the *Bacillus* species exhibited improved germination and seed vigour compared to the control. Among these species, *B. amyloliquefaciens* (Ka1), *B. subtilis* (Bbv 57), and *B.subtilis* (BST8) showed superior performance compared to other strains. These strains can be further used to develop a consortiumand exploit it against GBNV in tomato.

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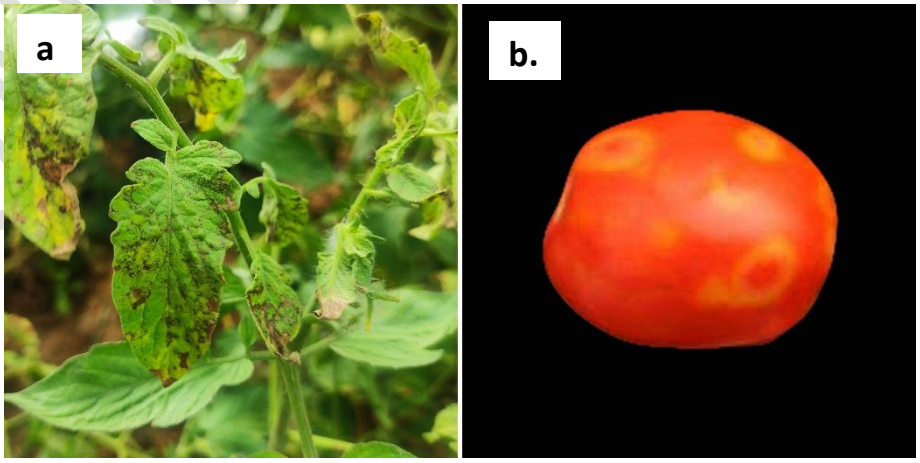


Fig. 1Tomato plants expressing GBNV symptoms collected from the field. a) necrotic ring spots on the leaves. b) chlorotic rings on the infected fruits**.**

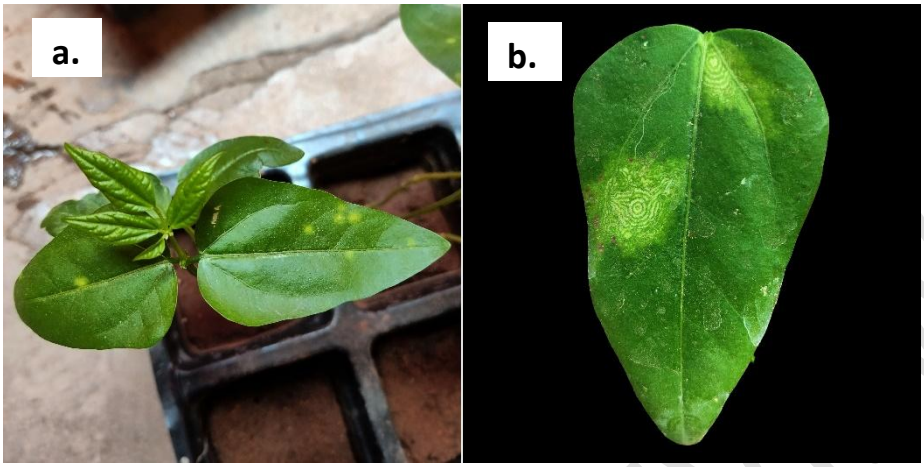


Fig. 2aCowpea plant inoculated with GBNV. a) leaves with chlorotic spots, b) six days old lesions on cowpea

Fig .2bSymptoms observed on tomato plants after inoculation of virus for pathogenicity test a) necrotic ring-like lesions on leaves, (b)necrotic streaks on stem, (c) necrosis of flower buds.

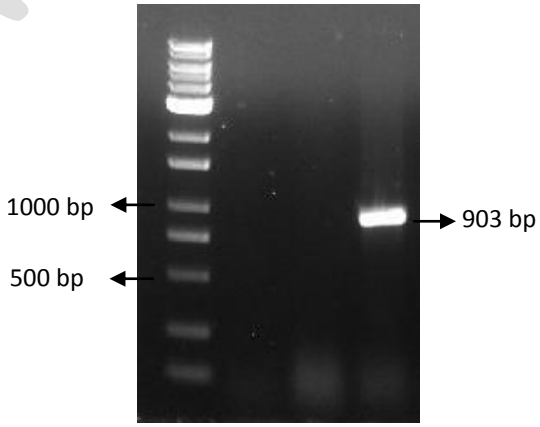


Fig .3 Amplification of movement protein gene of GBNV

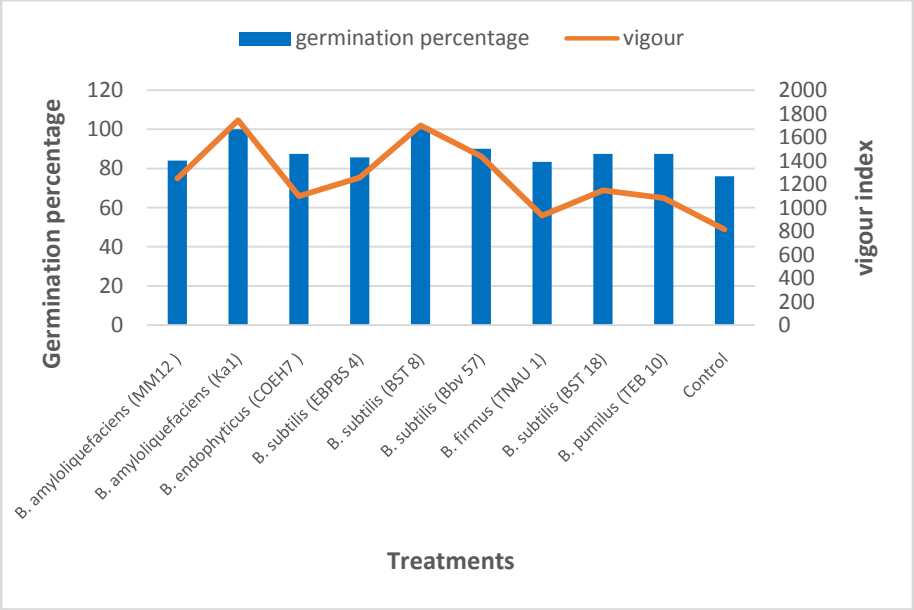


Fig.4. Growth promoting attributes of *Bacillus* sp. in tomato

Table.1. Vigour index: Root and shoot length

SI.NO	TREATMENTS	Average root length (cm)	Average shoot length (cm)
1	*B. amyloliquefaciens (MM12)*	9.25^{bcd}	5.6^{b}
2	*B. amyloliquefaciens (Ka1)*	11.48^{a}	5.96^{ab}

3	*B. endophyticus (COEH7)*	6.28^{e}	6.28^{c}
4	*B. subtilis (EBPBS 4)*	8.73^{cd}	5.93^{ab}
5	*B. subtilis (BST 8)*	10.43^{ab}	6.56^{a}
6	*B. subtilis (Bbv 57)*	9.81^{abc}	6.14^{ab}
7	*B. firmus (TNAU 1)*	6.55^{e}	4.63^{c}
8	*B. subtilis (BST 18)*	8.31^{cd}	4.82^{c}
9	*B. pumilus (TEB 10)*	7.75^{de}	4.64^{c}
10	Control	6.27^{e}	4.46^{c}

Values indicate the mean of five replicated experiments. A set of five plants was tested per treatment. Mean values of the same letter within each column are not significantly different according to Duncan's multiple range test ($p < 0.05$).

SI.NO	TREATMENTS	Average No. of lesions per leaf
1	*B. subtilis* (BST 8)	2.07^{dcb}
2	*B. firmus* (TNAU 1)	2.49^{edc}
3	*B. subtilis* (EBPBS 4)	5.52^{f}
4	*B. pumilus* (TEB 10)	3.34^{ed}
5	*B. subtilis* (BST 18)	4.12^{fe}
6	*B. amyloliquefaciens* (MM12)	2.38^{dc}

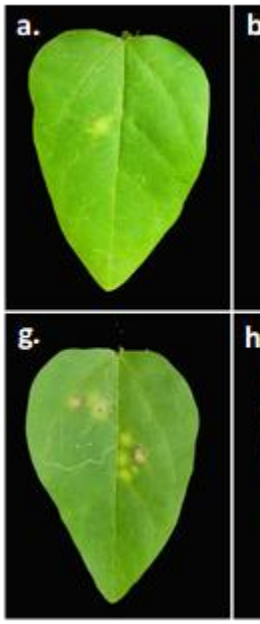
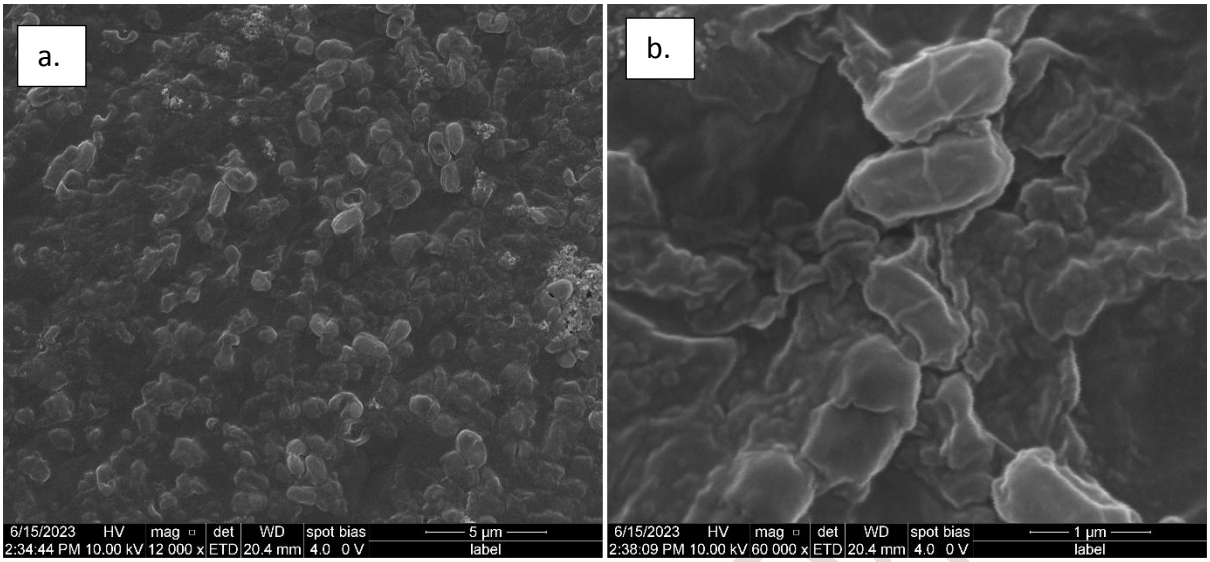


Fig 5. Antiviral efficacy of *Bacillus* sp. against GBNV in cowpea: Antiviral activity of various *Bacillus* sp. against GBNV in cowpea. (a) *B. amyloliquefaciens* (Ka1), (b) *B. subtilis* (Bbv 57), (c) *B. subtilis* (BST 8), (d) *B. amyloliquefaciens* (MM12), (e) *B. firmus* (TNAU 1), (f) *B. endophyticus* (COEH7), (g) *B. pumilus* (TEB 10), (h) *B. subtilis* (BST 18), (i) *B. subtilis* (EBPBS 4), (j) Inoculated control, (k) Healthy control, (l) Buffer control

Table.2. Antiviral efficacy of *Bacillus* sp. against GBNV in cowpea

7	*B. subtilis* (Bbv 57)	1.39^{cba}
8	*B. endophyticus* (COEH7)	3.14^{cd}
9	*B. amyloliquefaciens* (Ka1)	0.57^{ba}
10	Healthy control	0^{a}
11	Inoculated control	9.45^{g}

Values indicate the mean of five replicated experiments. A set of 5 plants was tested per treatment. Mean values of the same letter within each column are not significantly different according to Duncan's multiple range test ($p < 0.05$).



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444 **Fig. 6.SEM image of *Bacillus* sp. on the leaf surface after 24hrs of inoculation**: (a)
445 magnification-12000 X, (b) magnification-60000 X