Flagellin of Bacillus amyloliquefaciens *works as a resistance inducer against groundnut bud necrosis virus in chilli* (Capsicum annuum *L.*)

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ORIGINAL ARTICLE

Flagellin of *Bacillus amyloliquefaciens* works as a resistance inducer against groundnut bud necrosis virus in chilli (*Capsicum annuum* L.)

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Abstract

Groundnut bud necrosis virus (GBNV), a member of the genus *Tospovirus*, has an extensive host range and is associated with necrosis disease of chilli (*Capsicum annuum* L.), which is a major threat to commercial production. Plant growth promoting rhizobacteria (PGPR) have been investigated for their antiviral activity in several crops and for their potential use in viral disease management. However, the microbial mechanisms associated with PGPR in triggered immunity against plant viruses have rarely been studied. To understand the innate immune responses activated by *Bacillus* spp. against GBNV, we studied microbe-associated molecular pattern (MAMP) triggered immunity (MTI) in chilli using transient expression of the flagellin gene of *Bacillus amyloliquefaciens* CRN9 from *Agrobacterium* clones, which also induced the expression of *EAS1* gene transcripts coding for epi-aristolochene synthase, which is responsible for the accumulation of capsidiol phytoalexin. In addition, the transcript levels of WRKY33 transcription factor and salicylic acid (SA)-responsive defense genes such as NPR1, PAL, PO and SAR8.2 were increased. Jasmonate (JA)-responsive genes, *viz*., PDF, and LOX genes, were also upregulated in chilli plants challenged with GBNV. Further analysis revealed significant induction of these genes in chilli plants treated with *B. amyloliquefaciens* CRN9 and benzothiadiazole (BTH). The transcript levels of defense response genes and pathogenesis-related proteins were significantly higher in plants treated with *Bacillus* and BTH and remained significantly higher at 72 h post-inoculation and compared to the inoculated control. The plants treated with flagellin using the agrodrench method and exogenous treatment with *B. amyloliquefaciens* and BTH showed resistance to GBNV upon mechanical inoculation and a reduced virus titre which was confirmed by qPCR assays. Thus, transient expression of flagellin, a MAMP molecule from *B. amyloliquefaciens* CRN9, is able to trigger innate immunity and restrain virus growth in chilli via induced systemic resistance (ISR) activated by both the SA and JA/ET signalling pathways.

Introduction

Chilli (*Capsicum annuum* L.) a genus in the family Solanaceae known as red pepper, is an important condiment and vegetable crop in India. Capsicum is native to Central and South America, consisting of thirty species, however only five of these, including *Capsicum annuum*, have commercial importance. As per the latest statistics, India produces around 800,000 tonnes of dry chilli from an area of 930,000 hectares. The major chilli-producing states of India are Maharashtra, Karnataka Andhra Pradesh, and Tamil Nadu. Chilli is susceptible to infection by at least 10 viruses, which pose a serious threat to commercial cultivation of chilli and reduce its yield [1]. The viral disease complex of chilli can cause a 100 percent yield reduction during early stage of infection and often has social consequences [2, 3]. A recent outbreak of necrosis disease in chilli caused by groundnut bud necrosis virus (GBNV) has become as a major impediment to chilli production [4–7]. GBNV, a member of the genus *Tospovirus*, causes necrosis disease of vegetables in India and is an important crop pathogen in many countries, causing major economic losses in chilli cultivation and other vegetable crops [2, 8]. Necrosis disease caused by GBNV results in a variety of symptoms, including circular chlorotic and necrotic spots on leaves and stunting with misshapen leaves. Transmission of GBNV usually occurs through

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different species of thrips [4, 9]. In India, commercial cultivars are susceptible to viral pathogens whose biological diversity can lead to a breakdown of resistance. Microbe-associated molecular pattern (MAMP)-triggered immunity via bacterial endophytes involves a combination of specific enzymes and defense-related genes. Inducible plant defense responses suppress the multiplication of a wide variety of plant viruses, by producing antiviral compounds.

In recent years, plant-growth-promoting rhizobacteria (PGPR) have been exploited to manage fungal and bacterial diseases, but their effect on viral diseases has not been extensively studied. PGPR promote plant growth and induce host defense, and they have been shown to induce an induced systemic resistance (ISR) against tomato leaf curl virus (ToLCV) [10]. However, the role of MAMP molecules of PGPR in triggered immunity and their interactions with the signalling pathway mediated by the NPR1 are not well understood. MAMP molecules such as iturin, bacilysin, surfactin, bacillomycin, fengycin, mersacidin and mycosubtilin from *Bacillus* species have been reported to exhibit antiviral activity against tobacco streak virus infecting cotton [11]. Likewise, MAMP-triggered immunity enables the plants to acquire broad-spectrum antiviral capacity, including against viruses propagated by physical wounding and aphids. Plants treated with MAMP molecules have been shown to develop resistance to several viruses without much of a negative effect on plant growth. Flagellin, a major protein component of flagella in members of the genus *Bacillus*, is known to be a general elicitor that is recognized by plants and induces MAMP-triggered immunity. It is believed to activate signalling cascades, resulting in coordinated expression of a specific set of genes involved in plant defense. Regardless of the inherent mechanism of disease resistance, after exposure to PGPR, plants initiate expression of genes involved in plant defense mechanisms [12]. The levels of transcripts for a large complex of structurally diverse gene products called pathogenesis-related (PR) proteins, increase following exposure to PGPR and contribute to an overall defensive condition in plants [13]. In this study, exogenous application of *B. amyloliquefaciens* CRN9 to chilli seedlings was demonstrated to elicit a defense response. Triggered immunity was analysed by measuring the levels of transcripts of different systemic acquired resistance (SAR) and ISR genes and expression of defense-related compounds. Using this information, investigations were carried out to document the presence of GBNV in chilli and to increase our knowledge about the role of *B. amyloliquefaciens* CRN9 in triggering innate immunity involved in the suppression of GBNV in chilli.

Materials and methods

Plant material and virus isolate

Chilli pepper cultivar Syngenta Bullet plants showing susceptibility to the virus were used in this study. The plants were grown on a sterilized mixture of cocopeat, vermiculite, and perlite (3:1:1 ratio vol/vol/vol ratio) in an insect-proof controlled environment at 28 ± 2 °C with a photoperiod of 16 h day/8 h night. The virus isolate collected from infected plants showing characteristic symptoms of GBNV was used for inoculation and used for subsequent studies.

Inoculum preparation

A standard protocol for mechanical sap inoculation was applied for virus inoculation [14]. The virus extract was prepared by macerating infected chilli plant tissue in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% β-mercaptoethanol, using an ice tray. Inoculation was carried out by gentle rubbing with inoculum, using the broad end of a pestle, on the young leaves of one-month old chilli plants that had been dusted with 600-mesh carborundum powder. Three replications with 15 plants in each replication were performed, and post-inoculation observations were recorded according to Widana Gamage et al. [15]. Chilli plants showing characteristic symptoms of necrosis disease were tested by direct antigen coating-ELISA (DAC-ELISA) using a polyclonal antibody specific for GBNV as described by Hobbs et al. [16].

Amplification of the coat protein gene of GBNV

Total RNA was extracted from 100 mg of leaves of infected chilli using a TRIzol Plant Extraction Kit (Sigma Chemicals, USA) according to the manufacturer's protocol and resuspended in 50 µl of nuclease-free water. The total RNA isolated from virus-infected field chilli samples were subjected to PCR in a 50-µl reaction volume containing cDNA, 2 units of enzyme mix with primers specific for GBNV (CPF—ATGTCTAACGTYAAGCAGCTC; CPR—TTACAACTC TAGCGAAGGAC), to amplify the complete coding region of the CP gene. The PCR conditions included 35 cycles of denaturation at 94 °C for 2 min, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR reaction was carried out in an Eppendorf Mastercycler Gradient ES. The amplified products were analyzed in a 1% agarose gel, stained with ethidium bromide, and photographed using a UV gel documentation system (AlphaImager).

Isolation and molecular characterization of the bacterial antagonist

Bacterial endophytes were isolated from chilli plants following a protocol described earlier [17]. In order to characterize the bacterial antagonist, a standard cetyl trimethyl ammonium bromide (CTAB) method was used to extract the genomic DNA from bacterial endophytes, and amplification of 16S rRNA gene was performed using the universal primers 799F (AACMGGATTAGATACCCKG) and 1193R (ACGTCATCCCCACCTTCC) to obtain an product size of approximately 1500 bp [18]. The PCR reaction was carried out in a total volume of 50 µl in an Eppendorf Master Cycler (Germany). The amplified products were resolved on a 2% agarose gel at 50 V, stained with ethidium bromide (0.5 µg/ml), photographed and analyzed using a gel documentation system (Alpha Innotech Corporation, San Leandro, California). DNA sequencing was performed at M/s Chromous Biotec Pvt. Ltd., Bangalore, India.

Screening and selection of bacterial endophytes against GBNV

To test the antiviral activity of bacterial endophytes, individual bacterial isolates were inoculated into a 250-ml conical flask containing 100 ml of nutrient broth and incubated at room temperature (28 ± 2 °C) in an orbital shaker at 150 rpm for 48 h. Then, fully grown bacterial culture was mixed with 1% Tween 20 (10 ml), 1% glycerol (10 ml), and 1% polyvinylpyrrolidone (10 g). To ensure uniform mixing, the mixture was incubated in an orbital shaker at 200 rpm for 5 min. The concentration of the suspension was adjusted to a minimum of 2.5×10^{10} colony-forming units (CFU)/ml [19]. The inoculum of GBNV was multiplied in cowpea (CO7) plants maintained in the glasshouse under insect-proof conditions. A virus extract was prepared by macerating GBNV-infected leaves of cowpea plants in 0.1 M sodium phosphate buffer, pH 7.0, using an ice tray. In order to screen the bacterial endophytes, cowpea plants were treated separately with a 1% bacterial suspension of each isolate as a foliar spray following a standard protocol. After 24 h, the cowpea plants were challenge inoculated with the freshly prepared GBNV inoculum and incubated at 28 ± 2 °C in a glasshouse. The experiment was repeated three times with three plants per replication. A buffer-inoculated control and an uninoculated control were also included. The number of lesions was recorded to assess the antiviral activity of the bacterial endophytes.

Construction of pBIN-*flg* for transient expression

The binary vector pBIN-GFP was used to clone the gene encoding flagellin, a general elicitor of plant defense. The full-length flagellin gene was amplified from the effective isolate *B. amyloliquefaciens* CRN9 using the self-designed specific primers F (ATGAGAATCAACCACAATATC) and R (TTAACCTTTAAGCAATTGAAG). The PCR product contained restriction sites for *Xba*I and *BSP* to facilitate cloning into the vector pBIN-GFP. The amplified PCR products were separated in 1.2% agarose gels, purified, and cloned into pGEM-T Easy Vector (Promega). Subsequently, the products were excised from the pGEM-T Easy Vector and cloned to the pBIN-GFP vector. The positive clones with recombinant plasmid pBIN-*flg* containing the flagellin expression cassette were identified by restriction endonuclease digestion with *Xba*I and *BSP*. The expression cassette from plasmid pBIN-*flg* was mobilized into *Agrobacterium tumefaciens* strain LBA4404 via triparental mating using pRK2013 as a helper strain [20, 21] for use in agrodrenching for transient expression in chilli plants.

Agrodrenching of chilli plants for the transient expression of flagellin

Agrobacterium tumefaciens LBA4404 containing recombinant vector pBin-*flg* were cultured overnight to an optical density (OD) of 1 in 20 mL of LB broth containing 10 µg of rifampicin and 50 µg of kanamycin per mL. The overnight cultures were inoculated into 100 ml of fresh LB broth containing 10 µg of rifampicin and 50 µg of kanamycin per mL for 24 h. The culture was centrifuged at 4,000 rpm for 10 min to pellet the *Agrobacterium* cells, which were then resuspended in 100 ml of Murashige and Skoog (MS) basal medium without hormones. The suspension was mixed with 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES) and 100 µM acetosyringone, adjusted to an OD of 1 and kept at room temperature for at least 3 h. This cell suspension was used to inoculate the roots of chilli plants for transient expression of the flagellin gene. For agrodrenching, one-month-old chilli seedlings were grown in a sterilized mixture of coir pith, vermiculite and perlite (3:1:1) and maintained in glasshouse under insect-proof conditions. The collar region of each chilli plant was drenched with 5 ml of *Agrobacterium* strain containing pBIN-*flg* following the protocol described by Ryu et al. [22]. The transcripts of the flagellin gene in the freshly grown leaves were detected at different time points 0 to 9 days after inoculation, using the gene-specific primers.

Bacillus, BTH treatment and virus inoculation

Our isolate of *B. amyloliquefaciens* CRN9 showing antiviral activity was inoculated into a 250-ml conical flask containing 100 ml of nutrient broth and incubated at room temperature (28 ± 2 °C) for 48 h in an orbital shaker at 150 rpm to a final OD of 1. The bacterial suspension was then mixed with 1% Tween 20 (10 ml), 1% polyvinylpyrrolidone (10 g),

and 1% glycerol (10 ml) and incubated in an orbital shaker at 200 rpm for 10 min to obtain a homogenous mixture. Afterward, the mixture was adjusted to a concentration of 2.5×10^{10} CFU ml^{-1}. The prepared suspension was applied as foliar spray onto chilli seedlings until runoff with a maximum of 15 ml of suspension per seedling [19]. For hormonal treatment, chilli seedings were sprayed with 300 mM BTH in distilled water. Control plants were treated with sterile distilled water. The first applications of both *Bacillus* and BTH were carried out 24 h prior to virus inoculation, and later, the seedlings were challenged with the GBNV inoculum [13]. After 24 h of inoculation, seedlings were given a second application of *Bacillus* or BTH according to the treatment schedule. All experiments were conducted in a growth chamber, with three replicates, each with 30 plants. The control and treated samples were harvested at 0, 1, 3, 5, 7 and 9 days after treatment, frozen immediately in liquid nitrogen and preserved at -80 °C until use.

RNA isolation and cDNA synthesis

Total RNA was extracted from the collected frozen samples using TRIzol Reagent (Sigma Chemicals, USA) from 100 mg leaves, resuspended in 50 µl of nuclease-free water and treated with DNase I (Sigma Chemicals, USA). The purified RNA, with a 260/280 nm absorbance ratio ranging from 2.0 to 2.1, determined using a NanoDrop spectrophotometer (BioDrop, Germany), was used for cDNA synthesis. For cDNA synthesis, 1 µg of total RNA was annealed with 0.3 µM random primer at 70 °C for 10 min, after which 1 µl of RNase inhibitor (20 U), 2 µl of dNTPs (10 mM) and 4 µl of 5 × reverse transcriptase buffer (250 mM Tris–HCl, 250 mM KCl, 20 mM $MgCl_2$, 50 mM dithiothreitol [DTT]) were added to the reaction mixture. The reaction mixture was incubated at 37 °C for 10 min, 40 U of M-MuLV reverse transcriptase was added, and the mixture was incubated at 45 °C for 60 min. The reaction mixture was heated at 70 °C for 10 min to stop the reaction. The reaction was performed in Eppendorf Mastercycler Gradient ES.

Quantitative real-time polymerase chain reaction (qRT-PCR)

A tenfold dilution of the first-strand cDNA was used as a template for qRT-PCR as described by Mishra et al. [23]. The qRT-PCR analysis was carried out using the specific primers corresponding to defense-related genes (Table 1). The specificity of the primers was verified by RT-PCR and analysis of the amplified PCR products on an agarose gel. A total volume of 10 µl of reaction mixture was prepared for qPCR analysis, and the reaction mixture consisted of 5 µl of FAST-SYBR Green PCR mix (Sigma Chemicals, USA), 1 µl each of the forward and reverse primers (5 µM), 1 µl of template cDNA, and 2 µl of nuclease-free water. qPCR was performed on a real-time PCR system (Bio-Rad company, Germany) with a temperature plan consisting of 95 °C for 30 s (initial denaturation) followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. A melt curve from 65 to 95 °C was performed by using a cycle of 65 °C for 15 s. To check the PCR product specificity, the temperature was increased slowly to 95 °C at a rate of 0.2 °C/s. Ct values were calculated from three biological replicates for each sample with

Table 1 Sequences of gene-specific primers used for quantitative qPCR experiments

Gene	Forward and reverse primers
5-epi-aritolochene synthase gene (*EAS1*)	F-TGGCAGACTAAAGGAGTCTCTTG
	R-GTGGAGAAAGCGAGTGCATCTTC
WRKY33	F-GTCCTACCGGTGGCAATAGC
	R-TGCTTTGAAGCTTGGATCTTTG
NPR1	F-CTTTACCTTCCAGATCTCTGA
	R-GCAATCTCTCACATGCTTTAC
Lypoxygenase	F-TGGTGATCCTGCGAATGGTT
	R-CGTCCCAATCAAACGTGACA
Peroxidase	F-ACACGTCTGATTTGCCAGGCT
	R-GCTGAGGTCCCAATTGTGTGC
Phenylalanine ammonia lyase	F-AAGTCATTCGCGCTGCAACT
	R-CCACCGTGTAAGGCCTTGTT
Plant defensin 1.2 (PDF)	F-TCAATCCTTCAGGACCAACCA
	R-CCACCGGTAGGACTAGCACTCT
SAR 8.2	F-TGAGACTAAGAAAGTTGGAC
	R-ACCTCTATGGATTTCTGATC
Ubiquitin 3 (UBI3)	F-TCAAGCCTCCAAAGGTTGCT
	R- GGACTCCACTGCTCCTTGAGA

two technical replicates. Gene expression was normalized against the constitutively expressed, ubiquitin gene from *C. annuum*. The level of gene expression in the control sample was set to 1, and the relative repression of gene expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method [24].

Viral resistance assay in flagellin-, *Bacillus*- and BTH-treated plants

The progression of viral disease was assessed by visual observation of symptom development in flagellin, *Bacillus*- and BTH-treated plants. The symptom severity grade was assessed 10 days after challenge inoculation with GBNV, where 0 = no symptoms, 1 = mild symptoms, 2 = moderate symptoms, 3 = severe symptoms and 4 = very severe symptoms [25]. The number of plants with symptoms and the percent reduction in disease relative to the inoculated control were recorded. The results were analysed statistically to ascertain the significance of the results and compared. Further, the virus titre was assessed by DAC-ELISA using a polyclonal antiserum specific for GBNV [16]. The samples were collected 10 days after challenge with GBNV and were considered positive if the absorbance at 450 nm was more than three times the average of three healthy control samples. Subsequently, the abundance of GBNV in chilli plants was tested by quantitative PCR using a real-time PCR system (Bio-Rad, Germany). Sampling, standard curve preparation, and quantification of samples were performed using a standard protocol [26]. The normalized Ct values of unknown samples were compared to a standard curve. The threshold cycles were determined for the CP gene of GBNV from different treatments of chilli plants at 10 dpi and the number of copies per gram of plant tissue was calculated and compared.

Statistical analysis

The statistical significance of the results was analyzed by two-way analysis of variance (ANOVA). Multiple comparisons were done using the uncorrected Fischer's LSD test. Differences between mean values were considered statistically significant at $p < 0.05$.

Results

Virus isolate, coat protein (CP) gene characterization

Chilli showing characteristic symptoms of virus infection collected from a field was subjected to DAC-ELISA. The results revealed a strong positive reaction with an approximately fourfold increase in absorbance values compared to healthy samples against a polyclonal antiserum specific for GBNV. The positive samples were inoculated onto a local-lesion host, cowpea cv. C152, and the plants expressed characteristic circular lesions 4–5 days after inoculation. Subsequently, the inoculated cotyledonary leaves exhibited chlorotic and necrotic lesions with systemic veinal necrosis. cDNA derived from the extracted RNA of the chilli samples was amplified by PCR to produce an amplicon of approximately 840 bp, which was excised from a gel and cloned into pGEM-T Easy Vector. Independent clones were selected and confirmed by restriction analysis using EcoRI. The clones were sequenced in both directions using universal M13 primers and edited using BioEdit software to obtain the full-length nucleotide sequence of the CP gene. NCBI BLAST analysis showed that this sequence shared 99% nucleotide sequence identity to a sequence available in the NCBI database, confirming its identity as GBNV. The CP gene sequence of GBNV isolates were submitted to the NCBI GenBank database under accession no. MK424873.

Identification of potential antagonistic bacteria and screening against GBNV

A total of 50 bacterial isolates were isolated from the endosphere of chilli plants to test their antiviral activity against GBNV. Out of 50 bacterial endophytes, 10 isolates were tentatively identified as *Bacillus* spp. by biochemical tests, *viz.*, Gram staining, KOH test, citrate utilization test, catalase test, and gelatin hydrolysis. In order to identify the bacterial antagonist as *Bacillus* spp., a PCR reaction was performed to amplify its 16S rRNA. All ten isolates were positive by PCR, with a product size of ~ 1500 bp corresponding to 16S rRNA, and sequences of the amplified products were determined. BLAST searches of sequences against the NCBI database revealed that three out of the 10 antagonists were *Bacillus megaterium* (MK863555; MK875983 and MK876002), four were *Bacillus subtilis* (MK863567; MK863568; MK863569 and MK863570), two were *Bacillus amyloliquefaciens* (MK863573 and MK863574), and one was *Bacillus licheniformis* (MK863566), respectively. Screening of the 10 isolates of *Bacillus* spp. against GBNV reflected that *B. amyloliquefaciens* CRN9 effectively reduced the number of lesions from 32.00 lesions per leaf in the virus-inoculated control to 4.00 lesions per leaf. This was followed by *B. licheniformis* CRN3 and *B. megaterium* CRN2, which were effective in reducing the number of lesions to 6.67 and 7.67 lesions per leaf, respectively. The number of lesions in the other bacterial endophytes treated cowpea plants ranged from 8.00 to 13.67 lesions per leaf. (Fig. 1).

Amplification and cloning of the flagellin gene

Flagellin gene fragments were amplified with an amplicon size of 1.19 kb with restriction sites for *Xba*I and *BSP*.

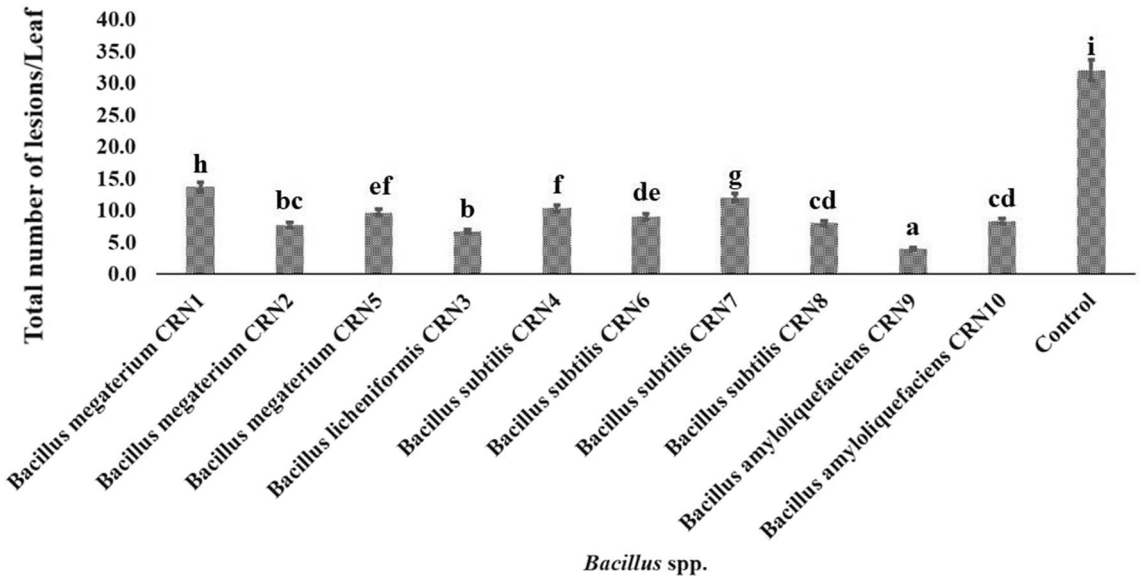

Fig. 1 In vitro efficacy of endophytic *Bacillus* spp. for the reduction of symptom expression upon artificial inoculation of GBNV. Error bars represent the standard deviation of the mean from three independent biological experiments. Different letters indicate significant differences between treatments

They were then cloned into pGEM-T Easy Vector and their sequence determined. In a BLAST similarity search, the flagellin clone showed more than 99% identity to a sequence from *B. amyloliquefaciens*, and the sequence was submitted to the GenBank database with the accession number MK947369. The resultant construct, pGEM-T (*flg*), was digested with the restriction enzymes *Xba*I and *BSP*, producing a 1.19-kb fragment containing the *flg* gene, which was subcloned into pBIN-GFP. The resulting plasmid, pBIN-*flg*, was digested with *Xba*I and *BSP* to produce a 1.19-kb fragment containing the flagellin gene. This gene construct was then mobilized into *Agrobacterium tumefaciens* strain LBA4404 via a triparental mating approach using pRK2013 as a helper strain (Fig. S1).

Transient expression of flagellin with consecutive defense gene analysis

The expression of flagellin and its ability to elicit the expression of MAMP-associated genes in chilli were investigated after agrodrenching with *A. tumefaciens* containing pBIN-*flg* versus challenge inoculation of GBNV. The results indicated a high level of expression of *flg*, with a strong band being visible from days 0 to 5 post-inoculation (dpi). The intensity of the band became very faint after 7 dpi. RT-qPCR data further revealed that the level of transcripts increased at 3 dpi and decreased at 7 dpi compared to the host reference gene UBI (Fig. 2). Meanwhile, the expression of MAMP-associated genes categorized as chemically induced, defense-responsive, pathogenesis-related (PR), and transcription factors was studied in chilli cv. Syngenta Bullet infected with GBNV at 0, 1, 3, 5, 7 and 9 dpi.

Differential expression of chemically induced and defense responsive genes

A relative expression analysis was performed to determine the transcript levels of seven defense-related genes that were induced in response to different signaling pathways. In plants treated with flagellin, the phytoalexin precursor gene *EAS1* was strongly upregulated as early as 3 dpi (1.38-fold), and reached a peak at 3 dpi (1.64- and 1.56-fold) in plants treated with *Bacillus* and BTH, respectively which was significantly higher than in the GBNV-inoculated control. Subsequently, the expression declined from 5 dpi. In correspondence with pathogen inoculation, inoculated control plants showed a 2.29-fold increase in gene expression at 1 dpi. Subsequently, the expression declined in all treatment groups at 5 dpi, except in agro and IC chilli plants at 9 dpi (Fig. 3a). *P*-values for all comparisons are provided in Supplementary Table S1.

Differential expression of transcription factor and non-expressor of pathogenesis-related protein 1 (*NPR1*)

A relative expression analysis of transcription factor WRKY33 gene revealed that the WRKY33 gene was upregulated as early as 1 dpi (0.45-fold) in plants treated with flagellin and reached a peak at 7 dpi (0.61-fold) in

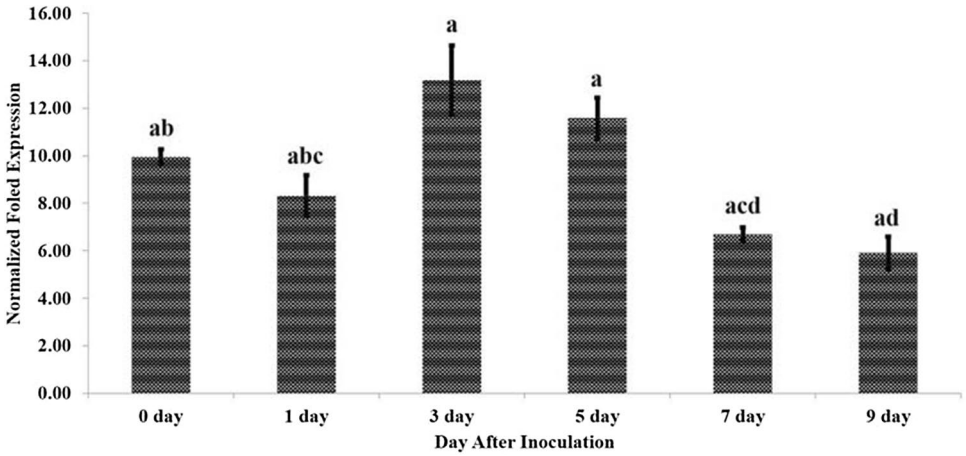

Fig. 2 Transient expression of flagellin in chilli plants. The chilli plants were agrodrenched with *A. tumefaciens* LBA4404 containing pBIN:flagellin. RT-qPCR was performed to determine gene expression of the flagellin at different times after inoculation filtration, and the samples were normalized against UBI3. The normalized fold expression of the flagellin (*flg*) gene in the healthy plant at time 0 was set equal to 1.0. Error bars represent the standard deviation of the mean from three independent biological experiments. Different letters indicate significant differences between treatments

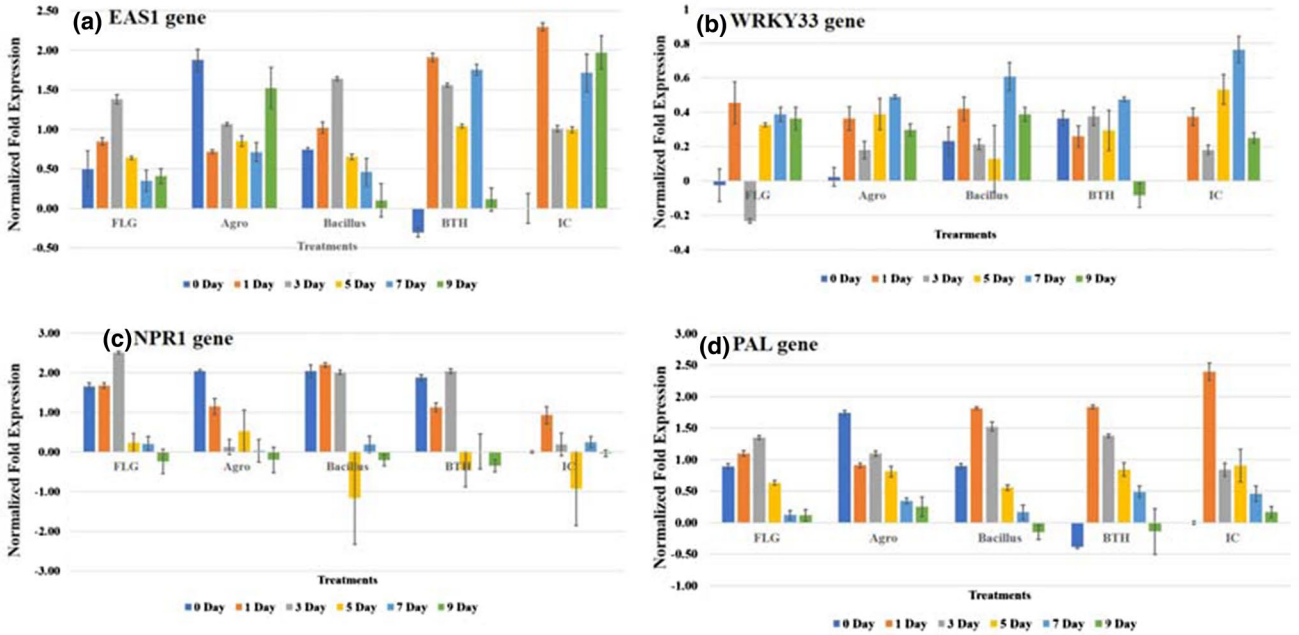

Fig. 3 Expression pattern of the a) EAS1, b) WRKY33, c) NPR1 and d) PAL genes after treatment (transient expression of flagellin, application of *Bacillus* and BTH) and challenge with GBNV inoculation. The expression level of genes was calculated relative to healthy control (HC) plants. The ubiquitin (UBI3) gene was used as an internal reference. The normalized fold expression of the genes at various intervals was set equal to 1.0 against HC. Error bars represent the standard deviation of the mean from three independent biological experiments

plants treated with *Bacillus*. Significant upregulation was also observed in all treatment groups at 7 dpi. Subsequently, the expression declined in all treatment groups at 9 dpi (Fig. 3b). The transcript accumulation for the NPR1 gene in *flg*-treated plants reached a peak at 3 dpi (2.5-fold) and a decreasing trend was observed from 5 dpi (0.23-fold). However, strong upregulation was observed in all treatment groups except the inoculated control as early as 0 dpi (Fig. 3c). Subsequently, downregulation of gene transcripts was observed in plants treated with *Bacillus* or BTH and in the inoculated control at 9 dpi.

Differential expression of salicylic acid (SA)-mediated defense responsive genes

The PAL gene plays a major role in priming defense-related genes in the SAR pathway. A transcript analysis revealed an increase in PAL gene transcript irrespective of treatment. The transcript level increased (1.10-fold) at 1 dpi and reached a maximum (1.35-fold) at 3 dpi in plants treated with the *flg* gene. The increase in the transcript level of PAL was very high in *Bacillus*-and BTH-treated plants (1.82- and 1.84-fold, respectively). Similarly, a high transcript level was observed in the inoculated control (2.39-fold) at 1 dpi, but it dropped to 0.9-fold after 5 dpi (Fig. 3d). For PO, the transcript levels increased by 1.73-fold in *flg*-treated plants as early as 1 dpi. A maximum peak of 2.39- and 2.25-fold was observed in *Bacillus*- and BTH-treated plants, respectively, at 1 dpi. However, irrespective of the treatment, the transcript level of PO in all cases decreased to 1.43-fold at 3 dpi, but it was significantly higher than the inoculated control (Fig. 4a). The transcript level of basic protein family (SAR 8.2), increased from 1.93-fold at 0 dpi to 2.0-fold after 3 dpi. It subsequently decreased to 0.61-fold after 3 dpi in *flg*-treated plants. However, downregulation of gene transcripts was observed at 0 dpi in plants treated with BTH and an increase was observed from 1 to 3 dpi. Compared to all the other transcripts, the level of SAR 8.2 in *Bacillus*-treated plants increased only marginally. In contrast, the transcript accumulation of the SAR 8.2 gene was constitutively higher in the inoculated control at 0 dpi (2.32-fold), and thereafter the expression declined from 1 dpi (Fig. 4b).

Differential expression of jasmonate (JA)-mediated defense-responsive genes

Application of agroinfectious clones of flagellin and *Bacillus* onto chilli plants increased the expression of lipoxygenase 3 (LOX3) and plant defensin 1.2 (PDF1.2) genes associated with JA biosynthesis. Agrodrenching with the *flg* gene increased the transcript of the LOX3 gene to 0.96-fold at 1 dpi. The transcript levels decreased from 3 to 5 dpi. The maximum level of transcript (2.07-fold) was observed in plants treated with *Bacillus*, and a strong downregulation of the transcript of the LOX3 gene (-0.08- and -0.97-fold) was observed at 3 dpi (Fig. 4c). Similarly, the plant defensin 1.2 (PDF1.2) gene transcript was strongly upregulated and reached a peak (1.16-fold) at 0 dpi immediately after the application of flagellin. A decreased level of transcripts was observed from 5 dpi onwards. Plants treated with *Bacillus* and *Agrobacterium* alone expressed higher levels of transcripts (1.75- and 1.71-fold) at 0 dpi. However, significant down regulation of transcripts level (-0.94-fold) was observed in plants treated with BTH at 0 dpi, a 1.2 fold increase in plant defensin was observed when flagellin and *Bacillus* were applied (Fig. 4d).

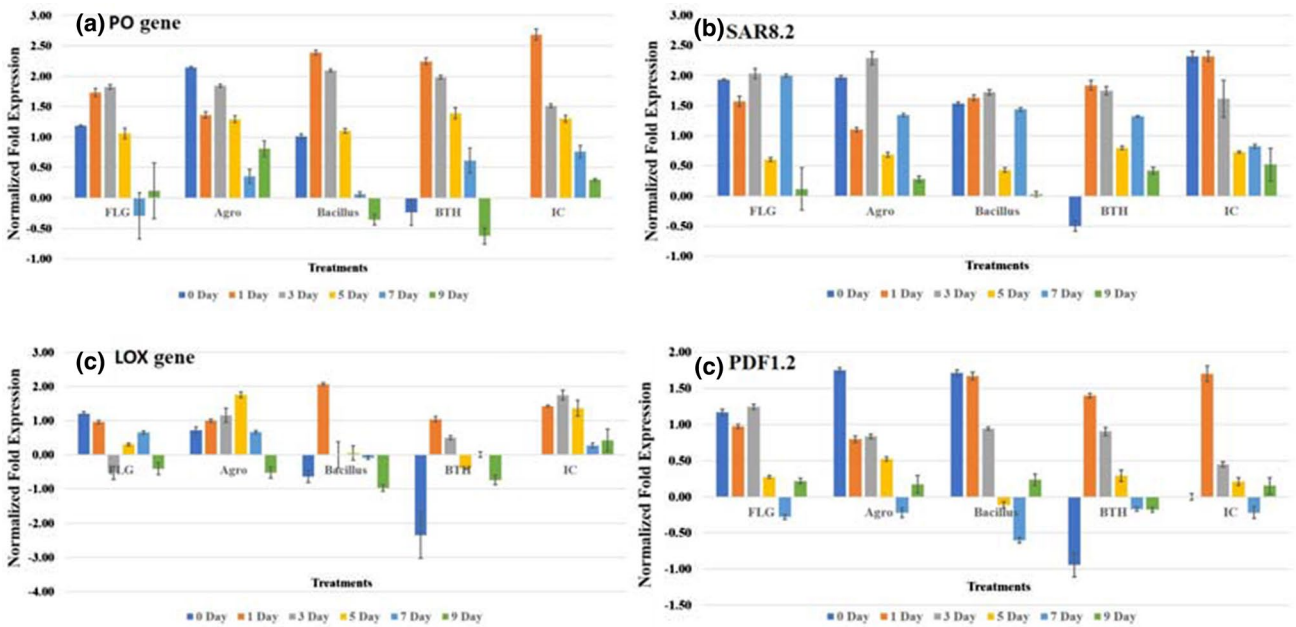

Fig. 4 Expression pattern of the a) PO, b) SAR8.2, c) LOX and d) PDF1.2 genes after treatment (transient expression of flagellin, application of *Bacillus* and BTH) and challenge with GBNV inoculation. The expression level of genes was calculated relative to healthy control (HC) plants. The ubiquitin (UBI3) gene was used as an internal reference. The normalized fold expression of the genes at various intervals was set equal to 1.0 against HC. Error bars represent the standard deviation of the mean from three independent biological experiments

Virus titer and disease assessment

To investigate the level of resistance against GBNV, plants responding to the applied treatments were selected and inoculated mechanically with GBNV at the four-leaf stage. The results revealed that transient expression of the flagellin gene and treatment with *Bacillus* effectively reduced the disease severity grade to 0.33 and 0.25, respectively, after 10 days of challenge inoculation with GBNV. Application of BTH was the most effective, reducing the disease severity grade to 0.39, whereas the inoculated control plant had a severity grade of 3.50 (Fig. 5). Characteristic symptoms of GBNV were observed in all of the virus-inoculated plants control group (12/12). The presence of virus was confirmed by DAC-ELISA, which showed a high virus titre at 405 nm. GBNV was detected in only two out of 12 plants that were agrodrenched with flagellin. Likewise, only one plant out of 12 treated with *B. amyloliquefaciens* CRN9 was observed to be infected with GBNV, and none of the BTH-treated plants gave a positive reaction for GBNV (Fig. 6; Table S2). qPCR was used to examine virus accumulation in parallel to expression of defense-related genes (Table 2). The lower concentration of target correlated with a higher C_t value. The qPCR results revealed a reduced accumulation of GBNV in chilli plants transiently expressing flagellin, with a minimum GBNV concentration of 1.42×10^5 copies when compared to virus-inoculated control at 9 dpi. Similarly, reduced copy numbers of GBNV (0.253×10^5 and 0.308×10^5) were observed in treated applied with *B. amyloliquefaciens* CRN9 and BTH, respectively, compared to the inoculated control, which had a copy number of 8.34×10^9.

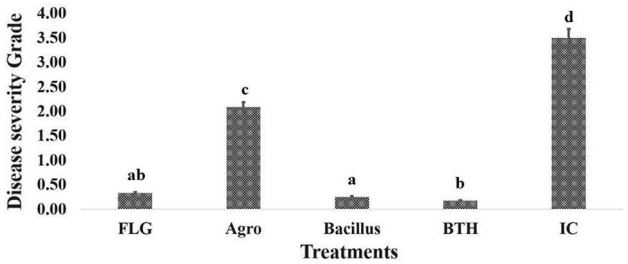

Fig. 5 Suppression of symptom expression in plants treated with flagellin, *Bacillus*, BTH and challenged with GBNV. Error bars represents the standard error of the mean. Different letters indicate significant differences between treatments

Table 2 Copy numbers of GBNV coat protein genes in plants treated with flagellin, *Bacillus*, or BTH and challenge inoculated with GBNV

Treatment	Transcript copy number (CP gene of GBNV)
Flagellin gene (transient expression)	1.42×10^5
Agrobacterium alone	1.26×10^8
B. amyloliquefaciens CRN9	0.253×10^5
BTH	0.308×10^5
Inoculated control	8.34×10^9

A lower concentration of target resulted in an increased C_t value. The unknown sample C_t values were correlated with a standard curve ($R^2 = 0.92$; $y = -2.7206x + 44.45$), which refers to a 10-fold dilution of a known concentration of the CP gene of GBNV

Discussion

Tospoviruses causing necrotic diseases are evolving as major viral pathogens in chilli and other vegetable crops under protected and field conditions in Tamil Nadu, India. GBNV is artificially transmitted through mechanical sap inoculation and is transmitted by insect vectors under field conditions. The major symptoms of necrosis disease include the presence of circular chlorotic and necrotic patches, necrotic streaks on the stem, stunting of plants, and failure to produce flowers if infection occurs in the early stages of the crop. We identified the cause of the necrosis disease to be a member of the genus *tospovirus* using an inoculation test on cowpea and testing samples using a polyclonal antiserum specific to GBNV. Our results are in agreement with those of Jain et al. [27], who made the GBNV-specific antiserum and detected the virus in infected chilli under field condition. Similarly, Gopal et al. [4] surveyed for GBNV infection in chilli under field condition and confirmed the infection using DAC-ELISA. Recently, Sharma and Kulshrestha [6] also detected

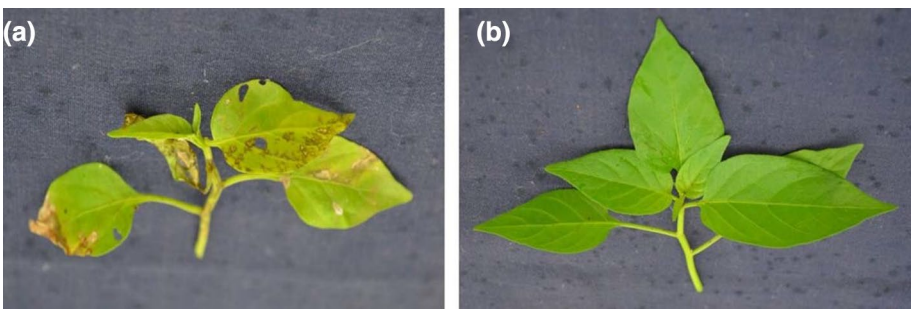

Fig. 6 Representative symptoms of GBNV in inoculated control plants (a) in contrast to the majority of the flagellin, BTH, and *Bacillus*-treated plants (b), which were symptomless

GBNV infection in bell pepper plants in Himachal Pradesh, using DAC-ELISA and DAS-ELISA with a polyclonal antiserum specific for GBNV. The pathogen was confirmed as GBNV by PCR, which produced an amplicon of 832 bp corresponding to coat protein gene of GBNV. Sequence analysis showed that this virus was similar to existing isolates from chili and tomato. Similar results were also obtained by Kunkaliker et al. [2], who characterized the CP gene of GBNV from chilli and tomato plants. Management of virus disease in crop plants can be achieved by PGPR, which has broad-spectrum of antimicrobial activity and promotes growth [28]. However, the defense mechanism triggered by the PGPR against virus disease remains unknown. In our study, endophytic bacteria were isolated from chilli and identified as *Bacillus* spp. based on the sequence of the 16S rRNA region. An isolate of *B. amyloliquefaciens* CRN9 inhibited the symptom expression in a local lesion host up to 87.50% compared to an untreated control. Similarly, Vinodkumar et al. [11] reported that *Bacillus* spp. had antiviral activity against necrosis disease of cotton caused by tobacco streak virus. PGPR and *Enterobacter asburiae* BQ9 reduced the severity of disease caused by tomato yellow leaf curl virus (TYLCV) under protected conditions, up to 52% promoted tomato plant growth, and induced resistance to TYLCV [29]. The MAMP molecules of PGPR act as elicitors, activating the first line of defense by interaction with pattern-recognising receptors at the plasma membrane, activating a signalling cascade that leads to the induction of immunity.

To study GBNV resistance in chilli, we investigated the priming effect of the *B. amyloliquefaciens* CRN9 and the role of flagellin against GBNV through the expression of defense-related genes in a susceptible cultivar due to lack of resistant commercial cultivars. In the present study, *Bacillus* induced the transcription of flagellin and other defense-signaling genes in chilli plants challenged with GBNV. Flagellin, a monomeric component of the flagellum, is the MAMP molecule involved in elicitation of a defense response in plants. The plant detects a specific epitope of flagellin known as flg22 during the host-microbe interactions, which is recognized by the plant receptor-like kinase (RLK) FLS2 [30, 31]. Flagellin binding leads to a cascade of signalling events triggering defense gene expression and production of reactive oxygen species [32]. Plant immunity induced by flagellin is associated with elevated levels of cytosolic Ca^{2+} and Ca^{2+}-dependent immune signalling that occurs as a result of interaction between the epitope-containing peptide flg22 and the FLS2 receptor. This is mainly dependent on influx of extracellular Ca^{2+} into the cytosol through the plasma membrane [33]. Recently, it was reported that transient expression of flagellin-derived PAMPs (flg22 and flgII-28) in tomato resulted in transcriptional reprogramming and enhanced defense gene expression.[34], suggesting that flagellin may interact with the FLS2 receptor in chilli plants, inducing the signalling pathway for SAR- and ISR- mediated resistance against invading GBNV.

The plant defense is not a constitutive and requires the accumulation gene transcripts in the infected plant to counter the risk posed by the invading pathogens. In the present study, the gene transcript of the epi-aristolochene synthase gene (*EAS1*) accumulated to a significantly higher level in plants treated with flagellin and *Bacillus* than in virus-inoculated control plants. The plant challenged with virus alone produced a higher accumulation of *EAS1* at 1 dpi, and a reduced level of accumulation was observed in the ensuing days, indicating the antiviral activity of phytoalexin capsidiol. 5-epi-aristolochene synthase catalyzes the formation of 5-epi-aristolochene, which is an immediate precursor of the bicyclic phytoalexin capsidiol and later on produces the bicyclic sesquiterpenic phytoalexin capsidiol [35]. Resistant genotypes of chilli produced more *EAS1* gene transcripts upon inoculation with capsicum chlorosis virus [15], suggesting that induction of *EAS1* gene expression in chilli plants might represent a part of the induced resistance mechanism, which can directly affect the degree of resistance against the pathogen.

Plant defense reactions against pathogen infection in plants can be triggered by the recognition of MAMPs, elicitors, exogenous application of plant hormone, and biotic or abiotic stresses. These hormonal induced defense responses in plants are mediated by SA or JA. In the present study, chilli plants treated with flagellin and challenged with GBNV showed a maximum accumulation of WRKY33 transcripts at 1 dpi when compared to the control. This was in line with the observations of Jingyuan et al. [36], who found that chilli plants sprayed with 5 mM SA, produced high levels of CaWRKY30 transcripts, which accumulated 2 h after treatment and reached a level that was 19-fold higher than in compared to the control. CaWRKY is a positive regulator of PR gene expression, a component of the L-mediated resistance response to tobacco mosaic virus in hot pepper [37]. Moreover, treatment with *Bacillus* or BTH induced the *NPR1* expression, the basic component of plant defense against virus infection. Consistent with previous reports, *Bacillus* treatment resulted in increased transcription of *NPR1*, indicating a primed state or induction of SAR [13]. In our study, plants treated with flagellin and challenged with virus had higher level of transcript accumulation at 1 dpi and 3 dpi when compared to virus-inoculated untreated plants. However, the levels decreased at 5 dpi, which indicated that up-regulation was more pronounced (2.20-fold) in flagellin-treated plants than in *Bacillus*- and BTH-treated plants.

In marker expression with *NPR1*, the PAL gene was significantly upregulated and peaked at 3 dpi in flagellin-treated plants. Interestingly, a higher level of transcript accumulation was observed at 1 dpi in plants treated with *Bacillus*

and BTH. A similar trend was observed for the transcripts of the PO, PPO, SAR 8.2 genes, which accumulated significantly. The significant upregulation of the PR genes in plants treated with flagellin, *Bacillus* and BTH indicated the induction of resistance through SA in chilli. The basal expression of PAL and PO genes, whose gene products belong to the family of PR-proteins, has been strongly associated with resistance against pathogens [23]. Likewise, a basic protein family called SAR 8.2 was induced locally and systemically in pepper and tobacco plants upon pathogen infection and salicylic acid treatment during the onset of SAR [38, 39]. Overexpression of the SAR 8.2 gene in *Arabidopsis* results in increased defense activity against *Fusarium oxysporum* f. sp. *matthiolae*, *Botrytis cinerea, Pseudomonas syringae* pv. tomato and abiotic stress. Transgenic lines of *Arabidopsis* plants overexpressing CaSAR82A exhibit high levels of expression of PR genes associated with high levels of transcripts in CaSAR8.2. The induction profile of CaSAR8.2 resembles that of PR proteins, and it is considered to be a member of a novel PR protein family [40, 41]. This suggests that MAMPs-triggered expression of PR genes is a prerequisite for initial suppression of the viral pathogen through the activation of the SAR pathway.

The lipoxygenase (LOX) pathway is crucial for lipid peroxidation and lead to the formation of JA from linolenic acid, which is a marker for JA-dependent signalling and induced systemic resistance against biotrophic pathogens [42, 43]. In our study, LOX gene expression was induced in plants treated with flagellin, *Bacillus* and BTH. Likewise, defensins, members of the PR-protein family (PR-12), contribute to defense signaling and serve as effectors of JA signaling and resistance in several plant species after infection by necrotrophic pathogens. In the present study, the PDF 1.2 gene was upregulated in plants treated with flagellin, *Bacillus* and BTH. The antiviral activity of defensins has been reported by Kachroo et al. [44], who found that inoculated leaves of Arabidopsis (Col-0) plants inoculated with turnip crinkle virus (TVV) exhibited enhanced basal-level expression of the defensin (PDF 1.2) gene and activation of both the MeJA and ethylene pathways, which are required for the expression of PR12 (PDF1.2). On the other hand, coexpression of the *PR-1* and *PDF1.2* genes in wild-type *Arabidopsis* Col-0 through the application of *B. amyloliquefaciens* EXTN-1, indicated that *B. amyloliquefaciens* EXTN-1 induces systemic resistance through the salicylic-acid- and jasmonic-acid-dependent pathways and leads to a counterattack against the invading viral pathogen [45]. It is manifested that there is a combined pathway leading to resistance against virus through specific communication with host components.

The results presented in this report show that exogenous application of *Bacillus* reduces the severity of necrosis disease caused by GBNV in chilli. Similar results have been reported by several workers investigating the reduction of the viral symptoms in crop plants though the application of several bioagents [46, 47]. Hongwei et al. [29] reported that *Enterobacter asburiae* BQ9 significantly reduced the severity of disease caused by tomato yellow leaf curl virus (TYLCV) under greenhouse conditions by up to 52% and promoted tomato plant growth. Similarly, foliar application of leaf-colonizing *B. amyloliquefaciens* has been shown to protect *N. benthamiana* and pepper plants against cucumber mosaic virus under field conditions [48]. Furthermore, while all the untreated control plants were positive when tested using a polyclonal antiserum specific for GBNV, only a few plants treated with *Bacillus* were positive for viral infection by DAC-ELISA, indicated the enhanced antiviral resistance of susceptible plants after treatment. Interestingly, agrodrenching with flagellin and *Bacillus* resulted in reduced virus accumulation at the early stages of infection and delayed virus detection in apical leaves, similar to treatment by exogenous application of BTH. This was confirmed by qPCR, which clearly showed a reduced accumulation of GBNV in agrodrench experiments with flagellin and *Bacillus* spp. This is in agreement with the observations of Beris et al. [13], who demonstrated that tomato plants treated with *B. amyloliquefaciens* strain MBI600 and SA had reduced virus titres, indicating enhanced antiviral activity against TSWV and PVY. Similarly, Vanthana et al. [49] found that tomato plants treated with *B. amyloliquefaciens* VB7 showed significantly reduced disease severity and GBNV titer. They also suggested a role of flagellin and elongation factor of *B. amyloliquefaciens* VB7 as inducers of MAMP-triggered immunity in tomato through expression of PR1, NPR1, MAPK and WRKY33. In our study, the flagellin gene of *B. amyloliquefaciens* CRN9, a native isolate from chilli, triggered the ISR-mediated defense mechanism of disease resistance through the activation of both salicylic acid and ethylene mediated pathways against necrosis of chilli caused by GBNV. Our results also showed that foliar application of *B. amyloliquefaciens* CRN9 during the early stages of crop growth regulated MAMP-triggered immunity against GBNV, which implies that it can be used for the biological control of necrosis disease in chilli and can play an important role in sustainable agriculture.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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