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Diversity and antiviral potential of rhizospheric and endophytic *Bacillus* species and phyto-antiviral principles against tobacco streak virus in cotton

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ABSTRACT

Plant growth promoting rhizobacteria arewidely exploited for the management of various fungal and bacterial diseases in plants. However, antiviral action of PGPR and their efficiency have been rarely investigated. In the present study, influential diversity of *Bacillus* species was related with tobacco streak virus (TSV) infection in cotton. The study revealed that, the population of *B. subtilis, B. licheniformis* and *B. velezensis* in rhizosphere of apparently healthy cotton plants were comparatively higher than TSV infected plants, indicating that they efficiently colonized the rhizosphere. Similarly, the population of the endophytic *B. cereus* and *B. licheniformis* were relatively higher in apparently healthy than TSV infected cotton plants. *In vitro* screening of rhizospheric, endophytic *Bacillus* species and phyto-antiviral principles revealed that rhizospheric *B. amyloliquefaciens* (VB7) and endophytic *B. licheniformis* (CoEH6) were effective in the suppression of TSV symptoms in indicator host (cowpea). The strain *B. amyloliquefaciens* (VB7) contained ten antimicrobial peptide genes responsible for the biosynthesis of antibiotics including, iturin, bacilysin, bacillomycin, surfactin, subtilin, and subtilosin. Moreover, the strain VB7 is reported to secrete pentadecenoic acid, heptadecenoic acid, octadecenoicacid, pyrrolo, piperazinedione and tetradecenoic acid, which would have together complemented in the antiviral activity. Upon simultaneous inoculation of the bacterium or phyto-antiviral principles with TSV in the indicator host plant, revealed that *B. amyloliquefaciens* (VB7) and *M. jalapa* were much effective and reduced the number of lesions up to 2.22/leaf and 3.00/leaf respectively compared with TSV inoculated control (25.28 lesions cm^{-2} area). Further, under field conditions, soil application and foliar spray of the *B. amyloliquefaciens* (VB7) resulted in 52 per cent reduction in TSV incidence. For reference TSV incidence in *B. amyloliquefaciens* (VB7) treated and untreated plots were 21.67 (PDI) and 45 (PDI) respectively. Moreover, *B. amyloliquefaciens* (VB7) improved seed cotton yield upto 149.45 g/plant compared to control (97.71 g/plant). Thus *B. amyloliquefaciens* (VB7) was exploited as an efficient antagonist for the management of TSV in cotton.

1. Introduction

Cotton regarded as white gold is an economically important crop in India. Major cotton producing states in India include, Gujarat, Maharashtra, and Telangana. Productivity of cotton is hampered due to various bacterial, fungal, and viral diseases. Among them, cotton necrosis caused by tobacco streak virus (TSV) is an emerging threat in India (Vinodkumar et al., 2017b). The species *Tobacco streak virus* (TSV) belonging to the genus *ilarvirus* is a multipartite, single-stranded, positive-sense, RNA virus. The host range comprises 200 plant species including, agricultural, horticultural crops and weeds (Fulton, 1948, 1985). In our previous study, severity of cotton necrosis was observed to be highest in Telangana with 51.1 percent disease incidence (PDI), compared to other states in India surveyed, which include, Andhra Pradesh, Maharashtra and Tamil Nadu (Vinodkumar et al., 2017b).

In plants, management of viral disease is a difficult task. Most of the plant viral diseases are transmitted through vectors. In the case of TSV, transmission has been confirmed through infected seeds and thrips species (Jagtap et al., 2012; Sharman, 2009). Besides, TSV infection is also reported to be systemic in cotton (Rageshwari et al., 2017). Vector control plays a crucial role in the management of viral diseases. However, due to indiscriminate application of chemicals results in the loss of

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non-targeted, beneficial, microflora. This have reoriented the scientists to lookup for the need for an alternate method of management. Under these circumstances induction of host defense mechanism is a promising solution. Host defense could be induced through biotechnological approaches by the production of resistant lines. Antagonistic bioagents also play a major role in the induction of host defense. *Bacillus* species have been widely exploited for the management of various fungal diseases, however against viral diseases they remain unexplored. Very few studies have reported the antiviral efficiency of *Bacillus* species against, cotton leaf curl virus (Ramzan et al., 2016), cucumber mosaic virus in tomato (Zhender et al., 2000), tomato mottle virus in tomato (Murphy et al., 2000) and tobacco mosaic virus in tobacco (Wang et al., 2009). *Bacillus* species have been reported with diverse anti microbial peptide (AMP) genes responsible for the biosynthesis of antibiotics like iturin, bacilysin, bacillomycin, fengycin, surfactin, mersacidin, ericin, subtilin, subtilosin, and mycosubtilin (Vinodkumar et al., 2017a). Antibiotics produced by the bacteria have specific modes of actions. Even though the efficacy and mode of action of ant-microbial peptides have been widely studied against various fungal and bacterial diseases, their interactive study with virus remains uninvestigated.

Phyto-antiviral principles including *Mirabilis jalapa, Harpullia cupanioides, Bougainvillea spectabilis,* and *Terminalia chebula* are also exploited. Antiviral activity of *M. jalapa* is well studied and proved to be effective against potato virus X, potato virus Y, potato leaf roll virus, and potato spindle tuber viroid (Vivanco et al., 1999). Renuka Devi et al. (2004) reported that *H. cupanioides* (HAP) was highly effective in inhibiting TSWV at 80% saturation. *T. chebula* has been reported with antiviral activity against human viruses (Kesharwani et al., 2017) however not tested against plant viruses.

Recognizing the impact of cotton necrosis and the need for an alternate management strategy, the following study was carried out. This study includes the population analysis and characterization of the bacterial community residing as endophytes and rhizosphere microorganisms in both, TSV infected and healthy cotton plants. Further, *Bacillus* species and phyto-antiviral principles were screened for their efficacy against TSV in the indicator host plant, cowpea. Effective strains were screened for their efficiency against TSV in cotton under open field conditions. The reduction in TSV incidence and growth promoting characteristics of the microorganisms were also recorded.

2. Materials and methods

2.1. Isolation and identification of rhizospheric and endophytic bacterial community

Rhizospheric and endophytic bacterial community was isolated from apparently healthy and TSV infected cotton plants (Islam et al., 2016; Egamberdieva et al., 2017). Apparently healthy plant denotes symptomless cotton plants, since TSV has been reported to be present even as systemic and latent manner. The soil and leaf samples were collected from one-month old cotton plants including, MCU12- variety and RCH659 – hybrid in the same field from five different plants each. Rhizosphere samples were subjected for serial dilution technique and 1 ml of the aliquot from 10^{-6} dilution was spread on to nutrient agar medium for the enumeration of culturable bacterial community associated with rhizosphere. The plates were incubated for 48 h at 28 ± 2 °C.

For the enumeration of culturable endophytic bacterial community associated with leaf, 1 g of the leaf sample from apparently healthy and TSV infected leaves were washed thoroughly in sterile water. Later, they were surface sterilized with 0.5% sodium hypo chlorite (NaOCl) for 2 min. The chemical traces were removed by washing the tissue in double sterile water for six times. The sixth wash was maintained as a sterile check. After washing, the samples were dried on sterile filter paper and ground with phosphate buffered saline (PBS) (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) in a sterile pestle and mortar. The ground samples were subjected for serial dilutions and 1 ml of 10^{-4} dilution were plated on nutrient agar medium. The plates were incubated for 48 h at 28 ± 2 °C.

After 48 h, representative of colonies that differed in size, shape and colour were selected and pure cultured on to fresh Petri plates containing nutrient agar. Further, the CFU of the representative bacterium per gram of soil or leaf tissue was also assessed.

The representative colonies were characterized by sequencing the 16S rRNA gene. DNA was extracted and the universal primers (Wilson et al., 1990), 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT -3′) were used for amplifying the 16S rRNA gene. PCR reaction was performed with GoTaq® DNA Polymerase (Promega Corporation, Madison, WI, USA) in a thermocycler (Eppendorf-Master Cycler nexus gradient S -Eppendorf, A G, Hamburg, Germany) (Crespo-Medina et al., 2014). The PCR products were sequenced at Chromous Biotech Pvt. Ltd., Bangalore, India. The sequences were compared with the previously submitted sequences available in NCBI. Further their identity was also compared with the sequences available in EzBioCLoud (Yoon et al., 2017). After comparison the sequences were submitted in NCBI and accession numbers were obtained. The newly obtained sequences were subjected to phylogenetic analysis with MEGA 7.0 (Kumar et al., 2016) by comparing with the 16S rRNA gene sequences retrieved from Genbank database. The diversity of the bacteria in different samples were assessed with Shannon - Wiener and simpson indices (Shannon and Weaver, 1949; Simpson, 1949). The CFU data for rhizospheric and endophytic bacteria were pooled for individual treatment and the analysis was performed.

2.2. In vitro screening for the antiviral activity of the bacterial community and phyto-antiviral principles against TSV in indicator host - cowpea (CO7)

Since mechanical inoculation in cotton plants are cumbersome *in vitro* screening was performed in cowpea (CO7) plants. As per our previous studies cowpea (CO7) was the most suitable indicator host with highest virus titre (Vinodkumar et al., 2017a,b). Four bacterial strains with the highest population (CFU g^{-1} of soil or leaf tissue), among all the strains isolated was used for the study. Further, seven effective bacterial strains with known anti microbial peptide (AMP) gene profile from our previous studies were also included for screening the antiviral activity. The strains from our earlier studies include, *B. amyloliquefaciens* - VB7 (KJ603234), *B. subtilis* - VB9 (KJ603236), *B. megaterium* – BmTNAU3 (KC540825), *B. pumilus* – BSC4 (JX036519), *B. licheniformis* - BlTNAU3 (KC540820), B. *cereus* – BSC5 (JX036520), and *Ochrobactrum* – BSD5 (JX036527). The strains were selected based upon their antagonistic performance with a range of plant pathogens and their ability to promote plant growth. *B. amyloliquefaciens* (VB7) comprised 10 anti microbial peptide (AMP) genes responsible for the biosynthesis of antibiotics including, iturin, bacilysin, bacillomycin, surfactin, subtilin, and subtilosin. GC/MS analysis of the non-volatile secretary metabolite confirmed *B. amyloliquefaciens* (VB7) to secreted wide ange of fatty acids including, pentadecenoicacid, heptadecenoicacid, octadecenoicacid, pyrrolo, piperazinedione and tetradecenoicacid (Vinodkumar et al., 2017a).

Along with bacterial strains, certain phyto-antiviral principles including, *Mirabilis jalapa* root powder, *Bougainvillea spectabilis* leaf extract, *Harpullia cupanioides* seed powder, and *Terminalia chebula* seed powder, were also screened for their efficiency in suppressing symptom expression in cowpea, upon TSV inoculation.

2.2.1. Preparation of cowpea (CO7) plants

Cowpea (CO7) plants were raised in pots containing sterilized pot mixture (red soil: sand: farm yard manure @ 1:1:1 w/w/w sterilized at 121 °C @ 15 psi for three times). The plants were incubated in ambient glass house environment with 25 ± 2 °C. The plants are ready for the experiment when it reached two leaf stage.