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Biological control of *Alternaria alternata* causing leaf spot disease of *Aloe vera* using two strains of rhizobacteria

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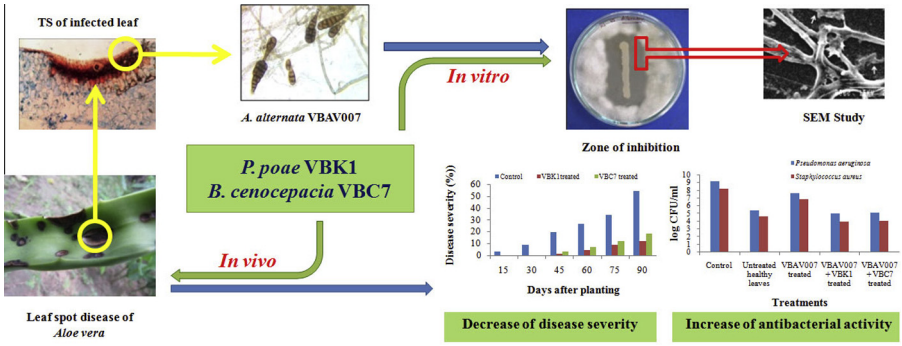
HIGHLIGHTS

- Leaf spot pathogen *Alternaria alternata* have been isolated from *Aloe vera*.
- Pathogen destructs leaf mesophyll tissue and decrease antimicrobial potential.
- Two biocontrol bacterial strains inhibit pathogenic growth both *in vitro* and *in vivo*.
- SEM study indicated mycelia breakage of pathogen by biocontrol bacterial strains.
- Biocontrol agents decrease disease severity and increase antimicrobial potential.

GRAPHICAL ABSTRACT



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ABSTRACT

Fungal pathogens causing the leaf spot disease of *Aloe vera* have been isolated from 40 randomly selected infected leaves, collected from different locations of Birbhum and Burdwan districts of West Bengal. All of the isolates showed similar morphological characteristics and the strain VBAV007, isolated from a severely infected *Aloe* leaf was identified as *Alternaria alternata* by D1/D2 region of 28S rRNA gene sequence homology. In addition to the destruction of leaf mesophyll tissues the pathogen also decreases the antimicrobial potential of *A. vera* gel. The commercially available fungicide mancozeb was effective at low concentration (100 μg/ml) to control the pathogen whereas it can tolerate 1000 μg/ml or more concentrations of bavistin. Two plant growth promoting rhizobacterial strains, viz. *Burkholderia cenocepacia* VBC7 and *Pseudomonas poae* VBK1 were able to produce prominent zones of inhibition against the pathogen in dual culture overlay plates. 89.3 ± 1.22% and 81.5 ± 2.67% inhibitions of conidial germination of the pathogen were noticed in the presence of cell free supernatant of VBK1 and VBC7 respectively. Radial growth assay also suggested prominent growth inhibition of VBAV007 by biocontrol strains. They induce mycelial breakage of pathogen as evidenced by scanning electron micrographs. Greenhouse challenge experiments also suggested excellent capabilities of biocontrol agents to reduce disease severity in good measure even after exposure to high concentration (3.1×10^4 conidia/ml) of pathogenic spores. During *in vivo* field experiments 54.25 ± 3.55% disease severity was observed for untreated plants, whereas only 11.69 ± 1.25% and 15.22 ± 2.64% disease severities were noticed in plants treated with VBK1 and VBC7 respectively. Since biocontrol organisms have the potential to decrease the disease severity, they also help to maintain the good health as well as antimicrobial potential of *A. vera* plants.

Abbreviations: CFU, Colony forming unit; DMSO, Dimethyl sulphoxide; ME, Malt extract; CFS, Cell free supernatant; SEM, Scanning electron microscopy.

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1. Introduction

Aloe vera is one of the important medicinal plants of the family liliaceae, It is a stem less succulent species with thick, fleshy, lance-shaped leaves. The color of the leaves varies from green to grey green. *A. vera* is mainly important for its gel, which is colorless, mucilaginous and is obtained from the parenchymatous cells in the *Aloe* leaves. The gel is viscous, odourless and transparent liquid and slightly bitter in taste. The gel contains 98.5% water and 0.3% carbohydrates (Grindlay and Reynolds, 1986). In addition to different polysaccharides, it also contains several amino acids, a numbers of enzymes, minerals, phytosterols and some vitamins (Surjushe et al., 2008). Nowadays not only the cosmetic industries but also the traditional systems of medicines make use of *A. vera*. *A. vera* products have proper wound healing properties (Dat et al., 2012). The aloesin derivative of *A. vera* act as an antioxidant and produces a strong anti-inflammatory effect (Yagi et al., 2002). It serves as an important moisturizing, anti-ageing and anti-acne agent (West and Zhu, 2003). It is highly effective on skin exposure to UV and gamma radiation (Roberts and Travis, 1995). *A. vera* has extremely useful antimicrobial properties to inhibit the growth of *Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Candida albicians* and *Penicillium sp.* (Renisheya et al., 2012).

Leaf spot disease of *A. vera* is a very serious ailment causing massive loss to *A. vera* production. This disease is marked by dark brown necrotic spots, circular to oval with grayish centers developing on either surface of the leaves. As the leaves get severely infected they start drying off from the tip leading to a significant reduction in the mucilaginous gel content of the leaves. It has already been reported that leaf spot disease is caused mainly by the fungal pathogen *Alternaria alternata* (Silva and Singh, 2012; Bajwa et al., 2010). On the other hand Zhai et al. (2013) reported another fungus *Nigrospora oryzae* as the pathogen of such type of leaf spots in *A. vera*.

Until now it has been possible to recommend only few chemicals to counteract the leaf spot disease of *A. vera*. Sharma and Amrate (2009) found two chemical fungicides Amistar and Score to be effective in inhibiting the spore germination of the pathogen at very low concentrations. Panwar et al. (2013) reported the antifungal potential of some fungal biocontrol agents against leaf spot pathogen of *A. vera*. Any suitable biological control of leaf spot disease of *A. vera* using bacterial antagonists is yet to be reported.

In the present work we have tried to isolate and identify the pathogen causing leaf spot disease of *A. vera*. We have also focused on the harmful activities of the pathogen in course of infection. Finally, we tried to find out safe strategies to control the pathogen of disease of *A. vera*, that use bacterial biocontrol agents while avoiding the use of chemical fungicides.

2. Materials and methods

2.1. Collection of samples

The pathogens causing leaf spot disease of *A. vera* were isolated from two locations in the districts of Birbhum and Burdwan respectively in West Bengal, India. In all 40 highly infected leaf samples with large number of leaf spots were collected randomly from diseased fields during the month of July. All the leaves were thereafter stored immediately at 4 °C for further exercises.

2.2. Study of infected A. vera leaves under light microscope

A small piece of infected leaf was taken and transverse sections through the infected regions were prepared with the help of a sharp blade. Then the samples were stained with cotton blue and finally mounted with lactophenol. It was observed with the help of light microscope under 10× and 40× magnifications.

2.3. Isolation of pathogen causing leaf spot disease of A. vera

All the infected leave samples were surface sterilized with 0.1% $HgCl_2$ solution and then washed thrice with sterilized distilled water to remove the $HgCl_2$. The leaves were cut into small pieces containing the infected regions and placed on sterilized malt extract (ME) agar plates and incubated at 28 °C for 72 h. Streptomycin (100 μg/ml) was incorporated with ME to avoid bacterial contaminations. After emergence of fungal mycelia they were further purified by streaking on ME agar plates and finally stored in ME slants at 4 °C for further studies.

2.4. Characterization and Identification of the isolated pathogen

Fungal pathogens were characterized morphologically under light microscope (Olympus CH2Oi) after staining with cotton blue and lactophenol. One of the pathogenic strains VBAV007, was then identified based on molecular technique using D1/D2 region of LSU (Large Sub Unit: 28S rDNA). Forward and reverse DNA sequencing reactions were carried out with DF and DR primers, using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Nucleotide sequence of D1/D2 region of 28S rDNA gene was generated and used for carrying out BLAST with the nrdatabase of NCBI GenBank database. On the basis of maximum identity score the first ten sequences were selected and the neighbor-joining phylogenetic tree (Saitou and Nei, 1987) was constructed, using MEGA 4 (Tamura et al., 2007). The evolutionary distances were computed, using the Kimura 2-parameter model (Kimura, 1980).

2.5. Effect of different fungicides on isolated pathogen

The effects of two commonly used fungicides, viz. mancozeb and bavistin, were checked at different concentrations (50–1000 μg/ml) against the isolated pathogen by agar well diffusion method (Fernández-Garayzábal et al., 1992). The zones of inhibition were measured and compared after 72 h of incubation at 28 °C. Conidial germination patterns were also studied at their different concentrations of fungicides on cavity slides (Saravanan et al., 2004).

2.6. In vitro activity of biocontrol bacterial isolates against isolated pathogen

Two different strains of plant rhizospheric bacteria viz. *Burkholderia cenocepacia* VBC7 (NCBI GenBank Accession No: HM042678) and *Pseudomonas poae* VBK1 (NCBI GenBank Accession No: GU384238) were used (Mukhopadhyay, 2010) for controlling the isolated pathogen *in vitro*. Antifungal activities of these two bacterial strains were checked against isolated pathogens by dual culture overlay assay (Magnusson and Schnürer, 2001) with at least three replicates. After 72 h of incubation at 28 °C the zones of inhibition were observed and compared.

The antifungal activities of both boiled (10 min in water bath) and unboiled cell free supernatants (CFS) of these two biocontrol bacterial strains were also checked against isolated pathogen by agar well diffusion method (Fernández-Garayzábal et al., 1992). Both the bacterial strains were grown in nutrient broth for 24 h with mild shaking (120 rpm) at 28 °C and centrifuged at 10,000 rpm for 20 min to get the CFSs. The effects of CFSs on spore germination of VBAV007 was also tested on cavity slides followed by microscopic observations (Saravanan et al., 2004) and the percentages of spore germinations were calculated. All the experiments were repeated and replicated thrice.