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Identification of a Novel *Streptomyces* Species for Management of Plant Disease

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Native isolates of groundnut stem rot casual organism, *Sclerotium rolfsii* Sacc. Were collected from major groundnut growing areas of Tamil Nadu. Selected isolates were screened, characterized and indentified the virulent isolate (GNSR1). Several native bacterial and fungal antagonists were isolated against GNSR1. Two antagonistic isolates, *viz.*, GNRAJK1 and GNRAVR14, were found to have antagonistic effects on GNSR1. Morphology and spore structure of isolated antagonists, GNRAJK1 and GNRAVR14, were studied under Light microscopy, Biochemical test, Thin layer chromatography and Biolog analysis. It confirmed the group of micro organisms as *Streptomyces* based on gram staining, medium specific growth, visual morphological characters, cell wall amino acid studies and spore forming chraracters. The genus and species level of the antagonists were identified by Fatty Acids Methyl Esters (FAME) Analysis. The isolate GNRAJK1 had a SIM index of 0.007 and was identified as *Streptomyces violaceusniger,* and GNRAVR14 had a SIM index of 0.013 and was identified as *Streptomyces exfoliatus*.

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Keywords: Streptomyces violaceusniger, Streptomyces exfoliates, Sclerotium rolfsii; groundnut; stemrot; biological control; FAME analysis.

1. INTRODUCTION

"The excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution and the development of pathogen resistance to fungicide. Microbial antagonists are widely used for the biocontrol of fungal plant diseases due to the lack of induction of pathogen resistance and reduction of chemical fungicide residues in the environment. Understanding the pathogen, developing and relay on a single antagonism becomes challenging and gives way to exploring and identifying the suitable alternate antagonist against the disease. *Streptomyces* are common inhabitants of the rhizosphere and act as beneficial microorganisms for plant growth and development" [1]. "The genus *Streptomyces* was well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi" [2]; [3]; [4]. In the present study, fatty acids methyl ester studies were conducted to identify the *Streptomyces* species by identifying 9 and 20 length carbon fatty acids and related compounds present in the antagonists by Sherlock MIS software.

2. METHODOLOGY

2.1 Isolation of Antagonist from the Rhizosphere of Groundnut Against *S. rolfsii*

Antagonistic fungi and bacteria were isolated from the rhizosphere soil collected from different groundnut growing areas of Tamil Nadu. The plants were pulled out gently with intact roots, and the excess soil adhering to the roots was removed gently. Ten grams of rhizosphere soil was transferred to a 250 ml Erlenmeyer flask containing 100 ml of sterile distilled water. After thorough shaking, the antagonist in the suspension was isolated using the serial dilution plate method. From the final dilutions of 10^{-3}, $10^{-5}$and 10^{-6}, one ml of each aliquot was pipetted out, poured in a sterilized Petri dish containing PDA medium, King's B medium, nutrient agar and yeast malt extract medium separately, and they were gently rotated clockwise and -anti-clockwise for uniform distribution and incubated at room temperature (28±2°C) for 24 hours. "The colonies were viewed under UV light at 366nm. Colonies with characteristics of *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp. were isolated individually and purified by streak plate method on Nutrient agar medium, yeast malt extract medium and King's B medium. Morphological identification was made through a light microscope at 40x, and pure cultures were maintained on respective agar slants at 4°C". [5]

2.2 Sample Processing

A 4mm loop was used to harvest about 40mg of bacterial cells from the third quadrant (second or first quadrant if slow-growing) of the quadrant streaked plate. The cells were placed in a clean 13x100 culture tube. The culture was subjected to Saponification**,** Methylation, Extraction and Base Wash.

2.3 Hardware Factors

The Sherlock MIS Software was used with the Agilent technologies 5890, 6890 or 6850 gas chromatographs. The Sherlock System's unique configuration was designed for optimal analysis of Fatty Acid Methyl Esters by gas chromatography.

2.4 Ultra 2 Column

A 25m x 0.2mm phenyl methyl silicone fused silica capillary column has both the chromatographic performance and the column lifetime desired for routine analysis of bacterial extracts. The column is required to have more than 4,000 theoretical plates per meter for peaks with k = 7 to 9. Since the stationary phase was cross linked to the silica tube, there was less noise and drift during temperature programmed runs.

2.5 Gas Chromatograph

The temperature programme ramps from 170°C to 270°C at 5°C per minute. Following the analysis, a ballistic increase to 300°C allows cleaning of the column during a hold of 2 minutes. The flame ionization detector allows for a large dynamic range and provides good sensitivity. Hydrogen was the carrier gas, nitrogen was the "makeup" gas, and air was used to support the flame.

2.6. Autosampler

The use of an autosampler allows the system to be operated unattended for up to 2 days at a time. Samples were logged into the computerized sample table, and all sampling (including STAT samples) was done automatically**.**

2.7. Computer

The electronic signal from the GC detector was passed to the computer, where the integration of peaks was performed. The electronic data was stored on the hard disk, and the fatty acid methyl ester composition of the sample was compared to a stored database using the Sherlock pattern recognition software.

2.8. Calibration and Peak Naming

The Sherlock MIS used an external calibration standard developed and manufactured by MICROBIAL ID, Inc. The standard is a mixture of straight chained saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and five hydroxy acids. All compounds were added quantitatively so that the gas chromatographic performance could be evaluated by the software each time the calibration mixture was analyzed. The hydroxy compounds are especially sensitive to changes in pressure/ temperature relationships and to contamination of the injection port liner. As a result, these compounds function as quality control checks for the system Retention time data obtained from injecting the calibration mixture was converted to Equivalent Chain Length (ECL) data for bacterial fatty acid naming. The ECL value for each fatty acid was derived as a function of its elution time in relation to the elution times of a known series of straight chain fatty acids

$$ECL_x = \frac{Rtx\text{-}Rtn}{Rt(n+1)\text{-}Rtn}$$

Where Rtx is the retention time of x; Rtn is the retention time of the saturated fatty sacid methyl ester preceding x; Rt(n + 1) is the retention time of the saturated fatty acid methyl ester eluting after x. Thus, it is possible, by comparison to the external standard, to compute the ECL value for each compound following an analysis. The GC and column allow windows to be set at 0.010 ECL unit wide giving great precision in the resolution of isomers. After naming the peaks in an unknown sample, Sherlock compares the ECL values for the most stable series (e.g., saturated straight chain or branched chain acids) to the peak naming table's theoretically perfect values and may recalibrate internally if sufficient differences are detected. This feature allows the system to be run for up to two days unattended without worrying about drift between runs.

2.9. Libraries

The Sherlock libraries consist of more than 100,000 analyses of strains obtained from experts and from culture collections. The cultures were collected from around the world to avoid potential geographic bias.

3. RESULTS AND DISCUSSION

The antagonists groups were further reconfirmed by identifying 9 and 20 length carbon fatty acids and related compounds present in the antagonists by Sherlock MIS software used with Agilent technologies 5890, 6890, or 6850 gas chromatography. Retention time data obtained from injecting the calibration mixture was converted to equivalent chain length (ECL) data for bacterial fatty acid naming. Based on the ECL derivation of 0.006 and SIM Index of 0.007, the antagonist GNRAJK1 was identified as *Streptomyces violaceusniger* - *violaceusniger* (Fig 1), and the closely related species were *Streptomyces lavendulae* and *Streptomyces halstedii* - *olivaceus*. The antagonist GNRAVR14 was identified as *Streptomyces exfoliatus* (Fig. 2) with ECL derivation of 0.003, and SIM Index of 0.013, and the closely related species were *Streptoverticillum-septatum* and *Streptomyces lavendulae*.

This result corroborated with the findings of [6,7,9,10,11,12], which enumerated that *Streptomyces* species exhibit biocontrol activity, and it correlates with their production of antibiotics. Though there was no report on *S. exfoliatus,* which produces polysaccharide degrading enzymes have practical applications in fields. Some groups of bacteria are more amenable to fatty acid composition analysis for identification.

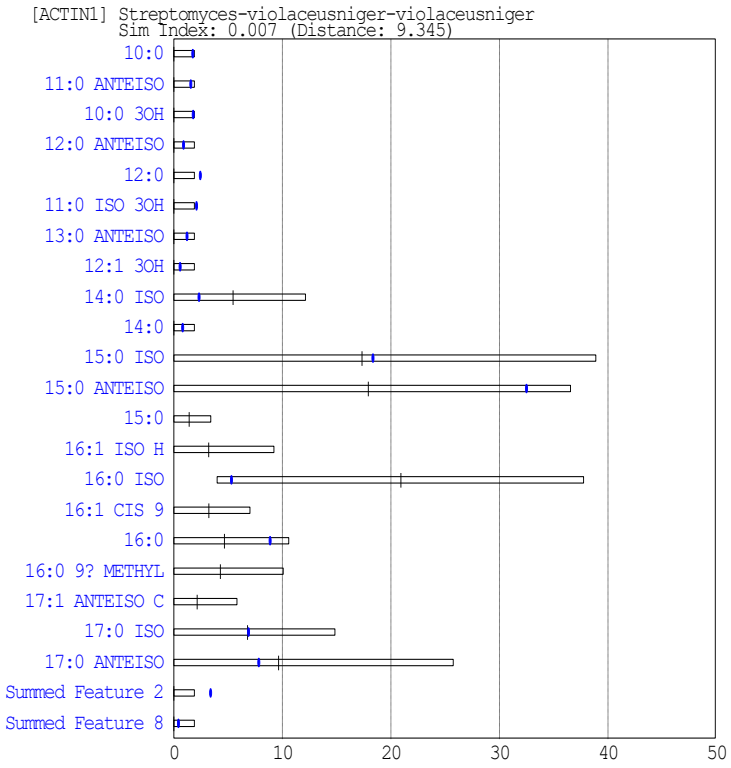

Fig.1. Sherlock Report

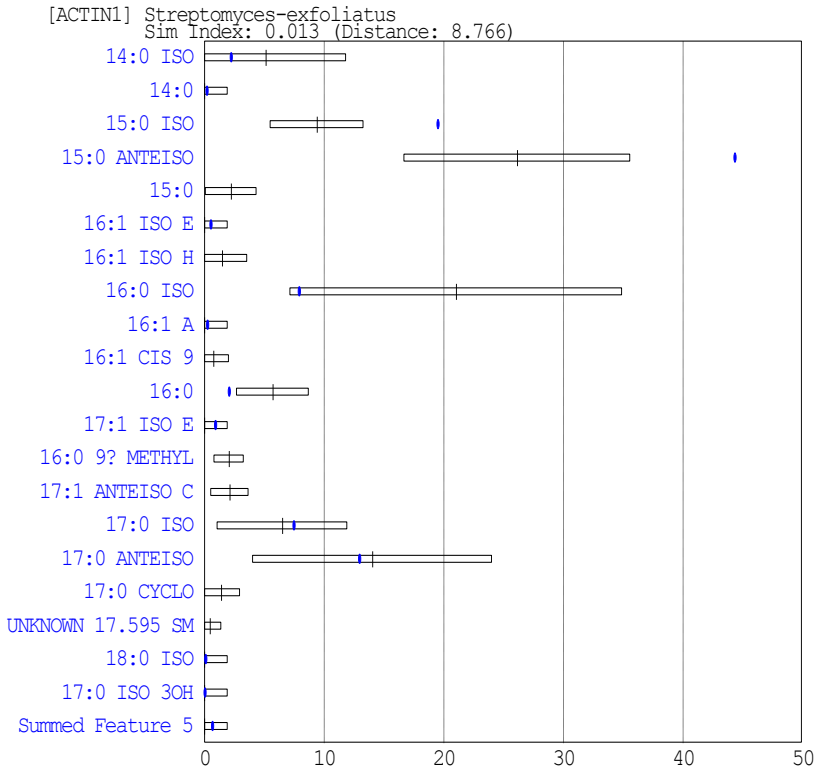

Fig. 2. Sherlock Report

Fig. 3. Dendrogram

4. CONCLUSION

The genus name of the unknown antagonists was identified as *Streptomyces*. Further, fatty acids methyl ester studies were conducted to identify the species by identifying 9 and 20 length carbon fatty acids and related compounds present in the antagonists by Sherlock MIS software. The antagonist GNRAJK1 was identified as *Streptomyces violaceusniger* - *violaceusniger* and GNRAVR14 as *Streptomyces exfoliatus* based on the equivalent chain length for bacterial fatty acids.The isolate GNRAJK1 had sim index of 0.007 and was identified as *Streptomyces violaceusniger,* and GNRAVR14 had SIM index of 0.013 and was identified as *Streptomyces exfoliates.*

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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