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Potential of *Microbispora* sp. V2 as biocontrol agent against *Sclerotium rolfsii*, the causative agent of southern blight of *Zea mays* L (Baby corn)–*in vitro* studies

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The study was undertaken with the aim of exploring novel and beneficial agro activities of rare actinomycetes like *Microbispora* sp. V2. The antagonistic activity of *Microbispora* sp. V2 was evaluated as a biocontrol agents against *Sclerotium rolfsii*, a soil-borne fungal plant pathogen. The methodology performed for evaluation of biocontrol agent was *in vitro* evaluation assay which comprised of three tests viz., cellophane overlay technique, seed germination test and Thiram (fungicide) tolerance of *Microbispora* sp. V2. The isolate was found to inhibit the fungal pathogen *Sclerotium rolfsii* to 91.43% in cellophane assay. In seed germination assay, *Microbispora* sp. V2 treated seeds resulted in 25.75% increased germination efficiency, as compared to seeds infected by *Sclerotium rolfsii*. The isolate *Microbispora* sp. V2 could tolerate 1000 µg mL^{-1} of Thiram (fungicide). The *in vitro* assay studies proved that *Microbispora* sp. V2 can be used as antifungal antagonist and thus posses' great potential as biocontrol agent against southern blight caused by *Sclerotium rolfsii* in *Zea mays* L (Baby corn) which causes large economical losses.

Keywords: Biocontrol agent, *Microbispora* sp.V2, *Sclerotium rolfsii, Zea mays*

As the problems in fungal disease control are increasing, a serious search is needed to identify alternative methods for plant protection, which are less dependent on chemicals and are more environment friendly. Microbial antagonists are widely used for the biocontrol of fungal plant diseases. Metabolites from microorganisms are important sources of molecules with antifungal properties. These microbial metabolites may help to overcome the resistance and pollution problems caused by chemical pesticides due to their versatility in structure and possible enhanced biodegradability[1]. One of the severe fungal plant pathogen is *Sclerotium rolfsii* Sacc (*Athelia rolfsii* (Curzi) Tu & Kimbroug). It causes the southern blight disease in a wide variety of crops.

The chemical fungicides in high concentrations have adverse environmental effects due the toxicity and biomagnification, causing health hazards to humans and other non-targets. Several disease management strategies employ biocontrol agents in combination with fungicide at reduced concentrations for disease control[2, 3]. Fungicide Thiram is applied popularly as seed dressing against *Sclerotium rolfsii*.

The objectives of this study are to (i) to check the ability of phenazine producing isolate *Microbispora* sp. V2 to inhibit pathogenic fungus *Sclerotium rolfsii* the causative agent of southern blight disease in baby corn (ii) and determination of tolerance towards Thiram for its successful survival in field conditions. The *Microbispora* sp. V2 has been thus evaluated for its biocontrol capabilities in order to gain potential inoculant to enhance crop yields.

Materials and Methods

Cellophane overlay technique—For determining antifungal activity of *Microbispora* sp. V2 as biocontrol agent against *Sclerotium rolfsii in vitro*, cellophane overlay technique was employed[4]. The strain of *Microbispora* sp. V2 was isolated from hot water spring at Vajrashwari near Mumbai, Maharashtra, India[5].

Test microorganisms—Fungal pathogen: The culture *Sclerotium rolfsii* (NCIM 1084), pathogen causing south blight of soybean was procured from CSIR-National Chemical Laboratory (NCL), Pune, India. The fungus was grown on Potato Dextrose Agar (PDA, Hi-Media) for 10 days at 30 ^{o}C.

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Antagonist Microbispora sp. V2—*Microbispora* sp.V2 was grown in optimized sesame oil cake broth[6] for 6 days at 30 °C. Sesame oil cake (7.5 g) was added to 100 mL tap water and boiled for 20 min. Final volume was adjusted to 100 mL with tap water and pH was adjusted to 7.0. It was then autoclaved at 121 °C for 15 min. The mycelial growth was harvested by centrifugation at 10,000 rpm for 15 min and was resuspended in sterile 0.02% teepol saline and turbidity of the homogenous suspension was adjusted to 0.5 McFarland standards (0.4×10^4 - 5×10^4 cfu mL^{-1}) CLSI (M38-A, 2002).

Culture medium for cellophane overlay assay—Optimized sesame oil cake agar medium was used for cellophane overlay assay. Both *Microbispora* sp. V2 and *S. rolfsii* were grown on optimized sesame oil cake agar medium till adequate growth was observed.

Cellophane overlay assay—For the cellophane overlay technique, cellophane membrane (Shivananda Marketing Private Limited, Delhi 110 006, India), 9 cm in diameter was boiled in distilled water interleaved with filter paper and autoclaved at 121 °C for 20 min. A cellophane membrane was placed on the sesame oil cake agar in each petri plate of 9 cm and dried in a laminar flow cabinet for 15 min. A 6-day old culture of *Microbispora* sp.V2 strain suspended in sterile 0.02% teepol saline was smeared on the entire surface of the cellophane. Care was taken to avoid touching the margins and puncturing the cellophane layer to prevent any direct contact between *Microbispora* sp. V2 and the agar. For controls, sterile 0.02% teepol saline was applied. The plates were incubated at 40 °C for 8 days, after which the cellophane membrane with adhering *Microbispora* sp. V2 was removed. A plug (5 mm diameter) of *S. rolfsii* was placed on the sesame oil cake agar plate previously occupied by the antagonist. The plates were then incubated in the dark at 25 °C for 8 days. The surface area of *S. rolfsii* colonies were recorded daily and compared with the control. The pathogen colony diameter was measured and the area was calculated. The percent growth inhibition was calculated using the formula $n = (a-b) / a \times 100$ where n is percent growth inhibition, a is the colony area of uninhibited *S. rolfsii* and b is the colony area of treatment. The data for 4 and 8 days after inoculation of the pathogen in cellophane technique were pooled and analyzed statistically[4].

Seed germination assay (pathogenicity test)—The extent of pathogenicity of *S. rolfsii* was determined by seed germination assay[7].

Preparation of sclerotia of S. rolfsii for disease infestation—S. rolfsii isolate was grown on PDA and sclerotia were formed rapidly (in about 10 days). Mass multiplication of *S. rolfsii* was carried out in Potato Dextrose Broth at room temperature for 3 weeks[8]. The sclerotia produced were used for infecting *Zea mays* seeds for germination assay.

Preparation of biocontrol agent—The biomass of isolate *Microbispora* sp. V2 was harvested after fourth day of fermentation in optimized sesame oil cake media for production of phenazine-1-carboxylic acid (75.24 mg L^{-1}) at 30 °C. The concentration was decided to restrict the phytotoxicity of phenazines in the biocontrol formulations[9,10]. *Microbispora* sp. V2 from such fermented broth (100 mL) with 5×10^7 cfu mL^{-1} was added with 1% carboxy methyl cellulose[11] (CMC, Merck).

Seed germination assay—Zea mays L (Baby corn) seeds (Sonal variety) were obtained from Amar Seeds Pvt. Ltd., Pune 411 016, India. The seeds were surfaced sterilized in 1% sodium hypochlorite solution (Qualigens, Fine Chemicals Pvt. Ltd., India) for 3 min and rinsed twice in sterile distilled water.

For pathogenicity testing, the surface sterilized seeds were soaked in sclerotial suspension with 2 sclerotia per seed for 2 h[12]. Seeds were then placed in sterile 90 mm diameter petri dishes lined with filter paper and allowed to germinate for 7 days at 30 °C. Four petri dishes were used with 10 seeds in each (four replications). The percent of germinated healthy and rotten seeds was calculated.

The effect of *Microbispora* sp. V2 on seed germination was also tested. *Microbispora* sp. V2 from optimized fermented broth with production of phenazines upto 75.24 mg L^{-1} was used for coating *Z. mays* seeds. *Microbispora* sp. V2 from such fermented broth (100 mL) with 5×10^7 cfu mL^{-1} was added with 1% CMC to which surface sterilized *Z. mays* seeds (5) were immersed for 2 h for seed treatment formulation[13]. The seeds were put in sterile 90 mm diameter petri dishes lined with filter paper. Four petri dishes were used with 10 seeds in each (four replications). Germination was observed for 7 days. Effect on seed germination was recorded. Seeds soaked in sterile water and treated with CMC as well as the unsoaked, dry, surface sterilized

seed served as control. These controls were not treated with antagonist and pathogen.

Fungicide tolerance of Microbispora sp. V2—The isolate was tested for its tolerance to Thiram [Vegfra Thiram (Pesticides India)]. Hence effect of various concentrations of Thiram on viability of *Microbispora* sp. V2 was checked out[14]. Thiram was purchased from the local market. Stock solution of 1000 µg mL^{-1} was prepared in distilled water. The *Z. mays* seeds were treated with 1 g of Thiram per kg of seeds. This corresponds to 1000 µg per g of seeds. Hence concentration range of Thiram was selected from 10 µg to 1000 µg mL^{-1}.

Antagonist Microbispora sp. V2—Microbispora sp. V2 was grown in optimized sesame oil cake broth for 6 days. The mycelial growth was harvested by centrifugation at 1000 rpm for 15 min and suspended in sterile 0.02% teepol saline. The turbidity of the homogenous suspension was adjusted to 0.5 McFarland standards (0.4×10^4 to 5×10^4 cfu ml^{-1}) with sterile 0.02% teepol saline [CLSI (M38-A, 2002)].

Fungicide tolerance assay—To determine the fungicide tolerance, in 100 mL of sesame broth, Thiram was added to obtain final concentrations of 5, 10, 20, 30, 50, 100, 250, 500 and 1000 µg mL^{-1} from the stock solution of 1000 µg mL^{-1}. 1 mL of *Microbispora* sp. V2 in 0.02% teepol saline was inoculated in each flask. Inoculated flasks were incubated for 6 days on a rotary shaker at 40 °C and 180 rpm. The growth of *Microbispora* sp. V2 in terms of dry cell weight (g L^{-1}), in each treatment was monitored at regular intervals of 24 h, in comparison to control without any fungicide. The experiments were repeated with three replications. The experiment was also carried out on sterile sesame agar medium in 500 mL conical flasks, Thiram was added to obtain final concentrations of 5, 10, 20, 30, 50, 100, 250, 500 and 1000 µg mL^{-1} from the stock solution of 1000 µg mL^{-1}. The sesame agar plates were prepared, each with 20 mL medium. *Microbispora* sp. V2 in 0.02% teepol saline was inoculated on these plates. The plates were incubated for 6 days at 40 °C. The growth of was monitored by microscopic observations at regular intervals of 24 h, in comparison to control without any fungicide. The experiments were repeated with three replications.

Results

Cellophane overlay technique—The isolate was found to inhibit the fungal pathogen *S. rolfsii* to 91.435±0.03% in cellophane assay (Fig. 1). As the isolate *Microbispora* sp. V2 could inhibit *S. rolfsii* in cellophane assay, the studies were conducted further to determine the efficiency of the isolate to inhibit *S. rolfsii* in seed germination assay and to find out the potential of isolate as biocontrol agent against the fungal plant pathogen.

Seed germination assay (pathogenicity test—In seed germination, seeds protected with *Microbispora* sp. V2 showed a very good response for germination. As compared to seeds infected by *S. rolfsii*, the *Microbispora* sp. V2 treated seeds showed 25.75% more germination efficiency (Table 1).

Fungicide (Thiram) tolerance of Microbispora sp. V2—The isolate *Microbispora* sp.V2 was tolerant to Thiram at all the test concentrations. By broth dilution, 12±57% reduction in growth of *Microbispora* sp V2 [dry cell weight (g L^{-1})] was observed in presence of Thiram as compared to control (Fig. 2). In case of agar dilution method, the microscopic studies revealed that the isolate could tolerate 1000 µg mL^{-1} of Thiram fungicide and also could grow at 20 and 30 °C. This tolerance of isolate will prove to be useful when it will be applied as biocontrol agent in near future to the soil contaminated with Thiram.

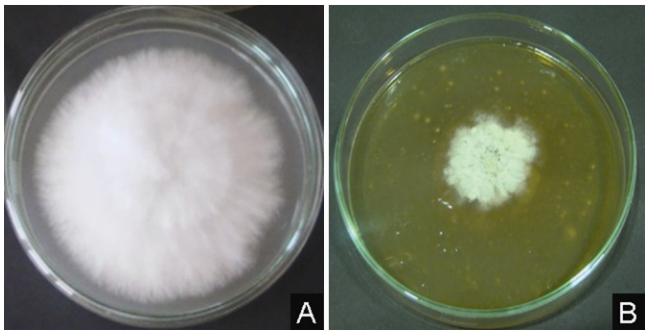

Fig. 1—Growth of *S. rolfsii* on sesame agar (cellophane overlay assay). [A: Control. B: Test growth of *S. rolfsii* on sesame agar plate pregrown with *Microbispora* sp. V2. incubated at 25 °C for 8 days].

Table 1—Effect of bacterization on germination of *Zea mays* L (Baby corn)

Replicate no.	Seed Germination (%)		
	Control	Seeds treated with	
		S. rolfsii	*Microbispora* sp.V2
1.	69	51	76
2.	68	50	76
3.	68	50	76
4.	68	50	75
Mean ± SD	68.25±0.57	50.25±0.50	76.00±0.00

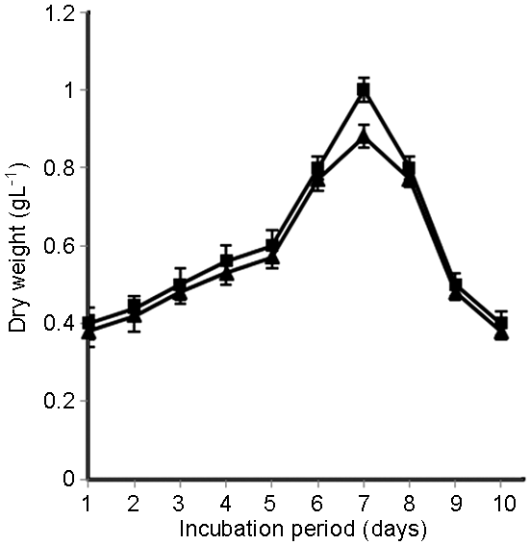

Fig. 2—Thiram (Fungicide) tolerance assay of *Microbispora* sp. V2. [Expressed in terms dry cell weight (g L^{-1})] (—■—) growth of *Microbispora* sp. V2 in absence of Thiram, (—▲—) growth of *Microbispora* sp. V2 in presence of Thiram (1000 µg/mL).

Discussion

This study focused on screening the rare actinomycete *Microbispora* sp. V2 in order to detect its ablility to control the phytopathogenic fungus *Sclerotium rolfsii*. The *in vitro* evaluation assay performed for testing of biocontrol agent comprised of cellophane overlay technique, seed germination test and fungicide tolerance of *Microbispora* sp. V2. As *Microbispora* sp. V2 was found to inhibit the fungal pathogen *S. rolfsii* to 91.43% in cellophane assay and increased germination efficiency by 25.75% in seed germination assay, it was proved to be capable of protecting *Z. mays* from southern blight caused by *Sclerotium rolfsii* under laboratory conditions. The results are in accordance to the results seen in case of *Pseudomonas cf. monteilii* 9 producing non-volatile diffusible antibiotic[15] which inhibited *S. rolfsii*. Compatibility studies of this isolate as potential biocontrol agent with fungicide Thiram revealed that it was compatible with Thiram even at 1000 µg mL^{-1} and pesticidal contamination in field soil will not affect the biocontrol agent effectiveness. The present *in vitro* study clearly proved that *Microbispora* sp. V2 can be easily applied in conjugation with the pesticide for the control of soil borne plant pathogens. Such integrated studies have been done for biocontrol agents *Trichoderma viride*, *Trichoderma harzianum*, *Pochonia chlamydosporia*, *Pseudomonas fluorescens* and *Bacillus subtilis*[16,17]. Hence *Microbispora* sp. V2 can be employed as antifungal antagonist against southern blight disease caused by *S. rolfsii* in *Zea mays* L (Baby corn) and can be successfully exploited in the integrated disease management in future.

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