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Self-fusion of *Streptomyces griseus* enhances chitinase production and biocontrol activity against *Fusarium oxysporum* f. sp. *lycopersici*

A. ANITHA[1] and M. REBEETH[2]

[1]Ph.D. Research Scholar, Department of Biotechnology, Kongunadu Arts and Science College, G.N. Mills Post, Coimbatore - 641 029 (India).
[2]Lecturer, Department of Biotechnology, Kongunadu Arts and Science College, G.N. Mills post, Coimbatore - 641 029 (India).

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ABSTRACT

Protoplasts were isolated from Streptomyces griseus (MTCC - *4734) strain using lysing enzymes and self-fusion of Streptomyces griseus protoplasts was carried out using 50% polyethylene glycol (MW 1000, Sigma Chemicals Co., USA) in protoplast buffer. The regenerated 8 self fused Streptomyces griseus were studied detailed for chitinase production and biocontrol activity. Parent strain (PSg) showed protein content of 2.7 mg/ml with chitinase activity of 120 IU/ml. High chitinase activity was measured in the culture filtrates of most of the self-fusants (87%) than the parent. Among the fusants, the strain SFSg 5 produced protein content of 7.8 mg/ml, maximum chitinase activity of 283.3 IU/ml with a two-fold increase as compared to the parent strain. All the self-fusants exhibited increased antagonistic activity against F. oxysporum f. sp. lycopersici than the parent. Maximum inhibition (82%, 80%) of mycelial growth of F. oxysporum was recorded with fusant of SFSg 5, SFSg 1 as against 61.1% with PSg. The result implies that, the self-fused Streptomyces griseus resulted in appreciable increase of chitinase production and biocontrol activity also the significance of the protoplast fusion technique, which could successfully be used to develop hybrid strains also for commercial formulation.

Key words: *Streptomyces griseus*; protoplast fusion; chitinase production and antagonistic activity.

INTRODUCTION

Tomato *(Lycopersicon esculentum*) is one of the most popular and important commercial vegetable crops grown throughout the world. It is rich in vitamins A, B and C. In India, it occupies an area of 0.54 million ha with a production of 7.60 million tonnes[1]. Many diseases and disorders can affect tomatoes during the growing season. *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is a highly destructive pathogen caused wilted plants, yellowed leaves and minimal or absent crop yield. There may be a 30 to 40% yield.[2]

Microbial antagonists have been widely studied as biological controls for the last few years. *Trichoderma* is one of the most exploited fungal biocontrol agents in agriculture for the management of plant diseases caused by a wide spectrum of fungal pathogens[3, 4]. *Streptomyces* sp. is a gram positive filamentous bacterium that produces a wide array of biologically active compounds is an attractive candidate for biological control[5]. Many of these biological control agents are still being tested and are not commercially available[6]. Although naturally occurring organisms provide a major source of chitinolytic enzymes, genetic improvement

plays an important role in their biotechnological applications. Among the different methods available, protoplast fusion is a preliminary important tool for bringing genetic recombination and developing hybrid strains[7]. Previous studies clearly demonstrated the feasibility of protoplast fusion and for improving biocontrol activity [8, 9]. But not much work has been focused in *Streptomyces* for increasing the production of chitinase enzyme using this technique. Therefore, the aim of the present study was to isolate and self-fusion of protoplasts from *Streptomyces griseus* for enhancing the chitinase production and antagonistic potential was studied and the results were presented.

MATERIAL AND METHODS

Organism

Chitinase enzyme producing *Streptomyces griseus* (MTCC - *4734) stain purchased from Microbial Type Culture Collection, Chandigarh, India was used for the current protoplast fusion and antagonistic activity studies. Soil borne pathogenic fungi *F. oxysporum* f. sp. *lycopersici* causing wilt disease, isolated from infected plants of major tomato growing areas in Coimbatore district was studied.

Isolation of protoplasts

About 1ml of spore suspension of *Streptomyces griseus* was inoculated into 100ml of Yeast extract Malt Extract (YEME) medium and incubated in a rotary shaker at 30°C for 36-40 hrs. After incubation, the culture was centrifuged and separated spores were separated. The collected spores were re suspended in 1.5 ml of 10.3% sucrose, centrifuged and the supernatant was discarded. The spores were re suspended in 0.4 ml of (lysing enzymes) lysozyme solution (1 mg/ml 0.1 M Phosphate buffer; pH 6.0) and it was incubated at 30°C for 15-60 min with mild shaking and the release of protoplast was monitored regularly under light microscope. Draw the solution in and out of 1 ml pipette three times, incubated at 30°C for 15 min. Then 0.5 ml of phosphate buffer (0.1M; pH 6.0) was added and repeated the above step. The suspension was filtered through cotton wool and centrifuged at 1000g for 7 min. The supernatant was discarded and sedimented protoplasts were suspended in 1 ml protoplast buffer.[10]

Fusion and regeneration

Self-fusion of protoplasts of *Streptomyces griseus* was carried out by the method of Hopwood *et al.*[10] Polyethylene glycol (PEG) (MW 1000, Sigma Chemicals Co., USA) prepared in protoplast buffer (Sucrose – 10.3g, K_2SO_4 – 0.025g, $MgCl_2.6H_2O$ – 0.202g, Trace element solution – 0.2 ml, Water – 100ml, Autoclaved and stored at 4°C) was used as fusogen. The suspended protoplast was sediment by centrifuged at 1000g for 7 min. The supernatant was discarded and the pellet was resuspended in remaining liquid. Then 5 ml of protoplast buffer was added and repeated the above step once again. Resuspended the pellet, added 0.8 ml of 50% PEG solution and immediately pipetting rapidly in and out of pipette once. The fused protoplast was serially diluted and the suspension were plated on 2% colloidal chitin agar (CCA) selective medium containing (g/l) colloidal chitin - 2.0; sucrose - 1.0; $NaNO_3$ -2.0; K_2HPO_4 - 1.0; KCl - 0.5; $MgSO_4$ - 0.5; $FeSO_4$ - 0.01; agar - 15; distilled water 1000ml at pH 6.5 for getting individual fused colonies. The plates were incubated at room temperature and the protoplast regeneration and development of colonies were observed. [9]

Preparation of culture filtrates of parent and fusants colonies

The parent and self-fusant strains of *Streptomyces griseus* colonies regenerated in plates were inoculated in to 50ml of colloidal chitin broth (CCB) and incubated at 30°C on a rotary shaker. After six days, the cultures were harvested, centrifuged at 10,000rpm at 4°C. The cell free culture filtrates of parent and fusants were used as enzyme source. The protein content of the enzyme source was estimated by Lowry's method [11] and chitinase assay was carried out by measuring the released reducing sugar of *N*-acetylglucosamine in reaction mixture.[12]

Antagonistic activity of parent and self-fusants *Streptomyces griseus* against *F. oxysporum* f. sp. *lycopersici* of tomato

Antagonistic activity was also observed directly on plates of YMA medium, using modification of hyphal extension–inhibition assay.[13] Actively growing self fusant cultures of *Streptomyces griseus* and *F. oxysporum* f. sp. *lycopersici* (pathogens) were streaked at the

opposite poles on YMA. The plates were incubated at room temperature and the mycelia growth of *Streptomyces griseus* strains (antagonists) and *F. oxysporum* f. sp. *lycopersici* (pathogens) was measured after four days and percent inhibition.[14] of pathogen was calculated. Parent stain of *Streptomyces griseus* streaked against fungal pathogen was used as reference.[15]

RESULTS

Isolation and self fusion of protoplast of *Streptomyces griseus*

Isolation and self-fusion of protoplasts of *Streptomyces griseus* was carried out by the method of Hopwood *et al.,*[10]. The conditions for releasing the protoplasts were reported. Initially swelling and rounding up of cell content were observed and the lysis of mycelium started after few min. Complete lysis of mycelium and release of protoplasts were observed at the end of 1h. (Plate 1) Then the self-fusion of protoplasts of *Streptomyces griseus* has been achieved using 50% PEG (Mol. Wt 1000), exposed for 1 min, after then the fused protoplasts were plated on high concentration (2%) of CCA for regeneration and further selection. Based on the growth, 8 fast growing self-fusant colonies were selected and designated as SF*Sg* 1– SF*Sg* 8.

The parent and self-fusant strains of *Streptomyces griseus* colonies regenerated in the plates were inoculated in to 50ml of colloidal chitin broth (CCB). After desired incubation the cell free culture filtrates were collected and protein content, chitinase enzyme activity was assayed. High protein content was recorded in culture filtrates of all the 8 self-fusants than the parent strain. Like wise enhanced chitinase enzyme production in self-fusants was confirmed by quantitative assay. As per Table 1, chitinase activity was remarkably increased in most of the self fusants compared to the P*Sg*. The maximum enzyme activity of 283.3 IU/ml, protein content of 7.8 mg/ml was measured in

Table 1: Protein content and enzyme chitinase activity in culture filtrate of parent and self fusants of *Streptomyces griseus*

S. No	Protein content (mg/ml)	Chitinase activity (IU/ml)
P*Sg*	2.7	120
SF*Sg* 1	7.4	270
SF*Sg*2	5.4	198.3
SF*Sg*3	6.8	240
SF*Sg*4	4.73	210
SF*Sg*5	7.8	283.3
SF*Sg*6	5.4	226.6
SF*Sg*7	6.0	254.9
SF*Sg*8	4.05	169.95

Values are mean of three replicates

Table 2: Anti fungal activity of self fused *Streptomyces griseus* against *Fusarium oxysporum* of tomato-Dual culture test

Bacterial strains	Zone of Inhibition (cm)	Mycelium growth (cm)	Percent Inhibition
P*Sg*	2.0	3.5	61.1
SF*Sg* 1	2.5	2.0	80.0
SF*Sg*2	3.5	2.5	75.0
SF*Sg*3	3.0	2.2	78.0
SF*Sg*4	2.3	2.3	77.0
SF*Sg*5	3.0	1.8	82.0
SF*Sg*6	2.8	2.2	78.0
SF*Sg*7	3.2	2.1	78.0
SF*Sg*8	2.5	2.3	77.0

Values are mean of triplicates

culture filtrate of the self-fusant SF*Sg* 5, which was 2.4 fold increase as compared to parent strain whereas; the minimum chitinase activity of only 169.95 IU/ml, protein content of 4.05 mg/ml was recorded in the self-fusant SF*Sg* 8.

Dual culture test of parent and self fusant of *Streptomyces griseus*

Antifungal activity was also observed directly on plates of YMA medium using dual culture[16], but using spread inoculation of the actinomycete instead of punctuate inocula. Like wise in the present dual culture study, improved antagonistic activity in self-fusant strains of *Streptomyces griseus* was observed against *F. oxysporum* f. sp. lycopersici. The diameter of zone of clearance was measured and percent inhibition was recorded in Table 2. As per the results maximum inhibition (82%, 80%) of mycelial growth of *F. oxysporum* was recorded with fusant of SFSg 5, SF*Sg* 1. The parent *Streptomyces griseus* (P*Sg*) showed zone of inhibition of 2.0 cm and percent inhibition of 61.1% against *F. oxysporum* f. sp. *lycopersici.*

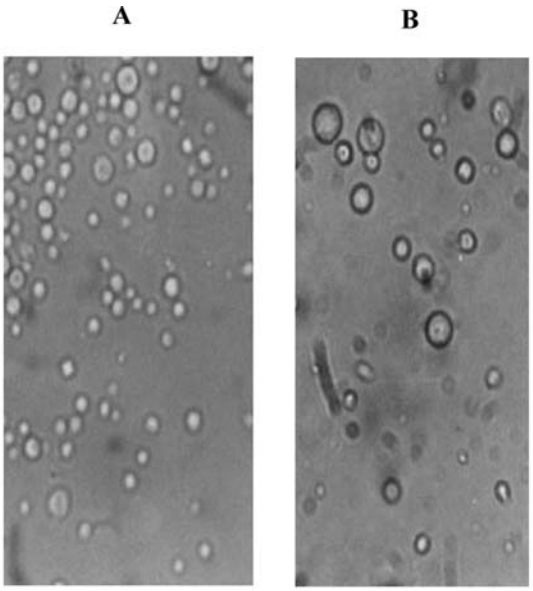

Plate 1: Digestion and release of protoplast from *streptomyces* grisues (A) Partial lysis of mycelium and release of protoplast (x 400) (B) Complete lysis of mycelium and release of protoplast (x 400)

DISCUSSION

In isolation of protoplasts from *Streptomyces griseus* complete lysis of mycelium and release of protoplasts were observed at the end of 1h.Where as in *T. harzianum* complete lysis of mycelium and release of protoplasts were observed after 3h.[9] The most common reagent for fusion of *Streptomycetes* protoplasts was PEG, whose various molecular weights have been used in different concentration. Among them, PEG 1000 at a concentration of 50% (w/v) is the most recommended as efficient one.[17, 18] In the present study, self-fusion of protoplasts in *Streptomyces griseus* has been achieved in the present study using 50% PEG (Mol. Wt 1000). Where as self-fusion of protoplasts in *T. harzianum* has been achieved by using 40% PEG.[9] Same concentration of PEG was reported as optimum for interspecies fusion of protoplasts between *T. harzianum* and *T. longibrachiatum.*[19]

The optimum time for protoplast to be exposed to PEG, has been suggested as 30–60 s.[20, 21] Therefore, in these experiments, protoplasts were treated with 50% PEG 1000 for 1 min, then the fused protoplasts were plated on high concentration (2%) of CCA. Based on the growth, 8 fast growing self-fusant colonies were selected and designated as SF*Sg* 1– SF*Sg* 8. Growth pattern on CCA indirectly indicated the enhanced production of extracellular chitinase and this could be directly related to strain improvement in *Streptomyces griseus*.

High protein content was recorded in culture filtrates of all the 8 self-fusants than the parent strain. Chitinase activity was remarkably increased in most of the self fusants compared to the P*Sg*. The maximum enzyme activity of 283.3 IU/ml, protein content of 7.8 mg/ml was measured in culture filtrate of the self-fusant SF*Sg* 5, which was 2.4 fold increases as compared to parent strain. Similar work was reported in *T. harzianum* by Prabavathy *et al.,* [9]. Nazari *et al.,* [22] applied intra-specific protoplast fusion of *Streptomyces griseoflavus,* showed higher Desferrioxamine B syntheis, which is used as a precursor for producing Desferal, a chelator that absorbs additional iron from the blood of thalassaemia patients.

In dual culture test, maximum inhibition (82%, 80%) of mycelial growth of *F. oxysporum* was recorded with fusant of SFSg 5, SF*Sg* 1. The parent *Streptomyces griseus* (P*Sg*) showed zone of inhibition of 2.0 cm and percent inhibition of 61.1% against *F. oxysporum* f. sp. *lycopersici.* Similarly improved antagonistic activity in self-fusant strains was observed against *R. solani* as compared to the parent strain was reported [9]. The antagonistic activity of *Streptomyces* RC1071 [23]; *actinomycetes* [13]; *Streptomyces lydicus* WYEC108 [24] and *Streptomyces* sp. [25] was cited previously against a wide variety of plant pathogenic fungal cultures.

From the above, the results suggested that self-fusion of protoplasts of *Streptomyces griseus* resulted in appreciable increase of chitinase production and biocontrol activity. It showed that *Streptomyces griseus* producing higher inhibition probably due to the presence of some inhibitory substance, antibiotics and other enzyme systems such as glucanases, protease essential for complete cell-wall lysis. There was no available data on enhanced chitinase production from *Streptomyces griseus* using self-fusion technique. Results illustrated significance of the protoplast fusion technique, which could successfully be used to develop hybrid strains. Also self fused *Streptomyces griseus* can be formulated and used as commercial agent for biocontrol of the pathogens.

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