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Full Length Research Paper

Antagonistic activity of an endemic isolate of *Streptomyces tendae* RDS16 against phytopathogenic fungi

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In this work, a wide survey and collection of agricultural soils from different habitats of the Al-Kharj region, Saudi Arabia, were conducted. 110 isolates of actinomycetes were isolated from these samples. Among them, 25 actinomycetes isolates were found to be antagonistic to *Fusarium oxysporum, Macrophomina phaseolina* and *Sclerotium rolfsii*. Only one isolate, denoted RDS16 had the strongest antagonistic activity against all of the tested fungi. This isolate was identified as *Streptomyces tendae* RDS16 based on morphological, cultural, physiological and molecular analyses. In addition, the optimal culture conditions for production of antifungal metabolites were investigated. The obtained results showed that incubation of *S. tendae* RDS16 for 4 days at 30°C and an initial pH of 7.5 on a fish-meal extract medium containing galactose as carbon source and casein as nitrogen source exhibited the highest production of antifungal compounds.

Key words: Antifungal activity, *Fusarium oxysporum*, *Macrophomina phaseolina* and *Sclerotium rolfsii*.

INTRODUCTION

Several fungal pathogens attack economically important plants causing high losses, especially in the subtropical and tropical regions (Brimner and Boland, 2003). *Fusarium oxysporum* Schlecht., *Macrophomina phaseolina* (Tassi) Goid. and *Sclerotium rolfsii* Sacc. cause *Fusarium* wilt, charcoal rot and root and stem-rot in a wide range of agricultural and horticultural crops, respectively (Yaqub and Shahzad, 2005; Ullah et al., 2011; Alwathnani and Perveen, 2012).

Research during the last two decades has led to the possibility of biological control as an increasingly realistic option for the management of plant pathogens. Biological control provides an environmentally safe and potentially stable alternative to chemical control (Gnanamanickam, 2002). Many researchers have investigated the management of plant pathogens by using different bacterial, fungal and algal bioagents (Yaqub and Shahzad, 2010; Abdel-Fattah et al., 2011; Sanei and Razavi, 2011; Galal et al., 2011). Among the common biocontrol agents, the genus *Streptomyces* has long attracted the attention of scientists due to its ability to produce a wide range of antibiotics. Indeed, *Streptomyces* is the largest antibiotic producing genus in the microbial world discovered so far (Watve et al., 2001). *Streptomyces* spp. have been actively studied and used as biocontrol agents against several plant pathogens (Al-Askar et al., 2011; Baz et al., 2012). However, there is still considerable interest in finding more efficient isolates since *Streptomyces* spp. strains differ considerably with respect to their biocontrol effectiveness.

This work was aimed to screen an antagonistic actinomycete isolate from a Saudi soil, to investigate the antagonistic activity of this isolate against *F. oxysporum*, *M. phaseolina* and *S. rolfsii in vitro*, and to study the optimal conditions for a maximum production of antifungal

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compounds by this microorganism.

MATERIALS AND METHODS

Microorganisms and culture conditions

Twenty-five (25) random rhizosphere soil samples were collected from different agricultural fields in Al-Kharj region, Saudi Arabia and stored in sterile plastic bags, labeled in the field and stored at 4°C until use. Soil samples (300 g) were carefully taken with spatula down to a 10 cm depth into the soil (around the plant root). 10 g of air-dried soil sample were suspended in 100 ml of basal salt solution (5 g/L KH_2PO_4 and 5 g/L NaCl) and shaken in a rotary shaker (150 rpm) at 28°C for 30 min. The soil suspension was then diluted and 1 ml of diluted soil suspension was spread onto starch-nitrate-agar plates (Waksman, 1961). The medium was adjusted to the initial pH 7 prior to sterilization using 0.1 N NaOH supplemented with 50 µg/ml of filter-sterilized cycloheximide to inhibit fungal growth, and incubated at 28°C for one week. Colonies of actinomycetes on the agar plates were picked on the basis of their morphological characteristics and purified and then transferred to starch nitrate/ NaCl slants for further use (Shirling and Gottlieb, 1966). The selected colonies were then transferred from the plates to starch nitrate/ NaCl slants and incubated at 28°C for 14 days. Slants containing pure cultures were stored at 4°C until further examinations.

The fungal strains of *F. oxysporum*, *M. phaseolina* and *S. rolfsii* were originally isolated from different naturally diseased plants collected from different agricultural fields in Riyadh region. All samples were pre-surface disinfected by immersing in sodium hypochlorite (2%) for 2 min, and washed several times with sterilized distilled water, then dried between two sterilized layers of filter paper. All fungi were cultured on potato dextrose agar (PDA) (Difco, USA) plates and incubated at 28°C for one week. Purification of the resulting isolates was carried out by using the hyphal tip or single spore techniques to obtain them in pure cultures. The detected isolates were then transferred into slants containing PDA and kept at 4°C for further studies. The pure cultures of the fungi were identified according to their cultural properties as well as to their morphological and microscopical characteristics (Domsch et al., 1980; Burgess and Liddell, 1983; Watanabe, 2002).

Screening of isolates for antagonistic activity

All isolates were screened for their antifungal activity *in vitro* against the three phytopathogenic fungi. The target fungus was pre-cultured on potato dextrose broth media and adjusted at 5 x 10^3 cfu/ml then added to starch nitrate agar plate before solidification. A disc (7 mm diameter) of a 5-day-old culture of the actinomycete isolate being tested was placed in the centre of the starch nitrate agar plate. Three replicates of each treatment were used and another set of untreated PDA plates was used as control. The experiment was arranged in a completely randomized block design. The starch-nitrate plates were then incubated at 30°C. The inhibition zone (mm of diameter), if any, was measured after two and five days of incubation.

Morphological characteristics

The morphological characteristics (the shape of the spore chains and the surface of spores) of the most promising *Streptomyces* isolate grown on starch nitrate agar medium at 30°C for five days were examined under scanning electron microscopy (JEOL Technics Ltd.).

Physiological and biochemical characterization of the most promising isolate

Amylase and cellulase (Cowan, 1974), lipase (Elwan et al., 1977), protease (Chapman, 1952), pectinase and egg yolk hydrolase (Hankin et al., 1971) and catalase (Jones, 1949) activities as well as production of melanin pigment (Pridham et al., 1957) were detected using standard methods. Degradation of both esculine and hypoxanthine (Gordon et al., 1974), nitrate reduction (Gordon, 1966) and production of hydrogen sulphide (Cowan, 1974) were also investigated. The use of different carbon and nitrogen sources (Pridham and Gottlieb, 1948) and cell wall hydrolysates (Becker et al., 1964; Lechevalier and Lechevalier, 1968) was studied. The cultural characteristics were studied in accordance to the guidelines established by the International Streptomyces Project (Lechevalier and Lechevalier, 1968).

Genotypic identification and characterization

The total genomic DNA was extracted according to Sambrook et al. (1989). PCR amplification of the 16S rRNA was conducted in a thermocycler (Perkin Elmer Cetus Model 480) by using the universal primers 27f (5'-AGA GTT TGA TCC TGG CTC AG -3') and 1525r (5'-AAG GAG GTG ATC CAG CC-3') under the following temperature profile: 94°C for 5 min, 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 90 s and a final extension step at 72°C for 5 min. The product was directly sequenced by a BigDye terminator cycle sequencing kit (PE Applied Biosystems, USA) on an ABI 310 automated DNA sequencer (Applied Biosystems, USA). Homology of the 16S rRNA sequence was analyzed by using the BLAST algorithm, available in Genbank (http: www.ncbi.nlm.gov/BLAST/).

Optimization of antifungal metabolite production

Effect of different growth media

In this experiment, starch nitrate, oat-meal extract, yeast-malt extract, glycerol asparagine, potato dextrose or fish-meal were used as growth media. Liquid cultures were prepared in 250 ml flasks containing 100 ml of sterile broth medium. The flasks were inoculated with the selected strain, denoted RDS16, at a concentration of 1 x 10^7 cfu/ml and incubated on a rotary shaker (160 rpm) at 30°C for 5 days. 5 ml of the culture were aseptically transferred to pre-made well in the centre of the plate. Three plates were used for each treatment for each fungus. The inhibition zone (mm of diameter), if any, was measured after 5 days of incubation.

Effect of incubation periods

Erlenmeyer flasks (250 ml) containing 100 ml of sterile starch-nitrate broth medium were inoculated with strain RDS16 at a concentration of 1 x 10^7 cfu/ml and incubated on a rotary shaker (160 rpm) at 30°C for different incubation periods (one, two, three, four, five, six and seven days). After each incubation period, 5 ml of the culture were aseptically transferred to pre-made well in the centre of the plate. Three plates were used for each incubation period for each fungus. The inhibition zone (mm of diameter), if any, was measured after five days of incubation. The experiment was arranged in a completely randomized block design.

Effect of incubation temperature

Erlenmeyer flasks (250 ml) containing 100 ml of sterile starch-nitrate medium were inoculated with strain RDS16 at a concentra-

Table 1. Classification of the obtained actinomycetes isolates according to color of spore mass.

Color of spore mass	Number of isolate	%	Active isolate	%
Gray	61	55.4	14	22.9
White	26	23.6	6	24
Red	13	11.8	5	38.5
Yellow	6	5.4	0	0
Green	4	3.6	0	0
Total	110	100	25	22.7

tion of 1 x 10^7 cfu/ml and incubated on a rotary shaker (160 rpm) for five days (optimum incubation period) at different temperatures (24, 26, 28, 30, 32 and 34°C). For each temperature, 5 ml of the culture were aseptically transferred to pre-made well in the centre of the plate. Three plates were used for each incubation temperature for each fungus. The inhibition zone (mm of diameter), if any, was measured after 5 days of incubation. The experiment was arranged in a completely randomized block design.

Effect of pH

Liquid cultures were prepared in 250 ml flasks containing 100 ml of sterile starch-nitrate medium. The medium was separately adjusted to different pH values (5.5, 6, 6.5, 7, 7.5, 8, and 8.5) using phosphate buffer before sterilization. Then, the culture media were inoculated with strain RDS16 at a concentration of 1 x 10^7 cfu/ml and incubated for 5 days at 30°C (optimum temperature). For each pH, 5 ml of the culture were aseptically transferred to pre-made well in the centre of the plate. The inhibition zone (mm of diameter), if any, was measured after 5 days of incubation. Three plates were used for each pH for each fungus. The experiment was arranged in a completely randomized block design.

Effect of carbon source

The carbon source from the starch-nitrate medium was substituted with glucose, galactose, fructose, sucrose, lactose or maltose (which contain the same total of carbon). Erlenmeyer flasks containing starch substituted starch-nitrate medium were inoculated with strain RDS16 at a concentration of 1 x 10^7 cfu/ml. The initial pH of the media was adjusted to 7.5 before sterilization and the flasks were incubated for 5 days at 30°C on a rotary shaker (160 rpm). For each medium, 5 ml of the culture were aseptically transferred to pre-made well in the centre of the plate. The inhibition zone (mm of diameter), if any, was measured after 5 days of incubation. Three plates were used for each carbon source for each fungus.

Effect of nitrogen source

The nitrogen source of starch-nitrate medium was substituted with casein, urea, peptone, ammonium sulphate or soybean (which contain the same total of nitrogen). Erlenmeyer flasks containing sodium nitrate substituted starch-nitrate medium were inoculated with strain RDS16 at a concentration of 1 x 10^7 cfu/ml. The initial pH of the media was adjusted to 7.5 before sterilization. The flasks were incubated for 5 days at 30°C on a rotary shaker (160 rpm). For each medium, 5 ml of the culture were aseptically transferred to pre-made well in the centre of the plate. The inhibition zone (mm of diameter), if any, was measured after 5 days of incubation. Three plates were used for each nitrogenous source for each fungus.

RESULTS AND DISCUSSION

A wide survey and collection of agricultural soils from different habitats of the Al-Kharj region, Saudi Arabia, were conducted in this investigation. These soils had been previously cultivated with leguminous crops because of the high soil fertility and wide distribution of beneficial rhizospheric actinomycetes, particularly *Streptomyces*.

110 isolates of actinomycetes were obtained from the rhizosphere soil samples. The isolates showed a wide variation in color (Table 1). These isolates were screened for their antagonistic effect against the three phytopathogenic fungi *F. oxysporum*, *M. phaseolina* and *S. rolfsii*. A high variation in antifungal activity was recorded between the isolates. Among the 110 actinomycete isolates, 25 were found to be antagonistic to the tested fungi with varying efficiencies. The antifungal activity was determined qualitatively by measuring the inhibition zones. Only one isolate, termed RDS16, had the strongest antagonistic activity against all of the tested fungi. This isolate was selected for further experiments.

In order to identify isolate RDS16, presumptive identification methods (traditional morphological and biochemical tests) were used. These included determination of growth rate, color of aerial and substrate mycelia, and diffusible pigments using seven recommended media as mentioned in the textbook of International Streptomycete Project (ISP) (Table 2). In addition, chemotaxonomical properties such as types of cell wall sugars and cell wall amino acids were analyzed. Data showed that RDS16 has LL- diaminopimelic acid. On the other hand, RDS16 has not a distinctive pattern of sugars, which is a characteristic of the genus *Streptomyces*. Therefore, RDS16 was preliminary characterized as belonging to *Streptomyces*. The physiological and morphological (Table 3) characteristics of the *Streptomyces* strain RDS16 were also determined. Morphological characteristics were determined by using scanning electron microscopy (SEM). SEM observation allowed observing the spiral shape of the spore chains (Figure 1) and the smooth surface of spores (Figure 2). RDS16 was identified as similar to *S. tendae* following the Bergey's Manual of Determinative Bacteriology

Table 2. Cultural characteristics of *S. tendae* RDS16 according to growth and colors of aerial and substrate mycelia as well as diffusible pigment.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Tryptone yeast extract broth	Poor	264 L.Gray	79 l.gy.YBr	No
Yeast-Malt extract agar	Moderate	10 Pk Gray	70 l.oy	No
Oat-meal extract agar	Good	263 L.Gray	76 l.yBr	79 l.gy.YBr
Inorganic salts starch agar	Moderate	10 Pk Gray	79 l.gy.YBr	No
Glycerol asparagine agar	Good	264 L.Gray	79 l.gy.YBr	No
Peptone yeast extract iron agar	Poor	10 Pk Gray	79 l.gy.YBr	No
Tyrosine agar	Poor	10 Pk Gray	79 l.gy.YBr	No

L. Gra, Light Gray; l.gy.YBr, light gray yellowish brown; PK. Gray, pinkish gray; l.oy, light orange yellow; l.y Br, light yellowish brown.

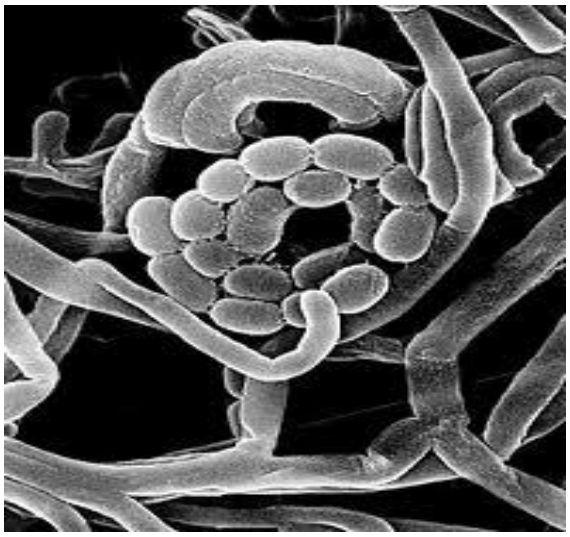

Figure 1. Scanning electron micrograph of *S. tendae* RDS16 showing the spiral shape of spore chain (X = 25000).

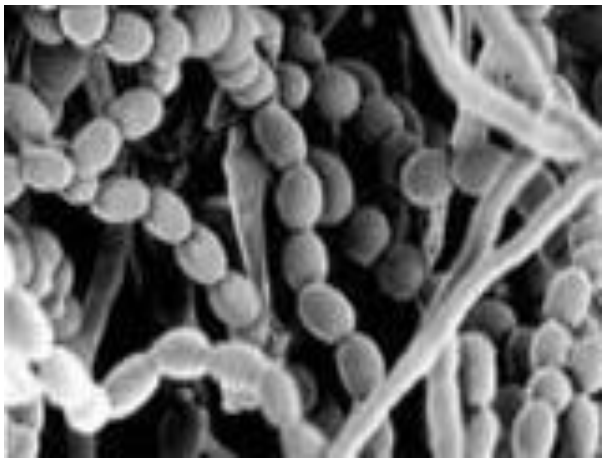

Figure 2. Scanning electron micrograph of *S. tendae* RDS16 showing the smooth surface and ellipsoidal shape of spores (X = 25000).

(Buchanan and Gibbons, 1974). When the 16S rRNA gene from the strain RDS16 was sequenced, it showed a 98% similarity with sequences from *S. tendae* strains. The strain was designated as *S. tendae* RDS16 (accession number HQ834291).

The nutritional sources like carbon and nitrogen, as well as the environmental factors such as incubation period, pH and temperature are known to have a profound effect on antibiotic production by actinomycetes (Himabindu and Jetty, 2006). Optimization of culture conditions is essential to obtain high yields of the antimicrobial metabolites. In the present study, we describe the optimization of culture conditions for the production of antifungal metabolites by *S. tendae* RDS16. Six broth media were tested to determine the best growth medium. As a result, we observed that the maximum antifungal activity was recorded when growing RDS16 in the fish-meal extract broth (Table 4). The highest antifungal activity observed in this medium was correlated with a very high content of carbon (glucose), calcium carbonate and raw material from Fish-meal.

Figure 3 shows the antifungal activity during the incubation period. The obtained results indicate that the antifungal activity of *S. tendae* RDS16 was initially observed at the 2nd day and reached the exponential phase which began from the last 4th day until the last 5th day of incubation; to introduce the maximum antifungal activity and then to stationary and decline phases respectively. The obtained result is in agreement with that reported by Srividya et al. (2012) who found that strain *Streptomyces* sp. 9p produced relatively high levels of the antifungal compound at day 4 of the incubation period.

The effect of different temperatures on the antifungal activity of *S. tendae* RDS16 against the tested fungi is shown in Figure 4. The results reveal that increases in temperature led to a higher antagonistic activity. The optimal temperature was 30°C above and any increase in temperature caused a decrease in the antagonistic activity. The obtained results are in agreement with those reported by Islam et al. (2009), who found that the maxi-

Table 3. Morphological and biochemical characteristics of *S. tendae* RDS16.

Characteristic		Result
Morphological characteristics	Spore chains	Spiral
	Spore mass	Gray
	Spore surface	Smooth
	Substrate mycelium	Yellowish brown
	Diffusible pigments	Yellowish brown
	Motility	Non motile
Cell wall hydrolysis	Diaminopimelic acid (DAP)	LL-DAP
	Sugar pattern	Not detected
Biochemical characteristics	Amylase, protease, lipase, cellulase production	+
	Catalase, Pectinase and egg yolk hydrolase production	-
	Melanoid pigment production	-
	Degradation of hypoxanthine and esculine	+
	H_2S production	-
	Nitrate reduction	+
Use of different carbon sources	D-glucose	+
	D-galactose	+
	Sucrose	+
	Mannitol	+
	L-arabinose	-
	Raffinose	+
	meso-inositol	+
	D-fructose	+
	Xylose	-
	Rhamnose	+
Use of different nitrogen sources	L-cystiene	+
	L-valine	+
	L-histidin	+
	L-alanine	+
	L-lysine	+
	L-leucine	+
	L-tyrosine	+
	L-phenylalanine	+
	L-proline	-
Tolerance to NaCl	1-7 %	+
	10 %	-

+, Growth; -, no growth.

Table 4. Effect of growth medium on the antifungal activity of *S. tendae* RDS16 against phytopathogenic fungi.

Medium	Inhibition zone (mm)		
	F. oxysporum	***M. phaseolina***	***S. rolfsii***
Starch nitrate	35	30	40
Oat meal extract	30	30	25
Yeast-malt extract	25	25	30
Potato dextrose	15	10	20
Glycerol asparagine	40	35	42
Fish meal extract	42	40	45

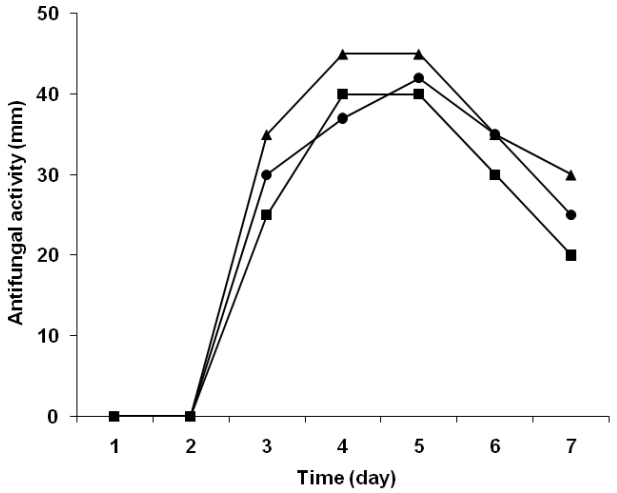

Figure 3. Effect of incubated period on the antifungal activity of *S. tendae* RDS16 against *F. oxysporum* (●), *M. phaseolina* (■) and *S. rolfsii* (▲).

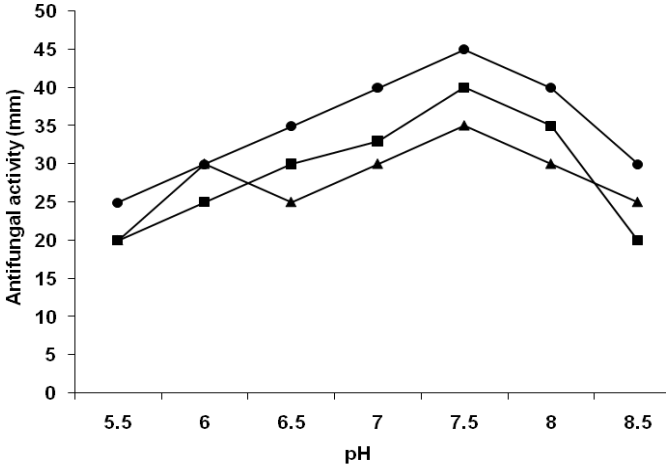

Figure 5. Effect of pH on the antifungal activity of *S. tendae* RDS16 against *F. oxysporum* (●), *M. phaseolina* (■) and *S. rolfsii* (▲).

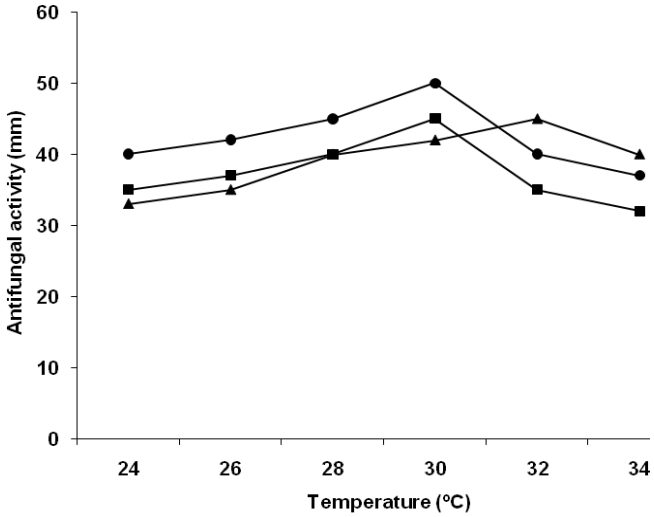

Figure 4. Effect of temperature on the antifungal activity of *S. tendae* RDS16 against *F. oxysporum* (●), *M. phaseolina* (■) and *S. rolfsii* (▲).

mum inhibition of the fungal growth was achieved when *S. albidoflavus* C247 was cultured at 30°C.

Figure 5 shows that increases in pH values led to an increased antagonistic activity up to certain limit above which any increase in pH caused a decrease in the antagonistic activity. A pH of 7.5 was found to be optimum for the antagonistic activity of *S. tendae* strain RDS16. These results are in agreement with those of Reddy et al. (2011), who recorded pH 7.5 as the optimum pH for both metabolite production and mycelium growth. Environmental factors such as temperature, pH and incubation period are known to have profound influences on growth and antibiotic production in *Streptomyces* species (Srinivasan et al., 1991). Changes in external pH affect many cellular processes such as regulation and biosynthesis of secondary metabolites (Sole et al. 1997)

Carbon compounds constitute the major requirement for growth. These compounds enter in different metabolic processes resulting in the production of primary and secondary metabolites, including antifungal compounds. *S. tendae* strain RDS16 was able to grow with all of the carbon sources tested (Figure 6). However, the highest yield was observed in cultures supplemented with galactose, followed by glucose and fructose. In the production of secondary metabolites, the interaction between growth metabolism and product secretion is critically influenced by growth-limiting nutrient concentrations (Kumar and Kannabiran, 2010). Galactose may be used less rapidly, and thus it is available during the phase of antifungal metabolite production. Optimal production has generally been achieved by cultivating organisms in media containing slowly used nutrient sources, or under conditions which allow a slow supply of these nutrients (Jonsbu et al. 2002).

Nitrogen constitutes the second major requirement for growth since it is involved in the synthesis of cell structural and functional proteins. Figure 7 shows that casein was the best nitrogen source for the antifungal activity exhibited by *S. tendae* RDS16. It may be due to its degradation product, which is the disaccharide lactose. This molecule is hydrolyzed to glucose and galactose where the later is the preferred carbon source.

We conclude that the ability to produce antimicrobial metabolites changes greatly under different culture and nutritional conditions. It can be concluded that the antifungal compound(s) produced by *S. tendae* RDS16 demonstrated obvious inhibitory effects against *F. oxysporum*, *M. phaseolina* and *S. rolfsii*. However, more studies should be conducted with regard to field applica-

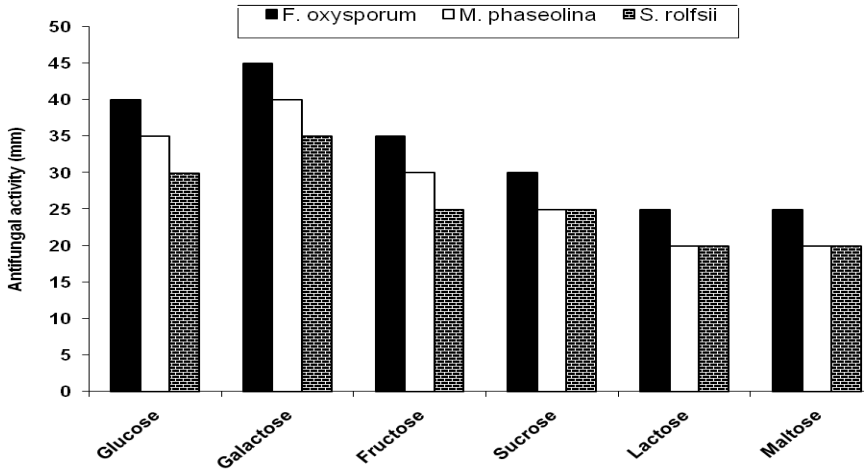

Figure 6. Effect of different carbon sources on the antifungal activity of *S. tendae* RDS16 against *the tested fungi.*

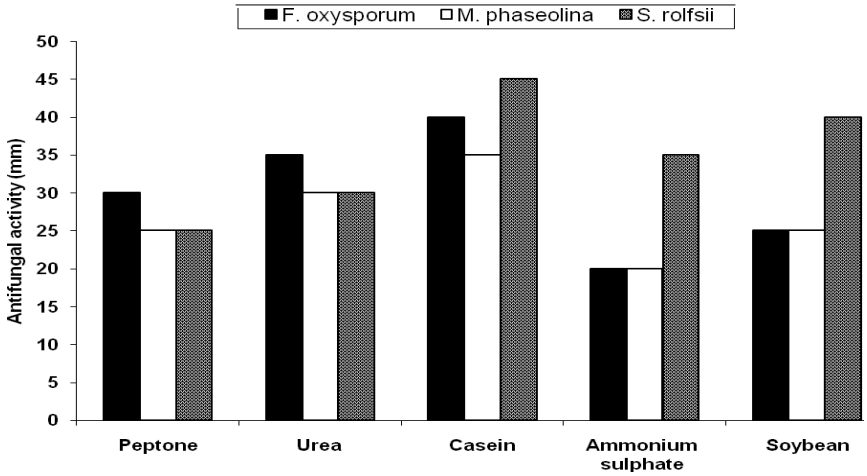

Figure 7. Effect of different nitrogen sources on the antifungal activity of *S. tendae* RDS16 against the tested fungi.

tion, formulation and mass production of the biocontrol agent, in order to develop a biofungicide that can be efficiently used for a large scale application.

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