ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Application of Broad-Spectrum of Marine *Streptomyces albidoflavus* as biofungicide and Plant Growth Promoting of tomato diseases

Wafaa M Haggag[1]*, SM Singer[2], and Mohamed DEH Aly[1].

[1]Department of Plant Pathology, National Research Center, Dokki, Cairo, Egypt.
[2]Vegetable Research Dept., National Research Centre, Dokki, Egypt.

ABSTRACT

Biological control of fungal plant pathogens can improve global food availability, one of the three pillars of food security, by reducing crop losses, particularly for low-income farmers. During a screening for microorganisms with the potential to be used as microbial fungicides, *Streptomyces albidoflavus* was isolated from a marine soil. The strain potently inhibited the mycelial growth of *Alternaria solani, A. alternate, Colletotrichum gloeosporioides, Fusarium oxysporum, F. solani , Rhizoctonia solani* and *Botrytis cinerea*. The culture filtrate of strain was extracted using various solvents (chloroform, n-butanol, diethyl ether, ethyl acetate, and n-hexane). The n-butanol extract demonstrated the highest activity against the test organisms, with a minimum inhibitory concentration (MIC) of 30.6 to 55.3 µg/ ml. *Streptomyce* produced β-1-3-glucanase, protease, chitinase and Indole-3-acetic acid. Under greenhouses, the efficacy of bio-fungicides was more significant to reduce tomato diseases incidence and enhanced plant growth. This study confirms that the selected *Streptomyces albidoflavus* strains have broad-spectrum biocontrol and Plant Growth Promoting (PGP) properties.

Keywords: biofungicide, fungal diseases, Marine Streptomyces albidoflavus , Plant Growth Promotion , tomato.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Fungal phytopathogens are the cause of many plant diseases and much loss of crop yields, especially in subtropical and tropical regions (Brimner and Boland, 2003). The management of plant pathogens was mainly addressed through chemical means for several decades, which lead to the development of fungicide resistance to a range of chemicals (and the environmental contamination (Kranthi *et al*., 2002). Due to the broad host range of these biotic constraints, the farmers are finding it difficult to grow these crops profitably. Also, with the increasing concern over environmental pollution as a result of injudicious usage of synthetic chemicals, there is a need for environment friendly methods of pest management. Hence, there is an urgent need to identify alternate environment friendly management options to control these important pathogens. Microorganisms as biological control agents have high potential to control plant pathogens and no effect on the environment or other non-target organisms (Haggag, Wafaa, 2008). There presently exist numerous reports on the potential use of biocontrol agents as replacements of agrochemicals (Haggag, Wafaa and Salme Timmusk 2008 and Yang *et al.,* 2008).

Broad-spectrum antifungal and anti-fungal pest biocontrol organisms are required for use in different cropping systems and also for the control of multiple diseases in a single crop. Actinomycetes have been exploited successfully for their biologically potential secondary metabolites (Haggag Wafaa and Abdall, 2011). Among the genus of Actinomycetes group, Streptomyces is the major and more than 500 species of this genus have been reported by Euzeby (2008). Almost two third of the naturally occurring antibiotics and enzymes (Haggag Wafaa and Abdall, 2011).

Marine actinomycetes are a potential source of novel compounds as the environmental conditions of the sea are entirely different from the terrestrial conditions (Meiying and Zhicheng, 1998). Many researchers have isolated novel antibiotics from the marine environment (Li *et al*., 2005 and Haggag, Wafaa and Radwan, 2014). The marine actinomycetes are the good source of enzyme inhibitors (Imade, 2005).

The main objective of the current investigation is to develop and evaluate broad-spectrum marine *Streptomyces albidoflavus* with multiple actions against tomato pathogens *Alternaria solani, A. alternate, Colletotrichum gloeosporioides, Fusarium oxysporum, F. solani , Rhizoctonia solani* and *Botrytis cinerea* and plant growth promotion so that one bio-control application can address more than one problem.

MATERIALS AND METHODS

Streptomyces strain

Streptomyces albidoflavus was previously isolated from from El Areash coast in red sea Seini, Egypt , identified in Plant Pathology Department, National Research Centre, Egypt (Haggag, Wafaa and Radwan, 2014) . Actinomycete isolate was purified by streak-plate technique and the pure cultures were maintained on starch casein agar (SCA) slants at 4 °C for further use. It was incubated at 30 °C for 5 days and after suitable growth the slants were sealed with paraffin and were maintained at 4 °C.

Filamentous fungi

Filamentous fungi were *Alternaria solani, A. alternate, Colletotrichum gloeosporioides, Fusarium oxysporum, F. solani , Rhizoctonia solani* and *Botrytis cinerea*.

Antifungal activity of *Streptomyces albidoflavus*

Streptomyces albidoflavus was evaluated for their antifungal activity against *Alternaria solani, A. alternate, Colletotrichum gloeosporioides, Fusarium oxysporum, F. solani, Rhizoctonia solani* and *Botrytis cinerea* by dual-culture assay. A fungal disk of pathogens of 6mm diameter was placed on one edge (1cm from the corner) of the glucose casamino acid yeast extract plate, and *Streptomyces albidoflavus* isolate was streaked on the other edge of the plate (1cm from the corner), followed by incubation at 26±2^{o}C for 4 days or until the pathogens covered the entire plate in the control plate. Inhibition of fungal mycelium around the *Streptomyces albidoflavus* was measured.

Extraction and testing of antifungal and compounds of strain *Streptomyces albidoflavus*

Streptomyces albidoflavus was cultivated in 500 ml of yeast malt extract agar (YM) broth, pH 7.0 and incubated at 28^{o}C with shaking at 125 rpm for 10 days**.** Cells were separated from the supernatant by centrifugation at 10,000 rpm for 15 min, and the supernatant was filtered **.** A range of extraction solvents was tested, including *n*-hexane, diethyl ether, ethyl acetate, chloroform and *n*-butanol. The organic extracts were concentrated by rotary evaporation. The antifungal activity of the crude samples was checked. Ten milligrams of samples were dissolved in 1 ml of dimethyl sulfoxide (DMSO), and 50 µl samples were loaded on to sterile filter paper discs (9 mm diameter). Fungal discs (9 mm diameter), from 7 days old cultures on PDA at 27ºC were placed at the center of PDA plates. The loaded filter paper discs were placed on opposite sides of the plates, 3 cm away from the fungal disc. The plates were incubated at 27ºC for 10 days. Bioactivity was determined by measuring inhibitory zones (mm).

Biological activity

Minimum inhibitory concentrations (MIC) of the *n*-butanol extract of strain *Streptomyces albidoflavus* could be determined by the cup assay method (Kavanagh, 1972)

Enzymatic activities and secondary metabolite production by the *Streptomyces albidoflavus*

Colloidal chitin was prepared freshly and used in the chitin agar as per the standard protocols of Hirano and Nagao (1988) and the chitinase production assay was conducted as described by Gadelhak *et al*.(2005). - β-1,3 glucanase activities were determined by measuring the amount of reducing sugar, released from laminarin for β-1,3 glucanase or carboxymethylcellulose (CMC) for β-1,4 glucanase. One unit of enzyme activity is defined as the amount of enzyme required to release 1.0 µmol of glucose equivalent per min . Protein concentration was determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as the standard. Protease production was done as per the protocols of Bhattacharya *et al*. (2009). One milliliter of the supernatant was mixed with 2 mL of Salkowski reagent; the appearance of a pink color indicated IAA production. Optical density (O.D.) was read at 530 nm. The level of IAA produced was estimated against the IAA standard.

Antifungal activity of the *Streptomyces albidoflavus* on tomato under greenhouse conditions

Streptomyces albidoflavus against *Alternaria solani, Colletotrichum gloeosporioides, Fusarium oxysporum, F. solani and Botrytis cinerea* was evaluated in protected tomato greenhouse grown under natural infested condaition during the seasons 2011 and 2012 . Actinomycete was inoculated by seeds soaking and inoculation of the seedlings after 30 days of transplanting (10 ml per seedling, 10^{7} CFU ml-1). Positive control, where no actinomycete was used . Each treatment was replicated three times in randomized complete block design and the row size was 3 rows of 2 m long with a row spacing of 50 cm and a plant-to-plant spacing of 25 cm. Tomato transplants were at a rate of 10 seedlings within each row. Incidence of diseases (number of plants showing symptoms to total number of plants in a plot) was recorded. Actinomycete population was also enumerated, as explexplained earlier, from the rhizosphere soils for all the treatments. Growth parameters ,yield and the disease incidence were determined .

Statistical Analyses

The effects of the treatments on disease severity were analyzed by using Duncan Multiple Range Test (SPSS software 16).

RESULTS AND DISCUSSION

Antifungal activity of *Streptomyces albidoflavus*

Streptomyces albidoflavus was previously isolated, screened for the antibacterial effects of the secondary metabolites produced by the Streptomycete isolates (Haggag, Wafaa and Rhdawan 2014). The results of primary screening indicated that the total isolate demonstrated have antimicrobial and antifungal activities. Data of the antifungal agent spectrum indicated that the agent is active against filamentous fungi . The diameter of zone of inhibition ranged between 20 to 25 mm (Fig. 1). The highest zone was obtained with *R. solani* and *F. solani.* The

ISSN: 0975-8585

metabolites of the *Streptomyces albidoflavus* exhibited various degrees of activities against filamentous Fungi (Fig.2). The highest zone was obtained with *R. solani* (28.3 mm) and *F. solani (*26.4mm*).*

Biological activities of the antifungal agent

The MIC of antifungal antibiotic was determined and the results showed that the minimum inhibitory concentration (MIC) of the compound against filamentous fungi *Alternaria solani* (51.0 µg/ ml), *Alternaria alternate* (52.0 µg/ ml), *Colletotrichum gloeosporioides* (55.3 µg/ ml), *Fusarium oxysporum* (32.28 µg/ ml), *F. solani* (36.0 µg/ ml), *Rhizoctonia solani* (30.6 µg/ ml) and *Botrytis cinerea* (38.55 µg/ ml) (Table 1).

Antifungal activity of the *Streptomyces albidoflavus*

Streptomyces albidoflavus strain produced β-1,3-glucanase , protesea and chitinase as well as IAA during the 10 days of observation (Figs 3 and 4). Results shows that the highest production of all enzymes (chitinase, B,1-3 glucanase and protease) and IAA (Fig.4) was noticed for *Streptomyces albidoflavus* and therefore it was used as biofungicide for borad spectrum pathogens and PGR.

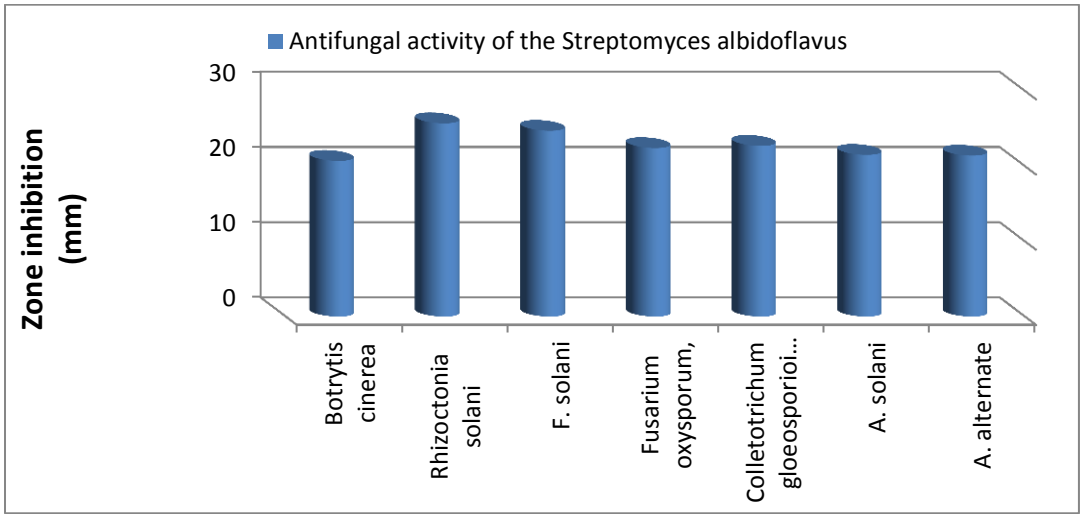

Figure 1: Antifungal activity of the *Streptomyces albidoflavus*

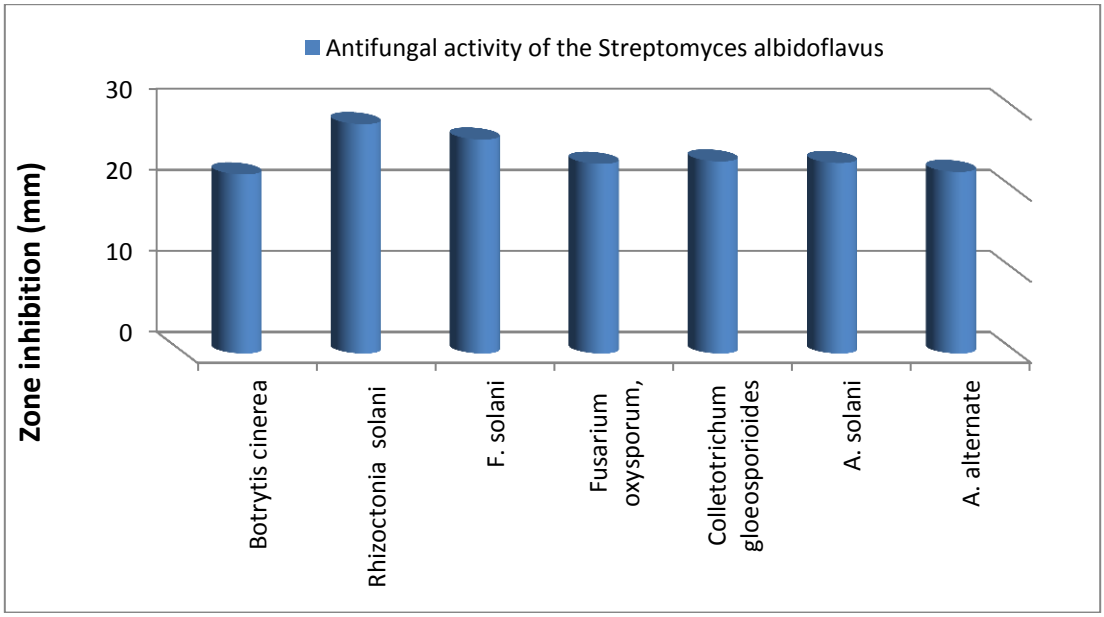

Figure 2: Antifungal activity of the metabolies *of Streptomyces albidoflavus*

ISSN: 0975-8585

Table 1: Biological activities (MIC) of the antifungal agent by paper method assay.

Test organisms	MIC µg/ ml) concentration
Alternaria solani	51.0
Alternaria alternate	52.0
Colletotrichum gloeosporioid	55.3
Fusarium oxysporum	32.28
F. solani	36.0
Rhizoctonia solani	30.6
Botrytis cinerea	38.55
LSD	0.8

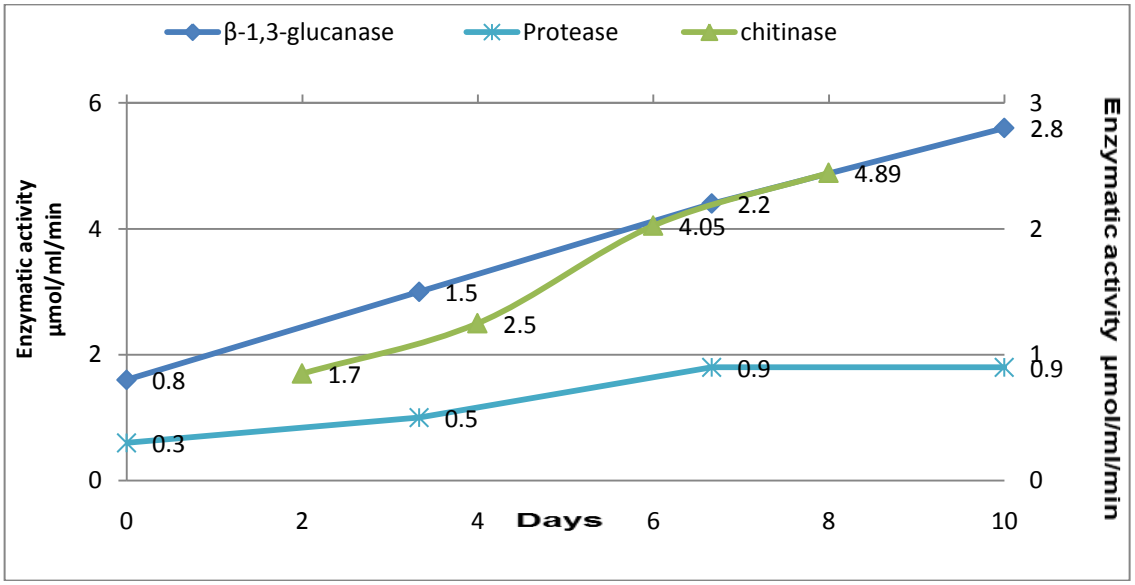

Figure 3: Evaluation of the *Streptomyces albidoflavus* strain for its enzymatic activities

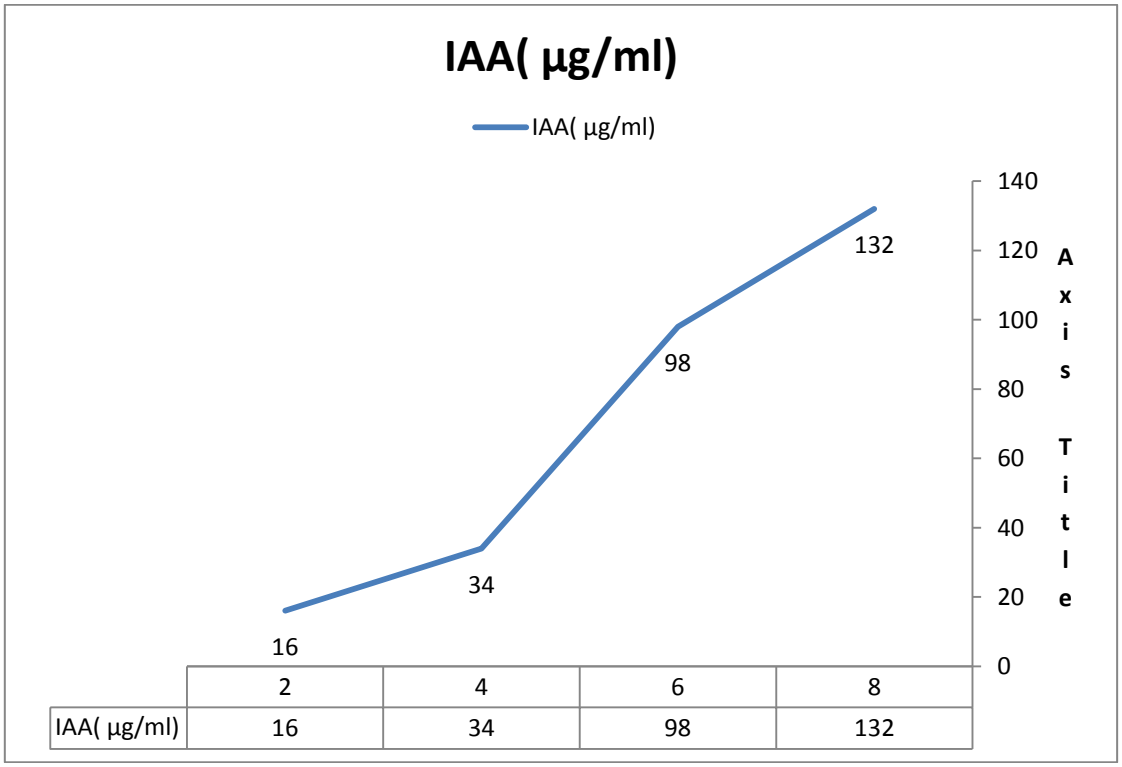

Fig. 4. The IAA production by *Streptomyces albidoflavus*

ISSN: 0975-8585

Effect of bio-fungicides on disease incidence under growth chamber experiments.

By applying bio-fungicides as seed soaking and foliar 30 days after transplanting, disease incidence was low for bio-fungicides treatment against all diseases in both seasons (Fig.5). *Streptomyces albidoflavus* promising biocontrol agents against *Alternaria solani, A. alternate, Colletotrichum gloeosporioides, Fusarium oxysporum, and Botrytis cinerea* in tomato grown under greenhouse conditions . Results obtained show that disease incidence has never exceeded 3.7 % and it reached 20.8 % with untreated control. *Streptomyces albidoflavus* isolate having broad spectrum of antifungal activity are of major importance for their study.

It was also demonstrated for their plant growth promotion (PGP) potential . Data presented in Table (2) revealed low values of growth parameters, (plant height, fresh , dry weight of plants and yield /plant) with the control treatment in comparison with other treatment. The growth parameters of tomato plants were significantly increased with the dual inoculation of *Streptomyces albidoflavus* compared with the fungicide The increase of plant growth could be attributed to the aforementioned role of both microorganisms present in dual inoculum. The promotion of tomato growth parameters by *Streptomyces albidoflavus* strain may be due to their abilities to produce phytohormones and solubilizing minerals besides, their role in direct inhibition of pathogen growth . Thus, this study confirms that the selected actinomycete *Streptomyces albidoflavus* have broad-spectrum biocontrol and PGP properties.

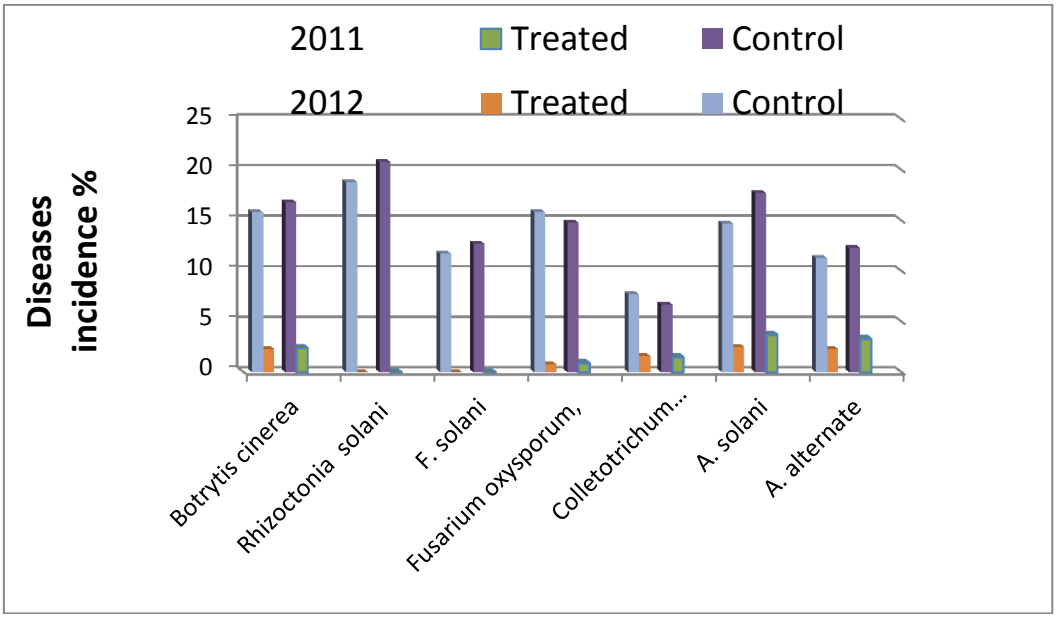

Figure 5: Antifungal activity of the *Streptomyces albidoflavus* for controlling tomato diseases under greenhouse conditions.

Table 2: Influence of biofungicide on growth characters and yield of tomato plants

Treatment	Av. plant height (cm)		Fresh weight (g/plant)		Dry weight (g/plant)		Fruit yield (kg/plant)	
	2011	2012	2011	2012	2011	2012	2011	2012
Biofungicide	85.5	75.7	420.0	432.4	88.9	84.7	1.9	1.8
Untreated control	67.4	64.6	343.0	361.7	49.4	45.7	1.3	1.0
Fungicide	72.5	76.7	387.4	398.1	55.4	53.5	1.4	1.3
LSD	4.5	4.7	6.8	6.8	5.3	5.2	0.2	0.3

Streptomyces is a major genus of actinimycetes, the Gram-positive terrestrial or marine bacteria found in both colony and mycelium forms. It has a wide host range and distribution and causes sheath blight in some field crops, such as corn, rice, lawn grass and cucumber (Huang Huang *et al.,* 2012). These actinomycetes, therefore, are likely to be potential candidates for the discovery of novel secondary metabolites which may be of importance for various PGP and biocontrol applications. Furthermore, identification of the mechanisms of action of these organisms may lead to the discovery of novel phenomena in PGP and biocontrol. Biological control of plant

diseases is slow, gives few quick profits, but can be long lasting, inexpensive and harmless to life (Haggag, Wafaa and Salme Timmusk 2008 and Haggag Wafaa ,2010). In sustainable agriculture natural biofungicides are safe and pro environment. Since most of synthetic fungicides do harm the ecosystem to some extent, their usage should be banned and switched to safer strategies as biological control techniques. Our findings suggest that extracellular secondary metabolites and/or hydrolytic enzymes including chitinase , glucanse and protesea play a crucial role in fungal growth inhibition. The strain potently inhibited the mycelial growth of *Alternaria solani, A. alternate, Colletotrichum gloeosporioides, Fusarium oxysporum, F. solani , Rhizoctonia solani* and *Botrytis cinerea* in vitro and in vivo . The culture filtrate of this strain had also the ability to inhibit all these pathogens . Several biofungicides are based on antibiotic metabolites and hydrolytic enzymes. *Streptomyces albidoflavus* has the ability to produce a significant amount of IAA in a tryptophan-supplemented medium. Thus culture filtrates of this isolate were most efficient in promoting seed germination and plant growth .

Prapagdee et al. (Li *et al.,* 2005 and Prapagdee *et al*., 2008) reported that the antifungal activity of *S. hygroscopicus* during exponential growth was mainly due to hydrolytic enzymes, while in the stationary phase it was due to secondary thermostable compound(s). In addition, there was a report on a positive correlation between chitinolytic and antagonistic activities of *Streptomyces* against the fungi *Collectotrichum sublineolum, Guignardia citricarpa, Rhizoctonia solani* and *Fusarium oxysporum*, but not in the oomycetes *Pythium* sp. and *Phytophthora parasitica*, which contain cellulose as a major cell wall component (Quecine *et al*., 2008).The present study demonstrates that a single bio-fungicide alone significantly reduced diseases incidence.

REFERENCES

[1] Bhattacharya A, Chandra S, Barik S. Ind J Agric Biochem 2009;22: 26-30.
[2] Bradford MA. Anal Biochem 1976;72:248-254.
[3] Brimner TA and Boland GJ. Agr Ecosyst Environ 2003;100(1): 3-16.
[4] Euzeby JP. 2008, http://www.bacterio.cict.fr/ s/streptomycesa.html.
[5] Gadelhak G Gadelhak, Khaled A. El- Tarabily and Fatma K. Al-Kaabi. Int J Agr Biol 2005;7:627- 633.
[6] Haggag Wafaa. Life Sci J USA 2000;7(2):57-62.
[7] Haggag Wafaa and Abdall AM. European J Scientific Res 2011;63(1):139-149.
[8] Haggag Wafaa M. Arch J Phytopathol Plant Prot (German) 2008;41(7): 477
[9] Haggag Wafaa and Radwan S. Int J Pharm Bio Sci 2014;5(4): (B) 527 – 536
[10] Haggag Wafaa and Salme Timmusk. J Appl Microbiol (UK) 2008;104(4): 961-969.
[11] Hirano SN Nagao. Biol Chem 1988;52:2111–2112
[12] Hong TY, Meng M. Appl Microbiol Biotechnol 2003;61:472–478.
[13] Huang X, Zhang N, Yong X, Yang X, and Shen Q. Microbiol Res 2012;167:135-143.
[14] Imade C. Atonie van Leeuwenhoek 2005; 587:59-63.
[15] Kavanagh F. 1972. Analytical Microbiology. Vol. 2, Acad. Press, New York.
[16] Kranthi KR et al. Crop Prot 2002;21: 449-460.
[17] Li F, Maskey R P, Qin S, Sattler I, Fiebig H H, Maier A, Zeeck A and Laatsch H. J Nat Prod 2005;68:349-353.
[18] Meiying Z and Zhicheng Z. J. Xiamen Univ Nat Sci 1998;37:109-114.
[19] Prapagdee B, C Kuekulvong and S Mongkolsuk. Int J Biol Sci 2008;4: 330-337.
[20] Quecine GWL Araujo, J Marcon, CS Gai, JL Azevedo and AA Pizzirani-Kleiner. Lett. Appl. Microbiol 2008; 47l:486-491
[21] Yang L, et al. World J Microbiol Biotech 2008;24(7): 909–915.