Biological Control of *Sclerotinia sclerotiorum* the causal agent of Lettuce Rot Disease by use of soil *Streptomyces*

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**ABSTRACT:** *Sclerotinia sclerotiorum* (Lib) De Bary, the causal agent of lettuce rot, is a soil borne fungal pathogen which can cause extensive damage to infected plants or crop and occurs world over. It is difficult to control this pathogen by normal cultural and chemical practices. Our studies have shown that the pathogen can be managed by use of bacteria belonging to the actinomycetes group. Preliminary screening of 40 soil isolates of actinomycetes from Kerman province of Iran was carried out for testing their ability to inhibit *Sclerotinia sclerotiorum* by using *in vitro* agar disk and dual culture evaluations. Among the tested isolates, Actinomycetes isolates B, F and S showed reasonable inhibitory capabilities in dual culture. These isolates were positive forchitinase, Amylase, Protease and Lipase activities but none of them produced HCN. Minimal inhibitory concentration of the active crude extracts varied from 1.25 to 0.312 mg/ml and the thermal inactivation point was determined between 90-120°C. All three isolates had fungicidal activity and only isolate F retained its antifungal activity after exposure to chloroform. Significantly, all three isolates reduced the severity of the disease in greenhouse evaluations.

**KEY WORDS:** Actinomycetes, Biological control, *Sclerotinia sclerotiorum*, Lettuce rot, Basal drop disease

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**INTRODUCTION**

*Sclerotinia sclerotiorum* (Lib.) De Bary is a soil borne fungal pathogen which can cause extensive damage to infected plants or crop and occurs world over (Boland and Hall, 1994; Purdy, 1979). Basal drop disease or lettuce rot caused by either *Sclerotinia minor* or *S. sclerotiorum* (Budge et al., 1991; Subbarao, 1998) is one of the most common and serious diseases of lettuce (*Lactuca sativa*) worldwide, causing major yield losses to growers (Patterson & Grogan, 1985; Subbarao, 1998). Early symptoms of disease include wilting and yellowing of lower leaves. The disease is characterized by a soft, watery rot of the crown and stem of lettuce (Abawi et al., 1985; Patterson and Grogan, 1985) and can occur at any stage of crop development. Infected plant parts may show cottony mycelial growth, at later stages dark colored sclerotia will be seen on the surface (Osoofe *et al*., 2005). Due to non-availability of proper management practices research has focused on use of bioagents belonging to the actinomycetes group. Studies show that most actinomycetes species belong to the genus of *Streptomyces* and this group have shown potential to suppress several phytopathogenic fungi (Saadoun and Al-Momani, 1997; Saadoun *et al*., 2000; El-Tarabily *et al*., 2000). In the present study experiments were carried out to select strains from the actinomycetes group that have potential for effective biological control of *Sclerotinia sclerotiorum*.

**MATERIALS AND METHODS**

**Culture media**

Case in Glycerol (or starch) Agar (CGA) was applied for screening and isolating actinomycetes and composed of: glycerol or starch, 10g; casein, 0.3g; KNO₃, 2g; NaCl, 2g; K₂HPO₄, 2g; MgSO₄·7H₂O, 0.05 g; CaCO₃, 0.02g; FeSO₄·7H₂O, 0.01g and agar 18g in 1L of distilled H₂O (pH 7.2) (Dhingra and Sinclair, 1995). The fungus was grown on potato dextrose agar (PDA, Difco, 39 g PDA per liter of distilled H₂O, pH of 7.2) at 25°C.

The minimal chitin agar medium (MCA) (colloidal chitin 0.4%, KH₂PO₄ 1.72 mM, K₂HPO₄ 5.14 mM, MgSO₄·7H₂O 2 mM, FeSO₄·7H₂O 0.035 mM, ZnSO₄·7H₂O 3.4 μM, MnCl₂·4H₂O 5 μM and agar 1.5 %, pH was adjusted to 8 to 8.5) was used to evaluate chitinase activity of *Streptomyces* strains (Hsu and Lockwood, 1975). Proteolytic activity was studied on medium containing glucose 1g, casein 3g, CaCl₂ 2g and agar 15g in 1 L of distilled H₂O (pH 7.0).
(Dunne et al., 2000). Lypoletic activity was assessed using a media containing peptone 10 g, NaCl 5 g, CaCl2 0.1 g, agar 15 g and Tween-80 10 ml in 1 L of distilled H2O (pH 7.0) (Sierra, 1957). The amidase activity was assayed on starch agar medium containing 0.2% soluble starch plus suitable nutrient agar basal medium (Society of American Bacteriologists, 1951).

Preparation of fungal isolate

Pure culture of *Sclerotiorum* was obtained from Mycology Lab, Dep. of Plant Pathology, College of Agriculture, Shahid Bahonar University of Kerman, Iran. All cultures stored at 4°C and sub-cultured as needed.

Preparation of *Sclerotinia sclerotiorum* inocula

A millet (*Panicum miliaceum*) seed-based inoculum was prepared by adding 50 g of seeds to 40 ml of distilled water in 500 ml conical flasks. Autoclaving of the flasks was done at 121°C for 30 min for two consecutive days. Ten agar plugs (6 mm dia) of actively growing culture of *S. sclerotiorum* were aseptically inoculated into the seed inoculum flasks and incubated at 25±2°C for 14 days. Periodic shaking of the flasks were carried out so as to enable uniform growth and cover of the pathogen. The control flasks had no pathogen. Autoclaved but uninoculated millet seeds were served as the control. The viability and purity of the prepared inoculum was tested by culturing of pathogen from colonized seeds on PDA plates before inoculation (Tahtamouni et al., 2009).

Greenhouse pathogenicity test

To assure pathogenicity of the fungus to be used in further evaluations, fungal colonized millet seeds—were mixed with the upper one third part of sterile soils in plastic pots at the ratio of 1% w/w. Lettuce seedlings (5-6 weeks old) were transplanted in infested soil. For healthy controls only sterile millet were seeds mixed into the soil at the similar ratio. All pots were maintained in a greenhouse at 22±3°C and watered regularly as needed. The pathogenicity test performed in triplicates. The fungus was reisolated from all inoculated plants, confirming Koch’s postulates.

Soil sampling and isolation of Actinomycetes

The methodology was as per Saadoun et al., 1999 and Saadoun and Gharaibeh, 2001. Random soil sampling was carried out in grasslands and vegetable fields located in the province of Kerman, Iran. Samples were collected at a depth of 10-20 cm from surface by use of an open-end soil borer (20 cm in depth, 2.5 cm in diameter) (Lee and Hwang (2002). The soil samples were air dried at ambient temperature for 7 to 10 days and later sieved using 0.8 mm mesh. The samples were kept at room temperature in polyethylene bags. 100 ml by adding sterile distilled water was added to 10 g soil and vigorously agitated for 1 h and allowed to settle for 1 h. 1 ml of the suspension was used for serial dilution and 1 ml aliquots from 10⁻¹-10⁻⁶ soil dilutions were spread plated onto CGA. Three replicates were maintained and the plates were incubated at 28°C for 7 days. Actinomycetes colonies were purified onto CGA slants and used for further studies.

In vitro inhibition studies

To assess the inhibitory potential of isolated *Streptomyces* spp inhibitory experiments were done using the agar disk method (Aghighi et al., 2004). Inhibition zone around *Streptomyces* agar disks were measured (Lee and Hwang, 2002; El-Tarabily et al., 2000).

Agar-disk method

From the refrigerated stocks, each Actinomycete isolate was freshly cultured on CGA medium (28±2°C for 4-6 days) as a single streak. Disks of the grown colonies (6 mm dia obtained using sterile cork borer) was transferred to PDA plates containing fresh growing culture of *S. sclerotiorum*. The PDA plates were incubated at 28±2°C for 6 days and the Diameter of Inhibition Zones (DIZ mm) was measured and analyzed (Shahidi Bonjar and Karimi, 2004).

Dual culture bioassay

Mycelial disc (6 mm diameter) from the peripheral region of 4-6 day old culture of *S. sclerotiorum* were placed in the center of the fresh PDA plates. Six mm discs of 5-7 day old cultures of *Streptomyces* isolates were placed at 3 cm distance from pathogen disc. Sterile agar discs were used as control at similar positions. All the plates were incubated at 28±2°C for 8 days (Dhingra and Sinclair, 1995). The intensity of inhibition at dual cultures was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist colony (γ) from the fungal growth radius (γ°) of a control culture to express rating as ∆γ = 5-9 mm, + (weak inhibition); ∆γ = 10-19 mm, ++ (moderate inhibition); and ∆γ >20 mm, +++ (strong inhibition) (Lee and Hwang, 2002; El-Tarabily et al., 2000).

Well diffusion method

In order to evaluate the antifungal activity of aqueous samples, wells (6×4 mm, 2 cm apart) were punctured by sterile cork borer in fresh PDA lawn or at 30 mm distance from plugs of *S. sclerotiorum*. Specific concentrations of Actinomycetes crude extracts were prepared in dimethyl sulfoxide:methanol (1/1; v/v) solvent (DM solvent) and then filled into each well. Plates were incubated at 28±2°C for 4-6 days for lawn culture method and 14 days in dual culture method. Disk-plug bioactivity was determined.
by measuring means of inhibitory zones (mm) from three replicates in comparison with controls which included DM solvent without test compounds (Aghighi et al., 2004).

SEM studies: Morphological characters of spores and mycelium of the isolated Streptomyces spp. were studied using scanning electron microscope (SEM) model Lutz 100A. 7-8 day old Streptomyces cultures grown on CGA were used for the study. Culture specimen was deposited on aluminum stubs and held by double stick scotch tape. Coating using the sputter was carried out for 2 min. Viewing was done at 6000-20000X and digital prints obtained.

Chloroform assay

Spores from pure cultured Streptomyces isolates were suspended in sterile water @ 10⁸ spores/ml. Drop dotting of these suspensions was done onto starch casein agar plates (5 dots/plate). The dotted plates were incubated for 3 days at 28± 2° C. The dotted cultures were inactivated by inverting open plates over watch glass containing 4mL of chloroform for 1h. The plates were then aerated in a fume hood to remove chloroform fumes. 10 mL of 1% water agar was overlaid onto each dot cultured plate and kept for 24h. S. sclerotiorum was spread plated onto each plate and incubated at 28± 2° C for 4-5 days. Inhibition zones around dotted culture were scored as positive (Davelos et al., 2004).

Submerged culture and crude culture extract

Streptomyces isolates were cultured in CG broth by using an incubator shaker at 28°C with 130 rpm. Maximum antifungal activity of crude extract was measured by the well diffusion-method where in small aliquots of the culture were drawn every 24 h for 28 days and tested. Crude extract was prepared by filtering out spores and mycelia with double layered cheese cloth. Extracts were prepared by taking into account the maximum activity time (Aghighi et al., 2004).

Estimation of minimum inhibitory concentrations (MIC)

MIC was measured by making two-fold dilutions of , 5, 2.5, 1.25, 0.625, 0.312, 0.156 mg/mL in DM solvent and tested by using the well-diffusion technique. The MIC will indicate the minimum concentration needed for fungal inhibition (Shahidi Bonjar and Karimi, 2004).

Estimation of Thermal Inactivation Point (TIP)

The crude extract obtained from different Streptomyces isolates were exposed to different temperatures and tested for bioactivity. Soluble crude extract samples (20 mg mL⁻¹) were exposed to a range of temperatures 40, 50, 60, 70, 80, 90 and 100° C for 10 min and cooled with ice. Bioactivity of exposed extracts were evaluated using the well diffusion method. Control included incubation of an untreated sample at 25°C (Shafii Baftii et al., 2005).

Evaluation for fungicidal and/or fungistatic activity

Agar plugs (1 mm³) were taken from inhibition zones of S. sclerotiorum and transferred onto fresh PDA plates and incubated at 28°C for 7 days. Visual as well as microscopic observations for growth and rejuvenated plugs were labelled as fungistatic and non rejuvenation represented fungicidal properties of the antagonist (Shafii Baftii et al., 2005).

Chitinase assay

Plain disks of Streptomyces isolates colony were inoculated to MCA plates and incubated at 28°C for two weeks. Then, chitinase activity was evaluated by observing the clear zones representative of enzymatic digestion of chitin in the vicinity of colonies of Streptomyces isolates (Sadeghi et al., 2006).

Proteolytic and Lypolytic activity tests

Specific culture media were prepared for testing proteolytic and lypolytic activity of Streptomyces isolates. After 4-5 days incubation at 28°C, development of clear zone was positive for protease activity and and halo of white precipitate around the colonies indicated lipase activity. (Sierra, 1957; Dunne et al., 2000).

Amylase assay

The Streptomyces isolates were inoculated on starch agar plate and incubate at 28°C for 3 day. Then Lugol’s solution (1% iodine in 2% potassium iodide) was added to the plates. The colonies which secretes amylase, produced zone of clearance or decolorization against the blue color background (Selvam et al., 2011).

Hydrocyanic acid (HCN) production

Streptomyces isolates were grown on CGA. One sterilized sheet of filter paper was soaked in reagent solution (0.5% picric acid in 2% sodium carbonate) for 1 min and attached to the undersurface of Petri dish lid. The lids were replaced and sealed with parafilm. Incubated for 4 days at 28°C. Development of cream, light brown or reddish brown color on the filter paper indicated positive for HCN production (Lork,1948).

Greenhouse studies

To evaluate in vivo activity of Streptomyces isolates, soil treatments were conducted under greenhouse condition. The treatments included: 1) control, 2) S. sclerotiorum,
3) *Streptomyces* isolate B, 4) *Streptomyces* isolate F, 5) *Streptomyces* isolate S, 6) *S. sclerotiorum* plus *Streptomyces* isolate B, 7) *S. sclerotiorum* plus *Streptomyces* isolate F and 8) *S. sclerotiorum* plus *Streptomyces* isolate S. Pathogen inocula was prepared and used as described previously in section of pathogenicity test. Antagonist inocula consisted of spore suspensions with concentration of $10^8$ CFU ml$^{-1}$ in distilled sterile water from which 10 ml thoroughly mixed with each Kg of soil in pot mix. Controls received neither pathogen nor antagonist. All treatments kept under greenhouse conditions and watered regularly. Disease symptoms recorded 14 days afterwards.

To compare the relative growth performance of the plants in the treatments, at the 14th day, plants were carefully uprooted, adhering soil removed and rinsed in tap water. Then shoots and roots cut apart and dried at 60°C for 72h and weighed with accuracy.

**Data analysis**

Antagonistic effects of *Streptomyces* isolates against *S. sclerotiorum* were statistically analyzed incomplete randomized design (CRD) analysis was done using SAS9.1.3 software and means were compared by Duncan’s multiple range test at the 0.01 level of confidence.

**RESULTS AND DISCUSSION**

**Screening and bioassays**

In screening for actinomycetes with antifungal activity, 40 isolates were screened from which 3 isolates B, F and S showed strong antifungal activity against *S. sclerotiorum* (Fig. 1).

**Scanning electron microscope studies**

Scanning electron micrographs of spore chains of active *Streptomyces* isolates are shown in Fig. 2.

**Chloroform assay**

Among effective *Streptomyces* isolates, only isolate F retained its antifungal activity after exposure to chloroform and produced inhibition zone (Fig. 3).
media at 28°C, clear zones was observed around all the three *Streptomyces* isolate colonies which were representative of their chitinase activity (Fig. 5).

Fig. 5. Chitinase activity of *Streptomyces* isolates on minimal chitin-agar medium. Clockwise from top: *Streptomyces* isolates B, S and F.

**Proteolytic and Lipolytic activities**

All three *Streptomyces* isolates exposed lipase activity by precipitating halo around the colonies which are representative of Tween-80 hydrolysis. Isolates B and F could also produce proteolytic enzymes as indicated by digesting protein of the medium and producing transparent zone around the their colonies (Fig. 6).

Fig. 6. Lipase (A) and Protease (B) enzyme activities of *Streptomyces* isolates. Clockwise from top in both plates: *Streptomyces* isolates B, S and F.

**Amylase activity**

All tested isolates could produce amylase enzyme as indicated by the transparent zone around their colonies after addition of iodine (Fig. 7).

Fig. 7. Amylase production by *Streptomyces* isolates in starch agar medium. Clockwise from top: *Streptomyces* isolates B, S and F.
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HCN production by the *Streptomyces* isolates

None of the three isolates were able to produce Hydrocyanic acid. The results are shown in Fig 8.

**Fig. 8.** HCN production assay results. Top row from right to left: control, *Streptomyces* isolates S, F and B. Lack of color change of filter papers impregnated with reagent (bottom row) is indicative of no HCN production.

**In vivo greenhouse studies**

Plants in soils not infested by the pathogen (Treatment 1) did not show any signs of the disease. Application of the antagonists in combination with pathogen (Treatment 6, 7 and 8), significantly (P = 0.01) reduced the incidence of lettuce rot disease compared with the treatment containing the pathogen alone (Treatment 2). In the absence of the pathogen, the *Streptomyces* isolates did not have any harmful effects on plant growth (Treatments 3, 4 and 5) and even seedlings inoculated with isolate F showed enhancement of plant growth recording significant increase in root and shoot height and shoot biomass compared with untreated controls (Figs. 9, 10 and 11). Regarding biocontrol action of *Streptomyces* isolates B, F and S against *S. sclerotiorum* significant difference in disease onset and symptom development was noticeable. In this regard, high level of antagonistic effect of *Streptomyces* isolates B, F and S against *S. sclerotiorum* was noticeable. Performance of the treated plants at three weeks period for all treatments are indicated in Table 1.

**Fig. 9.** *In vivo* greenhouse results in lettuce seedlings (a): In Untreated control plants, (b): Plants inoculated with *Streptomyces* isolate F alone, (c): plants inoculated with both *S. sclerotiorum* and the antagonist *Streptomyces* isolate F and (d): plants inoculated with the pathogen alone.

**Fig. 10.** *In vivo* greenhouse results in lettuce seedlings (a): In Untreated control plants, (b): Plants inoculated with *Streptomyces* isolate S alone, (c): plants inoculated with both *S. sclerotiorum* and the antagonist *Streptomyces* isolate S and (d): plants inoculated with *Streptomyces* alone.

**Fig. 11.** *In vivo* greenhouse results in lettuce seedlings (a): In Untreated control plants, (b): Plants inoculated with *Streptomyces* isolate B alone, (c): plants inoculated with both *S. sclerotiorum* and the antagonist *Streptomyces* isolate B and (d): plants inoculated with the pathogen alone.

The deleterious effects of fungicides on the environment has made biological control as an effective alternative to control fungal pathogens particularly, soil-borne pathogens. Many species of fungi and bacteria have been known as antagonists against *S. sclerotiorum* (Adams and Ayers, 1979) but most reports are based on laboratory observations and little information is available on *in vivo* and field conditions. Although some of them such as parasitic fungus *Coniothyrium minitans* has been presented commercially and is available as Contans® (Vrije et al., 2001). Actinomycetes have received considerable attention as biocontrol agents of soil-borne fungal plant pathogens as well plant growth promoters. Our findings represent the presence of potential antifungal behavior in *Streptomyces* isolates B, F and S. Since isolate B and S did not retain their antifungal activity after exposing to chloroform. Davelos et al., (2004) reported that antifungal activity is not from antibiotic characteristic of these *Streptomyces* isolates. Apparently isolate F has non-enzymatic antifungal behavior. The main components in antifungal activity are lytic enzymes such as chitinases, proteases, amylases or glucanases and...
they help in the in the degradation of fungal cell walls (Fernando et al., 2006). Tahtamouni et al., (2009) reported in vitro antifungal activity of some of the chitinolytic Streptomyces against S. sclerotiorum. Actinomycetes are also, protease-producers. Apart from their antagonistic activity actinomycetes have been known to be actively involved in the decomposition of organic matter, mineralization of nutrients and plant growth promotion. The proteolytic enzymes produced by actinomycetes have been shown to degrade the cell walls of as Phytophthora and Pythium spp. (Lima et al., 1998). Hence the enzymes produced by the antagonistic actinomycetes B and S are playing key role in inhibition of the tested fungal pathogen. Also reports show that amylase and lipase enzymes are produced by actinomycetes in large quantities and hence used industrially in food, biotechnological, oleochemical, pharmaceutical and detergent industries (Pandey et al., 2000; Schmid and Verger, 1998). It is well known that HCN plays an role in disease suppression (Wei et al., 1991). Haas et al., (1991) reported that HCN produced by certain strains of Pseudomonas fluorescens aided in inhibition of black root rot of tobacco. Keeland Défago (1996) also reported that HCN can inhibit Phytophthora citri on cucumber. However, none of our isolates were able to produce HCN. In green house study, similar to in vitro evaluations, all three isolates were effective in controlling S. sclerotiorum and the percent reduction in infection and mortality were significant on the lettuce seedlings. All three Streptomyces isolates enhanced growth factors such as biomass, height of root and shoot of treated plants. Nassar et al., (2003) showed infestation of soil with Streptomyces griseolueus promoted the growth of bean plants by production of polyamines such as putrescine, spermidine, spermine and endogenous plant growth regulators (PGRs) (indole acetic acid, IAA, and gibberellic acid). Hamdali et al., (2008) reported that Streptomyces griseus-related strain (BH7) stimulated aerial growth of wheat. Plant growth promotion by actinomycetes have also been reported on rice (Gopalakrishnan et al., 2013). Our findings are in agreement with aforesaid researches. Actinomycetes may promote plant growth either by direct stimulation such as phosphate solubilization, phytohormones (IAA and gibberelic acid) production and siderophore (El-Tarabily et al., 2008; Hamdali et al., 2008) or by indirect stimulation such as production of b-1, 3 glucanase, chitinase, protease, hydrocyanic acid, antibiotics and polyamine that suppress pathogens and induce resistance in host plants (Glick, 1995; Nassar et al., 2003; Gopalakrishnan et al., 2013). The wide range of antifungal physiological activities of the Streptomyces strains used in this investigation establishes the fact that several mechanisms (antibiosis, HCN, cell wall-degrading enzymes etc.) are involved in disease suppression. The identified actinomycetes species from this study could be potential candidates for preparation and development of novel formulatons containing the secondary metabolites expressed by them. Determination of the exact mechanisms of action of these biocontrol agents can help in further understanding the behavior of these eco-friendly antifungal agents.

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**REFERENCES**


BANIASADIPOUR and BONJAR


Sierra G. 1957. A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. Antonie Van Leeuwenhoek Ned. 23: 15–22.


