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Biological Control of White Cottony Stem Rot (*Sclerotinia sclerotiorum* (Lib.) De Bary) by Chitinolytic Actinomycetes under Laboratory and Greenhouse Conditions in Jordan

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ABSTRACT

Previously characterized isolates of chitinomycetes were used to control white cottony stem rot disease caused by the pathogenic fungus *S. sclerotiorum* (Lib.) De Bary on cucumber (*Cucumis sativus*) under laboratory and greenhouse conditions. The actinomycetes inoculum was added to the potted soil 7days in the laboratory and 14days in the greenhouse after soil inoculation with the pathogen in pre-planting treatments. The actinomycetes' isolates Ma3, Ju1 and Sa8 were found to reduce disease incidence to 0% and 20% under laboratory and greenhouse conditions, respectively. Disease severity was also reduced to zero (0) in the greenhouse and to 0.6 rating in the laboratory in contrast to 3 (dead plants) in the control. In addition, a tested non-chitinolytic isolate (Na5) reduced disease incidence to 50% and disease severity to 1.25 in the laboratory. Disease incidence and severity were also reduced to 40% and 0.8 in the greenhouse, respectively, by the same isolate. The present findings support the significance of actinomycetes as biocontrol agents against phytopathogenic fungi.

Keywords: Cucumber (*Cucumis sativus*), Fungi, Sclerotia.

INTRODUCTION

White cottony stem rot disease of field crops and vegetables is caused by *Sclerotinia sclerotiorum* Lib. De Bary; a soil-borne fungus that attacks a wide range of host plants at all stages of growth, including young seedlings, mature plants and their harvested products distributed worldwide (Agrios, 1997; Purdy, 1979). This disease of cucurbits is a problem of increasing concern to farmers in the Jordan Valley; an intensively cultivated region that is highly subjected to pesticide application for a long time (personal observation). Crop losses attributed to *S. sclerotiorum* ranged from moderate to 100%, where millions of dollars around the world are annually lost due to this disease (Purdy, 1979; Subbarao, 1998; Tahtamouni *et al.,* 2006).

A number of biological control methods to control this soil-borne plant pathogen were postulated using microbial antagonists (Adams and Ayers, 1982; El-Tarabily *et al*., 2000). During the past decades, chitinase producing organisms received more attention. Their potential in biocontroling phytopathogenic fungi was sought to be promising, since chitin is the major constituent of the cell walls of many plant pathogenic

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fungi including *S. sclerotiorum* (Gupta *et al.,* 1995; El-Tarabily *et al.,* 2000; Tahtamouni *et al.,* 2006). Many species in the order actinomycetales are characterized by the production of extracellular important bioactive compounds. Most of actinomycetes' species belong to the genus of *Streptomyces* which were promising biocontrol agents against several phytopathogenic fungi (Saadoun and Al-Momani, 1997; Saadoun *et al.,* 2000; El-Tarabily *et al.,* 2000; Tahtamouni *et al.,* 2006). Under greenhouse conditions, the incidence of cavity spot disease caused by *Pythium coloratum* was reduced through utilizing active antagonistic actinomycetes' isolates *in vivo*. *Streptomyces janthinus* and *Streptosporangium albidum* were found to be more effective in reducing the disease (El-Tarabily *et al.,* 1997). Isolates of the two chitinolytic bacteria *Paenibacillus* spp. and *Streptomyces* spp. were reported to suppress Fusarium wilt of cucumber caused by *Fusarium oxysporum* in non-sterile, soil-less potted medium (Singh *et al.,* 1999). Disease suppression of *Sclerotinia minor,* the basal drop disease agent of lettuce utilizing chitinolytic bacteria (*Serratia marcescens, Streptomyces viridodiasticus and Micromonospora carbonacea*) under field conditions was investigated (El-Tarabily *et al.,* 2000). The present research aimed at using previously isolated and characterized chitinase producing *Streptomyces*' isolates indigenous to Jordan and investigating their capability in controlling *S. sclerotiorum* infection on cucumber under laboratory and greenhouse conditions.

MATERIALS AND METHODS

Cultures of *S. sclerotiorum* and Actinomycetes

Subculture of *S. sclerotiorum* and four actinomycetes' isolates (Ma3, Ju1, Sa8 and Na5) were provided by the Plant Pathology and Mycology Laboratory, Department of Plant Production, Faculty of Agriculture, Jordan University of Science and Technology. Those microorganisms were previously characterized and identified during previous work (Tahtamouni *et al.,* 2006).

Preparation of *S. sclerotiorum* and Actinomycetes' Inocula

Millet (*Panicum miliaceum* L.) seeds, 30 g per 250 ml conical flask, were soaked in 40 ml of distilled water for 10 minutes inside each flasks and then autoclaved at 121°C for 30 minutes. Flasks were aseptically inoculated with 10 agar plugs (6 mm-in-diameter) cut off at the advancing edge of actively growing culture of *S. sclerotiorum* and incubated at 27°C for 10 days. The flasks were periodically shaken to ensure the uniformity of colonization. The viability and purity of the prepared inoculum were tested by plating the culture on PDA-plates before inoculation.

Starch-casein nitrate agar slant cultures (15-21 days old) of the chitinase active isolates (Ma3, Ju1 and Sa8) and a non-chitinolytic isolate (Na5) were used to prepare the inoculum for each one of those isolates. Suspension of spores and cells of each isolate was prepared by scraping the surface of their slant cultures with the aid of a sterile loop. Thirty grams of moistened wheat bran placed inside 250 ml conical flasks were autoclaved at 121°C for 30 minutes and aseptically inoculated with the 12 ml of spores and cells' suspensions. Inoculated flasks were then incubated at 27°C for three weeks with daily hand-shaking to ensure the uniformity of colonization.

Sand Inoculation of *S. sclerotiorum* under Laboratory Conditions

Pure washed and steam sterilized sand was filled into sterile plastic free-draining pots (300g/pot) and used as a rooting medium for cucumber. All pots were placed in the growth chamber illuminated with a set of fluorescent lamps to give a light intensity of 250 Lux at 25ºC±2°C under laboratory conditions. The rooting medium was infested by incorporating *S. sclerotiorum* inoculum at a

rate of 1% on a w/w basis as a pre-planting treatment. All pots were maintained moist close to their field capacity by daily irrigation with sterile distilled water.

Inoculation of Actinomycetes' Isolates into *S. sclerotiorum* Inoculated Sand

Inocula of the actinomycetes' isolates were introduced 7 days after the incorporation of the pathogen into the rooting medium at a 1% (w/w) ratio in the following combinations; untreated plant (negative control)**,** *S. sclerotiorum* alone (positive control), *S. sclerotiorum* followed by one of the isolates Ma3, Ju1, Sa8 and Na5 and the isolate Ma1, Ju1, Sa8 and Na5 alone. All treatments were replicated four times and arranged in a Completely Randomized Design (CRD) inside the growth chamber.

Seeds of cucumber (*C. sativus*, cultivar Reema), surface sterilized by dipping them in 70% ethanol for 2 minutes followed by 0.5% NaOCl for 1 minute and rinsing them five times with sterile distilled water, were sawn in a rate of 2 seeds/pot one week after inoculation. The number of seedlings was thinned down to one seedling per pot one week after emergence. All pots received elements of supplementary nutrients by irrigation with 20 ml of Hogland's nutrient solution (Hopkins and Huner, 2004) once a week and maintained around the field capacity throughout the experiment. After six weeks, the disease incidence of cottony rot was assessed using an arbitrary disease rating scale of 0 to 3, where; 0= no detectable symptoms.1= appearance of a 1-2 cm water-soaked lesion on the crown region of the plant. 2= appearance of a 2 cm water-soaked lesion covering the stem base of the plant. 3= plant completely dead (Ngugi *et al.,* 2002).

Greenhouse Experiment

A basin experiment was conducted under greenhouse condition. A sandy-loam field soil was sieved and pasteurized in oven at 72°C for three days. The sterilized soil was placed in clean free-drainage plastic basins (40cm-in-diameter and 15cm-in-depth). The soil was inoculated with *S. sclerotiorum* as previously described into the top 5cm of the potted soil at a rate of 1 % w/w and maintained at field capacity.

Two weeks after the pathogen inoculation of the soil, the wheat bran actinomycetes' cultures prepared as previously described were introduced into the inoculated soil at a rate of 1% w/w. Soil treatments were made in order to cover all combinations in the same manner made in the laboratory experiment. Each treatment was replicated five times and arranged in a Completely Randomized Block Design (CRBD) on the bench top inside the greenhouse.

Seeds of cucumber (var. Reema) were surface sterilized as previously described and sown in nursery trays filled with a sterilized potting mixture of peat moss and perlite (3:1 v/v) and allowed to grow inside the greenhouse for 2 weeks. Cucumber seedlings were then transplanted into plastic basins (40cm-in-diameter and15cm-in-depth) filled with soil, 2 seedlings per basin, one week after the addition of the antagonist's inoculums and thinned to one seedling one week after transplanting. Soil in all basins was maintained around its field capacity. The disease incidence was carefully observed and recorded 5 weeks after transplanting and at the end of the experiment. Disease severity was determined using the previously described 0-3 scale.

Data Analysis

Data were statistically analyzed using analysis of variance (ANOVA) procedure in the MSTATC program (Michigan State University, MI, USA). Probability of significance was used to indicate significant differences among treatment means. Least Significant Difference (LSD) test (at P=0.05) was used to compare means.

RESULTS

Effect of Actinomycetes' Isolates on *S. sclerotiorum* Infection of Cucumber Seedlings under Laboratory Conditions

Results of the growth chamber experiment showed that the actinomycete isolates (Ju1 and Sa8) have completely controlled the disease since there was no disease incidence (Table 1); similar to the non-inoculated control. The isolate Ma3, on the other hand, which is a chitinase active isolate did not significantly reduce the disease incidence and disease severity inflicted by *S. sclerotiorum* infection on cucumber, but, resulted in 75% decrease in the disease incidence and 67.5% decrease in disease severity compared to the positive control (pathogen alone, 100% and 2.0, respectively) (Table 1). Also, the non-chitinolytic actinomycete isolate Na5 prompted a 50% decrease in disease incidence and 62.5% decrease in disease severity compared to the positive control (pathogen alone, 100% and 2.0, respectively) (Table 1).

Table (1): Effect of actinomycetes isolates on disease incidence and severity caused by *S. sclerotiorum* on cucumber seedlings under laboratory conditions.

Treatment [a]	Disease Incidence (%)	Disease Severity[b] (0-3)
Untreated plant/plant alone	0 c[c]	0 c
S. sclerotiorum alone	100 a	2.0 a
S. sclerotiorum followed by Ma3	25 bc	0.75 bc
S. sclerotiorum followed by Ju1	0 c	0 c
S. sclerotiorum followed by Sa8	0 c	0 c
S. sclerotiorum followed by Na5	50 b	1.25 ab
Ma3 alone	0 c	0 c
Ju1 alone	0 c	0 c
Sa8 alone	0 c	0 c
Na5 alone	0 c	0 c

[a] Cucumber seeds were grown in sand infested with and without *S. sclerotiorum and* with or without antagonistic organisms inside the growth chamber.
[b] Disease severity rating scale: 0= no detectable symptoms; 1= appearance of 1-2 cm; 2= appearance of a 2 cm water soaked lesion covering the stem of the plant and 3= plants are completely dead (Ngugi *et al.,* 2002) .
[c] Means of 4 replicates, followed by the same letter are not significantly different at ($P \leq 0.05$).

Control of *S. sclerotiorum* under Greenhouse Conditions

Application of the actinomycetes' isolates (Ma3, Ju1, Sa8 and Na5) significantly reduced the disease incidence and disease severity of the cottony white rot on cucumber compared with that of the positive control (the pathogen alone). However, there were no significant differences among those actinomycetes in their suppression of the disease incidence and severity. The isolates Ma3, Ju1 and Sa8 prompted a 75% decrease in disease incidence and 80% decrease in the disease severity compared to the positive control values (pathogen alone, 80% and 3.0, respectively). The isolate Na5, which is the non-chytinolytic

actinomycete, prompted lower degrees of disease suppression (disease incidence 50% and disease severity 74%), even though it was not significantly different from the other isolates (Table 2).

Table (2): Effect of the chitinolytic isolates on the disease incidence and severity of *S. sclerotiorum* on cucumber under greenhouse conditions.

Treatment [a]	Disease Incidence (%)	Disease Severity[b] (0-3)
Untreated plant/plant alone	0 c[c]	0 c
S. sclerotiorum alone	80 a	3 a
S. sclerotiorum followed by Ma3	20 bc	0.6 bc
S. sclerotiorum followed by Ju1	20 bc	0.6 bc
S. sclerotiorum followed by Sa8	20 bc	0.6 bc
S. sclerotiorum followed by Na5	40 b	0.8 b
Ma3 alone	0 c	0 c
Ju1 alone	0 c	0 c
Sa alone	0 c	0 c
Na alone	0 c	0 c

[a] Cucumber seeds were grown in sand infested with and without *S. sclerotiorum,* and with or without antagonistic organisms inside the growth chamber.
[b] Disease severity rating scale: 0= no detectable symptoms; 1= appearance of 1-2 cm; 2= appearance of a 2 cm water soaked lesion covering the stem of the plant, and 3= plants are completely dead (Ngugi *et al.,* 2002).
[c] Means of 4 replicates, followed by the same letter are not significantly different at ($P \leq 0.05$).

DISCUSSION

Biological control became a highly desired practice, since there was growing concern about the use of chemical pesticides and their long-term adverse environmental effects. Therefore, searching the indigenous environment for novel and beneficial organism(s) significant as biocontrol agent(s) is very important to the sustainability of human life and environment (Higa, 1994; Knight *et al*., 1997). It is very legitimate to launch intensive efforts in searching for promising biological control agent(s) against *S. sclerotiorum*. The present study evaluated the potential of chitinase producing actinomycetes isolated from different soils in Jordan to control white cottony stem rot disease of cucumber. Taking into consideration that chitin is a constituent of the fungal cell wall of most fungal plant pathogens (Hus and Lockwood, 1975), production of chitinase could be considered as a criterion for the selection of potential biocontrol agents against the phytopathogen *S. sclerotiorum* (Hus and Lockwood, 1975; El-Tarabily *et al*., 2000).

The significant suppression of disease incidence and disease severity under laboratory and greenhouse conditions by isolates; Ju1 and Sa8 and to less extent by Ma3 may mainly be attributed to their chitinolytic activity since similar results were reported by Tahtamouni *et al.* (2006). However, the isolate Na5 prompted similar results even though it is characterized as a non-chytinolytic organism, indicating the diversity in the extracellular activity of this group of

microorganisms. The same isolates (Ju1, Sa8 and Ma3) were also capable to achieve similar results under greenhouse conditions indicating that they could be good candidates for future formulations of biocontrol product against *S. sclerotiorum* and the management of white cottony rot of cucurbits and other crops. These results confirmed what was previously reported by El-Tarabily *et al.* (2000) about the importance of the chitinase producing actinomycete isolates against *Sclerotinia minor in vivo* and are also in agreement with Adams *et al.* (1982) who showed the significance of the biocontroling process of lettuce drop disease caused by *Sclerotinia minor*.

The non-chitinolytic isolate (Na5) was also capable to reduce disease incidence under laboratory and greenhouse conditions. This result points out that possible inhibitory metabolites may be produced by the non-chitinolytic isolate and are also involved in the reduction of incidence and severity of the disease (Saadoun and Gharaibeh *et al.*, 2002). This finding is not in agreement with what was previously reported by El-Tarabily *et al.* (2000) in relation with the non-chitinolytic *Streptomyces* spp. isolate which failed to control basal drop disease of lettuce under greenhouse conditions and it could be attributed to the diversity among isolates of this group of microorganisms (Saadoun and Gharaibeh, 2002). However, the presence of chitinase activity stays the most significant one in the biological control of this fungal pathogen.

In conclusion, the present study emphasized the importance of using chitinolytic actinomycetes as promising *in vivo*-biocontrol agents of many phytopathogens. Further studies have to concentrate on the elucidation of the chemical characteristics and biological effects of actinomycetes' extracellular products on the phytopathogenic fungi and the plant crop. Also, formulation studies of novel product(s) based on utilizing certain actinomycetes into a bio-pesticide to control fungal plant diseases are recommended.

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المكافحة الحيوية لمرض التعفن القطني الأبيض (*Sclerotinia sclerotiorum* (Lib.) De Bary) باستخدام الأكتينومايسيتات (Actinomycetes) المحللة للكايتين تحت ظروف المختبر والبيت الزجاجي في الأردن

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ملخـص

استخدمت عزلات من الأكتينومايسيتات (Actinomycetes) سبق تشخيصها ومعرفة أنها منتجة للانزيم المحلل للكايتين (كايتينيز Chitinase) وأنها مضادة لنمو الخيوط الفطرية (mycelium) و مثبطة لحيوية الأجسام الحجرية (sclerotia) للفطر المسبب لمرض التعفن القطني الأبيض (*Sclerotinia sclerotiroum*) تحت ظروف التنمية المغلقة (*in vitro*) في مكافحة المرض على الخيار(*Cucumis sativus*) تحت ظروف التنمية المفتوحة في المختبر وتحت ظروف البيت الزجاجي، على التوالي .أضيفت لقاحات الأكتينومايسيتات إلى تربة الأصص 7 ايام في المختبر، و 14 يوماً في البيت الزجاجي، بعد إضافة لقاح الفطر الممرض إلى التربة في معاملات ما قبل الزراعة للخيار. تبين أن العزلات(Ma3, Ju1, Sa8) تمكنت من اختزال نسبة حدوث الإصابة بالمرض إلى 0% تحت ظروف التنمية في المختبر والى 20% تحت ظروف البيت الزجاجي. كما ان شدة الإصابة قد اختزلت إلى صفر (0) تحت ظروف المختبر والى 0.6 في البيت الزجاجي بالمقارنة مع أعلى تقدير(3، نباتات ميتة) في حالة الموت التام للنباتات في معاملة الشاهد. وبينت الدراسة أن العزلة Na5 غير المنتجة لانزيم كايتينيز كانت قادرة على تقليص نسبة حدوث الإصابة بمقدار 50% و40% تحت ظروف المختبر والبيت الزجاجي، على التوالي، وانها تسببت في تقليص شدة الإصابة إلى تقدير 1.25 في ظروف التنمية في المختبر و0.8 في البيت الزجاجي. تؤكد هذه الدراسة أهمية الأكتينومايسيتات المنتجة لانزيم كايتينيز كعوامل في المكافحة الحيوية للفطريات المرضية.

الكلمات الدالة: الخيار، الفطريات، الاجسام الحجرية السوداء.

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