

# Evaluation of *Streptomyces* spp. against *Fusarium oxysporum* f. sp. *ciceris* for the management of chickpea wilt

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**Abstract:** In this study, about 112 isolates of *Streptomyces* were isolated from chickpea rhizospheric soils. Among the isolated strains, five showed strong inhibitory effects against chickpea Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* *in vitro* using plate assay and selected for further studies. The selected strains were identified as *Streptomyces* spp. based on morphological and biochemical characterization as well as 16S rDNA sequences analysis. Our results assigned them to strains related to genus of *Streptomyces*. *In vitro*, antagonistic effects of *Streptomyces* strains against the disease were evaluated through the dual-culture method, volatile and non-volatile metabolites, siderophore, protease and chitinase production. All bacterial strains inhibited mycelial growth of the pathogen ranging from 26 to 44.2% in dual culture assay. The non-volatile extract of five of the *Streptomyces* strains inhibited more than 50% growth of the pathogen, whereas volatile compounds were less effective on mycelial growth inhibition (20.2 to 33.4%). The ability of the biocontrol agents to produce siderophore and protease were varied, whereas, production of chitinase was detected for all strains. Results of the greenhouse assay indicated that all biocontrol agents reduced disease severity (ranging from 38.7 to 54.8%). Accordingly, strain KS62 showed higher control efficacy (54.8%). In addition, the biomass of chickpea plants (plant height and dry weight) significantly increased in plants treated with *Streptomyces* strains compared to non-bacterized control. The results of this study showed that it may be possible to manage chickpea Fusarium wilt disease effectively by using *Streptomyces* species, as biocontrol agents. Therefore, evaluating their efficiency under field conditions is needed.

**Key words:** biocontrol, chickpea, *Fusarium*, *Streptomyces*, 16S rDNA

## Introduction

Chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato, is the most important disease in chickpea throughout the world, and it is the main yield-limiting factor in chickpea production in Iran (Navas-Cortés *et al.* 1998). Fungi survive saprophytically on crop debris in the soil. The pathogen infect the roots of a susceptible host, can colonize the vascular system of plants, produces toxins and kills plants by blocking xylem vessels that restricts water transport (Anjaiah *et al.* 2003). The uses of resistant cultivars are not always available or effective, because new races of the pathogen have appeared that overcome resistance in currently grown cultivars (Navas-Cortés *et al.* 1998). Furthermore, application of fungicides does not always prove economic against soil borne pathogens and it has led to environmental pollution, pathogen resistance, and increased risk to human and animal health (Li *et al.* 2012; On *et al.* 2015). In addition, excessive use of fungicides creates an imbalance in the microbial community in soil (On *et al.* 2015). The use of microbial biocontrol agents is an alternative to fungicides for the management of plant disease because biological control is one of the most environmentally viable and health-friendly approaches for replacing fun-

gicides in management programmes to control fungal pathogens (Li *et al.* 2012; Jiménez-Díaz *et al.* 2015). Numerous biocontrol agents have been reported to control Fusarium wilt of chickpea, such as *Pseudomonas fluorescens* (Anjaiah *et al.* 2003), *Trichoderma* species (Dubey *et al.* 2007; Moradi *et al.* 2012) and *Bacillus subtilis* (Moradi *et al.* 2012). Different mechanisms have been implicated in the suppression of fungal root disease by biocontrol agents, such as the competition for nutrients and the production of biocontrol metabolites (extracellular antibiotics, volatile substances, siderophores, lytic enzymes, hydrogen cyanide, etc.) (Das *et al.* 2008; Naureen *et al.* 2009; Erdogan and Benlioglu 2010).

Species of *Streptomyces* have been evaluated against plant pathogens and have exhibited great potential in suppressing plant diseases caused by fungal pathogens (Li *et al.* 2012; Sadeghi *et al.* 2012; Elango *et al.* 2015). Some isolates of *Streptomyces* can produce compounds with antifungal activity for plant protection (Tanaka and Omura 1993). Hence, they have been selected as efficient antagonists against soil-borne fungal pathogens (Sabaratnam and Traquair 2002).

The objective of this research was to isolate, characterize and evaluate the biocontrol potential of *Streptomy-*

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ces strains in chickpea *Fusarium* wilt causing *Fusarium oxysporum* f. sp. *ciceris* under *in vitro* and greenhouse conditions.

## Materials and Methods

### Pathogen and inoculum preparation

Wilt-infected plants of chickpea were collected from the major chickpea-growing region of Kurdistan Province, Iran. The pathogen, *Fusarium oxysporum* f. sp. *ciceris* (FOC) was isolated from infected tissues and cultured on Potato Dextrose Agar (PDA) medium. Fungi identification was based on morphological characteristics and microscopic observation on PDA and Carnation Leaf Agar (CLA) (Leslie and Summerell 2006). A pathogenicity test for the pathogen was carried out under artificially inoculated conditions. Then, the pathogen was re-isolated from the diseased tissues of chickpea. The fungus was grown on PDA medium for 7 days in Petri dishes at 25–27°C for mycelial production. The microconidial inoculum of FOC was produced in flasks (250 ml) containing 150 ml of sterile Potato Dextrose Broth (PDB) medium. Each flask was inoculated with 7-days-old culture on PDA. The flasks were shaken at 150 rpm at 25–27°C for 7 days. Next, the culture was filtered through sterile glass wool to collect spores to be used as inoculum. The concentration of microconidia was adjusted to  $1 \times 10^6$  spores  $\cdot$  ml<sup>-1</sup>.

### Bacterial isolates

*Streptomyces* isolates were isolated from the rhizosphere of healthy chickpea plants using the dilution plate technique on Starch Casein Agar (SCA) medium (g  $\cdot$  l<sup>-1</sup>: starch – 10, casein – 0.3, CaCO<sub>3</sub> – 0.02, FeSO<sub>4</sub> – 0.01, K<sub>2</sub>HPO<sub>4</sub> – 2, MgSO<sub>4</sub> – 0.05, NaCl – 2, Nystatin – 0.02 and agar – 15, pH 7.0±0.2) (Don *et al.* 1993; Dhanasekaran *et al.* 2005). For bacterial isolation, 5 g of rhizosphere soil of chickpea plants were suspended in 45 ml of sterile distilled water in an Erlenmeyer flask (250 ml). The flasks were shaken at 150 rpm at 26–28°C for 30 min. The bacterial suspension obtained was diluted by a factor of 10<sup>-3</sup>, 10<sup>-4</sup> and 10<sup>-5</sup> for selection (Dhanasekaran *et al.* 2005). Then, 50  $\mu$ l of each dilution were spread on SCA media and incubated at 26–28°C until colony development was observed (5–7 days). Among different types of bacterial colonies that grew, only those morphologically similar to *Streptomyces* were selected (Sinclair and Dhingra 1995). Purification of bacterial isolates was undertaken by streaking them onto SCA media to obtain well-separated single colonies. The *Streptomyces* strains were stored as glycerol stocks at –20°C.

### Screening for antagonistic bacterial isolates *in vitro*

The assessment of the inhibitory effects of all the isolated bacteria on the chickpea *Fusarium* wilt was performed using plate assay (four spots per plates) as described by Weller and Cook (1983). Four different isolates of bacteria were spotted on the edge of PDA plates. Each plate was inoculated with four droplets of 20  $\mu$ l bacterial suspen-

sion (10<sup>8</sup> cfu  $\cdot$  ml<sup>-1</sup>). The droplets were placed on four sites at equal distances from the center of plates. After 24 h incubation at 28°C, a single 5 mm-diameter plug of the pathogen from dilute PDA was placed in the center. After five days, the radius of each fungus colony was measured and zones of inhibition were calculated. Antagonism isolates showing the greatest inhibition by this test were selected for further studies.

### Phenotypic and molecular characterization of selected strains

Morphological and biochemical properties of bacterial strains were studied according to Shirling and Gottlieb (1966) and Schaad *et al.* (2001). Molecular characterization based on DNA sequencing of 16S rRNA gene was used for phylogenetic studies. Genomic DNA was extracted from pure cultures using the genomic DNA extraction kit from gram positive bacteria (QIAGEN) based on the manufacturer's instructions. Nearly full-length 16S rRNA gene sequences in target-specific screening strains were amplified with the universal primers RP1 (5'-AGAGTTT-GATCATGGCTCAG-3') and FD2 (5'-ACGGTTACCTT-GTTACGACTT-3') (Weisburg *et al.* 1991). Polymerase Chain Reaction (PCR) was carried out in a total volume of 50  $\mu$ l. Reaction mixture containing 25  $\mu$ l of Taq master mix (Genet Bio, Seoul, South Korea), 2  $\mu$ l of each of forward and reverse primers (10  $\mu$ M stock), 2  $\mu$ l of template DNA (50 ng  $\cdot$   $\mu$ l<sup>-1</sup>) and 19  $\mu$ l of PCR grade pure water. The reaction mixture contained for 4 min at 95°C and then amplification was performed in 35 cycles. For each cycle, DNA was denatured at 94°C for 1 min, annealed at 51°C for 1 min, and then extended at 72°C for 2 min. The final extension was at 72°C for 10 min. PCR products were purified using the QIAGEN PCR purification kit. PCR-purified 16S rRNA genes of the five isolates were partially sequenced in both directions using the same two primers. The results obtained via sequencing were compared with other related taxa in the GenBank databases using the NCBI BLAST ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The nucleotide sequences were aligned with multiple sequence alignment program CLUSTALW (Thompson *et al.* 1994). The phylogenetic tree was constructed by using MEGA version 6.0 software based on the neighbour joining method and Kimura two-parameter distance with 100 bootstrap replicates (Tamura *et al.* 2013).

### *In vitro* antagonism assessment

#### Dual culture method

The antifungal activity of the selected *Streptomyces* strains was assessed against FOC by dual culture method in Petri dishes on PDA media (Erdogan and Benlioglu 2010). The bacterial strains (3-days-old culture) were streaked on one side of the Petri dishes (9 cm diameter) containing PDA media. After 48 h a 5 mm diameter of plug from the leading edge of a 5-days-old fungal culture (FOC) was placed in the center of each Petri dish. Plates with FOC only were used as control. The Petri dishes were incubated at 28°C for 7 days (16 h light : 8 h dark). The percentage of growth

inhibition (zone of inhibition) was recorded using the following formula: [(diameter in control plate – diameter in treated plate)/diameter in control plate] × 100.

#### Effect of volatile inhibitors

To study the effects of volatile antibiotic on radial growth of FOC, 100 µl of antagonistic bacterial suspension ( $1 \times 10^8$  cfu · ml<sup>-1</sup>, 3-days-old culture) was placed at the center of a Petri dish (90 mm diameter) containing PDA media, and a 5 mm disk of a 5-days-old FOC was placed at the center of another Petri dish containing PDA. Both half Petri dishes were placed face to face preventing any physical contact between the pathogen and the bacterial suspension. The pairs of each Petri dish were sealed together with parafilm. Bacterial suspension was replaced with sterile water in the control Petri dish. All Petri dishes were incubated at 28°C for 7 days (Naureen *et al.* 2009). Then, the percentage of growth inhibition was measured as mentioned above.

#### Effect of non-volatile inhibitors

PDA media Petri dishes, covered with a 0.2 µm cellophane membrane, were inoculated in the center with 200 µl of *Streptomyces* strains suspension ( $1 \times 10^8$  cfu · ml<sup>-1</sup>, 3-days-old culture). Petri dishes were incubated at 28°C for 7 days. After 72 h, the membrane with the growth bacterial isolates was removed and a 5 mm disk of a pure culture of FOC (5-days-old) was placed in the center of the Petri dishes. Bacterial suspension was replaced with sterile water in control Petri dishes. Inhibition percentages were determined using the method previously described (Naureen *et al.* 2009).

#### Siderophore production

This experiment was undertaken based on the method described by Weller and Cook (1983). Firstly, King's medium B Agar (KB) containing 0, 100 and 1,000 mM of FeCl<sub>3</sub> was prepared. Then, 100 µl of bacterial strains were cultured on the surface of media and plates were incubated at 28°C for 5 days. Next, 0.5 ml suspension of *Geotrichum candidum* ( $1 \times 10^6$  cfu · ml<sup>-1</sup>) was sprayed on the surface of the media and incubated at 28°C for 72 h. Clear zones surrounding the bacterial strains colonies suggested siderophore production by biocontrol agents that caused inhibition of mycelial growth of *G. candidum*.

#### Protease production

Bacterial strains were tested for production of protease by growing them on Skim Milk Agar (SKM) (Chantawannakul *et al.* 2002). Plates were incubated at 28°C for 24 h. An ability to clear the SKM suspension in the agar was taken as evidence of the secretion of protease. Non-bacteria inoculated plates were used as the control.

#### Chitinase production

To assay the production of chitinase, *Streptomyces* strains were grown on medium containing chitin (0.4%), KH<sub>2</sub>PO<sub>4</sub> (0.3 g), K<sub>2</sub>HPO<sub>4</sub> (0.7 g), MgSO<sub>4</sub> · 5H<sub>2</sub>O (0.5 g), FeSO<sub>4</sub> · 7H<sub>2</sub>O (0.01 g), ZnSO<sub>4</sub> · 7H<sub>2</sub>O (0.001 g), MnCl<sub>2</sub> · 4H<sub>2</sub>O (0.001 g) and Agar (20 g · l<sup>-1</sup>), (pH was adjusted to 8.0–8.5). Petri dishes were incubated at 28°C for 7 days. Clear zones surrounding the bacterial colonies suggested chitinase activity on colloidal chitin agar plates (Hus and Lockwood 1975). All experiments were repeated twice with four replicates per treatment.

#### Greenhouse studies

Biocontrol efficacy tests were conducted in the greenhouse in pots (20 cm in diameter and 15 cm in height) with a pasteurized sand-peat-perlite mix (2 : 2 : 1, v/v/v). Chickpea seeds (cv. Kaka) were surface-sterilized with 2% sodium hypochlorite for 3 min, washed twice with sterile water and soaked in 30 ml suspension ( $1 \times 10^8$  cfu · ml<sup>-1</sup>) of bacterial-methylcellulose for 30 min. Then the treated seeds were dried under a laminar flow hood and sown in sterile soil in pots infested with the FOC (30 ml of 10<sup>6</sup> spores · ml<sup>-1</sup> added to each pots before planting). For the control tests, seeds were soaked in sterile water-methylcellulose instead of the bacterial spore suspension. All pots were transferred to a greenhouse and incubated at 27±2°C with a 16-h light : 8-h dark cycle and 60–70% relative humidity. Three seeds were sown in each pot and the treatments were replicated five times in a completely randomized design. Plants were watered weekly with sterile tap water and fertilizer solution (NPK 1 : 1 : 1) was added to pots twice a week at a rat of 3 g · l<sup>-1</sup>.

Disease severity was recorded for each plant 30 days after inoculation by a visual 0–4 scale according to the percentage of chlorosis, necrosis and wilt, as follows: 0 = healthy, 1 = 1–33%, 2 = 33.1–66%, 3 = 66.1–100%, 4 = dead plant (Campbell and Madden 1990). Disease incidence assessments were calculated using the method reported by (Cao *et al.* 2011) with the following formula:

$$\text{Disease incidence \%} = \left[ \frac{\text{(number of infected plants)}}{\text{total number of plants}} \right] \times 100.$$

Biocontrol efficacy was measured using the following formulas:

$$\text{Control efficacy \%} = \left[ \frac{\text{(disease index of control)} - \text{(disease index of treated)}}{\text{disease index of control}} \right] \times 100.$$

At the end of the experiments, effects of bacterial strains on shoot length (cm), root length (cm), dry weight of shoot and root (g) were determined for each plant. The experiment was repeated twice.

#### Statistical analysis

Experiments *in vitro* and greenhouse conditions were designed as a completely randomized design (CRD). All analyses were performed using SPSS (Statistical Product and Service Solutions) versions 12.0. The means were compared by Duncan's Multiple Range Test (DMRT) at  $p \leq 0.05$ .

## Results

### Pathogenicity test

Among ten isolates of *F. oxysporum* f. sp. *ciceris*, isolate number one was found to be significantly more pathogenic in chickpea (cv. Kaka) as all five plants from each replicate were diseased and symptoms of wilting, yellowing and discoloration of the vascular tissue were observed on diseased plants. The diseased index for infected plants in the F1 isolate was expressed as 3 which represent 66% wilt. Therefore, the F1 isolate was selected and used in this research. Isolates number 5 was a weak pathogen and showed significantly lower pathogenicity than all the other isolates (Table 1). The pathogen was re-isolated from the infected plants.

### In vitro antifungal activity

A total of 112 bacterial strains were isolated from the rhizosphere of healthy chickpea plants according to the isolation procedures described in material and methods. Of the 112 bacterial isolates, five (KS31, KS55, KS58, KS62 and KS112) were selected for further studies according to their high efficiency in *in vitro* antagonism (with inhibition zones > 25%) using the plate assay on PDA media (data not shown).

### Identification of selected strains

Identification of five of the selected strains, designated KS62, KS55, KS112, KS58 and KS31 was based on the morphological, cultural and biochemical characteristics, as well as molecular phylogenetic analysis. Results showed that all strains produced chalky/white colonies on yeast extract malt extract agar (ISP2) plates. Spore masses in four of the five strains were gray with linear spore chain. The strain KS62 had a white spore mass color with spiral chain. The strains were strictly aerobic, Gram-positive, catalase positive and they were all able to hydrolyze starch and casein. They were negative for H<sub>2</sub>S test, liquefaction of gelatine and nitrate reduction test. The isolates

were characterized by 16S rDNA sequencing and phylogenetic analysis. After sequencing DNA, the resulting sequences were submitted as BLAST queries to GenBank. BLAST search results indicated that related strains have relatively high homology (97–99%) with members of the *Streptomyces* genus.

To determine phylogenetic relationships between isolated strains and other related *Streptomyces* spp. that shared the highest sequence similarities, a phylogenetic tree was constructed (Fig. 1). The 16S rDNA sequences from strains KS62, KS55, KS112, KS58 and KS31 were deposited to Gene Bank databases under the accession numbers of KT358975, KT358977, KT358974, KT358978 and KT358976, respectively.

### Dual culture assay

All of the five selected bacterial strains showed antifungal activities in dual culture assay on PDA medium. Strain KS62 had maximum inhibitory effect (44.2%), followed by KS31 (44%) and KS112 (36.7%) on PDA medium. Strain KS58 had minimum inhibitory effect (26%) (Table 2).

### Effect of volatile compounds

Out of the five antagonistic bacterial strains, three strains (KS55, KS58 and KS112) were found to inhibit growth of FOC and gave maximum inhibition 33.4%, 32.2% and 27.7%, respectively. The other bacterial strains (KS62 and KS31) were weak for inhibition growth of the pathogen and reduced the radial growth of the pathogen which ranged from 20.2% to 23.4% (Table 2).

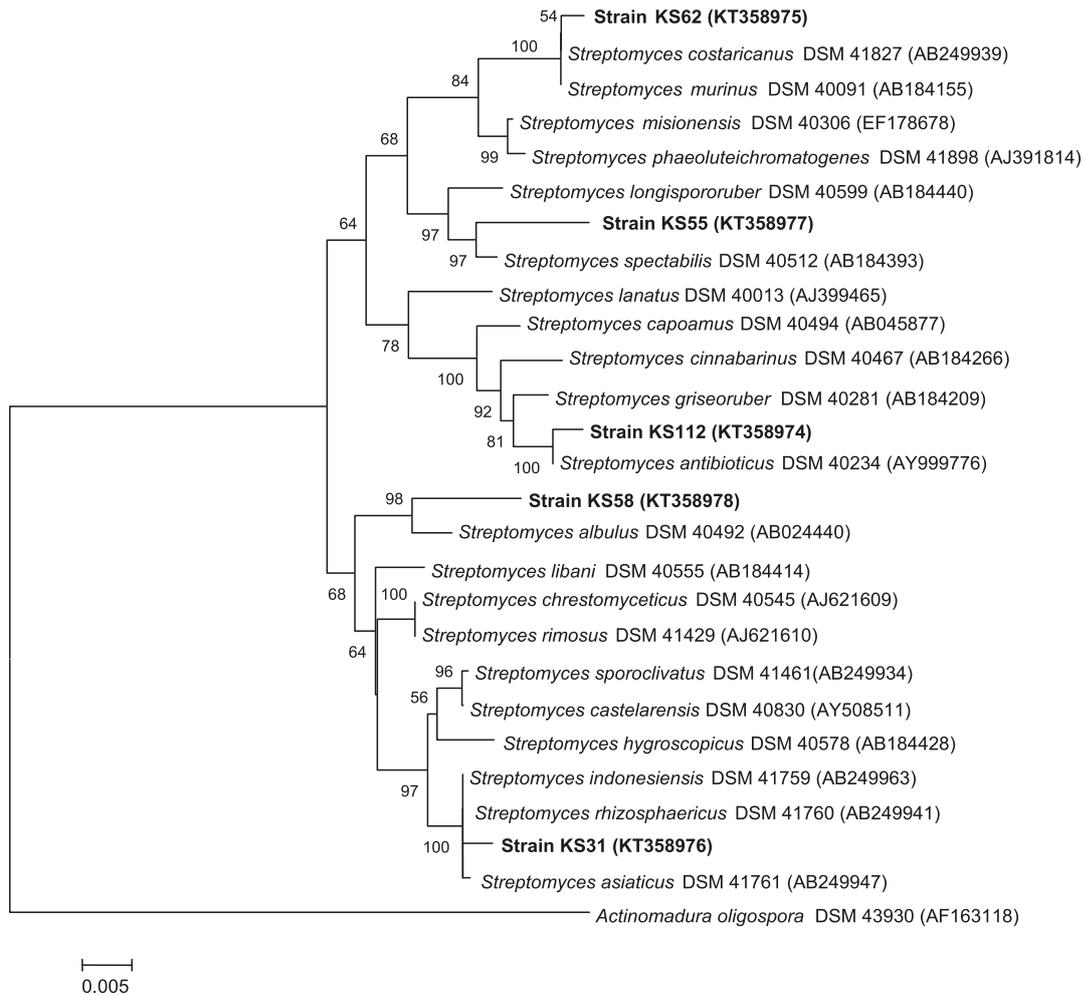
### Effect of non-volatile compounds

The evaluation of antagonistic activity of five strains of *Streptomyces* against FOC showed that four strains inhibited more than 50% growth of FOC. The maximum percent inhibition of growth of FOC was observed by strains KS62 (61.1%) followed by strain numbers KS112, KS55 and KS31 (ranging from 58.1% to 50.4) (Table 2).

**Table 1.** Pathogenicity of *Fusarium oxysporum* f. sp. *ciceris* isolates under greenhouse conditions

Isolates of pathogen	Disease severity	Disease incidence [%]
F1	3.00 a	100.0 a
F2	2.58 a	100.0 a
F3	1.33 bc	91.5 ab
F4	1.66 b	100.0 a
F5	1.00 c	91.5 b
F6	1.41 bc	91.5 ab
F7	1.58 b	91.5 ab
F8	1.66 b	100.0 a
F9	1.25 bc	91.5 ab
F10	1.25 bc	100.0 a
Control water	0 d	0 c

Different letters within columns indicate significant differences at  $p \leq 0.05$ , according to Duncan multiple-ranges test (DMRT). Data are means of five replicates



**Fig. 1.** Phylogenetic tree showing the position of the five isolated strains among the type strains of related representative species of the genus *Streptomyces*. The numerals show percentages from 100 replicate bootstrap samplings (frequencies of less than 50% are not shown). Scale bar, 5 substitutions per 1,000 nucleotide positions. Gene Bank accession numbers for 16S rRNA gene sequences are shown in parentheses. *Actinomadura oligospora* DSM 43930 was used as an out group

**Table 2.** *In vitro* activity of biocontrol agents against *Fusarium oxysporum* f. sp. *ciceris* and production of antimicrobial metabolites

The bacterial strains	DC%	Va%	NVa [%]	Siderophore	Protease	Chitinase
KS31	44±0.16 a	23.4±0.08 c	50.4±0.12 d	+	-	+
KS55	28.4±0.14 c	33.4±0.11 a	57.4±0.21 c	+	+	+
KS58	26±0.15 d	32.2±0.08 a	36.3±0.14 e	+	+	+
KS62	44.2±0.11 a	20.2±0.12 d	61.1±0.14 a	-	-	+
KS112	36.7±0.08 b	27.7±0.09 b	58.1±0.17 b	+	-	+

Different letters within columns indicate significant differences at  $p \leq 0.05$ , according to Duncan multiple-ranges test (DMRT). Data are means of four replicates ("±" = standard deviation): DC = percent growth inhibition in dual culture method, Va = percent growth inhibition in volatile antibiotics, NVa = percent growth inhibition in non-volatile antibiotic, "+" = positive, "-" = negative

### Siderophore, protease and chitinase production by *Streptomyces* strains

Strains KS55, KS58, KS112 and KS31 produced siderophores in media containing 100  $\mu\text{M}$   $\text{FeCl}_3$ . Two of the five *Streptomyces* strains, KS55 and KS58 produced protease. All of the five selected strains produced chitinase in media containing chitin 4% and produced a clear halo zone on media (Table 2).

### Greenhouse studies

All treatments significantly decreased disease severity and increased control efficacy compared to untreated control (pathogen alone) (Table 3). Fifty days after treatment, strains KS62, KS112 and KS31 significantly ( $p \leq 0.05$ ) reduced disease severity by 54.8%, 51.6% and 48.4%, respectively. Strains KS55 and KS58 had the least effect on disease severity 45.1% and 38.7%, respectively (Table 3). All

**Table 3.** Effect of *Streptomyces* strains on *Fusarium oxysporum* f. sp. *ciceris* under greenhouse conditions after 50 days

The bacterial strains	Disease severity	Control efficacy [%]	Disease incidence [%]
KS31	1.6±0.07 cd	48.4	100
KS55	1.7±0.16 d	45.1	100
KS58	1.9±0.15 e	38.7	100
KS62	1.4±0.10 b	54.8	100
KS112	1.5±0.07 bc	51.6	100
Non infested control	0±0.00 a	–	–
Control pathogen	3.1±0.10 f	–	–

Different letters within columns indicate significant differences at  $p \leq 0.05$ , according to Duncan multiple-ranges test (DMRT). Data are means of five replicates ("±" = standard deviation)

**Table 4.** The effects of *Streptomyces* strains on growth parameters of chickpea plants in seed treatments under greenhouse conditions

The bacterial strains	Shoot length [cm]	Root length [cm]	Shoot dry weight [g]	Root dry weight [g]
KS31	32.41±0.14 b	15.31±0.10 c	0.26±0.01 a	0.18±0.01 b
KS55	33.23±0.15 c	15.31±0.09 c	0.31±0.04 b	0.18±0.02 b
KS58	30.1±1.42 a	13.5±0.20 a	0.24±0.02 a	0.12±0.01 a
KS62	32.45±0.19 b	14.38±0.18 b	0.31±0.04 b	0.21±0.01 c
KS112	34.98±0.19 d	17.65±0.25 d	0.37±0.01 c	0.21±0.01 c
Control	30.18±0.21 a	13.55±0.15 a	0.25±0.01 a	0.13±0.01 a

Different letters within columns indicate significant differences at  $p \leq 0.05$ , according to Duncan multiple-ranges test (DMRT). Data are means of five replicates ("±" = standard deviation)

treatments showed disease incidence and there were not any significant differences among treatments.

The effects of all strains on shoot length, root length, dry weight of shoots and roots of chickpea plants were determined 50 days after inoculation by the pathogen. Strains KS112 significantly increased length of shoot (34.98 cm) and length of root (17.65 cm) of chickpea plants in seed treatments. Moreover, this isolate increased the dry weight of shoots (0.37 g) and the dry weight of roots (0.21 g) compared with the non-treated control. Minimum inhibitory effect was mediated by isolates KS58 in all of the above parameters measured (Table 4).

## Discussion

The present study was conducted to evaluate the efficacy of *Streptomyces* strains against chickpea Fusarium wilt. In this study, all bacterial isolates were selected from the rhizosphere of healthy chickpea plants at different locations of chickpea-growing regions. The use of microorganisms isolated from the rhizosphere of a specific crop might be better adapted to that crop. Hence, it may provide better control of diseases than organisms that are isolated from other plant species (Lucy *et al.* 2004). Of the 112 isolates, five isolates were selected according to their high efficiency in the plate assay. These results have been demonstrated in previous research (Tjamos *et al.* 2004; Zheng *et al.* 2011).

The sequence analysis of 16S rDNA region of selected isolates showed that all of the five strains belonged to the *Streptomyces* genus. *In vitro*, selected bacterial strains showed different degrees of antagonism. In dual-cul-

ture assay, the inhibition zone values of selected strains ranged from 26 to 44.2%. The *Streptomyces* strains inhibited the growth of pathogen significantly (more than 50%) by the production of non-volatile compounds ( $p \leq 0.05$ ). This result showed that non-volatile substances produced by *Streptomyces* strains were more – inhibitory to *Fusarium oxysporum* f. sp. *ciceris* than production of volatile substances (Table 2). The inhibitory effects of volatile and non-volatile compounds on different soil borne fungal pathogens were observed (Sabaratnam and Traquair 2002; Elango *et al.* 2015). The results of the present study demonstrate that non-volatile substances produced by *Streptomyces* strains have a significant effect on the mycelial growth of pathogen. In other studies, the effects of these compounds on different soil borne fungi pathogen of chickpea have also been observed (Abo-Elyour and Mohamed 2009). Non-volatile antifungal substances produced by *Streptomyces globisporus* JK-1 can inhibit the growth of *Magnaporthe oryzae* causing rice blast of rice (Li *et al.* 2011). Production of chitinase was observed in all selected *Streptomyces* strains. Production of non-volatile enzymes, such as chitinase by *Trichoderma harzianum* and *Talaromyces flavus* against a wide range of fungal species of *Aspergillus*, *Rhizopus*, *Moucor* and *Sclerotinia* have been reported (Menendez and Godeas 1998; Nampoothiri *et al.* 2004). Moreover, chitinase production by *T. harzianum* and *T. flavus* was effective for controlling soybean stem white rot and bean stem rot diseases caused by *Sclerotinia sclerotiorum* and *S. rolfsii*, respectively (Madi *et al.* 1997; Menendez and Godeas 1998). Chitinase inhibited the fungal growth through damaging cell walls (Xiao *et al.* 2002).

Four strains produced siderophore and two strains produced protease.

*Streptomyces* strains can protect plants from pathogens by different mechanisms, including antibiosis (Smith *et al.* 1990), competition for space and nutrients and (Tanaka and Omura 1993), like dimethyl disulfide, dimethyl trisulfide, acetophenone and 2-methylisoborneol (Li *et al.* 2010), competition for iron through production of siderophores (Sadeghi *et al.* 2012), induction of systemic resistance (Tarkka *et al.* 2008), production of extracellular enzymes such as chitinase (Srividya *et al.* 2012) and protease that can lyse cell walls of pathogen (Xiao *et al.* 2002; Srividya *et al.* 2012), and parasitism (Loliam *et al.* 2013).

Our study indicated that *Streptomyces* strains applied as seed treatment significantly reduced disease severity of chickpea Fusarium wilt caused by *F. oxysporum* f. sp. *ciceris* under greenhouse conditions compared to control (38.7 to 54.8%). Strains S62 and S112 showed the highest control efficacy 54.8 and 51.6%, respectively. Previous studies have illustrated that *Streptomyces* spp. effectively suppressed red root-rot disease of tea plants and red rust disease caused by *Poria hypolateritia* and *Cephaleuros parasiticus*, respectively (Elango *et al.* 2015). Of the five *Streptomyces* strains tested in the greenhouse, the highest degree of increment of plant biomass was observed by strains KS112 and KS55 (Table 4). Moreover, the *Streptomyces* strains significantly increased plant height and dry matter compared to the untreated control (Gopalakrishnan *et al.* 2013). Thus, these biocontrol agents have the capability to enhance the growth of plants by producing plant growth regulator hormones including cytokinins, gibberellins and auxins (Ajay *et al.* 2004).

## Conclusions

Our findings demonstrated that *Streptomyces* spp. have great potential to be used as biocontrol agents for management of *F. oxysporum* f. sp. *ciceris* causing chickpea Fusarium wilt as well as many other fungi pathogens that have been previously reported (Wan *et al.* 2008; Li *et al.* 2010; Elango *et al.* 2015). These biocontrol agents not only reduced the disease severity but also increased growth parameters of chickpea plant. Therefore, further study is needed to verify their effectiveness under different field conditions.

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