

## Biological Control of *Fusarium* root-rot of Sorghum

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**Abstract:** *Fusarium oxysporum* Schlectend causes root and crown rot in several crops including sorghum that result in low grain yield. All antagonists showed inhibition of mycelial growth of *F. oxysporum* and the maximum inhibition was recorded when *Bacillus subtilis* as biocontrol agent (67.7%). The *in vitro* root colonization study demonstrated that after four days of germination, the cell counts obtained from the roots have increased and the maximum count is achieved by *B. subtilis* ( $16.9 \times 10^5$  cfu/cm root). The greenhouse pot experiment demonstrated that *T. viride* and *B. subtilis* resulted in more than 80% suppression of root rot. The reduction in fresh weight of roots amounted to 93.6% in the control treatment inoculated with *F. oxysporum* alone, whereas 71.1% reduction in fresh root weight was recorded for the treatments inoculated with both the pathogen and *B. subtilis*; 66.8% reduction in fresh root weight was recorded for the treatments inoculated with both the pathogen and *T. harzianum*. Root dry weight of the control treatment inoculated with only *F. oxysporum* decreased by 94.5% in relation to the non-inoculated control. Among the potential biological control agents in this study, *B. cereus* resulted in 42.3 reduction in root dry weight compared to the 94.5% reduction recorded for the control inoculated with *F. oxysporum* alone. 100% of the roots from the control treatment (*F. oxysporum* only) rendered growth of *F. oxysporum* compared to an incidence ranging from 20 to 55% for plants treated with *B. subtilis*, *B. lecheniformis*, *B. cereus*, *T. harzianum* and *T. viride*. Both chlorophyll fractions increased when treated with antagonist and the maximum enhancement was recorded when *Bacillus subtilis* used as antagonist compared with that of control. The maximum values of the carbohydrate components were recorded when *Bacillus subtilis* used as antagonist relative to those of control.

**Key words:** sorghum, biocontrol, bacterial antagonists, fungal antagonist, root colonization, carbohydrate content

### INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) and maize are the most popular cereals consumed by both adults and infants in Africa, south of the Sahara<sup>[3]</sup>. Sorghum is a major source of protein and calories in the diets of large segment of the populations of Africa and Asia<sup>[50]</sup>.

*Sorghum bicolor* (L.) Moench, is grown in the southern part of Saudi Arabia as a staple food grain and/or fodder. It is the most commonly used crop in the region, covering about 85% of the cultivated land<sup>[5]</sup>.

Sorghum is used to prepare various dishes in different parts of the world. It can be used in production of fermented and unfermented bread, stiff porridge, thin porridge, steamed cooked products, boiled whole or pearled, snack foods, alcoholic beverages, and nonalcoholic beverages. The sorghum flour is used to prepared local bread know as Khamir in Gizan province, Saudi Arabia<sup>[17]</sup>.

Several members of the Genus *Fusarium* cause root diseases in sorghum leading to serious yield losses. Among the major pathogens in this group are *Fusarium oxysporum* Schlectend, *F. moniliforme* Sheld, *F. graminearum* Schwabe and *F. tricinctum* (Corda) Sacc<sup>[15]</sup>.

Chemical control of sorghum diseases is also unaffordable in most developing countries. The use of broad-spectrum fungicides further results in imbalances within the microbial community creating unfavorable conditions for the activity of beneficial organisms<sup>[47]</sup>.

Soil-borne diseases have been controlled more recently by means of certain beneficial bacteria that are indigenous to the rhizosphere of plants<sup>[42]</sup>. The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large and metabolically active groups of bacteria<sup>[46]</sup> known as plant growth promoting rhizobacteria (PGPR)<sup>[22]</sup>. PGPR are known to rapidly colonize the rhizosphere and suppress deleterious microorganisms as well as soilborne pathogens at the root surface<sup>[33]</sup>. These organisms can also be beneficial to the plant by stimulating growth<sup>[7]</sup>.

The aim of this study is therefore to screen some of bacterial and fungal organisms for *in vitro* and *in vivo* antagonistic activity against *F. oxysporum*, one of the major causes of root rot. This study may contribute to the introduction of PGPR systems in biological control of phytopathogenic fungi in sorghum and other crops.

## MATERIALS AND METHODS

**Fungal and Bacterial Inocula Preparation:** *Fusarium oxysporum* was isolated from diseased sorghum seedlings and maintained on Dox's medium. The antagonistic bacteria (*Bacillus cereus*, *B. lecheniformis* and *B. subtilis*) were maintained on nutrient medium; while the antagonistic fungi (*Trichoderma harzianum* and *T. viride*) were maintained on PDA medium.

The antagonistic bacteria were grown in nutrient broth on a rotary shaker at 28±2°C and 180 rpm for 24 h. The suspension was centrifuged at 5000 rpm for 15 min. The pellets were re-suspended in quarter strength sterile Ringer's (Merck) solution to give a final concentration of 100 cfu/ml using the viable plate count method. Also, spore suspension of fungal antagonists was prepared (100 cfu/ml).

**In Vitro Antagonistic Activity:** The *in vitro* inhibition of mycelial growth of *F. oxysporum* by the antagonist was tested using the dual culture technique as described by Paulitz *et al.* [31] and Landa *et al.* [24]. Two 50 µl drops from the 100 cfu/ml suspension were placed on the margins of PDA plates and incubated at 28±2°C for 24 h. A 4 mm agar disc from fresh PDA cultures of *F. oxysporum* was placed at the centre of the PDA plate for each antagonist and incubated at 28±2°C for seven days. The radii of the fungal colony towards and away from the bacterial colony were measured. The percentage growth inhibition was calculated using the following formula:

$$\% \text{mycelial inhibition} = \frac{(R - r)}{R} \times 100$$

where, *R* mycelial growth away from the antagonistic colony (the maximum growth of the fungal mycelia), *r* is mycelial growth towards the antagonist.

**In Vitro Root Colonization:** The antagonists were tested for their ability to colonize sorghum roots *in vitro*, using a modification of the methods by Patten and Glick [30] and Montealegre *et al.* [28]. Sorghum seeds were surface sterilized with 70% ethanol for 5 min and subsequently with 1% sodium hypochlorite for 1 min and rinsed two times in sterile distilled water. For each treatment, 15 seeds were transferred to a sterile moist chamber i.e. discs of filter paper placed in 9 cm diameter Petri dishes and moistened with sterile

distilled water. For inoculation, the bacterial and fungal inocula were prepared as described above. A 1 ml aliquot of each inoculum was added to the seeds in the moist chamber and the plates were incubated at room temperature for 1 h to allow binding of the bacteria to the seed coat. Both treated seeds and controls were then incubated at 30°C for 4-5 days in the dark for root development. One centimeter of root from each treatment was aseptically excised, one seed per treatment, and transferred to 0.1 M MgSO<sub>4</sub> solution, shaken and serially diluted. From each dilution, a 0.1 ml aliquot was plated on nutrient agar media for bacteria and PDA for fungi, and the plates were incubated at 30°C for colony counts. The number of antagonist isolates colonizing the root was calculated as colony forming units/cm root (cfu/cm root).

**Pathogenicity Tests and Fungal Inoculum Preparation:** *Fusarium oxysporum* isolated from infected sorghum roots. Pathogenicity of the isolate was confirmed under greenhouse conditions. Barely seed inoculum was prepared as follows: 150 g barely seed together with 100 ml distilled water was deposited in an conical flasks and autoclaved. After autoclaving each flask was inoculated with five 5-mm agar discs cut from a fresh PDA culture of *F. oxysporum*. The inoculum was incubated at 27±2°C for 7 days.

**Planting of Sorghum and Inoculation of Fungal and Bacterial Isolates:** Sorghum seeds were surface sterilized with 70% ethanol for 5 min, 1% sodium hypochlorite for 1 min and rinsed three times with sterile distilled water. The seeds were then pregerminated in sterile vermiculite for four days at 30°C in a growth cabinet. Prior to seedling transplanting, the barely seed inoculum of *F. oxysporum* was mixed into steam pasteurized soil in 12 cm by 10.5 cm diameter plastic pots at the rate of 30 g/kg. For the control treatments, sterile barely seed was mixed into the soil at the same rate. Eight sorghum seedlings were transplanted into each pot and the pots were maintained in the greenhouse at 30±2°C. One day after transplanting of seedlings, each pot was inoculated with 30 ml of each of the antagonist inocula (≈100 cfu/ml). Two successive antagonist applications were made a week apart. The treatments in the *in vivo* biocontrol experiment were: Plants inoculated with *F. oxysporum* and antagonist, a non-inoculated control (Control a) and plants inoculated with *F. oxysporum* on its own (Control b). The non-inoculated control was treated with sterile barely seed without fungal and antagonist inoculum. The plants were irrigated twice daily with tap water. All the *in vitro* and *in vivo* experiments were arranged in a randomized block design in three replications and each experiment was repeated twice.

**Disease Assessment:** Four weeks later, plants were removed from the soil and the roots washed with sterile distilled water. Roots were excised from the plants and data collected for analysis. Data included root and crown rot severity assessed on a rating scale of 0-4<sup>[9]</sup>. (0= no infection, 1= 1-25% infection, 2= 26-50% infection, 3= 51-75% infection and 4= 76-100% infection in the root region. Based on the disease severity index, the percentage suppression of root rot was calculated<sup>[47]</sup> as follows:

$$\% \text{Suppression} = \frac{(A - B)}{A} \times 100$$

where, A is the disease severity exhibited in the root region due to *F. oxysporum* alone and B is the disease severity exhibited in the root region after inoculation with both the pathogen and antagonists. Roots from six plants per treatment were excised and the fresh and dry weights were determined on three root systems per treatment. The remaining three roots were subsequently used to determine the incidence of *F. oxysporum*.

**Rhizosphere Colonization:** The survival of antagonist isolates in the rhizosphere of the sorghum plants was determined according to modification of the procedure described by Landa *et al.*<sup>[25]</sup>. Plants were carefully removed from the pots and roots were gently shaken to remove all but the tightly adhering soil. One gram of the adhering rhizosphere soil was collected and placed into 9 ml sterile 0.1 M MgSO<sub>4</sub> solution, shaken and serially diluted. From each dilution, a 0.1 ml aliquot was plated on nutrient agar media for bacteria and PDA for fungi, and the plates were incubated at 30°C for colony counts. The number of antagonist isolates colonizing the root was calculated as colony forming units/cm root (cfu/cm root).

**Extraction and Determination of pigment Content:** Photosynthetic pigments of sorghum shoot were extracted and determined according to the method described by Fedee<sup>[14]</sup>. The absorbance of each extract was measured using a spectrophotometer (Spectronic 20) at 644 and 622 nm. The contents of chlorophylls *a* and *b* were calculated using the equations outlined by Sestak *et al.*<sup>[36]</sup> for 100% acetone:

$$\begin{aligned} \text{Chlorophyll } a \text{ (}\mu\text{g ml}^{-1}\text{)} &= 9.78 E_{662} - 0.99 E_{644} \\ \text{Chlorophyll } b \text{ (}\mu\text{g ml}^{-1}\text{)} &= 21.4 E_{644} - 4.65 E_{662} \end{aligned}$$

The results were expressed as  $\mu\text{g pigment ml}^{-1}$  extract, then calculated as  $\text{mg pigment g}^{-1}$  fresh weight.

#### Carbohydrate Analyses:

**Extraction of Soluble Sugars:** Soluble sugars were extracted according to the method explained by

Upmeyer and Koller<sup>[45]</sup>. The clear extract contained soluble reducing and non-reducing sugars. The plant residue was oven dried at 60°C and stored to hydrolyze the insoluble carbohydrate.

**Hydrolysis of Non-reducing Sugar:** One ml of the soluble sugar extract was hydrolyzed with 1 ml 6N HCl for 12 min at 70°C on water-bath<sup>[16]</sup>. The pH of each sample was neutralized, then diluted to a known volume with 80% ethanol, and its total reducing value determined.

**Hydrolysis of Insoluble Carbohydrate:** The plant residue remained after the extraction of soluble sugar was hydrolysed with 0.2N H<sub>2</sub>SO<sub>4</sub> for one hour using a boiling water-bath<sup>[41]</sup>. The mixture was centrifuged for 15 min at 4000 g and the clear supernatant neutralized and completed to a known volume before determination of its reducing value.

**Statistical Analysis:** The data were subjected to analysis of variance using SAS-9.1 software<sup>[35]</sup>. Data on the *in vitro* root colonization and the survival of the bacterial isolates in the rhizosphere were log transformed before subjecting to analysis of variance (ANOVA). Mean values among treatments were compared by the least significant difference (LSD) test and Duncan's Multiple Range (DMR) test at 5% ( $p = 0.05$ ) level of significance.

## RESULTS AND DISCUSSION

The test bacterial and fungal antagonists showed variations in inhibition of mycelial growth, whereas *Bacillus subtilis*, *B. lecheniformis* and *B. cereus* resulted in 67.7%, 57.5% and 47.7% inhibition of mycelial growth of *F. oxysporum*, respectively (Table 1). The maximum inhibition achieved by *B. subtilis* was 67.7%. For fungal antagonists *Trichoderma harzianum* and *T. viride* resulted in 57.7% and 49.8% inhibition of mycelial growth of *F. oxysporum*, respectively. Control plates not treated with the bacterial isolates were completely covered by the phytopathogens showing no inhibition. The mean mycelial growth inhibition of the most effective bacterial and fungal isolates revealed that the inhibition was highly significant ( $p = 0.05$ ).

The *in vitro* root colonization study demonstrated that after four days of germination, the bacterial cell counts obtained from the roots have increased by  $16.9 \times 10^5$  cfu/cm root for *B. subtilis*, by  $0.4 \times 10^5$  cfu/cm root for *B. lecheniformis* and by  $16.1 \times 10^5$  cfu/cm root for *B. cereus* (Table 1) as compared to the control where counting was of 100 cfu/cm root length from the initial inoculum level of 100 cfu/ml (Table 1). Similarly, the fungal isolates colonized the roots and

the spore count, compared to the initial inoculum level increased by  $12.3 \times 10^5$  cfu/cm root length for *T. harzianum* and by  $1.0 \times 10^5$  cfu/cm root length for *T. viride* (Table 1).

Results from the greenhouse pot experiment demonstrated that *T. viride* and *B. subtilis* resulted in more than 80% suppression of root rot whilst *T. harzianum* and *B. cereus* resulted in disease reduction of more than 75% (Table 2). Control plants not treated with antagonists but inoculated with *F. oxysporum* alone rendered up to 100% root rot incidence with the majority of plants completely stunted or dead (Table 2, control b). Plants inoculated with neither the pathogen nor the antagonists also survived but rendered some infection (Table 2). This could probably be due to a low level of cross contamination by some *Fusarium* survival in the steam pasteurized soil, but internal contamination is unlikely in this case.

The fungus resulted in a pronounced decrease in the fresh and dry weight of the roots compared to the non inoculated control and to some of the treatments with the antagonists. The reduction in fresh weight of roots amounted to 93.6% in the control treatment inoculated with *F. oxysporum* alone, whereas 71.1%, 54.5% and 5.9% reduction in fresh root weight was recorded for the treatments inoculated with both the pathogen and *B. subtilis*, *B. lecheniformis* and *B. cereus*, respectively; 66.8% and 54.5% reduction in fresh root weight was recorded for the treatments inoculated with both the pathogen and *T. harzianum* and *T. viride* respectively. The least reduction in fresh root weight (5.9%) was recorded for the treatment inoculated with *B. cereus* (Table 2).

Root dry weight of the control treatment inoculated with only *F. oxysporum* decreased by 94.5% in relation to the non-inoculated control. Among the potential biological control agents in this study, *B. cereus* and *B. subtilis* resulted in 42.3 and 65.9% reduction in root dry weight respectively compared to the 94.5% reduction recorded for the control inoculated with *F. oxysporum* alone.

Percentage of roots of sorghum plants showing presence of *F. oxysporum* in the various treatments showed a reduction of the pathogen by the bacterial and fungal isolates. Almost 100% of the roots from the control treatment (*F. oxysporum* only) rendered growth of *F. oxysporum* compared to an incidence ranging from 20 to 55% for plants treated with *B. subtilis*, *B. lecheniformis*, *B. cereus*, *T. harzianum* and *T. viride* (Table 3).

The results represented in Table (4) showed that chlorophyll *a* and *b* of sorghum shoot grown in soil infested with pathogen and, pathogen and antagonists showed that all both chlorophyll fractions increased when treated with antagonist and the maximum enhancement was recorded when *Bacillus subtilis* used as antagonist compared with that of control.

The changes in soluble, insoluble and total sugars of 30-day old sorghum shoot plants grown in soil infested with pathogen and, pathogen and antagonists are represented in Table (5). The data showed an improvement in all plants and the maximum values of the carbohydrate components were recorded when *Bacillus subtilis* used as antagonist relative to those of control.

**Discussion:** The aim of this study is therefore to screen some of bacterial and fungal organisms for *in vitro* and *in vivo* antagonistic activity against *F. oxysporum*, one of the major causes of root rot. This study may contribute to the introduction of PGPR systems in biological control of phytopathogenic fungi in sorghum and other crops. The pathogenicity test conducted revealed that the barely seed inoculum used at a rate of 30 g/kg was sufficient for the pathogen to grow and cause infection in a few days.

The approach was hence to begin screening the bacterial and fungal isolates for their *in vitro* biocontrol activity against *F. oxysporum* and *in vitro* root colonization ability. The bacterial and fungal isolates used were initially isolated from the rhizosphere of sorghum plants in the sorghum growing fields. The approach provided an opportunity to select effective biocontrol strains capable of antagonizing soilborne pathogens in the same environment where they will be used commercially<sup>[24]</sup>.

It is known that *in vitro* assays have certain limitations in that the biocontrol efficiencies may not be equally expressed under gnotobiotic (axenic) and *in vivo* conditions<sup>[21]</sup>. However, the *in vitro* assays conducted in this study (pathogen inhibition and root colonizing ability) were used to screen and select potential biocontrol agents and subsequently test their ability to suppress *Fusarium* root rot of sorghum under greenhouse conditions.

The percentage of *in vitro* mycelial growth inhibition by the antagonists against *F. oxysporum* varied between 47.7% and 67.7%. This suggests that the mode of action exerted and/or the type of antifungal metabolite produced by the isolates may vary<sup>[49]</sup> but also that the antagonists are taxonomically different from each other<sup>[49]</sup>.

The PDA medium used for the dual culture assay is rich in nutrients, competition might be excluded as the mode of action for these isolates<sup>[24]</sup>. The antifungal metabolites produced seem to vary among the isolates tested in this study. In the dual culture assay for instance, some of the isolates not only inhibited the mycelial growth but also changed the appearance of the mycelia from white to reddish brown and red as was evident for isolates *B. lecheniformis*. This suggests that the fungal mycelia might have been inhibited not only by antibiosis but also by other antifungal metabolites

**Table 1:** Inhibition of mycelial growth of *Fusarium oxysporum* and *in vitro* root colonization of sorghum roots by antagonistic strains

Antagonist strain	Inhibition of mycelial growth (%)	<i>In vitro</i> colonization (cfu/cm rootsx10 <sup>5</sup> )
Control	0.0 <sup>a</sup>	0.3 <sup>a</sup>
Bacteria		
<i>Bacillus subtilis</i>	67.7 <sup>b</sup>	16.9 <sup>b</sup>
<i>B. lecheniformis</i>	57.5 <sup>b</sup> <sup>c</sup>	0.4 <sup>c</sup>
<i>B. cereus</i>	47.7 <sup>c</sup> <sup>d</sup>	16.1 <sup>b</sup>
Fungi		
<i>Trichoderma harzianum</i>	57.7 <sup>b</sup> <sup>c</sup>	12.3 <sup>d</sup>
<i>T. viride</i>	49.8 <sup>c</sup> <sup>d</sup>	1.0 <sup>c</sup>

Values within a column followed by the same letter are not significantly different ( $p= 0.05$ ) level according to Duncan's multiple range test.

**Table 2:** Sorghum disease suppression (%) of *Fusarium oxysporum* root rot and root biomass (g) under greenhouse conditions

Antagonist strain	Disease suppression (%)	Root biomass (g)	
		Fresh weight	Dry weight
Control (without pathogen)	72.6 <sup>abcd</sup>	0.187 <sup>ab</sup>	0.047 <sup>a</sup>
Control (with pathogen)	0.0 <sup>c</sup>	0.012 <sup>g</sup>	0.0012 <sup>h</sup>
Bacteria			
<i>Bacillus subtilis</i>	86.1 <sup>abc</sup>	0.054 <sup>efg</sup>	0.016 <sup>d<sup>c</sup>efg</sup>
<i>B. lecheniformis</i>	58.4 <sup>bcd</sup>	0.085 <sup>d<sup>g</sup></sup>	0.012 <sup>fgh</sup>
<i>B. cereus</i>	77.9 <sup>abcd</sup>	0.176 <sup>abc</sup>	0.027 <sup>bcd</sup>
Fungi			
<i>Trichoderma harzianum</i>	79.12 <sup>abcd</sup>	0.062 <sup>efg</sup>	0.012 <sup>fgh</sup>
<i>T. viride</i>	87.9 <sup>ab</sup>	0.085 <sup>d<sup>g</sup></sup>	0.014 <sup>efgh</sup>

Values within a column followed by the same letter are not significantly different ( $p= 0.05$ ) level according to Duncan's multiple range test.

**Table 3:** Efficacy of the antagonists as biological control agent in sorghum roots 30-day-old after inoculation with both antagonist and fungal pathogen *F. oxysporum*

Antagonist strain	Survival of antagonist in rhizosphere (log cfu/g soil)	Incidence of disease on root (%)
Control	0.0±0.0	100
Bacteria		
<i>Bacillus subtilis</i>	9.49±1.7	55
<i>B. lecheniformis</i>	5.22±0.15	18
<i>B. cereus</i>	8.16±0.94	22
Fungi		
<i>Trichoderma harzianum</i>	9.28±0.91	20
<i>T. viride</i>	9.09±0.92	20

**Table 4:** Effect of biological control of *F. oxysporum* on chlorophyll a and b (mg g<sup>-1</sup> fresh wt) 30-day-old sorghum shoot

Antagonist strain	Chlorophyll content		
	Ch a	Ch b	Ch a/b
Control (without pathogen)	0.95±0.02 <sup>c</sup>	0.43±0.01 <sup>d<sup>c</sup></sup>	2.21±0.08 <sup>a</sup>
Control (with pathogen)	0.77±0.03 <sup>a</sup>	0.36±0.03 <sup>b<sup>c</sup></sup>	2.14±0.09 <sup>a</sup>
Bacteria			
<i>Bacillus subtilis</i>	1.06±0.001 <sup>f</sup>	0.47±0.01 <sup>c</sup>	2.26±0.04 <sup>a</sup>

**Table 4:** Continue

<i>B. lecheniformis</i>	0.87±0.02 <sup>d</sup>	0.4±0.02 <sup>c</sup>	2.18±0.07 <sup>a</sup>
<i>B. cereus</i>	0.81±0.02 <sup>c</sup>	0.38±0.02 <sup>cd</sup>	2.13±0.06 <sup>a</sup>
Fungi			
<i>Trichoderma harzianum</i>	0.94±0.01 <sup>b</sup>	0.44±0.01 <sup>ab</sup>	2.14±0.02 <sup>a</sup>
<i>T. viride</i>	0.84±0.01 <sup>a</sup>	0.39±0.01 <sup>a</sup>	2.15±0.04 <sup>a</sup>

Values within a column followed by the same letter are not significantly different ( $p= 0.05$ ) level according to Duncan's multiple range test.

**Table 5:** Effect of biological control of *F. oxysporum* on sugar contents (mg g<sup>-1</sup> dry wt) 30-day-old sorghum shoot

Antagonist strain	Sugar content		
	Soluble	Insoluble	Total
Control (without pathogen)	15.6±0.5 <sup>d</sup>	20.4±0.43 <sup>d</sup>	36.0±0.19 <sup>c</sup>
Control (with pathogen)	12.4±0.07 <sup>b</sup>	17.4±0.12 <sup>b</sup>	29.8±0.06 <sup>c</sup>
Bacteria			
<i>Bacillus subtilis</i>	16.8±0.28 <sup>c</sup>	21.2±0.34 <sup>d</sup>	38.0±0.08 <sup>f</sup>
<i>B. lecheniformis</i>	14.7±0.31 <sup>d</sup>	19.2±0.13 <sup>c</sup>	33.9±0.21 <sup>c</sup>
<i>B. cereus</i>	13.5±0.17 <sup>c</sup>	18.3±0.35 <sup>c</sup>	31.8±0.18 <sup>a</sup>
Fungi			
<i>Trichoderma harzianum</i>	11.6±0.21 <sup>b</sup>	16.6±0.33 <sup>b</sup>	28.2±0.15 <sup>b</sup>
<i>T. viride</i>	10.6±0.39 <sup>a</sup>	15.4±0.22 <sup>a</sup>	26.0±0.18 <sup>a</sup>

Values within a column followed by the same letter are not significantly different ( $p= 0.05$ ) level according to Duncan's multiple range test.

such as siderophores, hydrogen ions and gaseous products including ethylene, hydrogen cyanide and ammonia<sup>[49,23,34]</sup>. Furthermore, the efficacy of a given biological control agent mostly results, not only from a single mechanism but from a combination of different modes of actions<sup>[1]</sup>.

In most biocontrol investigations, a large number of antagonists are commonly isolated in a short period of time and screened *in vitro* for antagonistic activity. However, tests based on *in vitro* mycelial inhibition and root colonization do not always correlate with biocontrol efficacy under natural conditions<sup>[31,49]</sup>. All promising isolates from the current study were therefore further evaluated under greenhouse conditions. The antagonist isolates which showed significant *in vitro* root colonization also colonized the sorghum rhizosphere effectively in the greenhouse experiment. Isolates *B. cereus* and *B. subtilis* maintained their initial level of population up to 30 days after inoculation. These results concur with those of others researchers<sup>[40]</sup> who reported that a culture based approach such as this can be an important tool to evaluate root colonizing bacterial communities.

However, all the isolates applied at an inoculum level of 100 cfu/ml and that inhibited the pathogen significantly didn't exhibit any negative effect on the sorghum.

The effective colonization of sorghum roots by isolates such as *B. cereus*, *B. subtilis* and *T. harzianum*

might have contributed to their capability to inhibit infection of sorghum roots by *F. oxysporum* and reduce root and crown rot. All four bacterial isolates inhibited *F. oxysporum* both in the dual culture assay and in the greenhouse experiments.

Among the potential biocontrol agents active in the rhizosphere, several members of the Genus *Bacillus* are reported to be effective in controlling a variety of fungal plant diseases<sup>[49,24,11]</sup>. Most of these agents were able to inhibit the mycelial growth of *F. oxysporum* effectively *in vitro*. A diversity of pathogenic *F. oxysporum* isolates including *F. o. ciciris*, *F. o. phasioli*, and *F. o. melonies* have been successfully suppressed by *Bacillus* spp. Isolated from chickpea rhizosphere<sup>[24]</sup>.

A strain of *Bacillus cereus* UW85 has previously been reported to suppress plant diseases caused by Oomycetes<sup>[37,19]</sup>. due to the production of the antibiotics Zwittermicine<sup>[27]</sup> and Kanosamine. Indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of phytopathogens through mechanisms such as antibiosis against the pathogens<sup>[2]</sup>.

*B. subtilis* also occurs in the soil surrounding the root and has often been reported as an antifungal agent against plant pathogens<sup>[26,4]</sup>. Fusarium wilt of chickpea caused by *F. oxysporum* f.sp.*ceciris* has been suppressed by *B. subtilis* isolate GBO3<sup>[20]</sup>. The same *B. subtilis* isolate has also been reported to activate an ISR pathway in Arabidopsis by the production of some

volatiles<sup>[12,8]</sup> demonstrated that certain root colonizing strains of *B. subtilis* play a role as biocontrol agents through induced tolerance of treated seedlings against attack by *F. oxysporum*. In another experiment<sup>[6]</sup> lysis and dissolution of fungal mycelium of *Aspergillus niger* strain have been associated with the chitinolytic property of *Bacillus subtilis* strain AF1.

*In vitro* trials with these isolates have demonstrated that strains of the two new species were able to antagonize a range of pathogenic fungi<sup>[13]</sup>, including *Gaeumannomyces graminis* (Sacc.) von Arx and Olivier var. *tritici* the causative agent of take-all, a serious disease of wheat and barley. These results suggested that these new species might be useful as biological control agents against take-all if *in vitro* antagonism can be matched with disease control in soil.

The significance of antibiotics in biological control, and more generally in microbial antagonism in natural disease-suppressive soils, often has been questioned in the past because of the indirect nature of the supporting evidence and the perceived constraints to antibiotic production in soil environments<sup>[18,32]</sup>. Because of the biotic and abiotic complexity of soils, there are several inherent difficulties in detecting antibiotics produced by microorganisms in soil and rhizosphere environments. Depending on the nature of the metabolite, recovery and detection may be hampered by chemical instability of the compound, irreversible binding to soil colloids or organic matter, or microbial decomposition<sup>[43]</sup>.

In this regard, both *B. circulans*<sup>[48]</sup> and *B. licheniformis*<sup>[44]</sup> produce the enzyme chitinase that degrade chitin. Because of their ability to degrade chitin, the major structural component of the cell walls of phytopathogenic fungi<sup>[39]</sup>, chitinolytic enzymes are considered important in the biological control of soilborne pathogens<sup>[38]</sup>.

Chitinolytic enzymes produced by *C. violaceum* have been shown to be involved in the biological control of *F. oxysporum*<sup>[29]</sup>. A strain of *C. Violaceum* ATCC 12472<sup>[10]</sup> selected from a variety of chitin utilizing bacterial species has previously been reported as the most active chitin degrading isolate. Although *C. violaceum* usually constitute only a small proportion of the total micro-flora in soil, the ability of certain strains to produce antibiotics, hydrogen cyanide, proteases and a number of chitinolytic enzymes<sup>[10]</sup>, indicates that *C. violaceum* have the potential as biological control agents.

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