In vitro SUPPRESSION OF PATHOGENIC FUNGI BY Streptomyces spp.

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ABSTRACT

The use of living organisms or natural enemies of pathogens to control their populations is called biological disease control. It involves harnessing and introduction of exotic species of microorganism in a natural form, with the intention of controlling pathogens that may exist naturally in the same ecosystem. Prospects for biological control of Aspergillus flavus, Fusarium oxysporum and Penicillium italicum were investigated using Streptomyces spp. isolated from Chinhoyi University of Technology Farm soils in Mashonaland West, Zimbabwe. Twenty seven Streptomyces spp were obtained from the soil, and screened for antimicrobial activity and antagonism in in vitro pathogen inhibition assays, replicated thrice. Although majority of the isolates tested elicited no effect on test pathogens, 22% of the Streptomyces isolates were able to effectively suppress A. flavus, F. oxysporum and P. italicum by at least 55%. There was a significant interaction between Streptomyces isolates and pathogen (A. flavus, F. oxysporum and P. italicum) (P<0.05) on fungal radial growth at days 7, 10 and 14 after pathogen-Streptomyces incubation. Antimicrobial potential against individual and multiple test pathogens was observed, with CUT-Streptomyces 4, CUT-Streptomyces 10, CUT-Streptomyces 11, CUT-Streptomyces 20 and CUT-Streptomyces 23 showing the greatest antimicrobial activity. CUT-Streptomyces isolates have the potential to suppress A. flavus, F. oxysporum and P. italicum in vitro.

Key Words: Aspergillus flavus, Fusarium oxysporum, Penicillium italicum

RÉSUMÉ

L’utilisation d’organismes vivants ou d’ennemis naturels d’agents pathogènes pour contrôler leurs populations est appelée contrôle biologique des maladies. Il s’agit d’exploiter et d’introduire des espèces exotiques de microorganismes sous une forme naturelle, dans le but de contrôler les agents pathogènes pouvant exister naturellement dans le même écosystème. Les perspectives de lutte biologique contre Aspergillus flavus, Fusarium oxysporum et Penicillium italicum ont été étudiées à l’aide de Streptomyces spp. isolées des sols agricoles de l’Université de Technologie de Chinhoyi à Mashonaland West, Zimbabwe. Vingt-sept Streptomyces spp ont été obtenus du sol et criblés pour l’activité antimicrobienne et l’antagonisme dans des tests d’inhibition des agents pathogènes in vitro, répliqués trois fois. Bien que la majorité des isolats testés ne provoquent aucun effet sur les
agents pathogènes testés, 22% des isolats de *Streptomyces* sont capables de supprimer efficacement au moins 55% des *A. flavus*, *F. oxysporum* et *P. italicum*. Il y avait une interaction significative entre les isolats de *Streptomyces* et l’agent pathogène (*A. flavus*, *F. oxysporum* et *P. italicum*) (*P* < 0.05) lors de la croissance radiale des champignons aux 7ème, 10ème et 14ème jours après l’incubation de l’agent pathogène-*Streptomyces*. Un potentiel antimicrobien contre des agents pathogènes individuels et multiples a été observé. CUT-*Streptomyces* 4, CUT-*Streptomyces* 10, CUT-*Streptomyces* 11, CUT-*Streptomyces* 20 et CUT-*Streptomyces* 23 ont montré l’activité antimicrobienne la plus élevée. Les isolats de CUT-*Streptomyces* ont le potentiel de supprimer *A. flavus*, *F. oxysporum* et *P. italicum* *in vitro*.

**Mots Clés:** *Aspergillus flavus*, *Fusarium oxysporum*, *Penicillium italicum*

**INTRODUCTION**

Le but de cette étude était de identifier des isolats de *Streptomyces* avec une activité antimicrobienne pour le développement de produits de contrôle biologique environnementalement friendly de plantes.

Les agents pathogènes testés, 22% des isolats de *Streptomyces* sont capables de supprimer efficacement au moins 55% des *A. flavus*, *F. oxysporum* et *P. italicum*. Il y avait une interaction significative entre les isolats de *Streptomyces* et l’agent pathogène (*A. flavus*, *F. oxysporum* et *P. italicum*) (*P* < 0.05) lors de la croissance radiale des champignons aux 7ème, 10ème et 14ème jours après l’incubation de l’agent pathogène-*Streptomyces*. Un potentiel antimicrobien contre des agents pathogènes individuels et multiples a été observé. CUT-*Streptomyces* 4, CUT-*Streptomyces* 10, CUT-*Streptomyces* 11, CUT-*Streptomyces* 20 et CUT-*Streptomyces* 23 ont montré l’activité antimicrobienne la plus élevée. Les isolats de CUT-*Streptomyces* ont le potentiel de supprimer *A. flavus*, *F. oxysporum* et *P. italicum* *in vitro*.

**Mots Clés:** *Aspergillus flavus*, *Fusarium oxysporum*, *Penicillium italicum*
In vitro suppression of pathogenic fungi by *Streptomyces* spp. friendly, biological control disease management strategies.

**METHODOLOGY**

**Soil sampling.** Soil samples were collected from five sites, including a fallow field, an orchard, two anthills, plant residues compost and natural woodland at Chinhoyi University of Technology Farm in Zimbabwe. To collect the samples, the top 4 cm of soil was scrapped off using a hand hoe to remove any plant or crop residues before collecting the soil residues. A sterilised 15 cm depth post hole auger was then used to collect soil. A composite sample was made by physically mixing five sub-samples from an area of 1 m radius at each location, with a clean shovel in a clean plastic bag, and thoroughly homogenised. From the composite sample, five -150 g soil samples were taken and placed in sterile poly bags, sealed tightly, stored at 5 °C in a cooler box with ice packs under, and transported to the laboratory for the isolation of *Streptomyces*.

**Isolation of *Streptomyces* spp. from soil and compost samples.** Soil and crop residue compost samples were dried overnight, under three layers of sterile muslin cloth, at room temperature approximately 23 °C. About 5 g soil or 2 g of compost was added to 50 ml sterile water in an Erlenmeyer flask, and shaken vigorously at 175 rpm for 60 minutes, on a orbital shaker. Serial dilutions of $10^{-1}$ – $10^{-5}$ were each spread on two plates of water agar.

Diluents 50-100 µl were each spread on two plates of water agar. Plates were overlaid with 5 ml of cooled molten starch casein agar (SCA), allowed to solidify and incubated at 28 °C for 5 days. The serial soil or compost wash dilutions were also plated on oatmeal agar (OA), amended with cycloheximide (50 µl ml$^{-1}$) to reduce a dense culture of cells to a more usable concentration, and incubated at 28 °C for 3-5 days. *Streptomyces* were identified through colony morphological traits. Twenty-seven *Streptomyces* isolates were selected randomly from the WA-SCA and OA plate, and purified on oatmeal agar by streaking.

**In vitro inhibition assays.** Individual *Streptomyces* isolates that grew as pure cultures on OA from WA-SCA and OA were streaked onto OA to produce high density cultures. After 10 days of growth, plugs with mycelium were removed from each plate, using a cork borer (7-8 mm). Three mycelium-containing plugs for each isolate were placed equidistant from each other, towards the edge of a fresh potato dextrose agar (PDA) plate; and the plate was sealed with parafilm and incubated for 3 days at 28 °C. Plugs from each *Streptomycete* isolate were transferred onto nine plates of PDA.

Plugs of growing test pathogens (*A. flavus, F. oxysporum* and *P. italicum*) obtained from the Plant Pathology Laboratory, Crop Science Department of the University of Zimbabwe, were each introduced into the centre of each plate so that the inhibition of each pathogen by each *Streptomycete* isolate could be assessed. Each pathogen-*Streptomycete* combination was replicated on three plates. The plates were sealed with parafilm and placed at random in an incubator at 28 °C (Shepherd *et al.*, 2010). Three control plates, each with a different test pathogen, but no *Streptomyces* isolate, were incubated under the same conditions as the pathogen-*Streptomycete* plates.

Inhibitory activities of *Streptomycete* isolates against each of the three pathogens were also determined at days 7, 10 and 14 of pathogen-*Streptomycetes* incubation, using a Veneer Callipers, by measuring the growth of the fungal pathogen alone using the control. Fungal pathogen radial growth data was recorded in mm at days 7, 10 and 14 of pathogen-*Streptomycetes* incubation.
**Statistical data analysis.** Data were subjected to Analysis of Variance using GenStat 14th edition software for windows. Mean comparisons were performed using the Standard Error of the Difference at P< 0.05.

**RESULTS**

**Isolation of Streptomyces spp. strains.** Twenty seven, white, cream, yellow, brown or grey colonies which phenotypically appeared to have a relatively smooth surface, but as growth progressed, developed a weft, granular, powdery or velvety aerial mycelium appearance were obtained in pure culture for *in vitro* inhibition assays.

**In vitro inhibition assays.** There was a significant effect (P<0.05) of *Streptomyces* isolates on the growth of *Aspergillus flavus* during the *A. flavus-Streptomyces* incubation period. *Aspergillus flavus* had a significant effect (P<0.05) on fungal radial growth at days 7, 10 and 14. There were also interactions (P<0.05) between *Streptomyces* isolates and *A. flavus* with fungal radial growth at 7, 10 and 14 incubation days (Table 1).

At day 7, radial growth for *A. flavus* was lowest in combination with *CUT-Streptomyces* 4, 6 mm and *CUT-Streptomyces* 11, 9.67 mm, which translated into radial growth inhibition of 84 and 75%, respectively. These were followed by *CUT-Streptomyces* 10, *CUT-Streptomyces* 17 and *CUT-Streptomyces* 20, where *A. flavus* radial growth was 12.22, 23.44 and 23.33 mm compared to the control with a radial growth of 38.67 mm.

On day 10, *A. flavus* in combination with isolates 4 and 11, had a radial growth of 9.7 mm and 11.1 mm, respectively. This translated in 77.5 and 74.2% radial growth inhibition. These were followed by isolate 10, which inhibited *A. flavus* radial growth by 67%.

Isolate 4 and 10 had the highest *A. flavus* radial growth inhibition of 64% (15.7 mm fungal growth) and 60% (17.22 mm fungal growth), respectively, on day 14. *CUT-Streptomyces* 11 was ranked 3rd with 53 % *A. flavus* radial growth inhibition.

**TABLE 1.** Effect of *Streptomyces spp.* on *A. flavus* radial growth (mm) at various times of *A. flavus*- *Streptomyces* incubation

| Treatments               | Day 7 | Day 10 | Day 14 |
|-------------------------|-------|--------|--------|
| *CUT-Streptomyces* 4    | 6     | 9.67   | 15.67  |
| *CUT-Streptomyces* 10   | 13    | 15     | 18.33  |
| *CUT-Streptomyces* 11   | 9.67  | 15     | 20.33  |
| *CUT-Streptomyces* 19   | 26    | 39     | 40.33  |
| *CUT-Streptomyces* 20   | 23.33 | 41     | 41.33  |
| *CUT-Streptomyces* 21   | 24.67 | 40     | 41     |
| *CUT-Streptomyces* 22   | 25.67 | 39     | 41     |
| *CUT-Streptomyces* 23   | 28.67 | 30.67  | 33     |
| Control                 | 38.67 | 43     | 43     |

P value <.001 <.001 <.001
± s.e.d 4.708 3.911 3.582
CV(%) 10.9 6.4 4.6

Data for some *Streptomyces* isolates not presented as their performance was more or less similar to the control.
For *Fusarium oxysporum*, there was a significant effect (P<0.05) of *Streptomyces* isolates on the growth for the duration of the *F. oxysporum*-*Streptomyces* incubation phase (Table 2). *Fusarium oxysporum* had a significant effect on fungal radial growth at days 7, 10 and 14. Also, there were interactions (P<0.05) between *Streptomyces* isolates and *F. oxysporum* with fungal radial growth being affected at 7, 10 and 14 assay days (Table 2).

On day 7, *CUT-Streptomyces 17* and *CUT-Streptomyces 7*, in combination with *F. oxysporum* had the highest fungal radial growth inhibition of 74% (4 mm Fusarial growth) and, 68% (5 mm Fusarial growth), respectively (Table 2). These were succeeded by *CUT-Streptomyces 20* and *CUT-Streptomyces 26*, which both had *F. oxysporum* radial growth of 5.67 mm in comparison to the control (15.67 mm).

Isolate 26 had the highest *F. oxysporum* radial growth inhibition of 74% (7.33 mm) on day 10 (Table 2). This was followed by *CUT-Streptomyces 20, CUT-Streptomyces 17, CUT-Streptomyces 4 and CUT-Streptomyces 24* with *F. oxysporum* radial growths of 8.7, 9, 10 and 12 mm, correspondingly.

At day 14, isolate 23 had the highest *F. oxysporum* radial growth inhibition of 56% (14.7 mm). This was followed by *CUT-Streptomyces 20, CUT-Streptomyces 4 and CUT-Streptomyces 26* with *F. oxysporum* radial growths of 15.33, 16 and 15.7 mm, respectively.

**TABLE 2. Effect *Streptomyces* spp. on *F. oxysporum* radial growth (mm) at various times of *F. oxysporum*-*Streptomyces* incubation**

| Treatments           | Day 7 | Day 10 | Day 14 |
|----------------------|-------|--------|--------|
| *CUT-Streptomyces 2* | 15    | 17.67  |        |
| *CUT-Streptomyces 4* | 10    | 16     |        |
| *CUT-Streptomyces 6* | 12    | 22     |        |
| *CUT-Streptomyces 7* | 13.33 | 25.67  |        |
| *CUT-Streptomyces 9* | 19    | 26     |        |
| *CUT-Streptomyces 10*| 14.33 | 18.33  |        |
| *CUT-Streptomyces 11*| 13.33 | 19.33  |        |
| *CUT-Streptomyces 17*| 9     | 18.33  |        |
| *CUT-Streptomyces 20*| 8.67  | 15.33  |        |
| *CUT-Streptomyces 21*| 20.33 | 30     |        |
| *CUT-Streptomyces 22*| 16.67 | 31.67  |        |
| *CUT-Streptomyces 23*| 14.67 | 14.67  |        |
| *CUT-Streptomyces 24*| 12    | 17.33  |        |
| *CUT-Streptomyces 25*| 13.67 | 22.33  |        |
| *CUT-Streptomyces 26*| 7.33  | 15.67  |        |
| *CUT-Streptomyces 27*| 20    | 21     |        |
| Control              | 29.67 | 33.33  |        |

**P value** $<.001$ $<.001$ $<.001$

**± s.e.d** 1.628 1.247 1.288

**CV (%)** 10.9 6.4 4.6

Data for some *Streptomyces* isolates not presented as their performance was more or less similar to the control.
There was a significant effect (P<0.05) of *Streptomyces* isolates on growth of *P. italicum* throughout the *P. italicum*-*Streptomyces* incubation stages (Table 3). *Penicillium italicum* had a significant effect (P<0.05) on fungal radial growth also at 7, 10 and 14 in vitro assay days. There were interactions (P<0.05) between *Streptomyces* and *P. italicum* with fungal radial growth being affected at days 7, 10 and 14 (Table 3).

At day 7, radial growth for *P. italicum* was the lowest in combination with isolates 2 and 8. These elicited fungal radial growth inhibition of 65% (3 mm fungal growth). These were trailed by *CUT-Streptomyces* 10, *CUT-Streptomyces* 11 and *CUT-Streptomyces* 22 with *P. italicum* radial growth of 3.33, 3.33 and 3.67 mm, respectively.

On day 14, *CUT-Streptomyces* 10, *CUT-Streptomyces* 23 and *CUT-Streptomyces* 25 had the highest *P. italicum* radial growth inhibition of 51% (8 mm), 45% (9 mm) and 45% (9 mm), respectively, at day 14. Ranked 4th and 5th were *CUT-Streptomyces* 14 and *CUT-Streptomyces* 17 with *P. italicum* radial growth of 9.333 and 9.887 mm, respectively.

Overall, data showed that *CUT-Streptomyces* 4 and *CUT-Streptomyces* 10 were

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**TABLE 3.** Effect *Streptomyces* spp. on *P. italicum* radial growth (mm) at various times of *P. italicum*- *Streptomyces* incubation

| Treatments               | Day 7 | Day 10 | Day 14 |
|--------------------------|-------|--------|--------|
| *CUT-Streptomyces* 2     | 3     | 11.67  | 12.333 |
| *CUT-Streptomyces* 4     | 4     | 6.67   | 13.667 |
| *CUT-Streptomyces* 8     | 3     | 11     | 14.333 |
| *CUT-Streptomyces* 10    | 3.33  | 7.33   | 8      |
| *CUT-Streptomyces* 11    | 3.33  | 8      | 12.333 |
| *CUT-Streptomyces* 12    | 4.33  | 11     | 13     |
| *CUT-Streptomyces* 14    | 5.33  | 6.33   | 9.333  |
| *CUT-Streptomyces* 15    | 4.33  | 11.67  | 11.667 |
| *CUT-Streptomyces* 17    | 8     | 10.33  | 9.887  |
| *CUT-Streptomyces* 18    | 7     | 9      | 13     |
| *CUT-Streptomyces* 19    | 4     | 6.33   | 13     |
| *CUT-Streptomyces* 20    | 4     | 7.67   | 13     |
| *CUT-Streptomyces* 22    | 3.33  | 7.33   | 11.667 |
| *CUT-Streptomyces* 23    | 8.67  | 9      | 9      |
| *CUT-Streptomyces* 25    | 3.67  | 9      | 9      |
| *CUT-Streptomyces* 26    | 7     | 9      | 13     |
| Control                  | 8.67  | 14.33  | 16.333 |

P value: <.001 <.001 <.001
± s.e.d: 0.964 1.411 1.303
CV (%): 10.9 6.4 4.6

Data for some *Streptomyces* isolates not presented as their performance was more or less similar to the control.
the highest performers in inhibiting A. flavus growth (Table 1). CUT-Streptomyces 2, CUT-Streptomyces 20 and CUT-Streptomyces 4 had the highest F. oxysporum growth. On P. italicum growth, CUT-Streptomyces 10, CUT-Streptomyces 23 and CUT-Streptomyces 25 had the highest inhibitory activity against the pathogen. Across all fungal pathogens, CUT-Streptomyces 2, CUT-Streptomyces 4, CUT-Streptomyces 10 and CUT-Streptomyces 23 performed the best. Additionally, Streptomyces had the highest growth reduction on A. flavus as compared to other fungal species tested.

DISCUSSION

Effect of Streptomyces spp. on A. flavus radial growth. CUT-Streptomyces 11 and CUT-Streptomyces 4 showed the highest antagonism to A. flavus at days 7 and 10 (Table 1). These bacterial isolates in particular had a significant reduction in A. flavus growth by more than 74% compared to the control. This suggests that these isolates (11 and 4) are quick producers of secondary metabolites (antibiotics) that control fungal plant pathogens (Golinska and Dahm, 2013). Secondary metabolites production enhances antifungal activity, suppressing pathogenic fungal plant diseases (El-Tarabily and Sivasithamparam, 2006; de Vasconcellos and Cardoso, 2009). However, because some Streptomyces colonies were slow-growing and that the production of secondary metabolites by Streptomyces usually corresponds with the aerial hyphal stage of development (Kieser et al., 2000), CUT-Streptomyces 10 ranked lower initially, but was able to surpass the antimicrobial performance of many other CUT-Streptomyces isolates by day 14.

Effect of Streptomyces spp. on F. oxysporum radial growth. CUT-Streptomyces 7, CUT-Streptomyces 10, CUT-Streptomyces 17, CUT-Streptomyces 20 and CUT-Streptomyces 26 exhibited the highest F. oxysporum antimicrobial activity at days 7 and 10 (Table 2). Van Driesche (1996) highlighted that Streptomyces have the ability to compete with the pathogen for nutrients and space. It was observed that CUT-Streptomyces 17, CUT-Streptomyces 7 and CUT-Streptomyces 10 grew faster than the fungal pathogen, thus depriving the fungus of nutrients and space. Research by Couteaudier and Alabouvette (1990) showed that when actinomycetes and fungal pathogens coexist in the same environment, they compete for minor elements, for instance, iron. In the present study, CUT-Streptomyces isolates deprived F. oxysporum of FeSO₄, MnCl₂ and ZnSO₄, which were amended in assay plates. By depriving F. oxysporum of these micro nutrients, CUT-Streptomyces were able to reduce the growth of the fungal pathogen.

Streptomyces species have the ability to secrete iron-binding ligands, called siderophores, having high affinity to sequester iron from the micro-environment (Pal et al., 2006). This deprives iron from other organisms, hence indirectly causing biological control of such organisms (Boukhalfa and Crumbliss, 2002).

At day 14, CUT-Streptomyces 23, CUT-Streptomyces 26, CUT-Streptomyces 20 and CUT-Streptomyces 4 had the highest F. oxysporum radial growth reduction by more than 50% (Table 2). Our observations show that these Streptomyces isolates are able to surround the fungus and appear to be degrading it.

Mycelium holes, disrupted and irregular growth patterns of F. oxysporum in in vitro pathogen inhibitory assays revealed that Streptomyces degraded the fungus (Table 2), similar to findings in a study by Patil (2010) on Streptomyces toxytricini antagonist activity against Rhizoctonia solani. The present study confirmed that Streptomyces are principal among actinomycetes that produce antifungal compounds and those that exhibit antagonistic potential against pathogens (Patil et al., 2010; Kaur et al., 2013).

Effect of Streptomyces spp. on P. italicum radial growth. Our results have shown that
CUT-Streptomyces and *P. italicum* have a significant interaction (P<0.05) on fungal pathogen radial growth at days 7, 10 and 14 (Table 3). It was observed that different CUT-Streptomyces isolates controlled *P. italicum* across the pathogen-Streptomyces inhibition time series. Observations were also made that *P. italicum* grew much slower; some CUT-Streptomyces isolates would grow quickly and cover assay plates before the fungus would establish itself. This may have been attributed to enzyme-producing *Streptomyces* which have the ability to destroy oospores of phytopathogenic fungi (El-Tarabily, 2006) and affect the spor germination and germ-tube elongation of phytopathogenic fungi (Rothrock and Gottlieb, 1984; Frankowski et al., 2001).

CUT-Streptomyces 2, CUT-Streptomyces 8, CUT-Streptomyces 10 and CUT-Streptomyces 14 were the best performers (Table 3). Earlier studies by El-Tarabily (2006) reported that *Streptomyces* had the capacity to produce extracellular cell wall degrading enzymes, which in turn were associated with biocontrol abilities of the producing bacteria. Such *Streptomyces* protect plants from plant pathogenic diseases, through antagonism mechanism of production of lytic enzymes such as glucanases, proteases, cellulases, and chitinases (Dunne, 1997). Through production of these enzymes, bacteria parasitise disease causing fungi.

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