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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

The use of living organisms or natural enemies of pathogens to control their populations is called biological disease control. It involves harnessing and introducing exotic species of microorganism in a natural form, with the intention of controlling pathogens that may exist naturally in the same ecosystem (Mukerji and Chincholkar, 2007). Mechanisms for controlling the pathogens are dependent on the target organisms. The mechanisms include parasitism, antibiosis, hypovirulence, competition for resources, exclusion from niches, plant-growth promotion and induction of systemic acquired resistance in plants; among others (Gupta *et al.,* 2014). Several microorganisms, including *Agrobacterium* spp., *Aspergillus* spp., *Bacillus* spp., *Gliocladium* spp., *Pseudomonas* spp., *Streptomyces* spp. and *Trichoderma* spp., have been developed into commercial biological control products, against plant diseases (Junaid *et al.,* 2013).

Streptomyces are filamentous Gram-positive bacteria that live in diverse environments like soils, water, plants and composts (Hasani *et al.,* 2014). They are well known for their unique characteristic of producing secondary metabolites, such as antibiotics and extracellular enzymes (Inbar *et al*., 2005). In soil, *Streptomyces* produce more than 60% of clinically useful antibiotics, and are involved in the decomposition of soil organic matter by degrading complex molecules like lignin, lignocellulose, cellulose, xylan and recalcitrant substances (Ding *et al.,* 2004).

Many *in vitro* and *in vivo* studies showed that these antinomycetes can be selected for biological control of plant pathogens and promotion of plant growth (Doumbou *et al*., 2001). These microorganisms possess antagonistic activity against pathogens, a result of their capacity to produce enzymes that have antimicrobial activity, siderophores and antibiotics, among others. Some *Streptomyces* spp. promote plant growth, through solubilisation of useful phosphates and competion with plant pathogens for nutrients (Cattelan and Hartel, 2000). Several antibiotic producing *Streptomyces* spp. are used to control various diseases. Some of the diseases include, potato scab caused by *Streptomyces scabies* (Liu *et al.,* 1995) and post-harvest diseases such as onion bacterial rot disease (Abdallah *et al.,* 2013); banana anthracnose caused by *Colletotrichum musae* (Ara *et al*., 2012); and *Penicillium digitatum* and *Geotrichum candidum* on lemons (Maldonado, 2010). There are several commercial *Streptomyces*-based biocontrol products to date, which include Actinovate® (Natural Industries, Inc) and Mycostop® (Verdara Oy, Finland). Furthermore, antibiotics from *Streptomyces* spp. that have been commercialised into fungicides and bactericides include kasugamycin, validamycin and polyoxin B and D (Doumbou *et al*., 2001). The objective of this study was to identify *Streptomyces* isolates with antimicrobial activity for the development of environmentally-

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-*Streptomyces* incubation, using a Veneer Callipers, by measuring the growth of the pathogen towards the *Streptomycete* isolate; and further comparing them to 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-*Streptomyces* 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	11	15	17.67
CUT-Streptomyces 4	7.33	10	16
CUT-Streptomyces 6	10.67	12	22
CUT-Streptomyces 7	5	13.33	25.67
CUT-Streptomyces 9	12	19	26
CUT-Streptomyces 10	4.67	14.33	18.33
CUT-Streptomyces 11	7.33	13.33	19.33
CUT-Streptomyces 17	4	9	18.33
CUT-Streptomyces 20	5.67	8.67	15.33
CUT-Streptomyces 21	13.33	20.33	30
CUT-Streptomyces 22	9.33	16.67	31.67
CUT-Streptomyces 23	11.67	14.67	14.67
CUT-Streptomyces 24	10.33	12	17.33
CUT-Streptomyces 25	10	13.67	22.33
CUT-Streptomyces 26	5.67	7.33	15.67
CUT-Streptomyces 27	11.33	20	21
Control	15.67	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.

CUT-Streptomyces 14 and *CUT-Streptomyces* 19 had the highest reduction in fungal pathogen growth of 56% (6.33 mm *Penicillium* growth) at day 10 (Table 3). These were followed on by *CUT-Streptomyces* 4, *CUT-Streptomyces* 22 and *CUT-Streptomyces* 20 with *P. italicum* radial growth of 6.7, 7.3 and 7.7 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

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 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 hyphae 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 $FeS0_4$, $MnCl_2$ and $ZnSO_4$, 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 spore 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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