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In vitro and *in vivo* tests of *Bacillus licheniformis* MGrP1 antibiotics culture filtrate as a potential biocontrol agent against bean anthracnose

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Abstract

Intensified agricultural production coupled with the heavy use of chemicals for crop protection has been practiced for a while as the only way through which food and nutrition security can be achieved for human welfare and economic growth. The excessive use of these broad-spectrum chemicals has resulted in a variety of harmful effects to the environment. Biological control involving the introduction of specific organisms that are natural antagonists or their metabolites to reduce disease, such as members of the genus *Bacillus*, represents the best alternative to disease control using chemicals.

Bacillus licheniformis strain MGrP1 produced antibiotics in liquid media containing soyabean meal and mannitol that Inhibited the growth of the plant pathogens of agricultural importance, namely: *Colletotrichum lindemuthianum* (Bean anthracnose), *C. kahawae* (Coffee berry disease), *Fusarium oxysporum* f.sp. *phaseoli* (Fusarium yellow) and *Alternaria solani* (Early blight). Paper chromatography combined with bioautography revealed two thermostable active compounds whose activity was optimal at pH 6. *In vitro* tests clearly displayed the antibiotics culture filtrate's ability to inhibit the growth of the phytopathogens on artificial medium. This was attested to *in vivo* when it significantly delayed and suppressed ($p \leq 0.05$) the development of bean anthracnose caused by *C. lindemuthianum* on bean leaves.

Key words: *Bacillus licheniformis*, antagonism, antibiotics culture filtrate, *Colletotrichum lindemuthianum*, bean anthracnose.

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Introduction

Biological control of plant pathogens involving introduction of specific organisms that are natural antagonists or their metabolites to reduce disease is of paramount importance, since the conventional use of chemical pesticides has been seriously questioned because of environmental and human health hazards (Burkhead *et al.,* 1995 Sharga, 1997). The suitability of this method is pegged on the fact that the biologically active secondary metabolites, which are mostly antibiotics, possess high specific activity, are target specific, are generally biodegradable and therefore hygienically safe to use.

Several members of the genus *Bacillus*, including *Brevibacillus* sp. and *Paenibacillus* sp. that produce various antimicrobial substances such as antibiotics have extensively been tested as potential biological control agents (BCA) (Edwards *et al.,* 1994; Giuliano *et al.,* 2002). *B. subtilis*, which produces more than 70 different antibiotics, is one of the major producers of these substances in the genus. *Brevibacillus brevis* (formerly known as *B. brevis*; Shido *et al.,* 1996) also produces antibiotics. In addition, a wide range of antimicrobial substances are also produced by *B. licheniformis, B. pumilus, B. circulans, B. cereus, Brevibacillus laterosporus*, *Paenibacillus polymyxa* (formally *B. polymyxa*; Ash *et al.,* 1994) and other species (Katz and Demain, 1977; Shoji, 1978; Smirnov *et al.,* 1986).

Whereas the dry bean is considered to form an integral part of the diet of the majority of people in developing countries, bean anthracnose, caused by *Colletotrichum lindemuthianum* poses a major set-back to its production and sales world-wide (Dillard, 1988). Production is reduced due to poor seed germination, poor seedling vigour and low yields. Marketing losses are attributed to seed spots and blemishes, which lower their quality rating and salability. Under favourable environmental conditions for disease development, losses of up to 100% could occur. Boyd (1942) reported complete destruction of a whole plot of beans with heavy losses occurring when a prolonged wet period provided ideal conditions for infection and disease spread.

Locally, control of bean anthracnose has been achieved through the use of fungicides such as benlate and dithio-carbamates. The toxic side effects caused by the use of these fungicides and the mounting pressure from stakeholders to reduce their use and revert to environmentally friendly methods of disease control, forms the rationale of carrying out this study. The study will undertake to develop biological control products for the control of bean anthracnose using antibiotics produced by *Bacillus* species.

Microbial antagonism has been established in an earlier study by Makumba *et al* (2008) (not published) where *B licheniformis* strain MGrP1, was statistically singled out as the best performer out of the initial 137 strains. The present study aimed to achieve the following objectives:

1) To produce an antibiotics culture filtrate of *B. licheniformis* strain MGrP1 by the fermentation process,
2) To test the culture filtrate produced for antimicrobial activity against selected phytopathogenic microorganisms,
3) To evaluate the efficacy of the culture filtrate in controlling bean anthracnose caused by *Colletotrichum lindemuthianum* on potted bean plants under greenhouse conditions.

Materials and Methods Isolates

The antagonistic bacterium *B licheniformis* strain MGrP1, was isolated and identified by Kiiru, (2002) and kindly provided by the

department of Botany, University of Nairobi. Plant pathogenic microorganisms used as antibiotic targets were: *C. lindemuthianum* (Sacc. and Magn.) Briosis and Cavara; isolated from a sporulating lesion on diseased bean pods, *Fusarium oxysporum* Schlecht. f.sp. *phaseoli* (Kendrick and Snyder); isolated from diseased bean stems, *C. kahawae* Waller and Bridge (Synon *C. coffeanum* Noack) and *Alternaria solani* kindly provided by The Department of Crop Protection, University of Nairobi.

Plant materials (Seeds)

Certified beans cv. Rosecoco-GLP-2 seeds were bought from the Kenya Seed Company.

Production of antibiotics in liquid media

Antibiotic production from the antagonistic *B. licheniformis* strain MGrP1 isolate was carried out in shaken modified Tschen's liquid medium consisting of: mannitol, 15 g; glycerol, 15 ml; soyabean meal, 15 g; ammonium sulphate $(NH_4)_2SO_4$, 5 g; yeast extract, 1 g; sodium chloride NaCl, 5 g; calcium carbonate $CaCO_3$, 5 g; distilled water 1000 ml; and pH 7.5.

A primary culture of 2.5×10^9 colony forming units (CFU) of the antagonistic *Bacillus* strain was inoculated in 100 ml of sterile Tschen's broth in 500 ml conical flasks in triplicates and incubated at 25°C on a rotary mechanical shaker at a speed of 120 revolutions per minute (r.p.m). Aliquots were taken after 36 h and thereafter daily until the 10^{th} day. Fermentation broths were then centrifuged at 7000 r.p.m for 15 minutes, supernatant decanted into sterile reagent bottles and tightly capped then stored at 4°C for later use (Wahome, 1998).

Plate assays of *B. licheniformis* strain MGrP1 culture filtrate for antibiotic activity

Sterile potato dextrose agar (PDA) was cooled to 45°C in a water bath and seeded with adjusted spore suspensions (10^6 spores ml^{-1}) of the phytopathogenic microorganisms (Loo *et al.*, 1945). Using sterile pipettes, 10 ml of the seeded media were carefully dispensed into sterile 9cm diameter Petri dishes and kept on a flat surface inside a lamina flow hood to maintain uniform medium depth.

Three autoclaved 11mm diameter Whatman No.1 filter paper discs were soaked in the *B. licheniformis* strain MGrP1 culture filtrate obtained at different incubation periods and then placed about 1cm from the periphery of the plate. The plates were incubated at room temperature for 72 h, after which the diameter of the clear zone around the filter paper discs measured using outside vannier calipers. The experiment was laid in a completely randomized design and replicated 3 times. Similar filter paper discs were soaked in sterile distilled water (SDW) and used for the control treatment.

Determination of the number of antibiotics in culture filtrates by paper chromatography

Whatman No. 1 chromatography paper strips (5 mm wide) were autoclaved and 5 µl of the *B. licheniformis* strain MGrP1 antibiotics culture filtrate harvested after the 7^{th} day of incubation applied 3cm from the lower edge of the chromatography paper strips and left to dry (Betina, 1973).

Solvent systems tested for elution of the antibiotic culture filtrates were; 1-butano1/acetic acid/water (4:3:3 v/v) and chloroform/methanol/water (65:25:4 v/v) (Wahome, 1998). Each solvent system (50ml) was placed in chromatography tanks and lined with filter papers to ensure saturation. The upper ends of the chromatography paper strips were attached onto the inner surface of the tank lids using

adhesive tape. The paper strips were immersed in each solvent to a depth of 1cm from the lower end. Ascending development of chromatograms was allowed for 30 minutes after which the chromatograms were removed, solvent front marked and then air-dried (Wahome, 1998).

Bioautography was done using *C. lindemuthianum* as the test phytopathogen and was carried out in 19.5 cm diameter Petri dishes. Thirty ml of sterilized PDA were poured into three baked petri dishes placed on a flat surface to make a uniform basal layer. After setting, 20 ml of PDA seeded with *C. lindemuthianum* was evenly spread on the unseeded layer and allowed to set before placing the developed chromatogram strips centrally over the seeded medium surface. The set ups were then incubated at 25^{o}C for not less than 72 h after which the position of the inhibition shown by clear zones on the medium were determined (Thornberry, 1950).

Partial purification of the antibiotics in culture filtrate

The fermentation broth from the antagonistic *B. licheniformis* strain MGrP1 harvested after the 7th day of incubation was mixed with n-butanol solvent at a ratio of 3:1 v/v (broth to solvent) then shaken thoroughly before putting in a separating funnel and left for 30 minutes for the two layers to separate. The broth was then run off into a beaker and the n-butanol layer containing the extracted antibiotics was put in a separate beaker. Using the same broth but with fresh n-butanol, the extraction process was repeated two more times to ensure that most of the antibiotics in the broth was extracted. The antibiotics extracted were then concentrated using the charcoal absorption process described by Loeffler *et al* (1986).

Dry finely ground charcoal was activated by heating at 200^{o}C for 1 hour in the oven and then cooled to room temperature (22 ± 2^{o}C). The extracted suspension, shown to contain antibiotics was mixed with 5% (w/v) of the powdered charcoal and shaken for 30 minutes on a mechanical shaker to allow for adsorption of the antibiotics into the charcoal particles. The mixture was then filtered through sterile Whatman No. 1 filter paper. The antibiotics containing charcoal left in the funnel was eluted with 80 ml solution of 80% acetone in water. The antibiotic acetonic filtrate was then concentrated invacuo using a rotary vaccum evaporator at 80^{o}C to about 20 ml. These partially purified antibiotics were then used to determine the minimum inhibitory concentration (MIC).

Determination of the minimum inhibitory concentration (M.I.C.) of the partially purified antibiotics

Sterile PDA at 45^{o}C was seeded with a spore suspension of *C. lindemuthianum*. Ten ml were poured into petri dishes and allowed to set. Serial dilutions using a base of 3 ml of the crude antibiotics culture filtrate were prepared (3^{-1}, 3^{-2}, 3^{-3}, 3^{-4}, 3^{-5}, 3^{-6}, 3^{-7}, 3^{-8}, $3^{-9}$and 3^{-10}). Starting with the highest dilution, 0.5ml of each dilution was pipetted and flooded on separate plates containing seeded medium with each dilution being replicated 3 times. The solutions were evenly spread using a flamed glass rod over the plates. The plates were then incubated at 25^{o}C for 72 h during which observations were made for the presence of growth of *C. lindemuthianum.*

Physical characterization of *B. licheniformis* strain MGrP1 antibiotics in culture filtrate

a) Stability at different pH levels

Five ml of the 7th day culture filtrate from the antagonistic *B. licheniformis* strain

MGrP1 were placed in sterile universal bottles and adjusted to pH levels: 1, 3, 5, 7,
9 and 11 using 0.1 N NaOH and 0.1 N HCI. The samples were then held at room temperature for 4 h then made to equal volumes using sterilised water. The different samples were then assayed for antibiotic activity against *C. lindemuthianum* using 3 sterile paper discs per plate. Triplicate plates were prepared for each treatment. The plates were incubated for 72 h for the inhibition zones to develop. These were measured as in the earlier experiments and compared to controls that had unadjusted pH levels.

b) Thermostability

Five ml of the 7th day crude antibiotics culture filtrate were placed in sterile universal bottles and held in a water bath at different temperatures as follows: + 4^{o}C, 20^{o}C, 50^{o}C, 80^{o}C, 100^{o}C, and 121^{o}C
(autoclaving) for 15 minutes each. The activity of the tested samples was determined by the size of the inhibition zones produced by paper discs on media seeded with *C. lindemuthianum*. Duplicate plates were prepared for each treatment that had 3 discs.

c) Shelflife of antibiotic culture filtrates over a period of time at different storage conditions

The 7th day active antibiotics culture filtrate (10 ml) was placed in sterile universal bottles, and properly closed with a screw cap to prevent contamination during storage. Half of the samples were placed at room temperature while the other in a refrigerator at +4^{o}C. The activity of the stored antibiotics against *C. lindemuthianum* was determined every 10 days for a period of three months. The size of the inhibition zones was recorded over the whole period of the assay.

Greenhouse/Pot plant studies

a) Potting Medium and Materials

One-litre pots were used in this experiment. A homogenized soil mixture composed of forest soil, manure, sand and ballast in the ratio of 2:1:1:1 respectively was prepared, sterilized for 2 h, allowed to stand for 2 days to release toxic gases such as ammonia that are capable of inhibiting germination of seeds, then ¾-filled into the pots (Isanda, 1994). Seven certified bean seeds of susceptible variety Rosecoco-GLP 2 were sown in each pot. Brigade**™**, an insecticide/miticide was sprinkled on the surface of each pot to control insect pests. After seed germination, the seedlings were thinned to leave 5 seedlings per pot. The plants were kept in the greenhouse and watered regularly.

b) Preparation of *C. lindemuthianum* culture medium, inoculum preparation and inoculation

Since *C. lindemuthianum* has been found to sporulate poorly in most artificial media (Muther *et al.,* 1950; Ramanowski *et al.,* 1962), enriched PDA media composed of bean pod extracts was prepared. Two hundred grams of green pods of cv. Rosecoco-GLP-2 were grounded in a warring blender to a homogenous broth. The homogenate was then filtered through a cheese cloth. The filtrate was diluted to 240 ml and incorporated in 39 g of PDA, made up to 1 liter and autoclaved. The media obtained was used for culturing the fungus. A spore suspension with a concentration of 10^{-6} spores ml^{-1} was prepared from 14 day old cultures. The adjusted inoculum was applied in the evening on both surfaces of all the first trifoliate leaves (about 2cm wide) and primary leaves present on the plants using a half litre atomizer held at a distance of 10-15 cm away until run-off. In each of
the bean pots, 2 trifoliate leaves were tagged

for scoring. To maintain high humidity, inoculated plants were covered with transparent polythene bags for 48 h then removed and observations for characteristic anthracnose symptoms on leaves made. An equal number of control plants were treated like the experimental ones, but sprayed with SDW.

c) Determination of the effectiveness of antibiotics culture filtrate in controlling bean anthracnose caused by *C. lindemuthianum* (Sacc. and Magn) Broisis and Cav

This was determined in the greenhouse by treating inoculated beans with active antibiotics culture filtrate. A conventional systemic fungicide Benlate 50% WP was used as a standard. Infected plants were placed on a greenhouse bench for yet another 48 h before treating them with various concentrations of the antibiotics i.e.- original culture filtrate, double concentrated culture filtrate and half concentration culture filtrate. Two control treatments and a non treated one were also set up. Experiments were arranged in the greenhouse in a completely randomized design layout with each treatment bearing six potted plants. Observations were made every week for 5 weeks (Wahome, 1998).

d) Data Collection

The tagged trifoliate leaves were assessed for anthracnose infection. The same plants were assessed for anthracnose severity for the entire growth period. The anthracnose severity on leaves was assessed using a modified CIAT scale (Schoonhoven and Corracks, 1987) and was based on the percent of leaf area infected. Scoring was done on weekly basis for 5 weeks. Phytotoxic effects of the culture filtrate were assessed on the basis of any morphological and colour changes on the test plants.

e) Insect pests and weed control

Red spider mites, aphid damage and white fly damage were controlled by the use of insecticide/miticide Brigade^{TM} 025EC. The insecticide/miticide was applied at a rate of 25ml/10liters. Pots were kept weed free by regular hand weeding.

Data Analysis

The experiment in the optimization of antimicrobial substances will be conducted in a completely randomized design (CRD). The experiments in the inhibitory concentration of antimicrobial substances against pathogenic fungi will also be conducted in a CRD. Greenhouse trials will be conducted in a completely randomized block design (CRBD). Recorded data will be analyzed with SAS® Software (SAS Institute, 2003). Treatment effect will be tested by Analysis of variance (ANOVA) and the means compared using the least significant difference (LSD) test and Duncan's Multiple Range Test (DMRT) at a 5% probability level (Gomez & Gomez, 1984).

Results

Assaying *B. licheniformis* strain MGrP1 culture filtrate for antibiotic activity

The primary culture of 2.5E+9 cfu/ml of the antagonistic bacterium—as determined by the plate dilution method—and inoculated in 100 ml of the modified Tshen's medium exhibited sustained growth for 7 days. The antibiotics culture filtrate obtained through centrifugation showed antibiotics activity owing to the production of inhibition zones on media seeded with the plant pathogens. Filter paper discs dipped in active culture filtrate gave clear zones of inhibition for all the test pathogens. Filter paper discs dipped in distilled water produced no clear zones. The clear zones were measured in mm after 72 h of incubation at room temperature.

Statistical analysis indicated that the zones of inhibition were significantly different

Table 1:

Inhibition zone diameter (mm) produced by active antibiotics culture filtrate of *B. licheniformis* strain MGrP1 after 7 days of incubation tested against the four fungal phytopathogens.

	Inhibition diameter (mm)			
Disc replicate	*C.lind*	*C.kaha*	*A. sol*	*F. oxy*
1	20	25.4	22.7	17.5
2	19.5	25.6	22	15
3	20	26	23.7	15.5
4	21	26.5	22.8	17.5
5	21.5	25.4	23.5	17.5
6	21.5	27	22.9	14.5
7	21	24.5	22.9	19.5
8	22	26	24.1	17.5
9	22	22.3	22.1	16
Means	20.94^{a}	25.4^{b}	22.97^{c}	16.72^{d}

Means followed by different letters are significantly different at $p \leq 0.01$

Key:

C. lind— Colletotrichum lindemuthianum;

C. kaha— Colletotrichum kahawae;

A. sol— Alternaria solani;

F. oxy— Fusarium oxysporum f.sp. *phaseoli*

The zones were clear with distinct boundaries and persisted for more than 7 days (Plates 1 and 2).

Plates:

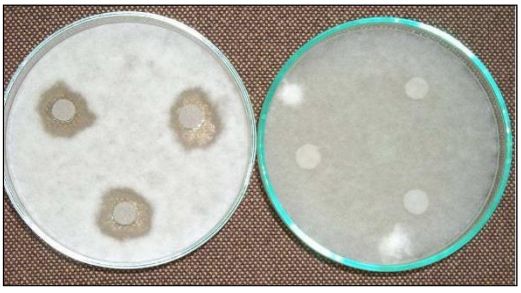

Plate 1

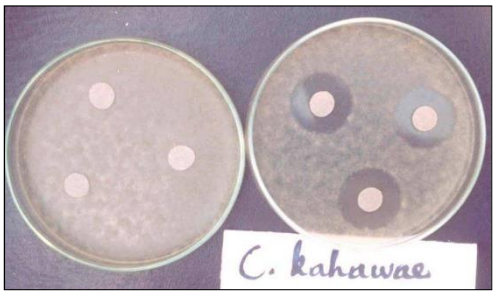

Plate 2

Plate 1: Zones of inhibition brought about by culture filtrate of *B. licheniformis* strain MGrP1 against *C. lindemuthianum*. Control on the right

Plate 2: Zones of inhibition brought about by culture filtrate of *B. licheniformis* strain MGrP1 against *C. kahawae*. Control on the left

The antibiotic activity of the culture filtrate obtained increased with increase in incubation time (Table 2).

Table 2:

Diameters (mm) of growth inhibition zones produced by antibiotic culture filtrate of *B. licheniformis* strain MGrP1 harvested at different incubation times and tested against *C. lindemuthianum*.

Disc replicate	**36hrs**	Growth inhibition diameter (mm) produced when harvested at day: **3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	11.6	13.6	17.1	19.2	22.1	20.1	19.6	20
2	0	11.9	14	17.3	19.1	20.9	20	19.9	19.9
3	0	12.4	14.2	17.2	19.1	20.7	20	20.2	19.8
4	0	11.8	13.1	18.1	19	21.1	19.7	21	20.1
5	0	11.7	12.9	18.6	18.9	21.3	19.5	21.2	20.5
6	0	11.9	12.8	18.4	18.9	21.4	19.6	20.9	20.3
Means	0^{h}	11.88^{g}	13.43^{f}	17.78^{e}	19.03^{d}	21.25^{a}	19.81^{c}	20.46^{b}	20.1^{c}

Means followed by different letters are significantly different at $p \leq 0.01$

The increase was steady upto the 7th day. Thereafter, fluctuations in inhibition zone values were noted. In the first two days of incubation, no visible zones of inhibition were seen. The 3rd to the 5th days produced zones that were faint and had diffuse boundaries compared to clear zones with distinct boundaries from culture filtrates harvested from the 6th day onwards upto the 10th day. Inhibition zones produced by culture filtrates harvested at day 3 to 6 were statistically different at $p \leq 0.01$ level from those harvested after day 7 to 10. These results indicate that the optimal incubation period for maximum production of antibiotics from *B. licheniformis* strain MGrP1 is 7 days.

Determination of the number of antibiotics in the Bacillus licheniformis strain MGrP1 culture filtrates by use of paper chromatography.

Antibiotics contained in the culture filtrate from *B. licheniformis* strain MGrP1 was only shown to be eluted by the solvent system containing a mixture of n-butanol, acetic acid and water. The elution of antibiotics on chromatography paper strips was evidenced by the formation of clear zones of inhibition at a distance from the original spot when using the bioautographic detection method. Two zones of inhibition after bioautographic detection were noted. This would imply that the culture filtrate of *B. licheniformis* strain MGrP1 contains two antibiotics that are active against *C. lindemuthianum*.

Plate 3

Plate 3: Zones of inhibition against *C. lindemuthianum* brought about by eluted antibiotics on a 19.5cm diameter petri-dish.

[The origin is indicated as the upper horizontal line. The lower line is 1 cm from the lower edge—the portion that was immersed in the solvent system].

The plate above clearly shows that the crude culture filtrate had two antibiotics eluted. At the origin, a large zone of inhibition is exhibited to indicate that the antibiotic eluted at this point whose molecular weight is high, has a strong activity of inhibiting the growth of *C. lindemuthianum*. Further up the chromatogram strip, is a small zone of inhibition. This was brought about by the activity of the second antibiotic whose activity against the growth *C. lindemuthianum* is weak and whose molecular weight is low.

Partial purification of antibiotics culture filtrate from *B. licheniformis* strain MGrP1

There was a noticeable increase in the activity of the purified antibiotics compared to the crude one. This was reflected by the increase in sizes of the inhibition zones produced which were also noted to persist for a longer period than those produced by the crude culture filtrate. They persisted for more than 10 days before the pathogen overcame them. Zones of inhibition produced by partially purified antibiotic culture filtrate had a mean diameter of 24.63 mm which was 3.71 mm (17.73%) greater compared to those produced by the crude antibiotic culture filtrate (Table 3).

Table 3:

Diameters (mm) of growth inhibition zones produced by crude and partially purified antibiotic culture filtrate of *B. licheniformis* strain MGrP1 harvested and tested against *C. lindemuthianum*.

	Growth inhibition diameter (mm) of Disc replicate:						
	1	**2**	**3**	**4**	**5**	**6**	**Means**
Partially purified	24.3	26.1	24	23.7	24.1	25.6	**24.63**
Crude	20.5	21.4	21.7	20.9	20.4	20.6	**20.92**

Determination of the Minimum Inhibitory Concentration (MIC) of *B.*

***licheniformis* strain MGrP1 antibiotics**

Plates flooded with a dilution of 3^{-4} (1:81) and lower did not show any growth of *C. lindemuthianum* while plates flooded with a dilution of 3^{-5} (1:243) and higher had fungal growth. Thus the minimum inhibitory concentration of antibiotics produced by *B. licheniformis* strain MGrP1 falls between $3^{-4}$and 3^{-5}.

Physical characterization of antibiotics in culture filtrate of *B. licheniformis* strain MGrP1

The antibiotics culture filtrate exhibited a very wide pH range only being most active at a pH of around 6 (Table 4).

Table 4:

Inhibition zone diameters (mm) produced by active culture filtrate of *B. licheniformis* strain MGrP1 adjusted to different pH levels and tested against *C. lindemuthianum*.

		Inhibition diameter (mm) at various pH values:						
Disc replicate	**Control**	**1**	**3**	**5**	**6**	**7**	**9**	**11**
1	21	0	16.4	17.5	23.4	18.9	14.6	13.2
2	20.6	0	16.5	16.9	21.3	18	12.6	13.6
3	17.8	0	17.1	17.2	20.6	19.3	13.9	13.4
4	19.2	0	16.9	18	21	17.8	15.6	12.4
5	19	0	17.2	18.1	19.6	20	12.5	12.6
6	18.5	0	16.6	17.5	20	18.2	13.1	12.5
7	16.6	0	17.3	16.9	19.9	17.5	13.6	13.7
8	17.9	0	17	19	21	20	13.9	13.5
9	20.1	0	17.3	18	19.9	19.3	13.6	13.6
Means	18.97^{b}	0^{e}	16.92^{c}	17.68^{c}	20.74^{a}	18.78^{b}	13.71^{d}	13.17^{d}

Means followed by different letters are significantly different at $p \leq 0.01$

Different thermal treatments did not affect the antibiotics activity of the crude supernatant, inhibition zones not being significantly different ($p \leq 0.01$) for aliquots subjected to different treatments and also for untreated aliquots, used as control (Table 5).

Table 5:

Inhibition zone diameters (mm) produced by active antibiotics culture filtrate of *B. licheniformis* strain MGrP1 subjected to different temperature levels and tested against *C. lindemuthianum*.

		Inhibition diameter (mm) at temperature level:					
Disc replicate	**Control**	**4ºC**	**20ºC**	**50ºC**	**80ºC**	**100ºC**	**121ºC**
1	20.8	19.6	20.5	20.1	19.8	20	0
2	21	20.1	21.1	20.3	19.7	20.1	0
3	19.8	19.8	20.5	21	20.1	20.3	0
4	20.1	19.4	19.7	19.9	18.7	19.9	0
5	18.9	20.3	18.9	20.1	20.2	20	0
6	19.3	20.4	20.2	19	19.9	20.3	0
Means	19.98^{a}	19.93^{a}	20.15^{a}	20.07^{a}	19.73^{a}	20.1^{a}	0^{b}

Means followed by different letters are significantly different at $p \leq 0.01$

The antibiotics activity of culture filtrate remained stable after storage at refrigeration (4^oC) and at room temperature (22 ± 2^oC) against *C. lindemuthianum* for a period of 3 months. Inhibition zone diameters obtained over the storage period remained constant with means of 20.28 mm and 20.00 mm respectively.

Determination of the effectiveness of antibiotic culture filtrates in controlling bean anthracnose caused by *C. lindemuthianum* (Sacc. and Magn) Broisis and Cav on potted bean plants under glasshouse conditions

Bean anthracnose was considerably controlled with the antibiotics culture filtrate. There was a delayed manifestation of the disease symptoms on the treated plants compared with the +ve control ones. Delayed disease development was also observed on plants sprayed with a fungicide Benlate® 50WP.

Suppression of bean anthracnose development on treated bean leaves remained consistent in the first two weeks for the original concentration culture filtrate, double concentrated culture filtrate and the chemical control. This was demonstrated in the disease severity scores recorded over this period that were not significantly different ($p \leq 0.05$). The double concentrated culture filtrate compared favourably with the chemical control in suppressing anthracnose symptoms through to the fourth week. Their disease severity scores did not differ ($p \leq 0.05$) as well. It is in the last week of assessment that a significant difference was noted between the two treatments. It is possible that the active antibiotics had started to undergo biodegradation and it would have been then appropriate to do a second application (Table 6

Table 6:

Effectiveness of the antibiotics culture filtrate of *B. licheniformis* strain MGrP1 at different concentrations in controlling *C. lindemuthianum in vivo* compared to Benlate 50WP.

	Mean disease severity scores for treatments:					
Week	Original culture filtrate	Half Conc.	Double Conc.	Benlate 50WP	+ve Control	-ve Control
1	0.58^a	0.75^a	0.58^a	0.42^a	1.17^b	0.92^c
2	1.08^d	1.50^e	0.92^d	0.67^d	2.33^f	1.58^g
3	1.67^h	2.08^i	1.33^j	1.00^j	3.67^k	2.00^i
4	2.08^l	2.42^m	1.58^n	1.17^n	5.17^o	3.25^p
5	2.17^q	2.42^r	1.75^q	1.17^s	6.17^t	4.67^u

Means followed by different letters in the same week are significantly different at $p \leq 0.05$

Discussion and Conclusion

The use of microorganisms for biological purposes has become an effective alternative to control plant pathogens. There are many examples of formulations using bacterial or fungal strains with biocontrol applications. Among them, members of the genus *Bacillus* are well known antibiotic producers

(Giuliano *et al.,* 2002).

Production of antibiotic substances usually contains a source of nitrogen, several salts and certain supplementary materials such as yeast extract (Campbell, 1989). The source of carbon is usually carbohydrates added in the form of glucose, mannitol, lactose, fructose, sucrose, starch etc. In this study, mannitol was used as the carbon source. Mannitol has been reported to increase the synthesis of Iturin antibiotics as opposed to glucose in *B. subtilis* (Besson *et al.,* 1978). $CaCO_3$ was added to provide Ca^{2+} that increase cell wall permeability of *B. licheniformis* strain MGrP1 to excrete the antibiotics (Petit-Glatron *et al.,* 1993).

Mechanical shaker speed, agitation and subculturing have been reported to affect antibiotic production in liquid media. Hanson *et al.,* (1965) while working with *Streptomyces rimosus*, reported that the widest inhibition zones resulted from the use of 7 to 10 day old culture. Loeffler *et al.,* (1986) produced fengimycin from *B. subtilis* strain F-29-3 on shake culture at 120 rpm and harvested after 7 days.

This study noted that inhibition zones produced by the antibiotics culture filtrate obtained after 7 to 10 days of shaking against *C. lindemuthianum* were greater than other incubation periods. Zones of inhibition were only observed from the 3rd day of shaking. Reports show that the synthesis of peptide antibiotics in liquid medium usually starts at the end of exponential growth; reaching maximum concentration after cell growth has ceased (Bodanzky and Perlman, 1969). It has been suggested that many microorganisms can synthesize antibiotics while in the growth phase (Haavik and Thomassen, 1973; Barr, 1975 and Haavik, 1976), which is in agreement with production kinetics in *B. licheniformis* strain MGrP1. The factor triggering the onset of antibiotic synthesis is more likely the exhaustion of a limiting nutrient required for cell growth. This limitation usually stimulates differentiation, which, for the case of Bacilli, means endospore formation. Sporulation is associated with the synthesis of a new cell wall and the degradation of that in the mother cell. This cell wall synthesis could furnish precursors for antibiotic synthesis (Giuliano *et al.,* 2002).

Antibiotics produced by *B. licheniformis* strain MGrP1 responded differently to the different physical conditions it was subjected to. Different sizes of clear zones of inhibition induced by the culture filtrate subjected to different temperature conditions, pH, storage conditions and fermentation incubation durations attest to the different responses. This showed that the antibiotic varied in stability, an observation also reported by Cladera-Olivera *et al.,* (2004) who noted that a bacteriocin-like substance produced by *B. licheniformis* strain P40 responded differently to different temperature conditions. The substance remained stable up to a temperature of 100°C but lost its activity when treated at 121°C and 1 bar for 15 minutes. The substance also retained stability within a wide range of pH (3-11), which is in agreement with the results obtained for *B. licheniformis* strain MGrP1.

The stability of antibiotics is usually determined by the chemical structure of the compound (Reymolds *et al.,* 1948). Low pH ranges and autoclaving temperatures significantly reduced the activity of the antibiotics. This was an indication that the antibiotics are capable of biodegradation. It is a positive attribute with regard to safety in the environment and makes the antibiotics to qualify as environmentally friendly compounds that can be recommended for use in agriculture.

The two storage conditions (at refrigeration temperatures of 4°C and room temperature

of 22 ± 2°C) and duration of 3 months did not have sharp effects on the activity of the antibiotic. This is attributed to the stability of the antibiotics chemical structure at these temperatures which had no adverse effect over the storage period. Storage can therefore be recommended at either of the two conditions for a period of 3 months.

During the process of partial purification, the antibiotics were shown to be adsorbable antibiotic culture filtrate. Similar results were also reported by Wahome (1998) and Kamote (1999) while working with bean anthracnose and using antibiotics produced by two *B. subtilis* isolates CA5 and CA10.

The culture filtrate obtained from *B. licheniformis* strain MGrP1 proved effective in inhibiting growth of *C. lindemuthianum* by ground charcoal and recovered by elution of the charcoal using organic solvent (80% acetone in water). This process enhanced the activity of the antibiotic culture filtrate following the prolonged persistence and greater size of inhibition zones produced by the purified antibiotic culture filtrate. Zones of inhibition produced were 17.73% greater compared to those of the crude

in vitro and was further subjected to *in vivo* trials in the greenhouse. Despite phytotoxicity problems at high concentrations on bean plants (Plate 4a),

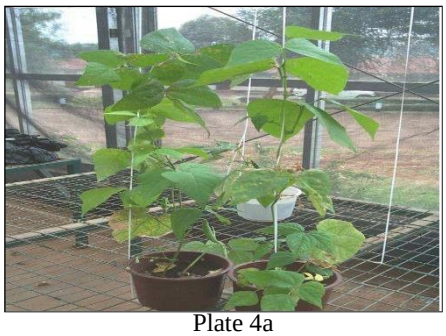

Plate 4a

Plate 4a): Bean plants exhibiting the phytotoxic effect of the *Bacillus* culture filtrate.

the antibiotic proved to be effective in decreasing the rate of disease progress and remarkably reduced disease severity on bean plants. The phytotoxic effects of the culture filtrate can be compared with non sprayed plants shown in plate 4b.

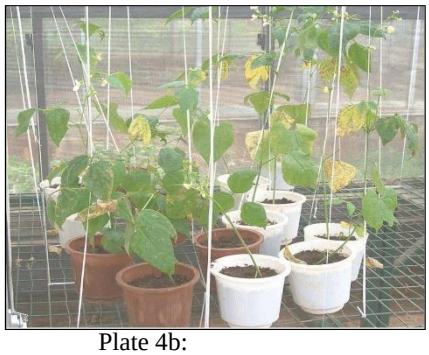

Plate 4b:

Plate 4b): Non-sprayed Bean plants.

The level of disease control compared favourably to that achieved by spraying with Benlate 50WP at a rate of 10g/l. Reduced disease severity was also reported by Wahome (1998) and Kamote (1999) while working with bean anthracnose and using antibiotics produced by two *B. subtilis* isolates CA5 and CA10. Effective disease control like the one achieved in this study using *Bacillus licheniformis* strain MGrP1 have been reported by several researchers. This study revealed that the antibiotic culture filtrate obtained, showed insignificant variability in activity when subjected to different storage conditions (refrigeration and room temperature) over a period of 3 months. This suggests that the antibiotic culture filtrate could be stored under any one of those conditions for a period not exceeding three months. It is possible that its shelf life exceeds 3 months. This needs to be researched further. Further research is needed to determine the chemical structure of the antibiotic produced by the *B. licheniformis* strain MGrP1 and *Rhizobium* or other members of the rhizosphere. The cloning of such genes directly into the plant cells will be an interesting option. It is appreciated that there is still a long way to go before a sound system is developed to protect plants from their predators without altering the ecological balance among species. The use of bioantagonists is certainly a very promising endeavour.

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Scharen and Bryan (1981) showed that metabolites of *B. licheniformis* produced in culture filtrate were antagonistic to *Pyrenophora teres*, the causal agent of net blotch of barley. Baker *et al.,* (1983) found that an isolate of *B. subtilis* gave greater than 95% reduction in the subsequent number of rust pastules when it was applied in liquid culture in the greenhouse 2 to 120 hours prior to inoculation with *Uromyces phaseoli* var. *typical* uredospores.
classify it. A thorough study on the kinetics of antibiotic production and the optimization of medium design and product recovery needs to be carried out. More knowledge is required on the mechanisms of biocontrol of phytopathogens to develop rational strategies for the application of the antagonists and their metabolites within the agroecosystem. Once such strategies are elucidated, genetic engineering can provide an efficient way of gathering desirable characteristics from different organisms in only one organism like

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