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Evaluation of Antibiotic Metabolites from Actinomycete Isolates for the Control of Late Blight of Tomatoes under Greenhouse Conditions

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Abstract: Antibiotic metabolites from two antagonistic actinomycete isolates coded 28P and CS35 were evaluated for the control of late blight of tomatoes in the greenhouse. Five concentrations namely; half strength, quarter strength, normal strength, one and half strength and double strength were evaluated for their efficacy in controlling late blight disease. The various concentrations of the metabolites were found to give a significant ($p \leq 0.05$) control in management of late blight. The antibiotic metabolites were found to be protective in nature and there was an average of 30.8 and 19.8% improvement in disease control when metabolites from isolate 28P and CS35, respectively were applied before inoculation compared to when the metabolites were applied after inoculation. Concentrating the culture filtrates increased the efficacy of the disease control without producing any phytotoxic effects. The disease incidence of tomato plants treated with culture filtrates from actinomycete isolate CS35 was 25.74, 17.93 and 15.01 for the normal, one and a half and two times concentrations, respectively. The antibiotic metabolites also delayed the onset of the disease and plants treated with the metabolites took relatively longer time (up to 2 more days) for the symptoms to develop. The actinomycetes have a great potential in the management of late blight.

Key words: Concentrations, disease incidence, *Phytophthora infestans*, sporulation

INTRODUCTION

Late blight of tomatoes caused by *Phytophthora infestans* is a very damaging disease in potatoes and tomatoes causing up to 90% of crop loss in cool and wet weather conditions (Hyang *et al*., 2005; Ghorbani *et al*., 2007). The disease is reported in all the production areas but it is more severe in humid and high rainfall areas and it occurs at a low intensity in dry areas (Lashomb and Richard, 1981; Denitsa and Naidenova, 2005; Agrios, 2005). Apart from yield losses, the disease reduces the market value of the crop due to the brown colour that forms on tomato fruits and rotting of potato tubers during storage (Hartman and Huang, 1995; Cao and Forrer, 2001; Ghorbani *et al*., 2007). Due to the devastating nature of the disease, it poses a threat to food security since many resource poor farmers cannot afford the numerous fungicide applications required to control it (Denitsa and Naidenova, 2005).

The conventional use of synthetic chemicals has limitations due to development of resistance (Mustafa *et al*., 2004), high costs, harmful effects to the users, environment and consumers of the treated produce (Taechowisan and Lumyong, 2003; Agrios, 2005). *P. infestans* has a high pathogenic variability and therefore, specific resistance has contributed little in controlling the disease and varietal resistance only helps in reducing the amount of fungicides required and the rate of disease development (Denitsa and Naidenova, 2005; Agrios, 2005). Due to all year round cultivation of solanaceous crops in the highland tropics, the disease and the infectious propagules are continuously present and the cultural control measures such as crop rotation cannot be relied upon to control the disease (Lehman *et al*., 2005; Ghorbani *et al*., 2007).

There is therefore a need to explore other strategies to supplement the existing measures to curb the heavy economic losses inflicted by the disease. Biopesticides (living organisms and the compounds naturally produced by these organisms) can be safer, more biodegradable and less expensive to develop. The actinomycetes, including the Streptomyces, are known producers of antimicrobial metabolites (Muiru, 2000; Sabaratnam and Traquair, 2002; Mustafa *et al*., 2004). Several actinomycete-produced antibiotics are being routinely used in an agricultural setting such as streptomycin and terramycin for fire blight control (Kuepper and Preston, 2004). Streptomycetes have demonstrated both *in vitro* and *in vivo* activity against

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plant pathogens such as *Fusarium*, *Cylindrocarpon*, *Pythium*, *Phytophthora megasperma* var. *sojae*, *Phytophthora capsici Phytopthora infestans* and *Colletotrichum* of pepper plant (Xiao *et al*., 2002; Sabaratnam and Traquair, 2003; Lim, 2005). However, there is no evidence of use of actinomycete metabolites in the control of late blight disease.

The antibiotic-producing actinomycete can be used as a suspension in a whole broth culture or as an antibiotic-containing supernatant obtained from a whole broth culture after centrifugation or after partitioning with solvents such as ethyl acetate and butanol (Muiru, 2000; Cao and Forrer, 2001; Lehman *et al*., 2005). Biocontrol of late blight using biocontrol agents such as actinomycetes have shown a great potential and commercial preparations for the management of diseases like Rhizoctonia damping off are in use (Cao and Forrer, 2001; Sabaratnam and Traquair, 2002; Taechowisan and Lumyong, 2003; Ghorbani *et al*., 2007). However, much work still needs to be done especially with the late blight of tomatoes since the disease is still causing much devastation on the crop. This study was undertaken with the objective of evaluating the antibiotic metabolites from actinomycete isolates for the control of late blight of tomatoes in the greenhouse.

MATERIALS AND METHODS

This research was carried out at the University of Nairobi and the actinomycete isolates used were isolated from soils collected during the dry months of January and February.

Isolation of actinomycete isolates: A modification of the dilution plate method by Herr (1959) was used in isolation of actinomycetes from the soil. The soil samples were spread on a laboratory bench and air dried for 7 days. The dried soil was then ground using a mortar and pestle and sieved through a 9 μ sieve. One gram of this soil was put in a vial containing 9 mL of sterile distilled water. This was stirred for 30 min using a magnetic stirrer and 1 mL of the suspension was drawn while still shaking and transferred into a 9 mL water blank. The suspension was shaken to give a dilution of 10^{-1} of the stock solution. Serial dilution was done to obtain 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilution levels. Aliquots of 0.1 mL of dilution 10^{-3} and 10^{-4} were transferred into plates containing 20 mL of sterile Czapeks Dox Agar ($NaNO_3$ 2 g, K_2HPO_4 1 g, $MgSO_4.7H_2O$ 0.5 g. KCl 0.5 g, $FeSO_4$ 0.01 g, Sucrose 30 g, agar 20 g, distilled water 1 L) prepared two days before by autoclaving at 121°C for 15 min at one bar pressure. These aliquots were spread evenly on the surface of solid medium of each plate using sterile bent glass rods. Four plates were prepared for each dilution level and arranged in a completely randomized design and incubated at 27°C. Incubation was done for 7-14 days and actinomycete colonies identified by their cultural characteristics like adherence to the media, dry surface with a conical appearance and presence of fringes of minute hyphae projecting into the medium. The actinomycete isolates were then purified by subculturing on fresh Czapeks Dox Agar.

Screening actinomycete isolates for antagonism: The preliminary screening for production of antagonistic metabolites was done using *Pythium* spp as the test pathogen following a modification of the method by Henis and Baker (1979). Four actinomycete isolates were spot inoculated 2 cm from the edge of the plate at four equidistant points. These plates were incubated for 3 days at 28°C in the incubator and arranged in a completely randomised design. On the third day, discs of *Pythium* sp. were cut using 6 mm cork borer from the edges of 4-7 days old pure cultures of *Pythium* and placed at the centre of the plates seeded with the actinomycete isolates. For the control, only the *Pythium* discs were seeded in the plates. These plates were then incubated at 28°C for 7-14 days and observations made daily. The actinomycete isolates that showed antagonism to *Pythium* sp. by producing an inhibition zone between them and the *Pythium* isolate were tested separately. Paired cultures of the *Pythium* and actinomycete were prepared by spot inoculating each actinomycete 2 cm from the edge of the plate at four equidistant points and incubated as above. The two actinomycete isolates CS35 and 28P were selected from a total of twenty one isolates that had consistently shown high levels of antagonism from the screening trials.

Isolation of *P. infestans* and production of inoculum: Late blight infected tomato leaves were collected from the University farm and washed in running tap water. The leaves were placed on moistened cellulose wadding in sandwich boxes and incubated for 24 h at room temperatures (24±2°C). Multiplication of the inoculum was done on V8 media and on potato tuber slices. Mycelium from the sporulating fungi was aseptically transferred onto the V8 based media (V8 juice 100 mL, $CaCO_3$ 1 g, β-sistosterol 0.05 g, Agar 15 g) and incubated at 18°C for 10-14 days. Harvesting of the inoculum was done following a modification of the protocol by Lehman *et al*. (2005). Cultures were flooded with 10 mL of sterile distilled water and the surface scrapped using sterile microscopic slides to dislodge the sporangia

from the mycelia. Filtration was done using sterile double layer of cheese cloth and the resultant sporangial suspension adjusted to 1.0×10^5 spores mL^{-1} using a haemocytometer.

A modification of the protocol reported in the CIP Manual (1997) was adopted in the multiplication of inoculum using potato tuber slices. Late blight infected tomato leaves were incubated in moist chambers as explained above for 24 h. Small sections (approximately 1 square centimetre) of leaves with sporulating mycelium were cut and placed beneath potato tuber slices 1 cm thick and 2 cm in diameter. Incubation was done on moist chambers which had been improvised by dispensing 10 mL of water agar in 9 cm diameter petri-dishes. The plates with the solidified media were inverted and the potato tuber slices with the leaf sections placed inside and incubation done for 7 days at 18°C. When harvesting the inoculum, 5 mL of sterile distilled water was added on the tuber slices and scrapped off using sterile microscopic slides. The resultant suspension was aseptically filtered using a double layer of cheese cloth and the sporangia suspension adjusted to 1.0×10^5 spores mL^{-1} using a haemocytometer.

Treatments preparation and application in the control of late blight: Tomato variety Cal J was sown in 20 cm diameter pots and placed in the greenhouse. The potting media used was loam soil and ballast at a ratio of 4:1 (v/v). The design used was completely randomized design replicated six times. Three pots planted with two tomato plants each served as the experimental unit. When the seedlings were 30 days old and with 3-4 fully expanded leaves, the different treatments were applied and the treatment were as follows.

Treatment 1: Tomato seedlings were sprayed until runoff with metabolites at double strength, one and a half times strength, normal, half and quarter strength concentrations from each of the actinomycete isolates and inoculated with *P. infestans* at the rate of 10^5 sporangia mL^{-1}. Inoculation was done twenty four hours after applying the metabolites on the seedlings.

Treatment 2: Tomato seedlings were inoculated with *P. infestans* at the rate of 10^5 sporangia mL^{-1} and 24 h later, sprayed with the various concentrations of metabolites as explained above. For the positive control, the tomato seedlings were sprayed with the standard chemical check (Dithane M45) before and after inoculation as explained above, whereas for the negative control, the seedlings were sprayed with sterile water before and after inoculation as explained earlier.

The lower concentrations of the metabolites were obtained by performing serial dilutions whereas the higher concentrations were arrived at by removing the water using the rotary evaporator. The paper disc method as outlined by Loo *et al.* (1945) was used to check for activity after concentrating and diluting the antibiotic metabolites. Data on incubation period, disease incidence, disease severity and phytotoxicity was recorded for a period of two months after inoculation. The data was analysed using Genstat Discovery edition 3 software and means separated using least significant differences.

The scoring key used in assessing disease severity was adopted from Lutaladio *et al.* (1996) and the key is as follows; 1 = No late blight observable, 2 = Late blight present. Maximum 10 lesion per plant, 3 = Plants look healthy but, lesions are easily seen at closer distance. Maximum foliage area affected by lesions or destroyed corresponds to more than 20 leaflets, 4 = Late blight easily seen on most plants. About 25% of foliage is covered with lesions or destroyed, 5 = Plot looks green; however, all plants are affected. Lower leaves are dead. About half foliage area is destroyed, 6 = Plot looks green; however all plants are affected. Lower leaves are dead. About 75% of each plant is affected. Leaves of the lower half of plants are destroyed, 7 = Plot neither predominantly green nor brown. Only top leaves are green many stems have large lesions, 8 = Plot is brown-colored. A few top leaves still have some green areas. Most stems have lesions or are dead and 9 = All leaves and stems are dead. (The description of symptoms was based on plants with 4 stems and 10 to 12 leaves).

RESULTS AND DISCUSSION

Production of the *P. infestans* inoculum: Use of both V8 and potato tuber slices supported high production of inoculum with each of them giving an average of 4.0×10^6 and 3.7×10^5 sporangia mL^{-1}, respectively. However, it took a relatively shorter time (7 days) to get the inoculum using the potato tuber slices compared to use of V8 where it took up to 14 days. It was observed that temperature was critical for the growth of the *P. infestans* and 18°C was the most ideal. Growth was very minimal at room temperature (24±2°C) and consequently no inoculum could be realized. Kerrs pink which is known to be more susceptible to *P. infestans* supported more sporangia production than the other three varieties namely, Dutch Robinson, Desiree and 38390.10 which are less susceptible. Potato slices cut from fresh tubers supported more sporulation than older tubers especially where solanin formation was evident.

Table 1: Percentage change in activity and water concentration of the diluted and concentrated antibiotic metabolites

Actinomycete isolate	Concentration of the metabolite	Change in water concentration of the metabolites (%)	Change in activity of the metabolites (%)
CS35	0.25N (Quarter strength)	300.0	-27.1
CS35	0.5N (Half strength)	100.0	-12.8
CS35	N (Normal strength)	0.0	0.0
CS35	1.5N (one and a half times strength)	-33.3	13.4
CS35	2N (double/two times strength)	-50.0	25.0
28P	0.25N(Quarter strength)	300.0	27.0
28P	0.5N (Half strength)	100.0	8.1
28P	N (Normal strength)	0.0	0.0
28P	1.5N (one and a half times strength)	-33.3	12.4
28P	2N (double/two times strength)	-50.0	23.2

N = Normal concentration of the antibiotic metabolites, - = Represents a decline or reduction

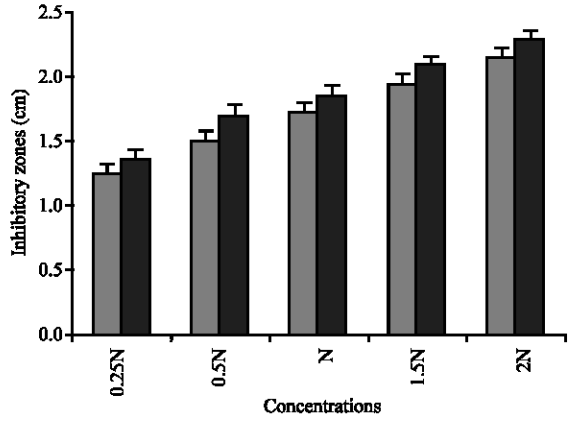

Fig. 1: Mean inhibitory diameters produced by the various antibiotic metabolites after dilution and concentration through addition and removal of water, respectively. N stands for normal concentration. Light gray bars represents isolate CS35 and dark gray bars represents isolate 28P. Vertical bars indicate standard error

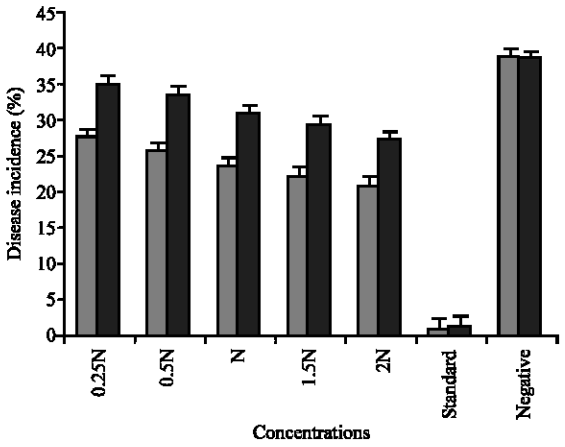

Fig. 2: Incidence of late blight on plants treated with various concentrations of metabolites from isolate 28P. N stands for normal concentration. Light gray bars represents disease incidence before inoculation and dark gray bars represents disease incidence after inoculation. Vertical bars indicate standard error

Evaluation of the antibiotic metabolites for the control of late blight in the greenhouse: The first appearance of late blight symptoms on the inoculated plants was noticed 4-5 days after inoculation, however the plants treated with the metabolites first before inoculation took relatively longer (6-7 days) for symptoms to appear. Application of metabolites before inoculation with the pathogen resulted to better control of the disease as evidenced by reduced disease incidence when the application was done before inoculation (Fig. 6, 7). For instance, disease incidences of 25.2, 26.5 and 29% were recorded when metabolites from isolate CS35 were applied before inoculation and disease incidences of 30.2, 34.8 and 36.4% were recorded when metabolites of the same isolate were applied after inoculation at normal, half and quarter strength concentrations, respectively.

The results also showed that, diluting the metabolites weakened their efficacy in controlling the disease (Fig. 2-5). Concentrating culture filtrates by removal of water enhanced the potency of these metabolites as shown by paper disc assay results (Fig. 1, Table 1). This enhanced potency was also reflected in the reduced disease incidences when these metabolites were used in controlling the late blight disease. Although the statistical analysis showed that, the metabolites had a significant effect ($p \leq 0.05$) in the control of late blight, this performance was comparatively inferior to the standard chemical check (Fig. 2-4).

The results showed that *P. infestans* can successfully be cultured on media however, it is fastidious and requires specific media and incubation at low temperatures for growth and sporulation to occur. The pathogen grows slowly on media and can easily be overrun by fast growing micro organisms such as fungi and bacteria and hence aseptic conditions is critical for successful culturing of the fungi. Other reports of culturing of *P. infestans* on media has been reported

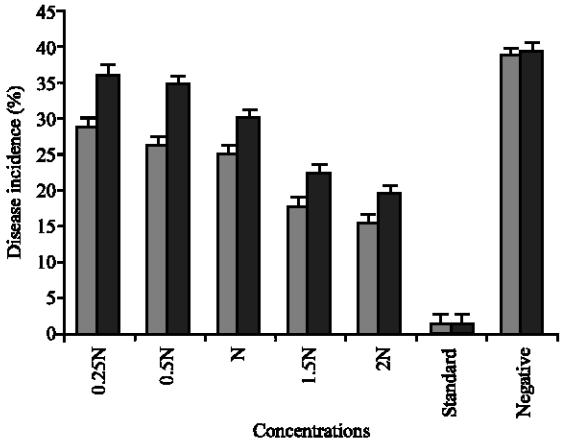

Fig. 3: Incidence of late blight on plants treated with various concentrations of metabolites from isolate CS35. N stands for normal concentration. Light gray bars represents disease incidence before inoculation and dark gray bars represents disease incidence after inoculation. Vertical bars indicate standard error

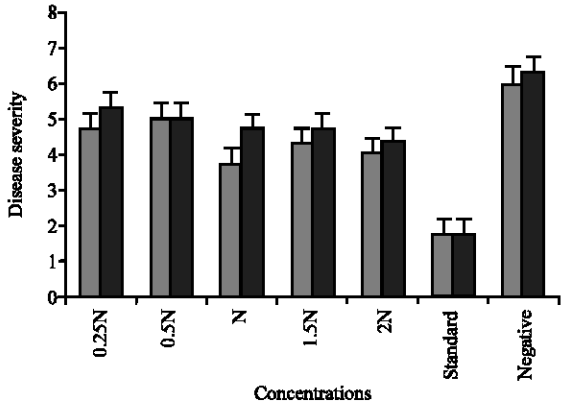

Fig. 4: Severity of late blight on plants treated with various concentrations of metabolites from isolate 28P. N stands for normal concentration. Light gray bars represents disease severity before inoculation and dark gray bars represents disease severity after inoculation. Vertical bars indicate standard error

(Lucas *et al*., 1991; Hartman and Huang, 1995; Lehman *et al*., 2005; Ghorbani *et al*., 2007) though this has been on other types of media such as rye agar, corn meal agar and lima bean agar. The disease requires cool conditions and high relative humidity for growth and sporulation and this was reflected in the *in vitro* studies. This agrees with other findings (Lehman *et al*., 2005).

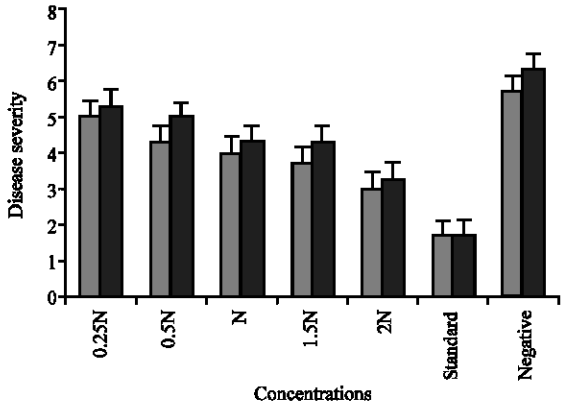

Fig. 5: Severity of late blight on plants treated with various concentrations of metabolites from isolate CS35. N stands for normal concentration. Light gray bars represents disease severity before inoculation and dark gray bars represents disease severity after inoculation. Vertical bars indicate standard error

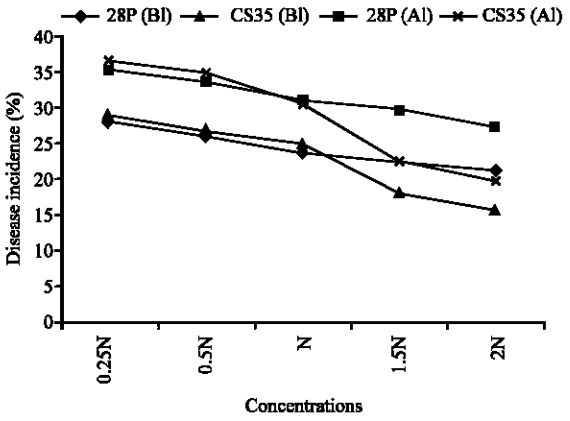

Fig. 6: Comparison of incidence of late blight on plants treated with antibiotic metabolites from isolate CS35 and 28P before and after inoculation. N stands for normal concentration. BI means before inoculation, AI means after inoculation

Incubation of the pathogen at low temperatures has the other advantage in that it can arrest the growth of some contaminants (Lucas *et al*., 1991).

The use of potato tuber slices to produce inoculum from *P. infestans* proved to be quite reliable and fast to accomplish, however soft rot caused by *Erwinia carotovora* can complicate the procedure. Other drawbacks associated with this method includes loss of pathogenicity if the pathogen is maintained on potato tuber slices (Toxopeus, 1954; CIP Manual, 1997;

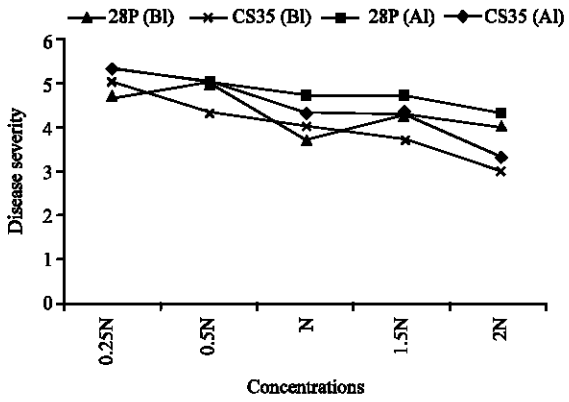

Fig. 7: Comparison of late blight severity on plants treated with antibiotic metabolites from isolate CS35 and 28P before and after inoculation. N stands for normal concentration. BI means before inoculation, AI means after inoculation

Ghorbani *et al*., 2007). The potato cultivar highly susceptible to late blight supported more sporangia production than the less susceptible ones. Many compounds such as solanide are reported to occur in the resistant potato tubers and this can be attributed to the low sporulation observed on such tubers. This agrees with research findings by Lucas *et al*. (1991). Potato tubers can be used to multiply the inoculum meant for inoculating tomato plants since the races of *P. infestans* infects both tomato and potato equally (Lim, 2005).

The metabolites from isolate coded 28P had the highest antagonistic activity after concentrating through the removal of water compared to the metabolites from isolate coded CS35. However, it performed dismally in the control of late blight in the greenhouse. This agrees with other findings that the activity of an antibiotic metabolite *in vitro* is not perfectly reflected in the *in vivo* conditions. The activity of an antibiotic may vary on application to the plants since the antibiotic metabolites can be rendered unstable or can be bound hence becoming unavailable (Turhan, 1981; Kuepper and Preston, 2004).

The different levels of concentration of the antibiotic metabolites had varied performance in terms of disease severity and disease incidence. Increasing the concentrations of these metabolites enhanced their efficacy as evidenced by the reduced disease severity and disease incidence. Removal of water from the metabolites raised the concentrations of the active compounds resulting to better disease control. Although the antibiotic metabolites were inferior in the control of late blight compared to the standard chemical check, they showed that they have a potential which should be exploited. Since phytotoxicity was not observed even at higher concentrations, these metabolites can be concentrated further and then evaluated for the management of late blight. This results corroborates findings by other workers (Cao and Forrer, 2001; Hyang *et al*., 2005; Lim, 2005) that actinomycetes possess a great potential in the management of late blight.

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