Suitable Medium for Increasing Biomass of Antagonistic *Streptomyces* spp. Against Bacterial Fruit Blotch Disease of Watermelon

Chanida Yaeram, Petcharat Thummabenjapone and Assanee Pachinburavan

Abstract

Eight selected *Streptomyces* isolates namely *Streptomyces* No.13, 15, 22, 33, 74, 78, 84 and 87, which were proved to be effective in controlling bacterial fruit blotch disease in watermelon, were cultured on three types of solid media, arginine-glycerol-mineral salt agar (AGMA),yeast extract-malt extract agar (YEME) and glycerol-arginine agar (GAA). It was found that all isolates tested grew rapidly on YEME and AGMA but not on GAA. Subsequently, their effectiveness in inhibiting the growth of *Acidovorax avenae* subsp. *citrulli* (Aac), a causal agent of bacterial fruit blotch disease, was tested by bioassay method. The results showed that seven tested isolates of *Streptomyces* except isolate No.74, which cultured on AGMA and YEME media had a great effect on inhibiting the growth of Aac. The inhibition zones created by 7 isolates were significantly different ($p<0.05$). The antibiosis activity against Aac of *Streptomyces* obtained in agar culture AGMA and YEME showed higher activity than in GAA. The biomass in liquid culture of four *Streptomyces* isolates (*Streptomyces* No.13, 15, 33 and 87) were evaluated. The highest ($p<0.05$) dry weights of biomass (ranging from 148.8 289.2 mg/100 ml) of *Streptomyces* isolate no.13, 15 and 33 were found in AGMB, while *Streptomyces* isolate No.87 with more rapidly produced biomass in YEME-broth was 144-376 mg/100 ml.

Keywords: *Acidovorax avenae* subsp. *citrulli*, Bacterial fruit blotch disease, biomass, *Streptomyces* spp., suitable medium

Department of Plant Pathology, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002.

Introduction

Bacterial fruit blotch (BFB) is a serious disease of watermelon and other cucurbits which was first observed in the United States in 1988 (Wall and Santos, 1988). It was first founded in Thailand in 1991 on seed production field at Khon Kaen province, Northease of Thailand (Sirithorn, 2001). *Acidovorax avenae* subsp. *citrulli* (Aac), the causal agent of bacterial fruit blotch disease, spreads rapidly throughout transplant houses and in the field to infect and eventually rot watermelon fruit. It is naturally contaminated and transmitted by seed which usually serves as the primary inoculum source for BFB outbreaks (Rane and Latin, 1992).

Biological control using antagonistic microorganisms to suppress plant diseases offers a powerful alternative method to replace synthetic chemicals. Filamentous soil bacteria belonging to the genus *Streptomyces* are widely recognized as important microorganisms because of their ability to produce many secondary metabolites, which have various activities, such as antifungal, antibacterial and antitumor (Demain, 1999). *Streptomyces* is a Gram-positive soil bacterium belonging to the order *Actinomycetales* and family *Streptomycetaceae.* They produce branching vegetative mycelia that form disperse units by sprouting spore-bearing airy branches on solid media (William et al., 1989). At least there were two species of *Streptomyces* which had been reported to control several economic plant diseases. They were *Streptomyces griseoviridis* and *S. lydicus* (Kortemaa, 1994 ; Mahadevan and Crawford, 1997; Tokala *et al*, 2002; Yuan and Crawford, 1995). The screening of *Streptomyces* from soil which have antagonistic activities against Aac was performed at Department of Plant Pathology, Khon Kaen University. The results from bioassay showed that several isolates of *Streptomyces* gave strong inhibition effect on Aac (Thummabenjapone and Pachinburavan, 2002). Some of *Streptomyces* isolates have a great potential to control Aac under green house condition (Wanking et al., 2004). For further development of *Streptomyces* biomass as biological control products, basic studies on the most suitable culture medium for all multiplication and maintenance of their antagonistic activities against Aac were investigated.

Materials and mathods

Microorganisms and culture conditions

Streptomyces isolates No.13, 15, 22, 33, 74, 78, 84 and 87 were kindly provided by P. Thummabenjapone (Department of Plant Pathology, Khon Kaen University). All *Streptomyces* isolates were stored in 30% glycerol frozen at -20 °C. Working cultures were obtained by streaking a loopful of spore stock onto arginine-glycerol-mineral salt agar (AGMA) plates. After incubation at 28 °C for 7 days,

colonies showing sporulation and filamentous aspect were employed in this experiment.

Isolate of *Acidovorax avenae* subsp. *citrulli* (Aac KK9) was used throughout this study. This virulent strain was kindly supplied by P. Thummabenjapone (Department of Plant Pathology, Khon Kaen University). The culture was maintained on nutrient agar (NA) slants at 4 ° C. Colonies were transferred to NA plates and incubated at 28 ° C for 48 h to prepare for the biological assay.

Media and morphological characterization

Agar culture: *Streptomyces* isolates were streaked on three different media including arginine-glycerol-mineral salt agar (AGMA; 1 liter consisted of : arginine, 1g; glycerol, 12.5 g; K_2HPO_4, 1 g; NaCl, 1 g; $MgSO_4 . 7 H_2O$, 0.5 g; $Fe_2 SO_4 . 7 H_2O$, 0.001 g; $CuSO_4 . 5 H_2O$, 0.0001 g; $ZnSO_4 . 7 H_2O$, 0.0001 g; $MnSO_4 . H_2O$, 0.0001 g; Bacto-agar, 15 g; pH 7), yeast extract-malt extract agar (YEME; 1 liter consisted of : yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; Bacto-agar, 16 g; pH 6.5) and glycerol-arginine agar (GAA; 1 liter consisted of : glycerol, 20 g; arginine, 1 g; NaCl, 1 g; $CaCO_3$, 0.1 g; $MgSO_4 . 7 H_2O$, 0.1 g; agar, 20 g; pH 7). Characteristics of colony's color, size and shape were recorded after incubation at 28 ° C for 7 days.

Biological assay of antibacterial activity

Antibacterial activities were tested by bioassay against bacterial pathogen Aac. The bacterium Aac was grown on NA plate and incubated for 48 h at 28 ° C. The Aac suspension was prepared in sterile distilled water and then added to melten NA (45 ° C) to obtain OD_{600nm} ~ 0.05, mixed well and poured into a sterile Petri-dish. The agar plate containing Aac was left at room temperature for 30 minutes. The disc cultures of *Streptomyces* isolates no.13, 15, 22, 33, 74, 78, 84 and 87) from various tested media (AGMA, YEME and GAA) were placed on Aac agar plate using AGMA disc as control. The plates were incubated for 48 hours at 28 ° C and examine for the inhibition of growth after 48 h. Diameter of inhibition zone was measured and analyzed using Duncan' Multiple Range Test (DMRT) with four replications.

Selection of medium for increasing biomass of Streptomyces spp.

Liquid culture: Four isolates of *Streptomyces* No.13, 15, 33 and 87 were cultured on AGMA medium. After incubation at 28 ° C for 7 days, three agar discs from each isolate were inoculated into a 250-ml Erlenmeyer flask containing AGMB, YEME-broth and GAB. After incubation at 28 ° C, 120 rpm on a rotary shaker for 7 days, microcolonies were filtered and then dried at 45 ° C for 24 hours. Total dry weight of biomass was measured and analyzed using DMRT with three replications.

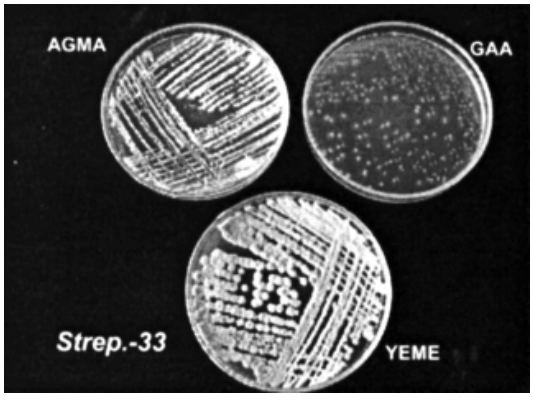
Results and discussion

Morphological characterization and antibacterial activity

All *Streptomyces* isolates grew well on AGMA and YEME, but not on GAA. Morphology, size and color of colonies of each *Streptomyces* isolates in each culture medium were different. Generally, the colonies were elevated, spreading and covered with white aerial mycelia as presented in Table 1 and Fig. 1.

Table 1 Cultural characteristics of *Streptomyces* isolates in 3 agar media, arginine-glycerol-mineral salt agar (AGMA), yeast extract-malt extract agar (YEME), and glycerol-arginine agar (GAA)

Isolates	Cultural characteristics					
	AGMA		YEME		GAA	
	Color of aerial mycelia	Growth	Color of aerial mycelia	Growth	Color of aerial mycelia	Growth
#13	white	well	grayish white	well, spreading	white	poor
#15	white	well, spreading	grayish white	well, spreading	white	poor
#22	white	well	grayish white	well	white	poor
#33	white	well, spreading	grayish white	well, spreading	white	poor
#74	yellow	well	grayish white	well, spreading	white	poor
#78	white	well	grayish white	well, spreading	white	poor
#84	white	well	grayish white	well	white	poor
#87	grayish white	well	grayish white	well	white	poor



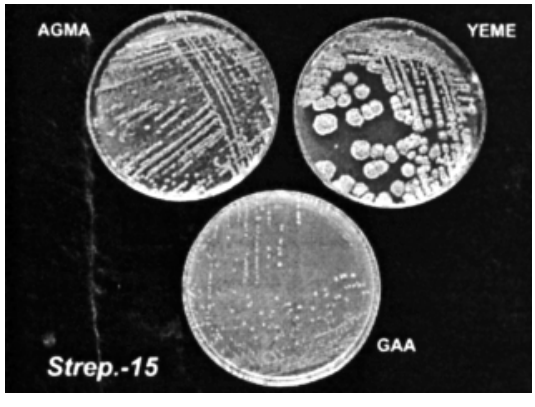
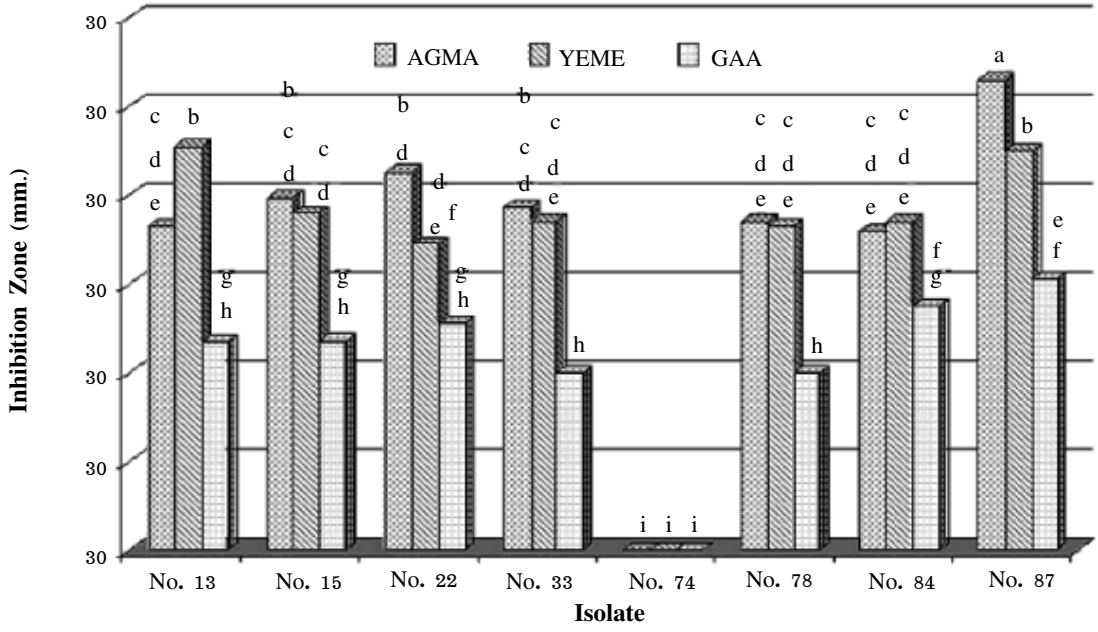


Fig. 1 Characteristic of *Streptomyces* colonies (7 days old) on 3 agar media, arginine-glycerol-mineral salt agar (AGMA), yeast extract-malt extract agar (YEME), and glycerol-arginine agar (GAA)

Most of tested *Streptomyces* isolates No.15, 22, 33, 78, and 87 grown on AGMA showed highest antibiosis activity against Aac. The level of antibiosis activity was decreased when *Streptomyces* isolates were cultured on YEME and GAA. For *Streptomyces* No.13 and 84, the best medium for antibiosis activity was YEME. The *Streptomyces* No.74 from three kinds of culture media did not produce antibiosis activity against Aac (Fig. 2, 3).



* Means with the same letter are not significantly different from each other according to Duncan's Multiple Range Test ($P<0.05$).

Fig. 2 Efficacy of *Streptomyces* isolates which cultured on AGMA, YEME, and GAA in inhibiting the growth of *Acidovorax avenae* subsp. *citrulli*. (Aac)

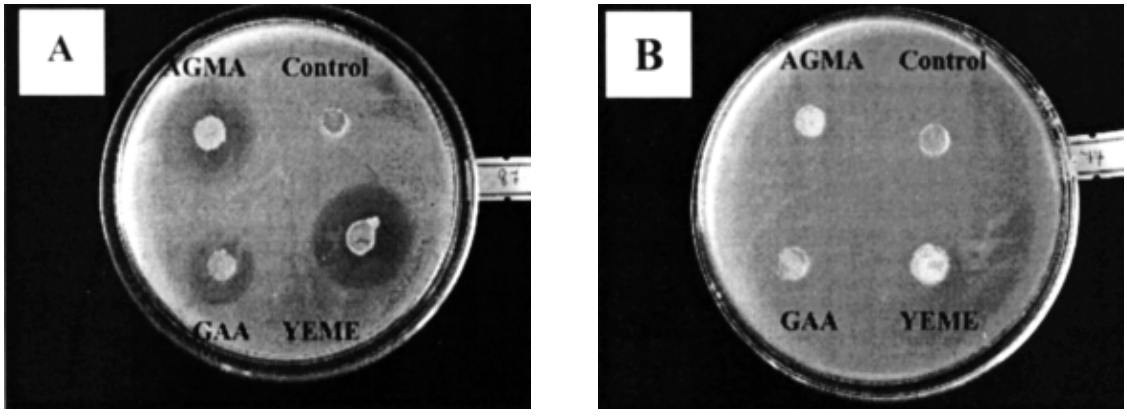


Fig. 3 Efficacy of *Streptomyces* isolate No.87 (A) and *Streptomyces* isolate No.74 (B) when cultured on AGMA, YEME, and GAA in inhibiting the growth of *Acidovorax avenae* subsp. *citrulli*.

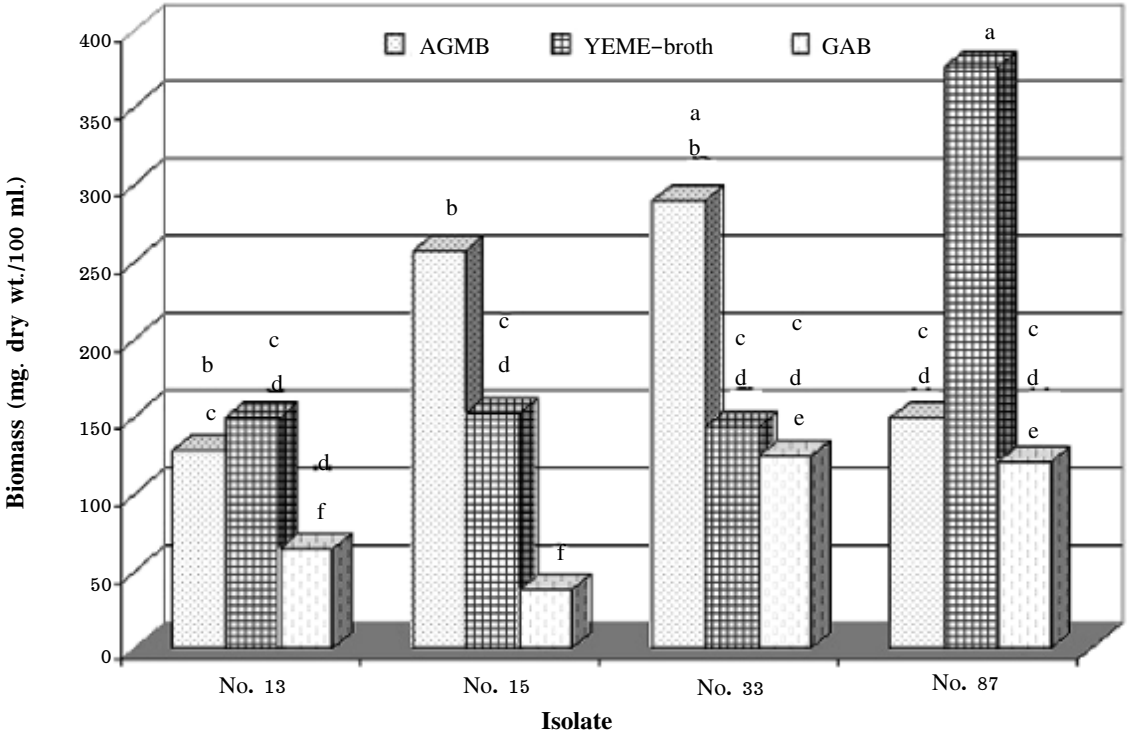
Different isolates of *Streptomyces* showed different effects on growth, colony morphology, and antibiosis activity. AGMA was found to be the most suitable medium that allow rapidly increase of *Streptomyces* population and enhance their antibiosis activity against Aac. Glycerol was included in AGMA to be a carbon source which is necessary for bacterial growth and antibiotic biosynthesis. In this study, it should be noted that antibiotic production from *Streptomyces* isolates grown in the medium that contained trace elements (AGMA) was higher than when grown in the medium lack of them (YEME and GAA). Mellouli *et al.* (2003) found that the highest antibacterial activities were obtained when starch was used as a carbon source in the presence of trace mineral oligoelements. This might explain why *Streptomyces* grown on AGMA could inhibit growth of Aac better than *Streptomyces* cultured on YEME and GAA. In addition, AGMA contained arginine which is the nitrogen source necessary for the secretion of proteins via the Twin-arginine translocation (Tat) pathway (Schaerlaekens *et al.*, 2001). An additional observation in YEME, it was found that the *Streptomyces* isolate no. 87 produced spore rapidly. This may be YEME contained glucose as a carbon source and yeast extract as a nitrogen source but lack of trace mineral elements that involved in antibiotic synthesis pathway.

Selection of a medium for increasing biomass of Streptomyces spp.

Biomass of *Streptomyces* isolate No. 87 increased most rapidly on YEME-broth while other tested *Streptomyces* isolates grew very well on AGMB. Biomass of *Streptomyces* isolate No. 87 was ranging from 144 - 376 mg. dry wt./100 ml YEME-broth whereas the biomass of isolate No. 13, 15 and 33 were ranging from 148.8-289.2 mg. dry wt./100 ml AGMB medium (Fig. 4.). The finest of microcolonies and color of culture filtrate of each *Streptomyces* isolate in different media also differed (Fig. 5). The *Streptomyces* isolate No. 87 grew better than the other isolates which were evidenced by its higher biomass in YEME-broth and well sporulation on YEME agar.

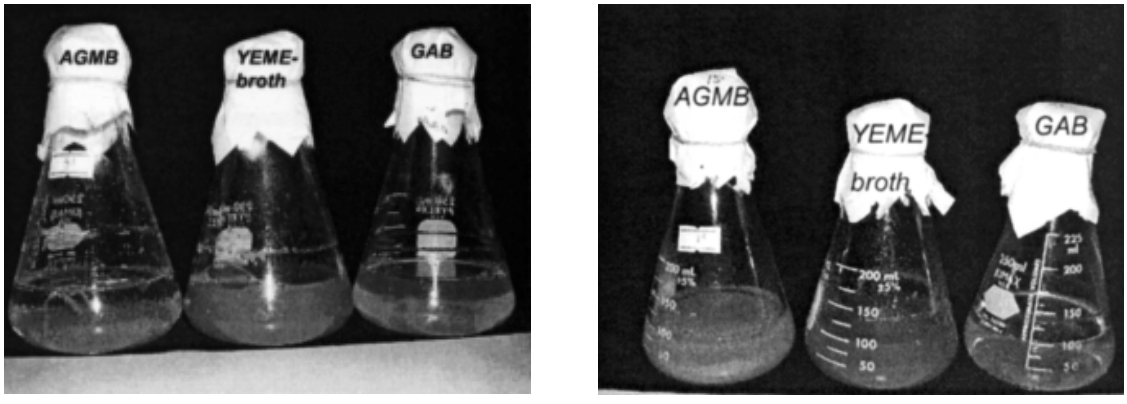
Conclusion

It can be concluded that all tested *Streptomyces* isolates exhibited different capacity in producing antibiotics against Aac, with *Streptomyces* isolate No. 87 giving the best result. AGMA is the most suitable agar medium for concurrently increasing *Streptomyces* biomass and producing antibiosis activities while YEME can be used to increase *Streptomyces* biomass only.



* Means with the same letter are not significantly different from each other according to Duncan's Multiple Range Test ($P<0.05$).

Fig. 4 Biomass of *Streptomyces* isolates when cultured in AGMB, YEME-broth, and GAB.



A **B**

Fig. 5 Cultural appearance of *Streptomyces* isolate No. 87 (A) and *Streptomyces* isolate No.15 (B) in liquid media, arginine-glycerol-mineral salt broth (AGMB), yeast extract-malt extract broth (YEME-broth), and glycerol-arginine broth (GAB).

Acknowledgement

This research has been financially supported by the Agricultural Biotechnology Project, Faculty of Agriculture, Khon Kaen University under the sponsorship of the Asian Development Bank Loan and the Thai Government, partly by RMUT's Staff Development Project, and The Agricultural Biofechnology Research Center for Sustainable Economy, Khon Kaen University.

References

Demain, A.L. 1999. Pharmaceutically active secondary metabolites of microorganisms. Applied Microbiology and Biotechnology 52: 455-463.

Kortemaa, H., H.Rita, K. Haahtela, and A. Smolander. 1994. Root-colonization ability of antagonistic *Streptomyces griseoviridis*. Plant and soil 163: 77-83.

Mahadevan, B. and D. Crawford. 1997. Propeties of the chitinase of the antifugal biocontrol agent *Streptomyces lydicus* WYEC 108. Enzyme and Microbial Technology 20: 489-493.

Mellouli, L., R.B. Ameur-Mehdi, S. Sioud, M. Salem, and S. Bejar. 2003. Isolation, purification and partial characterization of antibacterial activities produced by a newly isolated *Streptomyces* sp. US 24 strain. Research in Microbiology 154: 345-352.

Sirithon, P. 2001. Seed Pathology and exportation: Study case of bacterial leaf spot of tomato and bacterial fruit blotch of watermelon. In KKU Plant Pathological Review, Department of Plant Pathology, Faculty of Agriculture, Khon Kaen University. 69-81.

Rane, K.K. and R.X.Latin. 1992. Bacterial fruit blotch of with seed. Plant Disease. 76: 509-512.

Schaerlaekens, K., M. Schierov'a, E. Lammertyn, N. Geukens, Ann'e, and L. V. Mellaert. 2001. Twin-arginine translocation pathway in *Streptomyces lividans*. Journal of Bacteriology 183(23): 6727-6732.

Thummabenjapone, P. and A. Pachinburavan. 2002. *Streptomyces* potential biological control agent for bacterial fruit blotch and gummy stem blight disease in cucumbitsPage 175: in Summary of TPS 2002 The first international conference on tropical and subtropical plant disease. November 5-8, 2002. The Imperial Mae Ping Hotel, Thailand.

Tokala, R. K., J. L. Strap, C. M. Jung, D. L. Crawford, and M. H. Salove. 2002. Novel plant-microbe Rhizophere interaction involving *Streptomyces* lydicus WYEC 108 and the pea plant (Pisum sativum). Applied and Environmental Microbiology 68: 2161-2171.

Wall, G.C. and V.M. Santos. 1988. A new bacterial disease on watermelons in the Mariana Islands. (Abstr.) Phytopathology 78:1605.

Wanking, B., P. Thummabenjapone, and A. Pachinburavan. 2004. Potential of watermelon in green house. Page 480-490: Annual Agricultural Seminar for Year 2004. January 26-27, 2004. Faculty of Agriculture, Khon Kaen University.

William, S.T., M.Goodfellow, and G. Alderson. 1989. Genus *Streptomyces Waksman* and Henrici 1943, 399AL. In William, S. T., Sharpe, M. E., Holt, J. G. (ed) Bergey's manual of systematic Bacteriology, vol. 4, Williums & Wilkins, Baltimore, pp 2452- 2492.

Yuan, W. M. and D.L. Crawford. 1995. Characterization of *Streptomyces lydicus* WYEC 108 as a potential biocontrol agent against fungal root and seed rots. Applied and Environmental Microbiology 61: 3119-3128.