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RESEARCH ARTICLE

Weihong ZHANG, Wenxiang YANG, Qingfang MENG, Yaning LI, Daqun LIU

Screening and identification of antagonistic *Streptomyces* spp. against *Clavibacter michiganensis* subsp. *michiganensis* from tomato rhizosphere

Abstract The objectives of our present study were to isolate antagonistic *Streptomyces* from tomato rhizosphere, and evaluate the potential strain for the biological control of bacterial canker of tomato. One hundred and seventy strains of *Streptomyces* isolated from tomato rhizosphere were tested for antibiosis activity against *Clavibacter michiganensis* subsp. *michiganensis* on double-layer agar. Sixty-three isolates showed antibiosis activity with diameter of an inhibition zone ranging from 1.0–6.5 cm. Fifteen *Streptomyces* strains had strong antibiosis activity against *C. m*. subsp. *michiganensis* with diameter of the inhibition zone above 4.0 cm on double-layer agar. Especially, the strain named Z-L-22 showed the strongest antibiosis activity with 6.5 cm inhibition zone. The fermentation filtrate also showed a high inhibition activity against Gram-positive bacteria such as *Streptomyces scabies, Staphylococcus aureus*, and *Bacillus subtilis*. Morphological, physiological and biochemical tests combined with 16S rDNA sequence analysis were carried out to identify the strain Z-L-22. Characteristics of the Z-L-22 were similar to those of *Streptomyces setonii*, and the 16S rDNA sequence showed 99.4% homology to *S. setonii*. Based on the polyphase taxonomic views, the Z-L-22 was identified as *S. setonii*.

Keywords tomato bacterial canker, antagonistic *Streptomyces*, screening, identification

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Weihong ZHANG, Wenxiang YANG, Qingfang MENG, Yaning LI, Daqun LIU (✉)

Biological Control Center of Plant Diseases and Pests of Hebei Province, College of Plant Protection, Agricultural University of Hebei, Baoding 071001, China
E-mail: ldq@hebau.edu.cn

1 Introduction

Tomato bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* is a serious disease of field and greenhouse-grown tomatoes (*Lycopersicon esculentum*) in several countries (Strider, 1969; Gleason et al., 1993). It leads to vascular infections, wilting, chlorosis, and eventual death of the plant, and causes huge economic losses in tomato production. Recently, it was found in most tomato cultivated regions in China (Luo et al., 2004). At present, few commercially grown cultivars have significant tolerance or resistance to *C. m.* subsp. *michiganensis*. Also, control of the disease relies upon the use of antibiotics (such as streptomycin) or copper compounds (Gleason et al., 1993; Gartemann et al., 2003). Unfortunately, such chemical control methods are not very safe and efficient. Especially, the frequent use of pesticides and antibiotics against plant pathogenic bacteria has led to the selection of resistant bacterial populations against antibiotics. Biological control of *C. m*. subsp. *michiganensis* may be an alternative to chemical control.

The use of biological agents to control bacteria canker has been reported in recent research. Antagonistic bacteria, especially some fluorescent pseudomonads, could reduce the infection when applied as a seed treatment followed by a root treatment before transplanting (Boudyach et al., 2001). In fields pre-inoculation of the avirulent strain NCPP 3123 of *C. m*. subsp. *michiganensis* or application of its extracellular polysaccharides in young tomato plants some days before planting induced a systemic, persistent, and extreme resistance to highly virulent *C. m*. subsp. *michiganensis* isolates (Griesbach et al., 2002). The extract of medicinal plants could express bactericide activity on *C. m*. subsp. *michiganensis* (Morais et al., 2002).

Streptomyces can be found worldwide in soil and act an important symbiotic role within plant rhizosphere. They have the capability to synthesize many different biologically active secondary metabolites. Although many of

Streptomyces agents have been used in biocontrolling plant disease (Yuan and Crawford, 1995; Bélanger, 2002; Sabaratnam, 2002), and two end-products that respectively contained *Streptomyces griseoviridis* Strain K61 and *Streptomyces lydicus* WYEC 108 have been registered to the control of fungal diseases in greenhouse such as root rot, seed rot, damping-off, and powdery mildew (http://www.epa.gov/pesticides/biopesticides/ingredients/index.htm), few strains have been used in bacteria disease control. The objectives of the present study were to isolate antagonistic *Streptomyces* from tomato rhizosphere, and evaluate the potential strain for the biological control of bacterial canker of tomato.

2 Materials and methods

2.1 Soil samples

Nine soil samples were collected from tomato rhizosphere in different localities of Baoding, China. Samples were air-dried under room temperature for about 10 days prior to isolation.

2.2 Test microorganisms

The strain of *C. m*. subsp. *michiganensis* was obtained from the Chinese Academy of Agricultural Sciences. Antibacterial activities were tested for *in vitro* against phytopathogenic fungi and bacteria including *Botrytis cinerea*, *Alternaria solani, Verticillium dahliae, Fusarium oxysporum, Alternaria brassicae, Rhizoctonia solani, Streptomyces scabies, Bacillus subtilis, Staphylococcus aureus, Erwinia carotovora* var. *carotovora* and *Escherichia coli*, which were reserved in the Biological Control Laboratory of Agriculture University of Hebei.

2.3 Isolation of *Streptomyces*

Isolations of *Streptomyces* were performed by soil dilution plate technique (Fang, 1998). One gram of dried soil samples was suspended in 9 mL sterile distilled water, agitated for 1 min and subsequently allowed to settle for 1 h. The suspension was subsequently diluted to 10^2, 10^3, 10^4, 10^5 and 10^6 1000-fold dilution. 0.1 mL soil dilutions of 10^3 to 10^6 was separately spread on Gause's No.1 agar medium supplemented with 10^{-4} (w/v) potassium dichromate to suppress the growth of bacteria. The plates were incubated at 28°C for 7–14 d. Selected colonies of *Streptomyces* were transferred from mixed culture of the plates onto respective agar plates and incubated at 28ºC for 14 d. Suspension of spores in 20% glycerol was made and kept at −20ºC for further experiments.

2.4 Screening antagonistic *Streptomyces in vitro*

All the isolates were tested for inhibitory activity against *C. m*. subsp. *michiganensis* on double-layer agar. The suspension of spores (0.01 mL, 10^8 cfu·mL^{-1}) was spotted onto a paper disk (6 mm) on Oat Agar, and incubated at 28ºC. Three days after incubation the isolates were killed by chloroform, and subsequently overlaid with 15 mL 523 medium (sucrose 10.0 g, yeast extract 10.0 g, K_2HPO_4 2.0 g, $CaCO_3$ 5.0 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, agar 15.0 g, distilled water 1000 mL, pH 7.2) in which *C. m*. subsp. *michiganensis* grow well, and a suspension of *C. m*. subsp. *michiganensis* was spread over the double-layer agar. The plates were incubated at 28°C for another 3 days, and the antagonistic activity was evaluated by measuring the diameter of inhibition zones.

2.5 Fermentation filtrate preparation and inhibition test

The isolates showing high inhibition activity in plate were tested for inhibitory activity of the fermentation filtrate against *C. m*. subsp. *michiganensis*. The suspension of spores of *Streptomyces* isolates was inoculated in Tryptic Soy Broth (Difco) liquid medium, and incubated at 28°C for 36 h, then 1 mL pure culture was inoculated into 50 mL soybean extract medium (soybean cake 10.0 g, glucose 10.0 g, peptone 3.0 g, NaCl 2.5 g, $CaCO_3$ 2.0 g, distilled water 1000 mL, pH 7.2) and incubated at 28°C, 200 r·min^{-1} for 5 days. The culture broth samples were centrifuged at 5000 r·min^{-1} for 20 min, and 0.2 mL liquid fermentation filtrated by bacterial filter (0.22 μm) was spotted onto paper disk (1.2 cm) in the medium which was seeded with test microorganism, then incubated at 28°C for 3 days. Inhibitory activity against *C. m*. subsp. *michiganensis* or any other microorganism was noted by the size of the inhibition zone.

2.6 Identification of *Streptomyces*

The strain showing significant antimicrobial activity was characterized morphologically and physiologically following the directions given in the Taxonomy and Identification of Actinomycetes (Yan, 1992) and Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1984).

2.6.1 Morphological characteristics

Pure isolate was preliminarily identified according to the traditional morphological criteria, including cultural characteristics of isolates in various media, morphology of aerial and substrate mycelium, morphology of spores, pigment production and so on, which were recorded after incubation for 7 to 14 days at 28°C.

2.6.2 Chemical type of cell wall

The pattern of whole cell sugar of the strain was examined by the whole cell hydrolysate with TLC (Hasegawa et al., 1983).

2.6.3 Physiological and biochemical characteristics

Physiological and biochemical characteristics of the isolate was tested, including carbon source utilization, gelatin liquefaction, milk peptonization, cellulose decomposing, melanin production, nitrate reduction, and starch hydrolysis.

2.6.4 16S rRNA sequence analysis

Total DNA was prepared from the *Streptomyces* isolate as described by Practical *Streptomyces* Genetics (Kieser, 2000). The 16S rRNA gene was amplified using universal primers F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and R: 5′-AAGGAGGTGATCCAGCCGCA-3′ (Edwards et al., 1989). PCR conditions were 94ºC (4 min) followed by 30 cycles at 94ºC (1 min), 55ºC (1 min 30 s), and 72ºC (3 min) with a final extension step at 72ºC for 5 min. PCR fragments were purified and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The sequences were compared with known sequences in nucleotide database by using BLASTN.

3 Results

3.1 Isolation of *Streptomyces*

One hundred and seventy strains of *Streptomyces* were isolated from tomato rhizospheres in 9 different samples. Sixty-three isolates showed an antibiosis activity against *C. m*. subsp. *michiganensis*, and developed inhibition zones ranging from 1.0 cm to 6.5 cm on double-layer agar (Table 1). 34.9% isolates had a weak antibiosis activity at the inhibition zone (⩽2.0 cm), 42.9% isolates had a moderate antibiosis activity at the inhibition zone (2–4 cm), and only 22.2% isolates (fourteen in one hundred and seventy) had a strong antibiosis activity at the inhibition zone (⩾4.0 cm).

3.2 Screening antagonistic *Streptomyces in vitro*

The fourteen isolates that showed a strong antibiosis activity (inhibition zones⩾4.0 cm) not only were tested in double-layer agar, but also were tested for the inhibition of the fermentation filtrate *in vitro*. Most of the isolates showed a stable inhibition (Table 2) except isolates 7–2. The isolates 7–4 and 7–5 showed a significant inhibition with 6.5 cm inhibition zone on the double-layer agar and 5.8 or 5.7 cm inhibition zone with fermentation liquid. In morphology and antibiosis activity, there was no significant difference between the strains 7–4 and 7–5. Therefore, we only named 7–4 as Z-L-22 for further tests.

3.3 Inhibition test of *Streptomyces* strain Z-L-22

The inhibition of fermentation filtrate was tested against pathogenic germs including fungi, Gram positive bacteria and Gram negative bacteria. The results suggested that the fermentation filtrate showed a high inhibition towards the tested Gram positive bacteria; the strain showed a 4.5 cm inhibition zone to *Bacillu subtilis*, 4.0 cm inhibition zone to *Staphylococcus aureus* and 2.0 cm inhibition zone to *Streptomyce scabies*. However, no inhibition towards the tested fungi and Gram-negative bacteria was tested.

3.4 Identification of *Streptomyces* strain Z-L-22

The cultural characteristics of *Streptomyces* strain Z-L-22 on various media are presented in Table 3. Strain Z-L-22 grew well on most of the organic and synthetic media tested. Typically, the colonies were covered with pale yellow aerial mycelia and yellow-orange substrate mycelia, and orange soluble pigment was observed also. The scanning electron micrograph of strain Z-L-22 revealed

Table 1 The number of inhibition *Streptomyces* isolates from nine different soil samples and their antibiotic activity against *Clavibacter michiganensis* subsp. *michiganensis*

soil sample	No. of isolates	No. of inhibitory isolates	No. of isolates with inhibition zones⩽2.0 cm	No. of isolates with inhibition zones 2–4 cm	No. of isolates with inhibition zones⩾4.0 cm	the rate of inhibitory isolates
1	17	8	4	4	0	47.1
2	5	1	1	0	0	20.0
3	19	10	2	6	2	52.6
4	18	5	1	3	1	27.8
5	31	14	4	7	3	45.2
6	26	10	4	3	3	38.5
7	16	5	2	0	3	31.3
8	7	4	1	1	2	57.1
9	31	6	2	4	0	19.3
total	170	63	21	28	14	37.1

Table 2 The antibiosis activity of the isolates against *Clavibacter michiganensis* subsp. *michiganensis*

isolate	diameter of the inhibition zone in double-layer agar	diameter of the inhibition zone with fermentation liquid
7–4	6.5 a	5.8 a
7–5	6.5 a	5.7 a
7–2	6.4 a	0.0 c
3–10	6.0 ab	4.5 b
5–16	5.6 b	4.2 b
6–6	5.5 b	4.0 b

Note: Values followed by a different letter within a column are significantly different at $P < 0.05$.

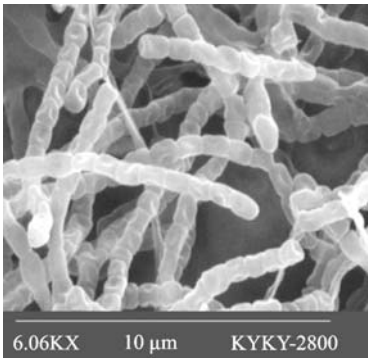

Fig. 1 Spores of *Streptomyces* strain Z-L-22 (SEM, 6060X)

that there were non-fragmented extensively branched substrate mycelia, and spore chains were rectiflexible, and the smooth-surfaced spores were spherical or ellipsoidal (Fig. 1). The amino acid composition of the cell-wall was identified as L, L-DAP (Diaminopimelic acid) and no amino sugar in the whole cell hydrolysate was detected by TLC. The cell wall chemotype belonged to Type I and the sugar pattern belonged to Type C.

Table 4 lists the physiological properties of *Streptomyces* strain Z-L-22. It was capable of liquefying gelatin, growing in cellulose, reducing nitrate and hydrolyzing starch. The strain Z-L-22 had a very broad pattern of carbon source assimilation (Table 4). Sucrose, arabinose, raffinose, xylose, inositol, rhamnose, fructose, mannitol and maltose were well utilized, and sorbose was not utilized.

1467 bp of the 16S rRNA genes of the strain were sequenced. Analysis of these 16S rRNA genes by BLAST confirmed that the strain Z-L-22 belonged to genus *Streptomyces*. It had 99.4% homology of the 16S rRNA gene sequence with that of *S. setonii* (D63872) and *S. caviscabies* (AF112160) in GenBank. 16S rRNA sequences of the strain Z-L-22 were used to construct a phylogenetic tree for phylogenetic analysis together with 9 reference sequences obtained from the GenBank database with MegAlign of DNAstar software package. Morphology, physiological and biochemical characteristics of the strain Z-L-22 were very similar to those of *S. setonii*, but distinct to *S. caviscabies* (Goyer et al., 1996). The result

Table 3 Cultural characteristics of *Streptomyces* isolate Z-L-22 on different media

medium	growth	aerial mycelium	substrate mycelium	soluble pigment
Gause' s No.1 agar	+	pale gray-yellow	yellow-orange	pale orange
starch ammonium agar	+	gray-yellow	yellow-orange	orange
glucose asparagine agar	+	gray-yellow	yellow-orange	pale orange
glycerol asparagine agar	++	gray-yellow	gray-yellow	pale orange
oat agar	++	gray-yellow	yellow-orange	orange

Note: "++" means aerial mycelia grow very well, "+" means moderate.

Table 4 Physiological and biochemical characteristics of *Streptomyces* isolate Z-L-22

characteristic	result	carbon source utilization	result
gelatin liquefaction	+	sucrose	+
milk coagulation and peptonization	−	arabinose	+
growth in cellulose	+	raffinose	+
H_2S production	−	xylose	+
melanin production	−	inositol	+
nitrate reduction	+	rhamnose	+
starch hydrolysis	+	fructose	+
		mannitol	+
		maltose	+
		sorbose	−

Note: "+" means positive, "−" means negative.

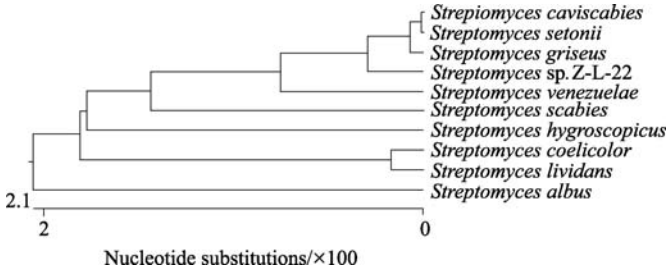

Fig. 2 Phylogenetic tree of 16S rDNA sequences of *Streptomyces* isolate Z-L-22 and its related strains

suggested that the strain isolated most frequently would be a member of *S. setonii*.

4 Discussion

Streptomyces is an important agent in plant disease biocontrol. It can be isolated from rhizosphere, rhizoplane and plant tissues. In our research, 37.1% isolates from tomato rhizosphere showed an antibiosis activity against *C. m*. subsp. *michiganensis in vitro*, and the antibiosis activity of fourteen isolates was strong. It was considered that the antagonistic *Streptomyces* against *C. m*. subsp. *michiganensis* widely existed in tomato rhizosphere. It was an effective means to search for potential biocontrol agents from rhizosphere to control tomato bacterial canker.

Despite that many isolates showed strong antibiosis activity *in vitro*, there was no distinct correlation between *in vitro* positive antagonism and *in vivo*. *In vivo*, besides the antibacterial activity of the isolates, the establishment, root colonization and environment conditions would affect the biocontrol activity (Crawford et al., 1993). For the biocontrol of bacterial canker of tomato with *Streptomyces*, much work should be done in greenhouse conditions. In addition, the tomato seed is known to be an important means of transforming *C. m*. subsp. *michiganensis* into new areas to survive from year to year; therefore, seed treatment with antibacterial metabolites of antagonistic *Streptomyces* may become an effective method to control tomato bacterial canker.

The significant isolate Z-L-22 showed a strong antibiosis activity not only against *C. m*. subsp. *michiganensis* but also against other Gram positive bacteria such as *Streptomyces scabies*, *Bacillus subtilis*, and *Staphylococcus aureus*. The prominent anti *C. m*. subsp. *michiganensis* activity of Z-L-22 strain highlights it as a candidate for further investigation in the biological control of this pathogen. Furthermore, we should test the bioactivity on more microorganisms widening the antimicrobial spectrum especially the drug-resistant strains and applying the isolates to medical exploration. Detailed studies of the biochemistry of the antibacterial metabolites (antibiotics, extracellular hydrolytic enzymes, etc.) are in process, which can be useful in understanding the mechanisms involved in biological control of the disease by *Streptomyces*.

Identifying *Streptomyces* is a tedious job. Traditional taxonomy of *Streptomyces* is based on morphological, physiological and biochemical tests, which need a long time to characterize the strains because the results are not very exact. With the development of molecular biology, 16S rRNA sequence is considered as one of the most important standards in bacteria identification. In our research, a phylogenetic tree constructed on the basis of 16S rRNA sequence data showed that the *Streptomyces* species including *S. setonii*, *S. acidiscabies*, and *S. griseus* constituted unique branches with high homology. Only based on the character could it not be identified. Based on the polyphase taxonomical views, Z-L-22 strain was identified as *S. setonii*.

S. setonii is seldom involved in plant disease biocontrol. In China, only one report claimed marine sponge-actinomyces Hmp-S14 identified as *S. setonii* had a bioactivity of anti-*Magnaporthe grisea* (Liu et al., 2004). Except for Larsen reporting a strain of *S. setonii* producing a pyrrole-ether antibiotic (Larsen et al., 1988), the antibiotic produced by *S. setonii* is less studied. Further experiments are needed to obtain antibacterial activities and metabolites of *S. setonii* Z-L-22.

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References

Bélanger R R, Jarvis W R, Traquair J A (2002). Formulation of a *Streptomyces* biocontrol agent for the suppression of *Rhizoctonia* damping-off in tomato transplants. Biological Control, 23: 245–253

Bergey (1989). Bergey's Manual of Determinative Bacteriology (Williams and Wilkins Co.). London: Baltimore

Boudyach E H, Fatmi M, Akhayat O, Benizri E, Ben Aoumar A A (2001). Selection of antagonistic bacteria of *Clavibacter michiganensis* subsp. *michiganensis* and evaluation of their efficiency in greenhouse. Biocontrol Science and Technology, 11: 141–149

Chet I, Ordentlich A, Shapira R, Oppenheim A (1990). Mechanisms of biocontrol of soil-borne plant pathogens by rhizobacteria. Plant and

Soil, 129: 85–92

Crawford D L, Lynch J M, Whipps J M, Ousley M A (1993). Isolation and characterization of actinomycete antagonists of a fungal rootpathogen. Applied and Environmental Microbiology, 59: 3899–3905

Edwards U, Rogall T, Blöcker H, Emde M, Böttger E C (1989). Isolation and direct complete nucleotide determination of entire genes. Nucleic Acids Research, 17: 7843–7853

Fang Z D (1998). Plant Pathology Technology. 3rd ed. Beijing: Chinese Agriculture Press, 243 (in Chinese)

Gartemann K H, Kirchner O, Engemann J, Gräfen I, Eichen-laub R, Burger A (2003). *Clavibacter michiganensis* subsp. *michiganensis*: first steps in the understanding of virulence of a Gram-positive phytopathogenic bacterium. Journal of Biotechnology, 106: 179–191

Gleason M L, Gitaitis R D, Ricker M D (1993). Recent progress in understanding and controlling bacterial canker of tomato in eastern north America. Plant Disease, 77: 1069–1076

Goyer C, Faucher E, Beaulieu C (1996). *Streptomyces caviscabies* sp. nov., from deep-pitted lesions in potatoes in Québec, Canada. International Journal of Systematic and Evolutionary, 46: 635–639

Griesbach E, Sotirova V (2002). Induction of resistance to *Clavibacter michiganensis* subsp. *michiganensis* by pre-inoculation of young tomato plants under field conditions in Bulgaria. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz, 109: 264–278

Hasegawa T, Takizawa M, Tanida S (1983). A rapid analysis for chemical grouping of aerobic actinomycetes. Journal of General and Applied Microbiology, 29: 319–322

Kieser T, Bibb M J, Buttner M J, Chater K F, Hopwood D A (2000). Practical *Streptomyces* Genetics. Norwich: The John Innes Foundation, 1–18

Larsen S H, Boeck L D, Mertz F P, Paschal J W, Occolowitz J L (1988). 16-Deethylindanomycin (A83094A), a novel pyrrole-ether antibiotic produced by a strain of *Streptomyces setonii*. Taxonomy, fermentation, isolation and characterization. The Journal of Antibiotics, 41: 1170–1176

Liu L, Hu J C, Wang S J (2004). Biological characteristics and identification of marine sponge-actinmyces Hmp- S14 with bioactivity of anti-*Magnaporthe grisea*. Journal of Shenyang Agricultural University, 35: 16–19 (in Chinese)

Luo L, Zhao T C, Li J Q, Zhang L, Li Y, Hasan B (2004). Progress in research on bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis*. Agricultural Sciences in China, 37: 1144–1150 (in Chinese)

Morais L A S, Carmo M G F, Viegas E C (2002). Evaluation of antimicrobial activity of extracts of medicinal plants on three tomato phytopathogens. Acta Horticulturae, 569: 87–90

Sabaratnam S, Traquair J A (2002). Formulation of a *Streptomyces* biocontrol agent for the suppression of Rhizoctonia damping-off in tomato transplants. Biological Control, 23: 245–253

Streder D L (1969). Bacterial canker of tomato caused by *Corynebacterium michiganense*: A literature review and bibliography. Technical Bulletin North Carolina Agricultural Experiment Station, 193

Werner N A, Fulbright D W, Podolsk R, Bell J, Hausbeck M K (2002). Limiting populations and spread of *Clavibacter michiganensis* subsp. *michiganensis* on seedling tomatoes in the greenhouse. Plant Disease, 86: 535–542

Yan X C (1992). Taxonomy and Identification of Actinomycetes. Beijing: Science Press (in Chinese)

Yuan W M, Crawford D L (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