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Sensitivities of *Ralstonia solanacearum* to Streptomycin, Calcium Oxide, Mancozeb and Synthetic Fertilizer

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ABSTRACT

Tobacco bacterial wilt caused by *Ralstonia solanacearum* is one of the most destructive diseases suffered to tobacco in China. Streptomycin, calcium oxide, mancozeb and synthetic fertilizer are four important non-target chemicals used frequently during tobacco growing period. This study has evaluated the activities of four this chemicals against *R. solanacearum*. Phenotypic pattern of *R. solanacearum* under pressure of those chemicals are also compared with control treatments. *Ralstonia solanacearum* was more sensitive to mancozeb (EC_{50} value of 3.80 μg mL^{-1}), less sensitive to streptomycin (EC_{50} value of 32.06 μg mL^{-1}) and not sensitive to calcium oxide and synthetic fertilizer (EC_{50} values >1000 μg mL^{-1}). Under greenhouse condition the disease severity was reduced by 94.96 and 63.03% with mancozeb and streptomycin treatment, respectively. Metabolic reaction of *R. solanacearum* on Biolog GEN III Microplates was not or poorly affected by synthetic fertilizer or calcium oxide while significantly inhibited by mancozeb and poorly inhibited by streptomycin.

Key words: Biolog GEN III MicroPlate, mancozeb, phenotypic reaction, tobacco bacterial wilt

INTRODUCTION

Tobacco bacterial wilt caused by *Ralstonia solanacearum* (Smith) is an important soil-borne disease in tobacco (*Nicotiana tabacum* L.) (Li *et al*., 2011). It has a worldwide distribution and a large host range of more than 200 species in 50 families (Kim *et al*., 2003). This disease is a major constraint in the production of tobacco throughout the world (Alvarez *et al*., 2010). China accounts for more than 39.6% of the total global tobacco production (Wang *et al*., 2013) and for more than 40% of the global tobacco consumption (British American Tobacco, 2012). In the last five years, the disease is wide spread and has become a serious problem for tobacco production in China, especially in flue-cured tobacco growing regions (Peng *et al*., 2007; Chen *et al*., 2013). Losses can reach 100% if disease management practices are not used.

In China, tobacco bacterial wilt is managed through the use of resistant cultivars, crop rotation, cultural practices and the antibiotic streptomycin. During tobacco production proceeding, farmers normally add calcium oxide to the soil to raise soil pH to the ideal range for tobacco production and use tobacco specific synthetic fertilizer with all three N:P:K plant nutrients (10:10:25) to provide nutrition for flue-cured tobacco plants (Table 1). The fungicide mancozeb has been frequently sprayed to leaves for bacterial disease management and is used together with metalaxyl for controlling tobacco black shank in China. Due to only protective activity and no systemic activity of this chemical, mancozeb has seldom been used for soil-borne bacterial disease management. Due to high aggressiveness and variation of *R. solanacearum* bacterial wilt is still a serious problem for farmers in China. Whether these chemicals could enhance the prevalence and severity of this disease is still unclear. So far, limited information actually

A1 Negative control	A2 Dextrin	A3 D-maltose	A4 D-trehalose	A5 D-cellobios	A6 Gentiobiose	A7 Scurose	A8 D-turanose	A9 Stachyose	A10 Positive control	A11 pH 6	A12 pH 5
B1 D-raffinose	B2 α-D-lactose	B3 α-D-lactose	B4 β-mthayl-D-glucose	B5 D-salicin	B6 N-acetyl-D-glucossamin	B7 N-acetyl-β-D-mamosamin	B8 N-acetyl-D-galactosamin	B9 N-acetyl neuraminic	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-glocose	C2 D-mannose	C3 D-fructose	C4 D-galactose	C5 3-methyl glucose	C6 D-fucose	C7 L-fucose	C8 L-rhamnose	C9 Inosine	C10 1% sodium lactate	C11 Fusidic acid	C12 D-serine
D1 D-sorbitol	D2 D-mannitol	D3 D-arabitol	D4 Myo-inositol	D5 glycerol	D6 D-glusose 6-PO_4	D7 D-fructose-6-PO_4	D8 D-aspartic acid	D9 D-serine	D10 Troleandomycin	D11 Rifamycin sv	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-prol	E3 L-alanine	E4 L-arginine	E5 L-aspartic acid	E6 L-glutamin acid	E7 L-histidine	E8 L-pyroglutamic acid	E9 L-serine	E10 Linocymcin	E11 guanidine Hcl	E12 Niaproof 4
F1 Pectin	F2 D-galaturonic	F3 D-galatonic acid lactone	F4 D-gluconic acid	F5 D-glucuronic acid	F6 Glucurona mide	F7 Mucic acid	F8 Quinic acid	F9 S-saccharic acid	F10 Vancomycin	F11 Tetrazolium violet	F12 Tetrazolium blue
G1 p-hydroxy-phenylatic acid	G2 Methyl pyruvate	G3 D-lactic acid	G4 L-lactic acid	G5 Citric acid	G6 α-keto-glutaric acid	G7 D-malic acid	G8 L-malic acid	G9 Bromo-succinic	G10 Nalidixic acid	G11 Lithium chloride	G12 Potassium tellurite
H1 Tween 40	H2 γ-amino-butryric acid	H3 α-hydroxy-butryric acid	H4 β-hydroxy-D lybutyric	H5 α-keto-butyric acid	H6 Acetoacetic acid	H7 Propionic acid	H8 Acetic acid	H9 Formic acid	H10 Aztreonam	H11 Sodium butyrate	H12 Sodium bromate

Fig. 1: Layout of assays in biolog GEN III MicroPlate

Table 1: Use dosage of four agronomic chemicals in flue-cured tobacco production seasons in Southwest China

	Chemical use rate		
Chemicals	Per application	Per season	Target diseases
Streptomycin	210-420 g ha^{-1}	≥3 applications	Tobacco bacterial wilt
Mancozeb	2000-2500 g ha^{-1}	≥3 applications	Tobacco black shank
Calcium oxide	750-3000 kg ha^{-1}	1 application	Tobacco bacterial wilt, tobacco black shank
Tobacco specific synthetic fertilizer	750 kg ha^{-1}	1 application	None

outlines the activities of calcium oxide, synthetic fertilizer, streptomycin and mancozeb to soil-borne pathogen of *R. solanacearum*. Lack of evidence for dosage use of calcium oxide and fertilizer guides bacterial wilt management in the field. Recently, the Biolog GEN III MicroPlate was introduced for characterizing of bacterial pathogen (GEN III MicroPlate™ Instruction for use) (Mienda and Huyop, 2013; Wang *et al*., 2012). The MicroPlate uses 94 biochemical tests (Fig. 1) to profile substrate utilization and metabolite profiling of the pathogen *R. solanacearum* under different chemical pressures and could be also used for mode of action studies of different chemicals from phenomics.

The objectives of this study were (1) To investigate *in vitro* activities of four agronomic chemicals (streptomycin, calcium oxide, mancozeb and synthetic fertilizer) against *R. solanacearum* and (2) To evaluate the metabolic reaction of *R. solanacearum* under pressures of those chemicals from phenomics. The outcome of this study will provide useful information to know the activity of *R. solanacearum* under pressure of those chemicals and will supply important information for tobacco bacterial wilt management in the field.

MATERIALS AND METHODS

Pathogen, media and chemical preparation: Isolates of *R. solanacearum* with wild-type sensitivity and pathogenicity to tobacco were selected as the species for assay in this study. They were collected and isolated by semi-selective medium (SMSA) (Engelbrecht, 1994) from diseased tobacco stem with typical bacterial wilt symptom in a tobacco commercial field of the Guizhou province of China in 2011. Bacterial suspension was incubated in a flask at 30°C in darkness with the speed of 170 rpm for 48 h, the final concentration was adjusted to 5×10^8 CFU mL^{-1}. Nutrient Agar (NA) and Nutrient Broth (NB) containing 0.3% beef extract, 0.5% peptone and with or without 1.5% agar was prepared according to Bonjar *et al.* (2006). Biolog Universal Growth medium plus Blood agar (BUG+B) (Catalog No. 71102-BUG agar with 5% sheep blood) and Inoculating fluid A (Catalog No. 72401-IF-A) were purchased from Biolog (Biolog Inc., Hayward, 1964). All culture media were autoclaved prior to use. Stock chemical solutions were prepared by dissolving technical grade streptomycin

(Sigma China Co., Ltd, Shanghai, China), technical grade calcium oxide (a.i. 85%, Jinfu Co., Ltd, Fuquan, China), technical grade mancozeb (a.i. 80%, DuPont (China) Co., Ltd) and tobacco synthetic fertilizer (45%, N:P:K = 10:10:25; Jinfu Co., Ltd, Fuquan, China) in distilled water. Chemical solutions were diluted as required and stored at 4°C to preserve chemical activity in the dark. They were added to test mediums after autoclaving when culture medium had cooled to approximately 50°C.

Molecular identification of *Ralstonia solanacearum*: A loopful of suspect colonies on SMSA plate was swabbed from the agar, re-suspended in 150 μL of distilled water, boiled for 12 min in an Eppendorf tube, cooled to room temperature on ice for 8 min, centrifuged at 9000 g for 2 min and the supernatant was utilized for PCR. The flagella C gene (flic) fragments (Schonfeld *et al*., 2003) were amplified using the *R. solanacearum*-specific primers as described by Schonfeld *et al*. (2003) which yielded a 400 bp product. The PCR amplifications were conducted by thermocycler (Biorad MyCycler™; BioRad, CA, USA) in a 30 μL reaction system that contained 6 μL of boiled supernatant, 3 mM $MgCl_2$, 0.5 μM of each primer, 1 unit of *Taq* DNA polymerase (Takara, Dalian, China), 200 μM (each) deoxynucleoside triphosphate (dNTP) in 1×PCR buffer. PCR conditions were performed as follows: 95°C for 10 min, 35 cycles of 30 sec at 95°C, 120 sec at 63°C and 10 min at 72°C and a final extension at 72°C for 10 min. Products (4 μL) were loaded on 1.0% agarose (Biowest®, Spain) gels, electrophoresed at 100 V for 30 min and checked under UV transillumination (254 nm).

Biovar determination: Strains of suspect pathogen were analyzed into biovars based on their ability to utilize three disaccharides (lactose, maltose and cellobiose) and three hexose alcohols (mannitol, sorbitol and dulcitol). The characterization of the biovars was conducted according to the method as outlined in previous reports (Hayward, 1964; He *et al*., 1983). One isolate (WJ1) was chosen randomly from the isolate collection for sensitivity detection, greenhouse experiment and metabolic reaction studies.

Sensitivity detection on nutrient agar plates: The qualitative sensitivities of *R. solanacearum* to chemicals were assayed on NA plates amended with different concentrations of the tested compounds previously dissolved in distilled water. A 30 mL of fresh bacterial liquid was dipped onto sterile round pieces of paper (4 mm in diameter). After inoculation, the paper was put in the center of a plate. The final concentrations tested were for streptomycin 0, 1, 10, 100 and 200 μg mL^{-1}, for calcium oxide 0, 125, 250, 500, 1000 and 2000 μg mL^{-1}, for mancozeb 0, 6.25, 12.5, 25, 50 and 100 μg mL^{-1} and for synthetic fertilizer 0, 31.25, 62.5, 125, 250, 500 and 1000 μg mL^{-1}. For each chemical at each concentration, three replicates were used. The plates were incubated at 30°C in darkness for 48 h and the colony growth of *R. solanacearum* around inoculated paper was observed (Xu *et al*., 2010). The experiments were conducted twice.

Sensitivity detection in the nutrient broth: The quantitative sensitivities of *R. solanacearum* to chemicals were assayed in NB amended with different concentrations of the tested compounds. One hundred microlitre of bacterial suspensions were added into a sterile tube amended with 5 mL of test nutrient broth at series concentrations. The final concentrations tested were for streptomycin 0, 6.25, 12.5, 25, 50, 100 and 200 μg mL^{-1}, for calcium oxide 0, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 μg mL^{-1}, for mancozeb 0, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 μg mL^{-1}, for synthetic fertilizer 0, 3.19, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 μg mL^{-1}. For each chemical at each concentration, three replicates were conducted. After inoculation, all tubes were incubated at 30°C in darkness with the speed of 170 rpm for 48 h. Absorbance values at 600 nm of test bacterial suspensions were measured by a turbidimeter. The experiments were conducted twice.

Metabolic reaction of *R. solanacearum* under pressure of chemicals: Metabolic reactions of *R. solanacearum* under different pressures of the chemicals were conducted by the Biolog system (Biolog Inc., Hayward, 1964). In preparation for analysis, *R. solanacearum* was incubated on (BUG+B) media plates at 30°C for 48 h, cells were swabbed from the plates and suspended in distilled water to a final concentration of 1×10^8 CFU mL^{-1}. Streptomycin, mancozeb, calcium oxide and synthetic fertilizer were added into inoculating fluid A to get a final concentration of 100, 20, 2000 and 2000 μg mL^{-1}, respectively. The GEN III MicroPlate test panel, containing 71 carbon source utilization assays (Fig. 1, columns 1-9) and 23 chemical sensitivity assays (Fig. 1, columns 10-12), were used for analysis according to the manufacturer's instructions throughout the experiment. Readings were recorded for 4 days and kinetic data were analyzed with OmniLog-PM software (Shrestha *et al*., 2013). This software generates time course curves for respiration and calculates differences in the areas for test pathogen under different chemical pressures.

Greenhouse experiment in planta: Experiments were conducted with highly susceptible cv. Honghua Da Jin Yuan in a greenhouse maintained at 20-30°C. Tobacco seedlings were cultivated and grown from seedlings at one plant per 0.2 L pot with sandy loam soil. They were used for test when six leaves were fully developed 5 weeks later. Forty milliliters of each chemical solution and of distilled water was added to the pot and 24 h later, 40 mL bacterial solution at a concentration of 1×10^8 CFU mL^{-1} was added to each pot. The distilled water treatment was regarded as control. The final concentrations tested were for streptomycin 1000 μg mL^{-1}, for calcium oxide 2000 μg mL^{-1}, for mancozeb 1000 μg mL^{-1} and for synthetic fertilizer 1000 μg mL^{-1}. After inoculation, tobacco plants were incubated at 30°C in the greenhouse for disease development. Twelve plants were treated per treatment and the experiment was conducted twice. Results were obtained 20 days after inoculation based on a 0 to 5 scale: 0, no visible disease symptoms; 1, leaves slightly wilted with brownish lesions beginning to appear on stems; 2, 30 to 50% of entire plant diseased; 3, 50 to 70% of entire plant diseased;

4, 70 to 90% of entire plant diseased and 5, plant dead (Wang *et al*., 2012). Disease incidences were calculated as number of diseased plants/number of total plants. Disease index was calculated as:

$$\frac{\text{Sum of (Number of the scaled plants}\times\text{Number of the scale)}}{5\times\text{number of plants detected}}\times 100$$

Percentage efficacy values were calculated as:

$$\frac{\text{Disease index (untreated)-Disease index (treated)}}{\text{Index (untreated)}}\times 100$$

Data analysis: Results from repeated experiments were combined for analysis since variances between experiments were homogeneous. All data were processed with the SIGMASTA Statistical Software Package (SPSS Science, Ver. 11). The concentration of each chemical causing 50% (EC_{50}) or 90% (EC_{90}) reduction in the growth of *R. solanacearum* compared to the absence of the compound was estimated from the fitted regression line of the log-transformed percentage inhibition plotted against the log-transformed compound concentration (Brandt *et al*., 1988; Wang *et al*., 2009). The values from the different replicates per treatment in each experiment were analyzed with Duncan's multiple comparison test ($p = 0.05$).

RESULTS

Isolation, identification of the pathogen and assignment to biovar: All suspect *R. solanacearum* strains were isolated from tobacco stems with symptoms of typical wilt. No more than two isolates per field site were conducted in our study. By incubation of the infected tissues nieces on selective media of SMSA, a total of 12 single tissue isolates of *R. solanacearum* were isolated. All isolates exhibited the typical colony morphology on the test medium. Moreover, all strains presented fragments of the expected targeting flic product (400 bp size). These results confirmed the correct assignment of the isolates from diseased tobacco stems to *R. solanacearum*.

The color changed from blue to yellow, indicating the oxidization of sugars by the tested bacterial isolates. Result in biovar test presented that all *R. solanacearum* isolates metabolized disaccharides (lactose, maltose and cellobiose) and sugar alcohols (mannitol, sorbitol and dulcitol) within several days' incubation. Therefore, all isolates of *R. solanacearum* tested in this study belong to biovar 3.

Sensitivity detection on nutrient agar plates: Four chemicals presented various sensitivities to *R. solanacearum* on NA plates. Among the agronomic chemicals tested, the bacterial growth of *R. solanacearum* was more sensitive to mancozeb, followed by streptomycin and less sensitive to calcium oxide and synthetic fertilizer. For 2000 μg mL^{-1} calcium oxide medium and 500 μg mL^{-1} synthetic fertilizer medium, the pathogen could still grow on the plate. In comparison, mancozeb and streptomycin exhibited higher activities to *R. solanacearum* on NA plates. In our test system, the bacterium could not grow on 50 μg mL^{-1} mancozeb plates and 200 μg mL^{-1} streptomycin plates.

Sensitivity detection in the nutrient broth: Inhibition of bacterial growth was highest with mancozeb and streptomycin, with EC_{50} values of 3.80 and 32.06 μg mL^{-1} and EC_{90} values of 21.48 and 151.32 μg mL^{-1}, respectively (Table 2). In this test system, bacterial growth was not affected by calcium oxide, with EC_{50} values of more than 1000 μg mL^{-1} (Fig. 2). In comparison, the pathogen growth could be enhanced by synthetic fertilizer (Fig. 2). With enhancement of the chemical, higher turbidity of bacterial suspension reached.

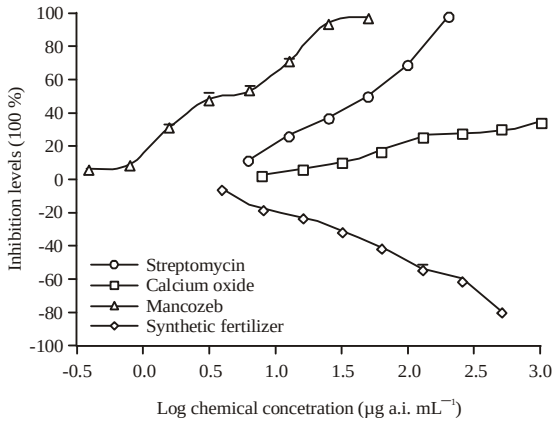

Fig. 2: Influence of dose on inhibition of the growth of *Ralstonia solanacearum*. The experiment was conducted in the nutrient broth at 30°C in darkness, absorbance values of each treatment were detected 48 h after incubation. Error bars represent one standard deviation for replicate treatments

Table 2: EC_{50}, EC_{90} and MIC values of four agronomic chemicals against the growth of *Ralstonia solanacearum*[a]

EC_{50}, EC_{90} and MIC values (μg.a.i mL^{-1})	Chemicals			
	Streptomycin sulphate	Calcium oxide	Mancozeb	Synthetic fertilizer
EC_{50}	32.06	>1000	3.80	-
EC_{90}	151.32	>1000	21.48	-
MIC	200.00	>2000	50.00	-

[a]EC_{50}, EC_{90} and MIC values are the concentrations of each chemical causing 50, 90% reduction and fatal dose, respectively, against the growth of *Ralstonia solanacearum* compared to the absence of a chemical, -: No effect

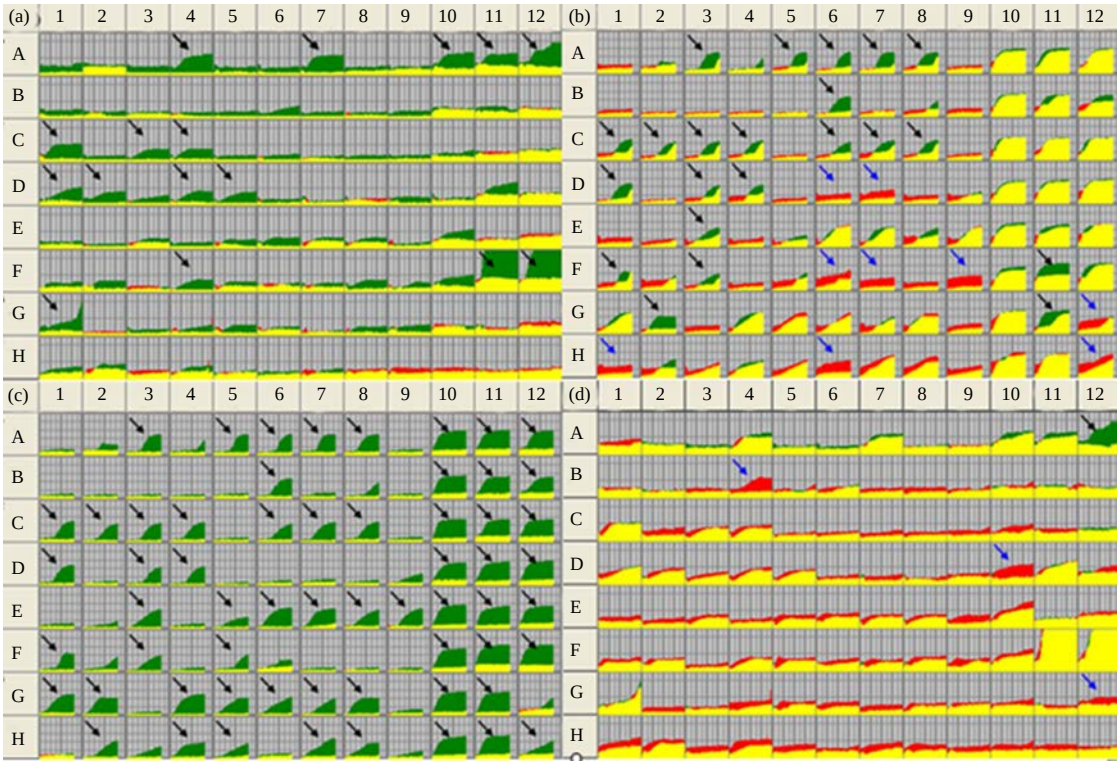

Fig. 3(a-d): Metabolic reaction comparisons of *Ralstonia solanacearum* between control treatment and four agronomic chemical treatments, (a) Comparison between control and 100 μg mL^{-1} of streptomycin, (b) Comparison between control and 2000 μg mL^{-1} of calcium oxide, (c) Comparison between control and 20 μg mL^{-1} of mancozeb and (d) Comparison between control and 2000 μg mL^{-1} of synthetic fertilizer. Green presented metabolic fingerprint of control treatments; red in (1), (2), (3) and (4) presented metabolic fingerprint of streptomycin, calcium oxide, mancozeb and synthetic fertilizer, respectively; yellow presented common fingerprints of both control and chemical treatments. The black arrow presented inhibition and the blue arrow exhibited promotion

Metabolic reaction of *R. solanacearum* under pressure of chemicals: Metabolic reaction of *R. solanacearum* was conducted by Biolog system. When incubated on GEN III MicroPlate without chemical, 46 out of 71 carbon sources supported its growth and all chemical sensitivity tests exhibited positive reaction (Table 3). The typical carbons metabolized by *R. solanacearum* included D-maltose, D-cellobiose, gentiobiose, sucrose, D-turanose, N-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-fucose, L-rhamnose, D-sorbitol, D-arabitol, myo-inositol, L-alanine, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, pectin, L-galactonic acid lactone, D-glucuronic acid, p-hydroxy-phenylacetic acid, methyl pyruvate, L-lactic acid, citric acid, α-keto-glutaric acid, D-malic acid, L-malic acid, γ-amino-butryric acid, β-hydroxy-D, L-butyric acid, α-keto-butyric acid, propionic acid and acetic acid (Fig. 3). In comparison, when incubation under the pressure of mancozeb at 20 μg mL^{-1}, none carbons in the GEN III plate could be utilized by the pathogen and all chemical sensitivity assays exhibited negative reaction (Fig. 3). when incubation under the pressure of streptomycin at 100 μg mL^{-1}, five carbons were poorly metabolized by the pathogen, the carbons were γ-amino-butryric acid, β-hydroxy-D, L. butyric acid, α-keto-butyric acid, propionic acid and acetic acid; additionally, four chemical sensitivity assays were inhibited by the chemical, including tests of pH 5, pH 6, tetrazolium violet and tetrazolium blue (Fig. 3). When incubation under the pressure of calcium oxide at 2000 μg mL^{-1}, the pathogen could metabolize 27 carbons (Fig. 3), all chemical sensitivity tests exhibited positive reaction. In comparison, when incubation under the pressure of synthetic fertilizer at 2000 μg mL^{-1}, the carbons utilized by the pathogen were the same as that of control and all chemical sensitivity assays exhibited positive reaction.

Greenhouse experiment in planta: In the greenhouse, mancozeb significantly ($p<0.05$) reduced the severity of tobacco bacterial wilt, followed by streptomycin (Table 4), whereas, calcium oxide and synthetic fertilizer exhibited much

Table 3: Carbon source utilization and chemical sensitivity assays of *R. solanacearum* under pressure of chemicals

	Chemicals				
Carbon sources	Control	Streptomycin sulphate	Calcium oxide	Mancozeb	Synthetic fertilizer
Dextrin	±	±	±	±	±
D-Maltose	+	+	±	-	+
D-Trehalose	-	-	-	-	+
D-Cellobiose	+	+	-	-	+
Gentiobiose	+	+	-	-	+
Sucrose	+	-	-	-	+
D-Turanose	+	+	-	-	+
Stachyose	-	-	-	-	-
D-Raffinose	-	-	-	-	-
α-D-Lactose	-	-	-	-	-
D-Melibiose	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-
D-Salicin	-	-	-	-	-
N-Acetyl-D-Glucosamine	+	+	-	-	+
N-Acetyl-β-D-Mannosamine	-	-	-	-	-
N-Acetyl-D-Galactosamine	±	±	±	-	±
N-Acetyl neuraminic acid	-	-	-	-	-
α-D-Glucose	+	-	±	-	+
α-D-Glucose	+	+	±	-	+
D-Fructose	+	-	±	-	+
D-Galactose	+	-	±	-	+
3-Methyl glucose	-	-	-	-	-
D-Fucose	+	+	±	-	+
L-Fucose	+	+	±	-	+
L-Rhamnose	+	+	±	-	+
Inosine	-	-	-	-	-
D-Sorbitol	+	-	±	-	+
D-Mannitol	-	-	-	-	-
D-Arabitol	+	+	±	-	+
myo-Inositol	+	-	±	-	+
Glycerol	-	-	-	-	-
D-Glucose- 6-PO_4	-	-	±	-	-
D-Fructose-6-PO_4	-	-	±	-	-
D-Aspartic acid	-	-	-	-	-
D-Serine	±	±	±	-	±
Gelatin	-	-	±	-	-
Glycyl-L-Proline	-	-	-	-	-
L-Alanine	+	+	±	-	+
L-Arginine	-	-	-	-	-
L-Aspartic acid	±	±	±	-	±
L-Glutamic acid	+	+	+	-	+
L-Histidine	+	+	+	-	+
L-Pyroglutamic acid	+	+	+	-	+
L-Serine	+	+	+	-	+
Pectin	+	+	±	-	+
D-Galacturonic acid	±	±	±	-	±
L-Galactonic acid lactone	+	+	±	-	+
D-Gluconic acid	-	-	-	-	-
D-Glucuronic acid	+	+	+	-	+
Glucuronamide	±	±	±	±	±
Mucic acid	-	-	±	-	-
Quinic acid	-	-	-	-	-
D-Saccharic acid	-	-	±	-	-
p-Hydroxy-Phenylacetic acid	+	-	+	-	+
Methyl pyruvate	+	+	-	-	+
D-Lactic acid methyl ester	-	-	-	-	-
L-Lactic acid	+	+	+	-	+
Citric acid	+	+	+	-	+
α-Keto-Glutaric acid	+	+	+	-	+
D-Malic acid	+	+	+	-	+
L-Malic acid	+	+	+	-	+
Bromo-Succinic acid	-	-	-	-	-
Tween 40	±	±	±	±	±
α-Amino-Butyric acid	+	+	+	-	+

Table 3: Continue

Carbon sources	Chemicals				
	Control	Streptomycin sulphate	Calcium oxide	Mancozeb	Synthetic fertilizer
α-Hydroxy-Butyric acid	±	±	+	-	±
β-Hydroxy-D,L-Butyric acid	+	+	+	-	+
α-Keto-Butyric acid	+	+	+	-	+
Acetoacetic acid	±	±	+	±	±
Propionic acid	+	+	+	±	+
Acetic acid	+	+	+	±	+
Formic acid	±	±	±	±	±
Chemical sensitivities					
pH 6	+	-	+	±	+
pH 5	+	-	+	±	+
1% NaCl	+	+	+	±	+
4% NaCl	+	+	+	±	+
8% NaCl	+	+	+	±	+
1% Sodium lactate	+	+	+	-	+
Fusidic acid	+	+	+	±	+
D-Serine	+	+	+	±	+
Troleandomycin	+	+	+	±	+
Rifamycin SV	+	+	+	±	+
Minocycline	+	+	+	±	+
Lincomycin	+	+	+	-	+
Guanidine HCl	+	+	+	-	+
Niaproof 4	+	+	+	±	+
Vancomycin	+	+	+	±	+
Tetrazolium violet	+	-	+	±	+
Tetrazolium blue	+	-	+	±	+
Nalidixic acid	+	+	+	-	+
Lithium chloride	+	+	+	-	+
Potassium tellurite	±	±	+	±	+
Aztreonam	+	+	+	±	+
Sodium butyrate	+	+	+	±	+
Sodium bromate	±	±	+	±	±

-: *Ralstonia solanacearum* cannot grow or grows poorly in the well tested, +: *R. solanacearum* grows well in the Well tested, ±: Pathogen grows at middle level in the well tested

Table 4: Effect of mancozeb, streptomycin, calcium oxide and synthetic fertilizer on the control of tobacco bacterial wilt under greenhouse conditions

Treatments	Disease incidence (%)[a]	Disease index[b]	Efficacy (%)[c]
Non-treated control	100.00[ad]	66.11[a]	-
Mancozeb	16.67[c]	3.33[c]	94.96
Streptomycin	63.89[b]	24.44[b]	63.03
Calcium oxide	100.00[a]	56.11[a]	15.12
Synthetic fertilizer	100.00[a]	62.22[a]	5.88

[a]Percentage disease incidences were calculated as number of diseased plants/number of total plants, [b]Disease index was calculated as 100×[summer of (number of the scaled plants×number of the scale)]/[5×number of plants detected], [c]Percentage efficacy values were calculated as 100×[disease index (untreated)-disease index (treated)]/incidence (untreated), [d]Means followed by different letters in the column are significantly different according to Duncan's multiple range test at p = 0.05

poor inhibition effect. Disease severity was reduced by 94.96% with mancozeb treatment and by 63.03% with streptomycin treatment, respectively (Table 4). There were no significant differences among the treatments of calcium oxide, synthetic fertilizer and distilled water. Additionally, no chlorosis or any other damage symptoms were observed on tobacco plants as a result of the chemical treatments throughout the experiments.

DISCUSSION

Bacterial wilt disease is endemic in all the tropical and subtropical areas and difficult to control because of the soil-borne nature of the bacterium. The most effective methods include the use of resistant crop varieties, crop rotation and chemicals (Feng *et al*., 2012). To establish an efficient disease management strategy for the control of tobacco bacterial wilt, it is important to know the interaction between environmental factors and the pathogen of *R. solanacearum*. As a first step, the activities of agronomic chemicals to the pathogen have to be established. Current analysis in this study has shown the strengths and weaknesses of four major agronomic chemicals (streptomycin, calcium oxide, mancozeb and synthetic fertilizer) against *R. solanacearum in vitro*, against bacterial wilt in planta and evaluated the metabolic fingerprints of the pathogen under different pressures of those chemicals to know more detail about their effects.

Bacterial wilt caused by *R. solanacearum* is a great threat to many important crops in China, especially for tobacco (Lu, 1998; Xu *et al*., 2009). Huge economic losses happened each year throughout the country (Lu, 1998; Xu *et al*., 2009). Isolates of *R. solanacearum* have been assigned to biovar 2, biovar 3, biovar 4, biovar 5 and biovar 6 in China (Xue *et al*., 2011), with host plants of peanut, tobacco, potato, casuarina, eggplant, ginger, mulberry, ramie, pepper, sesame, night shade, sweet potato and tomato. While for host of tobacco, just biovar 3 and biovar 4 of the isolates were detected in China (Liu *et al*., 2008; Xue *et al*., 2011). In our study, only biovar 3 isolates were found from tobacco in Guizhou province. This finding is in accordance with the results detected by other scientists mentioned above. Biovar 3 of *R. solanacearum* should be a major pathogen for bacterial wilt in tobacco in China.

Among the agronomic chemicals tested, the bacterial growth of *R. solanacearum* was more sensitive to mancozeb, followed by streptomycin and less sensitive to calcium oxide and synthetic fertilizer. Corresponding findings had also been found in metabolic reaction tests on Biolog GEN III MicroPlates. In our test, the GEN III MicroPlate which had 94 discrete substrates, containing 71 carbon sources utilization assays and 23 chemical sensitivity assays was introduced to test the metabolites of *R. solanacearum* under four agronomic chemical pressures. Culture growth of the pathogen was monitored by change in absorbance in each well. Phenotypic reaction was created with spotfire visualization software, data of each well were collected automatically during incubation and variations between control and treatments were compared. Data from the plates presented that calcium oxide, synthetic fertilizer and control exhibited no inhibition on carbon utilization and had no effect in chemical sensitivities test. However, at the pressure of 100 μg mL^{-1} streptomycin, the numbers of carbon sources that *R. solanacearum* used and of chemical sensitivity assay that showed positive reaction reduced greatly. Bacterial growth of the pathogen was less inhibited in those assays. In comparison, at the pressure of 20 μg mL^{-1} mancozeb, *R. solanacearum* could not use any carbon sources and showed negative reaction on all chemical sensitivity assays, indicating that mancozeb had high activity against *R. solanacearum*. Mancozeb is normally used as protectant fungicide with multisite inhibitory activity against pathogens that acts as a non-specific thiol reactant (Mills *et al*., 2004). Meanwhile, the protective efficacy of mancozeb against tobacco bacterial wilt in the greenhouse showed that it may have potential in tobacco bacterial wilt management in the field.

Streptomycin, an aminocyclitol glycoside antibiotic was first discovered in the 1950s (Sundin and Bender, 1993) and then used to control bacterial diseases of plants, including tobacco bacterial wilt and tobacco wildfire (caused by *Pseudomonas syringae* pv. *tabaci*). Its mode of action to bacteria is presumably the binding to the 30S ribosomal subunit, thereby interfering with polypeptide synthesis and thus inhibiting translation (Sundin, 2002). While activities of streptomycin have been investigated extensively to bacterial pathogens (Rooke and Shattock, 1983; Liu, 2001; Lee *et al*., 2005), the exact mechanism by which streptomycin influences the metabolism of bacteria is seldom reported (Chabane *et al*., 1996; Whittaker *et al*., 1996). After exposure to 100 μg mL^{-1} streptomycin, *R. solanacearum* showed lots of positive reaction on GEN III plates and could still use some carbon sources on the plates. Though in certain cases, toxicity may be different due to discrepancies between culture media, test concentrations, time of dosage and duration of contact with the pathogen. Results from this study still provided useful information for our understanding the activity of streptomycin against *R. solanacearum* and could in some case explain the poor efficacy of this chemical against bacterial wilt in the field.

Calcium is an important nutritional element that plays a major role in plant disease resistance system and various studies have demonstrated the mitigating effect of calcium for disease management (Bateman and Lumsden, 1965; Volpin and Elad, 1991). Tobacco in southwest China is mainly located in semi-arid areas over acid or neutral soils. The CaO and $CaCO_3$ are sometimes applied in the soil to enhance botanical composition and production of flue-cured tobacco. In our study, the result showed that calcium oxide at the concentration of 2000 μg mL^{-1} was not obvious inhibitory of *R. solanacearum in vitro*, *in planta*, nor its metabolism on carbon sources, indicating that calcium oxide cannot be directly applied to control of bacterial wilt and its role may be improving the soil environment of tobacco or induce the plant to get resistance against the pathogen (Jiang *et al*., 2013). Further research is needed to confirm the ability of calcium oxide to enhance bacterial wilt management by field trials. Additionally, to our best of knowledge, few researches have been conducted to the fungicidal value of synthetic fertilizer to *R. solanacearum*. In this test system, synthetic fertilizer had some promoting effect on the growth of *R. solanacearum* and did not affect the metabolism of the pathogen. It may provide some nutrition for the growth of the pathogen and may enhance the epidemic of bacterial wilt in the field, assays *in planta* in the greenhouse of this study presented the relationship between fertilizer and disease incidence.

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