Isolation of Methyl Gallate from *Toxicodendron sylvestri* and Its Effect on Tomato Bacterial Wilt

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Abstract

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We determined that the methanol extract of *Toxicodendron sylvestri* could significantly inhibit *Ralstonia solanacearum* in vitro and in planta. One compound responsible for the antibacterial activity was isolated from the ethyl ether extract and identified as methyl gallate (MG) based on its ^1H and ^{13}C nuclear magnetic resonance data as well as mass spectroscopy. MG displayed broad-spectrum activity against plant-pathogenic bacteria, and strong inhibitory effects on the growth of plant-pathogenic oomycetes. The half inhibition concentration of MG on *R. solanacearum* was 8.3 mg/liter, which was tested by the agar

dilution method. Disease control trials in planta showed that both natural and synthetic MG could effectively reduce the incidence of tomato bacterial wilt, and there was no significant difference between them in control efficacy. This is the first report on the use of MG for the control of a plant bacterial disease. Because of its broad-spectrum antimicrobial activity and significant control efficacy on tomato bacterial wilt in planta, MG shows potential to be used as a bactericide to control plant bacterial wilt.

Plant bacterial wilt, caused by *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1996, is an important disease distributed throughout the tropical, subtropical, and some warm regions of the world, and it can also occur in cooler climates, such as at high elevations in the tropics or high latitudes (12,23). Bacterial wilt is difficult to control, particularly race 1, due to its broad host range. Methods used to control other plant diseases fail with bacterial wilt. Breeding for resistance has not yet resulted in useful varieties (14), disease control through crop rotation fails with this broad-host-range pathogen (14), and biological control is still in its infancy (23). Grafting onto resistant root stocks can control this disease but it is expensive (29). Chemical control, which is used for many plant diseases, could aid in control of bacterial wilt but, at present, few bactericides are registered for control of bacterial wilt in China. It is desirable to develop new bactericides to manage the destructive disease.

A diverse range of plant natural products has been used, either directly or as lead molecules, for the development of new agrochemicals. These products have enormous potential to inspire modern agrochemical research (7). Numerous plant constituents, including antimicrobial protein, 2,β-D-glucogallin, protease inhibitor, α-(L)-terpineol, paeonol, 2-phenylethanol, and 2-phenylacetic acid, have been reported to inhibit the growth of *R. solanacearum* in vitro (15,18,19,21,32,36). Furthermore, soil treatment with thymol or palmarosa oil effectively reduces *R. solanacearum* population and bacterial wilt incidence (WI) on tomato in greenhouse experiments (28), and thymol treatment as biofumigant significantly reduces the disease incidence and increases tomato yield under field conditions (14). However, except for plant essential oils, few plant compounds have been used to control plant bacterial wilt in planta until now.

In preliminary screening of plants for antibacterial activity against three plant bacterial pathogens, we found that extract of the leaves of *Toxicodendron sylvestri* (Sieb. et Zucc.) O. Kuntze could

significantly inhibit *R. solanacearum* in vitro and in planta, and the application of the leaf extract could stimulate the growth of tomato (34,35). *T. sylvestri*, in the family Anacardiaceae, is widespread south of the Yangtze River in China and also is found in parts of Korea and Japan. It is a traditional folk medicinal plant with broad use in China, including promoting blood circulation and stopping pain, with anti-inflammatory and detumescence activity (9).

At present, there are no reports on the antimicrobial constituents of *T. sylvestri*. The objective of this study was to isolate and identify the compound responsible for the antibacterial activity against *R. solanacearum* from *T. sylvestri* leaf extract, and to evaluate the effects of selected compounds for control of tomato bacterial wilt in planta.

Materials and Methods

Strains tested. *R. solanacearum* strain Rs-T02 (phylotype I, sequevar 14) was used in the screening of prospective plant constituents and greenhouse experiments. For determining the antibiotic spectrum of the antimicrobial compound, plant pathogen strains were used as listed in Tables 1 and 2. These strains were isolated in Guangxi and provided by Plant Pathology Research Institute of Guangxi University, Nanning, China.

Preparation of the methanol extract of *T. sylvestri*. *T. sylvestri* leaves were collected from the Mashan district in Nanning, Guangxi, China in 2009. The leaves were air dried, then ground into powder (15,000 g) and soaked in methanol at room temperature. Methanol solution was evaporated under reduced pressure to obtain 2,400 g of crude extract.

Isolation and purification. The extract was suspended in water (1:1 by volume) at 40°C and extracted four times with petroleum ether. The petroleum ether extract was evaporated to remove the solvent, and the remaining aqueous layer was successively extracted with ethyl ether, ethyl acetate, and n-butanol. Each extract was concentrated to dryness and tested for antibacterial activity against *R. solanacearum* in vitro. The solvent extract portion, which had strong inhibitory effects on the pathogen, was subjected to silica gel column chromatography with successive elution with solvents at increasing orders of polarity (3,7). The fractions, giving similar thin-layer chromatography (TLC) profiles, were combined and tested for antibacterial activity in vitro. The putative antibacterial compound was purified by a recrystallization method, and the purity was determined with high-

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performance liquid chromatography (HPLC) by the peak area normalization method.

Structure identification. The chemical structure of the compound isolated from *T. sylvestre* was identified by using spectroscopic analysis. Mass spectrum was recorded by electrospray ionization mass spectrometry (ESI-MS). Nuclear magnetic resonance (NMR) spectra were recorded on a superconducting fourier transform NMR spectrometer (INOVA, 600 MHz; Varian, America) at 600 MHz for ^1H and at 150 MHz for ^{13}C , with TMS as an internal standard.

Determination of in vitro antibacterial activity. Antibacterial activity of the plant compounds was tested by the agar well diffusion method (2,4,35). About 25 ml of nutrient agar medium containing test bacteria (10^8 CFU/ml) was poured into 90-mm-diameter petri plates; 5-mm-diameter wells were punched out by a cork borer from the medium after solidification and filled with 50 μl of the compound solution. The impure mixtures were dissolved with 10% methanol and the pure compound was dissolved with 10% dimethyl sulfoxide (DMSO). Wells loaded with the same concentration of solvent served as controls. The width of inhibition zone in millimeters around the well in each treatment was measured at 48 h after incubation at 28°C (minus 5 mm). Mean inhibition zone measurements were calculated from three replicates of each of the bacterial species.

Determination of in vitro antifungal activity. Antifungal or anti-oomycete activity of methyl gallate (MG) was determined by a mycelial radial growth inhibition test (20). Oatmeal agar (for culturing pathogenic oomycetes) and potato dextrose agar (for culturing test fungi) plates containing the compound MG were prepared using 90-mm-diameter petri plates. MG was dissolved with DMSO and final concentration of DMSO in the medium was 0.1% (vol/vol). Discs (5 mm in diameter) of the test species were excised from the periphery of young cultures and placed in the center of the dishes with their mycelial surface down. The plates were incubated at 28°C . Control plates were treated with DMSO only at a concentration of 0.1% (vol/vol). When control plates were just covered with colony growth, the colony diameter of each treatment plate was measured. Mean growth measurements were calcu-

lated from three replicates of each of the fungal or oomycete species at doses of 0.5 mg/ml. Growth inhibition of the treatment compared with the control was measured by percentage, calculated by the equation $100 \times (C - T)/C$, where C is the colony diameter of the control and T is the colony diameter of the MG-treated plate.

Comparison of antibacterial activity against *R. solanacearum* between natural and synthetic MG. In order to compare the toxicity of the natural compound isolated from *T. sylvestre* and the synthetic compound against *R. solanacearum*, the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and half inhibition concentration (IC_{50}) were determined by the agar dilution method (2,24). The compound was diluted with 10% DMSO to give a series of concentrations. The bacterial inoculum concentration was adjusted to 10^3 CFU/ml, 100 μl of the bacterial broth culture was applied evenly on the plate containing the compound at different concentrations, and final DMSO concentration of any treatments in the medium was 0.1% (vol/vol). The control was prepared simultaneously in the same way without the compound but with 0.1% DMSO. The test plates were incubated at 28°C . MIC and MBC were taken as the lowest concentration of compound at which the bacterium tested did not show visible growth after 48 and 96 h, respectively. The degree of growth inhibition was calculated by the following equation: percent inhibition = $100 \times (C_c - C_s)/C_c$, where C_s was the number of colonies surviving on each plate with a series of compound concentrations and C_c was the number of colonies surviving on the control plate. A linear regression was performed, with compound concentration as the independent variable and percent inhibition as the dependent variable. LC_{50} was calculated using the SAS probit procedure.

Determination of disease control efficacy. The test was carried out in the greenhouse. Tomato plants at the seven- to eight-true-leaf stage of 'Jinkahong', which is highly susceptible to bacterial wilt, were used. The seedlings were grown in 26-cm-diameter pots (10 per pot) in a greenhouse at $30 \pm 5^\circ\text{C}$. Natural MG was diluted to four concentrations (0.05, 0.2, 0.5, and 1 g/liter) in 0.1% methanol for solution and 0.1% Tween 20 for uniform distribution of the emulsion, while synthetic MG was diluted to 0.5 g/liter in the same solvent. Potted plants were root drenched with 100 ml of an *R.*

Table 1. Antibacterial activity of methyl gallate (MG) against bacterial plant pathogens^y

Disease	Tissue isolated	Pathogen	Strain number	Inhibition zone width (mm) ^z
Tomato bacterial wilt	Stem	<i>Ralstonia solanacearum</i>	Rs-T02	31.5 a
Bacterial leaf streak of rice	Leaf	<i>Xanthomonas oryzae</i> pv. <i>oryzicola</i>	Xoo0602	30.3 a
Angular leaf spot of wax gourd	Leaf	<i>Pseudomonas syringae</i> pv. <i>lachrymans</i>	Ps109	19.8 b
Bacterial black spot of mango	Fruit	<i>Xanthomonas campestris</i> pv. <i>mangiferaeindicae</i>	Xcm0701	18.5 b
Citrus canker	Fruit	<i>Xanthomonas citri</i> pv. <i>citri</i>	Xcc0704	16.0 c
Pepper scab	Stem	<i>Xanthomonas vesicatoria</i>	Xv0701	15.8 c
Cassava bacterial blight	Stem	<i>Xanthomonas axonopodis</i> pv. <i>manihotis</i>	Xam05	13.3 d
Bacterial soft rot of Chinese cabbage	Leaf	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	Pcc08	11.3 e

^y Concentration of MG was 5 mg/ml.

^z Means within columns followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's least significant difference test.

Table 2. Antifungal activity of methyl gallate (MG) against plant fungal or oomycete pathogens^y

Disease	Tissue isolated	Pathogen	Strain number	Mycelial growth (mm)	Inhibition (%) ^z
Pythium cottony leak of cucumber	Fruit	<i>Pythium aphanidermatum</i>	Pa04	8.0	90.7 a
Litchi downy blight	Fruit	<i>Peronophythora litchi</i>	PI09	15.4	82.9 b
Root rot of tea seedling	Root	<i>Pythium spinosum</i>	Ps05	16.0	81.8 b
Tobacco black shank	Stem	<i>Phytophthora nicotianae</i>	Pn06	30.0	66.3 c
Jasmine branch blight	Stem	<i>Phoma putaminum</i>	Pp07	81.0	10.0 d
Southern corn leaf blight	Leaf	<i>Bipolaris maydis</i>	Bm08	90.0	0.0
Mango anthracnose	Fruit	<i>Colletotrichum gloeosporioides</i>	Cg0701	90.0	0.0
Watermelon Fusarium wilt	Stem	<i>Fusarium oxysporum</i> f. sp. <i>niveum</i>	Fon04	90.0	0.0
Rice sheath blight	Stem	<i>Rhizoctonia solani</i>	Rs08	90.0	0.0
Jasmine Sclerotium blight	Stem	<i>Sclerotium rolfsii</i>	Sr07	90.0	0.0

^y Concentration of MG was 0.5 mg/ml. Mycelial growth of control was 90.0 mm.

^z Means within columns followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's least significant difference test.

solanacearum suspension (10^7 CFU/ml) and were evenly irrigated with a 250-ml solution of the test compounds at 2, 10, and 18 days after inoculation. Plants treated with 0.1% methanol and 0.1% Tween 20 were served as controls, while agricultural streptomycin sulfate (0.5 g/liter) was used as the bactericide control. Each treatment consisted of three replicates and each replicate contained 10 plants. Bacterial WI and control efficacy were assessed 26 days after inoculation. WI was calculated as the percentage of plants that were completely wilted, and control efficacy was calculated using the formula control efficacy (%) = $100 \times (\text{WI of control} - \text{WI of treatment})/\text{WI of control}$.

Statistical analysis. Data were subjected to analysis of variance using SAS software (version 6.08; SAS Institute, Cary, NC). Mean comparisons were conducted using a least significant difference (Fisher's LSD) test at $P = 0.05$. Standard error and LSD results were recorded.

Results

Isolation and identification of an antibacterial compound from *T. sylvestre*. Solvent extracts of *T. sylvestre* were screened for their antibacterial activity to determine the most effective solvent extract. At a concentration of 50 mg/ml, petroleum ether, n-Butyl alcohol, and aqueous extract showed weak inhibitory activity against *R. solanacearum*, with inhibition zone width of 7.2, 9.7, and 5.7 mm, respectively. Ethyl ether extract was the most effective antibacterial extract in the test, with an inhibition zone width of 29.2 mm, followed by ethyl-acetate extract, with an inhibition zone width of 24.0 mm. Methanol (10%) had no effect on the growth of *R. solanacearum*. The ethyl ether extract was selected for further purification.

The ethyl ether extract was subjected to silica gel column chromatography with successive elutions with petroleum ether/acetone (20:1, 10:1, 5:1, 2:1, and 1:1 by volume) and, finally, methanol. Eluates were monitored by TLC and the fractions of F12 to F15, which showed high inhibitory activity on the growth of *R. solanacearum*, were pooled and then separated through a silica gel column using chloroform/methanol (10:1 by volume). F5 to F9, which showed inhibitory activity on the growth of the pathogen,

were further separated through a silica gel column using petroleum ether/ethyl acetate (2:1 by volume). Recrystallization from 100% ethyl ether gave white crystals of compound EE-1, and the purity was over 98% by HPLC analysis.

Compound EE-1 had a formula of $\text{C}_8\text{H}_8\text{O}_5$, as determined from the ESI-MS, which exhibited an ion peak at m/z 183.1 $[\text{M}-\text{H}]^-$ (Fig. 1A). The ^1H NMR and ^{13}C NMR data were as follows (Fig. 1B and C): ^1H NMR (600 MHz, acetone- d_6): δ 7.1 (each ^1H , S, H-2, 6), 3.8 (3H, S, $-\text{OCH}_3$). ^{13}C NMR (150 MHz, acetone- d_6): δ 167.3 ($-\text{COO}$), 146.1 (C-3, 5), 138.8 (C-4), 121.9 (C-1), 109.9 (C-2, 6), 52.0 ($-\text{OCH}_3$). Therefore, the structure of EE-1 was determined to be methyl-3,4,5-trihydroxy-benzoate (MG) (Fig. 1D) (16,22).

Antimicrobial activity of MG against plant pathogens. MG inhibited the growth of all test plant-pathogenic bacteria (Table 1). Of the test bacteria, growth of *R. solanacearum* and *Xanthomonas oryzae* pv. *oryzicola* were most strongly inhibited by MG, with inhibition zones exceeding 30.0 mm in width, which were followed by *Pseudomonas syringae* pv. *lachrymans* and *X. campestris* pv. *mangiferaeindicae*, with inhibition zone widths of 19.8 and 18.5 mm, respectively. *Pectobacterium carotovorum* subsp. *carotovorum* was the least sensitive to MG, showing an inhibitory zone of 11.3 mm in width. DMSO (10%) had no effect on any tested bacteria. MG also inhibited the growth of one fungus and four oomycetes (Table 2). The highest inhibition effect was observed with *Pythium aphanidermatum* (90.7%), followed by *P. spinosum* and *Peronophythora litchi* (82.9 and 81.8%, respectively). *Phytophthora nicotianae* was the least sensitive to MG among the test oomycetes. MG was slightly inhibitory to *Phoma putaminum* (10.0%) and ineffective against *Rhizoctonia solani*, *Bipolaris maydis*, *Colletotrichum gloeosporioides*, and *Fusarium oxysporum* f. sp. *niveum*. There were no effects of 0.1% DMSO on any tested fungi and oomycetes.

Toxicity comparison of natural and synthetic MG against *Ralstonia solanacearum*. The natural MG isolated from *T. sylvestre* was active against *Ralstonia solanacearum* at an IC_{50} of 8.3, MIC of 20, and MBC of 30 mg/liter. The synthetic MG gave a similar dose-response curve to that of natural MG, with an IC_{50} of 8.8 mg/liter and the same MIC and MBC values as that of natural

Fig. 1. A, Electrospray ionization mass spectrum; B, ^1H nuclear magnetic resonance (NMR) spectrum; C, ^{13}C NMR spectrum; and D, structure of compound EE-1

Table 3. Effect of methyl gallate (MG) and agricultural streptomycin sulfate on the incidence and control of tomato bacterial wilt under greenhouse conditions

Treatment	Concentration (g/liter)	Wilt incidence (%) ^{x,y}	Control efficacy (%) ^{x,z}
Natural MG	0.05	70.3 ± 3.7a	5.8 ± 7.0 c
	0.2	46.7 ± 1.9 b	38.5 ± 3.0 b
	0.5	26.5 ± 2.2 cd	65.2 ± 2.5 a
	1.0	16.4 ± 2.7 d	78.6 ± 2.9 a
Synthetic MG	0.5	30.0 ± 0.0 c	60.4 ± 1.9 a
Agricultural streptomycin sulfate	0.5	46.7 ± 1.9 b	38.5 ± 3.0 b
Control	...	77.6 ± 4.9 a	...

^x Wilt incidence (WI) was assessed 26 days after inoculation, and was calculated as the percentage of plants that were completely wilted.

^y Mean of three replications. Means within columns followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's least significant difference test.

^z Control efficacy was calculated using the formula control efficacy (%) = $100 \times (\text{WI of control} - \text{WI of treatment}) / \text{WI of control}$.

MG. The results of the experiment indicated that the antibacterial activities of the natural and synthetic compound against *R. solanacearum* in vitro were essentially identical.

Effect of MG on tomato bacterial wilt. In greenhouse experiments, typical tomato bacterial wilt symptoms were first observed 6 days after inoculation. Disease control efficacy of MG is positively correlated with concentration (Table 3). At the concentration of 0.2 g/liter, MG showed comparable efficacy in controlling bacterial wilt disease with the bactericide streptomycin sulfate at 0.5 g/liter. At same concentration (0.5 g/liter), the control efficacy of natural MG was 65.2%, which was significantly higher than that of agricultural streptomycin sulfate at a significance level of $P = 0.05$. The control efficacy of synthetic MG was 60.4%, and there was no significant difference between natural and synthetic MG.

Discussion

R. solanacearum appears to persist in soils or infested plant debris over long periods, which provide inoculum sources during whole crop growing seasons. Development of effective and safe bactericides to reduce soil populations of the pathogen has been the subject of increasing interest (14). Streptomycin has been used to control plant diseases caused by gram-negative bacteria since 1955 (25). It has been reported to be ineffective in controlling plant bacterial wilt in some countries (10) but it is used widely in China, where it can give some disease control (33). Our data showed that the control efficacy of streptomycin sulfate in greenhouse tests on tomato bacterial wilt was poor (38.5%). The variability in effectiveness of streptomycin sulfate at different sites may result from differences in physical, chemical, and biological properties of the soil.

In this study, MG, isolated from the methanol extract of *T. sylvestre* significantly inhibited the growth of *R. solanacearum* at an IC_{50} value of 8.3 mg/liter. Furthermore, there was no difference in the antibacterial activity of the natural and synthetic MG against *R. solanacearum* in vitro and in planta.

MG is a phenolic compound widely found in plant species, and its biological activities include antioxidant and antineoplastic activities (5). MG inhibits the growth of animal-pathogenic bacteria (5,6,11,17). According to our research results, in addition to *R. solanacearum*, some other plant bacterial pathogens, including *X. oryzae* pv. *oryzicola*, *X. citri* pv. *citri*, *X. vesicatoria*, *X. campestris* pv. *mangiferaeindicae*, and *Pseudomonas syringae* pv. *lachrymans*, were also sensitive to MG.

In addition to its antibacterial property, MG has been reported to have fungicidal and antiherpetic activities (5,16). MG isolated from *Galla rhois* (caused by Chinese sumac aphid on nutgall sumac tree) was suppressive to some plant fungal pathogens, including *Magnaporthe grisea*, *Botrytis cinerea*, and *Puccinia recondite*, in planta (1). Our findings also indicated that MG displayed a strong inhibitory effect on the growth of pathogenic oomycetes. Although MG displayed a broad spectrum of antimicrobial activities, the potential of using MG in planta for controlling plant bacterial diseases has not been reported prior to our studies.

We have shown that MG could significantly decrease the incidence of tomato bacterial wilt at concentrations over 0.2 g/liter in

greenhouse experiments. Although MG has shown potential as an effective bactericide in the control of bacterial wilt, it is not known whether the efficacy is sustained throughout the long growing season in different crop fields. Further research is required to assess how to optimally apply MG in the field to achieve maximum disease control efficacy and economic benefits.

MG belongs to alkyl gallate compounds, which are abundant gallic acid derivatives in organisms with a broad use. The alkyl chain length of gallate could play a key role in biological activities of alkyl gallate (13,30,31), and alkyl gallate shows a synergistic effect with antibiotics in preventing the growth of antibiotic-resistant bacteria (8,30,31). Moreover, MG and other gallic acid derivatives have been recognized as plant growth regulators and rooting promoters (26,27). Although we observed that the leaf extract of *T. sylvestre* could promote tomato growth (34), it is still unclear what role MG played.

We predict that minor structural variations of alkyl gallate may lead to major changes in their bioactivities, and screening for the derivatives of alkyl gallate might identify potent compounds that can be used alone or in combination with antibiotics to effectively control bacteria-elicited plant diseases. In addition, the antibacterial mechanism of the selected alkyl gallate against *R. solanacearum* at the cell and molecular levels needs to be studied, to provide a scientific basis for developing other novel compounds and improving efficacy of usage. These studies will strengthen the potential for applying MG and similar compounds as bactericides in cropping systems.

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