Indian Journal of Biochemistry & Biophysics
Vol. 56, April 2019, pp. 162-168

Natural anti-phytopathogenic fungi compound phenol, 2, 4-bis (1, 1-dimethylethyl) from *Pseudomonas fluorescens* TL-1

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Received 07 July 2018; *revised 20 December 2018*

A strain was isolated from tobacco phylloplane and preliminarily identified as *Pseudomonas fluorescens* TL-1, which had the visible inhibition against ten plant pathogenic fungi, *viz.*, *Curvularia lunata*, *Bipolaris maydis*, *Valsa mali*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Alternaria alternate*, *Fusarium oxysporum* and *Verticillium dahlia* in dual culture experiments. The ethyl acetate extract of nutrient broth seeded with *Pseudomonas fluorescens* TL-1 suspension was separated into fifty-nine fractions by the Sephadex LH-20 column and the antifungal activity of each fraction was tested with paper disc diffusion method against *Curvularia lunata*. The results showed that fraction 1 to 3 had the strongest inhibitory effects on *Curvularia lunata*. Furthermore, GC/MS analysis of the constituents of fraction 1 to 59 confirmed that phenol, 2, 4-bis (1, 1-dimethylethyl) was the active compound for the antifungal activity from *Pseudomonas fluorescens* TL-1.

Keywords: 2, 4-bis (1, 1-dimethylethyl), Column chromatography, Gas chromatography, Mass spectrometry, Phenol, *Pseudomonas fluorescens*

Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products annually[1]. Therefore the management of fungal plant diseases becomes a critical process for the achievement of the higher profits in agricultural production. Apart from the application of the agrochemicals in the control of fungal plant disease, more measures of biological controls are taken as an alternative to the use of agrochemicals with the advantages of greater public acceptance and reduced environmental impact[2]. It is well known that the bacteria are a number of biocontrol agents of plant pathogens, which include *Serratia plymuthica*[3], *Paenibacillus elgii*[4], *Pseudomonas fluorescens*[5,6], *Bacillus amyloliquefaciens*[7], *Chryseobacterium* sp.[8], *Pseudochrobactrum kiredjianiae*[9], *Pseudomonas azotoformans*[10], *Pseudomonas putida*[11], *Bacillus thuringiensis*[12], *Xanthomonas sacchari*[13], *Burkholderia cenocepacia* [14], *Lysobacter antibiocus*[15], *Bacillus cereus*[16], *Brevibacillus brevis*[17], *Mitsuaria* sp.[18] and so on from different environmental conditions. Among all of them, the genera of *Pseudomonas* and *Bacillus* are those of currently the most widely studied for the biocontrol of plant diseases[19,20] and various biocontrol mechanisms involved in the biological control of plant diseases have been addressed including the production of secondary metabolites such as antibiotics, siderophores, hydrolytic enzymes, volatile extracellular metabolites, hydrogen cyanide and competition for nutrients, promotion of plant growth and induced resistance within the plants.

The compound phenol-2, 4-bis (1, 1-dimethylethyl), is a precursor to many complex compounds and widely used as antioxidants, light protection agents or UV stabilizers and chemical intermediates for the synthesis of other chemical intermediates. While as the naturally antimicrobial compound, phenol-2,4-bis (1, 1-dimethylethyl) could be achieved from plant materials[21,22], animal materials[23,24] and the metabolites of microorganisms, such as *Streptomyces* sp.[25], *Shewanella algae*[26] and *Pseudomonas monteilii*[27], *Nocardiopsis* sp.[28], *Bacillus velezensis*[29], *Vibrio alginolyticus*[30], *Vibrio owensii*[31], *Vibrio* sp.[32], *Bacillus subtilis*[33], *Microbacterium mangrove*, *Sinomonas humi* and *Monashia flava*[34] and so on. Hitherto there have been still no reports of active compound phenol-2, 4-bis (1, 1-

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dimethylethyl) from *Pseudomonas fluorescens* against plant pathogenic fungi. This study validated the fact that phenol-2, 4—bis (1, 1-dimethylethyl) was a naturally antifungal compound from *Pseudomonas fluorescens* TL-1.

Materials and Methods

Isolation of strains

Nicotiana tabacum leaves were collected from Lingshan town, Jimo city, Shandong province, China in July 2015 and used for the isolation of epiphytic bacteria. Method for the isolation of bacteria was referred to the literature[35], but only a little adjustment was done in leaf disc size. The upper and lower side of the fresh leaf disc (28.3 cm^2) excised from the intact leaf was pressed on a petri dish with LB-agar (peptone 10 g, yeast extract 5 g, agar 15 g, deionized water 1000 mL) containing 50 mg actidione/L (Fluka, Neu-Ulm, Germany), in order to inhibit the growth of eukaryotic microorganisms such as yeasts and fungi. Petri dish was incubated at 28°C for several days to allow the growth of isolated bacteria. Single strains were sub cultivated on LB broth supplemented with 50 mg actidione/L. Stocks of all cultures were maintained at −80°C in LB medium containing actidione and 40% glycerol. Thirty leaf discs from the randomly intact leaf samples were used to isolate the bacteria.

Assay of antifungal effects of isolated bacteria

The antifungal activities of the isolated bacterial strains against the phytopathogenic fungus were investigated by a dual-culture plate method. The phytopathogenic fungi tested were *Curvularia lunata*, *Bipolaris maydis*, *Valsa mali*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Alternaria alternate*, *Fusarium oxysporum* and *Verticillium dahlia* and preserved in key lab, school of public health, Guizhou Medical University. Fungi preserved were grown on PDA (freshly peeled potato 200 g, dextrose 20 g, agar 15 g, deionized water 1000 mL) plate at 28°C for 4 days and then a small amount of mycelia from the advancing margin of plate cultures were transferred to the bilateral of other PDA plates, in the center of which the activated bacterial strains were inoculated concurrently. The plates were incubated at 28°C for 4 days and the clear zones of inhibition appeared as the criterion of antagonism. The bacterial strain of the most broad-spectrum inhibition was preliminarily identified according to the morphological and cultural characteristics, biochemical tests and 16S rRNA gene sequencing.

Separation of antifungal substance by column chromatography and assay of its inhibitory activity

The antifungal bacterium was grown in fifteen 1000 mL triangular flasks with 600 mL nutrient broth (containing beef extract 3 g/L, peptone 10g/L, NaCl 5 g/L, pH 7.4) each for 96 h at 28°C with constant agitation (120 rpm). The culture supernatant of the antifungal bacterium was obtained by centrifugation at 6000 rpm and 4°C for 20 min and then filtered by filter-sterilizer (aperture 0.22 μM, Millipore). Ulteriorly, the culture supernatant was extracted with ethyl acetate of the equal volume and then lyophilized to powder. 2 mL methanol (chromatographical grade) was added to the lyophilized powder to solubilize the extracts and then the methanol solute was applied to a methanol pre-equilibration glass column (2.5 × 200 cm) packed with Sephadex LH-20 of 190 cm height and eluted with 1000 mL methanol. Approximately at a flow rate of a drop percent second, different 10 mL fractions of chromatography were sequentially collected in the centrifugal tubes and lyophilized in a vacuum drier and further resuspended in 1 mL 50% methanol not affecting the fungal growth for antifungal activity test. The pathogen- *Curvulairia lunata* was used as the indicator and the analogous disc diffusion method was adopted[36] to assay the inhibitory activity of each fraction, but with a fresh colony mattress (φ 6 mm) of *Curvularia lunata* placed in the center of PDA plate instead of conidial spreading on PDA plate. Three sterile paper discs (φ 6 mm) of 3 cm distance from the center colony mattress in each PDA plate were loaded 20 μL of different fraction solutions, respectively. 50% methanol was used as a negative control (CK). Three parallel tests were performed for each fraction. A clear inhibition zone formed after incubation of 5 days at 28°C is considered to be inhibited.

Confirmation of antifungal substance by GC/MS

The methanol solutions (1 mL) filtered with a 0.22 μm filter membrane (Agela Technologies, China) of lyophilized powders of different fractions of chromatography were subjected to GC/MS analysis. GC/MS analysis was performed using Agilent 6890 gas chromatography coupled with an Agilent 5973 mass selective detector and a HP-5 MS (5% Phenyl Methyl Siloxane) capillary column (30 m × 0.25 mm; 0.25 μm film thickness). The chromatographic conditions were as follows: column oven program, 90°C (2 min, isothermal) to 200°C (2 min, isothermal) at 10°C/min, 200 to 280°C (6 min, isothermal) at 4°C/min. The injector and detector temperatures were

280°C and 250°C, respectively. Helium was the carrier gas (flow rate 0.80 mL/min) and the ionization voltage was maintained at 70 eV. The total ion chromatogram obtained was auto integrated by Chemstation and the constituents were identified by comparison with the mass spectral database (NIST and WILEY Library, 2005).

Results

Inhibitory effects of isolated strains against phytopathogenic fungi

Forty six bacterial strains were isolated from *Nicotiana tabacum* leaves and their inhibitory effects with dual-culture plate method against ten phytopathogenic fungi, *viz.* *Curvulairia lunata*, *Bipolaris maydis*, *Valsa mali*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Alternaria alternate*, *Fusarium oxysporum* and *Verticillium dahlia* showed that only one strain named TL-1 had the most obvious inhibition against all of the above-mentioned phytopathogenic fungi, while the other strains had no (93.5%) or less inhibitory effects on ten phytopathogenic fungi (4.3%).

Identification of strain TL-1

Strain TL-1 is Gram-negative, rod-shaped (0.4-0.7 × 0.7-2.0 µm) and motile. The colony on LB-agar appears brown, dry, flat and irregular, with lobate margins. The biochemical tests of strain TL-1 showed that positive results were catalase, hydrolysis of urea, Voges-Proskaeur, tryptophan deaminase, oxidase, reduction of nitrate to nitrite, hydrolysis of esculin, gelatin liquefaction starch hydrolysis, acid production from fructose; while negative results for indole production, *3-Ketolactose* production, malonate utilization, H_2S production, phenylalanine deaminase, utilization of citrate as a source of carbon, acid production from dulcitol, mannitol, rhamnose, lactose, maltose, arabinose, inulin, dextrin, xylose, sorbitol. The 16S rRNA gene sequence of strain TL-1 had a 99% identity with the type strain of *Pseudomonas fluorescens*, available in the public domain. The phylogenetic tree (Fig. 1) was constructed using 16S rRNA gene sequences and a close cluster was formed between strain TL-1 and the type strain *Pseudomonas fluorescens* (HQ420253.1). According to the morphological and cultural characteristics, biochemical tests and 16S rRNA gene analysis, the bacterial strain TL-1 was identified preliminarily as *Pseudomonas fluorescens*.

Inhibitory effects of chromatography fractions on *Curvularia lunata*

Approximately 1 g of yellow lyophilized powder was obtained from the extract of ethyl acetate. Fifty-nine fractions were collected, lyophilized and solubilized with 50% methanol for the assay of inhibitory effects with the analogous disc diffusion method. The results showed that only fractions 1st-3rd had strong inhibitory

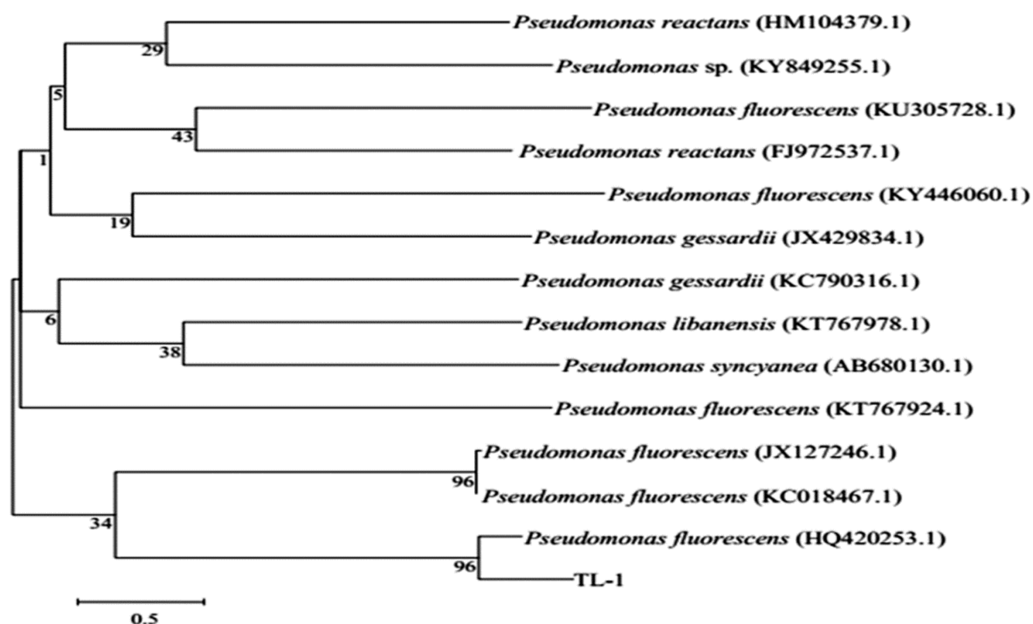

Fig. 1 — Neighbor-joining phylogenetic tree including stains of TL-1 and other related strains downloaded from GenBank based on 16S rDNA sequence (Bootstrap values n = 1000)

effects against *Curvularia lunata* compared with other fractions (Fig. 2A). Therefore, there was a need to achieve the chemical information about the antifungal substance from *Pseudomonas fluorescens* TL-1.

Confirmation of antifungal compound from Pseudomonas fluorescens TL-1

The metabolites of *Pseudomonas fluorescens* TL-1 were extracted, separated and concentrated and the analytes of all the fractions were then analyzed by GC/MS. The compounds from *Pseudomonas fluorescens* TL-1 in fractions 1st-6th, 58th-59th were identified and listed in table 1. Based on the corresponding information of chemical constituent (Table 1) and antifungal activity (Fig. 2B) of different fractions, especially for fraction 3, we draw the conclusion that phenol, 2,4-bis (1, 1-dimethylethyl) was really natural antifungal compound from *Pseudomonas fluorescens* TL-1, while the nearly ubiquitous p-xylene, o- xylene or m-xylene in all of the other fractions ranged from 1.2 to 100% did not display any inhibitory effects

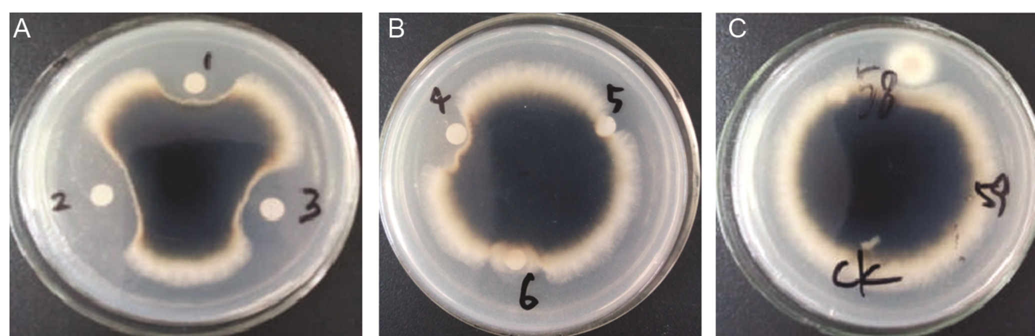

Fig. 2 — (A-C) Test on antifungal activity of fractions against *Curvularia lunata* (Number denoted the sequence of chromatographic solution collected from the beginning (1) to the end (59); CK (control)

Table 1 — Chemical constituents identified in the 1st-6th, 58th-59th fractions by GC/MS showing their RT, % of total and quality of compounds

Fraction number	Peak	Retention time	Identified compound	% of total	Quality
1	1	6.281	O-xylene	12.1	97
	2	8.044	Phenol	4.4	96
	3	14.216	Indole	3.3	95
	4	15.210	Phenol,2,4-bis(1, 1-dimethylethyl)	3.8	96
	5	25.714	L-leucyl-D-leucine	52.3	50
	6	25.978	2,5-piperazinedione,3,6-bis(2-methylpropyl)	19.8	43
2	1	6.284	O-xylene	28.9	94
	2	14.463	4-(3,4-dimethoxybenzylidene)-1-(4-nitrophenyl)-3-phenyl-2-pyrazolin-5-one	6.8	42
	3	15.210	Phenol,2,4-bis(1, 1-dimethylethyl)	4.9	87
	4	25.673	L-leucyl-D-leucine	8.0	38
3	1	6.284	P-xylene	14.8	97
	2	15.209	Phenol,2,4-bis(1, 1-dimethylethyl)	2.0	90
4	1	6.284	P-xylene	19.2	94
	2	15.211	Phenol,2,4-bis(1, 1-dimethylethyl)	1.2	87
	3	19.426	Carbamic acid,(4-chlorophenyl)-,1-methylethyl ester	2.9	27
5	1	6.280	O-xylene	14.8	97
	2	19.425	n-(2,7-dioxooctyl)acetamide	3.1	22
	3	25.952	2,5-piperazinedione,3,6-bis(2-methylpropyl)-	3.3	64
	4	35.483	1,2-benzenedicarboxylic acid,mono(2-ethylhexyl)ester	11.7	87
6	1	6.283	O-xylene	6.0	97
	2	25.968	2,5-piperazinedione,3,6-bis(2-methylpropyl)-	8.4	43
	3	35.489	1,2-benzenedicarboxylic acid,mono(2-ethylhexyl)ester	85.6	97
58	1	6.278	P-xylene	21.1	97
59	1	6.280	m-xylene	100	97

on *Curvularia lunata*, such as 21.1% p-xylene in fraction 58 and 100% m-xylene in fraction 59 (Fig. 2C).

Discussion

Plant pathogenic fungi are major threats to crops and plant production. Compared with the application of agrochemicals for the control of fungal diseases, that of *Pseudomonas* sp. represents another safe and environmentally friendly method for plant protection in agriculture. Nowadays, several commercial products based on various *Pseudomonas* species such as *P. syringae*[37], *P. chlororaphis*, *P. aureofaciens*[37], *P. fluorescens*[37] have been marketed as bio fungicides and most of the biocontrol efficacies are attributed to the active metabolites secreted by various *Pseudomonas* species, which include siderophores from *Pseudomonas* sp.[38] and *Pseudomonas fluorescens*[39]; phenazines from *Pseudomonas fluorescens*[40] and *P. chlororaphis*[41,42]; pyoluteorin from *Pseudomonas fluorescens*[43]; pyrrolnitrin from *Pseudomonas fluorescens*[44]; 2-hexyl, 5- propyl resorcinol from *P. chlororaphis*[45]; nunamycin and nunapeptin from *P. fluorescens*[46]; pyocyanin from *Pseudomonas fluorescens*[47]; 2,4-diacetylphloroglucinol (2,4-DAPG) from *Pseudomonas fluorescens*[48]; hydrogen cyanide (HCN) from *Pseudomonas fluorescens*[49] and *Pseudomonas chlororaphis*[50]; gluconic acid from *Pseudomonas* sp.[51]; rhamnolipid from *Pseudomonas aeruginosa*[52]; lipopeptides from *Pseudomonas* sp.[53-56]; piliferolide A from *Pseudomonas brassicacearum*[57]; cellulose and protease from *P. chlororaphis*[58] and so on. Besides its noble antagonistic property and diverse active metabolites against a wide variety of phytopathogenic fungi and bacteria, *Pseudomonas fluorescens* also shows the great potential application as biofertilizer in agriculture[59]. In the present study, *P. fluorescens* TL-1 from tobacco leaves had the strong inhibition against many plant pathogenic fungi and its antifungal substance was confirmed as 2, 4-bis (1, 1-dimethylethyl) with chromatographic separation, disc diffusion and GC/MS analysis. This is the first report of active compound phenol, 2, 4-bis (1, 1-dimethylethyl) deriving from *P. fluorescens* against phytopathogenic fungi.

As a natural compound, phenol, 2, 4-bis (1, 1-dimethylethyl) has been reported to have many functions for medicine, food and agriculture. In medicine, it has the antioxidant[60,61], anticancer[62], antifungal[63], antibacterial[21] properties and the protection against trimethyltin (TMT) -induced cognitive dysfunction[64]. In food, it has been proposed to prevent browning in fresh apple juices[65] and the growth of *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium chrysogenum* on wheat grains[66]. In agriculture, phenol, 2, 4-bis (1, 1-dimethylethyl) extracted from the rhizome of cogon grass (*Imperata cylindrica*) was found to have allelopathic effects on germination and seedling growth of weedy plants under soilless conditions[67] and it has also been reported as the defense compound of avocado root which prevents the root rot caused by *Phytophthora cinnamomi*[22], However in this pioneer study of *P. fluorescens* TL-1 producing the antifungal compound 2, 4-bis (1, 1-dimethylethyl), phenol, 2, 4-bis (1, 1-dimethylethyl) was expected to develop the green fungicides for the protection of fungal plant diseases in agriculture.

Acknowledgment

The authors acknowledge the Natural Science Foundation of Guizhou Province, China (NO. 2013 [2057]) and the First-Class Discipline Construction Project in Guizhou Province-Public Health and Preventive Medicine (NO.2017[85]) for their financial support

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