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*Research Article*

# Mining Agricultural Soils for Bacteria Antagonistic to Plant Pathogens

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## Abstract

Microbial biological control agents have been isolated from numerous substrates, but the reservoir of potential useful organisms has not been evaluated in soils that are exposed to herbicides during cropping practices. Therefore, we screened bacteria from soils from under field crops that had been routinely sprayed with herbicides for their ability to reduce the *in vitro* growth of two plant pathogens. Simple dual plate and paper disc diffusion assays were used to identify potential biocontrol agents. Among 115 bacterial isolates, 8 isolates showed antifungal activity against *Rhizoctonia solani* 1406 and 6 isolates had >50% inhibition. The most suppressive isolate was M59 (68% inhibition). From the 16S rRNA gene sequence, M59 had 100% identity to *Pseudomonas aeruginosa* strain JCM 5962$^T$. Results from antibacterial screening against *Xanthomonas axonopodis* pv. *citri* (*Xac*) revealed that isolate M93 has the potential to produce antibacterial compounds that can inhibit growth of the citrus canker pathogen *in vitro*. From a nucleotide BLAST search of the 16S rRNA gene sequence, M93 had 99.9% identity with *Bacillus subtilis* strain IAM 12118$^T$. Further research is necessary to identify candidate compounds and their antimicrobial efficacy in greenhouse trials.

**Keywords**: Antimicrobial activity, Plant disease, Herbicide-sprayed soil, *Pseudomonas, Bacillus*

## Introduction

Soil bacteria have a great impact in agricultural environments and plant production. In agricultural applications, biocontrol agents are often isolated from bulk soil, rhizosphere soil and plant tissues (Hallmann et al., 1997; Eichorst et al., 2007; Majeed et al., 2015). Conventional methods used for screening biologically active substances include the dual plate, agar well diffusion and paper disc diffusion assays (Sugita *et al*., 1998; Lertcanawanichakul & Sawangnop, 2008; Abeysinghe, 2009). From these approaches, bacterial strains of interest can be identified using the 16S rRNA gene region and their metabolites examined in detail. By way of an example, many *Bacillus* and *Pseudomonas* taxa have been shown to produce diverse classes of antimicrobial compounds and this has led to their use as biocontrol agents (Santoyo et al., 2012; Caulier et al., 2019).

Herbicides are routinely applied in modern broad-acre cropping in the world (Meena et al., 2020). It is often concluded that application of herbicide only has a minor and/or temporary impact on soil biota (Rose et al., 2016). However, organophosphate herbicides can have detrimental effects on the diversity of soil microbes (Usman et al., 2017). Whether herbicide-treated soils contain an untapped reservoir of useful organisms has so far not been studied in any detail. The aim of this study was to screen for antimicrobial activity of soil bacteria against *Rhizoctonia solani* 1406, a common fungal pathogen of horticultural and field crops. The most suppressive bacterial isolate was then assessed for *in vitro* activity against the bacterial pathogen *Xanthomonas axonopodis* pv. *citri*, the cause of citrus canker.

## Materials and Methods

### Isolation of bacteria from herbicide-sprayed soil

Ten farmer's paddy and fallow fields were randomly chosen and sampled in the wet season in Kamphaeng Phet, Chainat and Phichit provinces during June-August, 2016. The fields had a long history of applied glyphosate and/or paraquat herbicides. Within one rai, three soil samples (0-15 cm depth) were taken by hand and bulked into one mixed sample (10 grams). Two grams of each soil sample were used to isolate bacterial colonies and the 16S rRNA gene was amplified as described by Jumpathong & Masin (2018). The sequence data were deposited with accession numbers in GenBank.

### Screening for antifungal activity

A simple dual plate assay was used to determine extent of antifungal activity against the soil-borne pathogens *Rhizoctonia solani* 1406 and *Sclerotium rolfsii* 2357 isolated from root rot in chili. The bacterial isolates were streaked on one side of the potato dextrose agar (PDA) medium and incubated at 30ºC for 24 hours. After that, 6 mm-mycelial agar discs of each

fungal pathogen were placed on the other side of the Petri plates and they were incubated at 30ºC for a further 3-5 days. The experiment was performed in triplicate.

### Screening for antibacterial activity

*Xanthomonas axonopodis* pv. *citri* (*Xac*) isolated from kaffir lime (strain number DOABC856), lime (strain number DOABC956) and pomelo (strain number DOABC992) were obtained from the Plant Protection Research and Development Office, Department of Agriculture in Thailand.

One loop of *Bacillus* sp. M93 was scraped from nutrient agar and inoculated into nutrient broth before incubating on a shaking incubator at 150 rpm at room temperature (30±2 °C) for 24 hours. Then, 10% of bacterial seed culture was transferred to modified Wickerhams Antibiotic Test Medium (Jumpathong et al., 2019) and incubated in the same condition for 5 days.

To prepare the crude extract, 3 liters of cell-free supernatant were separated by centrifugation at 8,000 rpm for 20 minutes. The bacterial biomass was dried in a hot air chamber at 50 °C for 48 hours. Cell-free supernatant and dry biomass were extracted separately using ethyl acetate for three times. The ethyl acetate layer was evaporated using a rotary evaporator at 40°C under reduced pressure. Crude extracts were dried under $N_2$ for further study.

Twenty microliters of each crude extract (100 mg/ml) was loaded on each paper disc (diameter 6 mm), dried in a safety cabinet and then applied onto plates pre-inoculated with *Xac*. There were three replicates of each treatment. After 24-48 hours incubation, the inhibitory zones were measured and compared to the inhibition zone with chloramphenicol (20 µg/disc).

### Statistical analysis

Fungal and bacterial inhibition data were analyzed statistically using SPSS 17.0 software package (Trial License).

## Results and Discussion

### Bacterial isolates

One hundred and fifteen bacterial isolates were obtained from herbicide-sprayed soils and screened for antifungal activity against *Rhizoctonia solani* 1406 and *Sclerotium rolfsii* 2357, and antibacterial activity against *Xanthomonas axonopodis* pv. *citri* (*Xac*).

### Antifungal activity

Of the 115 bacterial isolates screened using the dual plate assay, 8 showed inhibitory activities against *Rhizoctonia solani* 1406. A nucleotide BLAST search against the GenBank database revealed that these 8 isolates belonged to *Bacillus, Brevibacillus*, *Lysinibacillus* and

*Pseudomonas* (Table 1). *Pseudomonas* sp. M59 had the highest inhibition percentage (68.1%) against mycelial growth of *R. solani* 1406 (Figure 1). The 16S rRNA gene sequence of M59 showed 100% similarity to *Pseudomonas aeruginosa* strain JCM 5962$^T$ (accession number MK796437) in the BLAST search. In a parallel study, supernatant from liquid cultures of M59 had weak antifungal activity against *S. rolfsii* 2357 and the supernatant did not inhibit growth of *R. solani* (Jumpathong & Masin, 2018). This indicates that extracellular lytic enzymes and other antifungal metabolites may be produced at very low levels in liquid culture in contrast to the agar challenge tests. This requires validation.

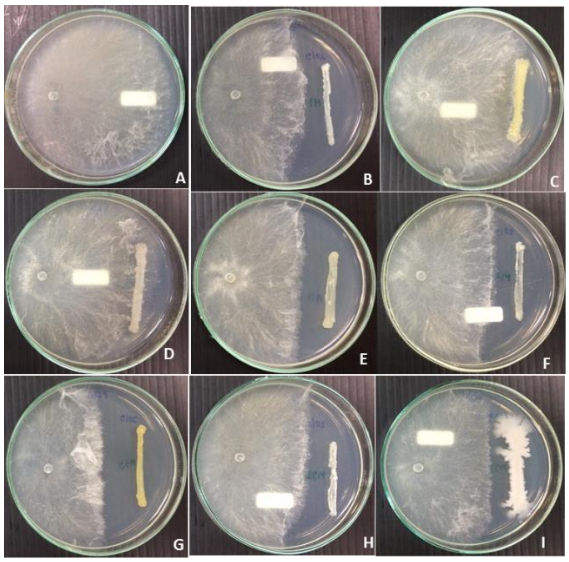


**Figure 1** Mycelial inhibition of *Rhizoctonia solani* 1406 (left side of plates) by bacterial isolates (right side of plates) using dual culture assay. (A) Control: no bacteria, (B) *Brevibacillus* sp. M1, (C) *Bacillus* sp. M3, (D) *Lysinibacillus* sp. M4, (E) *Pseudomonas* sp. M14, (F) *Brevibacillus* sp. M44, (G) *Pseudomonas* sp. M59, (H) *Brevibacillus* sp. M92, and (I) *Bacillus* sp. M93

Strains of *Pseudomonas* have been reported to produce a range of compounds such as siderophores, hydrogen cyanide, antimicrobials and extracellular lytic enzymes that are important in inducing systemic resistance and other responses (Etminani & Harighi, 2018; Kotasthane et al., 2017; Uzair et al., 2018). Many studies have demonstrated the use of *Pseudomonas* as biocontrol agents against root rot pathogens in green house trials (Durairaj et al., 2018; Kalantari et al., 2018; Al-Sman et al., 2019). Of the Bacillales, isolates M93 (*Bacillus* sp.) and M1 (*Brevibacillus* sp.) were nearly as effective as M59 in inhibiting mycelial growth of *R. solani* 1406 (Table 1). Members of the Bacillales such as *B. subtilis* (Todorova & Kozhuharova, 2010), *B. amyloliquefaciens* (Nastro et al., 2013), *B. thuringiensis* (Djenane et al., 2017) and *Brevibacillus laterosporus* (Saikia et al., 2011) exhibited antimicrobial activity against various bacteria and fungi.

## Antibacterial activity

*Bacillus* sp. M93 was selected to screen against *Xanthomonas axonopodis* pv. *citri* (*Xac*) in preference to *Pseudomonas* M59 because *Bacillus* produces endospores and is easier to apply in the field. Also, some *Pseudomonas* strains can cause opportunistic infections in humans. The nucleotide BLAST search of the 16S rRNA gene sequence of M93 exhibited 99.00 % identity with *Bacillus subtilis* strain IAM 12118.

**Table 1** Identity of 16S rRNA gene sequence of bacterial strains with antifungal activity (inhibition percentage) against *R. solani*

| Bacterial isolate[§] | Location[£] | Identity of 16S rRNA gene sequence, %[¥] | GenBank Accession Number | Antifungal Activity (Inhibition percentage)* |
|---|---|---|---|---|
| M1 | KP | *Brevibacillus laterosporus* strain NBRC 15654, 99.33% | NR112727 | 53.7 ± 0.38[bc] |
| M3 | KP | *Bacillus paramycoides* strain MCCC 1A04098, 98.04% | NR157734 | 42.8 ± 1.03[d] |
| M4 | KP | *Lysinibacillus fusiformis* strain DSM 2898, 99.60% | NR042072 | 29.2 ± 0.23[e] |
| M14 | PHS | *Pseudomonas aeruginosa* strain JCM 5962, 100% | MK796437 | 53.7 ± 0.38[bc] |
| M44 | KP | *Brevibacillus laterosporus* strain NBRC 15654, 99.45% | NR112727 | 50.6 ± 0.68[c] |
| M59 | KP | *Pseudomonas aeruginosa* strain JCM 5962, 100% | MK796437 | 68.1 ± 1.91[a] |
| M92 | KP | *Brevibacillus laterosporus* strain NBRC 15654, 99.45% | NR112727 | 51.4 ± 0.39[c] |
| M93 | KP | *Bacillus subtilis* strain IAM 12118, 99.93% | MK267098 | 55.3 ± 1.81[b] |

[§]Accession number for bacterial strains: M1(MK554648), M3(MK554656), M4(MK554657), M14(MK554652), M44(MK554650),M59(MK554655), M92(MK554651), M93(MK554667)

[£]Location: KP= Kamphaeng Phet Province, PHS= Phitsanulok Province

[¥]Homology searches using nucleotide BLAST software (NCBI)

*Different small letters in each column indicate significant differences from Duncan's multiple range test ($P<0.05$, n= 3).

The ethyl acetate crude extract from M93 biomass did not present any zone of inhibition when tested with *Xac*. By contrast, cell free supernatant extracts strongly inhibited *Xac* with the greatest effect against *Xac* from limes (Table 2). The inhibition zones were less than in the chloramphenicol treatments. This indicated that antibacterial molecules produced

and secreted in the supernatant. However, its secretion was relied on the growth phase, cultivation medium, and temperature (Saggese et al., 2018).

As many *Bacillus* species produce antibiotic compounds and resistant endospores (Piggot & Hilbert, 2004), they are often selected as biological control agents to control plant pathogens (Shafi et al., 2017). Also, some *Bacillus* species can induce systematic resistance in agricultural crops (Abriouel et al., 2011; Ashwini & Srividya, 2014). *Bacillus subtilis* produces antifungal agents such as iturin A and iturin A2 (Besson et al., 1978; Ye et al., 2012), mycosubtilin (Peypoux et al., 1976) and bacillomycin L (Besson et al., 1977). Fengycin synthesis in *B. subtilis* NCD-2 inhibited growth of *Rhizoctonia solani*, a soil-borne pathogen in cotton (Guo et al., 2013). Low cost commercial formulations have been manufactured from *B. subtilis* CPA-8 (Yánez-Mendizábal et al., 2012a; Yánez-Mendizábal et al., 2012b). Notably, *B. subtilis* also exhibited antimicrobial activity against various fungi and bacteria (Wang et al., 2015). Antagonistic activity tests of *B. subtilis* against *Xanthomonas campestris* pv. *glycines* revealed that strain 210 had potential biocontrol effect against bacterial pustule in soybean (Salerno & Sagardoy, 2003). Kwon & Kim (2014) isolated a cyclic lipopeptide from *B. subtilis* JW-1 culture broth that was able to control bacterial wilt caused by *Ralstonia solanacearum*. *Bacillus subtilis* CQBS03 isolated from citrus leaf surface was studied the antibacterial protein against citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) (Li et al., 2008). Islam et al. (2019) reported that the endophyte *B. thuringiensis* may be used as a biocontrol agent or source of antibacterial compounds to reduce citrus canker.

**Table 2** *In vitro* antibacterial test of crude extracts from bacterial strain M93 culture against *Xanthomonas axonopodis* pv. *citri* (*Xac*) from three citrus hosts

| | Inhibition zone (mm)* | | |
|---|---|---|---|
| **Treatment** | *Xac* (pomelo) | *Xac* (kaffir lime) | *Xac* (lime) |
| Chloramphenicol | 30.33±0.58Aa | 30.67±1.15Aa | 29.67±2.52Aa |
| Crude extract | | | |
| Biomass | 0.00±0.00c | 0.00±0.00c | 0.00±0.00c |
| Supernatant | 17.67±3.21Bb | 24.33±0.58Ab | 22.00±1.73Ab |

Notes * Values are presented as mean ± standard deviation.
Different lowercase letters in each column indicate significant differences from Duncan's multiple range test ($P<0.05$, n= 3).
Different capital letters in each row indicate significant differences from Duncan's multiple range test ($P< 0.05$, n= 3).

*Pseudomonas* are ubiquitous in agricultural soils and in the rhizosphere of crop plants (Andersen et al., 2001; Weller, 2007). Pseudomonads are an important group of bacteria that have potential for use in the control of *R. solani* 1406 and other plant pathogens (Schreiter et

al., 2018). Furthermore, *P. aeruginosa* produces phenazines, toxin compounds that can be employed to kill nematodes (Gallagher and Manoil, 2001; Cezairliyan et al., 2013).

## Conclusion

The present study suggests that agricultural soils affected by herbicides can harbor a useful source of bacteria with potential biocontrol attributes. Eight out of 115 bacterial isolates partially inhibited the mycelial growth of *Rhizoctonia solani* 1406 *in vitro*. This preliminary research indicates that comparative studies should be undertaken comparing soils with different chemical application histories. The efficacy of the most inhibitory isolates should now be evaluated *in vivo*. The present result also revealed the efficiency of the antibacterial metabolites produced by *Bacillus* sp. M93 as a good potential agent for controlling *Xanthomonas axonopodis* pv. *citri* (*Xac*). The strain may be used as a promising biocontrol agent in the future.

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