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Biocontrol of bacterial spot diseases of muskmelon using *Paenibacillus polymyxa* G-14

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***Paenibacillus polymyxa* strain G-14 (PpG14) isolated from the muskmelon rhizosphere, produces antibiotic(s) that are active against *Pseudomonas syringae* pv. *lachrymans* and *Acidovorax avenae* subsp. *citrulli* (two pathogens that cause bacterial spot diseases). Strain G-14 strongly inhibited the growth of *Pseudomonas syringae* pv. *lachrymans* and *Acidovorax avenae* subsp. *citruli* in a dual-culture plate assay. The biocontrol activity of PpG14 was examined by pot and field tests. Results show that the strain significantly reduced the development and suppressed the incidence of bacterial spot diseases. Moreover, the prevention treatment was better than the therapy treatment when using this strain. Based on its main bacteriological properties, identification using VITEK 32 and analysis of the 16S rDNA gene sequence, showed that strain G-14 belonged to *P. polymyxa*. Optimal growth was studied; temperature and pH were 28°C and 7, respectively.**

Key words: Muskmelon, bacterial spot diseases, *Paenibacillus polymyxa*, biocontrol.

INTRODUCTION

Bacterial spot diseases of muskmelon, which are caused by either *Pseudomonas syringae* pv. *lachrymans* or *Acidovorax avenae* subsp. *citrulli*, are serious diseases that can affect the growth of foliage, fruits and stems, and lead to a decrease in yield (Wang et al., 2008a, b; Yang et al, 2009). Biological control is a strategy that was proposed half a century ago at a symposium held in Berkeley in 1965 (Baker and Snyder, 1965). At present, measures to control melon bacterial diseases usually include planting resistant varieties, seed treatments, changes to the cultivation regime and comprehensive chemical control. In 1993, resistant varieties against the watermelon fruit rot bacteria (*A. avenae* subsp. *citrulli*) were investigated.

Liu et al. (1995) reported that inoculating cucumber with rhizobia could introduce the plant to resist bacterial angular leaf spot by activating the cucumber's own resistance systems (Liu et al., 1995). This system has also been tested on Hamimelon. Minzavage et al. (1995) used polymerase chain reaction (PCR) technology to find

a specific primer and basic experimental template that could be used to identify the presence of *A. avenae* subsp. *citrulli* (Liu et al. 1995). Walcott et al. (2000) used immunomagnetic separation and PCR to detect *A. avenae* subsp. *citrulli* in the seeds of watermelon. These studies showed that these methods could be used to rapidly and accurately detect infected seeds and thus improve infection source diffusion control (Walcott and Gitaitis, 2000).

Lu et al. (2004) isolated melon endogenetic bacteria and identified their populations, distribution and population size. They screened biocontrol bacteria that had antagonistic activity against bacterial leaf spot and fruit rot bacterial pathogens. This laid an important foundation for the use of endophytic biocontrol resources and for studying the molecular biology and genetic improvement of biocontrol mechanisms (Lu et al., 2004).

Muskmelon is a famous speciality crop grown in Xinjiang, China. However, bacterial spot diseases seriously restrict the development of muskmelon in Xinjiang. This study aimed to isolate strains that were strongly antagonistic towards bacterial spot pathogens. Sixty-three (63) strains, obtained from 86 different soil samples in the rhizosphere of muskmelon in Xinjiang,

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showed inhibitory activity against bacterial spot diseases after an initial antagonism test. The present paper describes the isolation of a newly isolated bacterial strain called G-14, which significantly inhibited the bacterial spot pathogens. The purpose of this study was to evaluate the potential of strain G-14 to reduce the development of bacterial spot diseases under greenhouse and field conditions.

MATERIALS AND METHODS

Bacterial strains

P. syringae pv. *lachrymans* P4 and *A. avenae* subsp. *citrulli* BFB were obtained from Shihezi University. *A. avenae* subsp. *citrulli* F159 was isolated from symptomatic muskmelon fruit by the Institute of Microbiology, Xinjiang Academy of Agricultural Sciences. The non-pathogenic *Paenibacillus polymyxa* G-14 was isolated from soil samples collected from muskmelon fields in Changji, Xinjiang.

Muskmelon fruit infected with bacterial spot disease and soil were collected and taken to the institute laboratory for isolation. The infected parts were surface sterilized with 70% absolute alcohol and a small piece of the infected portions were cut from the advancing margin of the lesions with a sterile scalpel. The tissue was washed thoroughly in two or three exchanges of sterile distilled water and placed in a petri dish with a drop of sterile water, teased apart with sterile needles and allowed to stand for 30 min after which the resulting suspension was then streaked on Sucrose Peptone Agar (SPA) using flamed wire loop. The culture media were placed upside down in the incubator and the temperature maintained at 27°C for period 48 h. A pure culture was obtained after sub culturing twice.

Isolation of antagonistic bacterial strains

Soil samples were collected from different soil layers in the muskmelon rhizosphere in Xinjiang, China. Diluted solutions of the soil samples were mixed on an orbital shaker with a broth containing the bacterial pathogen. The corresponding solutions were made up by extracting 1 ml of the mixed liquid. Then 200 μl of the mixed liquid was spread on a Luria-Bertani (LB) plate. After incubation at 28°C for 48 h, the inhibition zones were observed and recorded.

Screening of bacterial strains with antibacterial activities

In order to screen bacterial strains for antibacterial activity, the antagonistic bacteria and pathogens were fermented at 28°C for 48 h. The fermented pathogen solution was then coated onto a LB plate. The plate was perforated using a sterilized puncher and then bacteria antagonistic to the pathogen bacteria found in the fermented solution were drawn into the fermented solution through the hole in the plate. After incubation at 28°C for 48 h, the inhibition zones were observed and recorded (Zhao, 2004).

Identification of strain G-14

The G-14 strain was classified by morphology identification using VITEK 32 and analysis of the 16S rDNA gene sequence and culture characteristics (Gordon et al., 1983; Schaal, 1986; Holt et al., 1994; Dong et al., 2001).

Greenhouse and field tests

Experimental details

Muskmelon seeds of Hybrid Queen (susceptible to bacterial spot pathogens) were sown in a wet nursery in earthenware pots. After that muskmelon plants, which were clover period old, were planted by artificial spray inoculation. Field tests were established at an experimental station in Changji, Xinjiang using the muskmelon variety, Hybrid Queen.

In the preventative treatment greenhouse and field tests, bacterial suspensions were adjusted to a concentration using a spectrophotometer. Then potential biological control agents were spray-inoculated, using hand-trigger sprayers, onto the abaxial and adaxial leaf surfaces until run-off occurred. The plants were grown at a high relative humidity (RH) for 48 h, after which the plants were inoculated with either a suspension of *P. syringae* pv. lachrymans P4, *A. avenae* subsp. *citrulli* BFB, or *Acidovorax avenae* subsp. *citrulli* F159. The plants were then grown on at a high RH for a further five to six days until water-soaked lesions were visible on the inoculated foliage.

With the greenhouse and field tests therapy treatments, the plants were inoculated with a suspension of either *P. syringae* pv. *lachrymans* P4, *A. avenae* subsp. *citrulli* BFB, or *A. avenae* subsp. *citrulli* F159. The plants were grown at high RH for 48 h until water-soaked lesions were visible on the inoculated foliage. Potential biological control agents were then spray-inoculated, using hand-trigger sprayers, onto the abaxial and adaxial leaf surfaces until run-off.

Experimental observations

Bacterial spot disease scores for the diseased plants were recorded from the stable phase according to the classification standard evaluation system (0 to four scale) for muskmelon bacterial spot diseases. In addition, the disease index and the control effect were calculated for bacterial spot diseases. The disease index (DI) and the control effect (CE) were determined using the following formulas:

$$DI(\%) = \frac{\sum bacterial\,spot\,diseases\,scale \times number\,of\,the\,leaves}{the\,most\,serious\,bacterial\,spot\,diseases\,scale \times total\,number\,of\,leaves} \times 100$$

$$CE(\%) = \frac{Control\,DI - Treated\,DI}{Control\,DI} \times 100$$

The muskmelon bacterial spot disease scale is as follows: 0, No incidence; 1, lesions limited to lower 1/5 of leaf area; 2, lesions present on lower 1/3 of leaf area; 3, lesions present on more than 1/3 of leaf area; 4, lesions present on more than 2/3 of leaf area; 5, severe infection on all leaves.

RESULTS

Isolation of antagonistic bacteria

Between October and December 2006, 195 strains producing an antibacterial circle were obtained from 86 different soil samples using the agar plate method. After an initial antagonism test, 63 strains showed inhibitory

Table 1. Inhibitory activities of antagonistic bacteria to bacterial spot pathogen.

Strains no.	Percentage	Antagonistic diameter (mm)	Antagonistic activity
6	3.10	≥20	＋＋＋
29	14.90	≥10;＜20	＋＋
28	14.40	＜10	＋
132	67.60	0	－

+++, Strong; ++, middle; +, weak; -, nothing.

Table 2. Physiological and biochemical characters of the G-14strain.

| Characteristic	Value	Characteristic	Value	Characteristic	Value
Negative control	－	THRM	－	Palarinose	＋
Sucrose	＋	Maltose	＋	Oleandomycin	－
Tetrazolium red	＋	Trehalose	＋	Sodium Acetate	＋
Tagatose	－	Sorbitol	－	Amygdalin	－
Glucose	＋	N-Acetyl-D-Glucosamine	－	Inulin	＋
Inositol	－	Amylopectin	－	Arabinose	＋
Galactose	＋	Potassium Thiocyanate	－	Ribose	－
7%Sodium Chloride	－	Xylo	＋	Mandelic Acid	＋
Raffinose	＋	Mannitol	＋	Esculin	＋
Nalidixic Acid	－	Salicin	＋		

+, Positive reaction, -, negative reaction.
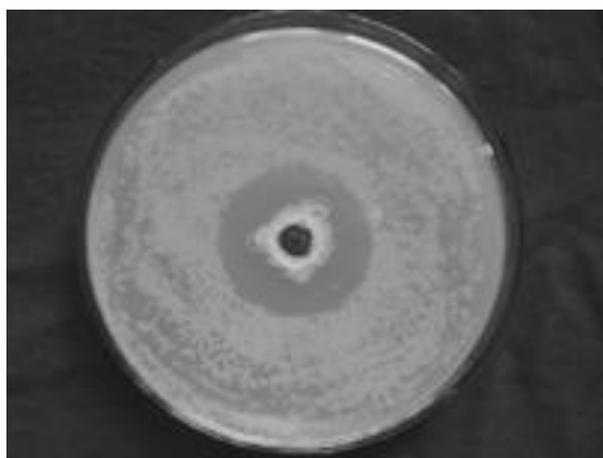
Figure 1. Inhibitory activities of G-14.

activities against the pathogens that cause bacterial spot diseases. The activity strength of the antagonistic bacteria was classified into three categories (strong, middle and weak) based on the diameter of the inhibition zone. Out of the 195 strains, six strains showed strong antagonism, 29 strains showed middle antagonism and the remainder showed weak antagonism (Table 1). The stain G-14, which was selected from the 63 strains showing inhibitory activity using the cup-plate method, displayed the greatest antagonism (Figure 1).

Identification of strain G-14

The identification results for G-14 from the biomerrieux VITEK32 automatic microorganism system are shown in Table 2. Comparing the identification results with the auto index database, the results show that strain G-14 belongs
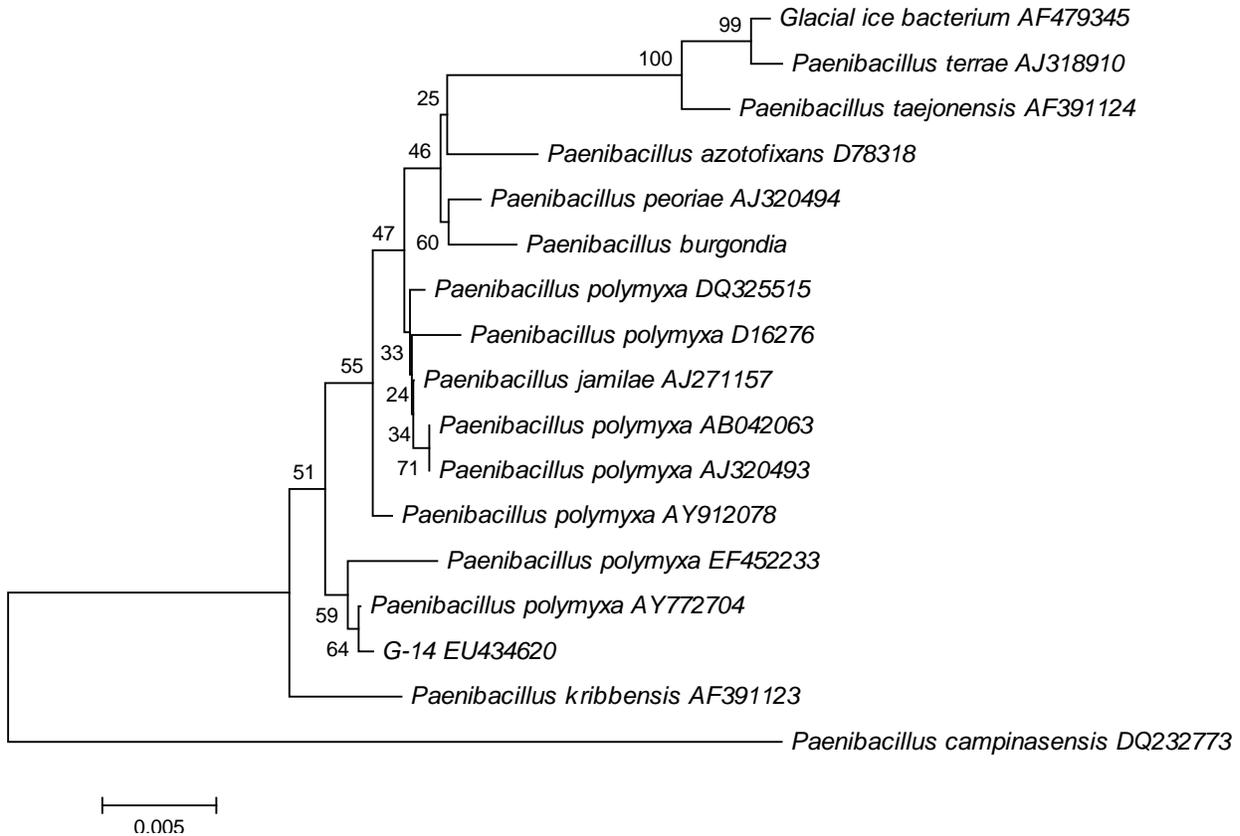
Figure 2. Phylogenetic tree based on G-14 16S rDNA.

to *P. polymyxa*. The total nucleotide sequence of the 16S rRNA gene from strain G-14 (accession no. EU434620) was 1463 bp long. The alignment of this nucleotide, through matching with 16S rRNA reported gene sequences in the gene bank using the basic local alignment tool (BLAST) program and phylogenetic tree analysis software to construct a phylogenetic tree (Figure 2), showed that the genetic distance of strain G-14 had the highest sequence similarity (99%) with *P. polymyxa* (AY772704 and EF452233). Based on the results of the physiological and biochemical characteristics studies into strain G-14 and on the analysis of the nucleotide sequence of the 16S rRNA gene, strain G-14 was identified as *P. polymyxa.*

Biological characteristics of strain G-14

The cell growth of G-14 was investigated under different temperatures (Figure 3). The maximal OD600 nm was achieved at 28°C. At the same time, the cell growth of G-14 was studied under different initial pH treatments (Figure 4). It was found that cell concentration was closely correlated with antagonistic activity against the pathogen. The optimal pH was 7.0, at which the highest antagonistic diameter was obtained.

Evaluation of strain G-14 against bacterial spot diseases in the greenhouse and field tests

Greenhouse test

Table 3 shows the abilities of P4, BFB and F159 to prevent pathogen infection, which can reach 60.58, 98.23, 57.42%, respectively, compared to the control. The results show that the antagonistic strain G-14 showed considerable control activity against bacterial spot diseases of muskmelon.

Field test

Table 4 shows that strain G-14 had a definite preventative effect on plant infection by muskmelon bacterial spot diseases. The results indicate that prevention of initial infection is better than control following infection.

DISCUSSION

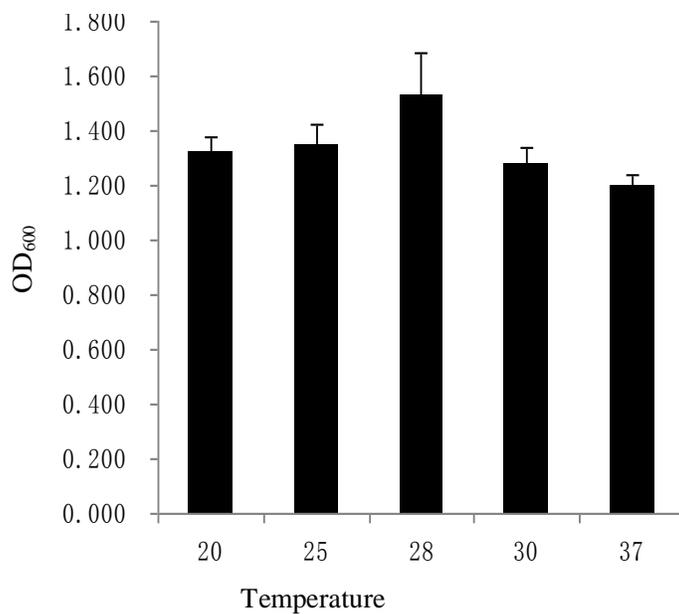There are many reports about the control of bacterial diseases using antagonistic microbes (Cui et al., 2003; Li

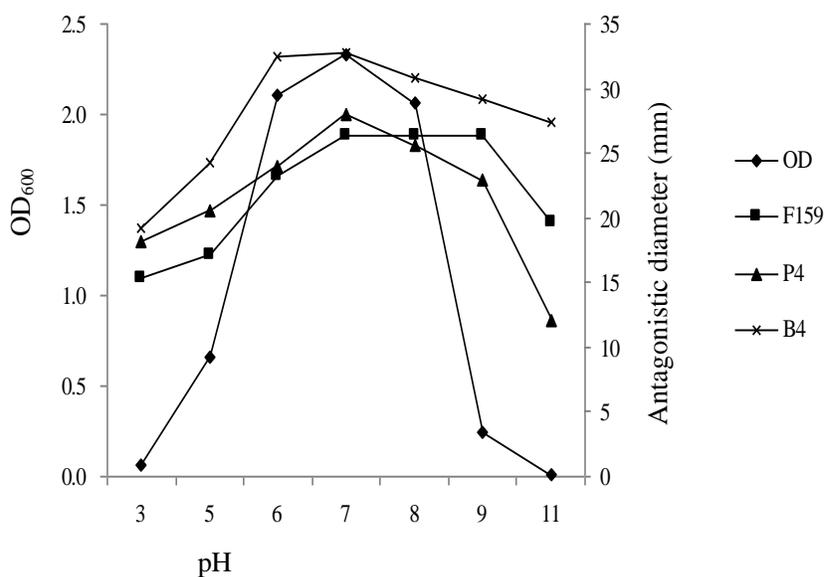**Figure 3.** The optimal growth temperature.

Figure 4. The optimal growth and antagonistic pH.

Table 3. Pot control effect of antagonistic strain G-14.

Treatment	P4		BFB		F159	
	DI	CE	DI	CE	DI	CE
Prevention	39.06[A]	60.58[B]	1.75[A]	98.23[B]	42.19[A]	57.42[A]
Therapy	66.07[B]	33.31[A]	33.30[B]	66.39[A]	40.00[A]	59.63[A]
Control	99.08[C]		99.08[C]		99.08[B]	

DI, Disease index (%): all values are means of five replications; CE, control effect (%). Control plants were sprayed with distilled water. Mean bars with different letters (A to C) significantly differs (p < 0.01).

Table 4. Field control effect of antagonistic strain G-14.

Treatment	P4		BFB		F159	
	DI	CE	DI	CE	DI	CE
Prevention	36.47[A]	59.42[B]	32.31[A]	64.05[B]	58.06[A]	35.40[B]
Therapy	43.93[B]	51.12[A]	46.56[B]	48.19[A]	67.85[B]	24.50[A]
Control	89.87[C]		89.87[C]		89.87[C]	

DI, Disease index (%): all values are means of five replications; CE, control effect (%). Control plants were sprayed with distilled water. Mean bars with different letters (A to C) significantly differs ($p < 0.01$).

et al., 2006; Raupach et al., 2000; Krishnamurthy et al., 1998; Wilson et al., 1989; Shahnaz et al., 2010; Hammami et al., 2012; Narayan et al., 2011) but the antagonistic microorganisms investigated and the test methods used to study the antagonistic effect vary. The study using the agar plate method concluded that through continual method improvement, compared to previous studies, they had successfully, quickly and accurately used the agar plate method to select antagonistic bacteria and actinomycetes. However, because the pH needed for the growth of the bacteria and fungi was different, the antagonistic fungi failed to grow at all.

Comprehensive analysis of morphology, bacteriological properties, biochemical characteristics and the 16S rDNA gene sequence of strain G-14 showed that strain G-14 belongs to *P. polymyxa*. Bacterial identification based on the analysis of 16S rDNA sequences is a common international identification technology. Generally, if the homology of the 16S rDNA sequences is less than 98%, then a variety can be considered different and if the homology is less than 93-95%, then a genus can be considered to be different (Fry et al., 1991). According to these criteria, the 16S rDNA sequence analysis of G-14 showed that the genetic distance was at a minimum when compared to *P. polymyxa* EF452233 and AY772704 and their homology was 99%, so strain G-14 can be identified as *P. polymyxa*.

Paenibacillus originally belonged to *Bacillus*. In 1994, Ash et al. (1994) established *Paenibacillus* and appointed *P. polymyxa* as the model species for this genus. There have been many studies into *P. polymyxa* as a biocontrol bacteria (Tong et al., 2004; Karpunina et al., 2003; Beatty et al., 2002; Yao et al., 2004) but this paper is the first to apply the antagonistic bacteria *P. polymyxa* in order to inhibit muskmelon bacterial spot diseases. These data indicate that the biological control agent *P. polymyxa* G-14 has moderate efficacy against both bacterial speck and bacterial spot of muskmelon. It is noteworthy that *P. polymyxa* G-14 was more effective against bacterial spot diseases under greenhouse conditions than under field conditions. This could be due to the poor survival of *P. polymyxa* G-14 in the field.

The study showed that the disease occurred in all tested cultivars but the degree of infection varied. So choosing varieties that showed improved resistance was an effective means of preventing the occurrence of the disease (Hopkins, 1993). Hopkins et al. (1996) showed that the infection ratio of melon seeds could be reduced from 61% to less than 1% if the seeds were dipped into a solution of fermenting biocontrol bacteria for 24 - 48 h and then subsequently washed in 1% HCl or 1% $CaOCl_2$, followed by water and then dried. This method was the most effective measure to eliminate seed contamination and did not affect seed germination (Hopkins, 1996).

It was found that G-14 had a broad spectrum antagonistic effect against pathogens, which suggested that there were a number of micro-organisms in the soil that could be used and modified to prevent or control plant diseases. The strains screened in this study were derived from the soil in the crop rhizosphere, so it should be easy to inoculate the rhizosphere soil with these strains and for these strains to survive and successfully perform biological control. Although this study undertook some preliminary investigations into the strain and found that it had the potential to develop into a biological pesticide, the purification of its antibacterial activity composition, the genetic improvement of the strain and the safety studies needed for the release of this strain, require further in-depth study.

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