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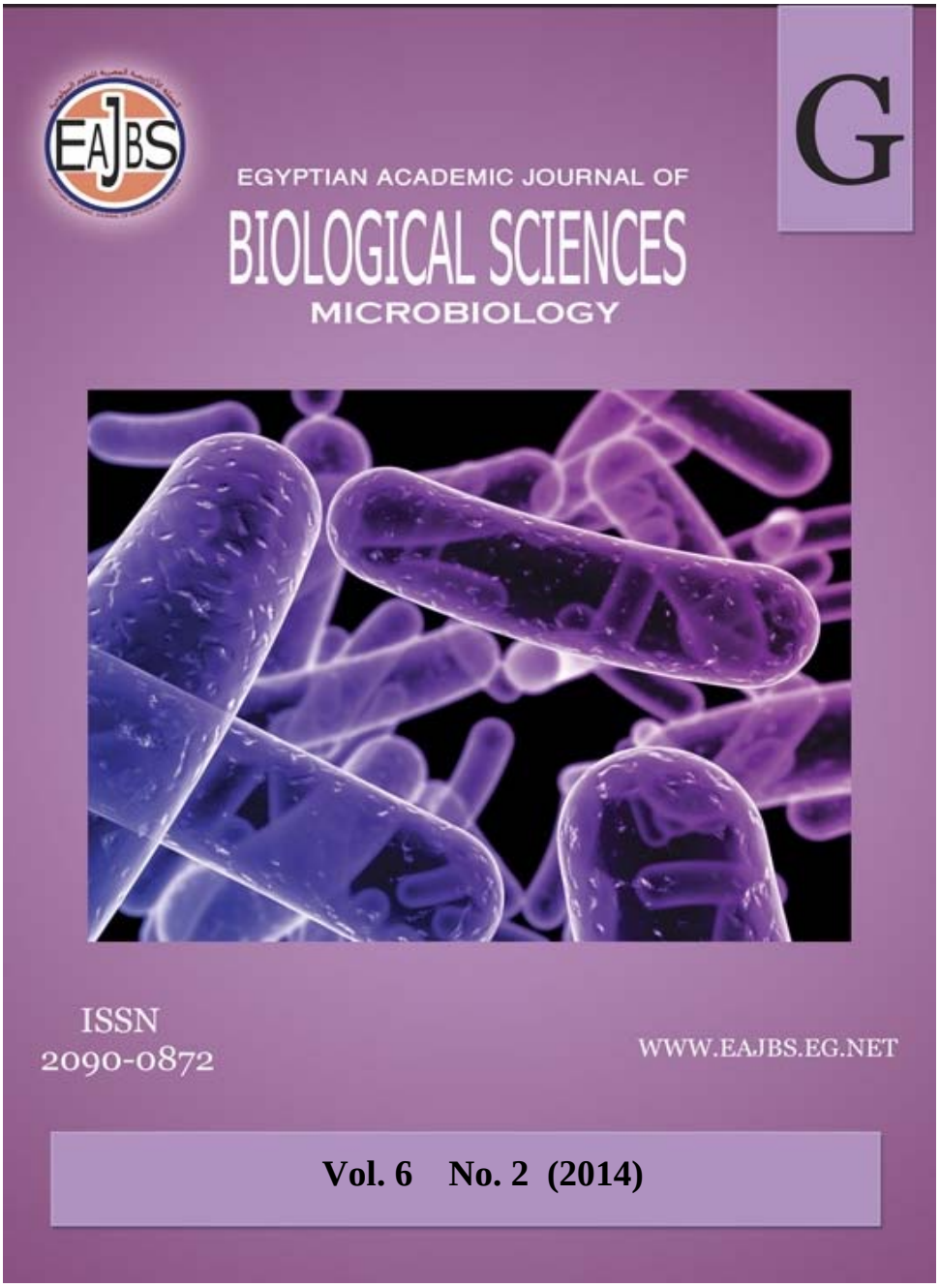

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Evaluation of Saudi Fluorescent Pseudomonads Isolates as a Biocontrol Agent against Citrus Canker Disease Caused by *Xanthomonas citri* subsp *citri* A*

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ABSTRACT

The goal of this study was to determine whether bacterial antagonists could be used to control *Xanthomonas citri* subsp *citri* (*Xcc*), the causal agent of bacterial citrus canker disease. A total of 22 potentially bacterial antagonists isolated as epiphytes from the phylloplane of healthy citrus trees were screened for their *in vitro* biological control capability against *Xcc*. These strains were identified as *Pseudomonas fluorescens* on the basis of biochemical and physiological tests and 16S rDNA. Out of these 22 potentially bacterial antagonists, five strains (KSA1, KSA9, KSA14, KSA17, and KSA20) showed high potential growth inhibition capabilities against *Xcc*. The KSA1 strain was selected for further studies to test its *in vivo* capability to control bacterial citrus canker pathogen. It was sprayed in a suspension of 10^7 CFU ml^{-1} on citrus leaves which were subsequently inoculated after 72 h with 10^8 CFU ml^{-1} suspension of *Xcc* strain JQ890095. According to the *in vivo* biocontrol tests, the putative antagonist KSA1 significantly reduced the symptoms on the leaves of Mexican lime seedlings compared with untreated inoculated plants.

INTRODUCTION

Citrus bacterial canker caused by *Xanthomonas citri* subsp *citri* (*Xcc*) is one of the most important diseases in many citrus growing countries including Saudi Arabia (Gottwald *et al.*, 2002; Ibrahim and Bayaa, 1989). The common well-known *Xcc* pathotypes are A, B, and C. The pathotype A (Asiatic form) is widespread throughout the world and affects the widest range of citrus hosts (Schubert *et al*., 2001; Juhasz *et al.*, 2013; Hoarau *et al.*, 2013). However, several groups within pathotype A with restricted host range have been identified. Group A* was reported for the first time by Vernière *et al.* (1998) in Southwest Asia. This group was isolated from Mexican lime trees in several countries in Southwest Asia; including Saudi Arabia, Oman, and Iran. Mexican lime, the most important fresh fruit citrus grown in Saudi Arabia, is the most susceptible citrus trees to *Xcc*-A* which is the common *Xcc* strain in Saudi Arabia.

Attempts to eradicate citrus canker disease in Saudi Arabia failed and it is now considered as an endemic disease in different Saudi citrus-growing areas. In addition, there are no highly effective canker disease control strategies to protect susceptible citrus cultivars, especially when environmental conditions are favorable to the disease development (Leite and Mohan, 1984). However, copper-based bactericides have been applied to control citrus canker disease in more than one citrus-growing area in Saudi Arabia. Copper-based bactericides can reduce bacterial populations on citrus leaf surfaces if multiple treatments are applied (Ibrahim *et al*. unpublished data). The problems with the continuous application of copper compounds are the accumulation and contamination of soils with potential phytotoxic compounds, in addition to the development of copper resistant *Xcc* strains (Alva *et al*., 1995). Biological control has emerged as one of the most promising and safest methods for the management of plant pathogens. Fluorescent pseudomonads are often selected as biological control agents because of their ability to utilize varied substrates under different conditions, short generation time, and motility that assist them to colonize plant roots (Bagnasco *et al*. 1998). The application of *Pseudomonas fluorescens* as a biological control agent against citrus canker in Iran showed a promising result. Since it reduced the number and size of canker lesions (Khodakaramian *et al.*, 2008). The purpose of this study was to evaluate the efficacy of bacterial antagonists on Mexican lime seedlings against bacterial citrus canker pathogen. The virulent *Xcc* strain JQ890095 previously isolated from diseased leaves of citrus trees from Jazan region in 2010 was used in all *in vitro* and *in vivo* experiments.

MATERIAL AND METHODS

Isolation of *P. fluoresecens*

In Spring 2012, leaf samples were collected from healthy citrus trees in Abha, Baha, Jazan, and Riyadh and then *P. fluoresecens* were isolated following the method of Vlassak, *et al.* (1992). After 24 hr incubation-period at 28°C, tentative Pseudomonads bacterial colonies were further purified on KB agar medium and pure cultures were preserved on KB slants at 4°C.

Identification of the Selected Bacteria:

Morphological and Biochemical Characterization of Bacterial Isolates

The morphological features: colony type, bacterial shape, and gram reaction were determined using King's B agar medium. Oxidase, catalase; and starch hydrolysis and levan formation tests were examined on media supplemented with 0.2% starch and 5% sucrose according to Goszczynska *et al.* (2000). Fluorescin production, gelatin liquefaction, salt tolerance, and carbohydrate utilization tests were performed following the methods of Goszczynska *et al*. (2000) and Pickett *et al*. (1991).

Molecular Characterization of Bacterial Isolates

The 16S rDNA region was amplified by PCR technology from bacterial isolates using 27f and 1492r primers according to Al Saleh *et al*. (2014) and Eden *et al.* (1991). PCR products were assayed on 1% agarose gel stained with ethidium bromide according to Sambrook *et al*. (1989). PCR products were cleaned and directly sequenced using 27f and 1492r primers at the Advanced Genetic Technologies Center (AGTC) (University of Kentucky, Lexington, KY, USA). The obtained DNA sequences were cleaned and manually edited using the BioEdit software (Hall 1999; http://www.mbio.ncsu.edu/Bioedit/bioedit.html). The cleaned sequences were searched against the GenBank database at the NCBI (http://www.ncbi.nlm.nih.gov/) to confirm the identity of isolated bacteria.

In Vitro Growth-Inhibition Capability of Bioagents against *Xcc*

All isolates of *P. fluorescens* were first screened for their antagonistic capability against *Xcc* on KB agar plates in dual culture assays (Ganesan and Gnanamanickam, 1987). KB plates were prepared by mixing

bacterial suspension scraped from 48-72 hour-old pathogen culture with cooled and molten KB agar (42°C). The agar suspension was then dispensed into Petri dishes and then spot inoculated with the tested antagonistic strain taken from a 24-hour-old culture (Skathivel and Gnanamanickam, 1987). The control KB agar plates were spot inoculated with sterile water. The plates were incubated at 28°C and observed for inhibition zones after 2 to 3 days.

Greenhouse Experiment

Plant Material

Nine-month-old seedlings of Mexican lime were used in this study. Rootstock of Mexican lime was planted on 40 cm-diameter plastic pots filled with sand. Plants were maintained in the greenhouse at 28-30°C.

Inoculum Preparation of *Xcc*

Xanthomonas citri subsp *citri* strain JQ890095, previously recovered from Mexican lime tree showing canker foliar lesions in Jazan region, Saudi Arabia (Al Saleh *et al.,* 2014), was grown on nutrient glucose agar (NGA) plates and incubated at 28°C for 24 h. Bacterial cells were scraped from these 24-h cultures with sterile distilled water and the resulting bacterial suspension was adjusted spectrophotometrically to approximately 10^8 CFU/ml^{-1} (OD660= 0.06).

Inoculum Preparation of *P. fluorescens*

Pseudomonas fluorescens strain KSA1 was selected for further *in vivo* experiments because it significantly restricted the growth of *Xcc* under laboratory conditions. The 48-h-old KSA1 culture on KB broth was centrifuged at 10,000 rpm for 10 min. Bacterial pellets were washed twice with sterilized distilled water using brief centrifugation. Then, the optical density (OD) of the bacterial suspension was adjusted to OD 0.45 at 610 nm to obtain $l0^7$ CFU- ml^{-1} (Mortensen 1999).

Evaluate the Efficacy of *P. fluorescens* against *Xcc* Pathogen under Greenhouse Conditions

The Mexican lime leaves were sprayed with $l0^7$ CFU- ml^{-1} KSA1 suspension before their inoculation with *Xcc* pathogen. Three days later, the antagonist-treated citrus plants were sprayed with $l0^7$ CFU- ml^{-1} *Xcc* suspension until run-off. Five plants were used for each treatment. Inoculated plants were immediately enclosed in plastic bag for 48 hours. Disease severity and disease intensity were evaluated two weeks post inoculation.

Statistical Analysis

The experiments were conducted as a randomized complete-block design and five plants were used for each treatment. Data were subjected to statistical analysis using analysis of variance and means were compared using L.S.D. test according to Gomez and Gomez (1984). The standard error (±SE) of each mean (n = 3) was calculated.

RESULTS

Isolation and Characterization of *Pseudomonas* Isolates

Biochemical properties of the *Pseudomonas* isolates are presented in Table 1. All the isolates were found to be gram negative and positive for oxidase and catalase tests. They were able to grow at 4°C but not at 41°C and tolerated NaCl concentration up to 2%. They utilized the tested carbohydrates where they produced yellow color on the medium indicating their ability to utilize these carbohydrates. In addition, they liquefied gelatin but failed to hydrolyze starch.

The bacterial strains were also confirmed at the molecular level by sequencing 16S rDNA gene. The 16S rDNA analysis could not discriminate Saudi Arabian strains. Comparison between the partial sequences of 16S rDNA of Saudi strains and the other sequences of 16S rDNA deposited in GenBank showed that isolates were *P. floursenses*.

Table 1: Morphological and biochemical characteristics of *P. fluorescens* bacterial strains.

	Characteristics of isolates			
	Abha group	Al Baha group	Jazan group	Riyadh group
Shape of cells	RS[a]	RS	RS	RS
Gram stain	-[b]	-	-	-
Motility	+[c]	+	+	+
Pigment Production	+	+	+	+
Gelatin liquefaction	+	+	+	±[d]
Starch hydrolysis	-	-	-	-
Levan production	+	+	±	+
Oxidase	+	+	+	+
Catalase	+	+	+	+
Nitrate reduction	+	+	+	+
Potato soft rot	-	-	-	-
Growth at 40°C	+	+	+	+
Growth at 41°C	-	-	-	-
Salt Tolerance				
1% NaCl	+	+	+	+
2% NaCl	+	+	+	+
3% NaCl	-	-	-	-

[a] RS = Rod Shaped
[b] - = negative reaction
[c] + = positive reaction
[d] ± = Intermediate reaction**.**

In Vitro Antibiosis

Out of the 22 fluorescent *Pseudomonas* isolates screened against *Xcc* on KB medium, only five were capable of inhibiting the growth of the pathogen while others did not show any antibiosis activity. The diameter of inhibition zones ranged from 45 (KSA14) to 62 mm (KSA1) (Table 2). The isolates KSA1 from Abha and KSA17 from Jazan showed the higest antibiosis activities.

Table 2: The *P. fluorescens* isolates that showed *in vitro* antibiosis activities against *Xcc* on KB medium.

Origin	Isolate name	No. of isolates tested	No. isolates with antibiosis	Inhabitation Zone (mm)
Abha	KSA1	6	1	62a*
Al Baha	KSA	5	1	52b
Jazan	KSA14	6	1 (KSA14)	45c
	KSA17		1 (KSA 17)	56b
Riyadh	KSA	5	1	50b

* Values marked with the same letter(s) are not significantly different according to Duncan Multiple range test ($p>0.05$).

Greenhouse Experiments

Application of *P. floursenses* significantly reduced citrus canker disease severity and intensity when compared with the untreated infected control. However, copper hydroxide was more effective than the bioagent *P. floursenses* in reducing the severity and intensity of citrus canker disease (Table 3). *P. fluorescens* reduced the canker lesion per leaf up to 72% compared with untreated infected control. A single application of 1.8 g/liter CuOH reduced significantly the number of canker lesions and disease severity by 90% compared to the untreated infected control (Table 3).

Table 3: Evaluation of the antagonistic capability of KSA1 strain as biological control agent against citrus bacterial canker pathogen under greenhouse conditions.

Treatment	***Xcc*[I]**	**1.8 g/L CuOH + *Xcc*[II]**	***P. fluorescens* + *Xcc*[III]**	***P***
Mean disease* intensity	3.1a**	1.8b	2b	< 0.001
Mean disease severity	15.8a	8.6b	10.6b	0.188

I Mexican lime seedlings infected with *Xcc* virlunet isolate
II Mexican lime seedlings treated with CuOH and then infected with *Xcc* virlunet isolate
III Mexican lime seedlings treated with *P. fluorescens* and after 72 hrs infected with *Xcc* virlunet isolate
*Disease intensity = lesion number per diseased leaf.
**Means within the same row followed by the same letter are not significantly different according to the Duncan *k*-ratio *t* test at $P = 0.05$.
Samples of 10 leaves per replicate were chosen and lesions were counted 40 days post inoculation.

DISCUSSION

In this study, a total of 22 bacterial isolates were recovered from leaf surfaces of Mexican lime trees and recognized as *P. fluorescens* based on the biochemical reactions (e.g., fluorescien production, levan formation, and certain carbohydrate utilizations) and morphological features (Palleroni, 1993). In addition, the results of oxidase, catalase, starch hydrolysis, gelatine liquefaction, growth temperature, salt tolerance, and carbohydrate utilization test confirmd the identity of these Saudi *P. fluorescens* isolates (Bossis *et al*., 2000). The ability of the Saudi *P. fluorescens* isolates to utilize different carbohydrate sources can help these bacteria to survive different environments and hence give them a competitive advantage over pathogenic organisms and make them as putative biocontrol agents. To confirm the identity of different Saudi strains, the 16S rRNA sequences showed that all strains were *P. flourseneses* with a similarity of 99%. Our results corroborate with the previous research that showed 16S rDNA gene was considered unsuitable for discriminating and identifying closely related strains due to the high levels of sequence similarity in this region (Fox *et al*. 1992).

The *in vitro* antibiosis experiments on KB agar medium showed that the largest inhibition zone of 62 mm diameter was obtained by KSA1 isolate. This local *P. fluorescens* isolate has more antagonistic capability than those isolates which applied against *Rhizoctonia solani* (maximum inhibition zone 40 mm) and *Ralstonia solanacearum* (maximum inhibition zone 280 mm) (Savithiry and Gnanamanickam, 1987; Anuratha and Gnanamanickam, 1990).

We have selected the most antagonistic isolates of *P. fluorescens* (KSA1) to conduct greenhouse experiments. The application of KSA1 decreased significantly the mean disease severity and disease intensity compared with untreated control. This suggests that this isolate can be used to improve the defense mechanism of citrus plants against bacterial citrus canker pathogen. In Iran, the application of *P. fluorescens* as a biological control agent against citrus canker reduced the number of lesion canker between 23.8 to 64% (Khodakaramian *et al*., 2008). The application of *P. fluorescens* as bioagents might induce plant systemic resistance or they may have a direct inhibitory effect on the pathogen (Kloepper *et al*., 1980; Aspiras and De-la Cruz, 1986; Van Loon *et al*., 1998). It has been demonstrated that isolates of *P. fluorescens* can induce systemic resistance in a variety of disease systems including wilt diseases, anthracnoses, bacterial, and viral diseases (Wei *et al*., 1991; Maurhofer *et al*., 1994; Liu *et al*., 1995). In conclusion, the isolation and application of indigeneous biological control agents such as isolates of *P. fluorescens*can offer an effective and safe control strategy against bacterial citrus canker in Saudi Arabia.

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