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The occurrence of apricot bacterial canker disease caused by *Pseudomonas syringae* pv. *syringae* in Saudi Arabia and factors affecting disease development

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Abstract

Bacterial canker of apricot (*Prunus armeniaca* L) has become a serious disease in many countries, including Saudi Arabia, but the causal agent in Saudi Arabia has not yet been sufficiently characterized. In the present study, bacterial strains were isolated from apricot fruits, showing bacterial canker symptoms, grown in Abha region, Saudi Arabia. Based on physiological, biochemical, and genetic characteristics, the pure bacterial cultures were identified as *Pseudomonas syringae* pv. *syringae* (Pss). The pathogenicity assays on *P. armeniaca* cv canino fruits developed canker symptoms that were similar to those observed on apricot fruits in Abha region. The 16S rDNA sequences were 99.9% identical to those of the Pss strains available in GenBank. Analysis of variances indicated that the temperature and inoculum concentration ($P < 0.0002$) significantly affect disease severity.

Keywords: Apricot trees, *Pseudomonas syringae* pv. *syringae*, bacterial canker, inoculum concentration.

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Introduction

Bacterial canker of stone fruit is a serious disease caused by the two related bacterial species *P. syringae* pv. *syringae* (Pss) and *P. syringae* pv. *morsprunorum* (Psm). Currently, the disease occurs in all regions of stone fruit production worldwide (Kennelly et al., 2007; Agrios, 1997; Hattingh & Roos 1995). The Pss pathovar causes canker disease on all commercially grown stone fruit tree species (Gavrilović et al., 2008), whereas the Psm primarily infects sweet cherry, sour cherry, plum and apricot (Bultreys & Kaluzna, 2010; Hattingh and Roos, 1995). Both pathovars are well adapted to different climatic conditions and cause severe damages in many stone fruit producing countries (Janse, 2006). The disease occurs on twigs and main trunk of the trees as well as buds, flowers, leaves and fruits (Janse, 2006). Bacteria can survive in high numbers within foliar, stem and fruit lesions. In addition, epiphytic populations of Pss are an important source of inoculum and can develop on host and non-host plants (Mansvelt & Hatting, 1986). Symptoms sometimes are not observed on host plants even when populations of Pss ($>10^4$ cfu g^{-1} of fresh tissue) are present. The need of a threshold population for infection has been suggested previously (Crosse, 1957), but factors determining the threshold levels of Pss require further studies. Environmental factors play an important role in the susceptibility of stone fruit plants to bacterial canker pathogens and may have a direct influence on resident populations of Pss and Psm (Gašić et al., 2012). In Saudi Arabia, some growers apply copper-based bactericides preventively while others apply them after the appearance of canker symptoms. Typical bacterial canker symptoms were observed on apricot trees in Abha region of Saudi Arabia where the disease had not been previously detected. In fact, bacterial canker pathogens of stone fruits are on the national A1 list of quarantine pathogens. The objectives of this study were to: (i) survey for the presence of *Pseudomonas syringae* pathovars on stone fruits tissues in the southwestern region of Saudi Arabia; (ii) identify and characterize the bacterial isolates recovered from apricot exhibiting canker symptoms, and (iii) study the effects of temperature and inoculum concentration on the infection and development of canker on apricot fruits.

Materials and methods

Pathogen isolation: Six locations belonging to three stone fruit-growing areas in the southwestern region of Saudi Arabia were surveyed for bacterial canker on commercial farms, backyard orchards and nurseries (Table 1). The disease incidence of bacterial canker for each block was calculated by expressing the number of diseased trees as a percentage of the total number of inspected trees. Immature fruits showing sunken brown-black regular lesions were collected. Each sample consisted of 4-6 symptomatic fruits per orchard. Tissue fragments were excised at the margin of the healthy and diseased tissues and first disinfected with 70% ethanol, then cut and crushed in a drop of distilled sterile water (DSW). After 20 min, a loopful of suspension was streaked on plates containing modified King's B (KB) medium supplemented with 1.5 mg/ml boric acid, 80 µg/ml cephalexin, and 200 µg/ml cycloheximide (Mohan & Schaad, 1987). After 2 days of incubation at 25°C, fluorescent bacterial colonies were selected and purified.

Identification of *Pseudomonas syringae* pathovars using biochemical tests: All bacterial isolates were tested for LOPAT (levan production, oxidase reaction, potato soft rot, arginine dihydrolase activity, and tobacco hypersensitivity) tests according to the methods described by Lelliot and Stead (1987) and Schaad et al. (2001). In addition, GATTa tests (gelatine liquefaction, aesculin hydrolysis, tyrosinase activity, tartrate utilization) were carried out for pathovar differentiation within *P. syringae* (Lelliot & Stead, 1987).

Presence of ice nucleation activity (INA): A loopful of each bacterial colony was suspended in 1 mL of DSW in sterile test tubes, and assayed for ice nucleation activity by the tube nucleation test (Paulin & Luisetti 1978). The ice nucleation test was carried out by placing the tubes containing the bacterial suspension refrigerated circulator water bath at -5°. Positive results were recorded if the bacterial suspension frozen within 10 min. Tubes with 5 ml NaCl were used as control.

16S rDNA sequence analysis: Total DNAs of *P. syringae* strains were extracted using the method described by Llop et al. (1999). DNA preparations were stored at -20°C until further use for PCR. The 16S rDNA region was amplified by using primers 27F and 1492R (Lane, 1991). PCR amplicons were cleaned and sequenced at the University of Kentucky Advanced Genetic Technologies Center (AGTC), College of Agricultural Sciences of the University of Kentucky (http://www.uky.edu/Centers/AGTC).
The obtained DNA sequences from *P. syringae* strains were compared with homologous sequences available in databases (GenBank, NCBI database).

Pathogenicity tests: Ten isolates were cultivated on KB media at 25°C. After 48 hrs, the plates were flooded with DSW and the resulting bacterial suspensions were adjusted to 1×10^8 cfu/ml^{-1} with spectrophotometer at wavelength of 660 nm and using serial dilution plating method. Immature apricot (*Prunus armeniaca* L) cv canino fruits were surface sterilized with 50% ethanol for 3 min, then rinsed thoroughly with DSW. Afterwards, fruits were wrapped with paper towel to remove the excess of water. Five fruits were pricked in two places with a sterilized needle dipped into 1×10^8 cfu/ml^{-1} bacterial suspension. The inoculated fruits were incubated in sterile plastic containers supplied with water-wetted piece of cotton to maintain high level of relative humidity at 25°C for four days. Fruits inoculated with DSW were used as negative control. After 48, 72 and 96 hrs of incubation, the symptoms developed around inoculation wounds were observed and pathogenic reactions were assessed by measuring the lesion diameter according to the following rating system: 0 = no symptoms, 1= lesion diameter between 0.1 and 1 mm, 2= lesion diameter between 1.1 and 2 mm, 3= lesion diameter between 2.1 and 3 mm, 4= lesion diameter between 3.1 and 4 mm, 5= lesion diameter over 4 mm (Xu and Gross, 1988). Re-isolations of the causal bacteria from infected fruits were performed on KB medium. The experiment was repeated once for all tested isolates with three replicates per strain.

Effect of inoculum concentration on disease development: The effect of inoculum concentration was investigated by inoculating five apricot fruits as previously described with different bacterial concentrations (1×10^3 to 1×10^8 cfu/ml^{-1}) of Psa3 strain. DSW was used as a negative control. The inoculated fruits were incubated in sterile plastic containers at 25°C for five days. The experiment was conducted twice with three replicate. Each replicate had five fruits.

Effect of temperature and inoculum concentration on disease development: Immature apricot cv. canino fruits were inoculated as previously described with an inoculum suspension of Psa3 strain, adjusted to 10^3 to 10^8 cfu ml^{-1}, and incubated at different temperatures (5, 10, 15, 20 and 25°C). Controls were treated similarly with DSW. Disease severity was determined after 5 days of incubation. This experiment was repeated twice with three replicate. Each replicate had five fruits.

Statistical analysis: Analysis were performed using SAS software system (SAS Inc., Cary, NC, USA, 2003). Data collected from the two experiments were pooled when no significant differences were found according to Bartlett's test ($P > 0.05$). Significant differences among means were determined using Tukey test.

Results

Orchard survey and bacterial isolation: Out of the six visited locations, bacterial canker symptoms were only observed on nearly 20% of apricot trees from the two sampled orchards in Abha (Table 1). No bacterial canker symptoms were observed on apricot and peach trees in both Al-Bahah and Jazan regions. The overall disease incidence on trees in Abha was 20% in one farm and 15% in the other one.

Identification of the causal pathogen: On modified KB medium, twenty-three bacterial strains developed creamy white, circular, flat, and translucent colonies and produced fluorescent pigment under UV light. Out of these 23 isolates, 10 formed levan from sucrose and were oxidase and arginine dihydrolase negative. These 10 isolates were also potato rot negative and induced hypersensitivity response (HR) on tobacco. Moreover, the 10 isolates were positive for ice nucleation activity. According to the results of LOPAT tests, the 10 bacterial strains were identified as *P. syringae*. *Pseudomona syringae* isolates were positive for gelatin and aesculin hydrolysis, but negative for tyrosinase activity and metabolism of tartrate. GATTa tests confirmed that all *P. syringae* strains were Pss. At the molecular level, the 16S rDNA sequences obtained from Saudi Pss isolates showed 99.9 % homology with other 16S rDNA sequences of Pss deposited in GenBank.

Pathogenicity tests: The results of pathogenicity tests showed that all Pss isolates were pathogenic on apricot fruits. Pss isolates significantly ($P < 0.0001$) caused disorder symptoms around wounds on fruits after 48, 72, and 96 hrs of inoculation. After four days from fruit inoculations with Pss isolates, deep black brown necrosis regions were observed with different degrees of

severity (Table 2). Generally, the severity of symptoms increased with increasing the incubation period throughout the experiments. Four days post inoculation, the isolates Pss3, Pss4 and Pss5 showed the highest virulence, whereas isolates Pss9, Pss6 and Pss7 showed the lowest disease severity (Table 2). No significant differences in disease severity were observed among Pss3 (5.3), Pss4 (4.8) and Pss5 (4.6) isolates. To fulfill the Koch's postulates, Pss bacteria were re-isolated from symptomatic fruit tissues and their identity was confirmed by biochemical and physiological characteristics. Overall, the most virulent Pss isolate was Pss3, and was selected for further pathogenicity tests. No symptoms were developed on control fruits treated with DSW.

Table 1: Region, location, host plant and disease incidence of stone fruit bacterial canker disease caused by *Pseudomonas syringae* pv. *syringae* in Saudi Arabia during 2014.

Region	Locations	Host plant	Disease incidence (%)
Al-Bahah	Al-Makhwah	Peach	0.0%
	Baljurshi	Peach, Apricot	0.0%
Jazan	Abu Arish	Peach	0.0%
	Sabya	Peach	0.0%
	Samitah	Peach	0.0%
Abha	Farm1	Apricot	20%
	Farm2	Apricot	15%

Table 2: Virulence of *Pseudomonas syringae* pv. *syringae* strains on apricot (*Prunus armeniaca* L) cv canino fruits using a disease severity scale from 0 to 5.

Strain*	Hours post inoculation		
	48	72	96
Pss1	2.1^{h}	3.2^{d-h}	4.1^{a-d}
Pss2	2.3^{f-h}	2.3^{f-h}	3.7^{b-e}
Psa3	2.4^{f-h}	3.1^{d-h}	5.3^{a}
Pss4	2.0^{h}	2.8^{e-h}	4.8^{ab}
Pss5	2.3^{f-h}	2.7^{e-h}	4.6^{a-c}
Pss6	2.4^{f-h}	2.6^{e-h}	3.5^{c-f}
Pss7	2.3^{f-h}	2.8^{e-h}	3.8^{b-e}
Pss8	2.2^{gh}	3.2^{d-h}	3.8^{b-e}
Pss9	2.1^{h}	2.6^{e-h}	3.2^{d-h}
Pss10	2.0^{h}	3.1^{d-h}	3.4^{c-g}
Control	0.0^{i}	0.0^{i}	0.0^{i}

*Mean value with same letter did not show significant difference in disease severity throughout experiments according to Tukey HSD Comparisons Test. Data were subjected to a two-way ANOVA using SAS PROC GLM.

Table 3: Effect of different inoculum concentrations of *Pseudomonas syringae* pv. *syringae* strain Pss3 on apricot (*Prunus armeniaca* L) cv canino fruits using disease severity scale from 0 to 5.

Inoculum conc.	Hours post inoculation		
	48	72	96
10^3	0.3^{f*}	0.6^{ef}	0.7^{ef}
10^4	0.3^{f}	0.6^{ef}	0.8^{ef}
10^5	0.4^{ef}	0.7^{ef}	0.9^{def}
10^6	0.4^{ef}	0.8^{ef}	1.2^{def}
10^7	1.6^{de}	2.9^{bc}	3.7^{ab}
10^8	2.1^{cd}	3.2^{bc}	4.6^{a}
Control	0.0^{g}	0.0^{g}	0.0^{g}

*Mean value with the same letter did not show significant difference in the disease severity development throughout the experiment according Tukey HSD Comparisons Test. Data were subjected to a two-way ANOVA using SAS PROC GLM.

Effect of inoculum concentration on disease severity: The results shown in Table (3) represent mean data from the two experiments to evaluate the effect of different Pss inocula on disease severity. Generally, significant differences in disease severity ($P < 0.0001$) were observed with different inoculum concentrations. However, no significant difference in disease development was observed between 10^8 and 10^7 cfu^{-1}inoculum concentrations. Disease severity significantly ($P < 0.0001$) increased in log regression/trend line throughout the time. Apricot bacterial canker lesions were observed in all treatments, except the control fruits treated with DSW. Inoculum concentrations of 10^7 and 10^8cfu resulted in 3.7 and 4.6 disease severities after 96 h post inoculation, while at 10^3 cfu ml^{-1} resulted only in 0.7.

Table 4: Effect of different inoculum concentration and temperature of *Pseudomonas syringae* pv. *syringae* strain Pss-3 on apricot (*Prunus armeniaca* L) cv canino fruit disease development after five days of inoculation using a disease severity scale from 0 to 5.

Inoculum conc. (cfu)	Temperature (^{0}C)				
	5	10	15	20	25
10^3	0.6^{j}	0.8^{ij}	0.8^{ij}	1.5^{g-j}	1.8^{f-j}
10^4	0.6^{j}	0.8^{ij}	1.1^{h-j}	1.8^{f-j}	2.2^{d-i}
10^5	1.5^{g-j}	2.6^{c-g}	2.6^{c-g}	2.0^{e-j}	2.8^{c-g}
10^6	2.1^{e-i}	2.8^{c-g}	3.2^{b-f}	2.8^{c-g}	3.4^{a-e}
10^7	2.3^{d-h}	3.2^{b-f}	3.8^{a-c}	3.6^{a-d}	4.3^{ab}
10^8	2.4^{c-h}	3.8^{a-c}	4.4^{ab}	4.6^{ab}	4.8^{a}
Control	0.0^{k}	0.0^{k}	0.0^{k}	0.0^{k}	0.0^{k}

*Mean value with the same letter did not show significant difference in the disease severity development throughout the experiment according Tukey HSD Comparisons Test. Data were subjected to a two-way ANOVA using SAS PROC GLM.

Effect of temperature and inoculum concentration on disease severity: Temperature significantly ($P < 0.0002$) affected disease severity on apricot fruits. Symptoms of bacterial apricot canker were observed at all evaluated temperatures. Disease severity ranged from 0.6 to 4.8, with an optimum temperature ranged from 15 to 25°C (Table 4). Analysis of variances indicated a significant effect of temperature and inoculum concentration ($P < 0.0002$) on disease severity. However, there was no interaction between inoculum concentration and temperature ($P = 0.051$). The mean disease severity obtained at 15°C was significantly different ($P < 0.05$) from the mean disease severity at 5°C according to Tukey's tests. The equation y = -0.0021x2 + 0.1373x + 1.0299 (R2= 0.9459) provided a description of the effect of temperature on disease severity (Fig. 1).

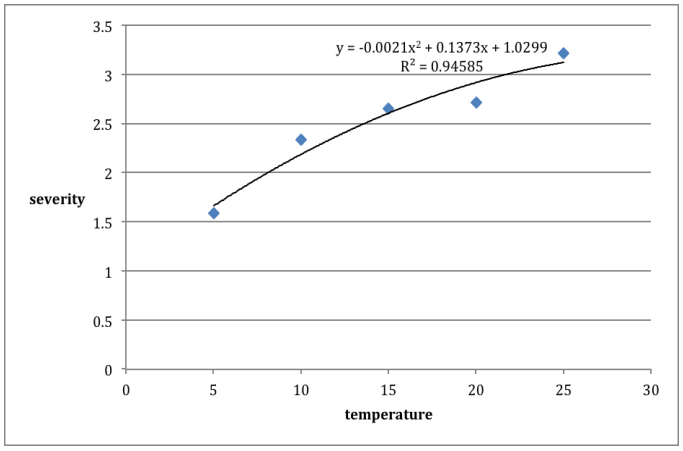

Figure 1: Trend line on temperature against disease severity of apricot (*Prunus armeniaca* L) cv canino inoculated with *Pseudomonas syringae* pv. *syringae* strain Pss3.

Discussion

Based on the results obtained from physiological and biochemical assays as well as the 16S rDNA sequence analysis, the symptoms on apricot trees in Abha, Saudi Arabia are caused by *P. s.* pv. *syringae*. This is the first report of Pss occurrence in stone fruit-producing orchards in the southwestern region of Saudi Arabia. In most of the plantations visited, only a small proportion of the apricot young trees showed symptoms of bacterial canker. This may be due to variable levels of resistance and/or older trees might be more resistant to bacterial canker. As apricot is generally planted as a proportion of a mixed plantation, this might also limit the spread and build up of inoculum. Our field observations suggest that low temperature in winter season followed by the appearance of the disease severity on twigs may be used as a reliable indicator of the damage that could happen during the following

growing season. The average annual mean temperature in Abha region is 18.3^{o}C with a maximum of 35^{o}C during July, and a minimum of 3^{o}C in January, with a relative humidity between 35% and 70% during July and August, respectively. Isolation of *P. syringae* from plant material showing bacterial canker symptoms was generally difficult. Semi-selective media was used in this study for isolation of *P. syringae* isolates from apricot fruits. Twenty-three isolates were obtained from fruits rather from branch cankers. This was probably related to the time of the year when plantations were visited. While bacterial cankers have a peak of development in spring and most bacteria die or become inactive during the summer. The bacterial strains isolated from the apricot tissues showing bacterial canker symptoms gave the typical characteristics of LOPAT assays (Lelliott et al. 1966), indicating that they belong to the fluorescent *P. syringae*. Lelliott et al. (1966) showed that among the fifteen tests used to determine and differentiate the fluorescent plant pathogenic *Pseudomonas* strains, LOPAT tests can differentiate five distinct pathogenic groups. Bultreys and Kaluzna (2010) also used LOPAT tests to differentiate the fluorescent plant pathogenic *Pseudomonas* spp. In addition, GATTa tests confirmed the identity of Pss strains *P. s. syringae*. Latorre and Jones (1979) reported that the GATTa tests were reliable enough to discriminate between pv. *syringae* and pv. *morsprunorum*. In addition to GATTa, other tests, such as ice nucleation and bacterial growth in sucrose nutrient broth, can also be used as differential tests between different pathovars. These tests are rapid, reliable, inexpensive and simple to perform. *Pseudomonas syringae* is a very heterogeneous group of bacteria. Strains of this pathogen can show differences in virulence when artificially inoculated in their host plants (Gilbert et al. 2009; Vicente et al. 2004; Scortichini et al. 2003). On the basis of physiological, biochemical or serological tests, isolates of pathogenic *P. syringae* cannot be distinguished from non-pathogenic ones (Vicente et al., 2004). Although tobacco hypersensitive reaction is a reliable discrimination method to identify pathogenic *P. syringae* strains, it is not a substitute for pathogenicity tests on susceptible host plants (Gašić et al., 2012, Latorre & Jones, 1979). Therefore, the pathogenicity of *P. syringae* strains should be evaluated on the host plants from which these bacteria were isolated to have a better idea about their pathogenic abilities. In this study, it was not feasible to evaluate the pathogenic ability of Pss strains on apricot trees as it would be time and money consuming, and consequently, the pathogenicity tests were conducted on immature apricot fruits. Many previous studies showed that pathogenicity tests on fruits were suitable for evaluating pathogenic ability of many pathogens and gave consistent results (Ivanović et al., 2012; Kałużna & Sobiczewski, 2009). In the present study, all Pss strains isolated from diseased apricot tissues induced typical symptoms of *P. s.* pv. *syringae* on an inoculated unripen apricot fruits after 48, 72, and 96 hrs post inoculation with significant differences. Similar to Wimalajeewa and Flett (1985), apricot fruits infection in this study increased when Pss concentration were higher than 10^3 cfu $m1^{-1}$. Resident populations of Pss higher

than 10^3 cfu $m1^{-1}$ may occur in the field in Saudi Arabia. Incubation temperatures played a significant role in apricot bacterial canker development. At low temperature (5°C), Pss growth was very slow, explaining the lowest infection ability on fruits. In contrast, in some studies, freezing temperatures can enhance bacterial infection through the physical damage of host cells leading to opening avenues for rapid bacterial penetration (Sule & Seemuller, 1987). At 20°C, bacterial growth was rapid and fruit infections were higher than at 5°C. Latorre et al. (2002) reported that the most favorable temperature for bacterial canker extension was 20°C. No significant differences were observed between 10°C to 25°C. These results suggest that temperature fluctuations (10 to 25°C) may be equally conducive to bacterial canker development, especially if bacterial populations are higher than 10^3 cfu $m1^{-1}$. Temperature appeared as the main determining factor for infection caused by Pss on fruits of apricot. Further molecular work is underway to characterize the Pss isolates from apricot, and to test the susceptibility of other apricot varieties.

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