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MOLECULAR IDENTIFICATION OF BACTERIAL CANKER AND BACTERIAL WILT DISEASES IN TOMATOES

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Abstract

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Severe bacterial symptoms were observed in tomato growing areas of Tokat province, Turkey. The tomato plants have asymmetric wilting appearing on leaves with brown discolorations of vascular systems. A total of 45 bacterial isolates were collected from all tomato grown areas of Tokat province. A potassium hydroxide (KOH) string test was applied to all the bacterial samples: 9 of the 45 bacterial colonies were gram positive and 36 colonies were gram negative. The gram positive bacterial colonies were analyzed with *Clavibacter michiganensis* subsp. *michiganensis* specific primers Cmm5 and Cmm6 in PCR assays of which 3 produced 614 bp amplification products and were identified as *C. michiganensis* subsp. *michiganensis*. The 36 bacterial gram negative colonies were analyzed with *Ralstonia* ssp. specific primers RsolfliC forward and RsolfliC reverse of which 4 colonies amplified 400 bp fragments and were identified as *Ralstonia* ssp. Pathogenicity tests with 7 tomato cultivars and 10 wild tomato accessions revealed that all the 7 tomato cultivars were susceptible to virulent Cmm2 isolate. However, 2 wild tomato accessions (LA1054, LA1318) were resistant, 6 accessions were susceptible and 2 accessions were enhanced susceptible to the Cmm2 isolate. The results of this study demonstrated express and reliable detection methods of bacterial canker disease in tomatoes and possible resistance sources to bacterial canker disease from wild tomato accessions.

Key words: tomato, bacterial canker, bacterial wilt, molecular diagnosis

Introduction

During the summer and early autumn 2008, 2009 and 2010 severe outbreaks of diseases were observed in tomato fields in Tokat, situated in the Black Sea region of Turkey. Characteristic symptoms were light brown discoloration of the vascular tissues, wilting of leaves, sometimes asymmetric and necrotic lesions on leaves, stems and petioles. These symptoms associated with bacterial pathogens caused significant crop losses under the high temperature and moist conditions. These suitable environmental conditions encouraged the plant pathogenic bacteria to cause epidemic diseases affecting whole tomato plant leaves, stems, and flowers, young or mature fruits damaging them irreversibly (Agrios, 1997; Cook and Sequeira, 1994). The quantitative yield losses specific to Tokat province have not yet been determined. Identification, characterization and quantification of the bacterial diseases are crucial to control the bacterial diseases.

Bacterial canker disease is a serious tomato disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). The pathogen can survive infested soil for short periods, in over-seasoned plant debris in the soil, on contaminated seeds and transplants. Cmm shows marginal necrosis on tomato leaves and stems,

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small dark spots on fruit surrounded with 'bird's-eye' spots, generally in seedlings stunting, wilting and stem splitting are characteristic of the bacterial canker disease (Agrios, 1997; Werner et al., 2002). The bacterial wilt disease caused by *Ralstonia solanacearum* (Rs) appears on young leaves with sudden and permanent wilting. The tomato roots and lower parts of stem have browning of their vascular system. The Rs infected tomato plants show signs of stunting, and adventitous root development on the main stems (Fegan and Prior, 2005). Bacterial wilt symptoms are distinguished from those of *Fusarium* wilt because of the rapidity of the wilt, under favorable conditions, for the former, and the drier, firmer stem rot of the latter (Elphinstone, 2005).

The genetic control of bacterial diseases has been promising. Tomato cultivars with some resistance or tolerance to bacterial canker have been introduced, but there is little significant tolerance in commercial tomato varieties (Gleason et al., 1993). Three cultivars of eggplant resistant to bacterial wilt have been used in India (Gopalakrishnan et al., 2005; Van Steekelenburg, 1985). Extensive international research has produced some highly resistant tomato breeding lines but the resistance is usually linked with undesirable traits like small fruit size. Some large-fruited resistant tomato cultivars (e.g. FL7514 and BHN 466) have become commercially available in recent years, and provide moderate resistance against bacterial wilt (Elphinstone, 2005). Resistance sources have been found in wild relatives of tomato to both bacterial diseases (Gleason et al., 1993; Coaker and Francis, 2004). Resistant tomato cultivars have not been investigated against both bacterial pathogens yet. Further work is needed to produce resistant tomato cultivars for tomato growers.

The bacterial pathogens cause severe disease and decline on the tomato yields in Worldwide. The bacterial pathogens cause between 10 to 80% yield losses depending on cultivar's susceptibility, environmental conditions and stage of infection (Agrios, 1997). Due to the economic importance of bacterial diseases in tomato production, the applications of new measures to control the agents of the diseases are crucial. Management of the disease is difficult and mainly based on chemical applications that include use of several copper compounds with agronomics practices to reduce inoculum's sources (Gleason et al., 1993; De Leon et al., 2008). The detection of tomato bacterial pathogens between infected or healthy seedlings at the time of transplanting is complicated, thus allowing undetected spread of the bacterial pathogens. One way to control the bacterial disease is to prevent the introduction of infected plants and to produce disease-free seedlings. Hence, highly sensitive, reliable and express detection techniques are essential for screening seeds and seedlings to contend with the bacterial diseases.

This paper aims to describe molecular identification of tomato plant bacterial pathogens, to distinguish two symptomatically similar bacterial pathogens, *Clavibacter michiganensis* subsp. *michiganensis* and *Ralstonia* spp., and to determine resistant source from wild and cultivated tomatoes.

Material and Methods

Bacterial strains: The reference strain of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm2) obtained from Prof. Dr. Hüseyin BASIM (Akdeniz University, Plant Protection Department, Antalya, Turkey) was used in these studies. The bacteria were grown on glucose yeast carbonate agar (GYCA) medium at 28 °**C (**Lelliott and Stead, 1987). The other reference strain of *Ralstonia solanacearum* biovar 2 was identified by Nursen USTUN (Plant Protection Research Institute, Izmir, Turkey) and used as positive controls in these studies (Ustun et al., 2008).

Plant materials: In pathogenicity tests, 7 tomato lines and 10 wild tomato accessions were used to assess their phenotypic reactions with Cmm2. Except NC84173, tomato seeds of all tomato lines (from EBR1 to EBR6) were kindly provided by Prof. Dr. Randy GARDNER (Horticultural Science, North Carolina State University, USA), a further 10 wild tomato accessions, and NC84173 tomato line seeds were obtained from Tomato Genetics Resource Center (Department of Plant Sciences, University of California at Davis, USA). The tomato seeds were sown in 6 cm × 30 cm × 50 cm plastic seed trays containing turf (Potground, Klasmann, Germany). The seed trays were placed in a glasshouse at 24±5 °C temperature with 50% relative

humidity, 16 h. day, 8 h. night conditions. Germinated seedlings were placed on turf containing pots at 4-5 real leaf stages of tomato plants.

Sampling and isolation: A total of 84 tomato fields in Tokat province were sampled. The disease ratio was determined by counting wilting plants per 100 plants per field. Tissue samples were collected from whole leaves, stems and fruits in a plastic bag and the samples were kept in a portable cool box. In the laboratory, whole plant tissues were cut (1-2 cm in length) at the margin of necrotic or wilting areas and placed in 5% sodium hypochloride (NaClO) for 45 seconds (s) and 70% ethanol solution for 45 s respectively, then rinsed three times in sterile distilled water. Thereafter, the samples were blotted dry on sterilized paper towels and plated on **n**utrient agar (NA) or glucose yeast carbonate agar (GYCA) according to Lelliott and Stead (1987), and incubated at 28 °C for 2-3 days. On the basis of typical colony morphology, 45 bacterial isolates were selected and further purified on NA or GYCA.

Bacterial culture media and plant inoculation: Individual bacterial strains collected from tomato fields were sub-cultured in NA (Merck, VM984550823) or GYCA medium containing 5 g glucose, 5 g yeast extract, 40 g calcium carbonate and 15 g agar per liter of water, pH 7.2. All strains were stored in 30% glycerol at –80°C until used. The inoculum of reference strain Cmm2 was the most virulent strain among those tested with our isolated strains in this study was prepared from 2-day-old bacterial culture on GYCA, the bacteria were picked using a sterile toothpick then the toothpick was immediately initiated into the plant stem at 4-5 real leaf stage. For the control plant's inoculation, the toothpick was dipped in distilled sterile water then was initiated into the plant stem. Inoculated plants were kept in a glasshouse at 23±5°C temperature with 60% relative humidity, 16 h day, 8 h night conditions.

Detection and characterization of bacteria: Symptoms of bacterial canker and bacterial wilt diseases are not easy distinguished on tomato seedlings. Each collected bacterial pathogen should be assessed to detect and characterize if the bacterium was either gram positive or gram negative. Hence, the obtained 45 bacterial isolates from diseased plants were tested with the KOH test according to Powers (1995). The gram positive and gram negative bacterial isolates were analyzed with *Clavibacter michiganensis* subsp. *michiganensis* and *Ralstonia solanacearum* specific primer sets in polymerase chain reaction (PCR) assays. The second bacterial pathogen *R. solanacearum* was further confirmed using biochemical and immunofluorescence (IF) tests.

Biochemical and immunofluorescence (IF) tests: The bacterial isolates were studied for identification of *Ralstonia solanacearum* by biochemical and immunofluorescence (IF) tests. The bacteria utilised maltose, lactose and D(+) cellobiose, but not mannitol, sorbitol and dulcitol in accordance with biovar 2. In IF tests, fluorescing cells with typical morphology were observed at antibody dilutions of 200–12 800 according to Ustun et al. (2008).

Primers: In PCR, a previously developed primer set for Cmm subspecies specific was used with the following sequences: Cmm5 forward 5'-GCGAATAAGCCCATATCAA-3' and Cmm6 reverse 5'-CGTCAGGAGGTTCGCTAATA-3' primers that yield an amplicon size of 614 bp for *Clavibacter michiganensis* subsp. *michiganensis* (Dreir et al., 1995; Santoz et al., 1997; Burokiene, 2006). For detection and identification of *R. solanacearum*, two primers sets were used: the first RalfliC primer set (RalfliC forward: 5'-CCTCAGCCTCAATASCAACATC-3' and RalfliC reverse: 5'-CATGTTCGACGTTTCMGAWGC-3') was specific for *R. picketti*, *R. solanacearum* and *R. syzygii* yielding an amplicon of 724 bp and the second RsolfliC primer set (RsolfliC forward 5'-GAACGCCAACGGTGCGAACT-3' and RsolfliC reverse 5'-GGCGGCCTTCAGGGAGGTC-3') was specific for *R. solanacearum* giving an amplicon size of 400 bp (Schonfeld et al., 2003).

Preparation of bacterial suspension for PCR: Each bacterial strain was grown on GYCA agar using a sterile loop, three full loops of bacteria transferred into 1 ml 25 mM sterile $MgCl_2$ (pH: 7.0) and mixed thoroughly. The number of colony-forming units (cfu) was determined for PCR using OD_{600} wavelength UV/V spectrophotometer (Biomate, USA). A 4 µl aliquot of the suspension was added to 16 µl PCR mixture.

PCR amplifications: The reaction was preformed in final volume of 20 µl containing 5× PCR buffer

(Promega Corp.), 4 mM $MgCl_2$, 100 µM each of dATP, dCTP, GTP and dTTP, 20 ng of primer, 1,2 units of Taq DNA polymerase and 4 µl bacterial suspension. After an initial denaturation step for 5 min at 94°C, amplification was performed by using 40 cycles. The cycles consisted of a 30 second (s) denaturation at 94°C, 2 min of primer annealing at 58°C for Cmm system, 60°C for RalfliC system and 63°C for RsolfliC system, and a 1 min primer extension at 72°C followed by a final extension step at 72°C (5 min) and stored at 8°C. The amplified products were separated on 1.5% agarose gels including ethidium bromide (0.5 µg ml^{-1}) for 2 h at 6 V cm^{-1} constant voltages in TBE buffer according to Sambrook et al. (1989).

Statistical analysis

Using the SPSS statistical package (SPSS for Windows 17.0), an analysis of variance (ANOVA) was performed to estimate genetic resistance.

Results

Bacterial sample collections: A total of 45 bacterial isolates were purified from 184 tomato samples that presented typical wilting and necrosis symptoms were collected from all tomato grown areas of Tokat province, Turkey. To determine gram positive bacterial colonies, shiny, highly viscous, yellow-colored colonies were analyzed using the KOH string test. Nine bacterial colonies out of the 45 were shown to be gram positive and the remaining 36 bacterial colonies gram negative. These KOH string tests revealed that 20% of collected bacteria were gram positive; however, 80% of the bacterial collection was gram negative. The KOH String Test therefore shows itself to be a reliable technique in classifying two distinct types of bacteria based on the structural differences of their bacterial cell walls.

Molecular detection of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm): The gram-positive 9 bacterial colonies were analyzed with the subspecies-specific primers Cmm5 and Cmm6 in PCR assays. The Cmm bacteria were confirmed with 614 bp amplification products (Figure 1). The other bacteria gave no amplification products with the same primer sets (Figure 1). Three bacterial isolates were found as *C. michiganensis* subsp. *michiganensis* in PCR analysis manifesting that 6.6% of tomato growing areas are infested with the bacterial canker disease at Tokat province, Turkey. These 3 bacteria were further tested on NCEBR3 homozygous tomato lines in pathogenic tests, wilting and canker symptoms were observed at 15 days post inoculation.

Plant phenotypic reactions to Cmm2: The Cmm2, the most virulent isolate and reference strain of *C. michiganensis* subsp. *michiganensis* was tested with 7 tomato cultivars and 10 wild tomato accessions in pathogenicity tests as described in material and methods. All the tested 7 tomato cultivars were enhanced susceptible to the Cmm2 isolate (Table 1), and the inoculated plants were died completely in 28 days post inoculation (dpi). However, the tested wild tomato accessions revealed various degrees of resistance and susceptibility. The wild accessions LA1054 and LA1318 were the most resistant wild tomatoes which set seeds without any wilting and canker symptoms on leaves and stems respectively (Table 1). The LA1362, LA0094, LA1912, LA1347, LA1579, LA1560 were susceptible and LA1391, LA1106 were enhanced susceptible to the Cmm2 (Table 1). Enhanced susceptible wild accessions died 14 dpi, the susceptible wild tomatoes died in 28

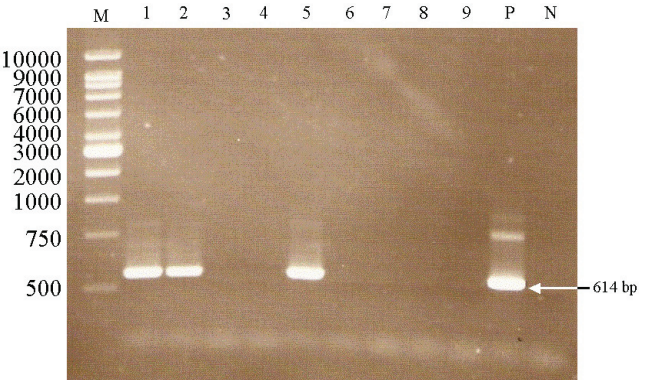

Fig. 1. PCR analysis of 9 gram-positive bacterial samples. A 614 bp band was detected from 3 bacterial samples showing the bacteria were *Clavibacter michiganensis* subsp. *michiganensis* from tomato plants. Lanes: M: 1 Kb DNA size marker, P: Positive control, N: Negative control

dpi and none of the accessions set any tomato fruits during the pathogenicity tests; therefore, they did not produce seeds.

Molecular detection of *Ralstonia solanacearum*: The remaining 36 isolates of bacteria were analyzed with the *Ralstonia* species flagella subunit specific RalfliC forward and RalfliC reverse primers in PCR assays. A 724 bp amplicon was found in 11 samples (Data not shown). The 11 bacterial samples were further analyzed with the species-specific primers RsolfliC forward and RsolfliC reverse in PCR analysis according to Schonfeld et al. (2003). The RsolfliC primer set produced 400 bp amplicon products in 4 samples but the other samples did not produce amplified bands (Figure 2).

Table 1
Pathogenicity test results of 7 tomato cultivars and 10 tomato wild accessions with *Clavibacter michiganensis* subsp. *michiganensis* Cmm2 isolate. The tested plants were monitored every day during first 7 days and 14, 21, 28 days post inoculation

Gene bank accession	Total number of plants*	Phenotypes			Tomato fruit production
		Enhanced Susceptible	Susceptible	Resistant	
NCEBR1	20	ES			No
NCEBR2	20	ES			No
NCEBR3	20	ES			No
NCEBR4	20	ES			No
NCEBR5	20	ES			No
NCEBR6	20	ES			No
NC84173	20	ES			No
LA1054	20			R	Yes
LA1318	20			R	Yes
LA1362	10		S		No
LA0094	10		S		No
LA1912	10		S		No
LA1347	10		S		No
LA1579	10		S		No
LA1560	10		S		No
LA1391	10	ES			No
LA1106	10	ES			No

* 50% of the number was used as control and 50% were inoculated.

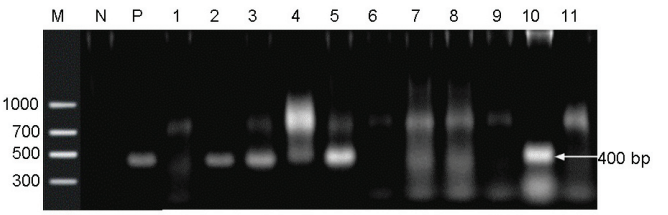

Fig. 2. PCR analysis of 11 gram-negative bacterial samples. A 400 bp band was verified the 4 bacterial samples were *Ralstonia solanacearum* from tomato plants. M: 1 Kb DNA size marker, P: Positive control, N: Negative control

Biochemical and immunofluorescens (IF) test results: The promising 4 bacterial samples were further studied in biochemical and immunofluorescens tests. The tested 4 bacterial isolates did not produce creamy-brown pigment on levan test and they were fermentative with oxidation/fermentation tests (Table 2). In IF tests with the 4 bacterial samples, fluorescing cells with typical morphology were not observed at antibody dilutions of 200–12800 (Table 2). The 4 bacterial isolates did not establish typical wilting symptoms on the inoculated tomato plants (cultivar Rio Grande) at 5-6 days post inoculation. These results revealed that the 4 bacterial isolates were not *Ralstonia solanacearum* but they were *Ralstonia* spp. according to the biochemical and the IF tests.

Discussion

In the last decade, tomato producers have chosen commercial tomato cultivars for economic reasons, the farmers have preferred to use either tomato seedlings or seeds from international companies located in southern part of Turkey such as Antalya. Seedling nursery companies are under pressure to provide millions of seedlings for farmers in a short time and these seedlings could be contaminated with seed born bacterial canker pathogens. The seedlings are imported from southern part of Turkey to other parts of Turkey without proper internal quarantine applications. It is important that farmers know if their purchased seedlings and seeds are free of or infected with the bacterial wilt and canker pathogens. At this critical stage plant protection de-

Table 2
Biochemical and immunofluorescens tests results for identification of *Ralstonia solanacearum*

Test	*R.solanacearum*	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Growth on SMSA	typical red colonies	-	-	-	-
Fluorescent pigment on King B	(-) creamy	- yellow	- yellow	- yellow	- yellow
Levan on NSA	- creamy (brown-red pigment)	-yellow	-yellow	-yellow	-yellow
Oxidation/ Fermentation	oxidative	fermentative	fermentative	fermentative	fermentative
Arginine	-	-	-	-	-
Aesculin	-	+	+	+	+
Gelatin	-	+	+	+	+
2% NaCl	+	+	+	+	+
IFAS	+ (1/100-1/12800)	-	-	-	-

partments of Universities need solutions to solve such dilemmas between seed or seedling companies and producers should provide important professional help. Hence, reliable and rapid test methods are requisite to identify the bacterial diseases.

Bacterial streaming could be a common diagnostic sign of *R. solanacearum*. When cut stem sections from infected plants are placed in water, threads of a viscous white slime can be observed streaming from the cut end of the stem within 10 min. This streaming test could be a valuable diagnostic tool for quick detection of bacterial wilt However, there are no common diagnostic signs of C. *michiganensis* subsp. *michiganensis* until advanced stage where brown stripes and cavities may appear on stems and bird's eye spots develop on fruits. These specific signs could not be observed on tomato seedlings before transplanting them into field. At this stage, a simple KOH string test could be applied to identify gram-negative and gram-positive bacteria. Subspecies-specific primers are able to identify gram-positive C. *michiganensis* subsp. *michiganensis* bacteria. However, this is not true for gram negative *R. solanacearum* bacteria. Previously, Schonfeld et al. (2003) identified that *R. solanacearum* flagella subunit specific RsolfliC primer set showed 82 strains of *R. solanacearum* in PCR analysis. In this study, we identified 4 gram negative bacterial isolates of *R. solanacearum* using the specific primer set in PCR analysis at molecular level but immunofluorescense and pathogenicity tests did not confirm these results. Therefore, sequencing could be a very simple procedure now and it is required for bacterial identification using both both specific primers as well as universal bacterial primers for 16SrDNA.

Both bacterial canker and bacterial wilt diseases cause significant crop losses on *Solaneceae* plants. Resistant plants are important to control the bacterial diseases. In this study, the pathogenicity tests exhibited at least two (LA1054, LA1318) resistance sources in wild tomato accessions to a virulent Cmm2 isolate of C. *michiganensis* subsp. *michiganensis*. The results of pathogenicity tests could provide substantial evidence to the bacterial canker disease.

The incidence of bacterial diseases in tomato fields has increased, and without good diagnostic tools this situation could result in significant epidemics in tomato fields in Turkey which will have devastating economic effects on the country. Farmers need not only need to use disease free seeds or seedlings but also resistant tomato plants to start appropriate tomato production. The present study could help to provide express and reliable methods to detect the bacterial diseases.

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