Bacterial leaf spot of lettuce caused by *Xanthomonas hortorum* pv. *vitians* in the Aegean Region of Turkey

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Abstract

Samples of diseased plants were collected from 12 lettuce fields in Aydin province of the Aegean Region of Turkey. A total of 8 bacterial isolates were identified by morphological characterization, sequence analysis of 16S rDNA and *gyrB* genes, and pathogenicity. The survey included twelve lettuce fields of three different cultivars [Yedikule (romaine type), Robinson and Chianti (iceberg type)]. The incidence of the disease ranged from 48 to 95%. Plants of cv. Chianti were the most severely infected cultivars. To our knowledge, this is the first report of the presence of *Xanthomonas hortorum* pv. *vitians* in lettuce plants in Turkey.

Keywords Bacterial leaf spot · Lettuce · *Xanthomonas hortorum* pv. *vitians*

Cultivated lettuce (*Lactuca sativa*), originated in the Mediterranean basin (including Turkey) and Near East (Davis et al. 1997), has been grown annually on about 21,000 ha area contributing 447,000 metric tons in Turkey (TUIK 2015). There are three main type lettuces commonly grown in Turkey: crisphead (iceberg), leaf, romaine or cos, with 14.4%, 35.3% and 50.3% of the total production, respectively. Aegean region is one of the seven geographical regions of Turkey and has the second largest lettuce cultivation area comprising about 18% of lettuce production after the Mediterranean region. Sixty percent of the production of commercial crisphead lettuce in Turkey occurs in the Aegean region (TUIK 2015).

Bacterial leaf spot (BLS) is a potentially serious seed-borne disease affecting lettuce production in the world. BLS outbreak, caused by *Xanthomonas campestris* pv. *vitians*, have been reported in many countries around the world and in different states of

US (Barak and Gilbertson 2003). Recently, Toussaint et al. (2016) reported that the occurrence of bacterial leaf spot on lettuce caused by *X. hortorum* pv. *vitians* in 376 lettuce samples collected from the main lettuce production area of Canada. Occurrence of BLS disease on lettuce in Turkey was reported for the first time in the Eastern Anatolia region in 1999, and the causative bacterium was identified as *X. campestris* pv. *vitians* (proposed name *X. axonopodis* pv. *vitians*) with the serological test and fatty acid analysis by Sahin (2000). Three years later, Mirik et al. (2007) reported that BLS was recorded on lettuce cultivars (Tasna, Arapsaci, and Yedikule) with 100% of incidence in commercial fields of the Eastern Mediterranean Region of Turkey. The authors identified 11 bacterial strains as *Xanthomonas axonopodis* pv. *vitians* by indirect ELISA with *Xanthomonas*-specific monoclonal antibodies and fatty acid analysis.

During the winter season in 2007, bacterial leaf spot-like symptoms were observed on plants in commercial lettuce fields (37°45' N 27°49.70'E) in Aydin province of Aegean region. The disease had variety symptoms that ranged from water-soaked lesions in the early stage to forming small brown spots scattered along the leaf surface and margins (Fig. 1a), and later becoming large necrotic (brown to black) regions (Fig. 1b). Coalescing lesions sometimes caused a papery appearance to older leaves (Fig. 1c). A disease survey was conducted in

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Fig. 1 Lettuce growing in the field showing bacterial leaf spot caused by *Xanthomonas hortorum* pv. *vitians*; a. plant with water-soaked lesions in the early stage to forming small brown spots scattered along the leaf

surface and margins; b. typical brown-black irregular spots later enlarging and coalescing into dark brown blotches; c. outer leaves with coalesced lesions showing a papery appearance

several localities of Aydin province in November and December of 2007. Disease incidence was determined as a percentage by counting the number of symptomatic plants in a sample of 4×25 plants selected from random locations in the field. The survey included twelve lettuce fields of three different cultivars [Yedikule (romaine type), Robinson and Chianti (iceberg type)] in Aydin province. The majority of the surveyed fields were affected by this disease with individual fields ranging from 48 to 95% disease incidence. Plants of cv. Chianti were the most severely infected cultivars and several farmers had to plow under entire fields of lettuce that were rendered unmarketable.

For isolation, symptomatic 1 to 2 cm² leaf pieces were surface-sterilized and macerated in sterile water and the resulting sap was subjected to streak on the King's B agar plates. Incubation at 28 °C for 3 to 4 days yielded yellow-pigmented *Xanthomonas*-like colonies that were mucoid, convex and straw-colored to yellow. A total of 8 bacterial strains from different fields and host plants were isolated, purified and preserved for pathogenicity and confirmatory tests. Pathogenicity tests were performed on greenhouse-grown 4–5-week-old lettuce plants cv. Yedikule as three replicates in 15 cm pots containing a steamed sand/peat/soil mixture. All plants as well as control (only with buffer) were foliar sprayed with the bacterial suspension (1×10^7 cfu/ml) in sterile phosphate-buffered saline and immediately placed in clear plastic bags, and incubated at 24 ± 2 °C. After four days, the bags were removed and the plants were observed for disease development at two weeks after inoculation. All bacterial isolates showed typical leaf spot symptoms on lettuce plants but no symptoms on the control plants. To fulfill Koch's postulates, bacteria were reisolated from symptomatic plant tissue and were identified as described in below-mentioned procedures.

The morphological and biochemical characterization of eight *Xanthomonas* isolates (Schaad et al. 2001) revealed that all of them were gram-negative, aerobic

rods, negative for oxidase, positive reactions for the presence of catalase, hydrolysis of starch and esculin. Hydrogen sulfide was produced from cysteine. No acid was produced in litmus milk, nitrates were not reduced and Indole was not produced. Unlike in previous reports in Turkey, our isolates were identified as *Xanthomonas axonopodis* pv. *carotae* with similarity indices ranging from 0.42 to 0.52% by fatty acid analysis (MIDI Inc., Sherlock Version 6.0B) (Supplement-1). PCR amplification with the specific primer pair B162 designed by Barak et al. (2001) for the identification of *X. campestris* pv. *vitians* yielded the expected c.700 bp DNA fragment from all our investigated isolates and reisolates. Primer Blast (NCBI) test indicated that B162 primer pairs virtually amplified a partial 661 bp sequences of *glycosyl hydrolase family 3* gene of *X. campestris* pv. *vitians*, *X. hortorum* and *X. gardneri* in silico PCR analysis.

Four isolates (1L1, 1L2, 2L3 and 2L4) in our local strains were later examined by sequencing the 16S rDNA with primers 27F (Weisburg et al. 1991) and Univ-1390R (Zheng et al. 1996). The partial nucleotide sequence of 16S rDNA of these four isolates (GenBank accession: MG262370, MG262371, MG262372, and MG262373) shared 100% nucleotide identity (921/921 bp) to the type strain of *Xanthomonas hortorum* (NR_026386). Since the *gyrB* gene is a rapid and efficient identification tool for the xanthomonad classification reported by Parkinson et al. (2007, 2009), the species-level identification of our 8 local strains was further confirmed by analyzing DNA sequences of partial *gyrB* gene amplified with the following designed primers: KBXGyrF1 (5'-TCGCCCCATCAAGGTGCTGAA-3') / KBXGyrR2 (5'-GATCGAGCGCGGCTACATCT-3') at an annealing temperature of 60 °C. The amplicons of the 786 bp fragment of the *gyrB* gene of 8 local isolates (1L1, 1L2, 1L4, 1L5, 2L1, 2L2, 2L3 and 2L4) were commercially sequenced at Macrogen (Korea) and were deposited in GenBank

Fig. 2 Maximum Likelihood tree derived from a ClustalW sequence alignment of *gyrB* sequence data of *Xanthomonas* isolates (701 bp) from lettuce in Aydin province of Turkey and equivalent sequences of the related *Xanthomonas* species available in GenBank. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura and Nei (1993) model. The tree with the highest log likelihood (−1928.2105) is shown. The percentage of replicate trees in

which the associated taxa clustered together in the bootstrap test (1000 replicates) and the values are positioned alongside the branches with values over 60%. *Stenotrophomonas maltophilia* were used as an out-group. The scale bar represents 5% genetic distance (0.05 substitutions per site). Phylogenetic analyses of sequence data were done using MEGA software version 7.0 (Kumar et al. 2016)

under the following consecutive accession numbers MG265992 to MG265999. A Maximum Likelihood tree derived from a ClustalW sequence alignment of *gyrB* sequence data of *X. hortorum* pv. *vitians* isolates and equivalent *gyrB* gene sequences of *Xanthomonas* strains available in GenBank. Phylogenetic analyses of sequence data were done using MEGA software version 7 (Kumar et al. 2016). Sequence comparison and phylogenetic analysis proved that all the eight isolates closely belonged to *Xanthomonas hortorum* pv. *vitians* (Fig. 2). Available data retrieved from the gene bank revealed that all our unique sequences of 16S rDNA and *gyrB* gene were closely affiliated with those of known, cultured taxa.

Pure cultures of eight bacterial strains have been deposited in the culture collection of the Plant Protection Department, Agricultural Faculty of Adnan Menderes University, Aydin, Turkey, with assigned numbers listed in Fig. 2, and also deposited in the ICMP culture collection (Landcare Research, Auckland, New Zealand)

under strains designation ICMP 21896 to 21903. To our knowledge, this is the first report of *Xanthomonas hortorum* pv. *vitians* causing bacterial leaf spot of lettuce plants in Turkey. The high rate incidence of BLS has been causing serious damage to the lettuce fields with overhead irrigation and threatening crop production in the Aegean region.

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