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Determination of bacterial leaf spot of lettuce caused by *Pseudomonas viridiflava* (Burkholder 1930) Dowson 1939 and the reactions of some lettuce cultivars

Marul yaprak lekesi etmeni *Pseudomonas viridiflava* (Burkholder 1930) Dowson'nın tespiti ve bazı marul çeşitlerinin reaksiyonlarının belirlenmesi

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ABSTRACT

Pseudomonas viridiflava (Burkholder 1930) Dowson 1939 is a multihost plant pathogenic bacterium all over the world and also in Turkey. The aim of this study was to identify the pathogenic bacteria causing the symptoms of water-soaked necrotic leaf spots, dark brown spots on leaves, tip burn of leaf in curly type lettuce (*Lactuca sativa* var. *crispy*) grown in the field conditions in Beypazarı region and to reveal the reactions of some lettuce cultivars against the bacteria. For this purpose, twelve isolates were obtained from 4 different locations. Pathogenicity tests were conducted on curly type lettuce and all isolates were found pathogenic on the host. Isolated bacteria were evaluated for Gram reaction, LOPAT tests, colony morphology on King's Medium B and carbon sources utilization by VITEK-2 system and identified as *Pseudomonas viridiflava*. The 16S rDNA region of a selected isolate representing the similar isolates in all tests was sequenced for genetic confirmation and the obtained sequence was submitted to the GeneBank under the accession number MN_539659. The result of the obtained sequence was 99.75% similar to the respective reference sequence after BLAST analysis. Reaction of the three different widely produced lettuce types, iceberg, curly and romaine types, with ten varieties against the causative bacterial agent was evaluated and the difference between the varieties found significant statistically. In this study, *P. viridiflava* was detected in lettuce in Central Anatolia for the first time.

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is a consumable leaf vegetable the member of the Asteraceae family. Lettuce has a great

market value for widespread usage of daily nutrition. There are different types of lettuce are consumed as a leaf

vegetable. Iceberg, curly and romaine types are the most common lettuce varieties on the Turkish market. According to the data of 2018, in our country, a total of 490.423 tons of lettuce, including curly, romaine and iceberg, are grown in approximately 220.500 da areas. Ankara has 50% of the production area and 52% of this production alone in Beypazarı region where has a significant role in lettuce cultivation (TUIK, 2018). A number of bacterial diseases affect lettuce production. Up to date, *Pseudomonas* spp. and *Xanthomonas campestris* pv. *vitians* (*Xcv*) have been reported as the bacterial pathogens of lettuce in the different regions of Turkey (Aysan et al. 2003, Mirik et al. 2004a, Mirik et al. 2011, Sahin 2000). *Xcv* was first reported in Oltu province of Erzurum (Sahin 2000). Mirik et al. (2004b) was reported *Xcv* on Arapsaci and Yedikule varieties in eastern Mediterranean region with 100% disease incidence. Mirik et al. (2011) reported *Pseudomonas cichorii* as the causal pathogen of lettuce leaf spot and bacterial rot of lettuce in the eastern Mediterranean region. In Hatay, *Pectobacterium carotovorum* subsp. *carotovorum* and *Enterobacter cloacae* as the root rot pathogens, *Xanthomonas campestris* pv. *vitians* as the leaf spot pathogen of lettuce were reported according to MALDI-TOF MS analysis results (Soylu et al. 2017).

Pseudomonas viridiflava (Burkholder) Dowson 1939 (*P. viridiflava*) is a multi-host plant pathogenic bacterium (Sarris et al. 2012) which is considered to be a member of *Pseudomonas syringae* (*P. syringae*) complex (Bartoli et al. 2014). Tomato (Aysan et al. 2005, Goumans and Chatzaki 1998, Ustun and Saygili 2001), eggplant (Goumans and Chatzaki 1998, Ustun 2012), melon (Aysan et al. 2003, Mirik et al. 2004a), chrysanthemum (Goumans and Chatzaki 1998), globe artichoke (Sanver et al. 2019) are some of economically important hosts of the bacterium. Necrotic areas on the leaf, stem necrosis and stem and root rot are common symptoms on different host plants (Bartoli et al. 2014). In Turkey, *P. viridiflava* was first detected on iceberg lettuce on the Black Sea region (Aksoy et al. 2018).

During the field surveys in summer 2018 and 2019 water-soaked, dark brown spots on lettuce leaves and tip burn of leaf were observed on lettuce fields of Beypazarı-Ankara. Infected lettuce was grown from plantlets and shortly after the head development, the symptoms occur. The aim of this study is to identify the causal pathogen in the symptomatic lettuce plants and the reaction of some common growth lettuce varieties to the isolated bacteria.

MATERIALS AND METHODS

Extensive surveys were carried out to the lettuce fields of Beypazarı region between June and September 2018 and 2019. Plants were sampled according to visible symptoms as water-soaked, dark brown spots on leaves, tip burn of leaf were collected from infected fields (Figure 1). The symptoms were observed on curly type lettuce. Four different fields were surveyed sized around between 15-120 da and three infected samples were collected from each. Each symptomatic plant was carried in a polyethylene bag and carried to the cold room at 4 °C in a cold carrier. Leaf samples were taken from each symptomatic plants and surface disinfected with 70% EtOH for 2 min and then rinsed 3 times with sterile dH₂O. To isolate the causal bacteria, approximately 5 mm pieces of the margin of the symptoms were cut with a sterile scalpel from each symptomatic plant and put into a sterile Eppendorf tube consists sterile 1 ml 0.9% NaCl and were macerated in a horizontal shaker at 2500 rpm for 30 minutes. The bacterial suspension was diluted 10⁴ and 50 µl of the suspension was streaked onto King's medium B (Proteose peptone #3: 20 g/l, K₂HPO₄: 1.5 g/l, MgSO₄·7H₂O: 1.5 g/l, glycerol: 15 ml/l) (King et al. 1954) by a sterile loop. The Petri dishes were incubated at 28 °C in an incubator and after 48 hours colonies were observed under a binocular (Leica, L2). Characteristic single colonies were selected and re-streaked onto King's medium B for bacterial purification. Selected colonies were taken to the NBY (Nutrient broth yeast) medium in cryotubes and sterile glycerol was added to each about 30% (v/v) and the cryotubes were stored in -86 °C for long term storage.



Figure 1. Water-soaked leaf spot symptom of isolated *Pseudomonas viridiflava* on the crispy lettuce (*Lactuca sativa* var. *crispy*)

Pathogenicity tests

Pathogenicity tests were conducted on curly type lettuce (*Lactuca sativa* var. *crispy*). Five replicates were used for each isolate. Healthy lettuce plantlets were potted in 0 no pots consist of sterile turf. Bacterial inoculum was growth from pure cultures on King's B medium at 28 °C for 24 hours.

Bacteria were diluted in dH₂O and the bacterial concentration was adjusted on 10⁸ cfu/ml with a spectrophotometer at OD:600. Bacterial suspension was pulverized on the lettuce plantlets by a hand spray. After the inoculation, inoculated plantlets were covered with a thin, transparent polyethylene cover to keep moisture in for 24 h and then the cover was removed. Plantlets were maintained in a controlled climate room (Digitech) at 24 °C, 75% humidity and 16 h light/8 h dark conditions. After 10 days the plantlets were evaluated for symptom incidence. Sterile dH₂O was used as a control. Isolation from inoculated plantlets was done to fulfill the Koch's postulates.

Characterization of the isolates

Obtained isolates were characterized by the following biochemical tests, Gram reaction (Schaad et al. 2002), LOPAT tests (Lelliot et al. 1966) (Levan production, oxidase test, pectolytic activity on potato slices, arginine hydrolysis and hypersensitive response on tobacco-HR), gelatin liquefaction, glucose fermentation, and carbon source usage and colony morphology on King's B medium. Carbon source utilization of the isolates was determined by VITEK-2 (Biomereux, France). 24 h culture of the isolates was suspended in sterile saline and the bacterial concentration was adjusted to 0.63 on the McFarland tool of the VITEK-2 system. GN cards were used for the analysis. *P. viridiflava* strain YA-649 was kindly provided from the bacterial culture collection of Çukurova University, Faculty of Agriculture, Department of Plant Protection to use as the reference for the biochemical tests.

The reaction of some cultivars

Cultivar reaction of some commonly growth lettuce varieties was handled on *Lactuca sativa* iceberg varieties Sumarnas, Ice Castle, Diskoa, Cartagenas; curly varieties Sementel, Mc Montana and Davidole; romaine varieties Cuore and Calorina and the red variety Bachus (Table 1). Four weeks old plantlets were inoculated with a selected virulent isolate P 5.1 of *P. viridiflava*. The bacterial inoculum was grown from pure cultures on King's B medium at 28 °C for 24 h. Bacteria were diluted in dH₂O and the bacterial concentration was adjusted on 10⁶ cfu/ml with a spectrophotometer at OD:600. Bacterial suspension was sprayed on the lettuce plantlets by a hand spray. After the inoculation, inoculated plantlets were covered with a thin, transparent polyethylene cover to keep moisture in for 24 h and then the cover was removed. Plantlets were maintained in a controlled climate room (Digitech) at 24 °C, 75% humidity and 16 h light/8 h dark conditions. After 2 weeks the plantlets were evaluated for symptom incidence. After 2 weeks the plantlets were evaluated for

symptom incidence. Disease score was assessed according to a qualitative rating scale as described by Thirthamallappa and Lohithaswa (2000) with some modifications on a 0-to-4 scale, in which 0: symptomless, 1: <10% of the leaf area, 2: 10-25% of the leaf area, 3: 26-49% of the leaf area, 4: 50-100% of the leaf area. The disease incidence (DI) was calculated as follows (Viriyasuthee et al. 2019):

$$DI (\%) = (\text{number of infected plant} / \text{total number of plants}) \times 100$$

Disease severity (DS) was calculated as follows:

$$DS (\%) = \Sigma [(\text{rating score} \times \text{number of plants in rating}) / (\text{total number of sampled plants} \times \text{highest rating})] \times 100$$

Obtained data was analyzed statistically by one-way ANOVA and Tukey's test at P≤0.05 using the Minitab Statistic Software Version 16.0 (Minitab Inc. 2017).

Table 1. Lettuce types and cultivars used for cultivar reaction to *Pseudomonas viridiflava* P5.1 strain in this study

Type of the lettuce	Iceberg	Curly	Romaine	Red
Cultivar	Sumarnas Ice Castle Diskoa Cartagenas	Sementel Mc Montana Davidole	Cuore Calorina	Bachus

Genotypic identification of the pathogen

Showing the same phenotypic features, an isolate was selected for 16S rRNA gene confirmation. Genomic DNA of the selected strain was isolated by ThermoFisher Scientific DNA extraction kit according to the instructions of the manufacturer. The concentration of the extracted DNA was measured with a nanodrop (Nano2000, Thermo Fisher) and 50 ng of genomic DNA was used as target for PCR. Universal 16S rRNA primer pair 63f/1387r (Marchesi et al. 1998) was used on PCR. PCR was performed with 12 µl GoTaq flexi master mix (Promega), 10 pmol from each primer, 8 µl dH₂O with a 25 µl final volume. The PCR reaction was conducted with the following steps, 35 cycles of 95 °C 3 min pre-denaturation, 95 °C 45 s for denaturation, 55 °C 30 s for annealing, 72 °C 45 s for elongation and 72 °C 10 min for final extension. Aliquot of 5 ml was analysed by electrophoresis on 1% agarose gel and visualized by staining with ethidium bromide and visualized under UV. The selected isolate was sequenced with 63f/1387r primer pair from both directions.

The obtained DNA sequence was aligned and a consensus sequence was obtained. The obtained sequence was blasted and submitted to the GeneBank.

RESULTS

Plant material and bacterial isolates

Surveys were conducted on different lettuce growing locations of Belpazarı region and 12 isolates were obtained from four different fields with the size between 15 da and 120 da. Dark brown water-soaked spots on the leaf and tip burn of the leaf were identified as the symptoms of *Pseudomonas viridiflava*.

Pathogenicity tests

Pathogenicity tests were conducted on curly lettuce (*Lactuca sativa* var. *crispy*) plantlets. After 10 days of inoculation, water-soaked symptoms were observed and re-isolated from the leaves. Isolation from inoculated plantlets was done to fulfill Koch's postulates. All of the obtained isolates were found as pathogens on lettuce plantlets.

Characterization of the isolated strains

The isolated bacterium was Gram negative, negative for levan, oxidase and arginine dihydrolase, pectolytic on potato slices, induce HR on tobacco leaves. Isolates have blue fluorescence

pigmentation on King's B medium. Glucose fermentation of the isolates was negative. VITEK-2 results showed that isolates can utilize citrate, malonate, D-glucose, coumarate and were able to alkalization of succinate and L-lactate but not able to use mannitol, arabitol, cellobiose and sucrose as carbon source (Table 2). All isolates were showed the same biochemical characteristics with the reference strain.

The reaction of some cultivars

Cultivar reaction of some common growth lettuce varieties was handled on *Lactuca sativa* iceberg varieties Sumarnas, Ice Castle, Diskoa, Cartagenas; curly varieties Sementel, Mc Montana and Davidole; romaine varieties Cuore and Calorina and the red variety Bachus. 5 replicates were used for each variety for cultivar reaction. Four weeks old plantlets were inoculated with a selected virulent strain P5.1 of *P. viridiflava*. All of the experimented varieties have disease symptoms on all replicates. Disease incidence was found 100% for *P. viridiflava* infection. When the iceberg varieties are evaluated, leaf spots were observed on all of the selected varieties. Sumarnas was found as the most sensitive to the *P. viridiflava* infection (Figure 2a). The leaf spots were dense and the infected plants were weak for 10 days post-inoculation. On Ice castle and Cartagenas leaf spots were observed in all of the five replicates. On Discoa variety, 3 of the inoculated plants had intense leaf spots, but the other two were weaker. For iceberg varieties it can be said that Sumarnas is the most sensitive, Discoa is a relatively tolerant variety among

Table 2. Carbon sources usage, LOPAT (* L: Levan, O: Oxidase, P: Pectolytic activity on potato, A: Arginine hydrolysis, T: Hypersensitive response on tobacco) test results and florescence pigmentation characteristics of the *Pseudomonas viridiflava* isolates

Isolate no	Carbon source utilization											LOPAT*	Florescence Pi gmentation
	Arabitol	Cellobiose	Citrate (Sodium)	Coumarate	D-Glucose	H2S production	L-Lactate	Malonate	Mannitol	Succinate Alcalisation	Sucrose		
P5.1	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.2	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.3	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.4	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.6	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.7	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.8	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.9	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.10	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.11	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
P5.12	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue
YA-679	-	-	+	+	+	-	+	+	-	+	-	- - + +	Blue

(-) negative reaction; (+) positive reaction



Figure 2. Water-soaked leaf spot symptom of isolated *Pseudomonas viridiflava* on the crispy type lettuce (*Lactuca sativa* var. *crispy*)

the infected iceberg varieties against *P. viridiflava* infection. When curly varieties evaluated, the leaves of the Davidole variety had dense leaf spot symptoms and the leaves were almost dry because of the infection. Semental had dense leaf spots on all replicates (Figure 2b). The leaf spots were less on Mc Montana variety than the other two varieties and it was found the most tolerant among the curly varieties. Both of the romaine type varieties, Calorina and Cuore, all five replicates had leaf symptoms of *P. viridiflava* and sensitive to the infection (Figure 2c). The red variety Bachus had dense leaf symptoms in all of the replicates (Figure 2d). The disease incidence was found 100%. None of the plants were symptomless. When the disease severity was evaluated, all the varieties had dense leaf symptoms and rated as 4 (50% of the leaf area except Discoa and McMontana). The disease severity was found 4 for three of the replicates and 2 for two of the replicates of Discoa. On McMontana the disease severity was evaluated as 3 for all of the replicates. The disease severity was calculated as 95.5%. When the statistical analysis evaluated, Discoa and McMontana varieties were grouped together and differentiated statistically significant ($p < 0.05$) from other varieties used in this study which had same score values. Moreover, the difference was not statistically significant ($p > 0.05$) between Discoa and Mc Montana varieties (Table 3).

Genotypic identification of the pathogen

Universal 16S rRNA primer pair 63f/1387r was used on PCR and approximately 1300 bp product was obtained from the

sequence analysis. The DNA sequence was aligned and a consensus sequence was obtained. The obtained sequence was blasted and the causal bacteria was found as *P. viridiflava* with a 99.75% similarity to the respective reference strain *P. viridiflava* ATCC 13223 and was submitted to GeneBank under the accession number MN_539659.

DISCUSSION

Lettuce is an important leaf vegetable and has a wide production area in Bepazari-Ankara. Like all other vegetables, lettuce has been infected by important plant pathogenic bacteria. Since the leaves are the consumable parts of the lettuce, any kind of spot causes a decrease in the market value. Previously, the causal bacterium was detected on curly type lettuce in greenhouse grown lettuce in Samsun (Aksoy et al. 2018). In this work, the bacterium was detected in field-grown lettuce. As a multi-host pathogenic bacterium, *P. viridiflava* may cause a threat in multi production agriculture systems in a vegetation time such as Bepazari region. In the field production, the spread of the plant pathogenic bacteria is a threat not only for other healthy plants but also for the inoculation of the soil by infected plant debris. Plant debris of the inoculated lettuce is a potential source for many host and non-host plants. Epiphytic survival of the bacterium has an important role in the life cycle. It can colonize not only on the host plants but also on non-host plants such as weeds without showing symptoms (Aysan and Uygur 2005, Yildiz et al. 2004). There is no effective management of bacterial diseases after the infection. Copper treatment may protect at some point but after infection, application of the copper-based compounds is not functional. Therefore, it is important to determine the inoculation source of the pathogen and the reaction of the cultivars to prevent it. Same as Aksoy et al. (2018)'s report, the bacterium was detected on the curly type lettuce in the field conditions in this study. The cultivar reaction results showed the disease severity was less on Discoa (iceberg) and McMontana (curly) varieties than other varieties. The disease incidence was found 95.5% and it showed that all lettuce cultivars from three different lettuce varieties tested in this work were susceptible to *P. viridiflava* infection in different degrees. Seeing infection only on curly type may cause the seeds to be infected before the plantlet stage. There is not any remarkable work on the reaction of the lettuce cultivars against the pathogenic *P. viridiflava*. This work exposes the cultivar reaction of different lettuce types and cultivars against the causal agent of bacterial leaf spot caused by *P. viridiflava*.

The LOPAT tests are known as the determinative physiological

Table 3. Comparison of experimental means and different significance groups of lettuce varieties based on analysis of variance (ANOVA) Tukey test ($P \leq 0.05$)

Lettuce varieties	Mean	St deviation	Grouping
Sumarnas	4.000	0.0000	a
Ice castle	4.000	0.0000	a
Diskoa	3.200	1.0954	b
Cartagenas	4.000	0.0000	a
Sementel	4.000	0.0000	a
McMontana	3.000	0.0000	b
Davidole	4.000	0.0000	a
Cuore	4.000	0.0000	a
Calorina	4.000	0.0000	a
Bachus	4.000	0.0000	a

test for the Genus *Pseudomonas*. In some case, *P. viridiflava* isolates may show different LOPAT characteristics. Because of the high variability between the genus, it is difficult to discriminate the species of the *Pseudomonas* by using only biochemical tests. 16S rRNA sequence is much more reliable for the identification of the species within the genus. Gonzales et al. (2013) reported *P. viridiflava* strains may show different LOPAT profiles such as yellowish mucoid colony structure on hyper-sucrose medium or variable pectinolytic activity on potato slices. Using the physiological test results and phenotypic features can be deceptive between closely related species within the same genus. In this work, the same phenotypic characteristics on King's medium B was observed and the physiological test results were the same for all isolates. Therefore, one representative isolate was selected as the reference for molecular characterization. *P. viridiflava* was detected on the lettuce and in central Anatolia for the first time with this work.

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ÖZET

Pseudomonas viridiflava (Burkholder 1930) Dowson 1939 tüm dünyada ve Türkiye'de çok konukçulu bitki patojeni bir bakteridir. Beypazarı ilçesinde tarla koşullarında yetiştirilen kıvrıkcık tip marullarda sulumsuz nekrotik lekeler, yapraklarda koyu kahverengi lekeler ve yaprak uçlarında yanıklık belirtileri bakterinin saptanması için incelenmiştir. Dört farklı tarladan 12 izolat elde edilmiştir. Patojenite testleri kıvrıkcık tip marul üzerinde yapılmış ve ele alınan tüm izolatlar konukçusu üzerinde patojen bulunmuştur. İzole edilen bakteriler Gram reaksiyonu, LOPAT testleri, King B besi yerinde oluşturduğu koloni morfolojisi ve VITEK-2 sistemi ile karbon kaynakları kullanımı yönüyle değerlendirilmiş ve *P. viridiflava* olarak tespit edilmişlerdir. Tüm testlerde benzerlik gösteren izolatları temsilen seçilen bir izolatın 16S rDNA bölgesi genetik doğrulama için sekanslanmış ve elde edilen dizi GenBank'a MN_539659 kodu ile kaydedilmiştir. Elde edilen sekans dizisinin sonucu BLAST analizi sonrası referans dizi ile %99.75 benzerlik oranı göstermiştir. Yaygın olarak üretimi yapılan iceberg, kıvrıkcık ve yedikule tipi üç farklı marul tipinden toplam on çeşit ile etmene karşı çeşitlerin reaksiyonu değerlendirilmiş olup çeşitler arasındaki fark istatistiksel olarak önemli bulunmuştur. Bu çalışma ile İç

Anadolu Bölgesi'nde *Pseudomonas viridiflava* marullarda ilk kez tespit edilmiştir.

Anahtar kelimeler: marul, yaprak lekesi, *Pseudomonas viridiflava*, çeşit reaksiyonu

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