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ELISA-based and Traditional Diagnosis Methods for Identification of *Pseudomonas cichorii* and *Pseudomonas corrugata* Causing Pith Necrosis on Tomato Plants

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Abstract

Pseudomonas cichorii and *Pseudomonas corrugata*, the causal bacterial agents of tomato pith necrosis, may cause severe losses on tomato worldwide. In Turkey, *P. cichorii* and *P. corrugata* were observed on tomato in the Mediterranean Region. The typical disease symptoms of pathogens were characterized as wilting, dark blotches on tomato stems, browning and hollowing of pith in greenhouse tomato plants in Adana and Mersin provinces. In this study, traditional and ELISA (**E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay)-based methods were performed for identification of isolates obtained from symptoms showing plants. In total, 15 of *P. cichorii* isolates and 1 of *P. corrugata* isolate were identified according to the diagnostic tests. In addition to traditional diagnosis methods, Indirect-ELISA that was made with pure bacterial cultures was evaluated as capable and reliable diagnosis method of rapid resulting for both pathogenic bacteria.

Keywords: *P. cichorii*, *P. corrugata*, traditional, indirect-ELISA, diagnosis

Domateste Öz Nekrozu Etmenlerinden *Pseudomonas cichorii* ve *Pseudomonas corrugata*'nın ELISA'ya Dayalı ve Geleneksel Tanı Yöntemleri ile Teşhisi

Öz

Domates öz nekrozuna neden olan bakteriyel etmenler *Pseudomonas cichorii* and *Pseudomonas corrugata* dünyada domates üretiminin yapıldığı yerlerde önemli kayıplara sebep olabilmektedir. *P. cichorii* and *P. corrugata* Türkiye'de Akdeniz Bölgesi'nde üretimi yapılan domateslerde tespit edilmiştir. Mersin ve Adana illerindeki sera domateslerinde, patojenlerin neden olduğu hastalığın simptomları solgunluk, bitki gövdesi üzerinde koyu renkte lekeler ve bitki özünde koflaşma ile karakterize edilmiştir. Bu çalışmada, simptom gösteren bitkilerden elde edilen bakteriyel izolatların tanısı geleneksel ve ELISA (**E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay)'ya dayalı tanı yöntemleri kullanılarak yapılmıştır. Test sonuçlarına göre 15 adet izolat *P. cichorii*, 1 adet izolat *P. corrugata* olarak tanılanmıştır. Her iki patojenin teşhisinde, geleneksel tanı testlerinin yanı sıra, saf bakteri kültürü kullanılarak yapılan indirekt-ELISA metodu hızlı sonuç veren duyarlı, güvenilir metot olarak değerlendirilmiştir.

Anahtar Kelimeler: *P. cichorii*, *P. corrugata*, geleneksel, indirekt-ELISA, tanı

Introduction

Pseudomonas cichorii (Roberts and Scarlett) and *Pseudomonas corrugata* (Swingle) are defined as pith necrosis bacterial pathogens of tomato plants (Lopez et al., 1994; Wilkie and Dye, 1974). These pathogens cause vascular disease that can affect tomato plants at any growth stage and in different crop systems, such as greenhouses and open fields all over the world (Cirvilleri et al., 2008). In Turkey, the first occurrence of *P. cichorii* was reported in 1985 (Demir and Gündoğdu, 1988), while *P. corrugata* was first reported in 1990 by Demir (1990).

P. cichorii was originally isolated from chicory (*Cichorium intybus* L.) by Swingle (1925). Afterwards, the pathogen was first isolated from tomato by Wilkie and Dye (1974). The symptoms of pith necrosis bacteria were defined with irregular water soaked lesions colored as dark green to brownish in 30 cm length. It was indicated that the first symptoms frequently could be confused with "*Verticillium* wilt disease" and bacterial canker agent "*Clavibacter michiganense* subsp. *michiganense*", since vessels coloring occurred on plant, however, the following symptoms could be distinguished obviously by browning and hollowing of pith on tomato plants. The following symptoms was defined as necrosis turning to dark brown and 1-3 mm dark brown spots on fruit at the point of calyx. The morphological and biochemical features of *P. cichorii* were characterized as gram negative, producing fluorescent pigment, giving hypersensitive reactions on tobacco leaves, positive for oxidase reaction and, producing levan, producing pectolytic enzymes, hydrolyzing the gelatin, positive for lipase, arginine dehydrolase and catalase tests.

P. corrugata was first isolated by Scarlett et al. (1978), and characterized as non-flurescent on King's B media, positive for oxidase reaction, giving hypersensitive reactions on tobacco leaves, non-producing pectolytic enzymes, hydrolyzing the gelatin, positive for arginine dehydrolase test, accumulating of poly-β-hydroxybutyrate. Since its curly growth on Nutrient Dextrose Agar, the name of "corrugata" which means curly was given to the bacterium and it is referred as "*Pseudomonas corrugata* Roberts and Scarlett".

Gouk et al. (1989), produced polyclonal antiserum by giving the dead cells of *P. cichorii* to rabbits and used it in ELISA analysis. They reported that there were no cross reaction occurred in ELISA analysis conducted with 48 bacterial isolates belonging to *Pseudomonas*, *Clavibacter*, *Erwinia* and *Xanthomonas* genus, hence, researchers evaluated ELISA as more sensitive and rapid diagnosis method compared to other biochemical and physiological ones for *P. cichorii.*

Fiori et al. (1983) carried on a study on identification of *Pseudomonas corrugata* by ELISA method as well as morphological and biochemical tests. In their study, 3 of antisera were used against *P. corrugata* isolates, 83 of isolates gave reaction against one antisera, 6 of isolates reacted against 2 antisera while 39 of isolates did not give any reaction against any of antisera. They indicated that serological methods are not suitable for routine usage, unless a general antigen was specified. Moreover, in their study among 98 of *P. corrugata* isolates, they determined more than 10 LPS (lipopolysaccharide) patterns which were related to serological reactions. Catara et al. (1997), compared 23 different originated *P. corrugata* isolates, variations in serological reactions were found in their study.

Most of the time, identification of stem necrosis-causing pathogens is difficult and time-consuming if more than one bacterial species is associated with the disease symptom. Even though, the result of traditional methods gives the most reliable methods, the application of diagnostic tests, in most cases the LOPAT (**L**: production of levan, **O**: oxidase reaction, **P**: soft rot in potato, **A**: arginin dihydrolase activity, **T**: hypersensitive reaction on tobacco) tests used for identification of bacterial pathogens, takes considerable time. Therefore, we aimed to find an alternative diagnosis method which is quick, reliable and accurate for identification of *P. cichorii* and *P. corrugata* in this study.

Material and Methods

Isolation of the Pathogen from Infected Plant Samples and Re-isolations

Infected tomato samples were collected from greenhouses in Adana and Mersin in the Eastern Mediterranean Region of Turkey. A small piece of infected pith was placed in a sterile mortar and macerated in sterile distilled water with a pestle. Loopfulls of the

suspension were streaked on plates of King's B medium. Plates were incubated at 25 ºC for 48 h. Intensively growing colonies were picked from the plates and transferred to King's B medium for purification. Tomato plants (*Lycopersicon esculentum* cv. H-2274) at the three-to-five leaf stage were inoculated with bacterial suspensions at the density of ~$1x10^8$ cfu ml^{-1} prepared from pure strain cultures by using a sterile syringe. For positive controls NCPPB 3802 (*P. cichorii*) and NCPPB 2445 (*P. corrugata*) from National Collection of Plant Pathogenic Bacteria-UK, were used and sterile distilled water used as negative control. Three tomato plants were inoculated for each bacterial strain. Plants were placed in a mist chamber for 24 h and then incubated in a climate room at 25 ºC and 70% humidity for 15 days. Whole plants were examined by cutting them vertically after symptom expression. Bacteria were re-isolated from infected areas.

Morphological, Physiological and Biochemical Characterization of Isolates

Strains were identified by the following tests; gram reaction using the nonstaining-KOH method, fluorescent pigmentation, levan formation, oxidase reaction, induction of soft rot on potato tubers, arginine dihydrolase activity, hypersensitivity test on tobacco leaves, and production of acid from sorbitol and sucrose, and utilization of some carbon source (Lelliot and Stead, 1987). NCPPB 3802 (*P. cichorii*) and NCPPB 2445 (*P. corrugata*) were used as positive control.

ELISA-based Diagnosis of Isolates

Indirect-ELISA tests performed with the polyclonal antisera of *P. cichorii* and *P. corrugata* that were developed in TÜBİTAK-MAM (Gebze, Kocaeli) with the framework of TARP-2364 numbered project. It was informed that the antisera did not give cross reaction with any bacteria of *P. viridiflava, P. mediterranea, P. fluorescens, E.c.* subsp. a*troseptica, E.c.* subsp. *carotovora,* and *E. chrysanthemi* (Aysan et al., 2002). Isolates that identified as *P. cichorii* and *P. corrugata* according to series of LOPAT tests were serologically analyzed by Indirect-ELISA as well. For positive controls, NCPPB 3802 and GSPB 2097 as reference cultures of *P. cichorii* were included while NCPPB 2445 and GSPB 1224 were used as *P. corrugata* reference cultures. PBS was used as negative control.

Indirect-ELISA was applied as defined by McLaughlin and Chen (1990). ELISA-plates were coated by 100 µl of bacterial cultures (~$1x10^6$ cfu ml^{-1}) with replication for each. The coated plates were incubated at 4 ºC overnight. Plates were rinsed up three times with washing buffer (Phosphate Buffered Saline +%0.05 Tween 20). Then 1% milk powder in PBS was added to wells and plates were incubated at 37 ºC for an hour. Plates were rinsed up three times with washing buffer. Antisera of both pathogens were diluted at rates (1/5000 for *P. cichorii* and 1/10000 for *P. corrugata*) specified by Aysan et al. (2002), and 100 µl of antisera was added to each plate well. Plates were incubated at 37 ºC for an hour and then rinsing was repeated as above. 100 µl of Alkaline phosphatase conjugated goat anti-rabbit immunoglobulin (diluted in PBS at 1/5000) was added to each well and incubated at 37 ºC for an hour. After incubation, rinsing was repeated 5 times. Para-nitrophenyl phosphatase in substrate buffer (1mM ZnCl2, MnCl2, 0.1 M glycine, pH: 10.4) was added to wells and incubated at room temperature for 45 min. The reaction measurements were performed at wavelength of 450 nm by a Bio-Tech ELISA-reader. Positive reaction was evaluated by an absorbance value ≥2x of the negative control.

Results

Isolation of the Pathogen from Infected Plant Samples and Re-isolations

As a result of isolations and on King's B medium from plants showing typical pith necrosis symptoms, non-fluorescent and fluorescent bacteria were obtained. All isolates had similar symptom on artificially inoculated tomato stems with black blotches on stems, browning of pith and vessels, and hollowing of the pith. Isolations were made from these plants and re-isolates were identified by traditional bacteriological methods as well as Indirect-ELISA technic. Totally, 14 of *P. cichorii* and 1 of *P. corrugata* isolates obtained from tomatoes with stem pith necrosis from greenhouse tomato plants in Adana and Mersin.

Morphological, Physiological and Biochemical Characterization of Isolates

There were non-fluorescent and fluorescent bacteria among the selected isolates. Strains were gram-negative. None of the strains produced levan on sucrose-amended nutrient agar. While both pathogen isolates were positive for oxidase reaction, *P. cichorii* isolates gave negative results for arginine dihydrolase reaction. Both pathogen isolates developed hypersensitive reaction on tobacco leaves in 24 h. *P. corrugata* isolates produced acid from sucrose but not from sorbitol. None of selected isolates caused soft rot in potato. In total, 14 isolates were identified as *P. cichorii* (MRX, MRX-sap2a, VCO1, Er-dom, HAG-5, İT-idc, İTÜ-ida, İTÜ-özc1, Mk-id, Mk-idc, Mk-özt, Mk-kök, M-Adb, M-Adf, Mcöz1), while 1 isolate was identified as *P. corrugata* (AA4). The result of traditionally identified isolates can be seen in Table 1.

Table 1. Traditional test results of *Pseudomonas cichorii and Pseudomonas corrugata* isolates obtained from infected tomato plants

Tests	***P. cichorii* (GSPB2097)**	**Tomato isolates (*P. cichorii*)**	***P.corrugata* (GSPB1224)**	**Tomato isolate (*P. corrugata*)**
Levan production	-	-	-	-
Oxidase Reaction	+	+	+	+
Pectolytic Activity	-	-	-	-
Arginine Dihydrolase	-	-	+	+
HR on Tobacco Leaves	+	+	+	+
Fluorescent Pigment on KB	+	+	-	-
Acid Production from Sugar Source				
Sorbitol	-	-	-	-
Sucrose	-	-	+	+

Table 2. Reactions of isolated bacteria with indirect-ELISA (reading at At A_{405} wavelength) using antisera against *Pseudomonas cichorii*

Isolate names	1. Replications	2. Replications	Means of Replications	Results
PBS (Negative Control)	0.153	0.159	0.156	-
GSPB 2097 (P. cichorii)	1.463	1.420	1.441	+
NCPPB 3802 (P. cichorii)	1.520	1.310	1.415	+
MRX	1.064	1.219	1.141	+
MRX-sap2a	1.417	1.500	1.458	+
VCO1	1.388	1.445	1.416	+
Er-dom	1.288	1.148	1.218	+
HAG-5	0.734	0.817	0.775	+
İT-idc	1.520	1.524	1.522	+
İTÜ-ida	1.989	1.974	1.981	+
İTÜ-özc1	1.431	0.927	1.179	+
Mk-id	1.752	1.232	1.492	+
Mk-idc	1.708	1.857	1.782	+
Mk-özt	1.720	1.717	1.718	+
Mk-kök	1.366	1.660	1.513	+
M-Adb	1.139	1.299	1.219	+
M-Adf	1.449	1.443	1.446	+
Mcöz1	1.449	1.451	1.450	+

ELISA-based Diagnosis of Isolates

Indirect-ELISA method with polyclonal antisera worked successfully for diagnosis of *P. cichorii* regional isolates. The average absorbance of negative control (only PBS) was recorded with value of 0.156, and the max average absorbance was obtained from İTÜ-ida named regional isolate with value of 1.981. The replication values, means of replications and the reading results can be seen in Table 2.

For identification of *P. corrugata* isolates, indirect-ELISA performed with polyclonal antiserum gave successful results. The average absorbance of negative control (only PBS) was recorded with value of 0.121, and the max average absorbance was obtained from AA4 named regional isolate with value of 2.924. The replication values, means of replications and the reading results can be seen in Table 3.

Table 3. Reactions of isolated bacteria with indirect-ELISA (reading at At A_{405} wavelength) using antisera against *Pseudomonas corrugata*

Isolate names	1. Replications	2. Replications	Means of Replications	Results
PBS (Negative Control)	0.120	0.122	0.121	-
GSPB 1224 (*P. corrugata*)	2.244	2.327	2.285	+
NCPPB 2445 (*P. corrugata*)	2.363	2.365	2.364	+
AA4	2.859	2.990	2.924	+

Discussion

In this study, the diagnosis of pith necrosis pathogens "*P.corrugata*" and "*P.cichorii*" was done by both traditional methods and Indirect-ELISA. Pith necrosis of tomato is caused by couples of *Erwinia* and *Pseudomonas* species including *P. cichorii* and *P. corrugata*. By traditional methods, identification of these plant pathogenic bacteria takes quite long time. Therefore, researchers focused on finding diagnostic methods that are quicker, reliable and capable. One of these fast identification methods is ELISA which based on antigen and anticore relationship. Nowadays ELISA is indispensable and used routinely for diagnosis of especially human disease. In addition to human disease, ELISA is applied for diagnosis of plant pathogens.

Gouk et al. (1989), produced polyclonal antiserum by giving the dead cell of *P.cichorii* to rabbit. The researchers detected *P. cichorii* by ELISA using this polyclonal antiserum and they indicated that no cross reaction occurred with 48 bacterial isolates belonging to *Pseudomonas, Clavibacter, Erwinia* and *Xanthomonas* genus. The antisera that were included in this study, were produced by Aysan et al. (2002), and it was informed that the antisera did not give cross reaction with any bacteria of *P. viridiflava, P. mediterranea, P. fluorescens, E.c.* subsp. a*troseptica, E.c.* subsp. *carotovora,* and *E. chrysanthemi* (Aysan et al., 2002). Janse (1987) determined the serological and pathological features of 19 of different originated *P. cichorii* isolates obtained from chrysanthemum. The researcher pointed out that the isolates were homogeneous biochemically, whereas they were showing serotypical variations.

In serological diagnosis of bacteria, epitopes, also known as antigenic determinant, is the part of an antigen that is recognized by the immune system, may vary in the same genus. This can cause non-detection of different isolates belonging to the same genus by an antiserum which developed against an isolate. Thus, Siverio et al. (1993) determined the applicability of indirect-ELISA and indirect-IFAs (Immunofluorescence Assays) by using 3 antisera developed against 3 different isolates of *P. corrugata*. Since the serological variations occurred among the 83 of *P. corrugata* isolates included in their study, they strongly indicated that unless developing a general antiserum for *P. corrugata*, the serological diagnosis of *P. corrugata* is not routinely applicable. Fiori et al. (1983), however, identified *P. corrugata* by ELISA and the researchers stated that ELISA is effective and sensitive test method for identification pathogen from both pure bacterial culture and infected plant materials.

In serological detection methods, sometimes, false positive results can be obtained, since cross-reactions or dead-cell recognitions by antiserum. So that, instead of using serological methods alone, combined testing methods including streaking on selective/semi-selective medium for identification is strongly recommended as easy, comparatively quick and reliable methods (EPPO, 1992).

Conclusion

ELISA is an inexpensive, sensitive and rapid test, which does not require excessive laboratory equipment and test procedures. The simplicity, speed and specificity of ELISA make it attractive for testing a large number of field samples. Furthermore, the pathogens can also be detected from frozen tissues as an added advantage, because collections made over an extended period can be safely stored and tested later (Rowhani et al., 1994). The results of the present study may suggest that polyclonal antisera specific to *P. cichorii* and *P. corrugata* should be used for diagnosis of tomato stem necrosis in addition to epidemiological studies of the strains of these pathogens isolated from various hosts in different regions.

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