

SHORT COMMUNICATION

EPIPHYTIC SURVIVAL OF *PSEUDOMONAS VIRIDIFLAVA*, CAUSAL AGENT OF PITH NECROSIS OF TOMATO, ON WEEDS IN TURKEY

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SUMMARY

Pith necrosis, caused by *Pseudomonas viridiflava* (Burkholder) Dowson, has been an important problem of greenhouse-grown tomatoes in the eastern Mediterranean region of Turkey since 1998. In the study, the epiphytic survival of *P. viridiflava* on weeds was investigated by using a semi selective agar medium (T-5) and indirect-ELISA in commercial greenhouses with a history of pith necrosis in three different locations of the region during 2001 and 2002. It is the first study showing that pathogenic strains of *P. viridiflava* on tomato may survive as an epiphytic population on the phyllosphere of *Amaranthus* spp., *Conyza canadensis*, and *Orobancha ramosa* in Turkey. The strains were identified as *P. viridiflava* by morphological, physiological, biochemical tests, indirect-ELISA and fatty acid methyl ester analysis, and were found to be pathogenic on tomato plants.

Key words: tomato, pith necrosis, weed, resident bacteria.

Pith necrosis of tomato (*Lycopersicon esculentum* Mill.) caused by *Pseudomonas viridiflava* (Burkholder) Dowson, is an economically important disease of greenhouse-grown tomato in many countries of the world (Lukezic *et al.*, 1983; Goumas and Chatzaki, 1998; Alippi *et al.*, 2003) including Turkey (Aysan *et al.*, 2004). *P. viridiflava* induces yellowing and wilting on tomato plants, brown-black spots limited to the pruning sites of the stem and canker of the petioles. The bacterium causes problems also on melon (Aysan *et al.*, 2003) and watermelon (Mirik *et al.*, 2004) in the region.

The ecology and epidemiology of *P. viridiflava* in Turkey has been studied recently (Yildiz *et al.*, 2004). Epiphytic populations of *P. viridiflava* are known to be important in its cycle of the disease. Bacteria can colonize non-host plants, including many weeds, without producing visible symptoms. In previous studies, the presence of

P. viridiflava on weeds has been screened on onion (Gittaitis *et al.*, 1998) and tomato (Mariano and McCarter, 1993). The elimination of this primary inoculum source is one of the strategies for managing the disease.

Knowledge of pathogen survival on weeds in tomato growing areas is still incomplete and further study is justified. In this study, we sampled weed species in greenhouses with a history of pith necrosis caused by *P. viridiflava* in our region. The aim of this study was to determine the role of epiphytic survival of *P. viridiflava* on the leaf surface of weeds as potential primary inoculum sources in greenhouses of the region in Turkey.

The first sampling (10 plants of each weed were randomly collected) was carried out at the end of January 2001 and at beginning of February 2002 when no visible pith necrosis symptoms were observed on tomato plants. Sixteen weed species (*Amaranthus viridis*, *Capsella bursa-pastoris*, *Conyza canadensis*, *Convolvulus arvensis*, *Cynodon dactylon*, *Fumaria officinalis*, *Lamium amplexicaule*, *Malva* sp., *Orobancha ramosa*, *Poa annua*, *Polygonum* sp., *Senecio vernalis*, *Stelleria media*, *Urtica urens*, *Veronica* sp., and *Oxalis cernua*) were sampled in the greenhouses.

The second sampling was carried out in April 2001 and 2002, when symptoms of pith necrosis were visible on tomato. Eighteen weed species in the greenhouses were sampled: *Alopecurus myosuroides*, *Amaranthus viridis*, *Avena sterilis*, *Bromus tectorum*, *Calendula arvensis*, *Capsella bursa-pastoris*, *Convolvulus arvensis*, *Crepis* sp., *Lactuca scariola*, *Lamium amplexicaule*, *Malva* sp., *Medicago scutellata*, *Ochthodium aegyptiacum*, *Rumex obtusifolius*, *Senecio vernalis*, *Sonchus oleraceus*, *Stelleria media*, and *Veronica* sp..

The third sampling was carried out on June 2001 and 2002 after the harvesting period. Ten weed species, *Amaranthus* sp., *Amaranthus retroflexus*, *Amaranthus viridis*, *Conyza canadensis*, *Echinochloa crus-galli*, *Medicago polymorpha*, *Plantago* sp., *Portulaca oleracea*, *Ranunculus* sp. and *Sonchus oleraceus*, were sampled in the greenhouses.

Weed leaves and stems (9 g) were placed in 90 ml of phosphate buffered saline (PBS; 10 mM K₂HPO₄, 10 mM KH₂PO₄, pH 7.2, 0.15 M NaCl) and Tween 20 (final concentration 0.02%). The samples were shaken for

3 h at 25°C at 200 rpm. The suspension was ten-fold serially diluted and three replicated 100- μ l aliquots from each dilution were spread uniformly onto King's medium B (King *et al.*, 1954) supplemented with penicillin (100 mg l⁻¹) and cycloheximide (100 mg l⁻¹) and T-5 medium (Gitaitis *et al.*, 1997). Fluorescent yellow-colored colonies on King's medium B and colonies with dark pink zones on T-5 medium were purified and used for further characterization.

Aliquots of shaken weed samples in PBS-tween were centrifuged for 20 min at 10,000 rpm. The pellet was re-suspended in 1 ml⁻¹ of PBS. The suspension was tested for the presence of *P. viridiflava* using indirect-ELISA with three replications according to methods described by Aysan *et al.* (2004). The suspension was also infiltrated into the stem of 3-5 leaves stage tomato plants (cv H-2274) as three replicates with a sterile syringe for pathogenicity tests.

Sterile distilled water and *P. viridiflava* GSPB 1685 (obtained from Georg-August University Göttingen, Germany) were used as a negative and positive control, respectively.

The strains were characterized by Gram reaction, production of fluorescent pigment on King's medium B, acid production from D(-)arabinose, L(+)-arabinose, D(-)mannitol, D(+)-xylose, fructose, glucose, lactose, maltose, melibiose, sorbitol, sucrose, trehalose, and utilization of D(-)tartrate and LOPAT tests (Lelliot and Stead, 1987),

indirect-ELISA, whole cell fatty acid methyl ester (FAME) analysis in Sherlock Microbial Identification System (Janse *et al.*, 1992) and pathogenicity test on tomato plants. The study of FAME was carried out in the Biotechnology Application and Research Center, Ataturk University, Erzurum, Turkey. *P. viridiflava* GSPB 1685 was used in all the tests as a reference strain.

P. viridiflava was recovered from the foliage of *Amaranthus viridis*, *Conyza canadensis*, and *Orobancha ramosa* in the greenhouses before the appearance of visible pith necrosis symptoms on tomato plants during the period of tomato cultivation in 2001, but it was not recovered from *C. canadensis* in 2002. The mean number of cfu in three replicates per g fresh weight on *A. viridis* foliage was 3.3·10³ and 4.3·10³ in 2001 and 2002, respectively. On *C. canadensis* the population was 1.0·10³ cfu g⁻¹. The populations on *O. ramosa* were 4.6·10³ and 3.3·10⁴ cfu g⁻¹ fresh weight in 2001 and 2002, respectively. *P. viridiflava* was not isolated from the other weeds sampled. The pellet of well-mixed suspension of samples of *A. viridis*, *C. canadensis*, and *O. ramosa*, inoculated into tomato plants, incited pith necrosis symptoms within 10-15 days after the inoculation. The suspension of foliage of the above mentioned weed species strongly reacted with the *P. viridiflava* specific polyclonal antiserum in indirect-ELISA tests. Mean absorbance values of three replications in ELISA tests were between 0.9640 and 1.1960 at A₄₀₅ wavelength (Table 1).

Table 1. Absorbance values obtained from an ELISA test applied to some weeds in greenhouses before the appearance of visible pith necrosis symptoms during the period of tomato cultivation in 2001 and 2002 in the eastern Mediterranean region of Turkey.

Weed Species	2001			2002		
	Absorbance values ¹	SE ²	Results ³	Absorbance values ¹	SE ²	Results ³
<i>Amaranthus viridis</i>	1.1960	0.049	+	1.0830	0.052	+
<i>Capsella bursa-pastoris</i>	0.3500	0.056	-	0.3240	0.025	-
<i>Conyza canadensis</i>	0.9799	0.060	+	0.2980	0.047	-
<i>Convolvulus arvensis</i>	0.3910	0.016	-	0.2110	0.018	-
<i>Cynodon dactylon</i>	0.2310	0.012	-	0.4010	0.024	-
<i>Fumaria officinalis</i>	0.3100	0.022	-	0.3190	0.053	-
<i>Lamium amplexicaule</i>	0.2830	0.030	-	0.2783	0.057	-
<i>Malva</i> sp.	0.3800	0.042	-	0.4021	0.029	-
<i>Orobancha ramosa</i>	1.0250	0.072	+	0.9640	0.151	+
<i>Poa annua</i>	0.1440	0.020	-	0.3670	0.040	-
<i>Polygonum</i> sp.	0.3310	0.011	-	0.2921	0.045	-
<i>Senecio vernalis</i>	0.3960	0.013	-	0.2770	0.013	-
<i>Stellaria media</i>	0.2750	0.012	-	0.2920	0.019	-
<i>Urtica urens</i>	0.3312	0.020	-	0.3410	0.033	-
<i>Veronica</i> sp.	0.1710	0.047	-	0.3670	0.050	-
<i>Oxalis cernua</i>	0.3691	0.080	-	0.1985	0.035	-
PBS (negative control)	0.2560	0.015	-	0.2945	0.028	-
GSPB 1685 (positive control)	1.2260	0.082	+	1.0623	0.095	+

¹ The mean absorbance values of three replications in indirect-ELISA tests.

² SE: Standard Error.

³ +: positive value was $\geq 2x$ the absorbance value of negative control; -: negative value was $< 2x$ the absorbance value of negative control.

In the second sampling of 2001 and 2002, *P. viridiflava* was detected only on *A. viridis* when visible symptoms of pith necrosis on tomato appeared. The epiphytic population on *A. viridis* was $1.6 \cdot 10^4$ and $2.3 \cdot 10^3$ cfu g⁻¹ fresh weight, in 2001 and 2002, respectively. The bacterium was not detected in all other weeds tested. Bacterial suspension obtained from *A. viridis* leaves induced pith necrosis symptoms on plants within 10-15 days after the inoculation. The mean absorbance values of suspension of *A. viridis* foliage were 1.9605 and 1.1580 at A₄₀₅ wavelength in ELISA tests in 2001 and 2002, respectively (Table 2).

In the third sampling, *P. viridiflava* was recovered from *Amaranthus* sp., *A. retroflexus*, and *A. viridis* after tomato cultivation. The epiphytic populations of the bacterium were $0.6 \cdot 10^3$, $1.3 \cdot 10^3$ and $0.6 \cdot 10^3$ cfu g⁻¹ fresh weight in 2001, respectively (Table 3). The bacterium was recovered in 2002 from *Amaranthus* sp., *A. retroflexus*, and *A. viridis* at $0.3 \cdot 10^3$, $2.3 \cdot 10^3$ and $1.6 \cdot 10^3$ cfu g⁻¹ fresh weight, respectively. The pathogen was not present in all other weeds tested. The pellets of well-mixed suspension of *Amaranthus* spp. were pathogenic on tomato plants within 10-15 days after inoculation. The mean absorbance values of three replications in indirect-ELISA tests were between 0.9685 and 1.1580 at

A₄₀₅ wavelength (Table 3). Our results show that three different test systems, isolation on semi-selective medium, pathogenicity, and ELISA tests, were in accordance.

Twenty-two isolates sampled over a period of two years were selected for further characterization. The isolates were fluorescent, yellow, mucoid on King's medium B, and negative for Gram-stain and levan, oxidase and arginin dihydrolase reactions. They were pectolytic on potato slices and induced a hypersensitive reaction on tobacco leaves. The isolates produced acid from sorbitol, fructose, glucose, L(+)-arabinose, D(+)-xylose, D(-)mannitol, but not from sucrose, trehalose, maltose, melibiose, lactose and D(-)-arabinose.

Utilization of D(-)-tartrate was positive. All isolates were pathogenic on tomato plants. Visible disease symptoms were recorded within 5-7 days after inoculation. Mean absorbance values of the strains were between 0.9280 and 1.2510 at A₄₀₅ wavelength in indirect-ELISA. All the test results were similar to those of reference strain GSPB 1685 of *P. viridiflava* used in this study. FAME analysis also confirmed the bacterial strains as a *P. viridiflava* with similarity index between 81 to 96%. The main 12 fatty acids detected included 10:0 3OH (2.98±0.66%), 12:0 (5.04±0.81%), 12:0 2OH (2.52±0.27%), 12:0 3OH (4.03±0.36%), 16:1 w7c

Table 2. Absorbance values obtained from an ELISA test applied to some weeds in greenhouses when visible pith necrosis symptoms appearance during the period of tomato cultivation in 2001 and 2002 in the eastern Mediterranean region of Turkey.

Weed Species	2001			2002		
	Absorbance values ¹	SE ²	Results ³	Absorbance values ¹	SE ²	Results ³
<i>Alopecurus myosuroides</i>	0.1028	0.047	-	0.2480	0.036	-
<i>Amaranthus viridis</i>	1.9605	0.102	+	1.1580	0.062	+
<i>Avena sterilis</i>	0.0960	0.035	-	0.3170	0.021	-
<i>Bromus tectorum</i>	0.3610	0.034	-	0.2110	0.042	-
<i>Calendula arvensis</i>	0.0750	0.011	-	0.3032	0.018	-
<i>Capsella bursa-pastoris</i>	0.2560	0.082	-	0.2094	0.024	-
<i>Convolvulus arvensis</i>	0.0753	0.010	-	0.1983	0.015	-
<i>Crepis</i> sp.	0.1450	0.021	-	0.0952	0.030	-
<i>Lactuca scariola</i>	0.0850	0.050	-	0.2372	0.024	-
<i>Lamium amplexicaule</i>	0.1850	0.030	-	0.2870	0.022	-
<i>Malva</i> sp.	0.3955	0.029	-	0.1980	0.054	-
<i>Medicago scutellata</i>	0.3560	0.075	-	0.3104	0.034	-
<i>Ochthodium aegyptiacum</i>	0.2880	0.021	-	0.2890	0.082	-
<i>Rumex obtusifolius</i>	0.3045	0.014	-	0.3150	0.061	-
<i>Senecio vernalis</i>	0.3204	0.020	-	0.3010	0.018	-
<i>Sonchus oleraceus</i>	0.2944	0.034	-	0.3044	0.022	-
<i>Stelleria media</i>	0.1250	0.009	-	0.2781	0.030	-
<i>Veronica</i> sp.	0.2590	0.024	-	0.3490	0.019	-
PBS (negative control)	0.2790	0.046	-	0.2926	0.018	-
GSPB 1685 (positive control)	1.1030	0.102	+	1.2795	0.061	+

¹ The mean absorbance values of three replications in indirect-ELISA tests.

² SE: Standard Error.

³ +: positive value was $\geq 2x$ the absorbance value of negative control; -: negative value was $< 2x$ the absorbance value of negative control.

Table 3. Absorbance values obtained from an ELISA test applied to some weeds in greenhouses after harvesting in 2001 and 2002 in the eastern Mediterranean region of Turkey.

Weed Species	2001			2002		
	Absorbance values ¹	SE ²	Results ³	Absorbance values ¹	SE ²	Results ³
<i>Amaranthus</i> sp.	1.1028	0.235	+	0.9980	0.074	+
<i>Amaranthus retroflexus</i>	0.9685	0.105	+	1.1580	0.068	+
<i>Amaranthus viridis</i>	0.9760	0.035	+	1.0307	0.040	+
<i>Conyza canadensis</i>	0.3610	0.023	-	0.2524	0.036	-
<i>Echinochloa crus-galli</i>	0.0750	0.018	-	0.2860	0.030	-
<i>Medicago polymorpha</i>	0.2560	0.030	-	0.1982	0.021	-
<i>Plantago</i> sp.	0.0753	0.041	-	0.1540	0.020	-
<i>Portulaca oleracea</i>	0.1450	0.016	-	0.2138	0.072	-
<i>Ranunculus</i> sp.	0.0850	0.009	-	0.1890	0.020	-
<i>Sonchus oleraceus</i>	0.1850	0.014	-	0.2224	0.010	-
PBS (negative control)	0.2021	0.043	-	0.1975	0.020	-
GSPB 1685 (positive control)	1.1420	0.058	+	1.1453	0.089	+

¹The mean absorbance values of three replications in indirect-ELISA tests.

²SE: Standard Error.

³+: positive value was $\geq 2x$ the absorbance value of negative control; -: negative value was $< 2x$ the absorbance value of negative control.

(36.95 \pm 2.55%), 16:0 (25.25 \pm 2.88%), 17:0 ISO (0.60 \pm 0.2%), 17:1 w8c (0.22 \pm 0.3%), 17:0 CYCLO (0.97 \pm 1.37%), 17:0 (0.38 \pm 0.11%), 18:1 w7c (21.67 \pm 2.77%), 18:0 (1.08 \pm 0.41%).

These results show that *P. viridiflava* occurs in epiphytic populations on some weed species in greenhouses. All *P. viridiflava* isolates obtained from different weeds in this study did not cause symptoms on the original host but were pathogenic on tomato. The weed species *Orobancha ramosa* presents a major problem in tomato greenhouses in our region (Orel-Aksoy *et al.*, 2001) acting as parasitic weed and/or alternative host of *P. viridiflava*.

Recent studies in the region showed that *P. viridiflava* can survive on plant debris, in soil and on tomato seeds (Yildiz *et al.*, 2004). Our study provides further evidence regarding the local inoculum sources of the pathogen in the region and that *Amaranthus* sp., *Conyza canadensis* and *Orobancha ramosa* are important in the epidemiology of the disease.

Once the pathogen is introduced *via* seed or soil, inoculum can become available for dissemination and survival in the greenhouses. The results clearly suggest that weed management is an important strategy in controlling pith necrosis disease in tomato growing areas.

ACKNOWLEDGEMENTS

The study was supported by a grant (ZF/2001/29) from Cukurova University, Adana Turkey. The authors wish to thank R. Cetinkaya-Yildiz (University of Cukurova, Adana, Turkey) for assistance in the ELISA

study, F. Sahin (University of Ataturk, Erzurum, Turkey) for FAME analysis by MIDI of *P. viridiflava* and critical review of the manuscript and E. Mavridis (Georg-August University Göttingen, Germany) for providing *P. viridiflava* strain GSPB 1685.

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Received 7 January 2005

Accepted 23 February 2005

