European Journal of Plant Pathology **104:** 181–188, 1998.

Characterization and host range evaluation of *Pseudomonas viridiflava* from melon, blite, tomato, chrysanthemum and eggplant

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Accepted 19 November 1997

Key words: bacteria, host range, identification, lession, new hosts, spot

Abstract

The fluorescent bacterium *Pseudomonas viridiflava* (Burkholder) Dowson was identified as the causal agent of bacterial leaf blight of melon, tomato, blite, eggplant and of pith necrosis of chrysanthemum plants. Koch's postulates were fulfilled on greenhouse-grown or on potted plants under controlled environmental conditions. Twenty seven cultivated and one weed species, when artificially inoculated, were found to be susceptible to a strain of the bacterium isolated from cucumber. In cross-inoculation tests on potted plants, each strain induced the disease symptoms, independently of the host of origin. To our knowledge, melon and blite have not been previously reported as natural hosts of *P. viridiflava*. Also this is the first record of the bacterium as a foliar pathogen of melon, tomato, eggplant and blite and as a stem pathogen of chrysanthemum in Greece.

Introduction

Symptoms of bacterial diseases have been frequently recorded throughout the island of Crete on melon (*Cucumis melo* L.), tomato (*Lycopercicon esculentum* Mill.), blite (*Amaranthus blitum* L.), eggplant (*Solanum melogena* L.) and chrysanthemum (*Chrysanthemum* sp.) plants grown in greenhouses and outdoors.

Initial streak-plate isolations made on King's medium B, from the affected plants parts, consistently resulted in essentially pure cultures of a fluorescent bacterium.

Infected plants of all hosts, except chrysanthemum, exhibited necrotic spots and/or necrotic lesions confined to leaves. During the early stages of disease, these appeared as small, yellow, watersoaked spots with chlorotic haloes on the older lower leaves, where water drops had collected. The spots enlarged and usually coalesced to cover a large area of the leaf with the center of the lesion becoming greyish and dry. Under favourable conditions, necrosis was extensive but rarely resulted in whole plant losses. The main symptom on chrysanthemum plants or cuttings was a brown discoloured pith that may or may not be hollow. Infected plants or cuttings died rapidly.

The objectives of the present work were to characterize the causal agent of these unreported bacterial diseases in Greece, to evaluate a possible host range of cucumber strain among several potential species and determine in cross inoculation tests whether isolates of the bacterium (from the above mentioned hosts) are able to cause similar symptoms on heterologous hosts. A preliminary report has been presented elsewhere (Goumas, 1992).

Materials and methods

Isolation and identification of isolates

Affected plant parts or whole plants were collected and maintained in plastic bags at 6 °C until isolations were performed. Samples from infected leaves were surface disinfested after being placed in 10% ethanol solution for 30 sec. After two thorough washings in sterile distilled water, small pieces taken from the margin of the lesions or the leaves of from brown discoloured pith

Table 1. Bacterial strains used in this study

	Collection no.	Host	Origin
Reference strains			
Pseudomonas	Pv400, Pv401	*Cucumis sativus*	Mesara Crete
viridiflava	Pv441, PV442	*Lycopersicon*	
		esculentum	Tymbaki Crete
	NCPPB[1] 1249	*Chrysanthemum*	
		morifolium	England (1962)
Pseudomonas syringe	Pss3, Pss5	*Citrus sinensis*	Fodele Crete
pv. *syringae*	Pss 11	*Citrus lemon*	Fodele Crete
	NCPPB 2778	*Pyrus communis*	France (1965)
pv. *tomato*	Pst 18, Pst 29	*Lycopersicon*	Antiscari Crete
	Pst 30	*esculentum*	Tymbaki Crete
pv. *phaseolicola*	20501 (INRA-V[2])	*Phaseolus vulgaris*	France
pv. *lachrymans*	Psl 110, Psl 119	*C. sativus*	Ierapetra Crete
	Psl 102	*C. melo*	Lasithi Crete
	NCPPB 541	*C. sativus*	Canada 1951
Isolated strains			
P. viridiflava	Pv612, Pv613	*C. melo*	Tymbaki Crete
	Pv614, Pv615		
	Pv616		
	Pv700, Pv709	*L. esculentum*	Antiscari Crete
	Pv710, Pv711		
	Pv712		
	Pv3005, Pv3006	*Solanum melogena*	Ierapetra Crete
	Pv3007, Pv3008		
	Pv3008		
	Pv5a, Pv6a, Pv6b	*Chrysanthemum* sp.	Ierapetra Crete
	Pv7a, Pv7b		
	Pv522, Pv527	*Amaranthus blitum*	St. Pelagia Crete
	Pv530, Pv531		
	Pv542		

[1] NCPPB, National Collection of Plant Pathogenic Bacteria.
[2] INRA-V, Institut Nationale de la Recherche Agronomique – Versailles.

(in the case of chrysanthemum) were triturated in a few drops of sterile distilled water. Loopfuls of the suspensions were streaked onto plates of NDA (Nutrient Dextrose Agar) and King's medium B (King et al., 1954). Plates were incubated for 48 h at 30 °C. Single colonies were subcultured, checked for purity and stored as slant cultures at 4 °C on NDA.

Isolation on King's medium B indicated that the isolated bacteria were fluorescent pseudomonads. Thus, many isolates were primary tested accordingly to the LOPAT tests (Lelliott et al., 1966). Twenty five representative isolates (five from each host, Table 1) were used for further characterization using the differential tests presented in Table 2. All methods had been previously described by Malathrakis and Goumas (1987). Reference bacterial strains are presented in Table 1. Each test was repeated at least twice. For further characterization, the following additional tests were performed: Gram stain (Schaad, 1988), glucose fermentation in Hugh and Leifson (1953) medium, and β-glucosidase on arbutin hydrolysis medium (Crosse and Garrett, 1963). The differential capacity of *P. viridiflava, P. syringae* pv. *syringae* and *P. syringae* pv. *tomato* strains to fluoresce on iron-deficient Misaghi & Grogan's medium (1969) containing sucrose, erythritol or DL-lactate as single carbon source, was also tested as described by Jones et al. (1986).

Table 2. Comparison of isolates differents hosts with isolates of *Pseudomonas viridiflava*, and some pathovars of *P. syringae* in differential tests

Tests	Isolates from: tomato eggplant melon blite chrysanthemum (25)[1]	*Pseudomonas viridiflava* (5)	*Pseudomonas syringae* pathovar		
			syringae (4)	*lachrymans* (3)	*tomato* (3)
Levan	-[2]	-	+	+	+
Oxidase	-	-	-	-	-
Potato rot	+	+	-	-	-
Arginine dihydrolase	-	-	-	-	-
Hypersensitivity	+	+	+	+	+
Nitrate reduction	-	-	-	-	-
Fluorescent pigment	+	+	+	+	+
Gelatin hydrolysis	+	V	+	NT	-
Pectate gel pitting	+[3]	+	-	NT	NT
Lipases	+	+	+	NT	NT
2-Ketogluconate	-	-	-	-	-
Use for growth					
D(-) Mannitol	+	+	+	+	+
D(+) Cellobiose	-	-	-	-	-
D(-) Sorbitol	+	+	+	+	+
D(+) Trehalose	-	-	-	-	-
D(+) Sucrose	-	-	+	+	+
i-Inositol	+	+	+	+	+
L(-) Rhamnose	-	-	-	-	-
D(-) Arabinose	-	-	-	-	-
Rhamnose	-	-	-	-	-
Erythritol	+	+	+	+	+
Betaine	+	+	+	+	+
Adonitol	-	-	-	-	-
DL-Lactate	+	+	+	+	+
L(-) Lactate	+	+	+	-	-
L(+) Tartrate	-[4]	-	-	-	-
D(-) Tartrate	+	+	-	-	+
Malonate	+	+	+	+	+
Benzoate	-	-	-	-	-
Aspartate	-	-	-	-	-
Anthranilate	-	-	-	-	-
L-Valine	-	-	-	-	-
β-Alanine	-	-	-	-	-

[1] ( ): Number of isolates used. Five isolates from each host were used (see Table 1).
[2] -: Negative reaction, +: Positive reaction, NT: not tested, V: variable reaction.
[3] Pectate gel pitting occurs at pH 7 and 8.3 for *Pseudomonas viridiflava*.
[4] Isolates from tomato are positives.

Pathogenicity tests

In preliminary studies all isolates were screened for their ability to induce a hypersensitive reaction on tobacco leaves and to rot potato slices by methods previously described (Malathrakis and Goumas, 1987).

For pathogenicity tests, two- to three-month old potted plants were used for inoculation. Ten plants of each host were inoculated with a homologous strain. Inocula were prepared from 48 h-old King's medium B agar plates cultures suspended in sterile distilled water and adjusted to approximately 10^7 CFU ml^{-1} by tur-

Table 3. Differential capacity of *P. viridiflava, P. synringae* pv. *synringae* and *P. syringae* pv. *tomato* strains to grow and fluoresce on a mineral base medium containing sucrose, erythritol or DL-lactase as single carbon course[1]

Bacteria	No of strains tested	Carbon source		
		Sucrose	Erythritol	DL-Lactate
Isolates from: tomato eggplant melon blite chrysanthemum	25	-[2]	+[3]	+
Pseudomonas viridiflava	5	-	+	+
Pseudomonas syringae pv. *syringae*	4	+	+	+
Pseudomonas syringae pv. *tomato*	3	+	-	-

[1] As described by Jones et al. (1985), on iron-deficient Misaghi & Grogan's medium (1969). No growth was occurred on basal medium without carbon source.
[2] -: Neither growth nor fluorescence.
[3] +: Growth and fluorescence.

bidity measurement with a spectrophotometer (A_{600}) and by dilution plate counts. A suspension of each isolate was sprayed onto leaves of plants until run-off with an airless electric paint sprayer. Control plants were sprayed with sterile distilled water. Stem inoculations of chyrsanthemum plants were made by stabbing with the tip of a sterile toothpick with an appropriate culture of *P. viridiflava* into the stem of the plant. Controls were similarly treated with sterile toothpicks. Plants were held under greenhouse conditions during winter (10–25 °C) with intermitent mist conditions (10 sec each hour). Symptoms were evaluated for one month after inoculation.

Cross-inoculation tests

One isolate per host (PV 542, PV 5a, PV 615, PV 3005, PV 709), and two others obtained from tomato with bacterial soft rot (PV 442) and cucumber with bacterial necrosis (PV 400), previously characterized as *P. viridiflava* (Malathrakis and Goumas, 1987; Goumas and Malathrakis, 1987), were used in cross-inoculation tests. Inocula were prepared, by the methodology described above in pathogenicity tests. A suspension of each isolate was sprayed onto seedling (4–5 true leaf stage) until run-off with an airless electric paint sprayer. Ten plants from each host (tomato, cucumber, eggplant, blite and melon) were inoculated with each isolate. Control plants were sprayed with sterile distilled water. Plants were predisposed to bacterial infection by being placed in a mist chamber for 24 h at 22 °C and 16 h photoperiod. Half of the plants from each combination were lightly wounded with a plastic brush while the rest remained uninjured. Stem inoculation of chrysanthemum and tomato plants were made by stabbing, into the stem of each plant above first true leaves with the tip of a sterile toothpick. The toothpick had been passed before through an appropriate Petri dish culture of *P. viridiflava*. Controls were similarly treated with sterile toothpicks. After inoculation, all plants were initially held in a mist chamber or in closed clear polyethylene bags for 48 h at 20 °C and afterwards transferred to glasshouse benches at 10–25 °C. Final disease observations were made 8–10 days later and pathogens were reisolated on King's medium B.

Host range

To determine host range, ten seedings (at 4–5 true leaf stage) from several potential host species (Table 4) were inoculated as before with a suspension (10^6 CFU ml^{-1}) of the PV 400 isolate originating from cucumber. The plants were treated as previously described and were assessed daily for ten days to record disease symptoms.

Bean pods test

Inoculations on snap bean (*Phaseolus vulgaris* L.) pods were also conducted as described by Cheng et al. (1989).

Table 4. Host range[1] and disease symptoms produced by *Pseudomonas viridiflava* isolate PV 400 (cucumber)

Family	Species name	Symptom(s) observed
Anaranthaceae	*Amaranthus blitum*	Leaf spot (Ls)
Chenopodiaceae	*Spinacia oleracea*	Ls
Compositae	*Chrysanthemum* sp.	Stem rot (Sr) and/or Leaf necrosis (Ln)
	Lactuca sativa	Ln
	Zinnia elegans	Ls
Cruciferae	*Brassica oleracea* var *botrytis*	Ls and/or Ln
	B. oleracea var *capita*	Ls and/or Ln
	B. oleracea var *italica*	Ls and/or Ln
Cucurbitaceae	*Cucumis melo*[2]	Ls and/or Ln
	C. sativus[2]	Ls and/or Ln
	Citrulus lanatus	Ls and/or Ln
	Cucurbita ficifolia	Ls and/or Ln
	C. foetidissima	Ls and/or Ln
	C. maxima	Ls and/or Ln
	C. pepo	Ls and/or Ln
	Ecballium elaterium (weed species)	Ls and/or Ln
	Luffa acutancula	Ls and/or Ln
	L. cylindrica	Ls and/or Ln
	Lagenaria sp.	Ls and/or Ln
	L. vulgaris	Ls and/or Ln
Lerguminosae	*Phaseolus vulgaris*	Ls and/or Ln
	Pisum sativum	Ls and/or Ln
	Vicia fabae	Ls and/or Ln
	Vigna unguiculata	Ls and/or Ln
Solanaceae	*Capsicum annuum*	Ls and/or Ln
	Lycopersicon esculentum[2]	Ls, Ln and/or Sr
	Solanum melogena	Ls
Vitaceae	*Vitis vinifera*	Ln

[1] At least ten plants of each species were inoculated. Inoculations were made by pressure spraying the leaf of hosts with a bacterial suspension of 10^6 CFU ml^{-1} or with the toothpick technique.
[2] No variation in susceptibility were observed among cultivars or hybrides of *Cucumis melo* (5), *C. sarivus* (10), and *Lycopersicon esculentum* (10) (unpublished data).

Results

Isolation and characterization of the pathogen

All isolations carried out on King's medium B, revealed consistently the presence, in essentially pure culture, of a fluorescent bacterium of the genus *Pseudomonas*. Furthermore *Erwinia carotovora* subsp. *carotovora* was often isolated from diseased chrysanthemum plants alone or in association with *Pseudomonas* spp. The colonies of *Pseudomonas*, on King's medium B, appeared opaque, convex, shiny, semifluid and produced a bright green-blue diffusible fluorescent pigment. On NDA medium the colonies were convex, smooth and whitish yellow. After five- to ten-days incubation on 5% sucrose nutrient agar, the centre of the colonies became greenish (Lelliott and Stead, 1987). In LOPAT tests (Lelliott et al., 1966) twenty-five tested isolates, together with five reference strains of *P. viridiflava* from cucumber and tomato gave similar results. Results of identification tests of isolates are presented in Table 2. These are in agreement with those obtained with the reference strains of *P. viridiflava*, except that isolates from tomato utilized L(+) tartrate. Other reference strains used in this work gave results which were consistent with their designated classifi-

cation (Malathrakis and Goumas, 1987; Hildebrand et al., 1988).

Quick identification of isolates as *P. viridiflava* was successful by using the pattern of Jones et al. (1986) for fluorescence on single carbon source media (Table 3).

On the basis of their morphological, physiological biochemical, and pathological characteristics, twenty-five representative isolates of *Pseudomonas* spp. (five from each host) were identified as *P. viridiflava* based on the determinative schemes proposed by Lelliott et al. (1966), Sands et al. (1970) and Billing (1970).

Pathogenicity tests

All isolates of *P. viridiflava* from the various host plants were pathogenic. Successful inoculations were made on tomato, eggplant, blite, melon and chrysanthemum under greenhouse conditions. In each host, the symptoms induced by the homologous strain were similar to those due to natural infections. On tomato, eggplant, blite and melon the disease started as a water-soaked spot which developed in 3–4 days into small (on tomato, blite and eggplant) or large (on melon) irregular lesions, usually with chlorotic haloes. The centre of the lesions later became dry and tan to black in colour. Usually the lesions coalesced and leaves appeared blighted. Chrysanthemum plants stab-inoculated into the stem, exhibited yellowing lower leaves, wilting, and a yellow to brown discoloured pith within 6–10 days. The stem often became hollow and split with bacterial slime exudation. Reisolations made from the artificially infected plants yielded pure cultures of *P. viridiflava*. Bacterial identification was confirmed by LOPAT tests.

Cross-inoculations

In cross inoculation tests, no differentiation was observed between strains of *P. viridiflava* from different hosts. In these, one strain of *P. viridiflava* per host and two others isolated from bacterial soft rot of tomato and bacterial necrosis of cucumber were tested. Each isolate induced disease symptoms independently of host of origin. Also, all strains after stab inoculation of the stem, developed bacterial soft-rot on tomato (Malathrakis & Goumas, 1987) or brown discoloured pith that was sometimes hollow in chrysanthemum plants. On wounded plants the symptoms were more severe and developed more rapidly than on non-wounded plants. Nonwounded plants with pre- and post-inoculation mist treatments showed symptoms 8–10 days later while wounded plants developed symptoms within 4 days.

Host range

Twenty-seven cultivated and one weed species (*Ecballium elaterium*) were found to be susceptible to the pathogen after inoculation with strain PV 400 from cucumber. Results of host-range tests are presented in Table 4.

Bean pods test

Finally, all isolates of *P. viridiflava* and reference strains of the bacterium, caused rust-coloured lesions within 48 h on excised pods. Strains of *P. syringae* (pv. *syringae*, pv. *tomato* and pv. *lachrymans*) caused a necrotic reaction whereas the strain of *P. syringae* pv. *phaseolicola* caused green, water-soaked, not sunken lesions, that remained without necrosis for at least 4 days.

Discussion

Results of this study suggest that the bacterium consistently isolated from diseased melon, tomato, eggplant, blite and chrysanthemum plants in Crete is *P. viridiflava*, which has been characterized as an opportunistic pathogen (Billing, 1970; Burkholder, 1930; Wilkie et al., 1973) to a wide variety of plants (Bradbury, 1986).

In Greece *P. viridiflava* was frequently found to cause severe problems on tomatoes and cucumbers grown in plastic houses or outdoors. The bacterium has been reported as the causal agent of pith necrosis of tomato (Alivizatos, 1986), bacterial soft rot of tomato (Malathrakis and Goumas, 1987), bacterial rot of eggplant (Goumas and Malathrakis, 1985) and bacterial blight of cucumber (Goumas and Malathrakis, 1987). *P. viridiflava* is recognized as a real or an opportunistic pathogen on many other hosts such as kiwi (Young et al., 1988), radish (Shakya and Vinther, 1989), bean (Billing, 1970), sweet onion (Gitaitis et al., 1991), alfalfa (Lukezic et al., 1983), parsnips (Hunter and Gigna, 1981), poinsettia (Suslow and McCain, 1981), and tomato (Jones et al., 1984; Malathrakis and Goumas, 1987). To our knowledge melon and blite which were found to be infected, in Crete, have not been previously reported as natural hosts of *P. viridiflava* (Bradbury, 1986). Also this is the first record of the bacterium as a foliar pathogen of melon, tomato,

eggplant and blite and as a stem pathogen of chrysanthemum in Greece. The disease on melon and tomato crops usually causes extensive tissue necrosis, resulting in losses of up to 50% of the foliage. On eggplants and blite plants the disease occurs sporadically, without severe economic losses. On chrysanthemum, during 1991, the bacterium had caused the total destruction of ten thousand plants in a nursery and the insidence of the disease was estimated up to 20% at an area of 0.4 hectare.

Inoculum of *P. viridiflava* induced diseases in Crete, may have originated from infected tomato and cucumber plants. Thus, *P. viridiflava* may be a well established pathogen of the region. Melon crops in Crete are planted in greenhouses from the mid to late winter as a second crop following curcumber or tomato. Eggplant and blite (outdoor crop) are grown in the areas of Ierapetra and St. Pelagia, respectively, where cucumber or tomato are the main crops in the same season. Primary inoculum may also originate from water used to clean pots or tablets in the nursery, as concentrations of 10^4 CFU ml^{-1} have been detected (Goumas, unpubl.).

Since 1985, the outbreak of these diseases, has been associated with favourable environmental conditions that aid invasion by the pathogen. Cool and humid conditions in greenhouses during winter, the succulence and vigour of the plants due to high nitrogen fertilization, prolonged wet conditions, low night temperatures ($< 15\,^{\circ}C$) and reduced illumination, combined with the absence of copper compound applications, create ideal conditions for disease initiation. *Ecballium elaterium* is a common weed specie in and near fields where susceptible hosts are cultivated. Since this weed can be infected by *Pseudomonas viridiflava* its role in the epidemiology of these diseases should be investigated. On the other hand, the epiphytic survival of the bacterium has been also well documented (Billing, 1970; Young et al., 1988; Mariano and McCarter, 1993).

During our experiments it was observed that many isolates of the bacterium lost their mucoid growth habit after repeated sub-culture on media. Also, most of them failed to produce typical hypersensitive reactions on tobacco. The same phenomenon was observed during artificial inoculations with non mucoid strains on the original host. This may partially be explained by the fact that bacterial exopolysaccharides can act as a determinant of pathogenicity (El-Banoby and Rudolph, 1979). However, Feet et al. (1989) reported that a soft rotting strain of *P. viridiflava* can produce alginate, an acidic exopolysaccharide. However, the role of this acidic exopolysaccharide in pathogenicity of this strain has not been determined. On the contrary the single extracellular pectate lyase produced by *P. viridiflava* determines pathogenicity (Liao et al., 1988).

The occurrence of the disease on many different hosts, its reappearence in the same greenhouses, the favourable conditions predisposing infection during winter season in Crete and the results of our inoculation experiments under similar conditions, support the view of the majority of previous reports (Gitaitis et al., 1991; Hunter and Cigna, 1981; Jones et al., 1984; Lucezic et al., 1983; Shakya and Vinther, 1989; Suslow and McCain, 1981; Young et al., 1988) which refer to *P. viridiflava* as an opportunistic pathogen. Also, observations made throughout recent years in Crete, show that efficient aeration of the greenhouse and protective applications of copper compounds can result in effective control of the diseases caused by the bacterium.

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