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Detection of *Pseudomonas syringae* pv. *aptata* in irrigation water retention basins by immunofluorescence colony-staining

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Abstract

Bacterial blight of cantaloupe (*Cucumis melo*) caused by *Pseudomonas syringae* pv. *aptata* was first observed in south-western France and has since spread to all cantaloupe-growing areas of this country. Use of pesticides registered for this disease has proved ineffective and no commercial cultivars of cantaloupe are resistant to this blight. To develop control strategies for this disease, the principal sources of inoculum were investigated. Among the different sources of inoculum studied, we report the isolation of *P. syringae* pv. *aptata* from irrigation water retention basins in south-western France using the immunofluorescence colony-staining (IFC) method. In this study, the pathogen was detected at a low concentration (12 and 70 cfu l^{-1}) in two different retention basins. These results suggest that *P. syringae* pv. *aptata* can survive in water used to irrigate cantaloupe crops and could be a source of inoculum for epidemics of bacterial blight. To develop control strategies for this bacterial disease, the importance of water retention basins as sources of inoculum for bacterial blight of cantaloupe needs to be evaluated relative to other potential sources such as seeds, plants from nurseries and plant debris in the soil.

Introduction

Some of the most frequently employed and effective measures for controlling bacterial diseases of plants are based on hygiene. The procedures to produce clean seed or vegetative propagation material and the recommendation of weeding and culling, for example, have been based on the identification of the principal sources of bacterial inoculum (McCarter et al., 1983; McDonald, 1995). Hence, the search for sources of inoculum and the evaluation of their impact on disease epidemiology are essential steps in developing control strategies for bacterial diseases of plants.

Since the early 1990s, cantaloupe (*Cucumis melo* var. *cantalupensis* Naud.) producers in France have faced serious outbreaks of a newly emerging bacterial blight caused by *Pseudomonas syringae* pv. *aptata* (Morris et al., 2000). Use of pesticides registered for this disease has proved ineffective and no commercial cultivars of cantaloupe are resistant to this blight. As part of our research on the potential strategies for control of this disease, we are attempting to identify the principal sources of inoculum of *P. syringae* pv. *aptata*. Among the different inoculum sources that are currently being investigated, we report the isolation of *P. syringae* pv. *aptata* from irrigation water retention basins in south-western France. We have been particularly interested in these basins as a possible source of *P. syringae* pv. *aptata* because of the characteristic topography of the south-western cantaloupe production region. These retention basins are generally situated in valleys and may receive run-off from the cantaloupe fields on the surrounding slopes. The water in these basins is used for irrigating cantaloupe and other crops and is also used in the preparation of pesticide and herbicide solutions. Although irrigation water has been suggested as a means of disseminating *P. syringae* and other species of epiphytic plant pathogenic bacteria

(Hirano et al., 1995), the occurrence of *P. syringae* in irrigation waters used in commercial production has, to our knowledge, never been reported.

To detect where plant pathogenic bacteria occur naturally, a wide range of approaches has been used. These have involved the culture of bacterial colonies on selective or semi-selective media (Schaad, 1988), detection of bacterial cells with specific antisera (Van Vuurde et al., 1983; Franken et al., 1993), detection of bacterial nucleic acids with oligonucleotide probes (Prosen et al., 1993; Audy et al., 1996; Manulis et al., 1998; Ojeda and Verdier, 2000) and various combinations of these (Schaad et al., 1995; Van der Wolf et al., 1998). In the present study, we chose to use IFC, as described by Van Vuurde (1987) because it is highly sensitive, readily allows the quantitative determination of the concentration of bacteria in the inoculum source and also allows for isolation of strains. Furthermore, antisera and semi-selective media have been developed in previous studies (Mohan and Schaad, 1987; Saunier et al., 1996) that have proved to be applicable to *P. syringae* pv. *aptata*. Here the combined use of these antisera and the selective medium has allowed us to determine whether irrigation water is a source of inoculum for bacterial blight of cantaloupe.

Materials and methods

Bacterial strains and growth conditions

One hundred and sixty strains of *P. syringae* pv. *aptata* isolated from cantaloupe (*C. melo*), seven strains of saprophytic fluorescent *Pseudomonas* spp. isolated from soil, one strain of *P. syringae* pv. *aceris* (CFBP2339), two strains of *P. syringae* pv. *aptata* (CFBP1617 and CFBP1906), one strain of *P. syringae* pv. *pisi* (CFBP1745) and one strain of *P. syringae* pv. *syringae* (CFBP1323) were used. Strains labelled 'CFBP' were reference strains from the French National Collection of Plant Pathogenic Bacteria and were kindly provided by L. Gardan, INRA Angers, France. Strains labelled 'CC' were isolated as described by Morris et al. (2000). All strains of *P. syringae* pv. *aptata* were virulent on cantaloupe (Morris et al., 2000). The bacterial strains were grown on King's medium B (KB medium) (King et al., 1954) for 48 h at 25 °C. Strains were stored in 40% glycerol at −80 °C and in sterile potassium phosphate buffer (8.75 g of K_2HPO_4, 6.75 g of KH_2PO_4 per litre, pH 7) at 4 °C.

Antisera

Polyclonal antisera were produced as described by Saunier et al. (1996). Nine rabbits were immunised with three strains of *P. syringae* pv. *aptata* (stains CC04, CC73 and CFBP1617). These strains belong to three of the different O-serogroups (APTPIS, PERSAVTOM and MOP6) (Saunier et al., 1996) (Table 1) and represent a large proportion of the serotypes of cantaloupe strains as determined in a preliminary study (Morris et al., 2000). The bacterial cells were prepared (Saunier et al., 1996) and bacterial suspensions (10^8 cells ml^{-1}) were administered to adult rabbits (Fauve de Bourgogne race) in four intravenous injections within six weeks. For rabbits immunised with strain CC04, five intravenous injections were made. The animals were bled 6–13 days after the last injection. Antisera were titrated by Ouchterlony double-diffusion and were then stored in glycerol (1 : 1, vol : vol). Immunoglobulins (IgG) were purified from crude antisera by protein A-Sepharose CL-4B (Pharmacia) affinity chromatography. After purification, the IgG fraction collected was stored in glycerol (1 : 1) at −20 °C.

The specificity of polyclonal antisera was tested with pure cultures of strains of the pathogenic and saprophytic bacteria listed above by indirect immunofluorescence staining (IF) (Guillorit-Rondeau et al., 1996). The rabbit antisera were used as the primary antibody and an anti-rabbit IgG labelled with fluorescein isothiocyanate (Sigma) as the secondary antibody. Stained slide preparations were observed with an Olympus B × 60 microscope equipped with a halogen lamp,

Table 1. Distribution of cantaloupe strains of *P. syringae* pv. *aptata* into three O-Serogroups

O-Serogroups[a]	Number of cantaloupe strains
APTPIS	58
PERSAVTOM1	61
MOP6	17
Not typed[b]	15
Cross-reaction[c]	9

[a]Saunier et al. (1996). The serum for the APTPIS serogroup was prepared with strain CFBP 1617, for PERSAVTOM1 with strain CC73 and for MOP6 with strain CC04.
[b]Strains belonged to none of the three O-serogroups.
[c]Cross-reactions observed between two antisera. Four strains cross-reacted with APTPIS and MOP6, four strains cross-reacted with PERSAVTOM1 and MOP6, and one strain cross-reacted with APTPIS and PERSAVTOM1.

a 450–480 nm excitation filter, and a 515 nm barrier filter. Bacterial cells were observed under ultraviolet light with a 100× objective.

Semi-selective medium for recovery of P. syringae *pv.* aptata

The recovery of *P. syringae* pv. *aptata* strains on KBC medium (Mohan and Schaad, 1987) was tested for 26 strains isolated from cantaloupe. Aqueous bacterial suspensions of 48 h cultures were adjusted to 0.3 at OD_{620} corresponding to approximately 2×10^8 cells ml^{-1}. Dilutions of these suspensions were spread on KBC and KB media and incubated for 48 h at 25 °C. Three replicates of each dilution were plated for each strain. The percentage recovery of cantaloupe strains on KBC medium was calculated relative to that on KB medium. The ability of KBC medium to inhibit the saprophytic background microflora of water was also determined. Dilutions of each water sample described below were plated on tryptic soy agar (1.7 g of tryptone, 0.3 g of Bacto-Soytone, 0.25 g of glucose, 0.5 g of NaCl, 0.5 g of K_2HPO_4, 15 g of agar and 50 mg of cycloheximide per litre) (TSA) and on KBC medium. Three replicate plates of each dilution were plated for each water sample. Plates KBC and TSA were incubated at 25 °C for 48 and 72 h, respectively and the total number of colonies on plates of the two media was compared.

Water samples

Water samples were collected on two different dates and at seven different sites located in a commercial cantaloupe-growing region in south-western France. The first sampling was conducted in March 2001 from five water retention basins just prior to transplantation of cantaloupe seedlings in the field. The second sampling was conducted in June 2001 from three retention basins including one basin that was sampled in March. For each site, a water sample (3 litre) was collected by submerging clean plastic bottles to a depth of approximately 15 cm and then allowing them to fill. The bottles of water were transported to the laboratory in a cooler and kept at 15 °C until the water was analysed. For each water sample, total bacteria were enumerated by dilution plating on TSA. Plates were incubated for 72 h at 25 °C before colonies were counted.

Detection of bacteria by immunoflorescence colony-staining (IFC)

Water samples were concentrated (by a factor of up to 50×) by filtration through a 0.22 μm (pore diameter) nitrocellulose filter. The filter was then placed in 20 ml of sterile distilled water to resuspend bacteria. The concentration factor used was limited by the microflora in the water sample.

Concentrated water samples were pour-plated in semi-solid KBC medium (0.8% agar in the poured medium). The temperature of the medium and water sample did not exceed 45 °C during pouring. Thirty, 35-mm-diameter Petri dishes were poured per sample. Each dish contained 900 μl of the mixture that included 500 μl of the concentrated water sample. After the mixture solidified, the agar surface was covered with an additional 500 μl of sterile semi-solid KBC medium to protect colonies on the surface of the first layer from being washed away in the subsequent steps. As a positive control, a suspension of a 48-h-culture of strain CC40 of *P. syringae* pv. *aptata* was incorporated into aliquots of the concentrated water samples from basins 1 and 2 (Table 2) at an expected density of 2 cfu ml^{-1}. Plates were incubated for 48 h at 25 °C. The plates were then opened and the agar was dried to a thickness of 2 mm under a laminar flow hood for 2 h.

Microcolonies were stained by addition of 1 ml per plate of polyclonal antiserum at 0.1 mg ml^{-1}. After 16 h incubation at room temperature with orbital agitation, the agar film was washed two times by addition of 2 ml phosphate buffered saline with 0.1% of Tween-20 (PBST) (8.8 g of NaCl, 2.9 g of Na_2HPO_4, 12 H_2O, 0.36 g KH_2PO_4, 0.2 g of KCl and 1 ml of Tween-20 per litre, pH 7.2) for 2 h. In the second step, microcolonies were stained by addition of 1 ml anti-rabbit IgG antiserum labelled with fluorescein isothiocyanate (Sigma) diluted 1 : 50 in PBST. After 18 h incubation at room temperature with agitation, the agar film was washed three times by addition of 2 ml PBST and incubation for 3 h. Agar films were observed with an Olympus B × 60 microscope equipped with a halogen lamp, a 450–480 nm excitation filter, and a 515 nm barrier filter. Fluorescent colonies were located and counted with a 2× objective.

Identification of IFC-positive colonies

IFC-positive bacterial colonies were harvested with sterile capillary pipettes and suspended in sterile distilled water. Bacteria were isolated and purified on

Table 2. Detection of *P. syringae* pv. *aptata*, pathogenic to cantaloupe, in irrigation water from retention basins

Retention basin	Concentration factor[c]	Date	Mean number of colonies of total flora ml^{-1} of water		Psa (CFU ml^{-1})[a]
			TSA medium	KBC medium	
1	50	March 2001	1.63×10^4	0.60	0.070
2	50	March 2001	8.17×10^5	38.3	ND[b]
3	50	March 2001	1.31×10^4	14.1	ND
4	50	March 2001	3.08×10^3	3.47	ND
5	50	March 2001	6.16×10^3	<0.2[d]	ND
1	5	June 2001	4.56×10^5	6.0×10^3	ND
6	50	June 2001	4.00×10^4	1.87	0.012
7	NC	June 2001	4.2×10^5	1.1×10^3	ND

[a]Detection of *P. syringae* pv. *aptata* by immunofluorescence colony staining.
[b]*P. syringae* pv. *aptata* not detected.
[c]Water samples were concentrated by filtration on membranes by a factor of 5×, 50×, or were not concentrated (NC).
[d]Detection limit of *P. syringae* pv. *aptata* on KBC medium.

KBC medium. Purified strains were tested for induction of a hypersensitive response in tobacco by infiltration of suspensions (10^8 cells ml^{-1}) of 48-h-bacterial cultures into leaves of tobacco. Strains that induced a hypersensitive reaction were characterised for production of fluorescent pigment, presence of arginine dihydrolase and cytochrome oxidase, and for their pathogenicity to cantaloupe (Morris et al., 2000). For pathogenicity tests, inoculum consisted of aqueous suspensions of 48-h-bacterial cultures from KB medium adjusted to an OD_{620} of 0.3. An aliquot of 50 µl of each bacterial suspension was injected into the petiole of cantaloupe seedlings. Three plants were inoculated with each isolate and three plants were inoculated with sterile distilled water as controls. Plants were incubated in a plastic chamber to assure high relative humidity for 7 days. The plants were evaluated at 2 and 7 days after inoculation using the following scale: 0 (plants without symptoms), 1 (hypersensitivity-like reaction), 2 (expansion of the necrotic zone to the leaves and the petiole) and 3 (dead plant).

Results

Recovery of cantaloupe strains and saprophytic microflora on KBC medium

For the 26 cantaloupe strains of *P. syringae* pv. *aptata* tested, between 90% and 135% of the CFU that developed on KB medium were recovered on KBC medium. The mean recovery rate was 108%. Colonies of *P. syringae* pv. *aptata* observed on KBC medium were 2 mm in diameter, flat, circular, white in colour and showed a blue fluorescence under UV light (325 nm) after 3 days of incubation at 25 °C. Evaluation of the selectivity of KBC medium with water samples collected in south-western France showed that this medium inhibited the growth of more than 99% of the saprophytic flora from irrigation water (Table 2).

Specificity of polyclonal antisera and distribution of cantaloupe strains into O-serogroups

Three antisera (APTPIS, MOP6 and PERSAVTOM1) were tested against each of the 160 strains of the cantaloupe pathogen and against other saprophyte and reference strains as described above. For *P. syringae* pv. *aptata* isolated from cantaloupe, 145 strains (94%) reacted positively with at least one of the antisera. The number of cantaloupe strains reacting with each of the antisera is presented in Table 1. Most strains (136) reacted with only a single antiserum. Fifty-eight strains (36%) belonged to the APTPIS O-serogroup, 61 (38%) belonged to the PERSAVTOM O-serogroup and 17 (11%) belonged to the MOP6 O-serogroup of Saunier et al. (1996). Nine strains cross-reacted with two of the antisera and 15 strains did not react with any of the antisera tested. All reference strains of *P. syringae* pv. *aptata* and pv. *syringae* reacted positively with the antisera. None of the antisera reacted with saprophytic *Pseudomonas* spp. isolated from soil, with the strains of *P. syringae* pv. *aceris* or with the strain of *P. syringae* pv. *pisi*.

Detection of P. syringae *pv.* aptata *in irrigation water*

Seven retention basins were examined for the presence of *P. syringae* pv. *aptata* by IFC. The pathogen was detected in samples from two different basins (Table 2). Bacteria found in the water sample from basin 1 were detected with the antiserum to the serogroup PERSAVTOM1. Those from basin 6 were detected with antisera to the serogroups PERSAVTOM1 and MOP6. Thirty-two IFC-positive colonies from these basins (27 from basin 1 and 5 from basin 6) were isolated, purified and characterised. Among these 32 strains, 22 (19 from basin 1 and 3 from basin 6) gave an intensely fluorescent IFC reaction. All of these 22 strains were identified as *P. syringae* pv. *aptata* based on the results of tests of production of fluorescent pigment on KB medium, for the presence of cytochrome oxidase and arginine dihydrolase, and for induction of hypersensitivity in tobacco and a compatible reaction in cantaloupe. The other 10 strains corresponded to colonies that were weakly fluorescent in the IFC test. These strains did not induce a hypersensitive response in tobacco and they reacted positively for the presence of cytochrome oxidase.

For all water samples, the saprophytic populations enumerated on TSA medium varied from 3.80×10^3 to 8.17×10^5 cfu ml^{-1}. In both samples in which *P. syringae* pv. *aptata* was detected, the number of colonies on KBC medium was low (<1000 colonies per 35 mm diameter plate) and samples had been concentrated 50-fold. For the control water samples in which the strain CC40 was incorporated, the bacterium was detected at the expected concentration of 2 cfu ml^{-1} in the 15 ml of each sample analysed.

Discussion

We have demonstrated that water retention basins used for irrigating and preparing chemical treatments of commercial cantaloupe production fields in south-western France can harbour cells of *P. syringae* pv. *aptata* virulent on cantaloupe. This bacterium was detected at concentrations as low as 70 cfu of culturable cells per litre of surface water in two of seven basins examined. If *P. syringae* pv. *aptata* cells enter a viable but non-culturable state, then the frequency of contaminated basins and the concentration of bacteria in basins reported here may have been underestimated. The most obvious source of contamination of retention basins by *P. syringae* pv. *aptata* in south-western France is water run-off from adjacent diseased cantaloupe fields. This suggests that this bacterium can survive in these waters during the six-month autumn and winter period between cantaloupe seasons. However, if *P. syringae* pv. *aptata* is an epiphyte on weeds, wild plants or crops that persist during the autumn and winter, these sources might foster continuous contamination of basins.

The sensitivity of the IFC technique permitted us to detect low concentrations of bacteria. The sensitivity was obtained by coupling the use of (i) a culture medium that was sufficiently selective (Mohan and Schaad, 1987), (ii) a specific antiserum (Saunier et al., 1996; Morris et al., 2000) and (iii) by concentrating the bacteria by filtration. The sensitivity reported here is similar to that reported by Van Vuurde et al. (1995). The sensitivity of this technique was nevertheless limited when the total microbial load of irrigation waters was high, and it was therefore difficult to concentrate the water samples. Occasionally, saprophytes cross-reacting with the three antisera used here were detected. These colonies were less intensely stained and their morphology was also different. The biological and physiological characterisation of bacteria from weakly IFC-positive colonies revealed that all these colonies corresponded to saprophytic microflora. Similar results were observed by Van der Wolf et al. (1998). Furthermore, water collected in the late spring (June) tended to have higher numbers of background flora able to grow on KBC medium than did water collected in March. This suggests that sampling date is critical for optimising the sensitivity of IFC for detecting *P. syringae* pv. *aptata* in irrigation water.

Numerous sources of inoculum have been reported for diseases caused by the species *P. syringae*. These include weeds and nursery seedlings harbouring epiphytic populations, seeds and soil (McCarter et al., 1983). Our results constitute the first report of naturally occurring populations of this species in irrigation water. Rain and irrigation water are well know vectors of epiphytic plant pathogenic bacteria (Hirano et al., 1995; Farag et al., 1999). They disseminate bacterial cells coming from other sources. For example, rain can scrub aerosolised cells of *P. syringae* from the air and deposit them on crop plants along with rain drops (Lindemann and Upper, 1985). Irrigation water disseminates epiphytic cells on leaves of crop plants (Hirano et al., 1995). However, there are few known examples of plant pathogenic bacteria surviving in water. *Ralstonia solanacearum* has been detected in irrigation water at concentrations of 10^5–10^6 cfu l^{-1}

(Elphinstone et al., 1998; Wenneker et al., 1999). Under these conditions, the survival of this pathogen was apparently assisted by its colonisation of the roots of certain aquatic weeds or weeds found on the banks of lakes, ponds, etc. (*Solanum dulcamara*, *Urtica dioica*). However, *R. solanacearum* has also been detected in irrigation water at concentrations ranging from 10^2 to 10^6 cfu l^{-1} in the absence of plant hosts (Farag et al., 1999). Soft rot *Erwinia* spp. have also been found in water samples collected from rivers, lakes, drains and ditches (McCarter-Zorner et al., 1984; Harrison et al., 1987; Cother and Gilbert, 1990). These studies have shown that irrigation water may be a significant source of pectolytic bacteria in potato fields (McCarter-Zorner et al., 1984; Harrison et al., 1987).

What is the potential epidemiological impact of water retention basins as sources of inoculum for bacterial blight of cantaloupe? Clearly, overhead irrigation using contaminated water ensures that *P. syringae* pv. *aptata* will be disseminated to the leaf surface. However, the number of bacteria that are likely to be deposited on plants via irrigation needs to be estimated. Cantaloupe fields are irrigated at a rate of about 2×10^5 l ha^{-1}. Commercial cantaloupe fields contain about 9000 plants ha^{-1} and these plants have about 150–450 leaves per plant during the fruit production period when the need for watering is at a peak. For retention basins containing 100 cfu l^{-1} of *P. syringae* pv. *aptata*, over 2000 cfu of this bacterium could be added to the microbial populations of each plant each time the field is watered. The number of bacteria actually deposited on each leaf of the plants depends on the exposure of each leaf to irrigation water, the proportion of the total volume of water applied that falls on plants rather than on the ground, and run-off from the leaves. The bacteria disseminated via irrigation will have an impact on the total population size of *P. syringae* pv. *aptata* on leaves and eventually on disease outbreaks depending on the subsequent survival and growth rates of deposited bacteria. An essential question to address is the number of bacterial cells necessary to incite disease. For other pathovars of *P. syringae* as few as 10^4–10^5 cfu constitute the median effective dose (Knoche et al., 1987; Cazorla et al., 1998). Furthermore, application of non-bactericidal pesticides prepared with contaminated water may also be a means of disseminating *P. syringae* pv. *aptata* to cantaloupe leaves, but the total volume of water used for such treatments is small compared to that applied during irrigation.

The importance of water retention basins as sources of inoculum for bacterial blight of cantaloupe also needs to be evaluated relative to other sources of inoculum. Although there are numerous potential sources – including seeds, plants from nurseries and plant debris in the soil – we have not yet been able to demonstrate the presence of *P. syringae* pv. *aptata* in any of these. In spite of the high level of sensitivity of IFC, technical and sampling constraints may still be a limiting factor in confirming whether these other potential sources are important. For example, the number of *P. syringae* pv. *aptata* cells from plant debris that survive overwinter in soil is likely to be very small and unevenly distributed. Hence, a considerable quantity of soil may need to analysed to detect this bacterium. The physical and chemical properties of the soil may also pose constraints to detection. IFC was extremely powerful for detection of *P. syringae* pv. *aptata* in water, in part because of the ease of concentrating the bacteria in water samples. Methods to concentrate cells from other sources will need to be developed to render IFC useful for detection of *P. syringae* pv. *aptata* in these sources.

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