

## SEASONAL FLUCTUATIONS IN KIWIFRUIT PHYLLOSPHERE AND ICE NUCLEATION ACTIVITY OF *PSEUDOMONAS VIRIDIFLAVA*

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### SUMMARY

Seasonal fluctuations of *Pseudomonas viridiflava*, the causal agent of bacterial blight, were investigated on the kiwifruit phyllosphere in relation to ice nucleation activity, at different sites in central Italy (north of Latium), from October 1995 until September 1996. The pathogen was detected during the different seasons on leaves, twigs, buds and fruits with population values ranging between  $1.1 \times 10^1$  and  $2 \times 10^6$  cfu cm<sup>-2</sup> with maxima in spring and autumn. The pathogen was also present in significant amounts in several winter and summer months. No *P. viridiflava* was detected on flowers. Most of the bacterial isolates showed ice nucleation activity, manifested at relatively low concentrations ( $10^4$  cfu ml<sup>-1</sup>) and at temperatures slightly below 0°C (-3°C). Epidemiological implications are discussed.

### RIASSUNTO

FLUTTUAZIONI STAGIONALI NELLA FILLOSFERA DI ACTINIDIA ED ATTIVITÀ CRIOGENA DI *PSEUDOMONAS VIRIDIFLAVA*. Nel centro Italia (Nord del Lazio), dall'ottobre 1995 al settembre 1996 in due differenti zone, sono state studiate le fluttuazioni stagionali nella fillosfera di actinidia e l'attività criogena di *Pseudomonas viridiflava* agente causale della maculatura batterica dell'actinidia. Il patogeno, durante le diverse stagioni, è risultato presente su foglie, tralci, boccioli e frutti con valori di popolazioni batteriche variabili tra  $1.1 \times 10^1$  e  $2 \times 10^6$  ufc cm<sup>-2</sup>. I valori massimi sono stati riscontrati in primavera e in autunno. Il patogeno è risultato presente con valori di popolazioni batteriche significative anche in alcuni mesi invernali ed estivi. Nessuna cellula di *P. viridiflava* è stata invece isolata dai fiori. Molti degli isolati batterici hanno manifestato la capacità di nucleare il ghiaccio. L'attività criogena si è evidenziata a concentrazioni relativamente basse ( $10^4$  cfu ml<sup>-1</sup>) ed a tempera-

ture di poco inferiori a 0°C (-3°C). Vengono discusse le implicazioni epidemiologiche.

*Key words:* *Pseudomonas viridiflava*, kiwifruit, epiphytic survival, ice nucleation activity.

### INTRODUCTION

From the end of the sixties, kiwifruit has gradually been planted almost everywhere in Italy. Since 1990 Italy has been the first world producer with more than 300,000 t produced on more than 21,000 hectares (Alvisi, 1996).

*Pseudomonas viridiflava* (Burkholder) Dowson, the cause of bacterial blight of kiwifruit (Fig. 1), was first isolated from leaves and flowers in New Zealand in 1973 (Wilkie *et al.*, 1973). It was later recorded from other continents (Luisetti and Gagnard, 1987; Conn and Gubler 1993), and was first isolated in Italy in 1990 (Varvaro *et al.*, 1990). *P. viridiflava* is an ubiquitous micro-organism recorded in more than 14 countries and on 31 plant species (Bradbury, 1986). It can survive epiphytically on the host and can infect plants when environmental conditions are suitable (Young *et al.*, 1988; Varvaro *et al.*, 1990).

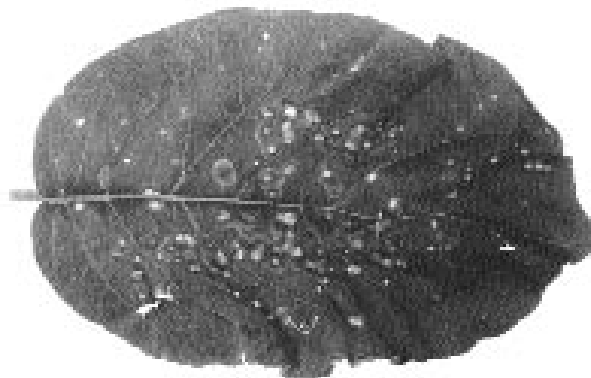


Fig.1. Natural bacterial blight symptoms caused by *Pseudomonas viridiflava* on kiwifruit leaf.

Young (1987) and Varvaro *et al.* (1990) report considerable *P. viridiflava* populations on the kiwifruit phyllosphere in autumn and spring and that several isolates show ice nucleation activity (INA) (Paulin and Luisetti, 1978).

The aims of this research were to study the presence and the seasonal fluctuations of *P. viridiflava* populations on the kiwifruit phyllosphere and its INA.

## MATERIALS AND METHODS

**Experimental plots.** Two 8-year-old orchards of kiwifruit var. 'Hayward' (A and B) were chosen, characterised by different geographic and meteorological conditions.

Area A is in the Viterbo district, with cold winters and mild summers (means of 5°C and 22°C, respectively). Area B is near Bolsena Lake, with a humid winter and hoar-frosts; rainfall is heavy in autumn (65 mm in November) and the summer is hot (mean 30°C). Relative humidity in area A is between 50% and 70%, and always over 70% in area B due the influence of the lake.

**Detection of epiphytic bacterial populations.** Samples of leaves, twigs, flowers, buds and fruits were taken monthly at random from kiwifruit plants with typical symptoms produced by *P. viridiflava* and from apparently healthy plants, from October 1995 until September 1996. Sampling was carried out on 20 kiwifruit plants per area. From each plant and at each time 10 twigs 7 cm long (all months), 10 leaves (all months except December), 20 buds (March-April), 20 flowers (May) and 10 fruits (June-September) were collected.

The samples were placed in sterile flasks with appropriate quantities of sterile distilled water (SDW) and washed for two hours using an orbital shaker at 150 rpm.

Ten-fold dilutions of the washing water were made and 0.1 ml of each dilution was plated, in duplicate, on Petri dishes containing nutrient agar supplemented with 5% sucrose (NAS) and with 2.5 mg l<sup>-1</sup> of crystal violet. After 48 h of incubation at 25±1°C the plates were observed under a stereomicroscope to count the colonies developed.

Colonies suspected to be *P. viridiflava*, were purified on NAS and then transferred to nutrient agar with 2% glycerol (NAG) slants.

Bacterial isolates were checked on King's medium B (KB) (King *et al.*, 1954) for production of fluorescent pigments, and the following tests were performed: Gram staining, motility, presence of oxidase, soft rot on potato slices, hypersensitivity on tobacco leaves, hydrolysis of arginine, production of 2-ketogluconate, reduc-

tion of nitrate and production of acid from sucrose (Lelliott *et al.*, 1966; Sands *et al.*, 1980; Fahy and Hayward, 1983; Lelliott and Stead, 1987).

Strains of *P. viridiflava* (PFM16) and *P. syringae* pv. *syringae* (van Hall) (PSSB3), from the Collection of the Department of Plant Protection, University of Viterbo, were used as controls.

Pathogenicity tests were performed on 1-year-old kiwi plants, cv. 'Hayward', kept in a greenhouse at 25±2°C with 70-80% RH, as described by Varvaro *et al.* (1990).

The number of *P. viridiflava* colonies counted on plates was averaged and then related to the number of flowers and buds or the area (cm<sup>2</sup>) of leaves, twigs and fruits, in the sample.

**Ice nucleation activity of *P. viridiflava* isolates.** To assess ice nucleation activity of *P. viridiflava* isolates, bacterial suspensions were prepared as described by Varvaro and Fabi (1992), then 100 µl of each suspension containing 10<sup>8</sup> cfu ml<sup>-1</sup> were placed in sterile test tubes that were half-immersed in a bath containing 30% ethylene glycol.

Temperature was adjusted to 0°C and then lowered by 1°C every half hour to reach -10°C. At each temperature ice formation was evaluated (Makino, 1982).

*P. viridiflava* isolates showing ice nucleation activity at -3°C were subsequently tested to assess the influence of bacterial concentration from 10<sup>8</sup> to 10<sup>4</sup> cfu ml<sup>-1</sup> on INA at -3, -5 and -7°C.

## RESULTS

Bacteria identified as *P. viridiflava* had white-yellow colonies, 2-3 mm in diameter, with dark-green centres. They were levan negative, rod-shaped, Gram negative, motile. They were negative for presence of oxidase, hydrolysis of arginine, production of 2-ketogluconate, reduction of nitrate, production of acid from sucrose. They were positive for soft rot on potato slices, hypersensitivity on tobacco leaves and production of fluorescent pigment on KB medium.

Bacterial isolates and the positive control (PFM16) *P. viridiflava* gave typical symptoms of bacterial blight on leaves within 2 weeks after inoculation on young kiwi plants. No symptoms occurred on leaves inoculated with the *P. s.* pv. *syringae*.

According to these criteria, 138 isolates were identified as *P. viridiflava*.

During the period of study we isolated *P. viridiflava* strains in both orchards from twigs, leaves, buds and fruits, but not from flowers.

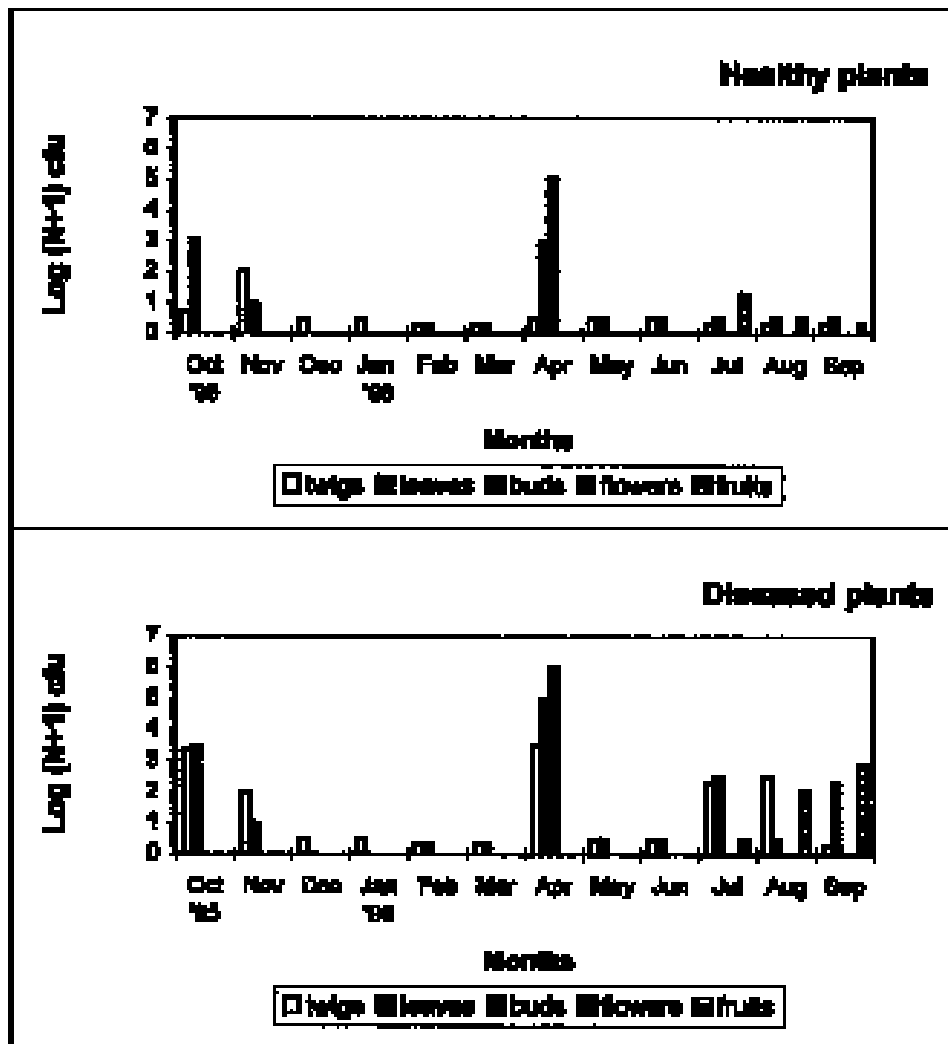


Fig.2. Seasonal fluctuations of *Pseudomonas viridiflava* populations on healthy and diseased kiwi plants in area A (see text).

The highest *P. viridiflava* populations were obtained from area A (Fig. 2), and considerable differences between healthy and diseased plants were obtained, particularly on twigs in October, where values were below  $10^1$  cfu  $\text{cm}^{-2}$  on healthy plants and  $2.5 \times 10^4$  cfu  $\text{cm}^{-2}$  on diseased ones.

In area A, the maximum value of the pathogen was  $2 \times 10^6$  cfu for buds on diseased plants in April. On the other vegetative parts, differences in bacterial populations between the healthy and diseased plants, highest in April, respectively  $1 \times 10^3$  and  $1 \times 10^5$  cfu  $\text{cm}^{-2}$  on leaves and  $1.7 \times 10^5$  and  $1 \times 10^6$  for buds. Few of the bacteria were isolated from fruits in August and September on healthy plants, while values of  $1 \times 10^2$  and  $8 \times 10^2$  cfu  $\text{cm}^{-2}$ , respectively, were recorded on diseased plants.

In area B, differences in populations on healthy and

diseased plants were also observed. Noticeable differences were recorded in April and June on leaves, in January and June on twigs and in July on fruits (Fig. 3). The highest value on healthy plants in this area was  $2.7 \times 10^4$  cfu  $\text{cm}^{-2}$  on twigs in June.

In both areas, *P. viridiflava* populations were much reduced during the summer and winter months (Figs 2 and 3).

80% of the *P. viridiflava* isolates were active in ice nucleation at  $-7^\circ\text{C}$  and most of them (97 isolates) showed activity at  $-3^\circ\text{C}$  (Fig. 4).

At a concentration of  $10^4$  cfu  $\text{ml}^{-1}$  from healthy and diseased plants, the cumulative percentages of the INA bacteria obtained in October was 42% at  $-3^\circ\text{C}$ , 75% at  $-5^\circ\text{C}$  and 94% at  $-7^\circ\text{C}$ . Increasing the bacterial concentration to  $10^6$  cfu  $\text{ml}^{-1}$ , almost 90% were active at  $-3^\circ\text{C}$  (Fig. 5).

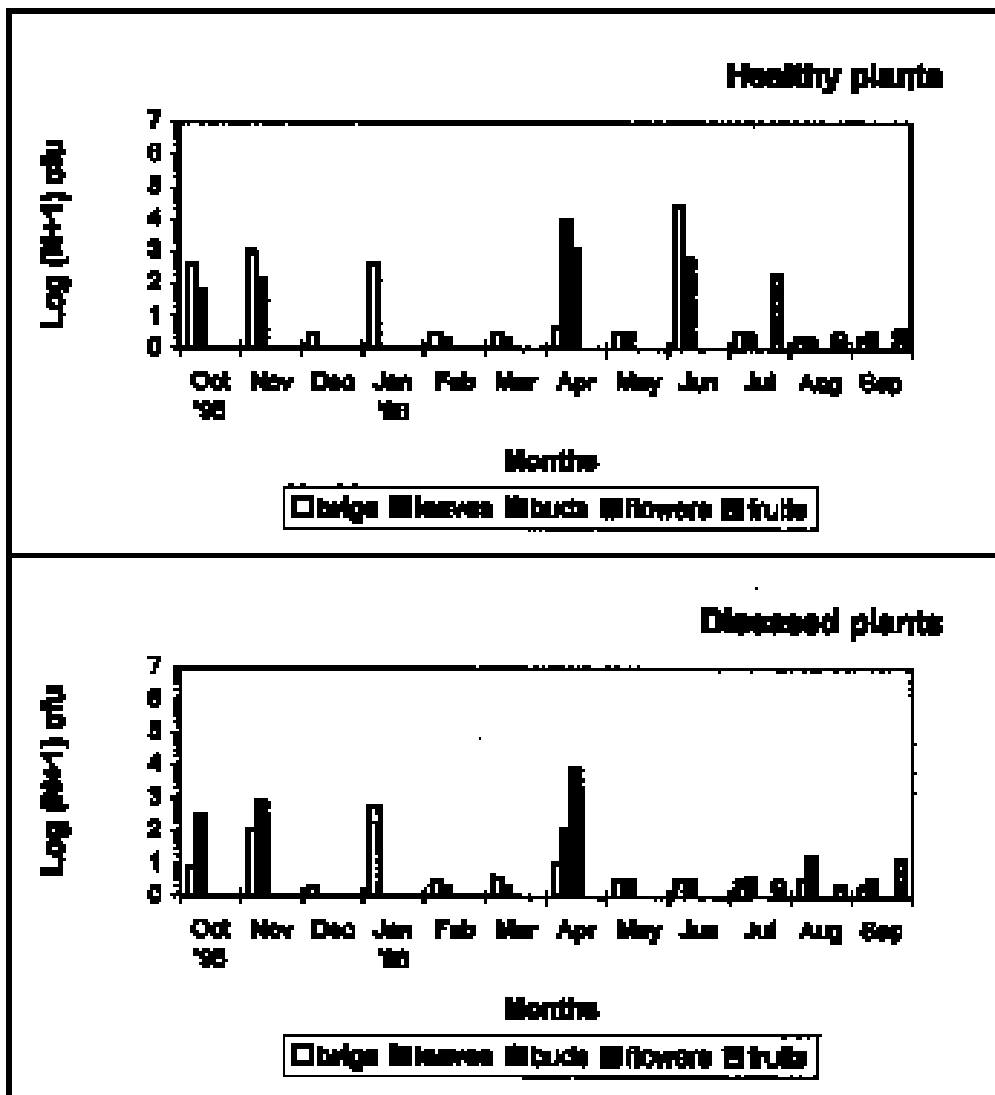


Fig.3. Seasonal fluctuations of *Pseudomonas viridiflava* populations on healthy and diseased kiwi plants in area B (see text).

Isolates obtained in April did not show INA at -3°C at 10<sup>4</sup> cfu ml<sup>-1</sup>, but INA was found at -5°C (30%) and at -7°C (40%).

*P. viridiflava* isolates obtained during the other months were not active at concentrations of 10<sup>4</sup> and 10<sup>5</sup> cfu ml<sup>-1</sup> at -3, -5, or -7°C. Only at concentrations of 10<sup>6</sup> cfu ml<sup>-1</sup> were 37% of them active at -5°C and 54% at -7°C.

**DISCUSSION**

The present research confirms the ability of *P. viridiflava* to establish populations on the kiwifruit phyllosphere, on both diseased and healthy plants throughout the year. High populations of *P. viridiflava* were found on leaves, buds, twigs and fruits, but none were isolated

from flowers, probably due to poor nutrient availability.

Temperature and rainfall certainly influenced bacterial survival especially in autumn, but also in spring and summer, when populations were still considerable; relative humidity seemed also to play a fundamental role, as reported by Balestra and Varvaro (1997).

Large populations were recorded in this study on the kiwifruit phyllosphere, during the spring and autumn, as earlier reported by Balestra and Varvaro (1997) and Young *et al.* (1988).

In the spring, good weather and nutrient availability favour the multiplication of epiphytic bacteria, creating a potential disease inoculum.

The pathogen was also recorded during winter (January) on leaves and during summer (July) on fruits, and was clearly able to survive during particularly unfavourable months.

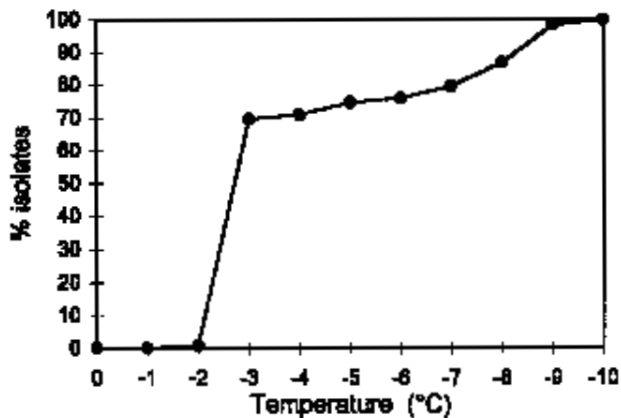


Fig.4. Cumulative percentages of *Pseudomonas viridiflava* isolates that were active in ice nucleation as a function of temperature.

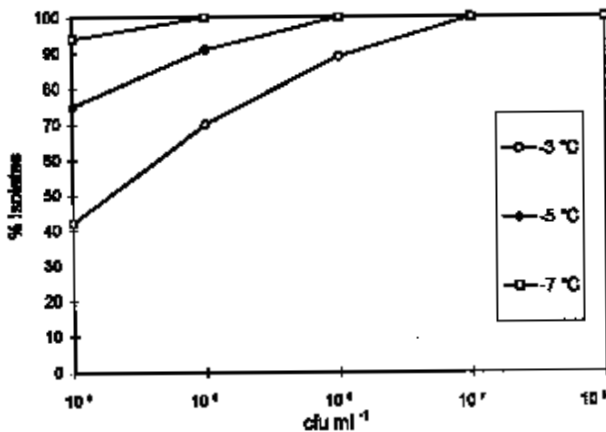


Fig.5. Cumulative percentages of *Pseudomonas viridiflava* isolates, obtained in October, active in ice nucleation at different temperatures as a function of different bacterial concentrations.

During hot and cold periods the epiphytic survival of *P. viridiflava* is reduced and few bacteria remain, apparently sheltered in particular niches on the host surface.

Probably the high bacterial populations in autumn allow them to colonise host surfaces (Varvaro, 1994b) in such a way that at least some *P. viridiflava* are able to survive during the winter.

As Billing (1970) stated, *P. viridiflava*, is a weak parasite causing disease on plants already damaged for different biotic and abiotic reasons. However, considering its now known ice nucleation activity, the presence of this pathogen on the kiwifruit phyllosphere should not be underestimated. In fact it is known (Lindow *et al.*, 1978; Varvaro and Fabi, 1992; Varvaro, 1994a), that

risks of damage caused by *P. viridiflava* increase when temperatures fall to -3 or -7°C.

Sudden decreases of temperature below 0°C in spring can also cause micro-lesions on aerial parts of kiwifruit plants so that *P. viridiflava* can start infect with subsequent development of symptoms.

Moreover, the ice nucleation activity of *P. viridiflava* could also enhance frost damage at temperatures near 0°C, on outer surfaces when a water film is present, in the intercellular spaces and on the fruit, in which the bacterium spreads through the flesh from the stem end (Pyke *et al.*, 1985).

*P. viridiflava* isolates, especially those obtained in October, showed noticeable INA at -3°C. Because almost half of them were found active at low bacterial concentrations and considering their substantial presence on the kiwifruit phyllosphere, it is likely that they play an important role, through INA, both in frost injury (Varvaro and Fabi, 1992) and in the severity of bacterial blight experienced in the field.

The ability of this bacterium to survive on kiwifruit twigs is very important for the epidemiology of bacterial blight because the leaves are deciduous. The few bacteria surviving during winter can multiply on the phyllosphere when favourable conditions return; they then rebuild high populations, as also noted in *P. syringae* on olives (Varvaro and Ferrulli, 1983).

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