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Occurrence in Portugal of stem necrosis on argyranthemum plants caused by *Pseudomonas cichorii*

por

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RESUMO

Assinala-se pela primeira vez em Portugal uma severa doença bacteriana que ataca os caules de plantas de *Argyranthemum* sp., em cultura protegida. As plantas infectadas apresentavam extensas lesões nos caules, de coloração azulada escura a negra. Não foram observados quaisquer sintomas a nível das folhas. A partir dos tecidos infectados, foi invariavelmente isolada uma bactéria verde fluorescente que se revelou patogénica, quando inoculada em plantas sãs de *Argyranthemum* sp.. Com base nas características morfológicas, culturais, bioquímicas, fisiológicas e de patogenicidade, os isolamentos obtidos foram identificados como pertencentes à espécie *Pseudomonas cichorii*. Apresentam-se, ainda, os resultados referentes à inoculação experimental de *P. cichorii* em diversas espécies de plantas ornamentais.

SYNOPSIS

A serious bacterial disease characterized by stem necrosis of argyranthemum plants (*Argyranthemum* sp.) grown under greenhouse conditions is reported for the first time in Portugal. Infected plants showed an extensive dark-blue to black discolouration of stems not followed by foliar lesions. Isolations made from infected tissues consistently yielded a green fluorescent bacterium, which was demonstrated by subsequent inoculation in healthy plants to incite the disease. On the basis of their morphological, cultural, biochemical, physiological and pathological properties, the isolates were identified as *Pseudomonas cichorii*. Results of experimental inoculation of *P. cichorii* into different ornamental plants are also presented.

1. INTRODUCTION

In the winter 1993/94, a serious disease of argyranthemum plants (*Argyranthemum* Webb ex Sch. Bip), grown under polyethylene-covered greenhouse was observed. Symptoms of infected plants consisted of black elongated lesions, mainly located at the lower stem. Young lesions appeared as water-soaked and later as dark-blue to black. When cut, some plants showed brown-discoloration of pith and vessels. Severely infected plants wilted and died, while others, although developing brown streaks along the stem, did not showed to be greatly affected, concerning growth and flowering. In both cases, however, no foliar symptoms were observed.

Although grown under identical greenhouse conditions, some cultivars of *Argyranthemum* sp. plants showed to be more susceptible to disease than others. The environmental conditions that preceded infection were long periods of high relative humidity, high soil moisture, low irradiance and mild temperatures. When greenhouse ventilation was increased and soil moisture reduced, disease spreading almost ceased.

A close similarity between the symptomatology here described and that referred by Jones *et al.* (1983) for a severe stem necrosis of florist's chrysanthemum (*Dendrathema grandiflora* Tzvelev., formerly *Chrysanthemum* × *morifolium Ramat.*) was found. In both cases, no foliar lesions were observed. The pathogen of that unusual disease was identified by Jones *et al.* (1983) as *Pseu-*

domonas cichorii (Swingle) Stapp.

The main objective of the present study is to identify the causal agent of the severe stem necrosis showed by *Argyranthemum* sp. plants. Also, results of inoculation studies with the pathogen on other ornamental plants are reported.

2. MATERIALS AND METHODS

2.1 ISOLATION

Diseased stems of different cultivars of *Argyranthemum* sp. plants were washed in tap water, rinsed in sterile distilled water (SDW) and cut into small slices at the transition area of brown discoloured and green tissues, including both the outer and the inner infected tissues. For bacterial isolation, tissues were macerated in SDW, streaked on King's medium B (KMB) (King *et al.*, 1954) and incubated at 26 °C. After 48 hours growth, green-fluorescent bacteria were selected from isolation plates and streaked again on KMB to obtain pure colonies.

For fungal isolation, fragments of similar infected tissues were surface-sterilized with 0.5% NaOCl for 3 minutes, rinsed in SDW and plated on potato-dextrose agar (PDA) amended with 0.5% KCSN. After 4-5 days of incubation at 24 °C, plates were checked for fungal growth.

2.2. CHARACTERIZATION OF ISOLATES

Characteristics of bacterial colonies were determined after 48 hours growth, at 26 °C, on nutrient agar (NA). Fluorescent pigment production was recorded on KMB and Gram reaction was made according to Schaad (1988). Standard bacteriological methods were used to study: oxidase reaction, presence of catalase, gelatin hydrolysis, anaerobic breakdown of L(+)arginine, levan production, potato soft rot and hypersensitivity reaction on *Nicotiana tabacum* (Lelliott *et al.*, 1966). Oxygen requirement was studied according to Hugh & Leifson (1953) method. Utilization

of mannitol, erythritol, sorbitol, trehalose, sucrose, L(+)tartrate was determined on minimal medium (MM) of Ayers *et al.* (1917) and repeated on Misaghi and Grogan's mineral medium (Misaghi & Grogan, 1969), using replica plating. Carbon sources were filter-sterilized (0.22 μm Millipore) and included in MM at the final concentrations recommended by Lelliott & Stead (1987) and also in Misaghi and Grogan's medium as described in the original receipt (Misaghi & Grogan, 1969). For comparative purposes, an authentic culture of *Pseudomonas cichorii*, PD 1701 (Culture Collection of Plant Protection Service, Wageningen, Netherlands) was included in this study.

2.3 PATHOGENICITY TESTS

Argyranthemum sp. plants, cultivar Snow Storm, were produced from healthy rooted cuttings growing on plastic pots containing steam-sterilized soil mixture, and maintained in a glasshouse under natural light and temperature of 24 ± 2 °C. Plants were inoculated at the beginning of the flowering stage. Cultures of 24-hours-old, grown on NA at 26 °C, were used to inoculate stabbing stems with a needle charged with each of six isolates. In another inoculation experiment, five superficial lesions were made in stems of each test plant, with a scalpel, and a drop of the bacterial suspension with approximately 10^8 colony-forming units per milliliter (cfu/ml) was placed on each lesion with an artist's brush. In both experiments, four plants were inoculated with each of six isolates, or treated with SDW as control plants. After inoculation, plants were individually covered with polyethylene bags to maintain high humidity conditions and transferred to a glasshouse under natural light and temperature of 24 ± 2 °C. Isolates obtained from artificially infected plants were also characterized according to their morphological, cultural, biochemical and physiological properties.

2.4. HOST-RANGE TEST INOCULATIONS

The following ornamental plants were tested: *Chrysanthemum coronarium, Dendrathema grandiflora* (formerly *C. morifolium*), *Gerbera jamesonii, Leucanthemum maximum* (formerly

C. maximum), *Lyrium* sp., *Pelargonium* × *hortorum*, *Spathiphyllum wallisii*. Ornamental plants were produced from healthy rooted cuttings or seeds. All seeds were surface sterilized in 1% NaOCl for 30 minutes, rinsed three times in SDW and pre-germinated in Petri plates containing moistened cotton and filter paper. The remaining procedure was similar to that already described for *Argyranthemum* plants.

Two isolates, one obtained from *Argyranthemum* plants (PC 2) and the reference strain PD 1701, were grown at 26 °C on KMB for 24 hours. The bacterial growths were suspended in SDW and adjusted to about 10^8 cfu/ml. Plants were wounded with a sterilized needle (three punctures in each of three leaves and stems, per plant), inoculated by spraying until runoff and wrapped individually in polyethylene bags for 72 hours. Three plants were inoculated with each bacterial isolate or treated with SDW as control plants. As the inoculation method (spraying) was different from those used in pathogenicity tests, *Argyranthemum* plants were also included in this study. Inoculated and control plants were maintained at day/night temperatures of approximately $24 \pm 2/20 \pm 2$ °C and natural light. Symptoms were recorded 72 hours after inoculation.

A completely randomized experimental design was used and the results were subjected to analysis of variance.

3. RESULTS

3.1 ISOLATION AND CHARACTERIZATION OF ISOLATES

Several cultivars of *Argyranthemum* sp. plants, grown under similar environmental conditions showed different degrees of susceptibility to stem necrosis. Estimates made during a survey suggest that Snow Storm, Vancouver, Peach Cheers and Snow Flake cultivars are more susceptible to disease than Jamaica Cooler, Compact White, Prado or Jamaica Primrost (Table 1 and Figure 1 A).

A green fluorescent bacterium was consistently isolated from the outer and the inner stem lesions of naturally infected plants on

KMB. Otherwise, no fungi were isolated on PDA amended with KCSN.

TABLE 1

Survey of disease incidence and plant mortality caused by Pseudomonas cichorii on some cultivars of Argyranthemum sp. plants grown under greenhouse conditions

Argyranthemum sp. cultivars	Disease incidence (%)	Plant mortality (%)
Compact White	20	10
Jamaica Cooler	40	10
Jamaica Primrost	100	10
Peach Cheers	100	80
Prado	80	5
Snow Flake	100	50
Snow Storm	100	50
Vancouver	100	90

Cells of the bacterium were gram-negative rods, motile by several polar flagella. After 48 hours growth at 26 °C on NA, colonies were circular (1.2-1.5 mm in diameter), white-to-cream colour, slightly raised, with irregular margins.

All isolates showed to be obligate aerobes. They were oxidase and catalase positive, produced an alkaline reaction on litmus milk and hypersensitivity in tobacco leaves. No isolate hydrolysed gelatin and L(+)arginine or produced levan from sucrose. Growth occurred on MM and Misaghi and Grogan's mineral medium supplemented with mannitol, and L(+)tartrate; while erythritol, sorbitol, trehalose and sucrose were not used as carbon source (Table 2).

3.2. PATHOGENICITY STUDIES

For both methods of inoculation, *Argyranthemum* potted plants (cv. Snow Storm) developed water-soaked dark lesions on

FIGURE 1

Stem necrosis caused by Pseudomonas cichorii on: (A) naturally infected argyranthemum plants, cultivar Vancouver; (B) argyranthemum plant of cultivar Snow Storm, 72 hours after inoculation; (C) stem of argyranthemum plant, cultivar Snow Storm, showing bacterial exsudate; (D) artificially infected stem and petioles of Pelargonium × hortorum

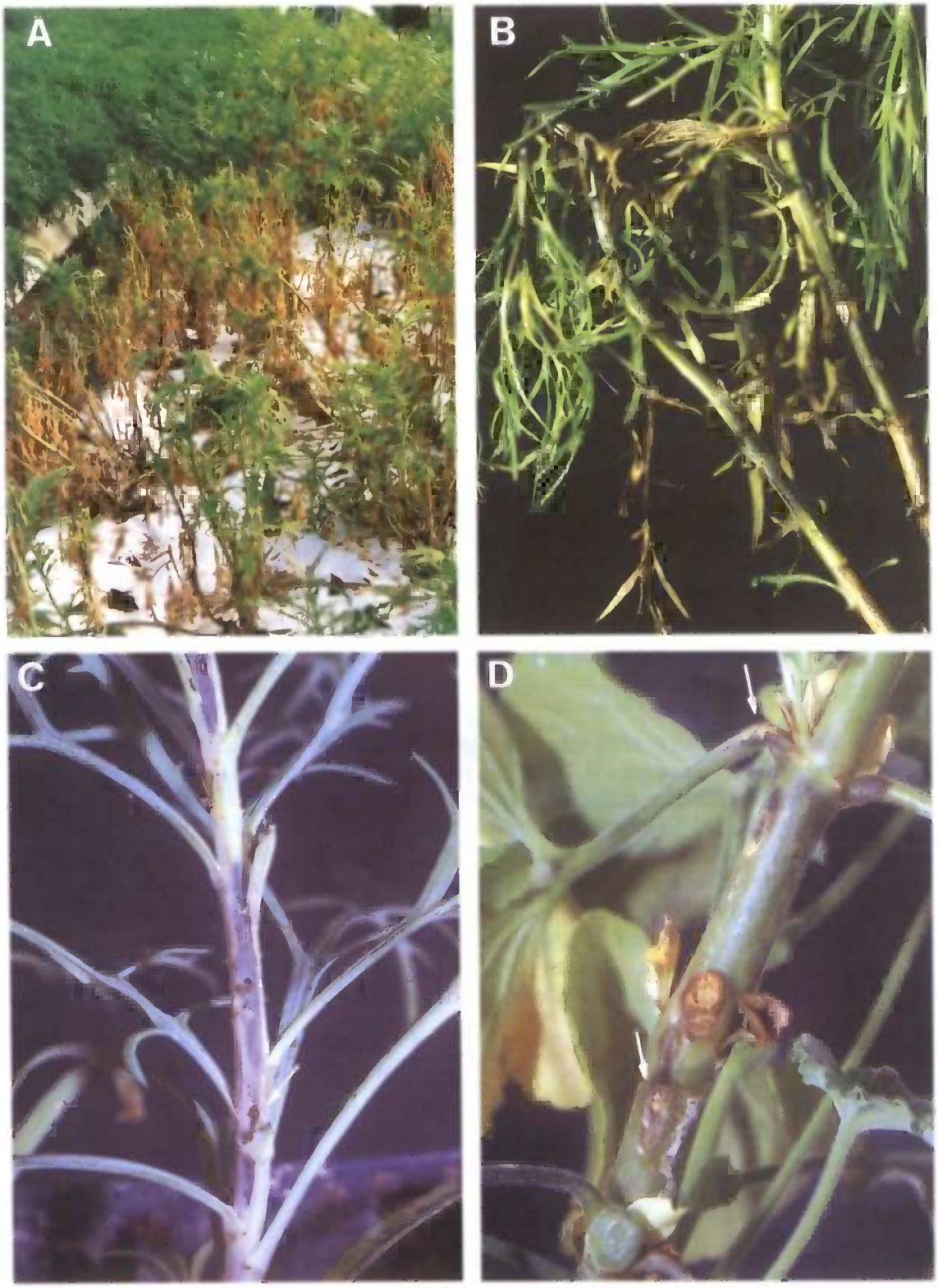

TABLE 2

Biochemical and physiological properties of six isolates obtained from Argyranthemum sp. plants and those of an autentic Pseudomonas cichorii

Tests	Isolates from *Argyranthemum* sp. plants	*P. cichorii* (PD 1701)
Fluorescent pigment	+	+
Levan production	-	-
Oxidase	+	+
Arginine dihydrolase	-	-
Potato soft-rot	-	-
Litmus milk	Alk.	Alk.
Gelatin hydrolysis	-	-
Utilization of:		
Mannitol	+	+
Erythritol	-	-
Sorbitol	-	-
Trehalose	-	-
Sucrose	-	-
L(+)tartrate	+	+
Tobacco hipersensitivity	+	+

+ = positive reaction; - = negative reaction; Alk. = alkaline reaction.
PD - Culture Collection of Plant Protection Service, Wageningen, Netherlands.

stems, 24 hours after inoculation. However, the size of lesions was greater (average 0.7 cm) in plants inoculated with the needle charged with the culture than with the drop of bacterial suspension (0.2 cm). Forty-eight hours after inoculation, lesions had in average 2.2 cm and 1.3 cm length, respectively, and 72 hours after inoculation some of them had already coalesced forming extensive black elongated streaks along the stems (Figure 1 B and 1 C).

Under high humidity conditions (100% RH), achieved in polyethylene bags, stem lesions continued to expand but ceased to develop in a low humidity environment (60-70% RH), when bags

were removed. In addition, under high humidity conditions lesions girdled the stems that finished to collapse. No symptoms on leaves were observed. Control plants remained symptomless. *P. cichorii* could only be reisolated from infected stems of inoculated plants.

3.3. HOST-RANGE TEST INOCULATIONS

All inoculated plant species showed to be susceptible to *P. cichorii* (Table 3). However, different levels of susceptibility were observed among hosts and host tissues. Stems of *Argyranthemum* sp., *C. coronarium* and *P.* × *hortorum* were affected while those of *D. grandiflora* remained healthy (Table 4).

On the outer tissues of stems, lesions were elongated with a water-soaked appearance and later they became brown to black coloured. When cut, they showed brown discoloration of pith and vessels, extending a few centimetres up and down the puncture level.

TABLE 3

Symptoms caused by Pseudomonas cichorii isolated from Argyranthemum sp. on stems and leaves of some ornamental plants

Hosts	Stem tissues		Leaves	
	Outer	Inner	Petiole	Blade
Argyranthemum sp.	+	+	-	-
Chrysanthemum coronarium	+	+	+	-
Dendrathema grandiflora	-	-	-	+
Gerbera jamenosii	nd	nd	+	+
Leucanthemum maximum	nd	nd	+	+
Lyrium sp.	nd	nd	-	+
Pelargonium × *hortorum*	+	+	+	+
Spatiphyllum sp.	nd	nd	-	+

+ = positive reaction; - = negative reaction; nd = not determined (plants without a well developed stem).

TABLE 4

Pathogenicity of Pseudomonas cichorii isolates on stems of some ornamental plants, 72 hours after inoculation

Tested plant	Stem lesions length (mm)	
	PC 2	PD 1701
Argyranthemum sp.	10.0*	3.6
Chrysanthemum coronarium	32.9*	2.3
Dendrathema grandiflora	0.0	0.0
Pelargonium × *hortorum*	13.8	8.1

Values are the mean of three lesions of each of three replicates.
PC 2 = Portuguese isolate; PD 1701 = reference strain.
* = for each host, strains significantly different at $P \leq 0.5\%$.

In some hosts, as *C. coronarium* and *P.* × *hortorum*, lesions progressed from stems to the petioles causing leaf abscission (Figure 1 D). Severely affected plants finished to die 7 days after inoculation. Excluding on *D. grandiflora*, PC2 isolate caused an extensive stem infection in all plants having a well-developed stem (Table 4). Stem infections on *Argyranthemum* sp. and *C. coronarium* were significantly ($P \leq 0.5$) more severe in plants inoculated with PC 2 isolate than with the reference strain (PD 1701).

Early symptoms on leaves were characterized by individual water-soaked black spot development mainly from wounded sites. However, leaves of *Argyranthemum* sp. and *C. coronarium* were not affected by *P. cichorii* and those of *S. wallisii* only developed minute lesions around the puncture sites (Table 5).

On leaves of *P.* × *hortorum*, spots had small raised tan centers, that appeared as "eyes", surrounded by a dark margin and a chlorotic halo, 72 hours after inoculation. Later, and in the most severe cases of infection, spots enlarged along the veins and coalesced into irregular lesions, covering large leaf sections. Lesions also developed at unwounded sites.

Concerning leaf infection significant differences ($P \leq 0.5\%$) between isolates were observed for *D. grandiflora, Lyrium* sp. and *P.* × *hortorum.*

P. cichorii was also reisolated from all diseased tissues of inoculated plants.

TABLE 5

Pathogenicity of Pseudomonas cichorii isolates on leaves of some ornamental plants, 72 hours after inoculation

Tested plant	Leaf lesion diameter (mm)	
	PC 2	PD 1701
Argyranthemum sp.	0.0	0.0
Chrysanthemum coronarium	0.0	0.0
Dendrathema grandiflora	3.6*	1.3
Leucanthemum maximum	2.7	2.2
Lyrium sp.	3.4	9.8*
Gerbera jamesonii	7.4	6.1
Spathiphyllum wallisii	1.0	1.2
Pelargonium × *hortorum*	2.6	5.4*

Values are the mean of nine counts for each of three replicates.
PC 2 = Portuguese isolate; PD 1701 = reference strain.
* = for each host, strains significantly different at $P \leq 0.5\%$.

4. DISCUSSION

Results of morphological, cultural, biochemical and physiological characteristics of the causal agent of stem necrosis of *Argyranthemum* sp. plants are in agreement with those reported for *Pseudomonas cichorii* (Bradbury, 1981) and with those obtained for the reference strain (PD 1701).

The six isolates obtained from *Argyranthemum* sp. infected plants induced an extensive stem necrosis when inoculated on healthy plants, reproducing completely the symptoms originally observed.

P. cichorii was first described from chicory (Swingle, 1925) and hereafter reported on several other hosts, including many ornamental and vegetable crops (Bradbury, 1986). Recently, the pathogen was detected in Portugal, from lettuce plants presenting varnish-spot symptoms but, until now, no other hosts were

referred to be infected by the pathogen in the country (Ferreira-Pinto & Oliveira, 1993). On chrysanthemum plants and on other hosts the pathogen has been more frequently associated with leaf spot and bud blight symptoms rather than with stem necrosis (McFadden, 1961; Janse, 1987). Lesions on stems, when occur, have been considered of minor importance. Nevertheless, Jones *et al.* (1983) reported a severe outbreak on *C. morifolium* caused by *P. cichorii*, whose symptoms were restricted to stems. Symptoms described by these authors are closely in agreement with those observed in the present work on naturally and artificially infected *Argyranthemum* plants. However, PC 2 and PD 1701 isolates were unable to infect stems of artificially inoculated *D. grandiflora* (= *C. morifolium*), although had incited typical leaf spot lesions on this host. On *Pelargonium*, the isolates induced leaf lesions which are in agreement with the descriptions made by others (Engelhard *et al.*, 1983; Jones *et al.*, 1984). Also, they affected the outer and the inner tissues of stems of this host plant.

In our conditions, disease epidemic was not developed under temperatures raising the optimum referred to this pathogen (28 °C), but high relative humidity, high soil moisture, low irradiance and close planting might have been the most decisive factors for disease development. Within certain limits (20-28 °C), temperature appeared to be less critical than moisture on disease development (McFadden, 1961; Jones *et al.*; 1984; Janse, 1987). Under identical temperature conditions (24 ± 2 °C), stem lesions of inoculated *Argyranthemum* plants ceased to develop at 60-70% of relative humidity, while plants maintained at 100% finished to die, 7 days after inoculation.

Like Jones *et al.* (1983), we did not find a good explanation to justify why a severe stem necrosis of *Argyranthemum* plants was not followed by other typical symptoms, which includes leaf spot and bud blight. Isolates of *P. cichorii* obtained from *Argyranthemum* plants showed to be not host specific. They were able to induce stem necrosis and/or typical leaf spot symptoms in several ornamental plants.

As there are evidences that *P. cichorii* survive in host debris, in soil-buried infected leaves and on roots of nonhost and host weeds (Ohata *et al.*, 1982; Bazzi *et al.*, 1984), the pathogen may be a threat for subsequent susceptible crops.

REFERENCES

AYERS, S.H.; RUPP, P.; JOHNSON, W.T. (1917) — A study of the alkali-forming bacteria found in milk. *United States Department of Agriculture Bulletin*, 782: 1-39.

BAZZI, C.; PIAZZA, C.; MAZZUCCHI, U. (1984) — Survival in the field of *Pseudomonas cichorii* (Swingle) Stapp, causal agent of lettuce varnish spot. *Phytopathologische Zeitschrift*, 111: 251--258.

BRADBURY, J.F. (1981) — *Pseudomonas cichorii*. CMI Descriptions of Pathogenic Fungi and Bacteria no. 695, Commonwealth Mycological Institute, Kew, 2 pp.

BRADBURY, J.F. (1986) — *Guide to Plant Pathogenic Bacteria*. Commonwealth Mycological Institute, Kew, 332 pp.

ENGELHARD, A.W.; MELLINGER, H.C.; PLOETZ, R.C.; MILLER, J.W. (1983) — A leaf spot of florists'geranium incited by *Pseudomonas cichorii*. *Plant Disease*, 67: 541-544.

FERREIRA-PINTO, M.M.; OLIVEIRA, H. (1993) — A new bacterial disease of lettuce in Portugal caused by *Pseudomonas cichorii* (Swingle) Stapp. *Phytopathologia mediterranea*, 32: 249-253.

HUGH, R.; LEIFSON, E. (1953) — The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *Journal of Bacteriology*, 66: 24--26.

JANSE, J.D. (1987) — Biology of *Pseudomonas cichorii* in chrysanthemum. *EPPO Bulletin*, 17: 321-333.

JONES, J.B.; ENGELHARD, A.W.; RAJU, B.C. (1983) — Outbreak of a stem necrosis on chrysanthemum incited by *Pseudomonas cichorii* in Florida. *Plant Disease*, 67: 431-433.

JONES, J.B.; RAJU, B.C.; ENGELHARD, A.W. (1984) — Effects of temperature and leaf wetness on development of bacterial spot of geraniums and chrysanthemums incited by *Pseudomonas cichorii*. *Plant Disease*, 68: 248-251.

KING, E.O.; WARD, M.K.; RANEY, D.E. (1954) — Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine*, 44: 301-307.

LELLIOTT, R.A.; BILLING, E.; HAYWARD, A.C. (1966) — A determinative scheme for the fluorescent plant pathogenic pseudomon-

ads. *Journal of Applied Bacteriology*, 29: 470-489.

LELLIOTT, R.A.; STEAD, D.E. (1987) — *Methods for the Diagnosis of Bacterial Diseases of Plants*. Blackwell Scientific Publications, Oxford, 216 pp.

MCFADDEN, L.A. (1961) — A bacterial leaf spot of florists' chrysanthemums, *Chrysanthemum morifolium*. *Plant Disease Reporter*, 45: 16-19.

MISAGHI, I.R.; GROGAN, G. (1969) — Nutritional and biochemical comparisons of plant-pathogenic and saprophytic fluorescent pseudomonads. *Phytopathology*, 5: 1436-1449.

OHATA, K.; SERZAWA, S.; SHIRATA, A. (1982) — Infection source of the bacterial rot of lettuce caused by *Pseudomonas cichorii*. *Bulletin of the National Institute of Agricultural Sciences., Series C*, 36: 75-80 (*In Review of Plant Pathology*, 61: 6125).

SCHAAD, N.W., ed. (1988) — *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. 2nd ed., American Phytopathological Society, St. Paul, 164 pp.

SWINGLE, D.B. (1925) — Center rot of "French endive" or wilt of chicory (*Cichorium intybus* L.). *Phytopathology*, 15: 730.