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Pseudomonas for biological control of Dutch elm disease. II. Further studies on the localization, persistence and ecology of Pseudomonas isolates injected into elms

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Abstract

For biological control of Dutch elm disease by *Pseudomonas* spp. a population of these bacteria has to be present within the tree. Gaining insight in the distribution of such bacteria, both spatially within the tree and over time, was one goal of this study, determining limiting factors of the habitat and selection bacteria better adapted to it, the other.

Immunofluorescence microscopy showed bacteria only along the walls of the xylem vessels. Apparently the xylem is the only habitat of the introduced bacteria. The xylem lumen is low in nutrients, the pH proved to be above the minimum value critical for growth of *Pseudomonas* spp., but the water potential may reach dangerously low values for bacteria. An isolate better adapted to a low water potential proved to establish itself better within elm than the parent strain. Spatial distribution studies suggested that the bacteria did not escape from the vessels in which they were introduced, but bacteria were consistently isolated from the new annual ring in the second growing season. Probably the wound tissue or the root system served as an inoculum source.

Additional keywords: Ophiostoma ulmi, immunofluorescence microscopy, xylem fluid pH, water potential.

Introduction

Biological control of Dutch elm disease, recently reviewed by Scheffer and Strobel (1988), may be feasible. It was shown that *Pseudomonas* spp. could be effective as a prophylactic treatment against *O. ulmi* (Scheffer, 1983a; Murdoch et al., 1986). Questions remained about the spatial distribution and longevity of the bacterial population within the tree, and about the duration of the protective effect.

For *O. ulmi* the xylem is the exclusive habitat, as long as the defense mechanisms of the elm are functioning (Pomerleau, 1970). If, after injection into a tree, *Pseudomonas* spp. would spread over a considerable distance within that tree, it would most probably be by the xylem vessels, which in the trunk of a mature elm have a length of several meters (Newbanks et al., 1983). Assuming that *Pseudomonas* spp. would become established in the xylem after a bacterial treatment of the tree, the bacteria would share this environment with *O. ulmi*, and a direct interaction between bacteria and pathogen would be possible. Antagonism by an antimycotic produced by *Pseudomonas* spp. was

suggested by Myers and Strobel (1983) and tested in vivo by Lam et al. (1987) using non-antibiotic producing mutants as controls. For *P. fluorescens* WCS374 it was shown in part one of this study (Scheffer et al., 1989) that a stable local population could develop within elms close to the inoculation site.

For *Pseudomonas* spp. the xylem is not a very 'friendly' environment. Whether the oxygen requirements of the strictly aerobic bacteria are met with is a first questionable point, as the oxygen pressure in the xylem fluid is estimated as approximately 10 % of saturation (Dimond, 1962). Furthermore, it is an environment low in nutrients, especially from mid to late summer: Kessler (1966) found only traces of sugar, although Singh and Smalley (1969) found 325 to 650 mg l^{-1}. Ammonia and many amino acids, amides and organic acids were reported from elm xylem sap in low concentrations, in the order of 1-2 mM for all nitrogen-containing compounds combined (Elgersma, 1969; Singh and Smalley, 1969). Xylem sap is usually considered to be slightly acidic; for elm a pH of 5.6 was reported (Elgersma, pers. comm.), but no more comprehensive data were available. Another factor of major importance for survival of *Pseudomonas* spp. could be the water potential inside the xylem vessels which may reach as low as minus 2 MPa (−20 bar) on a hot summer day. Such water potentials may even result in temporary breakage of water columns in the wider vessels, disrupting the flow of water and nutrients (Boyer, 1985; Zimmermann, 1983).

In part one of this study emphasis was on labeling and identification methods for the *Pseudomonas* isolates used as biological control agents. It was found that markers, the transposon Tn903 or a plasmid-based metabolic marker, were not as useful for in vivo studies as expected. Upon inoculation with the wild type *P. fluorescens* WCS374, a rather stable bacterial population was detected in elms during the season. However, derivatives of the same *P. fluorescens* labeled with a transposon or the plasmid-based metabolic marker disappeared altogether from mature elms within three months. A comparison of several identification methods showed that immuno-agglutination was a reliable routine test to identify *P. fluorescens* WCS374 (Scheffer et al., 1989).

The purposes of this part two of the study were to more clearly establish the habitat of the *Pseudomonas* spp. introduced into elm, to gather data on bacterial populations in elm, both spatially within the tree and over time, and if possible to select a bacterium better adapted to this habitat. In part three then data on the actual disease-controlling effects of *Pseudomonas* injections will be presented.

Materials and methods

Micro-organisms. All micro-organisms used in this study are summarized in Table 1. *Pseudomonas* cultures were maintained on 'King B' (medium B of King et al., 1954) agar slants. Cultures used for the inoculation of trees were grown in modified King B as described in part one of this study (Scheffer et al., 1989).

O. ulmi cultures were maintained on potato dextrose agar slants. Conidial suspensions needed for inoculation of elms were prepared from shake cultures as described (Scheffer, 1983a; Scheffer et al., 1989).

Adaptation of P. fluorescens to low water potentials. For adaptation studies, bacteria were grown in modified King B or nutrient broth amended with NaCl. Water poten-

Table 1. Micro-organisms used in this study.

Isolate code	Relevant description	Reference/source
Ophiostoma ulmi		
H6	aggressive (NAN)	Gibbs et al., 1975
H106	aggressive (NAN)	obtained from Dr C.M. Brasier, UK
Pseudomonas fluorescens		
WCS361	wild type from potato soil	Geels & Schippers, 1983a, b
WCS374	wild type from potato soil	Geels & Schippers, 1983a, b
WCS374 RJS101	adapted to low water potential	this study
P. putida		
WCS085	wild type; from potato soil	Geels & Schippers, 1983a, b
WCS358	wild type; from potato soil	Geels & Schippers, 1983a, b
P. syringae		
M27+	wild type; from barley	Myers & Strobel, 1983

tials of media were determined using a Hewlett-Packard 301A Vapor Pressure Osmometer. The conversions, based on those given by Wiebe et al. (1971) for NaCl and which are nonlinear, are given in Fig. 1.

Bacterial treatments of elms. The gouge pistols (Scheffer, 1983a) were used for treating trees with bacteria for all experiments except those where twigs were inoculated for detection of bacteria by immunofluorescence microscopy. Trees were injected at a height of 1.2 - 1.4 m every 5 cm of circumference with 1 ml of shake culture.

Twigs of young elms of the clones *U.* × *hollandica* 'Belgica' and 390 at the nursery of the institute at Baarn were inoculated near their base with a 3 mm-wide chisel. Three times 20 μl of a shake culture of WCS374 or WCS374 RJS122 was applied to each twig. Controls received modified King B.

Inoculation with O. ulmi. Inoculations with *O. ulmi* were performed with a 1 : 1 mixture of conidial suspensions of the aggressive isolates H6 and H106; 5×10^6 conidia ml^{-1}. Stems were inoculated with two times 50 μl at opposite sites (Scheffer, 1983a); twigs similarly at their base using a 3 mm wide chisel.

Qualitative reisolation. For qualitative reisolation of *Pseudomonas* spp. from elms King B agar was amended with 100 mg l^{-1} cycloheximide, 50 mg l^{-1} ampicillin and 12.5 mg l^{-1} chloramphenicol. For trunk samples a 'window' was cut out of the bark with a chisel. Subsequently several wood chips were cut out and placed in a petri dish containing the medium. After 3-5 days at 23 °C bacterial colonies were transferred to agar slants for further determination. Branch or twig samples were surface-sterilized with ethanol, the bark was peeled back and wood chips were cut out and treated as above.

Quantitative reisolation. Quantitative reisolation of *P. fluorescens* was performed by homogenizing pieces of wood taken from root or stem by saw and chisel. Big wedges were taken from the tree. After surface sterilization with ethanol the bark was removed, smaller pieces of wood comprising the current and last year's growth ring were collected and ca. 1 g was clipped into chips. The chips were homogenized with an Ultra-Turrax homogenizer for 30 s in 10 ml of a 0.1 % proteose peptone solution (Straka and Stokes, 1957). The homogenate was aseptically filtered through nylon gauze and plated in a dilution series on modified King B plates for assessing the *Pseudomonas* density.

Xylem fluid pH. For determination of the pH of xylem fluid, the xylem fluid of twigs, inoculated at their base, was collected as before with a French Press (Scheffer, 1981).

Immunofluorescence. Fluorescein iso-thiocyanate conjugated goat anti-rabbit serum (Nordic, Tilburg, the Netherlands) was used for the indirect staining technique (Elgersma and Steerenberg, 1978).

Bacterial smears on microscope slides were air-dried and fixed by heating the slide for an instant in a flame. The bacterial smears were then incubated in PBS with 2 % BSA (bovine serum albumin; Poviet, Oss, the Netherlands) for 5 min, with the same rabbit anti-WCS374 serum as mentioned above (1 : 400) for 30 min, rinsed twice for 5 min with PBS + 2 % BSA, incubated with the conjugate (1 : 70) for 30 min, rinsed again twice for 5 min with PBS + 2 % BSA and mounted in p-phenylenediamine/buffered glycerol (Johnson and de C. Nogueira Araujo, 1981). Slides were examined under incident blue light (490 nm) with a Leitz Orthoplan microscope at 275-1190 times magnification.

Wood samples were used fresh or were fixed in 5 % glutaraldehyde in 50 mM cacodylate buffer, pH 7.2. Sections (transverse, radial and tangential) of 15 - 40 μm were made in 70 % ethanol and stained and observed as described above.

Results

The water potential (Ψ) of a system is composed of the sum of the solute potential (Ψ_s), the matric potential (Ψ_m), the pressure potential (Ψ_p), the gravitational potential (Ψ_g) and in some cases the overburden potential (Ψ_Ω; Duniway, 1976; Nobel, 1974; Parr et al., 1981). Of these five components,only the solute and the matric potential, the sum of which is often referred to as the osmotic potential ($\Psi_\pi = \Psi_s + \Psi_m$), are relevant in shake culture. Growth and initial lag phase of two *Pseudomonas* isolates in media of decreasing osmotic potential are shown in Fig. 1. Similar data as presented for *P. fluorescens* WCS374 were obtained for *P. putida* WCS358 and *P. fluorescens* WCS361 (not shown). *P. fluorescens* WCS374 could not survive after logarithmic growth at a water potential under −4.0 MPa, *P. syringae* M27+ not at a water potential under −3.0 MPa. Cultures that were kept in logarithmic growth under low water potential by frequently transferring cells to fresh modified King B + NaCl (up to 1 M NaCl; total −5.7 MPa) yielded substrains that grew 30 % faster and did not show the considerable lag phase. One of these substrains, WCS374 RJS101, was used in several field studies. DNA restriction fragment pattern, cell envelope protein pattern and lipopolysaccharide pattern of this substrain were identical to that of the parent strain WCS374,

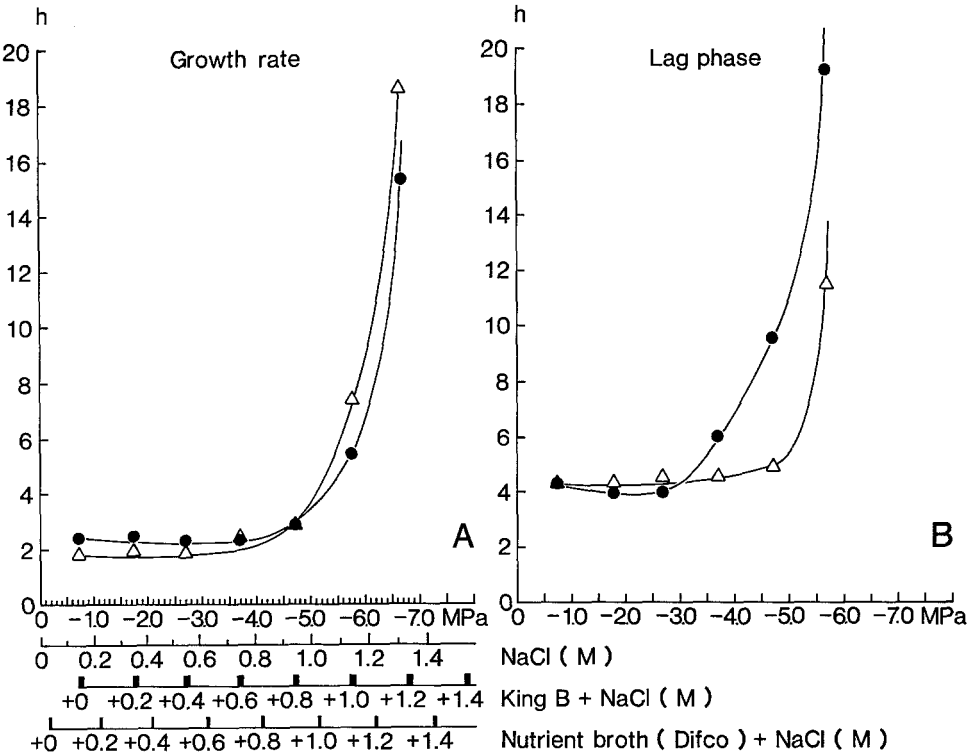

Fig. 1. A. Growth rate of *P. fluorescens* WCS374 (●) and *P. syringae* M27+ (△) as hours in which the A_{600} doubled during logarithmic growth of shake cultures in modified King B with sodium chloride. Water potential for the various media was determined with a vapor pressure osmometer.

B. The initial lag phase of such cultures, defined as the period after inoculation during which the A_{600} did not increase.

and also in the immunological assays reactions comparable to WCS374 were observed (cf. Scheffer et al., 1989).

Growth rate of *Pseudomonas* spp. (M27+, WCS374, WCS358, WCS361) in modified King B or King B acidified with HCl was not affected by pH variations ranging from 7.5 to 5.5, but decreased steeply at a pH lower than 5.0. In a twig experiment comparable with those for quantitative reisolation of *Pseudomonas* spp. from twigs (Scheffer et al., 1989) the pH of xylem fluid was followed during one summer for six cultivars (Table 2). The lowest pH detected in any sample was 5.61.

Spatial distribution of *Pseudomonas* spp. in 15 year old 'Commelin' elms is presented in Fig. 2. *P. fluorescens* WCS374 RJS101 was isolated up till 8 m high in the season of inoculation. The root system apparently also became colonized. The wild type, WCS374, did not spread as far within the tree as compared with its derivative adapted to a low osmotic pressure. For 'Vegeta' elms comparable data (not shown) were obtained. Routine agglutination tests with anti-374 serum confirmed that 80 - 90 % of the fluorescent colonies developing on King B agar were WCS374 (cf. Scheffer et al., 1989). In the same plot the population densities of *P. fluorescens* WCS374 and WCS374 RJS101

Table 2. The pH of elm xylem fluid after various treatments. Data given are the average from three series of samples, every sample comprising 10 twigs, taken 5, 12 and 26 days after inoculation with *O. ulmi*. As no trends were observed in the data in time, only the gross average is given.

1st treatment	Water	WCS374 RSJ101	WCS374 RSJ101	– – –	Modified King B
2nd treatment	– – –	– – –	*O. ulmi*	*O. ulmi*	– – –
U. × *hollandica* 'Belgica'	6.06	6.13	5.95	6.06	6.22
U. × *hollandica* 'Commelin'	6.22	6.20	6.10	6.06	—
U. glabra	6.16	6.19	6.25	6.20	6.30
U. 'Lobel'	5.88	5.86	5.79	5.80	5.88
U. 'Sapporo Autumn Gold'	5.85	5.74	5.91	5.86	—
U. americana[1]	5.85	5.82	5.70	5.79	—

[1] Only one series of samples, taken 12 days after *O. ulmi*.

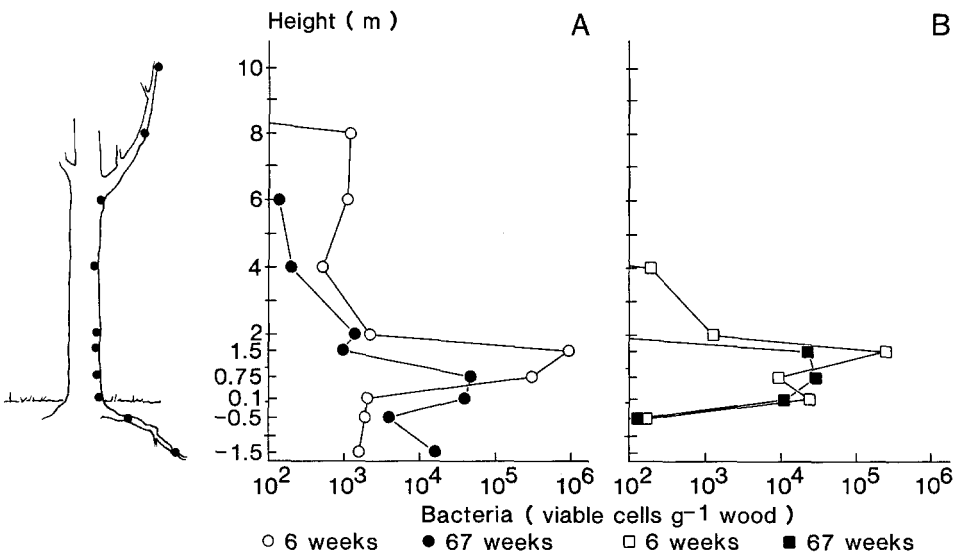

Fig. 2. Spatial distribution of *P. fluorescens* WCS374 RJS101 (A) and WCS374 (B) in mature 'Commelin' elms, inoculated at a height of 1.2 - 1.4 m, after six weeks and at the end of the second growing season, after 67 weeks. Data show *P. fluorescens* concentrations (viable cells g^{-1} wood) at various locations in the tree.

in elm were compared. The bacterial treatments were followed 10 days later by an inoculation with *O. ulmi*, or with sterile water for control groups. The bacterial population densities in stem samples taken 0.5 m above the site of injection after 13 weeks, by the end of the growing season, were consistently higher for WCS374 RJS101 than for WCS374 (Fig. 3).

The trees used for two experiments described earlier (Scheffer, 1983a,b) were 'Com-

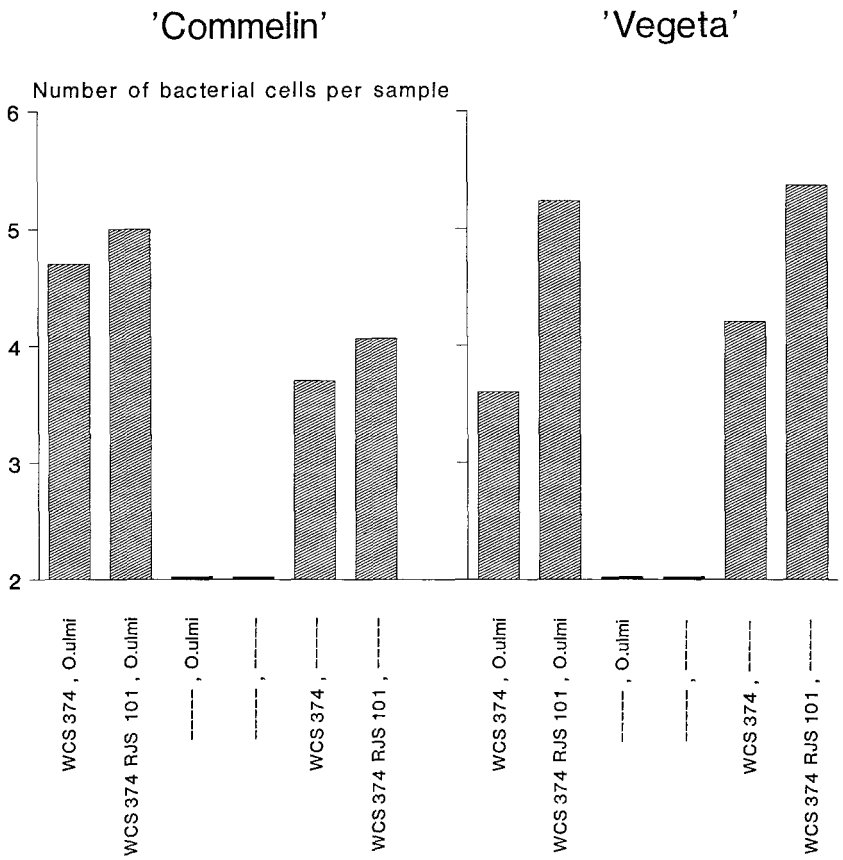

Fig. 3. Concentrations of *P. fluorescens* WCS374 and WCS374 RJS101 (viable cells g^{-1} wood) in 'Commelin' and 'Vegeta' elms 13 weeks after inoculation. Samples were taken 0.5 m above the site of inoculation, which was at a height of 1.2 - 1.4 m.

melin' and 'Groeneveld' elms of ca. six year old, with an average diameter of 6 cm (DBH) and a height of 6 m. *Pseudomonas* spp. (M27+, WCS374, WCS361 or WCS085) could be recovered qualitatively from every stem sample taken from a height of 1 m to 1.5 m in the year of inoculation and the year after. From twig samples, collected from high up in the crown (4-5 m high) by the end of the second growing season after inoculation, *Pseudomonas* spp. could be isolated from only 6 of 160 'Commelin' twigs and not at all from 170 'Groeneveld' twigs.

Similarly, from 40 older elms injected with the same four *Pseudomonas* isolates in the City of Rotterdam, branch samples taken of every tree in the year of inoculation and in the year after yielded only three *Pseudomonas* isolates. Using classical taxonomic tests (Doudoroff and Palleroni, 1974), these could not be identified as belonging to the same species as the isolate used for inoculation of the tree. From branches sampled from 18 elms in the City of Amsterdam inoculated with *Pseudomonas* spp. a year before no *Pseudomonas* isolates could be obtained.

Stem samples from trees at several locations inoculated with *Pseudomonas* spp. less

than two growing seasons before yielded *Pseudomonas* spp. from over 95 % of all samples. No clear differences between the four *Pseudomonas* isolates used in the experiments (cf. Scheffer, 1983 a,b) were apparent pertaining to reisolation from elm.

Separating annual rings from a tree segment close to the height of inoculation, in order to assess the *Pseudomonas* concentrations in the individual rings, was attempted. From a 'Groeneveld' elm inoculated in 1982 with WCS374 no *Pseudomonas* spp. could be isolated from the annual rings 1984/1985 by the end of 1985. *Pseudomonas* spp. isolated from older rings could not be identified as WCS374, using classical taxonomy (Doudoroff and Palleroni, 1974), immuno-agglutination with anti-WCS374 serum, total DNA restriction fragment pattern analysis (see Scheffer et al., 1989, Fig. 1), or immunofluorescence. From a 'Belgica' elm, inoculated with WCS374 in 1984, *Pseudomonas fluorescens*, probably WCS374 (classical taxonomy only; Doudoroff and Palleroni, 1974) could be isolated by the end of 1985 from the annual ring of '85 (concentration 4×10^4 g^{-1} wood), '84 (concentration 7×10^4 g^{-1} wood) and '83 (concentration 7×10^4 g^{-1} wood). Isolation from the annual ring of '82 was doubtful: few colonies were obtained. From several other trees *Pseudomonas* spp. could be consistently recovered from the growth ring before the one formed in the year of inoculation in that same year of inoculation and in the year after. In that year *Pseudomonas* spp. could be recovered from the new growth ring too (data not shown).

Immunofluorescence microscopy was used to detect *P. fluorescens* WCS374 and its derivatives in elm wood. Some representative images are shown in Fig. 4. Autofluorescence or non-specific binding of antisera did not interfere with detection of bacteria. Only in the lumen of ray cells often some FITC-fluorescence was observed, but this fluorescence was diffuse and no bacterial cells were observed. In contrast, bacteria were observed in large quantities along the walls of some xylem vessels. The only sections in which bacteria were detected outside xylem vessels were those from inoculation wounds. A bacterial population apparently persisted in and around the complex regenerating tissue.

Discussion

Thre parameters of the elm xylem, its pH, water potential and oxygen pressure, were considered to be potentially critical for *Pseudomonas* spp. Of these, the pH probably is not critical: for the *Pseudomonas* isolates tested, a pH of 5.6 as found experimentally for several elm species and clones is well above critical values for growth, which were around pH 5.0. Although the isolates tested could survive water potentials of at least −3 MPa, a derivative of *P. fluorescens* WCS374 adapted to a low osmotic potential seemed to have a better spatial distribution in elms than the wild type. For this derivative

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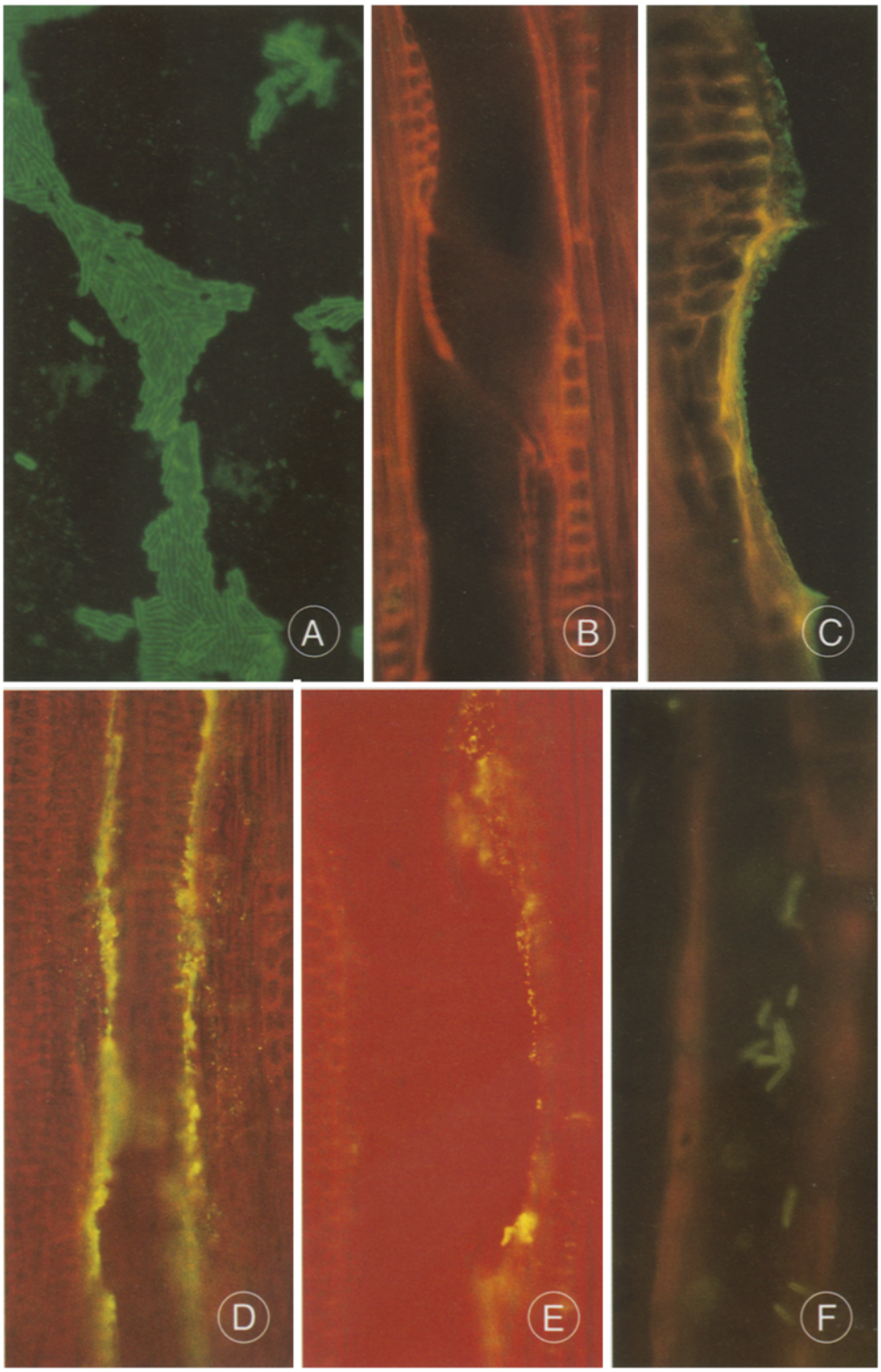
Fig. 4. Immunofluorescence microscopy of a smear of a pure culture of *P. fluorescens* WCS374 (A) and of elm wood sections (B-F). In control sections such as B) (not inoculated with WCS374) no fluorescent bacteria were observed; autofluorescence was present, but did not prevent positive identification of FITC-labeled bacteria. FITC- labeled bacteria were observed along xylem vessel walls: C) in 'Commelin' elm; D,E) in 'Belgica' (in D some bacteria have drifted from the xylem vessel on top of the section during the staining process)and F) in clone 390. Magnification: A and F 1080×; B-E 250×.


A
B
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F

also higher population densities in elm were recorded than for the wild type. To what extent oxygen pressure could be critical, data are not clear: in shake culture WCS374 was very sensitive to decreasing oxygen pressure, but *P. syringae* M27+ grew even anaerobically reasonably well. Some pseudomonads can use nitrate as an electron acceptor under anaerobic conditions, and a low concentration of nitrate was found in elm xylem sap (Elgersma, 1969). However, both WCS374 and M27+ did not show denitrification in tests according to Stanier et al. (1966). Probably M27+ could effectively use an alternative respiration pathway (Sweet and Peterson, 1978).

In part one of this study (Scheffer et al., 1989) it was found that *P. fluorescens* populations in elm twigs initially declined steeply upon inoculation of the twigs, but that they became rather stable within three months, up till the end of the second season when the experiments were terminated. Higher up in the twigs much lower bacterial population densities were recorded than close to the site of inoculation at the base of the twigs.

In an experiment comprising 47 different elm clones no, or hardly any bacteia were isolated from 88% of the samples of the new year's shoots. If any *Pseudomonas* spp. were isolated, the concentration was always lower than 1 % of that near the point of inoculation (Scheffer et al., 1989).

These data suggest that *P. fluorescens* probably cannot escape from the xylem vessels in which is was introduced.

For the spatial distribution within mature elms (Fig. 2) the same explanation is suggested. The differences between the wild type and the derivative adapted to a low water potential may be explained by a faster initial spread within the vascular system, which reacts to a certain extent to the bacterial invasion by forming barriers such as tyloses, and probably by a better distribution within the narrower xylem elements, which are certainly less prone to cavitation than the large earlywood vessels.

Fluorescence microscopy suggested that a *Pseudomonas* population only became established within the xylem vessels. This would fit well with the distribution patterns that can be explained by assuming that the xylem indeed is where *Pseudomonas* spp. can establish themselves. Large amounts of *Pseudomonas* cells were observed in the differentiating tissue around the inoculation wounds made by the gouge pistol. In the year after that in which the trees were inoculated these spots of bacteria-infested tissue may well act as sources from which the newly formed xylem vessels become colonized. If the root system becomes colonized by the bacteria, as in Fig. 2 for WCS374 RJS101, this could be such a source too, as the separation of annual rings is less pronounced underground than above. In that case, colonization of the new xylem may probably be expected for several years; if the differentiating wound tissue is the major source, than only one following year such a colonization may be expected, as the wound tissue usually gets covered by a entirely closed cambium layer during the season following bacterial treatment.

The prophylactic effect of one bacterial treatment therefore may last for two seasons, probably longer if the root system of the tree becomes colonized.

Acknowledgments

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Samenvatting

Biologische bestrijding van de iepeziekte met Pseudomonas. II. Nadere studie over de localisatie, persistentie en oecologie van de in de iep geïnjecteerde Pseudomonas isolaten

Voor biologische bestrijding van de iepeziekte door *Pseudomonas* spp. is een zekere populatiedichtheid van deze bacteriën binnen de boom vereist. Doelen van deze studie waren het verwerven van inzicht in de verspreiding van deze bacteriën door de boom en het verloop van de populatiedichtheid in de tijd, het bepalen van de beperkende faktoren van de habitat van de bacteriën en de selektie van isolaten die beter aan deze habitat zouden zijn aangepast.

Immunofluorescentie-microscopie toonde dat de in de iep geinjecteerde bacteriën uitsluitend langs de wanden van de houtvaten voorkwamen. Blijkbaar is het xyleem de enige habitat van de in de boom gebrachte bacteriën. Deze xyleem-inhoud is arm aan voedingsstoffen, de pH bleek voor de getoetste *Pseudomonas* isolaten steeds boven de voor de groei kritische waarden te blijven, maar de waterpotentiaal kan soms voor *Pseudomonas* spp. gevaarlijk lage waarden bereiken. Een bacterie-isolaat dat beter aangepast was aan een lage waterpotentiaal bleek het in de iep beter te doen dan het wildtype. De verspreiding van de bacteriën door de boom bleef beperkt. De bacteriën leken niet te ontsnappen uit de vaten waar ze ingebracht waren. Wel werden in het tweede jaar consistent bacteriën uit de nieuwe jaarring geïsoleerd; wondweefsel of het wortelstelsel leverden mogelijk het inoculum.

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