Neth. J. Pl. Path. 95 (1989) 305-318

# Pseudomonas for biological control of Dutch elm disease. III. Field trials at various locations in the Netherlands

R.J. SCHEFFER

Willie Commelin Scholten Phytopathological Laboratory, Javalaan 20, 3742 CP Baarn, the Netherlands

Accepted 1 May 1989

**Abstract**

The prophylactic effect in elm of one treatment with a *Pseudomonas* isolate was monitored in two types of field trials. In one type only natural Dutch elm disease infections were monitored and hence large numbers of trees were necessary due to the low incidence of natural occurring infections. In the other type trees were artificially infected.

The large-scale field trials in which only natural infections were monitored, were based on expected annual losses due to Dutch elm disease of approximately 2 %. As a result of the Dutch sanitation program, which was based on the prompt removal of every weakened or diseased elm, the actual losses were generally threefold lower. Dutch elm disease incidence was 22-45 % lower in the trees treated with a *Pseudomonas* isolate in the year of treatment and the year after. The results of the biocontrol treatment were negatively influenced because on several locations trees were felled that showed initial signs of Dutch elm disease, which probably would have disappeared during the season.

The advantage of artificial infections with *Ophiostoma ulmi* was a reproducable development of symptoms and the possibility to maintain diseased trees, at least till the first signs of elm bark beetle breeding. For 'Commelin' elms an increase in symptoms was observed with increasing *O. ulmi* dose till 3000 conidia per tree; the standard 500 000 conidia used for most experiments was well above this critical value. No decrease in effectiveness of the bacterial pre-treatment was observed with increasing *O. ulmi* inoculum. Different bacterial treatments suggested that injections at a smaller interval (i.e. more injections per tree) may result in a better prophylactic effect, but the significance of the correlation remained doubtful. A comparison of several elm species and clones showed the importance of the host tree. Prophylaxis as a result of one bacterial treatment was shown repeatedly in 'Commelin' elms; the numbers of trees showing symptoms by the end of the second year were 10 to 85 % lower in the bacteria-treated groups in comparison with the controls. Also in one experiment with 'Belgica' elms prophylaxis was observed, resulting in a 84 % decrease in the number of trees showing symptoms by the end of the second year after the prophylactic treatment followed by inoculation with *O. ulmi*. In 'Vegeta' symptom development was only less severe and in field elms (*Ulmus carpinifolia*) some prophylactic effect was observed in one experiment, but no effect in two others.

*Additional keywords: Ophiostoma ulmi, Ulmus*

## Introduction

The possibilities to achieve biological control of plant diseases, including Dutch elm disease, are currently widely studied (recently reviewed in Mukerji and Garg, 1988),

but the use of biological control measures has been limited up till now because of the inherent complexity of control measures based on biological interactions between plant, pathogen and the biocontrol agent. However, the potential advantages of biological control methods, such as a low environmental impact and reasonable costs, warrant further research.

Development of Dutch elm disease symptoms was significantly suppressed in Commelin elms (*Ulmus* × *hollandica* 'Commelin') when they had been injected with a bacterial suspension before inoculation with the pathogen, *Ophiostoma ulmi* (Scheffer, 1983a). Curative bacterial treatments were not successful in this experiment. Similar results were obtained by Murdoch et al. (1986) in the state of Maine (U.S.A.) using *U. americana*. Others obtained comparable (Myers and Strobel, 1983; Strobel and Myers, 1982) but also conflicting results (Shi and Brasier, 1986).

Because of the remarkable success of some of the preventive treatments, studies on the localization, persistence and ecology of the injected *Pseudomonas* isolates were performed (Scheffer et al., 1989a,b). The present study reports on further field trials that were initiated to examine the effect of such bacterial treatments on Dutch elm disease, also in elms other than 'Commelin', and the duration of the protective effect. For the latter, two country-wide trials were initiated in close cooperation with the Dutch Plant Protection Service and the City of Amsterdam. Monitoring naturally occurring Dutch elm disease infections thus not only provided information on the effect of a bacterial treatment on disease incidence, it also yielded data on the Dutch elm disease epidemic in the Netherlands, which was most probably affected by the country-wide sanitation program (Water, 1983).

## Materials and methods

*Bacteria and inoculum preparation.* Bacterial isolates were maintained on medium B of King et al. (1954). The *Pseudomonas fluorescens* isolates WCS361 and WCS374, rifampicin-resistant WCS374R, and WCS374FLU - were obtained from F.P. Geels and B. Schippers (Geels and Schippers, 1983a,b). WCS374FLU – was a non-fluorescent mutant which did no longer synthesize hydroxamate-type siderophores. WCS374 RJS101 was a derivative of WCS374 which was better adapted to low water potentials, WCS374 RJS112 and 122 were derivatives marked with a transposon and a plasmid construct, respectively, to allow for positive identification of the bacterium after re-isolation from the tree (Scheffer et al., 1989a,b). *P. syringae* M27+ was obtained from G.A. Strobel (Montana State University, Bozeman, Montana, U.S.A.). Other bacteria were (re-)isolates made in the autumn of 1981 from elms inoculated with WCS361 (L15), WCS374 (B13 II), M27+ (D105, D107, L7 III/IV) and from an untreated control tree (L12 I).

Shake cultures of *Pseudomonas* spp. used for injection of trees were grown for 40 h at 24 °C in a modification of medium B of King et al. (1954) in which $K_2HPO_4$ (1.5 g $l^{-1}$) was replaced by $KH_2PO_4$ (1.2 g $l^{-1}$; Scheffer et al., 1989a).

*Pathogen and inoculum preparation.* Isolates H6 and H106 of the aggressive strain (North American NAN race) of *O. ulmi* were grown, and inocula were prepared as described in part one of this study (Scheffer et al., 1989a). The final concentration used for inoculation of elm trees was adjusted to $5 \times 10^6$ conidia $ml^{-1}$, except where indicated otherwise.

*Field trials.* Experimental details of the various field trials are summarized in Tables 1 and 2. In the country-wide and the Amsterdam trials only natural Dutch elm disease infections could be used; in all other trials the trees were artificially inoculated with *O. ulmi*. The experiments in which trees were inoculated with *O. ulmi* were always planned for two growing seasons. Leaving experiments longer than two growing seasons was virtually impossible as invariably trees were granted because they had to be removed within a relatively short period. Moreover, progressing disease, for instance in controls, could have rendered trees suitable for breeding of elm bark beetles, turning these trees into a threat for other elms in the vicinity.

Generally trees were grouped at random, except for the country-wide and the Amsterdam trials where blocks of ten trees were used. These country-wide field trials were carried out in cooperation with the Dutch Plant Protection Service and the Department of Parks and Greens of the City of Amsterdam. Elms were treated with bacteria by staff members of these organizations at over 70 locations throughout the Netherlands, see Fig. 1. All trees in the country-wide field trials were *Ulmus* × *hollandica* clones:

Table 1. Details of field trials with elm trees naturally infected with *O. ulmi* after one preventive treatment with a *Pseudomonas* isolate.

| Site/year | Number of trees | | | Injection period | Duration of trial (years) |
|---|---|---|---|---|---|
| | untreated | injected with | | | |
| | | WCS374 | M27+ | | |
| Country-wide/1982 | 6607 | 3014 | 3335 | May 25 - June 24, 1982 | 6 |
| Country-wide/1986 | 3233 | 3363 | — | June 3 - 26, 1986 | ? |
| Amsterdam/1984 | 3136 | 3486 | — | July 4 - 20, 1984 | 3 |
| Amsterdam/1985 | 1046 | 1048 | — | June 5 - 21, 1985 | 3 |

Table 2. Details of field trials with elm trees inoculated with *O. ulmi* after one preventive treatment with a *Pseudomonas* isolate.

| Site | Elm clone/ species | Age of trees (years) | Total number of trees | Number of trees per group | Dates of bacterial treatment | Date of inoculation with *O. ulmi* |
|---|---|---|---|---|---|---|
| Zuurdijk | Belgica | 50 | 55 | 5 | June 28-29, 1982 | July 14, 1982[1] |
| Bloemendaal | carpinifolia | 15-25 | 88 | 8-9 | June 11,18, 1982 | July 8, 1982 |
| Horst-Huissen | carpinifolia | 12-20 | 194 | 10-22 | June 23, 28, July 7, '83 | July 12, 1983 |
| Lichtmis | Groeneveld | 16 | 142 | 16-19 | May 30, June 4, 1982 | July 7, 1982 |
| Larserbos | Vegeta | 14 | 33 | 11 | June 17-19, 1985 | June 27, 1985 |
| Larserbos | Commelin | 14 | 57 | 11-12 | June 12-19, 1985 | June 27, 1985 |
| Schiphol | Commelin | 12 | 50 | 10 | July 6, 1983 | July 13, 1983 |
| Hollandse Hout | Commelin | 15 | 69 | 9-10 | June 12, 16, 1986 | June 27, 1986 |

[1] In the Zuurdijk trial also curative bacterial treatments were performed at July 14-15, 1982, which were preceded by inoculation with *O. ulmi* at June 29.

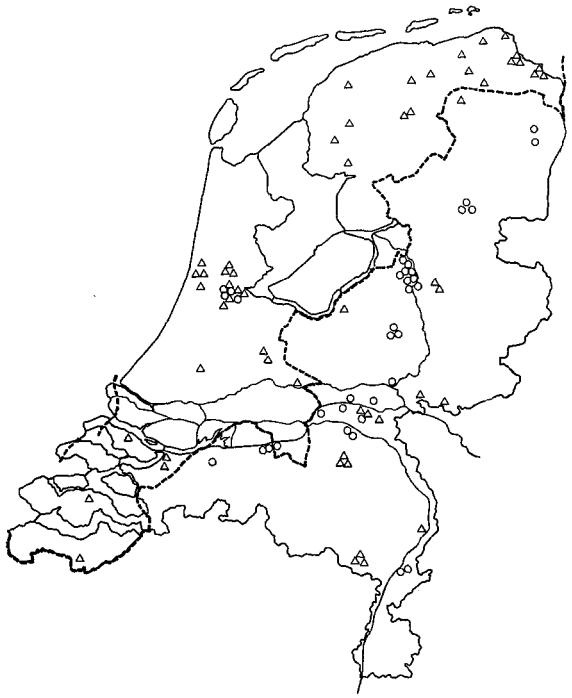

Fig. 1. Location of elm plots in the country-wide field trials started in 1982 (△) and 1986 ( ○ ). The dotted line indicates a sanitation program border: from 1985 on only in the Northwest part of the coutry elms were promptly removed upon showing any disease symptoms.

'Commelin' (55 %), 'Vegeta' (35 %), 'Belgica', 'Groeneveld', 'Bea Schwarz' and 'Sarniensis'. All trees were checked for Dutch elm disease symptoms twice every growing season.

*Bacterial treatments and inoculations with O. ulmi.* For all experiments gouge pistols were used to inject trees with bacteria. If not otherwise stated, trees were injected at breast height every 5 cm of circumference with 1 ml of a late-log shake culture (Scheffer, 1983a).

Inoculations with *O. ulmi* were performed with the aggressive isolate H6 in the stem of the tree in all experiments summarized in Table 2, except at Zuurdijk and Hollandse Hout, where two or three branches per tree were inoculated. Two times 50 $\mu$l of a conidial suspension was introduced into the tree at two opposite sides of a stem or branch using a Stanley trim knife. In the Larserbos and Hollandse Hout trials a mixture of two aggressive isolates, H6 and H106, was used. In the latter trial the precise concentration of viable conidia was critical, as various concentrations were used of which the lower ones were probably too low for reproducible symptom development. Therefore the suspensions used for inoculation were also plated out on agar media (Elgersma and Heybroek, 1979). Over 90 % of the conidia germinated, which confirmed that the expected conidial concentrations were indeed precise. Both *O. ulmi* isolates used were originally identified as belonging to the North American (NAN) race, but probably

H106 is a hybrid between the North American (NAN) and the Eurasian (EAN) race (Jeng et al., 1988).

*Scoring disease incidence.* Disease severity was evaluated using a disease-index, ranging from 0: healthy, to 5: leaves fallen and/or brown, shoots and tips of branches dead and crooked (Scheffer, 1983a). In trees that were more seriously affected in the first year of the experiment, usually recurrence of Dutch elm disease occurred in the second year, whilst lightly affected trees sometimes remained entirely symptomless in the second year. Most data presented in this study were acquired by the end of the second growing season. By that time the situation (i.e. the level of disease incidence) was not changing rapidly anymore.

*Statistical analysis of data.* The use of disease indices for rating disease incidence in trees precluded the use of standard parametric tests, as such data do not conform to normal distributions. MSUSTAT (a statistical package from Montana State University, Bozeman, Montana) was used for pair-wise comparisons (Wilcoxon and chi-square analysis). As such comparisons are considered doubtful for experiments comprising more than two groups, because of rapidly increasing real confidence limits (Conover, 1971; Hollander and Wolfe, 1973) Dunn's distribution-free multiple comparison procedure was used for multiple comparisons within experiments (Dunn, 1964; Hollander and Wolfe, 1973).

## Results

*Natural infections.* The large field trials depending on natural infections were initially based on an expected annual loss due to new Dutch elm disease infections of approximately 2 %. The observed losses were threefold lower, most probably due to the efficacy of the Dutch sanitation program, which, by realizing an immediate removal of every weakened or diseased elm, resulted in a strong suppression of the vector populations. However, this low incidence of Dutch elm disease infections also brought these experiments to the limits for any statistically significant differences. Table 3 summarizes the annual losses due to new Dutch elm disease infections in the experiment initiated in 1982; the overall results of the experiment are presented in Fig. 2A. Dutch elm disease incidence was 22-45 % lower in the treated trees in the year of treatment and the year after (analysis of covariance, $p = 0.05$). One *Pseudomonas* isolate, WCS374, was used for more comparable trials; one more country-wide trial, started in 1986 (See Fig. 1) and three more trials in the City of Amsterdam, started in 1984, 1985 and 1986. In Table 4 the annual losses of trees in these experiments in which WCS374 was used, are summarized; Fig. 2B gives the overall results. Dutch elm disease incidence was lower in the treated trees in the first and second year (analysis of covariance, $p = 0.05$).

In some cases the Dutch elm disease infection in these experiments was apparently due to root contact: such trees were standing next to an earlier Dutch elm disease victim and instead of gradually progressing symptoms starting at one or more twigs a major part of the crown wilted synchronously. These trees are omitted from the results, as the treatment is most probably ineffective as a curative one or against root graft transmission (Scheffer, 1983a,b and unpublished data). As trees were also lost from the experiments due to other cases, such as road reconstructions, the numbers of trees

Table 3. Field trials monitoring natural occurring Dutch elm disease infections in untreated control trees and trees injected once with *Pseudomonas* spp. in 1982. Plots were scattered throughout the Netherlands.

| Year | Number of trees at beginning of year and losses due to new Dutch elm disease infections in that year (in parentheses the losses as percentage of the current number of trees) | | | | | |
|---|---|---|---|---|---|---|
| | untreated | | WCS374 | | M27+ | |
| | number | losses | number | losses | number | losses |
| 1982 | 6607 | 47 (0.71) | 3014 | 17 (0.56) | 3335 | 18 (0.54) |
| 1983 | 6543 | 44 (0.67) | 2982 | 11 (0.37) | 3318 | 15 (0.45) |
| 1984 | 5698 | 40 (0.70) | 2532 | 21 (0.83) | 2926 | 20 (0.68) |
| 1985 | 5403 | 23 (0.43) | 2503 | 10 (0.40) | 2667 | 12 (0.45) |
| 1986 | 4349 | 21 (0.51) | 1947 | 3 (0.15) | 2097 | 6 (0.29) |
| 1987 | 4146 | 20 (0.49) | 1839 | 5 (0.27) | 2009 | 9 (0.45) |

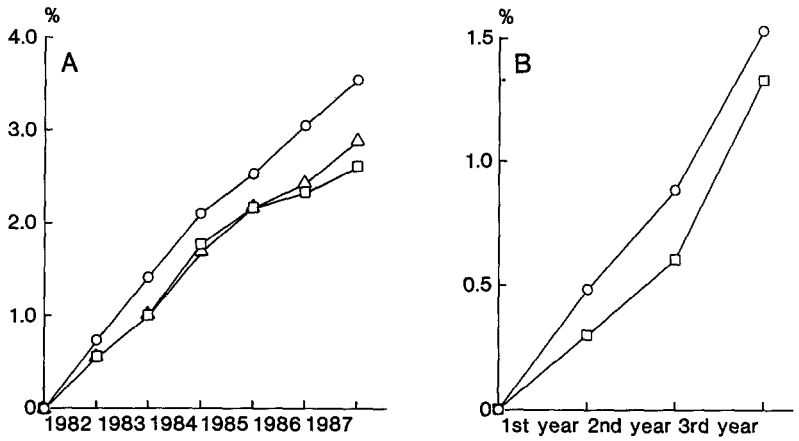


Fig. 2. Losses due to natural Dutch elm disease infections in elms at various locations in the Netherlands shown in Fig. 1. Treated trees were injected once with *P. fluorescens* WCS374 (□) or *P. syringae* M27+ (△) at the beginning of the experiment. Untreated trees, intermixed with the treated ones, served as controls (○). A: losses in the first country-wide trial over 1982-1987. B: losses in all four trials combined (initiated in 1982, 1984, 1985 and 1986) in which trees were injected with WCS374.

at the beginning of the consecutive years declined faster than could be explained from the losses solely due to Dutch elm disease.

*Artificial infections: effect of the O. ulmi doses.* Inoculations with the pathogen had the major advantage that most, if not all, control trees inoculated with only *O. ulmi* showed clear Dutch elm disease symptoms two to three weeks after inoculation with a conidial suspension. Usually in such trees the disease progressed slowly during the

Table 4. Summarized data of field trials started in 1982, 1984, 1985 and 1986 monitoring natural occurring Dutch elm disease infections in untreated control trees and trees injected once with *P. fluorescens* WCS374. Plots were scattered throughout the Netherlands.

| Year | Number of trees at beginning of year and losses due to new Dutch elm disease infections in that year (in parentheses losses as percentage of the current number of trees) | | | |
|---|---|---|---|---|
| | untreated | | WCS374 | |
| | number | losses | number | losses |
| 1st year | 14022 | 67 (0.48) | 10911 | 33 (0.30) |
| 2nd year | 13658 | 55 (0.40) | 10568 | 32 (0.30) |
| 3rd year | 12224 | 84 (0.69) | 9507 | 71 (0.75) |

first year, and recurrence of the disease, resulting in more severe symptoms, was observed in the second year.

Reliable symptom expression was reported for inoculum doses exceeding 200 spores per tree (Tchernoff, 1965). In the Hollandse Hout trial the effect of various *O. ulmi* doses in conjunction with preventive treatments with *Pseudomonas* spp. was tested by inoculating 'Commelin' elms in one branch per tree with 100, 1000, 10000 or (the standard) 500000 conidia (Fig. 3A). From linear regression on raw data 3000 conidia per tree emerged as the threshold level above which Dutch elm disease symptoms did not increase. Up to 67 % of the control trees showed symptoms in this experiment by the end of the second year. One preventive treatment with 1 ml of a *P. fluorescens* WCS374 RJS101 suspension per 2.5-3 cm tree circumference reduced the percentage of trees showing symptoms to 10 %.

*Artificial infections: effects of various bacterial doses.* Both the number of bacterial injections and the amounts per injection were varied in several experiments (Fig. 3B, 4 and 5). In the Schiphol experiment, on 'Commelin' elms, 1 ml per 2.5 cm tree circumference gave better results than 1 ml per 5 cm. The 1 ml WCS374 per 2.5 cm treatment was the only treatment resulting in significantly fewer symptoms than observed (Fig. 3B) in the control trees in pair-wise comparisons (Wilcoxon, two-tailed, $p < 0.01$), although the multiple comparison test (Dunn, $\alpha = 0.05$) did not show significant differences. In this experiment all control trees showed heavy symptoms by the end of the second year, whilst 60 % of the bacteria-treated trees (1 ml/2.5 cm) remained healthy.

In the Bloemendaal trial, for which a natural stand of field elms was used, disease development upon inoculation with the pathogen was slower than in any other experiment (Fig. 4). Only the lowest bacterial dose (0.2 ml per 10 cm) and the highest one (2 ml per 2.5 cm) did not result in any disease controlling effect. Multiple comparisons (Dunn, $\alpha = 0.05$) did not prove differences, but the statistical procedure is questionable in this experiment where a trend was expected. Pair-wise comparisons indicated that the control group and the groups that received the lowest and the highest bacterial dose may differ from the other groups ($p = 0.02$ to 0.06).

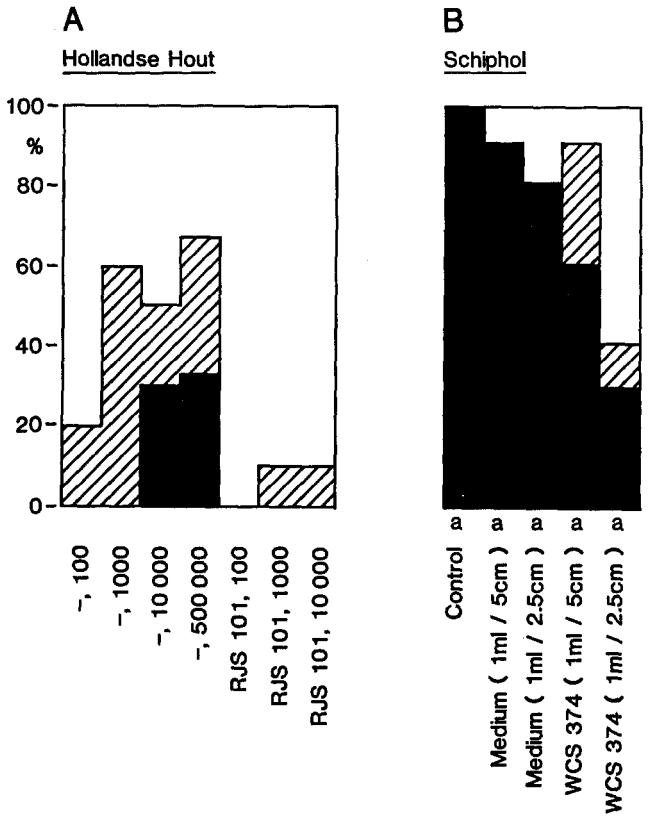


Fig. 3. Comparison of the effect on Dutch elm disease symptom development in 'Commelin' elms of (A) the amount of *O. ulmi* inoculum and of preventive treatments with *P. fluorescens* WCS374 RJS101 preceding the *O. ulmi* inoculations by two weeks and (B) the frequency of the injections with medium or bacterial suspensions around the stem, applying a standard 1 ml per injection, compared with a untreated control group, inoculated with *O. ulmi* only. Disease ratings were made in the second year, at Aug. 21, 1987 and July 31, 1984, respectively.

Unshaded is the percentage of trees not showing symptoms of Dutch elm disease (disease index less than 2). Black is the percentage of dying and dead trees (disease index over 4). The shaded area represents the percentage of trees not (yet) dying but clearly showing symptoms. Groups with a different letter differed significantly.

The Horst-Huissen trial was conducted simultaneously on two forest plots of planted field elms (Fig. 5). Pair-wise comparisons (Wilcoxon, two-tailed, $p < 0.01$) did not show differences for the comparable treatments in both plots. Therefore the combined experiment was evaluated using multiple comparisons (Dunn, $\alpha = 0.05$). Only one pair different from each other was found: control vs. medium (without bacteria) in the Huissen plot, but not in the other one. None of the bacterial treatments resulted in any effect. Because of the overall severe symptom development, this trial was evaluated only by the end of the first year, after which the trees were incinerated.

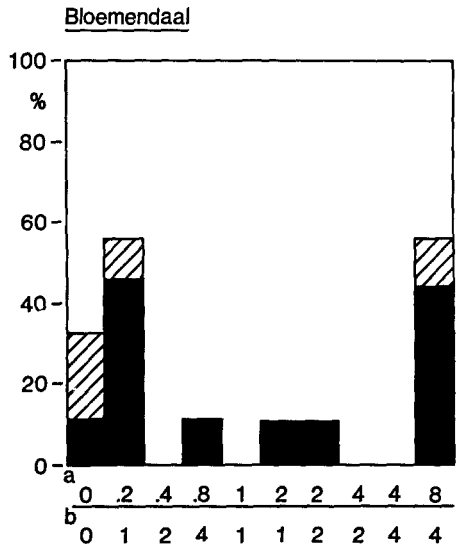


Fig. 4. Comparison of the effect in field elms (*U. carpinifolia*) on Dutch elm disease symptom development after inoculation with *O. ulmi* of various bacterial doses (*P. syringae* M27+) for which both the volume introduced into the tree per injection and the number of injections were varied. Disease ratings were made in the second year, at Sept. 28, 1983. See legend of Fig. 3 for further explanation.
[a] bacterial dose in ml per 10 cm tree circumference
[b] number of injections per 10 cm tree circumference

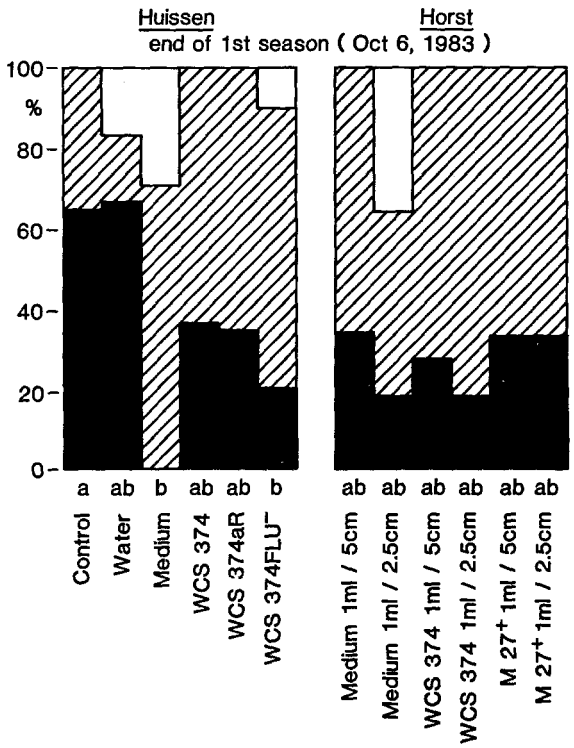


Fig. 5. Comparison of the effect on Dutch elm disease symptom development in field elms (*U. carpinifolia*) of preventive bacterial treatments followed by inoculation with *O. ulmi*. Because of severe symptom development in many of the trees disease ratings were made by the end of the first growing season only (at Oct. 6, 1983). See legend of Fig. 3 for further explanation.

*Artificial infections: additional data.* Three additional, straightforward control experiments are presented in Fig. 6. In the Zuurdijk trial 11 bacterial treatments were compared using 'Belgica' elms (Fig 6A and Table 5). Curative treatments did not result in any Dutch elm disease control, confirming other observations (Scheffer, 1983a,b; Shi and Brasier, 1986). All bacterial treatments gave comparable results. This is in concordance with other data presented here (Fig. 6B and C) and with data obtained in an experiment with large groups of 'Groeneveld' elms at Lichtmis (Table 5). In this latter experiment too, all bacterial treatments resulted in comparable disease ratings in the second year (Dunn multiple comparisons, $\alpha = 0.05$). Unfortunately, the entire control group was lost, preventing proper analysis of the disease-controlling effect of the treatments. Based on the observations that apparently different bacterial treatments generally had comparable effects, the curative treatments in the Zuurdijk trial were combined with the controls and compared with the combined preventive treatments. These large groups were significantly different (Wilcoxon, $p = 0.0000$).

The Larserbos trials were carried out simultaneously on both elm clones planted in that forest: the moderately resistant 'Commelin' and the more susceptible 'Vegeta' elm

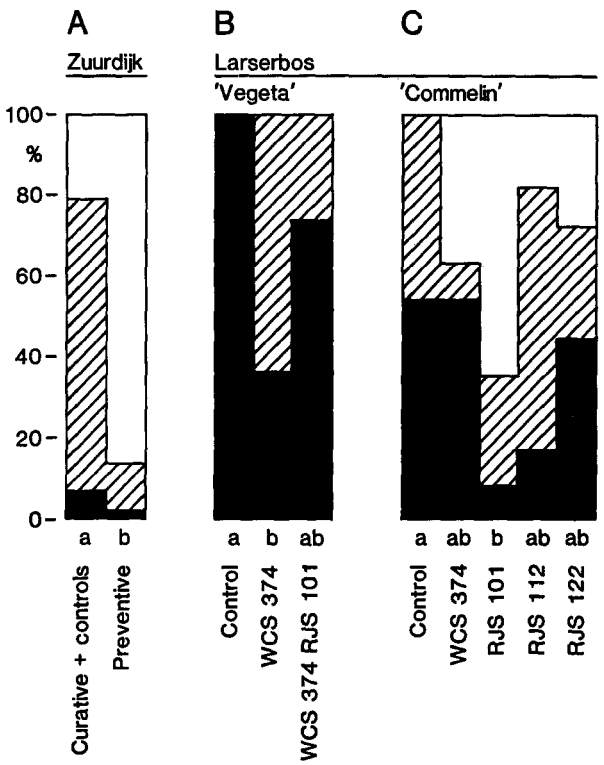


Fig. 6. Comparison of the effect of preventive bacterial treatments on Dutch elm disease symptom development after inoculation with *O. ulmi* in (A) 'Belgica' elms, (B) 'Vegeta' elms and (C) 'Commelin' elms. Disease ratings were made in the second year, at July 13, 1983 (A) and Aug. 13, 1986 (B,C). For the experiment with 'Belgica' elms at Zuurdijk combined data of the treatments are presented; see Table 5. See legend of Fig. 3 for further explanation.

Table 5. Disease indices in the second growing season (July 13, 1983) of two experiments with inoculated elms in which various *Pseudomonas* treatments were compared. Trials were at Zuurdijk ('Belgica' elms, 5 per treatment) and at Lichtmis ('Groeneveld' elms, 16-19 per treatment).

| Bacterial treatment | Zuurdijk | | Lichtmis |
|---|---|---|---|
| | curative | preventive | preventive |
| M27+ | 1.9 | 0 | 3.0 |
| WCS361 | 3.2 | 0 | 1.1 |
| WCS374 | 2.5 | 0.2 | 0.9 |
| M27+ + WCS361 + WCS374 | 1.3 | 0.8 | |
| WCS361 + WCS374 | 2.3 | 0.8 | |
| D105 | 2.7 | 0.6 | 1.6 |
| D107 | 1.6 | 1.4 | 1.8 |
| L7I | | | 2.9 |
| L7IV | 2.8 | 0.2 | 1.6 |
| L12I | 2.5 | 0.7 | 1.2 |
| L15 | 3.4 | 0.9 | |
| B13II | 2.7 | 0.2 | |
| none (*O. ulmi* only) | 2.2 | | |
| average | 2.5 | 0.5 | |

(Fig. 6B, C). The results show the difference in the rate with which disease developed in the two clones: by the end of the second year all 'Vegeta' control trees and 55 % of the 'Commelin' controls were dead or dying. Multiple comparisons (Dunn, $\alpha$ = 0.05) showed clear differences between 'Vegeta' controls and 'Vegeta' elms preventively injected with WCS374 and between 'Commelin' controls and 'Commelin' elms preventively injected with WCS374 RJS101. The other bacterial treatments resulted in intermediate disease levels. In 'Vegeta' the differences were only in the level of disease; none of them remained healthy. In 'Commelin' no healthy control trees were left, but 64 % of the trees preventively treated with WCS374 RJS101 (the WCS374 derivative better adapted to low water potentials) remained healthy.

## Discussion

The lower incidence of Dutch elm disease in elms treated with *Pseudomonas* spp. in both a 'natural' situation and in experiments where elms were inoculated with *O. ulmi* suggests a potential practical use of this treatment. Such a practical use of *Pseudomonas* may prove to be restricted to certain species and clones of elms. In 'Commelin' a good control was achieved, but in field elm (*U. carpinifolia*) no suppression of Dutch elm disease was obtained in the experiment where disease progressed quickly. Similar results were obtained in England, with the susceptible *U. procera* (Shi and Brasier, 1986). However, although *U.* × *hollandica* 'Belgica' is also susceptible to Dutch elm disease, the results strongly indicate a preventive effect. Experiments with the susceptible *U. americana* also indicated a suppression of Dutch elm disease development, even if the

trees were treated curatively (Strobel and Myers, 1982; Myers and Strobel, 1983; Murdoch et al., 1986).

In one experiment, in which the trees were artificially infected, several derivatives of *P. fluorescens* WCS374 were compared (Fig. 6B, C). No significant differences in protective effect of the various bacteria were recorded, although it was shown that the bacterial concentrations in the trees varied. The labeled derivatives of *P. fluorescens* WCS374, RJS112 and RJS122, could not even be detected in 'Commelin' elms 13 weeks after the bacterial treatment (Scheffer, 1989a). However, the trees were inoculated with *O. ulmi* within two weeks after the bacterial treatment; the protective effect of any of the bacteria used apparently was not affected significantly by any ecological advantage or disadvantage on such a short (two weeks) time span.

A major point of debate is the duration of the protective effect to be expected. Bacterial populations in elms initially declined rapidly after injection, but in the year after the year of injection bacteria were presumably still present in the xylem elements (Scheffer et al., 1989a, b). The infection of the new growth ring probably took place through the bacteria-infested regenerating wound tissue or from the root system. Indeed, in the country-wide experiments, reduced losses due to Dutch elm disease were consequently recorded in the first and the second year.

It seems plausible that the effect of the bacterial treatments will be underestimated from the data presented: in the trials in which only natural infections were allowed generally every elm showing any signs of Dutch elm disease was removed. However, in the experiments with inoculations it was sometimes observed that in control trees the disease progressed steadily, while in trees treated with *Pseudomonas* spp. only some initial symptoms developed. Such control trees showed disease recurrence in the second year, but generally trees that only developed some initial symptoms remained healthy in the second year.

This study (including Part 1 and 2; Scheffer et al., 1989a,b) warrants the conclusion that one *Pseudomonas* treatment may result in a prophylactic effect against Dutch elm disease for at least two growing seasons, but that the elm clone or species distinctly influences the ultimate effect of the treatment. This last observation, and the observations that injection into elm of many different bacterial isolates resulted in a comparable prophylaxis of elms against Dutch elm disease, suggest that the major mechanisms explaining this biological control are most probably related to induced or enhanced resistance in the tree rather than to a direct antibiosis between bacterium and pathogen.

**Acknowledgments**

This study was financially supported by the EEC in the context of the EEC Dutch Elm Disease Research Program, by the Dutch State Forestry Service and by the City of Amsterdam. Trees were made available by many Dutch authorities of which especially the Dutch State Forestry Service, Schiphol Amsterdam Airport, the recreational body Nulde-Horst and the municipalities of Bloemendaal and Huissen should be mentioned. The author is indebted to the staff members of the Plant Protection Service and Parks and Greens of the City of Amsterdam for their enthousiastic participation in some of the experiments.

**Samenvatting**

*Biologische bestrijding van de iepeziekte met Pseudomonas. III. Veldexperimenten op diverse plaatsen in Nederland*

De mogelijke bescherming tegen de iepeziekte, verkregen door injectie van de boom met bacteriën van het geslacht *Pseudomonas*, werd gemeten in twee soorten experimenten. In het ene soort werden natuurlijke infecties gemeten, waardoor grote proefgroepen nodig waren. In het andere soort werden de iepen kunstmatig geinfecteerd.

De grootschalige veldexperimenten waarbij natuurlijke iepeziekte-infecties werden gemeten, waren gebaseerd op een verwachte jaarlijkse uitval van 2 %. Als gevolg van de landelijke bestrijdingscampagne bleken de verliezen slechts ongeveer een derde hiervan te zijn. Er kwam minder iepeziekte voor in de met *Pseudomonas* geïnjecteerde bomen in het jaar van injectie en in het jaar daarna. Een storende invloed op de resultaten had het effect dat ook met bacteriën geïnjecteerde bomen soms beginnende symptomen vertonen na infectie met *Ophiostoma ulmi*, symptomen die in de loop van het seizoen soms weer verdwijnen. Als gevolg van de bestrijdingscampagne werden zulke bomen toch geveld.

Het voordeel van kunstmatige infecties met *O. ulmi* was een voorspelbaar verloop van de symptoomontwikkeling en de mogelijkheid om zieke bomen te laten staan tot er iepespintkevers in kwamen. In 'Commelin' iepen bleken de symptomen toe te nemen met een tot 3000 conidiën per boom toenemende dosis *O. ulmi*. De gebruikelijke 500000 conidiën die in de meeste experimenten werden gebruikt lagen ver boven deze kritische waarde. Er werd geen effect van een toenemende dosis *O. ulmi* op de effectiviteit van een bacteriebehandeling waargenomen.

Uit variaties in de diverse bacteriebehandelingen kwam naar voren dat injecties met een kleinere tussenruimte (dus meer injecties per boom) mogelijk het effect verbeterden, maar de significantie van deze correlatie bleef twijfelachtig.

Vergelijken van diverse iepen toont dat soort en kloon type een belangrijke rol speelt bij deze bestrijdingsmethode. Bescherming tegen de iepeziekte als gevolg van een bacteriebehandeling werd diverse malen aangetoond in 'Commelin' iepen; het aantal bomen met iepeziekte-symptomen was aan het eind van het tweede seizoen in de met bacteriën behandelde groepen 10 tot 85 % lager dan in de controlegroepen. Ook in een experiment met 'Belgica' iepen werd een goede bescherming gemeten. In 'Vegeta' werd slechts een verminderde symptoomontwikkeling gemeten en in veldiepen (*U. carpinifolia*) werd enige bescherming gevonden in één experiment, maar geen effect in twee andere.

**References**

Conover, W.J., 1971. Practival nonparametric statistics. John Wiley & sons, Inc., New York.

Dunn, O.J., 1964. Multiple comparisons using rank sums. Technometrics 6: 241-251.

Elgersma D.M. & Heybroek, H.M., 1979. Spread and survival of an aggressive and a non-aggressive strain of *Ophiostoma ulmi* in elms. Netherlands Journal of Plant Pathology 85: 235-240.

Geels, F.P. & Schippers, B., 1983a. Selection of antagonistic fluorescent *Pseudomonas* spp. and their root colonization and persistence following treatment of seed potatoes. Phytopathologische Zeitschrift 108: 193-206.

Geels, F.P. & Schippers, B., 1983b. Reduction of yield depressions in high frequency potato crop-

ping soil after seed tuber treatments with antagonistic fluorescent *Pseudomonas* spp. Phytopathologische Zeitschrift 108: 207- 214.
Hollander, M. & Wolfe, D.A., 1973. Nonparametric statistical methods. John Wiley & sons, Inc., New York.
Jeng, R.S., Bernier, L. & Brasier, C.M., 1988. A comparative study of cultural and electrophoretic characteristics of the Eurasian and North American races of *Ophiostoma ulmi*. Canadian Journal of Botany 66: 1325-1333.
King, E.O., Ward, M.K. & Raney, D.E. 1954., Two simple media for the demonstration of pyocyanin and fluorescin. Journal of Laboratory and Clinical Medicine 44: 301-307.
Mukerji, K.G. & Garg, K.L., 1988. Biocontrol of plant diseases. CRC Press, Inc., Boca Raton, FL.
Murdoch, C.M., Campana, R.J. & Hoch, J., 1986. Development of Dutch elm disease inhibited by fluorescent pseudomonads. Biological and Cultural Tests 1: 71.
Myers, D.F. & Strobel, G.A., 1983. *Pseudomonas syringae* as a microbial antagonist of *Ceratocystis ulmi* in the apoplast of american elm. Transactions of the British mycological Society 80: 389-394.
Scheffer, R.J. 1983a. Biological control of Dutch elm disease by *Pseudomonas* species. Annals of Applied Biology 103: 21-30.
Scheffer, R.J., 1983b. *Pseudomonas* treatments as a possible control method for Dutch elm disease. Proceedings 24e Colloque de la Société Française de Phytopathologie, Bordeaux, May 26 - 28, 1983. Les Colloques de l'INRA no. 18, p. 131-136.
Scheffer, R.J., Elgersma, D.M., Weger, L.A. de & Strobel, G.A., 1989a. *Pseudomonas* for biological control of Dutch elm disease. I. Labeling, detection and identification of *Pseudomonas* isolates injected into elms; comparison of various methods. Netherlands Journal of Plant Pathology 95: 281-292.
Scheffer, R.J., Elgersma, D.M. & Strobel, G.A., 1989b. *Pseudomonas* for biological control of Dutch elm disease. II. Further studies on the localization, persistence and ecology of *Pseudomonas* isolates injected into elms. Netherlands Journal of Plant Pathology 95: 293-304.
Shi, J.L. & Brasier, C.M., 1986. Experiments on the control of Dutch elm disease by injection of *Pseudomonas* species. European Journal of Forest Pathology 16: 280-292.
Strobel, G.A. & Myers, D.F., 1982. Bacterial antagonism as a strategy for the treatment of Dutch elm disease. In: Kondo, E.S., Hiratsuka, Y. & Denyer, W.B.G. (Eds), Proceedings of the Dutch Elm Disease Symposium and Workshop, October 5-9, 1981, Winnipeg, Manitoba, p. 46-54. Manitoba Department of Natural Resources, Winnipeg.
Tchernoff, V., 1965. Methods for screening and for the rapid selection of elms for resistance to Dutch elm disease. Acta Botanica Neerlandica 14: 409-452.
Water, J.K., 1983. Dutch elm disease control in the Netherlands. In: Burdekin, D.A. (Eds), Research on Dutch elm disease in Europe, p. 17-18. Her Majesty's Stationery Office, London.