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Fluorescent Pseudomonas isolate E11.3 as biocontrol agent for Pythium root rot in tulips

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Abstract

Fluorescent *Pseudomonas* isolates were obtained from Pythium-diseased tulip roots or rhizospheres. A selection of these isolates was tested for root rot-suppressing capabilities, using tulip cultivar Paul Richter (ice-tulip) as host and *Pythium ultimum* P17 as pathogen. With isolate E11.3 root rot suppression was consistently found, but the extent of the effects varied from experiment to experiment. Beneficial effects were obtained after introduction of the bacteria either by mixing them through the soil or by dipping the bulbs in a bacterial suspension, immediately before planting. Application of bacteria in methylcellulose also reduced disease, but is of no practical value as methylcellulose by itself increased disease. In steamed soil, disease was more severe than in natural soil. In both circumstances, however, beneficial effects of bacterization with E11.3 were observed.

Additional keywords: Pythium ultimum.

Introduction

In the Netherlands, growing bulbs for bulb and flower production is economically very important. Among these bulbous crops, tulips have a predominant place, a production of a 600 million flowers a year being achieved. In these crops, root and bulb rot caused by *Pythium* spp. frequently occurs. This disease can give serious problems, especially in greenhouse-forced tulips, because it leads to hampered growth of the crop and reduction in flower production. *Pythium ultimum* Trow is the *Pythium* species most frequently isolated from diseased roots, but also *P. intermedium* de Bary, *P. irregulare* Buisman and *P. dissotocum* Drechsler are associated with this disease (Bergman et al., 1983).The disease was observed in as early as 1930 and the first report ascribing the disease to *Pythium* appeared in 1937 (Moore and Buddin,1937).

The general practice for control of root rot is steaming of the soil in combination with application of fungicides. This last method, however, will soon be out of the question. Commonly used fungicides will not be produced anymore, or will no longer be permitted by the authorities. Furthermore, *Pythium* spp. showed resistance against phenylamide fungicides (Koster and Vink, 1984; Koster et al., 1986), as do other oomycetous fungi (e.g.Crute et al.,1987).Because of this and because of environmental and health hazards associated with the use of fungicides, other means of control of Pythium root rot are urgently needed.

Introduction of suitable antagonists is a possibility for control of soil-borne fungal diseases. Different kinds of organisms are under investigation in this respect, including fungi and bacteria. At present, increasing attention is paid to bacteria of the *Pseudomonas fluorescens-P. putida* group. This group of bacteria have disease-suppressive abilities, sometimes associated with natural disease-suppressive soils, and growth-promoting abilities, which are ascribed to interference with deleterious micro-organisms or promotion of mineral nutrition of the plants. Production of siderophores and of a variety of antibiotics, and their preferential proliferation in, on or near the roots are considered to be the most important factors determining these abilities (Burr and Caesar, 1984; Cook, 1986; Cook and Baker, 1983; Deacon, 1988; Kloepper et al., 1988; Schippers et al., 1987a, b; Suslow, 1982; Weller, 1988).

Biocontrol of Pythium diseases with introduced pseudomonads has been achieved with several economically important hosts, including cotton, sugar beets, cucumber and wheat (Becker and Cook, 1988; Cook et al., 1987; Elad and Chet, 1987; Howell and Stipanovic, 1980; Howie and Suslow, 1986; Lee and Ogoshi, 1986; Loper, 1988; Walther and Gindrat, 1988; Yuen et al., 1987). With wheat as an exception, these investigations concerned pre- and post-emergence damping-off.

This report investigates the possibilities of the use of fluorescent pseudomonads to control Pythium root rot in tulips.

Materials and methods

The tulip cultivar Paul Richter, prepared as ice-tulip, was used as host. Ice-tulips are tulips whose bulbs, after the development of flower and leaf initials and after the required period at low temperature, are being frozen in moist peat dust at -2 °C till use. In this way the bulbs can be kept for about one year and experiments can be performed during all seasons. Two days before use the frozen bulbs were put at 5 °C to insure a slow thawing.

The pathogen, *Pythium ultimum* Trow, isolate P17, was isolated from Pythium-diseased tulip roots. This isolate only infects the roots and is not able to infect the bulbs. The fungus was kept in culture on cornmeal agar. As inoculum a soil-cornmeal culture was prepared. Sandy soil (200 g) was put in Petri dishes (14 cm diameter), sterilized at 120 °C for 1 h on three successive days and supplemented with cornmeal extract (25 ml) before the last sterilisation. Tufts of aerial mycelium from a cornmeal agar culture were put on this soil and the ensemble was incubated at 23 °C for 3.5 weeks.

Pseudomonas isolates were obtained by plating root washings and rhizosphere soil suspensions from Pythium-diseased tulip roots on King's medium B (King et al., 1954) supplemented with cycloheximide (100 μg ml^{-1}), chloramphenicol (12.5 μg ml^{-1}), antimycin (50 μg ml^{-1}) and 8-hydroxyquinoline (0 – 200 μg ml^{-1}). Individual colonies were isolated and kept in culture on King's medium B. In vitro, antagonistic activities against *P. ultimum* P17 were determined on King's medium B and on the same medium supplemented with 100 μM FeNa-EDDHA according to the method described by Geels and Schippers (1983), with the exception that the bacteria were allowed to grow for 4 days before *Pythium* was added. A limited number of isolates with in vitro antagonistic activities was used for exploring their root rot-suppressing capabilities.

For preparing bacterial suspensions, a dilute bacterial suspension, obtained by suspending a small amount of a 3-day old culture on King's medium B in sterile water, was

plated on King's medium B (1 ml per Petri dish). After 2 days at 25 °C, the bacteria were collected in 0.1 M $MgSO_4$ (5 ml per Petri dish). The number of bacteria was counted using a haemocytometer and the number of viable bacteria was checked by plating dilutions on King's medium B. The suspensions were diluted with 0.1 M $MgSO_4$ to the desired concentration.

The root rot-suppressing capabilities of the selected pseudomonads were assessed in pot experiments, using pots or PVC tubes. Natural sandy soil, with a low content of organic materials and a pH($CaCl_2$) of c. 7, was obtained from the bulb-growing fields near Lisse, the Netherlands. The soil was mixed with the *Pythium* soil-cornmeal culture. Depending on the experiment, the *Pseudomonas* suspensions were also mixed thoroughly through the soil to a concentration of 10^8 cells per g dry soil before planting the bulbs, or the bulbs were dipped in a bacterial suspension of 2×10^9 cells per ml, with or without 1% methylcellulose, immediately before planting. For every treatment 10 bulbs were used, with 1 bulb per pot or tube. The tulips were grown in the greenhouse at 18-21 °C.

At the end of the growing period (c. 24 days) of the tulips, almost after flowering, the effect of bacterization was measured by determining the root rot index on a scale from 1 (healthy) to 5 (all roots severely affected), the fresh weight of the roots and the shoot, the length of the shoot (from the tip of the bulb to the tip of the highest leaf), the length of the flower stem (from the tip of the bulb to the underside of the flower) and an index for the stage of the flower on a scale from 1 (no flower present) to 8 (the petals begin to fall down).

Results

Effect of Pythium root rot on development of tulips. To find the most adequate parameter for measuring disease and effects of bacterization on disease development, root rot index, root- and shoot fresh weight, shoot and flower stem length and stage of flowering were determined. A clear correlation between these parameters at the end of the growing period was observed (Fig. 1). Infection of the roots by *Pythium* was reflected almost immediately in a reduction in root fresh weight. Reductions in length and fresh weight of the shoot, however, became discernable only when more than 30-50% of the roots had been rotted away. With increasing damage to the roots, the effects on the shoots increased dramatically, finally resulting in a very short crop without any flower development.

Within the group of diseased tulips with severely affected roots, indexed as 5, large differences in shoot development could be found (Fig. 1). These differences very probably reflect differences in time of infection of the roots, particularly in relation to shoot development. Results obtained in an experiment in which *Pythium* inoculum was applied in layers of 1.5 cm at increasing distances from the bulbs, corroborated this explanation. Roots of bulbs placed directly on the inoculum layer were infected within 3 days and the crop developed poorly (Fig. 2). All other plants became infected not before 7 days after planting. The shoots of these plants developed similarly to those of non-infected plants (Fig. 2b), whereas root fresh weight appeared to correlate with time of infection (Fig. 2a).

Based on these observations it was concluded that root fresh weight and shoot length are the most suitable parameters for measuring disease and effects of bacterization on

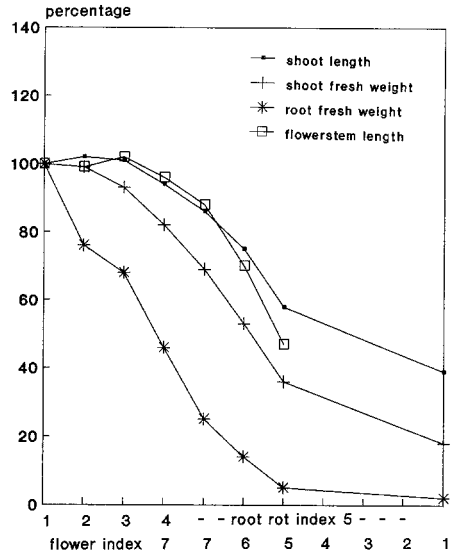

Fig. 1. Relationship between the parameters used for measuring disease in tulip cultivar Paul Richter caused by *Pythium ultimum* P17. Values are given as percentages of those of healthy tulips (100%).

disease development, root fresh weight giving the most information at low disease and shoot length at high disease incidence. Therefore, the results presented are based on these two parameters.

Effect of Pseudomonas isolates on Pythium root rot-screening experiments. Twenty-six *Pseudomonas* isolates with in vitro antagonistic activities against *Pythium ultimum*

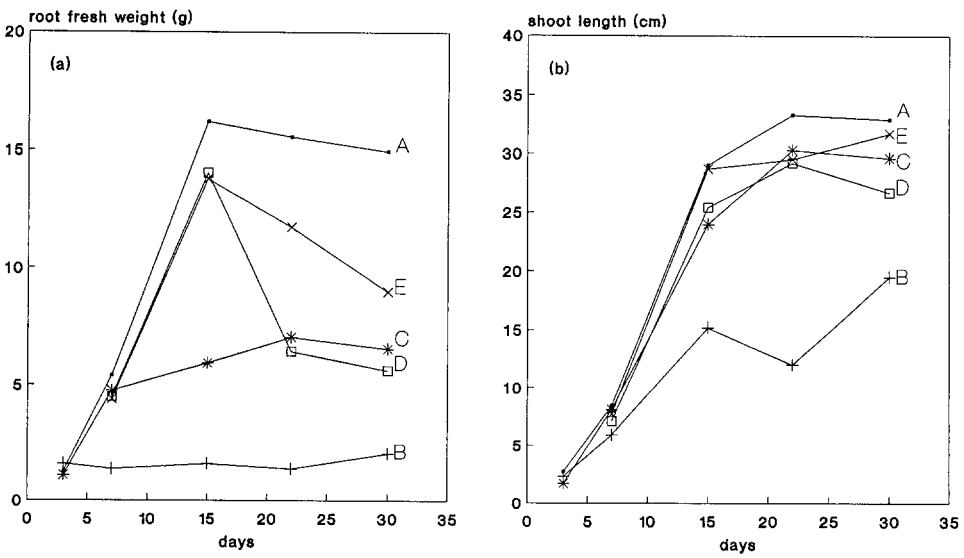

Fig. 2. Root fresh weight (a) and shoot length (b) of tulip cultivar Paul Richter infected with *Pythium ultimum* P17. No inoculum applied (A), or inoculum applied in a layer of 1.5 cm directly under the bulb (B), at 4-5.5 cm (C), at 7.5-9 cm (D) or at 11.5-13 cm (E) under the bulb.

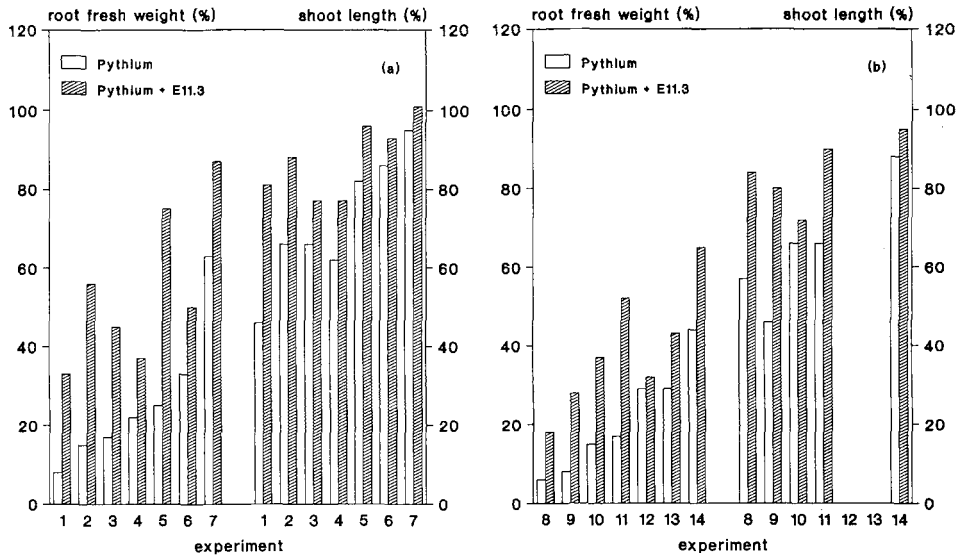

Fig. 3. Effect of bacterization with *Pseudomonas* isolate E11.3 on root fresh weight and shoot length of tulip cultivar Paul Richter grown in soil infested with *Pythium ultimum* P17. Values are given as percentages of those of healthy tulips (100%). Bacteria were either mixed through the soil (a) or applied by dipping the bulbs in a bacterial suspension (b). Results are arranged in order of decreasing root rot.

P17, were examined for their root rot-suppressing capabilities either by mixing them through the soil or by applying them on the bulbs immediately before planting. Some of these isolates showed root rot suppressing activities. Because of the initial positive effects obtained with isolate E11.3, this isolate was included in the ensuing experiments. This isolate appeared to be very consistent in its root rot-suppressing activities (Fig. 3). Generally, its effect exceeded the effects of other isolates. Mixing E11.3 through the soil was generally more effective than applying E11.3 to the bulbs. The differences between these two methods of introduction of the bacteria, however, were not statistically significant (Wilcoxon test, $a = 0.10$). Although these root rot-suppressing activities were consistently observed, shoots similar to those of non-infected tulips only sporadically developed.

Effect of bacterization in methylcellulose on Pythium root rot. For protecting bacteria during the time between bacterization of plant material and planting, bacteria are usually applied with a carrier or an adhesive. One of those compounds, methylcellulose, was tested in the tulip-*Pythium*-*Pseudomonas* system. *Pseudomonas* isolates were applied by dipping the bulbs in a suspension of bacteria in methylcellulose. The bulbs were grown in *Pythium*-amended soil.

In the presence of methylcellulose Pythium root rot appeared to be enhanced. Although *Pseudomonas* isolate E11.3 clearly suppressed root rot when applied with methylcellulose, E11.3 could not counteract the negative effects of methylcellulose (Fig. 4).

Effect of steaming of the soil on Pythium root rot and on effects of bacterization. The

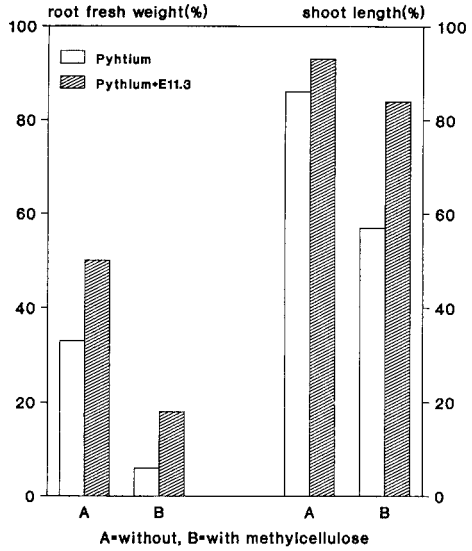

Fig. 4. Effect of bacterization with *Pseudomonas* isolate E11.3 applied in methylcellulose on root fresh weight and shoot length of tulip cultivar Paul Richter grown in soil infested with *Pythium ultimum* P17. Values are given as percentages of those of healthy tulips (100%). In the presence of methylcellulose differences due to bacterization were statistically significant (*t*-test; $p = 0.05$).

effect of steaming of the soil was investigated by comparing growth of tulips in natural soil and steamed soil, both with and without addition of *Pythium*. Within each of these treatments, part of the tulips was treated also with *Pseudomonas* isolate E11.3. Results were analyzed by analysis of variance, followed by orthogonal decomposition (Table 1).

As could be expected, the deleterious effect of *Pythium* was very pronounced and statistically highly significant. In the soils not treated with *Pythium*, tulips were somewhat shorter in the steamed soil than in the natural soil, whereas differences in root fresh weight were not statistically significant. Bacterization had no effect in both steamed and natural soil. In the *Pythium*-amended soils, on the other hand, root rot

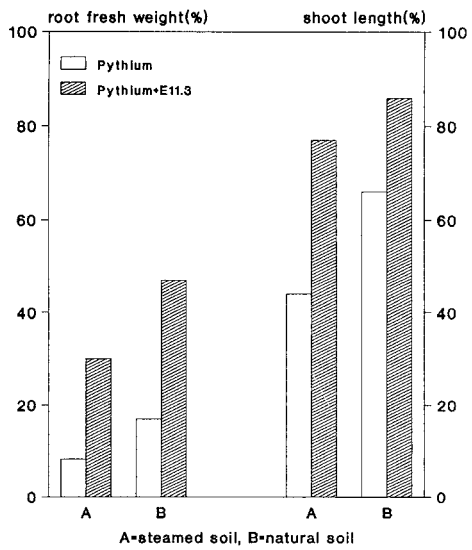

Fig. 5. Effect of bacterization with *Pseudomonas* isolate E11.3 on root fresh weight and shoot length of tulip cultivar Paul Richter grown in soil infested with *Pythium ultimum* P17. Comparison of effects in natural and steamed soil. For statistical analysis of results see Tables 1 and 2.

Table 1. Analysis of variance and orthogonal decomposition of the results on root fresh weight and shoot length of tulip cultivar Paul Richter grown in natural or steamed soil, without or with added inoculum of *Pythium ultimum* P17 and without or with addition of *Pseudomonas isolate* E11.3 (day 26; end of growing period).

Source of variation	df	MS	Fs	*p*	Mean values	Numbers compared
Root fresh weight						
Among groups	11	295.75	35.08	0.001		
A_1*A_2 [1)]	1	3001.12	356.04	0.001	14.58* 4.20	73*45
$A_1(B_1*B_2)$	1	13.32	1.58	ns	14.99*14.14	38*35
$A_1B_1(C_1*C_2)$	1	0.20	0.02	ns	14.91*15.06	18*20
$A_1B_2(C_1*C_2)$	1	3.30	0.39	ns	14.49*13.87	15*20
$A_2(B_1*B_2)$	1	55.90	6.63	0.025	5.39* 3.16	21*24
$A_2B_1(C_1*C_2)$	1	85.52	10.15	0.010	2.54* 6.82	7*14
$A_2B_2(C_1*C_2)$	1	54.28	6.44	0.025	1.21* 4.32	9*15
Within groups	106	8.43				
Shoot length						
Among groups	11	363.72	31.43	0.001		
A_1*A_2	1	2581.54	233.11	0.001	33.86*24.23	73*45
$A_1(B_1*B_2)$	1	107.26	9.27	0.005	35.05*32.60	38*35
$A_1B_1(C_1*C_2)$	1	17.77	1.54	ns	34.31*35.68	18*20
$A_1B_2(C_1*C_2)$	1	0.47	0.04	ns	32.47*32.70	15*20
$A_2(B_1*B_2)$	1	291.11	25.16	0.001	26.95*21.85	21*24
$A_2B_1(C_1*C_2)$	1	214.88	18.57	0.001	22.43*29.21	7*24
$A_2B_2(C_1*C_2)$	1	698.62	60.38	0.001	14.89*26.03	9*15
Within groups	106	11.57				

[1)] Explanation of symbols:
A_1,A_2: without and with Pythium inoculum added, respectively.
B_1,B_2 : natural and steamed soil, respectively.
C_1,C_2 : without and with Pseudomonas isolate E11.3 added, respectively.

was clearly more severe in steamed soil than in natural soil, as expressed by root fresh weight and shoot length (Fig. 5; Table 1). In both of these soils the introduced *Pseudomonas* isolate E11.3 suppressed the disease to a large extent, the resulting crop in natural soil being better than the crop in steamed soil (Fig. 5). An additional effect of Pythium root rot is an earlier start and an increase in incidence of stem topple. This disorder appeared to be reduced by the introduced E11.3 as well. The beneficial effects of bacterization in the presence of *Pythium* could already be discerned at 15 days after planting of the bulbs, a moment when the flowers had just reached the saleable stage (Table 2). Due to bacterization the number of saleable flowers was increased from 0% to 40% in steamed soil, and from 29% to 79% in natural soil.

Discussion

Pseudomonas isolates, particularly E11.3, clearly appeared to have capabilities to suppress Pythium root rot in tulips. Introduction of E11.3 in *Pythium*-infested soil resulted

Table 2. Analysis of variance and orthogonal decomposition of the results on shoot length of tulip cultivar Paul Richter grown in natural or steamed soil, without or with added inoculum of *Pythium ultimum* P17 and without or with addition of *Pseudomonas* isolate E11.3 (day 15).

Source of variation	df	MS	Fs	*p*	Mean values	Numbers compared
Among groups	11	218.00	23.40	0.001		
$A_1 * A_2$ [1)]	1	1216.75	130.58	0.001	28.50*21.89	73*45
$A_1(B_1 * B_2)$	1	103.86	11.15	0.005	29.64*27.26	38*35
$A_1B_1(C_1 * C_2)$	1	2.24	0.24	ns	29.39*29.87	18*20
$A_1B_2(C_1 * C_2)$	1	5.15	0.55	ns	27.70*26.92	15*20
$A_2(B_1 * B_2)$	1	179.47	19.26	0.001	24.02*20.02	21*24
$A_2B_1(C_1 * C_2)$	1	170.01	18.24	0.001	20.00*26.04	7*14
$A_2B_2(C_1 * C_2)$	1	601.91	64.59	0.001	13.56*23.90	9*15
Within groups	106	9.31				

[1)] Explanation of symbols: see Table 1.

in higher amounts of root fresh weight and longer shoots. Although the extent of the bacterization effects varied from experiment to experiment, root rot suppression by E11.3 was consistently observed.

Variability in the degree of bacterization effects is commonly observed. In this case, the main sources of the observed variability probably may be ascribed to the condition of the host and to slight differences in environmental conditions between experiments, especially in soil water content. Although ice-tulips can be used the year around, the time required for full develoment of the plant decreases from c. 4.5 weeks in January to less than 3 weeks in December. This difference in development has its bearing on the susceptibility of the tulips to *Pythium* and on the period during which protection against infection is required. Soil water content influences the activity of both pathogen and introduced bacteria. From other results it is clear that the impact of environmental conditions on the outcome of bacterization experiments has not to be underestimated (Cook, 1988; Howie and Suslow, 1987; Nelson, 1988).

The observation that bacterization of the bulbs resulted in root rot suppression indicates that E11.3 exerts its beneficial influence in the rhizosphere. The average effect obtained with soil bacterization was somewhat better. This may suggest an additional effect occurring outside the rhizosphere, but the possibility of a better colonization of the rhizosphere and the roots cannot be excluded. Roots of tulips and especially of ice-tulips increase in length very fastly. The bacteria, therefore, have to grow and spread fastly to colonize the roots when applied to the bulbs. With soil bacterization a continuous colonization of the roots from the bulk soil may be envisaged. The degree of root colonization, however, was not determined.

In steamed soil root rot was more severe than in natural soil, indicating that the natural soil microflora is of importance in delimiting the disease. This is a general phenomenon with Pythium diseases, as is exemplified by disease suppressiveness of Mexican chinampa soils (Lumsden et al., 1987), of container media (Chen et al., 1988) and of certain soils in Hawai (Kao and Ko, 1986a, b), where suppressiveness of soil is primarily correlated with a high general microbial metabolic activity. In these cases the suppressive effects

are probably brought about by nutrient competition, depleting *Pythium* of the necessary ingredients for germination, growth and infection.

Interference of E11.3 in disease development may be due to effects on the tulips, on *Pythium* and on the resident microflora. The observation that E11.3 was also effective in Pythium-amended steamed soil indicates a direct effect on the host-pathogen system.

As yet, no attempts have been made to determine the means by which E11.3 exerts its root rot-suppressive effects, but tentatively these effects may be ascribed firstly to competition for nutrients with *Pythium*. This was also suggested to be part of the mechanism of Pythium disease suppression in cotton by introduced pseudomonads, the main mechanism of which appeared to involve either siderophores (Loper, 1988) or antibiotics (Howie and Suslow, 1986) and to be the primary mechanism for six biocontrol agents, a.o. *Pseudomonas* spp. for *Pythium* control in a variety of crops including cotton, cucumber and wheat (Elad and Chet, 1987). Furthermore, E11.3 is capable of producing siderophores. The possible involvement of these compounds in suppression of different diseases is sufficiently documented (see Introduction). As far as known, E11.3 does not produce antibiotics active against *Pythium*, but it is able to produce HCN. HCN inhibits mycelial growth of Pythium in vitro (results not shown). Moreover, HCN influences the physiology of the root. In the case of suppression of root rot of tobacco caused by *Thielaviopsis basicola*, HCN production by the introduced bacteria appeared to be responsible for the observed effects (Voisard et al., 1989). Root rot suppression by E11.3 may thus in part be due to production of HCN, influencing the pathogen, the host or both. However, although E11.3 consistently reduced disease in the experimental system used, some caution is needed with this isolate. Deleterious effects of bacteria have been observed too (Bakker and Schippers, 1987; Schippers et al., 1987a; Suslow, 1982), and these effects may ascribed to bacterial HCN production. Moreover, active defence against pathogens tends to decrease with increasing amounts of HCN present (Lieberei, 1989).

The current investigations were carried out to find a way for biological control of Pythium root rot in tulips. The initial results obtained are promising, as consistent root rot suppression was found with E11.3. However, the use of pseudomonads in tulip-growing practices is as yet a long way off. Environmental conditions for optimal effects have to be determined, for which insight in the mechanisms of root rot suppression by E11.3 will be of great help. Combination of different isolates with different mechanisms may be useful (Burr and Caesar, 1984), amplifying the root rot-suppressive effects and the conditions under which root rot suppression will occur. The most practical way for introduction of the bacteria has to be found. Application of the bacteria with methylcellulose will not do, as this compound by itself increased disease by *Pythium*. Whether the beneficial effects of E11.3 will extend beyond the model system of tulip cultivar Paul Richter and *P. ultimum* P17 has to be investigated. Furthermore, in devising a method for biological control of Pythium diseases in tulips, the importance of natural microbial populations in delimiting disease should not be neglected, no more than the prospects of other kinds of organisms, whose values as biocontrol agents of Pythium diseases of other hosts have been proven (Brown, 1987; Harman and Hadar, 1983; Martin and Hancock, 1987; Nelson, 1988; Paulitz and Baker, 1987; Walther and Gindrat, 1988).

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Samenvatting

Onderdrukking van Pythium wortelrot in tulpen met het fluorescerende Pseudomonas isolaat E11.3

Fluorescerende pseudomonaden werden geïsoleerd van tulpewortels of uit de rhizosfeer daarvan. Een aantal van deze isolaten is getoetst op wortelrot-onderdrukkend vermogen in een experimenteel systeem met tulpecultivar Paul Richter (vriestulp) als waardplant en *Pythium ultimum* isolaat P17 als pathogeen. Wortelrotonderdrukking werd consequent waargenomen na bacterisatie met *Pseudomonas* isolaat E11.3; de mate waarin rotonderdrukking optrad verschilde echter van experiment tot experiment. Bacterisatie vond plaats òf door de bacteriën door de grond te mengen òf door de bollen vlak voor het planten in een bacteriesuspensie te dompelen. Met beide methoden werden positieve resultaten bereikt. Toedienen van bacteriën in methylcellulose leidde ook tot reductie van de ziekte, maar heeft geen practische betekenis aangezien methylcellulose op zich de ziekte doet toenemen. Wortelrot was ernstiger in gestoomde dan in niet-gestoomde grond, maar in beide omstandigheden werkte E11.3 wortelrotonderdrukkend.

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