Biocontrol of Fusarium Wilt of Radish in Commercial Greenhouse Trials by Seed Treatment with *Pseudomonas fluorescens* WCS374

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ABSTRACT

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In 1989, research was initiated on biological control of Fusarium wilt of radish by the plant growth-promoting rhizobacterium strain, *Pseudomonas fluorescens* WCS374, in a commercial greenhouse. During 4 consecutive years, radish seeds with one of three treatments were drilled into naturally infested greenhouse soil for a number of successive crops of radish. The three seed treatments were control, film-coating control, and film-coating with bacteria. At harvest time of each successive crop of radish, the disease incidence, yield, and root colonization by the applied strain were monitored in 16 fixed plots (0.5 m^2) per treatment. In six out of 11 crops of radish, *P. fluorescens* WCS374 significantly ($P \leq 0.05$) suppressed Fusarium wilt disease, compared with the film-coating control. The relative reduction of disease by the bacterial treatment compared with the film-coating control treatment ranged from 18.6 to 68.3%, with an average of 42.6%, whereas the relative increase of yield ranged from 19.5 to 100.1%, with an average of 44.7%.

In The Netherlands, radish (*Raphanus sativus* L.) is cropped in greenhouses throughout the year, with approximately nine harvests each year. During the summer, the growth period for radish is 3 weeks, gradually increasing to approximately 11 weeks in winter (19). Throughout the year different radish cultivars are used, each cultivar being adapted to the growth conditions of that season.

Fusarium wilt of radish is a vascular disease caused by *Fusarium oxysporum* Schlecht. f. sp. *raphani* Kendrick & Snyder (formerly called *Fusarium oxysporum* Schlecht. f. sp. *conglutinans* [(Wollenw.) Snyder & Hansen] race 2 Armstrong & Armstrong) (3,5,10). The symptoms are browning and/or blackening of the xylem tissue of the root and radish, and yellowing of leaves, which turn brown and brittle. The disease occurs during periods (May to November) with high soil temperatures (22 to 24°C); low water potential of the soil is also favorable for disease development (4). In general, the disease recurs annually at the same locations in the greenhouse. The area in the greenhouse with wilted plants expands either slowly or very fast. Slow spread of the infested area in certain greenhouses may be explained by soil suppressiveness of a biotic nature as described for the Châteaurenard soil where strains of nonpathogenic *Fusarium oxysporum* are considered to be responsible for the soil suppressiveness (1,18,20), and for the Salinas Valley soil where fluorescent pseudomonads are thought to be the prime organisms of the soil suppressiveness (12,21). Rapid spread of the radish pathogen through the greenhouse is often recorded after steam disinfestation of the soil, making this treatment very ineffective for control of the disease (19). Soil fumigation with metham sodium has also been used to control this disease, resulting in plant growth enhancement and a simultaneous suppression of Fusarium wilt disease (19). However, this method is not very reliable, is rather expensive, and is only available with a special permit for radishes, which allows a maximum of 7.5 liters/100 m^{-2}. For the control of Fusarium wilt of radish this quantity is considered to be insufficient. Before 1992, 15 liters/100 m^{-2} was commonly used (Ing. M. G. J. M. Onderwater, Dienst Landbouwvoorlichting, Horst, The Netherlands, *personal communication*). In 1993, two highly resistant radish cultivars (Arista, Nunhems Zaden B.V., Haelen, The Netherlands, and Furabella, Nickerson-Zwaan B.V., Barendrecht, The Netherlands) were introduced on the Dutch market. Whether or not these cultivars will become a consistent success is not yet clear, because a high level of resistance to Fusarium wilt is only one of several important cultivar characteristics.

Biological control of soilborne plant pathogens with bacteria has been studied as an alternative or complementary approach to physical and chemical disease control measures for over 70 years (26). Root-colonizing bacteria that have a beneficial effect on plants are termed plant growth-promoting rhizobacteria (PGPR) (13). PGPR can improve plant growth either through direct stimulation of the plant or suppression of pathogens. The known modes of action for the disease-suppressive effects of the PGPR strains are competition for substrate (e.g., carbon, nitrogen, and ferric iron), niche exclusion, antibiosis, and induction of resistance (17,23,26). Reports of growth-promoting effects on radish by fluorescent pseudomonads were first published by Kloepper and Schroth (13) and later by Geels et al. (9). Seed bacterization of radish and seed tubers of potato with *Pseudomonas fluorescens* WCS374 resulted in significant plant growth-promotion in high frequency radish- and potato-cropping soil (8,9). In these studies, siderophore-mediated iron deprivation of deleterious microorganisms was considered the mode of action for the observed growth promotion (2). Biological control of Fusarium wilt of radish by fluorescent pseudomonads, through competition for ferric iron by bacterial siderophores, was reported by Scher and Baker (22). Recent studies have demonstrated that WCS374 induces systemic resistance in radish against Fusarium wilt (15).

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Biological control in the field by inundative introduction of selected microorganisms often has been inconsistent and therefore not commercially feasible (6,26). The objective of this investigation was to study the effects of repeated seed application of the PGPR strain *Pseudomonas fluorescens* WCS374 on Fusarium wilt and yield of radish, in successive radish crops in a commercial greenhouse. For studies on biological control, this model has advantages over many other models: (i) the cropping of radish in summer takes only 3 weeks, so the biological control only needs to last for a relatively short period; (ii) up to six commercial crops of radish can be harvested each year in the period of May to November, when Fusarium wilt occurs, offering the possibility of collecting multiple field data sets in one cropping season; (iii) radish is cropped in greenhouses where growing conditions can be controlled; (iv) the Fusarium wilt pathogen of radish causes distinct disease symptoms that are easy to measure; (v) radish is a direct-seeded crop that allows seed application of the bacterium in commercial greenhouse trials; (vi) radish is a small-sized plant and therefore easy to handle; (vii) Fusarium wilt control by common cultural practices is unsatisfactory and expensive; and (viii) growth-promotion, and suppression of Fusarium wilt of radish, by pseudomonads has been demonstrated.

MATERIALS AND METHODS

PGPR strain. *Pseudomonas fluorescens* strain WCS374 was isolated from potato rhizosphere (7). The bacteria were cultured overnight on King's Medium B (11) at 27°C. Bacterial suspensions were prepared by flooding the plates with 0.01 M $MgSO_4$ and scraping off the bacteria.

Seed treatments. At the end of August 1989, radish seeds (cv. Hilo, susceptible to Fusarium wilt, Nunhems Zaden B.V., Haelen, The Netherlands) were subjected to one of three treatments: (i) seeds disinfected with the fungicide thiram T-80% (2 g per kg^{-1} seeds, 1 kg ≈ 125,000 seeds), a standard commercial treatment (control); (ii) seeds not disinfected with thiram and film coated with 1% methyl cellulose (MC) without bacteria (film-coating control); or (iii) seeds not disinfected with thiram and film coated with *P. fluorescens* WCS374 (10^5 to 10^6 cfu per $seed^{-1}$) in MC (film-coating with bacteria) (Table 1). Seeds were coated manually (9) and afterwards dried in front of an air flow and stored at 6°C. The crops of cv. Hilo were followed by thiram-treated cv. Topsy (moderately resistant to Fusarium wilt, Nunhems Zaden B.V.) and cv. Tarzan (susceptible to Fusarium wilt, Enza Zaden B.V., Enkhuizen, The Netherlands) (Table 1). From April 1990 on, instead of thiram-treated seeds, nontreated seeds were used in the control treatment, the MC film-coating was replaced by a polyvinylacetate (PVA) film-coating, and WCS374-treated seeds were sown in each successive crop of radish. The PVA film-coating was applied in a vertical, fluidized-bed, spray coater (S & G Seeds B.V., Enkhuizen, The Netherlands). In this process, the seeds remained dry, gained only 1% in weight, and retained their original shape. Treated radish seeds cv. Saxa × Nova (moderately resistant to Fusarium wilt, S&G Seeds B.V., Enkhuizen, The Netherlands) were used in each successive crop of radish throughout the remainder of the trials, being replaced only in the winter period (October to March) by treated seeds of cv. Tarzan or cv. Rondar (susceptible to Fusarium wilt, S&G Seeds B.V.).

TABLE 1. Seed treatments in successive plantings of radish, 1989 to 1992

Year	Months of harvest	Radish cultivar	Seed treatment[a]		
			Control plots[b]	Film-coating control plots[c]	Film-coating WCS374 plots[d]
1989	Aug.[e]	Hilo	Commercial	Commercial	Commercial
	Sept.	Hilo	Commercial	MC	MC + WCS374
	Nov.	Topsy	Commercial	Commercial	Commercial
1990	Jan., Mar.	Tarzan	Commercial	Commercial	Commercial
	April through Oct.	Saxa × Nova	Untreated	PVA	PVA + WCS374
1991	Jan., Mar.	Tarzan	Untreated	PVA	PVA + WCS374
	April through Oct.	Saxa × Nova	Untreated	PVA	PVA + WCS374
1992	Jan., Mar.	Rondar	Untreated	PVA	PVA + WCS374
	April through Oct., Dec.	Saxa × Nova	Untreated	PVA	PVA + WCS374

[a] Commercial = seeds disinfected with fungicide thiram, a standard commercial treatment; Untreated = seeds not disinfected with thiram; MC = seeds not disinfected with thiram and film coated with methyl cellulose; PVA = seeds not disinfected with thiram and film coated with polyvinylacetate; WCS374 = *Pseudomonas fluorescens* WCS374 applied in film-coating
[b] Fixed plots assigned to the control treatment.
[c] Fixed plots assigned to the film-coating control treatment.
[d] Fixed plots assigned to the film-coating with WCS374 treatment.
[e] Harvest just before trials started.

Design of the trials. In a commercial greenhouse in Wellerlooi (The Netherlands) (7,000 m^2), continuous cropping of radish started in 1978. Fusarium wilt of radish first occurred in the summer of 1982. In this commercial greenhouse, Fusarium wilt of radish was localized and the areas with wilted plants expanded slowly. Prior to the drilling of the first treated crop of radish at the end of August 1989, the Fusarium wilt incidence was recorded in a commercial crop of radish at fixed plots in the greenhouse. For each treatment, 16 fixed sampling plots (0.5 m^2) were assigned in a 180-m^2 area. Seeds (220 to 235 seeds m^{-2}) were pneumatically drilled into the sandy soil (5% organic matter). After the harvest in August 1990, the soil was fumigated with metham sodium for the first time in its history, because the area of the locations infested with Fusarium wilt of radish exceeded the economic threshold.

At the harvest of successive crops of radish in the periods September to November 1989, May to September 1990, July to October 1991, and June to October 1992, the percentage of Fusarium-wilted plants, the percent yield of marketable radishes, and root colonization by bacteria were recorded in the fixed plots.

Determination of Fusarium wilt of radish. The percentage of Fusarium-wilted radish plants was determined by counting the wilted seedlings, and the plants with external (yellow/brown leaves) and/or internal disease symptoms. Internal disease symptoms were determined by making a cross-section through the base of the radish. Infection was visible as a black spot in the center.

Root colonization by pseudomonads and the applied strain. Root colonization by *P. fluorescens* strain WCS374 and the total population of fluorescent pseudomonads were recorded in eight fixed plots per seed treatment. Root parts (approx. 5 cm each) from directly below the radish, were pooled from approximately three plants (approx. 0.3 g fresh weight). These root parts were shaken vigorously in a test tube containing 5 ml of 0.01 M $MgSO_4$ and 0.5 g of glass beads (0.17 mm).

In another series of drillings, separate from the trials on disease suppression and yield increase, root colonization of *P. fluorescens* WCS374 was recorded at 16 fixed plots at three different depths: 0 to 10; 10 to 20; and 20 to 30 cm below the radish. In these trials, strain WCS374 was applied on cv. Saxa × Nova seeds in the PVA film-coating for three successive drillings. To check the survival of WCS374 in soil and on old roots remaining in soil after harvest, the colonization of new roots was monitored in two successive drillings of untreated seeds.

Immunofluorescence colony-staining (IFC) (24,25), which was adapted for the detection of fluorescent pseudomonads by Leeman et al. (14,15), was used to assess the number of viable cells of the introduced WCS374 in the root washings. King's Medium B agar (11), supplemented with ampicillin (50 $\mu g/ml^{-1}$), chloramphenicol (13 $\mu g/ml^{-1}$), and cycloheximide (100 $\mu g/ml^{-1}$) (KB+), was used for semiselective growth of the fluorescent *Pseudomonas* spp. for IFC.

Data analysis. Per harvest, data on the relative percentages of disease incidence and yield were, after arcsine square root transformation, analyzed for significance using analysis of variance (ANOVA) (SAS software, SAS Institute, Cary, N.C.). The data on the relative percentage of disease of the harvests in September 1990 and July and October 1991 were analyzed with the nonparametric Wilcoxon's two-sample rank sum test, because of nonnormal distribution of the data and heterogeneity of variances, also after arcsine square root transformations. Over the total period the relative percentages of disease incidence and yield were analyzed by the paired *t*-test for pairs of variables. Root colonization data were log transformed and analyzed by ANOVA followed by Fisher's least significant difference test.

RESULTS

Effect of *P. fluorescens* WCS374 on disease incidence. Before the trials started in August 1989, there was no significant difference ($P = 0.05$) in the percentage of diseased plants between the groups of fixed plots that were assigned to the different seed treatment trials (control 42.6%, film-coating control 37.7%, film-coating with WCS374 41.3%). The Fusarium wilt disease incidence in the fixed film-coating control plots fluctuated in the course of the trials between 0 and 50% in the successive crops of radish. Disease incidence in the untreated control plots was never significantly different from the film-coating control plots. Every year, the highest level of disease incidence was observed in the September harvest (Fig. 1). The year 1990 was an exception; the disease started very early in May, and in July the disease severity reached its maximum. In September 1990 and all of 1991, Fusarium wilt disease never exceeded the 5% level, probably due to the soil fumigation after the harvest of August 1990.

The bacterial treatment significantly ($P \leq 0.05$) reduced the relative percentage (relative to before the trials started in August 1989) of Fusarium wilt disease at the harvests in May and August 1990, in July, September, and October 1991, and in June, August, September, and October 1992, when compared with the film-coating control treatment. The plots with the bacterial treatment showed a decline in disease compared with the film-coating control plots in June 1990 ($P < 0.08$) and in July 1992 ($P < 0.06$), although not significant with the statistical procedures used.

Significant disease suppression by the bacterial treatment was demonstrated in nine out of 16 harvests, compared with the film-coating control. The average relative reduction of Fusarium wilt of radish by WCS374 over the 4-year period amounted to 50.7% (ranging from 18.6 to 90.5%), compared with the film-coating control (significant difference, $P < 0.0001$, paired *t*-test for pairs of variables). If the harvests of September 1990 and all 1991 harvests are not considered, because disease incidence was below 5%, significant disease suppression by WCS374 was demonstrated in six out of 11 harvests, compared with the film-coating control. In that case, the average relative disease control by the bacterial treatment amounted to 42.6% (ranging from 18.6 to 68.3%) (significantly different from film-coating control, $P < 0.0001$, paired *t*-test for pairs of variables). Other harvests during the course of the trials are not reported, because disease did not exceed the 5% level.

Effect of *P. fluorescens* WCS374 on yield. Before the trials started in August 1989, there was no significant difference ($P = 0.05$) in percentage of marketable radishes (yield), between the three groups of fixed plots that were assigned to the different seed treatment trials (control 47.1%, film-coating control 52.3%, film-coating with WCS374 49.3%). In the successive trials, the yield of the untreated control was never significantly different from the film-coating control.

At the harvests of June 1990 and June, July, August, September, and October 1992, WCS374 significantly ($P \leq 0.05$) increased the relative percentage (relative to before the trials started in August 1989) of yield, compared with the film-coating control (Fig. 2). There was a nonsignificant increase in the relative percentage of yield in the plots with bacterial treatments at the harvest of September 1989 ($P < 0.09$) and August 1990 ($P < 0.06$), compared with the film-coating control treatment. In September 1990 and all of 1991, when disease incidence was below 5%, no significant increases in the relative percentage of yield due to WCS374 were observed.

Significant yield increase by WCS374 was demonstrated in six out of 16 harvests, compared with the film-coating control. The relative increase of yield by WCS374 over the 4-year period amounted to 36.4% (ranging from 10.4 to 100.1%), compared with the film-coating control (significant difference, $P < 0.0001$, paired *t*-test for pairs of variables). If the harvests with a low (<5%) level of disease incidence are not considered, the relative increase in yield due to WCS374 was 44.7% (ranging from 19.5 to 100.1%) (significantly different from film-coating control, $P < 0.0001$, paired *t*-test for pairs of variables). At the harvests of July, August, and October 1991 more than 30% of the uninfected radishes was not marketable, because of unknown factors. A loss of approximately 10% is considered normal.

Root colonization by *P. fluorescens* WCS374 and indigenous pseudomonads. The fluorescent pseudomonad population den-

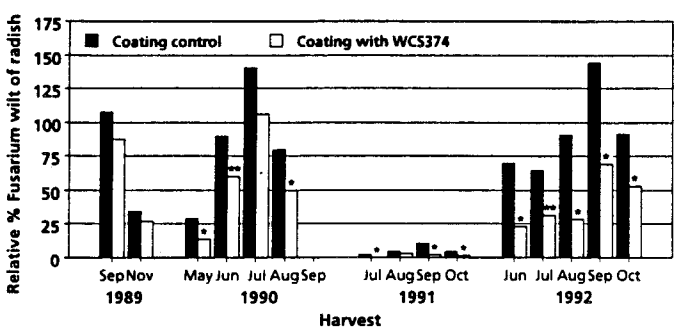

Fig. 1. Relative percentage of Fusarium wilt of radish at a number of successive harvests in summer periods of 1989 to 1992, compared with the situation before trials started, when the percentage of disease in plots assigned to film-coating control and film-coating with bacteria was 37.7% and 41.3%, respectively. Seeds received film-coating without bacteria (film-coating control) or film-coating with *P. fluorescens* WCS374 (film-coating with WCS374). Soil was fumigated with metham sodium in August 1990. (Per harvest, bars with one asterisk differ significantly from the corresponding film-coating control at $P \leq 0.05$; bars with two asterisks differ significantly at $P \leq 0.08$, analysis of variance, except for harvests of September 1990 and July and October 1991, which were analyzed by Wilcoxon's rank sum test.)

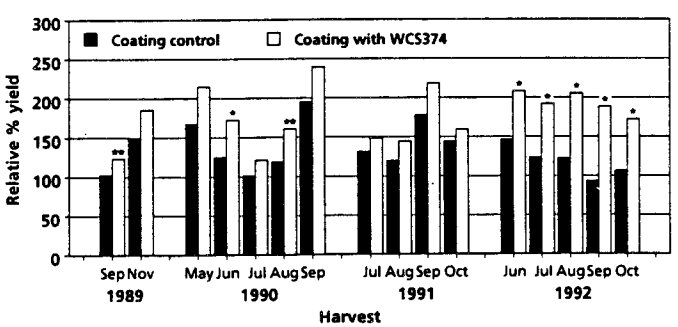

Fig. 2. Relative percentage of yield at a number of successive harvests in summer periods of 1989 to 1992, compared with situation before trials started, when the percentage of marketable radishes in plots assigned to film-coating control and film-coating with bacteria was 52.3% and 49.3%, respectively. Seeds received film-coating without bacteria (film-coating control) or film-coating with *P. fluorescens* WCS374 (film-coating with WCS374). (Per harvest, bars with one asterisk differ significantly from the corresponding film-coating control at $P \leq 0.05$; bars with two asterisks differ significantly at $P \leq 0.09$, analysis of variance.)

sity (KB+ population) varied in all treatments between 5 and 7 log cfu per g^{-1} of root fresh weight (Fig. 3). The introduced *P. fluorescens* strain WCS374 colonized the roots of radish resulting in population densities between 4 and 6 log cfu per g^{-1} of root fresh weight. Root colonization at harvest time and disease reduction/yield increase were not correlated. At the harvest of November 1989 (untreated seeds for this crop were drilled at the end of September 1989), *P. fluorescens* WCS374 was recovered (4.6 log cfu per g^{-1} of root) on the roots, although the strain had been delivered on the seeds of the previous crop of radish, which were drilled at the end of August and harvested in September 1989. The applied strain WCS374 was never isolated from the roots of plants coming from the control or film-coating control. On average, the applied strain contributed 3.9% (ranging from 0.4 to 9.8%) to the total fluorescent pseudomonad population.

In the separate series of drillings in the winter of 1991, the root colonization was monitored at three depths. At the first harvest (January), strain WCS374 was recovered only from the upper root part (0 to 10 cm) (Fig. 4). At the harvest of the second and third crop of radish (March and April), the introduced strain could be detected on all depths. However, the highest population density was still detected at the upper root part. At the fourth harvest (May), the first untreated crop of radish, the introduced strain was still present at all depths, although the population densities were reduced in the upper root parts. One drilling later (June), WCS374 was no longer recovered. This experiment was repeated in another period of the year with similar results (not shown).

DISCUSSION

Due to seasonal influences on the development of Fusarium wilt of radish, a continuous fluctuation in disease incidence and in yield of marketable radishes was observed over the year. This variability in disease incidence interferes with studying the effects of bacterization. For this reason, the effects of the different seed treatments were studied in fixed plots in the greenhouse throughout the trials and were analyzed for significant differences by comparing the relative (relative to before the trials started in August 1989) percentages of disease and yield of the treatments.

Seed bacterization with *P. fluorescens* WCS374 significantly reduced the relative percentage of diseased plants by 42.6% and correspondingly increased the relative percentage of yield by 44.7% compared with the film-coating control, at those harvests in which the percentage of diseased plants was above 5%. The yield increases in the bacterial treatment were probably due to disease suppression and not to direct growth-promotion, because no significant increases in yield were observed when the disease incidence was below 5%. Additional testing with this experimental approach was done in a series of five drillings at another commercial greenhouse in 1992. WCS374, applied in the PVA film-coating, resulted in a reduction of the relative percentage of disease of 13.4%, and an increase of the relative percentage of yield of 16.1% (R. J. Scheffer, *unpublished*). These numbers are comparable with the results obtained in the trials described in this paper, which were observed in the year of the first application of the bacterial treatment (relative disease reduction 1989: 20.5%; relative yield increase 1989: 21.8%). The disease control over the 4-year period was rather consistent. At six out of 11 harvests, in which disease incidence was above 5%, the bacterial treatment significantly reduced the relative percentage of Fusarium wilt disease, compared with the film-coating control treatment. At two additional harvests, disease was suppressed, although not significantly. Compared with the relative inefficiency and/or inconsistency of disease control in other field or commercial greenhouse trials (26), the results described here are very promising for commercial application of *P. fluorescens* WCS374.

The mechanisms by which WCS374 reduced disease in the commercial greenhouse remain to be elucidated. In a bioassay on rockwool, WCS374 suppressed Fusarium wilt of radish by the induction of systemic resistance (15,16). Also in a bioassay with naturally infested soil from the commercial greenhouse, evidence was collected for WCS374-mediated induced resistance (16). Thus, induced disease resistance may have been involved in the commercial greenhouse trials.

Soil fumigation with metham sodium after the harvest of August 1990 suppressed Fusarium wilt of radish, not only for the remainder of that year, but also for 1991. This metham sodium treatment, in combination with the repeated seed bacterization that started in March 1990 and continued directly after fumigation, possibly resulted in a reduction of the usual rapid build-up of the pathogen after soil fumigation. This resulted in the highest disease reduction over the 4-year period, observed at the harvests of the last year, 1992.

The relatively low yields in July, August, and October 1991, when Fusarium wilt disease was almost absent, were due to the high variability in size of the radishes in all treatments. This variability may have been caused by environmental factors (e.g., temperature, moisture) and/or cultural practices (e.g., fertilization).

In November 1989, WCS374 was recovered from the roots of radish that developed from untreated seeds. This is best explained by the fact that after the harvest old roots, which remained in the soil, acted as an inoculum source for the new roots that developed from the untreated seeds of the successive crop of radish. The trials on root colonization by WCS374 at different depths also demonstrated that the strain remained present in the soil (old roots) after harvesting, and was able to colonize the first untreated successive crop of radish. At the first application, the introduced

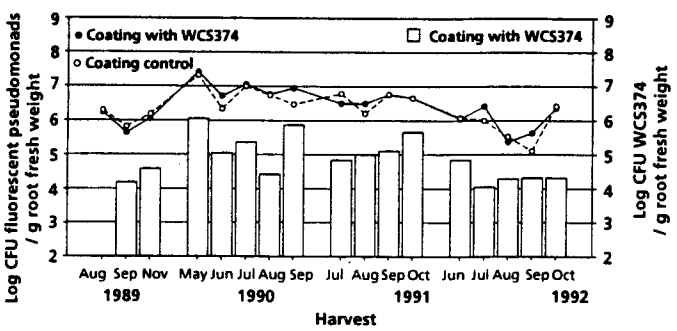

Fig. 3. Root colonization by fluorescent pseudomonads (KB+ population, KB+ = King's Medium B agar [11], supplemented with ampicillin [50 μg/ml^{-1}], chloramphenicol [13 μg/ml^{-1}], and cycloheximide [100 μg/ml^{-1}]) and *P. fluorescens* WCS374 at a number of successive harvests in summer periods of 1989 to 1992. Seeds received film-coating without bacteria (film-coating control) or film-coating with *P. fluorescens* WCS374 (film-coating with WCS374). (August 1989 harvest represents situation before trials started.)

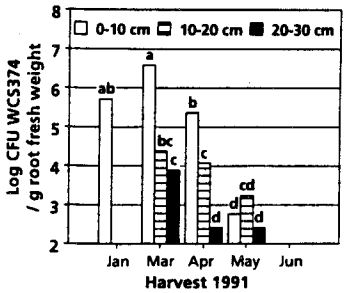

Fig. 4. Root colonization by *P. fluorescens* WCS374 at different depths (0 to 10, 10 to 20, 20 to 30 cm), at a number of successive harvests in winter of 1991. Saxa × Nova seeds of crops harvested in January, March, and April were film coated with bacteria in polyvinylacetate; successive crops harvested in May and June were untreated. Detection limit for introduced strain was 2 log cfu per g of root. (Bars with same letter are not significantly different at $P \leq 0.05$, analysis of variance followed by Fisher's least significant difference test.)

strain only colonized the upper part of the root (0 to 10 cm below the radish). In successive drillings with WCS374-bacterized seeds, the strain was also recovered from lower root parts (10 to 20 and 20 to 30 cm below the radish). Again, this may be explained by the fact that colonization occurred from colonized roots of the previous drillings, which remained in the soil and were mixed through the soil by the milling machine. However, root colonization densities of WCS374 in the successive crops of radish were the highest in the upper part of the roots (0 to 10 cm below the radish). This root part probably was mainly colonized from the bacterized seed.

It is concluded that seed film-coating with *P. fluorescens* WCS374 can result in a consistent and significant suppression of Fusarium wilt of radish in the commercial greenhouse. To grow radish commercially, an average harvest of 80 bunches (20 radishes each) of radishes m^{-2} $year^{-1}$ is required. A 10% loss of yield due to Fusarium wilt can already result in a nonprofitable situation. Therefore, for the commercial grower of radish, the oberved yield increase due to seed film-coating with WCS374 is very attractive. Starting in 1995, radish seeds with "BioCoat" will be commercially available from S&G Seeds B.V.

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