Investigations on Crop Developments and Microbial Suppressiveness of *Pythium aphanidermatum* after Different Disinfection Treatments of the Circulating Nutrient Solution

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Abstract

In this study results are presented of investigations on the development of a cucumber crop in a closed rockwool system and on the potential change in microbial suppressiveness due to different disinfection methods after inoculating part of the plants with the fungal pathogen *Pythium aphanidermatum*. Two trials were executed at which the core treatments were disinfection (UV, slow filtration and no disinfection as control), while additional treatments were priming half of the systems before planting either with an "old" nutrient solution from a former cucumber crop (spring trial) or an actinomycetes suspension (autumn trial). Crop developments (yield, disease and root development) and composition of the microflora were recorded. Non-inoculated plants showed that disinfection is needed either with UV or slow (sand) filtration to avoid spread of the pathogen; yield and root development was better and disease symptoms were less compared to the control. The hypothesis that passive disinfection might realise a more suppressive environment against *Pythium aphanidermatum* could not be proved, since inoculated plants showed a similar yield, disease and root development after the different disinfection treatments. Priming the nutrient solution with an "old" solution did not show significant differences in the composition of the microflora either by plate-counts or by PCR-DGGE. However, the addition of actinomycetes before planting resulted a 100 to 1000 fold increase of the actinomycetes population in the cropping system up to the end of the trial. In the spring trial, differences within the microflora as a result of the disinfection treatments were only detected for pseudomonads. In the autumn trial, differences in numbers of pseudomonads, actinomycetes and fungi were significant in the treated nutrient solution, but not in the drain. With PCR-DGGE, differences in the bacterial profile were detected in autumn in both the drain and treated solution; i.e. less bands (corresponding to certain bacterial species) occurred in the UV treatment compared to slow filtration and control.

INTRODUCTION

Growers attempt to overcome problems of pathogen dissemination in the circulating nutrient solution by disinfecting the water. Disinfection can take place either by "active" methods (heat treatment (Runia et al., 1988), ozone treatment and UV-radiation (Runia, 1996)) at which the nutrient solution is sterilised, which may result in an unbalanced recolonisation or by "passive" methods (slow sand filtration (Wohanka, 1995; Van Os et al., 1997; Runia et al., 1997), lava and membrane filtration) at which at least part of the resident microflora survives the treatment and may be able to suppress certain pathogens (McPherson et al., 1995; Postma et al., 2000). To optimise a microbial-balanced growing system it is necessary to study the microflora in nutrient solutions in relation to different "passive" or "active" water disinfection methods, while the potential for microbial suppression can be studied by addition of certain micro-organisms at the start of cropping. A solution from another cucumber crop is supposed to have a suppressive microflora, which could be used to prime the new solution. Another priming

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could be done by application of actinomycetes, which are suggested to play a role in disease suppression, since their presence correlated with disease suppressiveness in used rockwool, as well as in soil systems (Workneh and Van Bruggen, 1994) and compost (Tuitert et al., 1998).

Summarized, the following aims of this study are:

- to investigate the crop developments (yield, disease symptoms, root development) after inoculation with the pathogen *Pythium aphanidermatum*;
- the potential change in microbial suppressiveness due to different disinfection methods (UV as "active", slow filtration as "passive" method and no disinfection as control) in a cucumber crop;
- the spread of the pathogen to the non-inoculated part, as well as the development within the inoculated part;
- to study the effects on the microflora by priming the system with an additional microbial population either by using an "old" nutrient solution (from a former cucumber crop) or by adding specific micro-organisms.

MATERIAL AND METHODS

Treatments

Two trials (spring and autumn) with a cucumber crop grown on rockwool were conducted in a 300 m^2 greenhouse. In total there were 12 independent closed systems, which means that the superfluous nutrient solution was continuously reused. Before both trials all systems were cleaned with a quaternary ammonium compound. There were three core treatments based on disinfection of the nutrient solution:

- "active" disinfection: continuous treatment by a 36 W Ultra Violet lamp, realising a total dosage of 250 mJ cm^{-2};
- "passive" disinfection: continuous treatment via slow sand filtration (sand characteristics: 0.15-0.3 mm; D_{10} = 0.23 mm; D_{60} = 0.30 mm; Uniformity Coefficient = 1.33). The flow rate was set at 300 L m^{-2} h^{-1};
- control: no disinfection of the nutrient solution.

Each core treatment existed of four independent closed systems. Additionally there were the following treatments:

- Spring: two closed systems were filled with "fresh" and two systems were filled with "old" nutrient solution for each mentioned disinfection treatment. The "old" nutrient solution originated from an apparently healthy three month-old cucumber crop. All slabs of the treatment were initially filled with the "old" solution (10 L per slab).
- Autumn: two closed systems were filled with a "fresh" nutrient solution and two systems were filled with an additional actinomycete suspension for each mentioned disinfection treatment. For this, eight filamentous actinomycetes, originally isolated from soilless culture systems, were grown on chitin oat agar. Spores were scraped from the agar and suspended in water. This spore suspension was added to the initial nutrient solution to fill the rockwool slabs at the start of the experiment. In total, $9x10^{10}$ cfu were added per system ($1.2x10^{10}$ cfu streptomycetes and $7.8x10^{10}$ cfu micromonospora type actinomycetes). Since each system contained about 120 L nutrient solution, about $8x10^5$ cfu ml^{-1} nutrient solution were added.

In spring, cultivar Sudica was used and planted on May 8, 2000, while the trial was finished at July 10, after 64 days. In autumn cultivar Kjell was used and planted at August 15. The trial was finished after 80 days on November 3. For both trials, plants were risen in a small greenhouse at an average temperature of 23°C and planted on the slabs at an age of 21 days. Seven days after planting, a 50 ml suspension of the pathogen *Pythium aphanidermatum*, isolate 89, was added on top of the rockwool blocks of the young plants (approx. $2x10^4$ cfu $plant^{-1}$). Of each row, only the half of the plants standing closest to the drain were inoculated. The suspension was obtained by blending 80 agar plates (potato dextrose agar without antibiotics) grown for 8 days at 25°C, in 8 L of tap water.

Observations

To assess the effects of the different treatments and the inoculation of the plants with *Pythium aphanidermatum* the following observations were made:

- Yield: numbers and weight of 1st and 2nd quality cucumber fruits were recorded per treatment and for inoculated and non-inoculated plants during the two trials.
- Disease detection:
  - Number of zoospores: one week before inoculation and thereafter every 2 or 3 weeks samples were collected of the nutrient solution in the "drain", directly after flowing out of the trough, and in the container collecting the treated nutrient solution. Colony forming units of *Pythium aphanidermatum* were enumerated on a selective medium after the propagules were concentrated on a filter (Postma et al., in preparation).
  - Stem rot and wilting symptoms: number of diseased plants (i.e. brown stem base, wilted or dead plants) was counted separately in the inoculated and non-inoculated part. In general counting took place once a week.
- Root development (coverage and browning): at the end of the experiment the plastic cover of the rockwool slabs was removed and the slabs were turned-over.
  - Coverage was scored per slab using an index with a scale from 0 (few roots) to 3 (many roots) to distinguish the differences between the treatments.
  - Discoloration was also scored per slab using an index with a scale from 0 (none of the roots were brown discoloured) to 3 (almost all roots were discoloured) to distinguish the differences between the treatments.
- Microflora: at five dates, samples for plate counts and PCR-DGGE (polymerase chain reaction followed by denaturing gradient gel electrophoresis) were taken from the drain and of the treated solution to assess the dynamics of the microflora during cropping.
  - Plate counts on semi-selective media were used to quantify colony forming units (cfu) of the following groups of micro-organisms: total aerobic bacteria (R2A), fluorescent pseudomonads (KB), filamentous actinomycetes (COA with a filter), fungi and yeasts (¼ PDA).
  - PCR-DGGE profiles of the bacterial population were prepared following the method described by Postma et al. (2000).

Statistical analyses were performed by using the statistical programme Genstat 5 (1999), release 4.1 at a significance level of $p=0.05$. Disease percentages were analysed on square root transformed data to balance the residuals. Plate counts (cfu) were analysed on the ^{10}log scale to balance the residuals.

RESULTS

Plants in the different disinfection treatments inoculated with *Pythium aphanidermatum* did not have any significant difference in number of fruits (13.3 and 21.6 pieces m^{-2} on average in spring and autumn, respectively) and total weight m^{-2} (7.40 and 8.65 kg m^{-2} on average in spring and autumn, respectively), neither in the spring nor in the autumn trial. No significant difference was obtained between the "fresh" and "old" solution and in the fresh solution with or without addition of actinomycetes.

Between the non-inoculated plants in the different core and additional treatments there appeared significant differences in yield (Table 1). In the spring trial no disinfection (control) realised lower yields (number and weight) than with filtration and UV. The use of an "old" or primed solution may give advantages for filtration and control. In the autumn trial results were somewhat different. Without addition of actinomycetes (comparable with "fresh" in spring), UV realised similar (number) or lower (weight) yields than filtration and control. Addition of actinomycetes decreased yield in the control (number and weight).

In spring, around 200 cfu L^{-1} of zoospores could be detected in the drain of a system (at day 28 and 49). No significant differences in *Pythium* propagules between old and fresh solution could be detected. In the treated solution a significant difference could

be detected at day 28 and 49 ($LSD_{0.05}$ is 157 and 183, respectively). It shows that UV and slow filtration significantly removed *Pythium*. In autumn the total amount of propagules was lower than in spring: on average around 40 cfu L^{-1} in the drain and around 20 in the control in the container after treatment. Again, UV and slow filtration significantly removed *Pythium*.

The efficiency of the UV treatment was tested by counting the number of total aerobic bacteria on agar plates in samples taken immediately after the UV lamp (sampling every 2-3 weeks during cropping). In spring, between 96.4 and 99.7% of the bacteria were killed; in autumn between 74.1 and 98.7%. It appeared that only few types of bacteria survived, since only 2-5 colony types were detected. In week 3 of the autumn trial, the efficacy of the UV was suddenly low in two systems. Both phenomena, the high number of *Pythium* spores and the low efficacy of UV, may have their cause in an accidentally passage of a large particle behind which spores hide themselves and survived the UV treatment. This was followed by multiplication in the container before sampling. No other explanation such as dirty lamps or a continuous high turbidity could be found.

The weekly scoring of *Pythium* symptoms on the stem base and wilting of the plants have been elaborated in Fig. 1. The percentage of diseased plants in the inoculated part appeared to be higher in spring (about 60% of the plants) than in autumn (around 40%). In the non-inoculated part (receiving only treated solution) the control treatments can be recognised, because of a high percentage of diseased plants in spring. The percentage is even higher than in the inoculated part, due to a sudden outbreak of *Pythium*, most of the plants in one row died within a fortnight. UV and slow filtration significantly reduced the number of diseased plants compared to the untreated control at day 49, 56 and 63 ($LSD_{0.05}$ is 50, 48 and 38 for day 49, 56 and 63, respectively). On day 65 of the autumn trial the actinomycetes inoculation caused a higher percentage of diseased plants in the UV compared to the treatment without addition of actinomycetes. On day 85 significancy disappeared again.

Turning round of the slabs at the end of the crop showed differences in the coverage and the discoloration of the slab, especially in the non-inoculated part. In table 2 it can be seen that in spring, the values for coverage and discoloration in the inoculated part are similar per treatment: moderate coverage and high discoloration. In the non-inoculated part the control treatments were similar to the inoculated part, while for UV and slow filtration root development improved. In the autumn trial values were similar. The additional treatments ("fresh"/"old" in spring and with or without addition of actinomycetes in autumn) did not give significant differences.

In both trials, bacterial numbers were fluctuating around 10^6 cfu ml^{-1} during the whole experiment. Numbers of fluorescent pseudomonads decreased, whereas numbers of fungi increased significantly during crop growth. The number of yeasts is supposed to be high because of the total "sterilisation" of the whole growing system before planting. The number of actinomycetes was very low in the spring trial and increased, because of their application, in the autumn trial. It is remarkable that both groups, streptomycetes and micronospora type actinomycetes, kept alive during the entire trial after a single application before planting.

In Figure 2, an overview is given of the microflora in the nutrient solution in the drain and in the collecting tank after the disinfection treatment detected by plate counts at 6 weeks after planting in the spring trial. Here, significant differences between the microflora as a result of the disinfection treatments were only detected for pseudomonads. In the autumn trial lower numbers of pseudomonads, actinomycetes and fungi were present in the UV treated nutrient solution compared to the control. These differences did not occur in the drain. Differences in the bacterial profile were detected with PCR-DGGE in autumn in both the drain and treated solution; i.e. less bands (corresponding certain bacterial species) occurred in the UV treatment compared to slow filtration and control.

DISCUSSION

The hypothesis that passive disinfection might realise a more suppressive

environment against *Pythium aphanidermatum* could not be proven. For plants inoculated with *Pythium*, yield, disease symptoms and root development were similar for all three disinfection treatments. Plants not inoculated with *Pythium*, proved that disinfection is needed. UV treatment and slow filtration gave better root development and less symptoms than the control. In spring, this resulted in more yield for UV and slow filtration compared to the control, whereas differences in autumn were less clear probably due to the lower level of disease. The additional treatments, priming with an "old" nutrient solution or addition of actinomycetes did not show significant differences compared to the treatments without these additions for most of the observations.

In general the *Pythium* symptoms in spring were more severe than in autumn. In spring about 70% and in autumn about 40% of the inoculated plants showed symptoms. Yield (number of fruits) was reduced by 18% in spring and 4% in autumn (differences between the mean of healthy treatments and mean of inoculated treatments). The higher level of disease in spring than in autumn was probably caused by differences in temperature in combination with day length and plant load (Paternotte, 1992). It was also shown that not all *Pythium* symptoms immediately cause a decrease in yield.

The microbial composition (plate counts and PCR-DGGE) did not show significant differences between a "fresh" solution and a solution primed with an "old" solution from a former cucumber crop. Such differences were expected, since fungal as well as filamentous actinomycetes populations generally increase during crop growth. Previous experiments showed that nutrient solution taken from used rockwool slabs, which were suppressive to *Pythium*, contained more fungi (Postma et al., 2000) and actinomycetes (Postma et al., unpublished data). The addition of a mix of filamentous actinomycetes isolates clearly changed the microbial composition, since 100 to 1000 times higher actinomycetes populations were present up to the end of the trial. Although these additional actinomycetes did not increase the suppressiveness of the system, the unexpected good survival of both streptomycetes and micromonospora type actinomycetes is hopeful. Possibly they can balance the microbial population in other situations such as after replanting or more antagonistic isolates can be found when screening more isolates.

Differences in the composition of the microflora due to the disinfection treatments were detected in the treated solution, which is used as irrigation water for the crop. One could expect this, since bacteria in the nutrient solution had been killed for 99% by the UV treatment and recolonisation will result in an altered microbial population. Slow filtration only removes a minor part (up to 10%) of the bacterial population, but causes a shift in the composition (pseudomonads, actinomycetes and fungi are removed to a larger extent) (van Os and Postma, 2000). After the nutrient solution had passed the rockwool slabs containing plant roots (i.e. in the drain) the differences present in the treated solution often disappeared. Thus plant roots created partly their own microbial composition. Nevertheless, few differences could still be detected in the drain: less pseudomonads (spring) and missing bands in the PCR-DGGE profile of the bacterial population (autumn) in the UV treatment compared to slow filtration and control.

From all these data it can be concluded that, although differences in disease suppression did not occur, the disinfection treatments had a small but significant effect on the microbial composition in the irrigation water as well as in the drain. However, yield in the different disinfection treatments was not influenced dramatically. Plant roots create in the substrate their own environment with its own microflora. Results do not give occasion to change the practical guidelines to increase yield or to improve the quality of the product by introducing certain groups of micro-organisms.

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Tables

Table 1. Yield of cucumber fruits per m^2 in non-inoculated core (disinfection) and additional ("old", addition of actinomycetes) treatments in spring and autumn trial.

		Spring[z]		Autumn[z]	
	Disinfection	"fresh"	"old"	"fresh"	+ actinomycetes
Number	UV	17.0^{c}	16.3^{bc}	21.0^{ab}	22.6^{b}
	Filtration	14.9^{b}	16.8^{c}	22.6^{b}	23.7^{b}
	Control	12.8^{a}	14.8^{b}	22.2^{b}	20.2^{a}
Weight	UV	10.01^{d}	9.27^{cd}	7.72^{a}	8.89^{b}
	Filtration	8.52^{bc}	9.40^{cd}	9.06^{bc}	9.78^{c}
	Control	6.88^{a}	7.83^{ab}	8.95^{b}	7.77^{a}

[z] Means with the same letter within each trial did not differ significantly ($p<0.05$).

Table 2: Root development per treatment for inoculated and non-inoculated plants.

	Inoculated Spring		Non-inoculated Spring		Inoculated Autumn		Non-inoculated Autumn	
	"fresh"	"old"	"fresh"	"old"	"fresh"	+actino-mycetes	"fresh"	+actino-mycetes
Coverage								
UV	1.58	1.29	1.92	2.67	1.33	1.50	2.58	2.42
Filtration	1.75	1.75	2.50	2.58	2.08	1.83	2.50	2.08
Control	1.63	1.67	1.42	1.38	1.83	1.67	2.50	1.67
Discoloration								
UV	2.25	2.67	1.38	0.96	2.58	2.75	0.92	1.08
Filtration	2.54	2.29	0.92	1.50	2.92	2.75	2.42	1.92
Control	2.29	2.67	2.29	2.42	2.67	2.92	2.25	2.42

$LSD_{0.05}$ is 1.1 and 1.0 for coverage and discoloration, respectively, in spring and 1.1 and 0.6 for coverage and discoloration, respectively, in autumn.

Figures

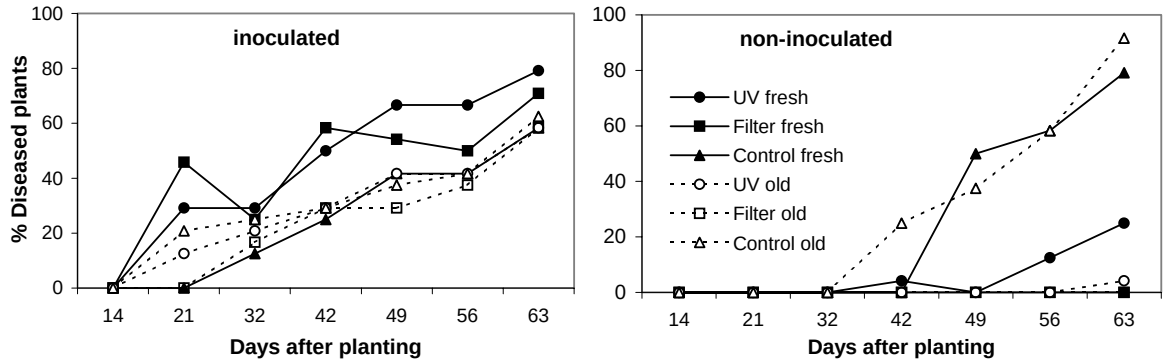

Fig. 1. Number of diseased plants in the inoculated and non-inoculated part per treatment in spring trial.

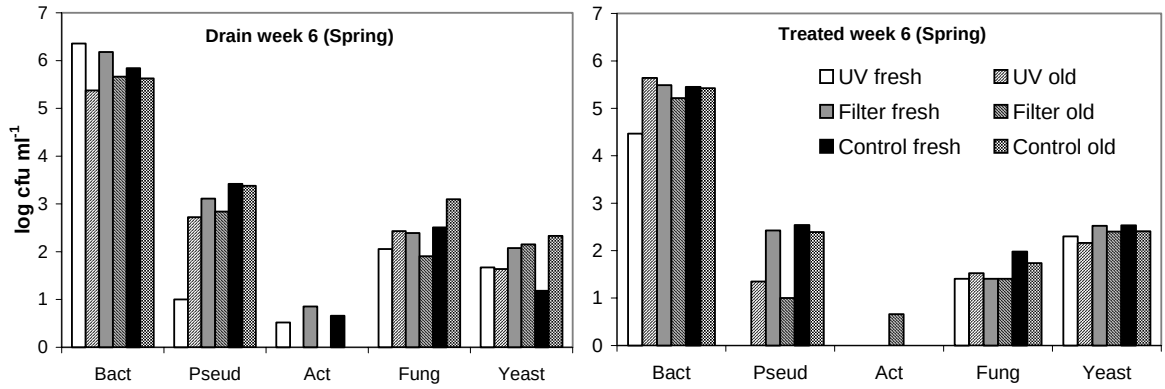

Fig. 2. Population of micro-organisms (log cfu+1 ml^{-1}) collected from different semi-selective media 6 weeks after planting, before (drain) and after disinfection treatment in spring trial. $LSD_{0.05}$ is 0.56 for total bacteria, 0.86 for fluorescent pseudomonads, 0.30 for actinomycetes, 0.66 for total fungi, and 0.56 for yeasts.