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Evaluation of a method for quantification of *Pythium aphanidermatum* in cucumber roots at different temperatures and inoculum densities

Evaluierung einer Methode zur Quantifizierung von *Pythium aphanidermatum* in Gurkenwurzeln bei verschiedenen Temperaturen und Inokulumdichten

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Summary

A method for the quantification of *Pythium aphanidermatum* density in cucumber roots based on an indirect enzyme-linked immunosorbent assay (ELISA) was established and tested. This approach was applied in three experiments under various environmental conditions. In addition, different inoculum densities were applied to vary the disease severity but also, to search for suitable inoculum densities in long-term epidemiological studies. Cucumber plants were grown in containers with aerated nutrient solution in a growth chamber at air temperatures of 15, 20, 25 and 30°C, and were inoculated with 0, 10, 10^3 and 10^5 oospores of *P. aphanidermatum* per litre nutrient solution. The pathogen density in the roots increased with inoculum density and temperature and resulted in growth reduction of the cucumber plants. Clearly, low temperatures delayed the development of the disease in the plant, while high temperatures combined with high inoculum densities led to sudden death of some plants. Therefore, inoculum densities not higher than 10^3 oospores per litre should be applied in long-term experiments. Independent of inoculum density and temperature, correlations were established between the mycelium density in the roots and the crop biomass, indicating that the indirect ELISA produces robust estimates of *P. aphanidermatum* density in cucumber roots under various conditions.

Key words: hydroponic, indirect ELISA, nutrient solution, oospores, plant growth, polyclonal antiserum

Zusammenfassung

Eine Methode zur Bestimmung der Dichte von *Pythium aphanidermatum* in Gurkenwurzeln wurde durch Anwendung eines indirekten enzyme-linked immunosorbent assay (ELISA) entwickelt und in drei Experimenten mit unterschiedlichen klimatischen Bedingungen getestet. Dabei wurde auch die Inokulumdichte variiert, um weitere Abstufungen der Erkrankung zu erhalten, aber auch, um geeignete Inokulumdichten für Langzeitstudien zur Epidemiologie herauszufinden. Gurkenpflanzen wurden in Gefäßen mit belüfteter Nährlösung in Klimakammern bei Lufttemperaturen von 15, 20, 25 und 30°C kultiviert und mit 0, 10, 10^3 and 10^5 Oosporen von *P. aphanidermatum* pro Liter Nährlösung inokuliert. Die Pathogendichte in der Wurzel stieg mit der Inokulumdichte und der Temperatur, wodurch sich das Wachstum der Pflanzen verringerte. Bei 20°C war die Krankheitsentwicklung deutlich verzögert, während 25 und 30°C in Kombination mit hoher Inokulumdichte zum vorzeitigen Absterben einiger Pflanzen führten. Deshalb sollten Inokulumdichten von mehr als 10^3 Oosporen je Liter in Langzeitexperimenten nicht verwendet werden. Über alle Inokulumdichten und Temperaturen wurden Korrelationen zwischen der Myzeldichte in der Wurzel und der Pflanzenbiomasse gefunden. Diese Korrelationen zeigen, dass der indirekte ELISA eine robuste Schätzung der Dichte von *P. aphanidermatum* in Gurkenwurzeln unter verschieden Bedingungen liefert.

Stichwörter: Hydroponik, indirekter ELISA, Nährlösung, Oospore, polyklonales Antiserum, Wachstum

1 Introduction

Hydroponic systems are often infested by plant pathogens, leading to root diseases which ultimately result in yield losses in various crops, e.g. lettuce (Utkhede et al. 2000), tomato (Jenkins and Averre 1983), pepper (Owen-Going et al. 2003) and cucumber (Moulin et al. 1994). Zoosporic pathogens can easily infect and then spread in the nutrient solution (Stanghellini and Rasmussen 1994). *Pythium aphanidermatum* (Edson) Fitzp., which causes root rot, is the most widely spread pathogen in greenhouse-grown cucumber (Menzies et al. 1996). Biocontrol products have specifically been developed against *Pythium* (McCullagh et al. 1996; Paulitz and Bélanger 2001; Punja and Yip 2003). However, environmental conditions are known to affect the population dynamics both of the pathogen and of the antagonists, and thereby may modify the efficiency of biocontrol products (Ehret et al. 2001).

Although the biology and ecology of *P. aphanidermatum* has been widely studied (Martin and Loper 1999; Stanghellini et al. 1983), the knowledge on environmental effects on the epidemiology is limited. For *Pythium* spp., only a few studies report on how environmental conditions can affect disease severity and most of these have examined the effect of temperature (Owen-Going et al. 2003; Paternotte 1992; Raftoyannis and Dick 2002). Therefore, disease development of *P. aphanidermatum* is still rather unpredictable (Postma et al. 2001). In such cases, mathematical models of population dynamics of the pathogen would provide valuable information on the relationship between pathogen and host plant (Paulitz 2000). However, such models require many data on population development and plant response under various environmental conditions. Measured data must therefore be robust not only in qualitative terms (i.e. the stronger the disease severity, the lower the yield), but also in quantitative (e.g., under given environmental conditions, an increase of the pathogen density of x % leads to a yield decrease of $y = f(x)$ %). The latter, however, is difficult to achieve due to the lack of methods for measuring absolute values of pathogen densities. Current methods to measure pathogen densi-

ties of oomycetes in the plant tissue like culture plating on selective media are labour-intensive, cumbersome and require taxonomic experience. Nested PCR has been performed with *P. aphanidermatum*, but the focus was to evaluate population dynamics of zoospores in the nutrient solution (POSTMA et al. 2001). Indirect ELISA has been used for the rapid detection of oomycetes in plant tissues (e.g., MACDONALD et al. 1990; THORNTON et al. 1999; YUEN et al. 1998). To date, most of the antibodies developed against oomycetes are genus-specific (ALI-SHTAYEH et al. 1991; LYONS and WHITE 1992), although species-specific ELISA systems have been developed for *P. ultimum* (YUEN et al. 1993) and *P. sulcatum* (KAGEYAMA et al. 2002) identification. ESTRADA-GARCIA et al. (1989) developed a species-specific monoclonal antibody against *P. aphanidermatum*, which is highly sensitive to epitopic sites on the cell surface components of zoospores and cysts of the fungus. However, to date, only few studies have used an indirect ELISA approach for pathogen quantification (MOULIN et al. 1996; SHANE 1991). Overall, studies estimating the pathogen density in the plant tissue based on (semiquantitative) ELISA absorbance readings are few (TAKENAKA and ARAI 1993) as are studies on how pathogen density affects plant growth or yield.

We aimed to develop a reliable tool to estimate mycelium density of *P. aphanidermatum* in cucumber. To this end, an indirect ELISA approach was adapted to quantify the mycelium density of *P. aphanidermatum* in cucumber roots. The robustness of this method was tested in growth chamber experiments under various temperature and inoculum density conditions, well known to influence disease development (MENZIES et al. 1996; PATERNOTTE 1992). At the same time, the study focussed on determining suitable inoculation densities that cause disease symptoms, but do not lead to sudden plant death in long-term epidemiological studies.

2 Materials and methods

2.1 Cultivation of Pythium aphanidermatum

P. aphanidermatum isolate BBA 70417 was used in all experiments for the evaluation of the indirect ELISA approach. The fungus was routinely maintained on carrot agar plates (30 g ground carrots, 15 g agar, 1 l distilled water, pH 5.0) at 18°C.

Sterile mycelium mats used to standardise the ELISA and oospores for the inoculation of cucumber plants were produced in carrot broth (juice of 30 g carrots, 1 ml wheat germ oil, 1 l distilled water) according to ELAD and CHET (1987). One-hundred ml carrot broth in 300 ml Erlenmeyer flasks were inoculated with mycelial disks from a seven-day old *P. aphanidermatum* culture and then incubated at 28°C with shaking at 90 rpm. Sterile mycelial mats for the ELISA standardisation and mycelial mats containing oospores were harvested two days and three weeks later, respectively. To obtain the oospores, mats were washed using running tap water, suspended in 0.3% NaCl solution and homogenised with a turbo blender (Moulinex, Ecully, France) for 5 min. Oospores were enumerated using a haemocytometer.

2.2 Indirect enzyme-linked immunosorbent assay (ELISA)

The immunoglobulin G (IgG) fraction 238 of a polyclonal antiserum raised against *P. aphanidermatum* mycelium extracts was used in this study. Previously performed ELISAs using this antiserum established intensive cross-reactions with antigens of all related oomycetous fungi tested, e.g. *Phytophthora citrophthora* and *P. nicotianae*. Extracts of all non-oomycetous fungi, such as *Alternaria solani*, *Botrytis cinerea*, *Fusarium oxysporum*, *F. oxysporum* f. sp. *radicis-lycopersici* and *Rhizoctonia solani*, failed to react (unpublished).

Mycelium extracts of *P. aphanidermatum* were prepared for standardisation of the ELISA by washing sterile mycelium mats with running tap water and harvesting mycelium extract using a roller press (MEKU Erich Pollähne GmbH, Wennigsen, Germany). Samples were stored at −20°C.

Cucumber root samples from the different experiments were stored at −20°C and root extract was later extracted using a roller press and diluted with phosphate-buffered saline (PBS) pH 6.8. The indirect ELISA was performed as described by GABLER and URBAN (1995):

(1) Coating of the wells of microtiter plates (Nunc) with 200 µl antigen aliqouts per well for 16 h at 4°C;
(2) blocking was carried out using 1% skimmed milk powder (SMP) for 30 min at 37°C;
(3) incubation with IgG 238 (1 µg ml^{-1}) was done in blocking solution for 120 min at 37°C;
(4) incubation with enzyme-labelled secondary antibody (goat antirabbit-IgG conjugate at a 1:2000 dilution) was performed in 0.05 M Tris-HCl pH 8.0 + 1% SMP for 90 min at 37°C; and
(5) substrate reaction with p-nitrophenylphosphate (1 mg ml^{-1}) was carried out in 10 mM diethanolamine buffer pH 9.8 for 60 min (except for experiment a) at room temperature.

The absorbance (A) was detected at a wavelength of 405 nm (Microplate reader Anthos hT III Version 1.21 E, Anthos, Salzburg, Austria). Microplates were washed two to nine times with distilled water and one to four times with PBS containing 0.05% Tween-20 (PBST) between each reagent step. Unless stated otherwise, all ELISA measurements were carried out in duplicate.

2.3 Evaluation of the ELISA

The substrate reaction time of the indirect ELISA was determined using a dilution series of *P. aphanidermatum* mycelium extract in 30, 45 and 60 min incubations (experiment a). To produce the dilution series, the extract was diluted by 1 part to 5 and then a one to tenth dilution series was made both from the diluted 20% and undiluted 100% extracts. Four aliquots of each dilution step were measured and the absorbance values were standardised by subtracting the absorbance of PBS. The density of the *P. aphanidermatum* mycelium in the extract was calculated by multiplying the dilution ratio and the efficiency of extract production, i.e. the ratio of mycelium mass to mycelium extract. Three experiments were performed to evaluate the variation of pathogen density as measured by indirect ELISA: (1) one dilution series was measured using ten duplicate samples (experiment b); (2) the mycelium from 30 Erlenmeyer flasks was divided into three aliquots (10 flasks each) and three separate dilution series were measured (experiment c); and (3) the extract of one mycelium cultivation was prepared and measured on different days (experiment d).

2.4 Pathogen density in cucumber roots

Dilution series of the extracts of root samples of the inoculated and uninoculated treatments and of the standard mycelium extract were carried out in microtiter plates. The *Pythium* absorbance function specific for the microtiter plate was estimated from the mycelium dilution series as:

$$[1] \quad A_{405} = \frac{a_0}{1 + e^{a_1 + a_2 \log D_{myceliumaliquot}}},$$

where A_{405} denotes the absorbance of the mycelium aliquot and $D_{myceliumaliquot}$ (g l^{-1}) the density of the mycelium in the aliquot. Parameters a_0, a_1 and a_2 were estimated by nonlinear regression analysis (Levenberg-Marquardt procedure). To exclude any growth condition effects (here: temperature),

absorbance values of cucumber roots of the treatments inoculated with *P. aphanidermatum* were blanked with those of the same dilution step of the corresponding (parallel) control treatment. Thus, the remaining absorbance ($A_{405\,blanked}$) is due to mycelium density in the roots (D_{root}, mg g^{-1}) which can be calculated as:

[2]

$$\log D_{rootextract} = \frac{\ln\left(\frac{a_0}{A_{405\,blanked}} - 1\right) - a_1}{a_2} + \log(f_{dilution}),$$

$$D_{root} = f_{rootextract} \cdot D_{rootextract},$$

where $D_{rootextract}$ (g l^{-1}) is the mycelium density in the root extract, $f_{dilution}$ is the dilution factor of the root extract in the measured aliquot, and $f_{rootextract}$ is the ratio of extract volume obtained to root mass used in the root extract preparation ($f_{rootextract} \approx 0.5$ ml g^{-1}). In this study, density calculations are based on the 1:100 dilution of the cucumber root extract ($f_{dilution} = 100$).

2.5 Pathogen distribution in cucumber roots

Cucumber plants were grown in a growth chamber and nutrient solution was inoculated with 10^5 l^{-1} oospores of *P. aphanidermatum* as described in the next subsection. A plant was harvested 7, 10, 12 and 14 days after inoculation. The root system of each plant was divided lengthwise into three subfractions and two root samples were taken from each fraction. Pathogen density in these samples was measured using indirect ELISA and the pathogen density distribution was evaluated by ANOVA and Fisher's F-test.

2.6 Cultivation of cucumber plants

Cucumber (*Cucumus sativus* L. cv. Corona) seedlings were grown in gravel. At the second true leaf stage, 16 plants were transferred to a growth chamber (York, Mannheim, Germany; 2.5 m x 4.0 m), and the gravel was washed from the roots and they were transplanted into 12-l containers each containing 11 l aerated nutrient solution. Cucumbers were cultivated at a constant air temperature of 25°C, a relative humidity of 80% and a CO_2 concentration of 350 µmol mol^{-1}. Plants were grown on a 16:8 h light:dark cycle at a photosynthetically active radiation (PAR) of 300 µmol m^{-2} s^{-1} (high-pressure sodium lamps, Philips SONT-T 400W, Philips, Eindhoven, The Netherlands). At the start of the light cycle, radiation increased gradually over 30 min from zero to 300 µmol m^{-2} s^{-1}, and similarly decreased to darkness in the evening. The composition of the nutrient solution was based on those recommended for cucumber production in hydroponic growing systems (De Kreij et al. 1997). The electrical conductivity (EC) of the solution was 2.0 dS m^{-1} and the pH was 5.5. The volumes of nutrient solution taken up by the plants were replaced three times per week. When the tenth true leaf had unfolded, air temperature in the chamber was gradually changed over two days to 30°C (experiment 1), 20°C (experiment 3) and 15°C (experiment 4), while in experiment 2, it was kept at 25°C. *P. aphanidermatum* inoculum (produced as described above) was added to the nutrient solution to approximate final densities of 0 (non), 10 (low), 10^3 (moderate) and 10^5 (high) oospores l^{-1}. Each treatment included four replications that were arranged in four blocks. Plants were topped at 20 leaves. All side shoots, except for the upper two, were removed as they appeared. Nine fruits were allowed to grow in the leaf axes 6, 7, 8, 11, 12, 13, 16, 17 and 18, while all others were removed. Fruits were harvested when they reached a mass of about 300 g. The experiments were terminated after having harvested all stem fruits. Thus, long growth periods were achieved at low temperature enabling disease severity and plant response to be studied. Parts of the roots were stored at -20°C and their *Pythium* mycelium content was later estimated using ELISA as described above. Plants were harvested and masses of the roots, stem, leaves and fruits were then recorded. Dry matter contents were measured after drying at 80°C for two days. In cases when plants suddenly died, they were also immediately examined as described above. Root samples of the uninoculated plants were checked for possible infection by plating on carrot agar plates.

Data were evaluated by two-way analysis of variance using Fisher's F-test. However, as the temperature effect was interconnected with the effect of growing time, inoculum density effects were evaluated for every temperature separately by Tuckey's T-test. Significance level in all tests was 0.05.

2.7 Correlation of pathogen density and plant characteristics

Plant-characteristic data were standardised within the experiments, i.e. values of inoculated plants were divided by the mean values of the pathogen-free treatment of the corresponding temperature experiment. The relationships between the mycelium density in the roots and the standardised plant characteristics were evaluated by correlation analysis.

3 Results

3.1 Evaluation of the ELISA

The absorbance of the dilution series of the *P. aphanidermatum* mycelium extracts increased with substrate incubation time. After 60 min, pathogen densities for aliquots inoculated with 10 g l^{-1} and above reached the maximum absorbance, whereas full absorbance was not achieved after incubation times of 30 and 45 min for any pathogen density (Fig. 1a). The variation of the absorbance for one dilution series of mycelium extract was low when duplicates were measured on one plate (Fig. 1b) as well as when extracts were produced independently from the same mycelium cultivation and measured on the same plate (Fig. 1c). The standard deviation of repeated measurements was zero at maximum absorbance, about 0.3 at medium absorbance and about 0.02 at absorbance lower than 0.1 (Fig. 1b). Thus, an absorbance reading below 3 x 0.02 = 0.06, which corresponds to a mycelium density of about 0.05 g l^{-1} or to a 1 to 100000 dilution of the mycelium extract, could not be distinguished significantly from zero. Significant differences in the absorbance occurred when dilution series of the same mycelium cultivation were prepared and measured on different days (Fig. 1d).

Using this ELISA approach, infection by *P. aphanidermatum* of the cucumber root system could be measured just seven days after inoculation (not shown). Up to two weeks after inoculation, mycelium densities in the root biomass of 6.0, 7.8 and 8.8 mg g^{-1} were measured respectively in the bottom, middle and top fractions of the root system and were seen to be quite uniform (no significant differences, Fishers F-test, significance level $\alpha = 0.05$).

3.2 Effect of inoculum density on pathogen density in cucumber roots and plant growth at different temperatures

Due to the finishing criteria of the experiments, i.e. the completion of the harvest of all stem fruits, cucumbers were cultivated for 19, 23 and 31 days after pathogen inoculation at 30, 25 and 20°C, respectively. Experiment 4 at 15°C was completed 41 days after inoculation, which was prior to the finishing criteria due to very slow fruit growth but immense

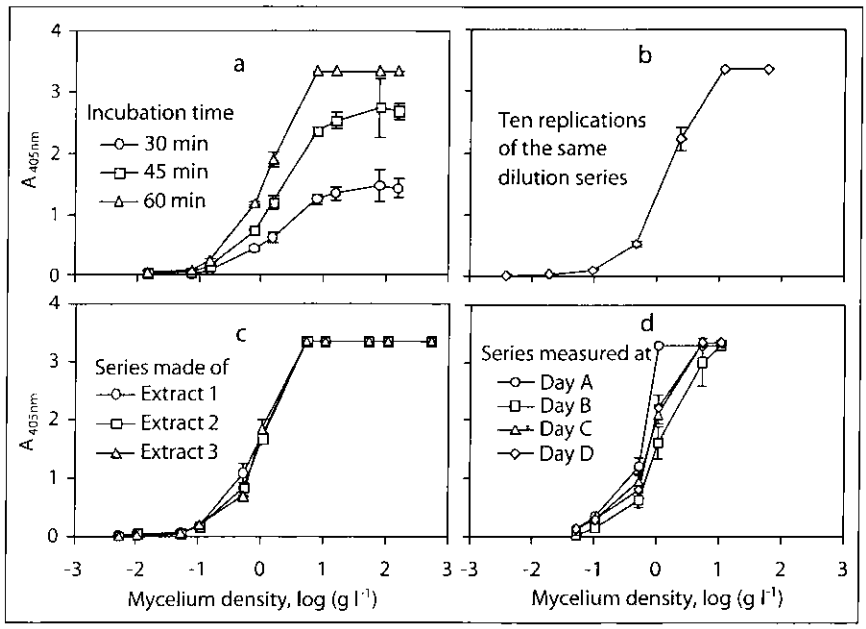

Fig. 1: Absorbance of dilution series of *Pythium aphanidermatum* mycelium extract in PBS at wavelength 405 nm (A_{405nm}). (a) Different substrate incubation lengths. (b) Ten duplicates of one dilution series. (c) Three dilution series of one mycelium cultivation. (d) Dilution series measured of one mycelium cultivation on four different days. Symbols represent the mean and bars the standard deviation when the latter is greater than the symbol.

side shoot growth and root growth in the small container. In control plants, after plating on carrot agar, no pathogens were detected in all root samples, except for one plant in the experiment at 15°C which was excluded in the data analysis. On the other hand, *P. aphanidermatum* was observed in the cucumber roots at all inoculation densities and temperatures investigated using the indirect ELISA approach (Fig. 2). The absorbance of the 1:100 diluted extract of healthy uninoculated roots after subtracting the absorbance of PBS was lowest when plants were cultivated at 15°C (0.05) and highest at 30°C (0.19).

Two-way ANOVA showed that both factors, the experiments (temperature plus growing time) and the inoculum density, affected significantly the pathogen density in the roots, the root mass, the shoot dry matter and the yield (Table 1). Moreover, for root mass and yield, significant interactions were observed. Two general tendencies could be observed. The pathogen density in the roots increased with inoculum density and, when excluding the experiment at 15°C, with temperature; although the propagation time for the pathogen was shorter with increasing temperature (Fig. 2).

Two plants which were inoculated with 10^5 oospores l^{-1} of *P. aphanidermatum* at 25 and 30°C and one plant in the 10-oospores treatment at 30°C died before the end of the experiments. Root mass, shoot dry matter and yield diminished with increasing inoculation density compared to uninoculated plants. Root growth was reduced at all four temperatures (Fig. 3, top). Moreover, reduction in shoot dry matter was observed at 20, 25 and 30°C (Fig. 3, middle), but yield reduction was only found at 25 and 30°C (Fig. 3, bottom).

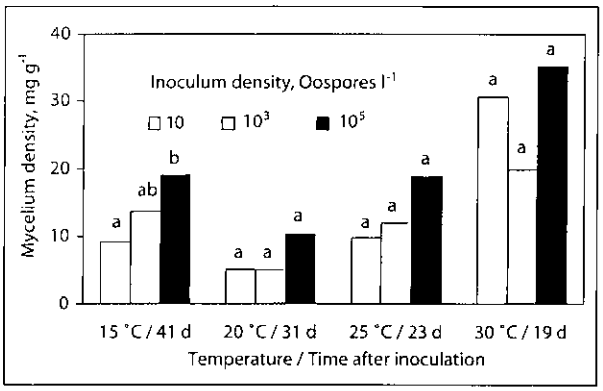

Fig. 2: Density of *Pythium aphanidermatum* mycelium in cucumber roots measured by indirect ELISA for different inoculum densities and temperatures. Bars represent averages of four replications. Bars followed by the same letters within each temperature level are not significantly different according to Tukey's HSD test at significance level $\alpha = 0.05$.

Table 1: Two-way ANOVA of the factor effects of the experimental conditions temperature and growing time, and the inoculum density on mycelium density in the cucumber roots, root mass, shoot dry matter and yield. Probability levels were calculated using Fisher's F-procedure. Treatments included four replications

Characteristic	Factor	Degree of freedom	Probability level
Mycelium density	Experiment	3	0.000026
	Inoculum density	2	0.034
	Interaction	6	0.64
Root mass	Experiment	3	< 0.000001
	Inoculum density	3	< 0.000001
	Interaction	9	0.00020
Shoot dry matter	Experiment	3	< 0.000001
	Inoculum density	3	0.000060
	Interaction	9	0.074
Yield	Experiment	3	< 0.000001
	Inoculum density	3	0.0015
	Interaction	9	0.049

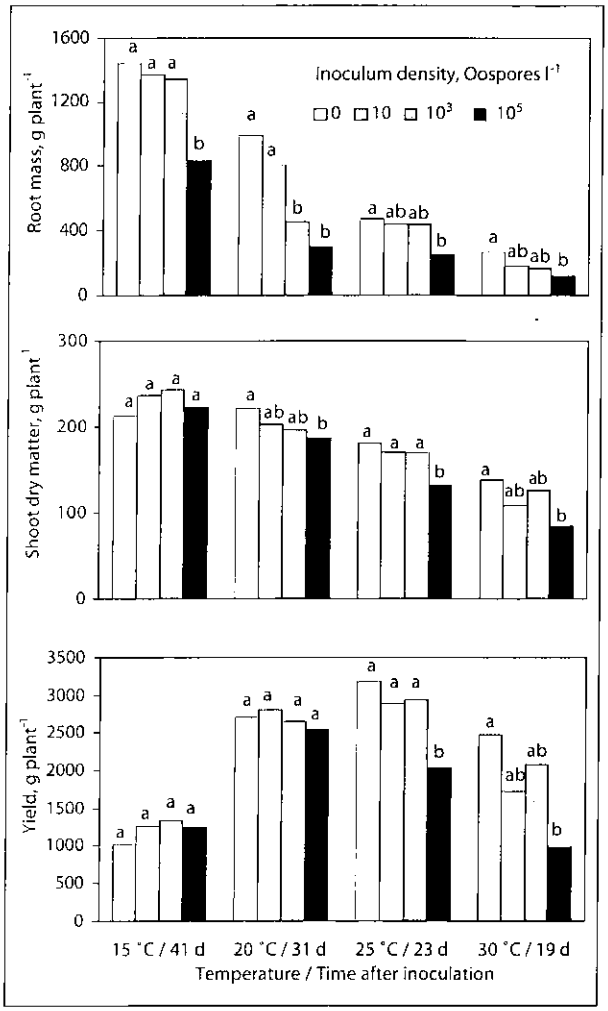

Fig. 3: Root mass (top), shoot dry matter (middle) and yield (bottom) of cucumber for different inoculum densities and temperatures. Bars represent averages of four replications. Bars followed by the same letter within each temperature level are not significantly different according to Tukey's HSD test at significance level $\alpha = 0.05$.

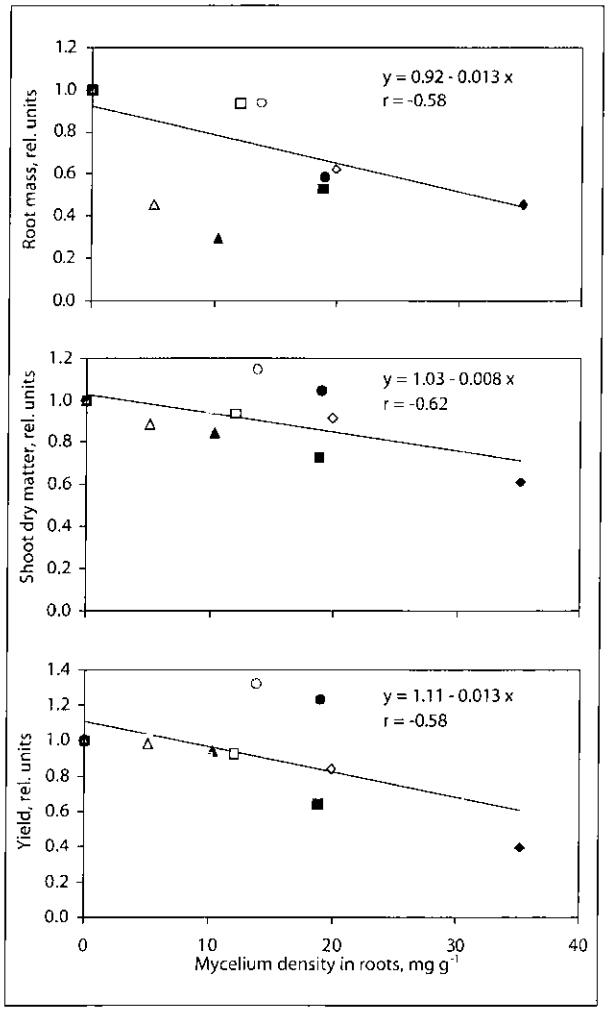

Fig. 4: Correlation of *Pythium aphanidermatum* mycelium density in cucumber roots measured by indirect ELISA and relative root mass (a), relative shoot dry matter (b) and relative yield (c) based on the means of the treatments. Relative data were obtained by dividing the values of the inoculated treatments by those of the uninoculated of the corresponding experiment (temperature). Thus, uninoculated treatments are by definition equal to one. Circles, triangles, squares and diamonds stand for 15, 20, 25 and 30°C, respectively; and open, grey, grey with black border, and black symbols for inoculation densities of 0, 10, 10^3 and 10^5 oospores l^{-1}, respectively. Every symbol represents the average of four replications.

3.3 *Correlation of pathogen densities and plant characteristics*

Root mass, shoot dry matter and yield decreased with increasing density of *P. aphanidermatum* in the roots (Fig. 4). Corresponding correlations explained 34, 38 and 34%, respectively, of the variation of these characteristics by the variation of the pathogen density in the roots. All coefficients of correlation were significantly different from zero. Excluding the data for 15°C, a temperature unsuitable for cucumber growth, 75% of the variation of the shoot dry matter and 84% of the variation of the yield, but only 30% of the variation of the root mass could be explained. In particular, root mass at 20°C failed to fit this correlation.

4 Discussion

A major challenge to developing an accurate quantification approach is to find an antibody that provides a good absolute and robust measure for the severity of the disease in the plant. In particular, oomycete biomass in plant roots may be composed of various organs such as mycelium, sporangia, oogonia or oospores; however, to develop and work with a set of antibodies specific for each organ is currently impracticable. In addition, even if it would be possible to estimate the density of all the different organs, it will be difficult to interprete them in terms of their contribution to the disease severity. Therefore, we used the less specific polyclonal antiserum Pythium-IgG 238 and focussed on the quantification of the mycelium, which is expected to be the largest biomass fraction of *P. aphanidermatum* in the plant roots (Zheng et al. 2000).

The disadvantage of this genus-specific reaction is assumed to be small in experiments with inoculated plants, where the presence of other oomycetes may be assumed to be negligible. A possible distortion of the ELISA due to the presence of other oomycetes in the experiments must be tested in the uninoculated treatments by plating of root samples on agar. If these control tests are negative, even though other oomycetes could be present but below the detection limit, their density can be expected to be very low and therefore to have negligible effect

on the quantification of *P. aphanidermatum* in the inoculated treatments. Additional information on the population of *P. aphanidermatum* can be obtained in hydroponic systems by measuring the density of the zoospores in the nutrient solution as proposed by POSTMA et al. (2001).

The course of the substrate solution absorbance over the dilution steps of the mycelium extract was similar to that reported for a *P. aphanidermatum* antiserum used by MOULIN et al. (1996). Interestingly, our dilution series, which were prepared and analysed on different days, resulted in different absorbance courses; however, this observation corresponds to findings of OTTEN et al. (1997). Otten's group emphasised the necessity to incorporate a standardised mycelium dilution series on every microplate and to relate the absorbance readings given by samples to that standard series produced on the same microplate. TAKENAKA and ARAI (1993) reported that plant proteins could interfere with the *Pythium paddicum* and *Typhula incarnata* antigen reaction in barley roots and leaves. To overcome any discrepancies due to this observation, they proposed to produce the fungal reference curve with dilution series of healthy leaf or root extracts. However, the absorbance of extracts of healthy plant material depends on the growing conditions and this makes standardisation difficult. Therefore, we attempted to prevent distortions by blanking the absorbance readings of inoculated roots with those of the uninoculated roots of the corresponding growing conditions. Nevertheless, the interference with plant proteins of the root tissue will be an uncertainty when using the ELISA approach. Additionally, when the disease progresses, root tissue dies and may be partly decomposed by secondary parasites, which may also change the background absorbance. Moreover, the ELISA does not distinguish between living and dead mycelium, while the digested mycelium is not detected.

Up to two weeks after inoculation, no preferred location for the pathogen infestation was observed, and therefore, root samples can be taken randomly from the root system of a plant. In older plants, however, it is possible to have all kind of roots from nearly decomposed to newly growing white ones. Here, an approach to obtain representative samples needs to be further developed.

Despite all problems discussed above, significant correlations between pathogen density in the roots and plant growth characteristics were established; although data were obtained in four experiments at different temperatures and after different growing period lengths (Fig. 4). These correlations are an indication of the robustness of the quantification approach presented. We acknowledge that the estimated mycelium mass in the roots is still a kind of semiquantitative or virtual value as it stands for the absorption caused by all organs of *P. aphanidermatum* in the root extract. However, such correlations are also lacking for other oomycetal root pathogens, although a few studies on *Pythium* spp. have presented data to show a correlation between inoculum density and disease severity for single experiments (MARTIN and LOPER 1999). However, in general, regression models are limited when describing the pathogen-host interaction for a wider range of environmental conditions because they do not take into account the loss of root function against time and overall plant growth. Simulation models however do offer some distinct advantages (KLÄRING et al. 2001), but require complex measurements to estimate the pathogen development and plants reaction to root diseases. Here, the indirect ELISA approach proved to be a fast, robust and powerful method for pathogen quantification to provide the data that are necessary for the development of such models.

The cucumber plants proved to be very sensitive to *P. aphanidermatum* even at 15°C, although disease severity considerably decreased with dropping temperature. This phenomenon has also been observed for cucumber seedlings (BEN-YEPHET and NELSON 1999), adult cucumber plants (PATERNOTTE 1992), sugar beet seedlings (RAFTOYANNIS and DICK 2002) and tomato seedlings (GROSCH and SCHWARZ 1998). PANOVA et al. (2004) reported an increase of *P. aphanidermatum* density in tomato roots and in the nutrient solution with increasing nutrient solution temperature; however, contrary to cucumber, tomato tolerated an infection at a nutrient solution temperature of 20°C during the whole experimental period of six weeks following an inoculation of 10^6 oospores l^{-1}. Also in spinach, root and shoot growth of infected plants decreased with increasing temperature but was not reduced at 17°C three weeks after inoculation (GOLD and STANGHELLINI 1985).

There was a time delay as regards the effects of the root disease on shoot growth. The higher the temperature, the faster and more aggressively the pathogen damaged the root and reduced its mass (Fig. 3a). However, even a reduced root system could still facilitate the water and nutrient uptake necessary for unlimited transpiration, photosynthesis and shoot growth. In particular, at 15 and 20°C, root growth was strong and even the mass of infected roots was relatively high, but transpiration was smaller due to the lower vapour pressure deficit of the air. In the shoot, available assimilates were preferentially distributed to the cucumber fruits as they are stronger sinks for assimilates compared to leaves and stems (MARCELIS 1996).

One difficulty regarding epidemiological studies on *P. aphanidermatum* in cucumber is that severely diseased plants may suddenly die during the experiment due to lower stem rot. This may occur shortly after inoculation when the conditions are favourable for *P. aphanidermatum* (STANGHELLINI et al. 1996). On the other hand, no effect of the disease on plant growth could be measured in short-term experiments at conditions moderate for *P. aphanidermatum* development. Under these circumstances, the density of the pathogen could be even below the detection limit of the quantification method applied. Thus, visual observations of the roots detected no symptoms of the disease in our experiments three weeks after inoculation at 15°C, whereas at 25 and 30°C some plants already had been died within three weeks. Therefore, no fixed inoculum densities can be recommended for long-term epidemiological studies as the environmental conditions influence the pathogen's development. The main difficulty is, however, that the effect of these conditions on pathogen development is often the subject of the study and hence, is unknown at the beginning of the experiments. In our experiments with cucumber, inoculation densities around 10^3 *P. aphanidermatum* oospores l^{-1} proved to be suitable at temperatures between 20 and 25°C, which is the common average temperature in greenhouse cucumber production.

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Literature

ALI-SHTAYEH, M.S., J.D. MACDONALD, J. KABASHIMA, 1991: A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water. Plant Dis. **75**, 305-311.

BEN-YEPHET, Y., E.B. NELSON, 1999: Differential suppression of damping-off caused by *Pythium aphanidermatum*, *P. irregulare*, and *P. myriotylum* in composts at different temperatures. Plant Dis. **83**, 356-360.

DE KREIJ, C., W. VOOGT, A.L. van den BOS, R. BAAS, 1997: Nutrient solutions for the growth of tomato in closed systems (In Dutch). Proefstation voor Bloemistrij en Glasgroente. Naaldwijk, The Netherlands.

EHRET, D.L., B. ALSANIUS, W. WOHANKA, J.G. MENZIES, R. UTKHEDE, 2001: Disinfestation of recirculating nutrient solutions in greenhouse horticulture. Agronomie **21**, 323-339.

ELAD, Y., I. CHET, 1987: Possible role of competition for nutrients in biocontrol of *Pythium* damping-off by bacteria. Phytopathology **77**, 190-195.

ESTRADA-GARCIA, M.T., J.R. GREEN, J.M. BOOTH, J.G. WHITE, J.A. CALLOW, 1989: Monoclonal antibodies to cell surface components of zoospores and cysts of the fungus *Pythium aphanidermatum* reveal species specific antigens. Exp. Mycol. **13**, 348-355.

GABLER, J., M. URBAN, 1995: Evaluation of resistance differences between tomato cultivars of *Phytophthora nicotianae* by indirect ELISA. Z. Pflanzenkrankh. Pflanzensch. – J. Plant Dis. Protect. **102**, 275-283.

GOLD, S.E., M.E. STANGHELLINI, 1985: Effects of temperature on *Pythium* root rot of spinach grown under hydroponic conditions. Phytopathology **75**, 333-337.

GROSCH, R., D. SCHWARZ, 1998: The effects of an infection by *Pythium aphanidermatum* on root morphology of tomato. (In German) *In*: Merbach, W. (ed.): 8th Borkheider Seminar on Ecophysiology of the Rhizosphere, pp. 65-72. B.G. Teubner, Stuttgart and Leipzig.

JENKINS, S.F., C.W. AVERRE, 1983: Root diseases of vegetable in hydroponic culture systems in North Carolina greenhouses. Plant Dis. **67**, 968-970.

KAGEYAMA, K., M. KOBAYASHI, M. TOMITA, N. KUBOTA, H. SUGA, M. HAYKUMACHI, 2002: Production and evolution of monoclonal antibodies for the detection of *Pythium sulcatum* in Soil. J. Phytopathol. **150**, 97-104.

KLÄRING, H.-P., R. GROSCH, E. NEDERHOFF, D. SCHWARZ, 2001: A model approach to describe the effect of root pathogens on plant growth and yield. Acta Hortic. **548**, 235-241.

LYONS, N.F., J.G. WHITE, 1992: Detection of *Pythium violae* and *Pythium sulcatum* in carrots with cavity spot using competition ELISA. Ann. Appl. Biol. **120**, 235-244.

MACDONALD, J.D., J. STITES, J. KABASHIMA, 1990: Comparison of serological and culture plate methods for detecting species of *Phytophthora*, *Pythium* and *Rhizoctonia* in ornamental plants. Plant Dis. **74**, 655-659.

MARCELIS, L.F.M., 1996: Sink strength as a determinant of dry matter partitioning in the whole plant. J. Exp. Bot. **47**, 1281-1291.

MARTIN, F.N., J.E. LOPER, 1999: Soilborne plant diseases caused by *Pythium* spp.: ecology, epidemiology, and prospects for biological control. Crit. Rev. Plant Sci. **18**, 111-181.

MCCULLAGH, M., R. UTKHEDE, J.G. MENZIES, Z.K. PUNJA, T.C. PAULITZ, 1996: Evaluation of plant growth-promoting rhizobacteria for biological control of *Pythium* root rot of cucumbers grown in rockwool and effects on yield. Eur. J. Plant Pathol. **102**, 747-755.

MENZIES, J.G., D.L. EHRET, S. STAN, 1996: Effect of inoculum density of *Pythium aphanidermatum* on the growth and yield of cucumber plants grown in recirculating nutrient film culture. Can. J. Plant Pathol. **18**, 50-54.

MOULIN, E., P. LEMANCEAU, C. ALABOUVETTE, 1994: Pathogenicity of *Pythium* species on cucumber in peat-sand, rockwool and hydroponics. Eur. J. Plant Pathol. **100**, 3-17.

MOULIN, E., P. LEMANCEAU, C. ALABOUVETTE, 1996: Suppression of Pythium root rot of cucumber by a fluorescent *Pseudomonad* is related to reduced root colonization by *Pythium aphanidermatum*. J. Phytopathol. **144**, 125-129.

OTTEN W., C.A. GILLIGAN, C.R. THORNTON, 1997: Quantification of fungal antigens in soil with monoclonal antibody-based ELISA: Analysis and reduction of soil-specific bias. Phytopathology **87**, 730-736.

OWEN-GOING, N., J.C. SUTTON, B. GRODZINSKI, 2003: Relationships of *Pythium* isolates and sweet pepper plants in single-plant hydroponic units. Can. J. Plant Pathol. **25**, 155-167.

PANOVA, G.G., D. GROTE, H.-P. KLÄRING, 2004: Population dynamics of *Pythium aphanidermatum* and response of tomato plants as affected by root-zone temperature. Z. Pflanzenkrankh. Pflanzensch. – J. Plant Dis. Protect. **111**, 52-63.

PATERNOTTE, S.J., 1992: Influence of growing conditions on disease development of *Pythium* in glasshouse cucumbers on rockwool. Meded. Fac. Landbouwwet. Rijksuniv. Gent **57**, 373-379.

PAULITZ, T.C., 2000: Population dynamics of biocontrol agents and pathogens in soils and rhizospheres. Eur. J. Plant Pathol. **106**, 401-413.

PAULITZ, T.C., R.R. BÉLANGER, 2001: Biological control in greenhouse systems. Annu. Rev. Phytopathol. **39**, 103-133.

POSTMA, J., P.J.M. BONANTS, E. van Os, 2001: Population dynamics of *Pythium aphanidermatum* in cucumber grown in closed systems. Meded. Fac. Landbouwwet. Rijksuniv. Gent **64**, 431-440.

PUNJA, Z.K., R. YIP, 2003: Biological control of damping-off and root rot caused by *Pythium aphanidermatum* on greenhouse cucumbers. Can. J. Plant Pathol. **25**, 411-417.

RAFTOYANNIS, Y., M.W. DICK, 2002: Effects of inoculum density, plant age and temperature on disease severity caused by pythiaceous fungi on several plants. Phytoparasitica **30**, 67-76.

SHANE, W.W. 1991: Prospects for early detection of *Pythium* blight epidemics on turfgrass by antibody-aided monitoring. Plant Dis. **75**, 921-925.

STANGHELLINI, M.E., L.J. STOWELL, W.C. KRONLAND, P. BRETZEL, 1983: Distribution of *Pythium aphanidermatum* in rhizosphere soil and factors affecting expression of the absolute inoculum potential. Phytopathology **73**, 1463-1466.

STANGHELLINI, M.E., S.L. RASMUSSEN, 1994: Hydroponics: a solution for zoosporic pathogens. Plant Dis. **78**, 1129-1138.

STANGHELLINI, M.E., S.L. RASMUSSEN, D.H. KIM, P.A. RORABAUGH, 1996: Efficacy of nonionic surfactants in the control of zoospore spread of *Pythium aphanidermatum* in a recirculating hydroponic system. Plant Dis. **80**, 422-428.

TAKENAKA, S., M. ARAI, 1993: Dynamics of three snow mold pathogens *Pythium paddicum*, *Pythium iwayamai*, and *Typhula incarnata* in barley plant tissues. Can. J. Bot. **71**, 757-763.

THORNTON, C.R., T.M. O'NEILL, G. HILTON, C.A. GILLIGAN, 1999: Detection and recovery of *Rhizoctonia solani* in naturally infested glasshouse soil using a combined baiting, double monoclonal antibody ELISA. Plant Pathol. **48**, 627-634.

UTKHEDE, R.S., C.A. LEVESQUE, D. DINH, 2000: *Pythium aphanidermatum* root rot in hydroponically grown lettuce and the effect of chemical and biological agents on its control. Can. J. Plant Pathol. **22**, 138-144.

YUEN, G.Y., M.L. CRAIG, F. AVILA, 1993: Detection of *Pythium ultimum* with a species-specific monoclonal antibody. Plant Dis. **77**, 692-698.

YUEN, G.Y., J.O. XIA, C.L. SUTULA, 1998: A sensitive ELISA for *Pythium ultimum* using polyclonal and species-specific monoclonal antibodies. Plant Dis. **82**, 1029-1032.

ZHENG, J., J.C. SUTTON, H. YU, 2000: Interactions among *Pythium aphanidermatum*, roots, root mucilage, and microbial agents in hydroponic cucumbers. Can. J. Plant Pathol. **22**, 368-379.