Enzyme-linked immunosorbent assay (ELISA) as a method for detection of Phytophthora fragariae Hickman in strawberry roots

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Abstract

ELISA was utilized to detect Phytophthora fragariae Hickman in roots of strawberries in experiments under controlled and field conditions. First the specificity of the antiserum was examined. The antiserum was not specific in its reactions with different P. fragariae isolates. Other Phytophthora species reacted with the antiserum but much less than P. fragariae. The tested Pythium species gave no ELISA values. Under controlled conditions detection of the pathogen in strawberry roots with ELISA was positive even if inoculum density of the substrate was low. Under field conditions P. fragariae could be detected in inoculated strawberry plants in spring and summer.

Zusammenfassung


Red stele root rot, caused by Phytophthora fragariae Hickman, has become more and more important in strawberry culture. The typical symptom of the disease is the red core of the roots. In general the discoloration only appears from autumn to spring (HICKMAN 1940, MONTGOMERIE 1977, SEMMÜLLER 1980). During summer months typical symptoms are not to be seen and isolation of the fungus makes difficulties (MONTGOMERIE 1977, DUNCAN 1980). This is very important for disease diagnosis specially with plant propagation of strawberries for P. fragariae is spread internationally by marketing of latent infected plants. The most common indirect test to detect P. fragariae is the root tip test by DUNCAN (1976, 1980) with Fragaria vesca var. semperflorens as a bait plant. This test is very sensitive but it takes about three to five weeks.

The present study is aimed to prove ELISA for the detection of P. fragariae in strawberry roots under controlled and under field conditions. With this test within two days a great number of samples can be examined. Only for a few years serological techniques are used for detection of fungal pathogens (AMOS and BURRELL 1967, CASPER und MENDGEN 1979, MAC DONALD and DUNIWAY 1979, MORLEY and JONES 1980, IANNELLI et al. 1982, JOHNSON et al. 1983, KOUGH et al. 1983, AGUELON and DUNEG 1984, BANOWETZ et al. 1984, MUSGRAVE 1984, WALCZ et al. 1985, EL-NASHAAR et al. 1986 a. o.). Up to now there has been a limited number of serological experiments with Phytophthora species (BURRELL et al. 1966, SUERTH and VARNET 1968, GILL and POWELL 1969, MAC DONALD and DUNIWAY 1979).

1 Material and methods

1.1 Production of antiserum

The antigen used was mycelium because this structure can be identified first microscopically in roots after inoculation with P. fragariae. Since it is not known which are the antigenic components it seems useful to take a mycelium homogenat as antigen.

For antigen preparation an isolate (L1) from naturally infected strawberry plants was taken. This isolate (P. fragariae L1) was incubated for 21 days at 20°C in the dark on oat meal-V8-CaCO₃-agar as described in WERRES (1987). Mycelium was scraped under steril conditions from the medium and freeze dried for 72 hours. For preparing an injection solution the freeze dried mycelium was mixed in a 0.85 % sodium chloride solution and emulsified for the first injection with Freund adjuvant complete (DIFCO 0638-59). With this solution a rabbit was given a series of 5 injections, each injection with about 0.2 mg freeze dried mycelium. Bleeding for preparing the antisera followed 15 weeks after the first injection. From this antiserum the antibodies (y-globuline) were prepared as written in CLARK and ADAMS (1977). The optical density (OD₂₅₀) was regulated to 1.4 (equal to 1 mg/ml). For ELISA antibodies were conjugated with alkaline phosphatase (Boehringer CIP 567525).
1.2 Culture and preparing of fungal material for ELISA

Serological studies were initiated with different Phytophthora fragariae isolates from strawberry roots (isolates L1, 64130; PF43, PF44, PF47 from West-Germany; isolates PF43, PF44 from Scotland), other Phytophthora species (P. citricola Sawada, P. cinnamomii Rand, P. cryptogea Pethybridge et Lafferty, P. drechsleri Tucker, P. nicotianae var. nicotianae van Breda de Haan, P. nicotiana var. parasitica Waterhouse, P. parasitica var. nicotianae Tucker) and two Pythium species (Pythium ultimum var. ultimum Trow, Pythium paroecanthrum Drechsler). All fungi were cultivated in liquid oat meal-V8-CaCO3 medium as described in WERRES (1987). 100 ml of this medium were filled in 300 ml flasks and inoculated with 20 discs from an agar culture of the fungus. The flasks were incubated at 20°C (± 1°C) in the dark. After 20 days from all P. fragariae isolates or after 10 days from all other Phytophthora species and Pythium species mycelium was collected, filtered in a Büchner filter, carefully washed in sterile aqua dest. and freeze dried for 72 hours. The freeze dried mycelium was stored for at least 4 weeks at -30°C.

1.3 Preparing of inoculum for plant experiments

Vermiculite was soaked in a red kidney bean solution and then drained. 600 ml of this substrate were filled in flasks and sterilized. The vermiculite was then inoculated with 40 mycelium disks from P. fragariae L1, each flask and incubated for three weeks at 20°C in the dark (WERRES 1987). After this period the vermiculite was overgrown exclusive with mycelium. The control inoculum was prepared with sterile disks from the medium.

1.4 Culture of strawberry plants and inoculation method

For experiments under controlled conditions runners of strawberry cultivar Tenira grown in the greenhouse were cultivated at 20°C. After 2 weeks they were planted in contaminated soil in 8 cm diameter pots. Contaminated soil was prepared by mixing 1 part of inoculum and 19 parts of autoclaved soil mixture (WERRES 1987) 24 hours before transplanting. The plants were inoculated at +16°/+14°C, daylength 10 h and relative air humidity 80%. Soil moisture was held at 60% of field capacity during the whole experiment as described in WERRES (1987). The control plants were planted in non contaminated soil. To prove sensitivity of ELISA two different inoculum levels were used: for high inoculum level substrate and inoculum was mixed as described, for low inoculum level inoculated vermiculite was mixed with the substrate at a rate of 1:40.

For experiments under field conditions tissue cultured strawberry plants cultivar Tenira were planted in soil not contaminated with P. fragariae. Soil was fumigated before planting (WERRES 1987) and examined for P. fragariae with the bait test of DUNCAN (1976). The test did not show any contamination. The plants were planted on August 1985 in randomised blocks with 30 plants per replication. Inoculation of plants with P. fragariae followed in October. For inoculation the root system of each plant was bored with a soil-borer and the holes were filled with a spoonful of inoculum. Holes in the root system of non-inoculated plants were filled with sterile vermiculite. Plant protection and cultivation were done like commercial practice (WERRES 1987). Runners from strawberry plants growing in the glasshouse and cultivated in containers in the glasshouse were taken as control plants.

1.5 Sampling and preparation

For testing freeze dried mycelium was mixed with sample buffer 1:200 and spatula tip Celite (Serva 535, 10-40 μm) in a mortar and diluted once more with this buffer up to 1:20,000. The mortars were emersed in ice.

Samples from experiments under controlled conditions were collected by washing the entire root systems of plants, cutting them into pieces of about 1 cm and mixing them. Field samples of root material from strawberry plants were obtained with a soil-borer. The samples from each variant were mixed. The root pieces were carefully washed free of soil. From all samples a part were fixed in a formalin-alcohol-acetic acid solution for microscopical observation (WERRES 1987). For ELISA the rest of the root samples were freeze dried for 72 hours and stored at -30°C. The freeze dried root material was macerated in sample buffer (1:15). It was then pressed through a cheese cloth and diluted with sample buffer if necessary.

For testing plant material with P. cactorum (crown rot) the crowns of diseased strawberry plants were cut into small pieces and freeze dried as described above. For ELISA it was then diluted and macerated like the root material.

Microscopical examination, calculation of dead plants and measuring vegetative plant growth in field experiments were realized as described in WERRES (1987).

1.6 ELISA standardisation

In all tests the double sandwich enzyme immunoassay as described by CASPER and MEYER (1981) was used. All tests were realized in immunol plates (Dynatech) and measured at 405 nm in a photometer Titertek Multiskan Plus (Flow Laboratories). All ELISA values from controls and samples were adjusted with the sample buffer (blindvalue). For standardization of the test a concentration plate for every antigen (γ-globuline and enzyme-linked antigen (conjugate) preparation was measured. Freeze dried mycelium of P. fragariae L1 was diluted 1:20,000. Those dilutions of γ-globuline and conjugate were chosen, where this mycelium dilution gave a value about 0.65 one hour after addition of substrate. For the control in each test there was carried along freeze dried mycelium of P. fragariae L1 and freeze dried growth medium diluted 1:20,000. In every ELISA the samples were measured, when the mycelium dilution of P. fragariae L1 reached a value about 0.65.

Fig. 1. Reactions of different P. fragariae isolates with the antisera against P. fragariae L1 (dilution 1:20,000).

![Graph](image-url)
Enzyme-linked immunosorbent assay (ELISA)

**Fig. 2.** Reactions of different Phytophthora and Pythium species with the antiserum against *P. fragariae* L1 (dilution 1:20,000).

**Fig. 3.** Reactions of *P. cactorum* mycelium (a) and diseased crown tissue (crown rot, b) with the antiserum against *P. fragariae* L1 (dilution of mycelium 1:20,000).

**Fig. 4.** Influence of inoculum density on the detection of *P. fragariae* L1 in strawberry roots cv. Tenira using microscopic and ELISA procedures (dilution for ELISA 1:15).

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0.65 one or 1.5 hours after addition of substrate. Growth medium must show no reaction with the antibodies.

1.7 Statistics

All experiments besides the field experiment were done twice. For statistical analysis of vegetative growth mean values were calculated over time. They were exploited univariately. Differences between two mean values were calculated with STUDENT-test, differences between more than two mean values with TUKEY-Test (SACHS 1974).

2 Results

2.1 Specificity of antisera

With all the isolates tested of *P. fragariae* only the isolate PF44 differed significantly in the ELISA value from the standard *P. fragariae* L1 (fig. 1). All tested Phytophthora species *P. citricola* and *P. cactorum* cross-reacted highest with the antisera. But all ELISA values of the tested species were significantly less than that of *P. fragariae* L1 (Fig. 2, 3a). The diseased crowns from strawberries infected with *P. cactorum* cross-reacted with the antisera, but the ELISA values were significantly lower than those of the standard *P. fragariae* L1 (Fig. 3b). Both Pythium species tested did not react with the antisera (Fig. 2).

2.2 Sensitivity of ELISA

The roots of strawberry plants from both concentrations low ("+"), and high ((+++)) inoculum density reacted with the antisera. ELISA values of roots "++" increased drastically 15 days after potting (Fig. 4). Reaching a maximum 21 days after potting microscopic evidence of fungal structures was found in the roots. Up to the end of this experiment the ELISA values of the "+++" plants dropped continuously, while the part of roots with microscopically identified fungal structures increased. Roots from plants potted in substrate with "++" reacted with the antisera 24 hours after the beginning of the experiment. Up to 13 days after potting the ELISA values did not exceed over 0.2 (Fig. 4). From the 15th day up to the end of the experiment the values of the following samples showed great variability. They were always lower than that of "+++" samples. A microscopical identification of any fungal structure of *P. fragariae* was not successful in this variation. All control plants tested with ELISA did not react with the antisera.

2.3 Detection of *P. fragariae* in strawberry fields

In the field experiment the root samples of inoculated plants harvested in April, May and June reacted with the antisera of *P. fragariae* L1 (Fig. 5). The samples from the plants inoculated with sterile vermiculite gave low ELISA values, too. The root samples from plants growing in the glass house did not react with the antisera. Microscopical examination of the root material in April, May and June did not show any infection of the roots. Evaluation of vegetative plant growth indicated significant different only in the number of leaves calculated over time between inoculated and none-inoculated plants (Tab. 1). Measuring dead plants gave no significant difference. At the beginning of the new growing season (April) 3.3% of the inoculated and none of the non-inoculated plants had died. This did not change through the completion of the experiment.

3 Discussion

Testing different isolates did not establish specificity of the antisera against *P. fragariae* L1. In both repetitions only the isolate PF44 reacted significantly higher with the antisera than the isolate L1 itself. There is no explanation for this value. To judge isolate or race specificity more than one antisera against *P. fragariae* L1 must be proved. Some authors confirm low race specificity of *P. fragariae* and other Phytophthora species (GILL and POWELL 1969, MAC DONALD and DUNIWAY 1979). Perhaps, it is best to think whether high race specificity is desirable for diagnostic purpose in growers strawberry fields. Usually the race mixture of a diseased strawberry field is unknown. A serological examination of those plants is only helpful if the antisera reacts with all races.

The values of ELISA with other Phytophthora species showed, that the antisera of *P. fragariae* L1 is not species specific. Cross-reactions in serological tests with fungi are well known (GILL and POWELL 1969, MAC DONALD and DUNIWAY 1979, KOUGH et al. 1983, AUGELO and DUNEZ 1984). Although the values of all tested Phytophthora species were significantly lower than that of *P. fragariae* L1 the cross-reactions could give an incorrect positive result when testing plant material. Above all that is important for *P. cactorum* (crown rot). Up to now only this Phytophthora species attacks strawberry plants under natural conditions, too. But it prefers the crown of the plants, while *P. fragariae* grows favourably in the roots and only roots are taken for sample to look for red core disease. Besides it is not unmistakably proved that *P. fragariae* and *P. cactorum* (crown rot) attack the same plant at the same time. Nevertheless cross-reactions of the antisera of *P. fragariae* L1 with other Phytophthora species should be eliminated for all tests with plant material.

No reaction with the antisera could be noted with the tested Pythium species. Testing *Pythium oligandrum* with an antisera of *P. cactorum* in agar gel diffusion test, BURRELL et al. (1966) could not note any reaction. Nevertheless it would be better to test more Pythium species to make sure that there will not be any cross-reaction with these fungi. Diseased strawberry roots with red core from growers fields very often show secondary infections with Pythium species. For detection of latent infections of strawberry roots with *P. fragariae* the

Fig. 5. Detection of *P. fragariae* in strawberry roots cv. Tenira in a field experiment (planting date 16 Aug., 1985, inoculation 16 Oct., 1985, n = 120, sample dilution 1:15, the samples were taken at different times after inoculation with *P. fragariae* L1).
standardisation of ELISA proved to be good. As shown in the present experiments and in \textsc{W}erres and \textsc{Casp}er (1987) and \textsc{W}erres (1987) ELISA detects root infections before any microscopic and macroscopical detection. Even if inoculum density is not very high, roots gave positive ELISA values 24 hours after potting the plants in infected substrate. As the present field experiment shows, roots infections could be detected by ELISA during summer months when red core disease is latent.

To use ELISA for routine testing there must be set a positive threshold below which plants are looked upon to be free of the pathogen. It is suggested that the threshold value be established for an accurate colormetric “zero” value (\textsc{C}lark 1981). For experiments under controlled conditions ELISA values from roots of none-inoculated strawberries proved to be good for “zero” value. Growing under field conditions roots of none-inoculated plants reacted with the antiserum, while the roots from control plants from the glasshouse did not. As shown by \textsc{B}anowetz and \textsc{M}ac\textsc{g}ra\textsc{ve} (1984) and other Glomus species. 

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{inoculated with} & \multicolumn{2}{|c|}{\textbf{P. fragariae L. I.}} & \multicolumn{1}{|c|}{\textbf{surface area of plant canopy (cm²)}} & \multicolumn{1}{|c|}{\textbf{number of leaves per plant (absolutely)}} & \multicolumn{1}{|c|}{\textbf{plant height (cm)}} \\
\hline
\hline
\textbf{–} & & & & & & & & & \\
\textbf{+} & & & & & & & & & \\
\hline
\textbf{–} & & & & & & & & & \\
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\end{tabular}
\caption{Vegetative growth of tissue-cultured strawberry plants cv. Tenara in a field free of \textit{Phytophthora fragariae} after inoculation with the pathogen (conditions for experiment see fig. 5).}
\end{table}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{P. fragariae L. I.} & \textbf{surface area of plant canopy (cm²)} & \textbf{number of leaves per plant (absolutely)} & \textbf{plant height (cm)} & \textbf{percentage} & \textbf{percentage} & \textbf{percentage} \\
\hline
\textbf{–} & & & & & & & & & \\
\textbf{+} & & & & & & & & & \\
\hline
\textbf{–} & & & & & & & & & \\
\textbf{+} & & & & & & & & & \\
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\end{tabular}
\caption{Values of healthy plants and different stages of diseased plants. Nevertheless fungal structures than mycelium reacts with an antiserum of \textsc{Aguelon}, while the roots from control plants from the glasshouse did not react evidently less than mycelium itself. Though free of the pathogen. During autumn and/or spring, too. \textsc{A}bula\textsc{t}i\textsc{d}o and other \textsc{Glom}us species. 

\textbf{Literature}

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