Monoclonal Antibody-Based Double-Antibody Sandwich-ELISA for Detection of *Verticillium* spp. in Ornamentals

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ABSTRACT

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Monoclonal antibodies (MAbs) were raised against extracellular molecules from *Verticillium dahliae* to detect *V. dahliae* and *V. albo-atrum* in rose and chrysanthemum. Eight selected MAbs recognized *V. dahliae* and *V. albo-atrum* isolated from these hosts. One of these MAbs performed well in a monoclonal double-antibody sandwich enzyme-linked immunosorbent assay. With this assay, *V. dahliae* and *V. albo-atrum* could be detected in roses and chrysanthemums that were artificially or naturally infected. Sap of healthy plants did not react, and cross-reactions with six other fungi isolated from rose were not observed. The distribution pattern of *V. dahliae* in the plant was determined by testing stem parts taken at different heights. *V. dahliae* was detected predominantly in the stem-base, although it occurred throughout the whole plant and not in every stem. This implies that the test should include a number of small samples of each plant.

Additional keywords: ACP-ELISA, DAS-ELISA, hybridoma, serology.

In current horticultural practice, detection and identification of systemic plant pathogenic fungi is increasingly important to ensure the health of propagation material. One of the main prerequisites for the reduction of pesticide applications is the availability of rapid, sensitive detection methods. Conventional methods for detection and identification are laborious and time-consuming. Identification of fungi on agar demands expertise, and a bioassay with test plants requires greenhouse facilities and considerable time (24,26). A serological assay with monoclonal antibodies (MAbs) would be a suitable alternative. Such tests are rapid and specific and do not require the expertise necessary for conventional methods.

In the Netherlands, Verticillium wilt is a major problem in field- and glasshouse-grown roses and chrysanthemums. The disease is caused by the vascular pathogens *Verticillium dahliae* Kleb. and *V. albo-atrum* Reinke & Berthold. Curative control of the disease is difficult to achieve. Symptoms appear during periods of stress, such as drought during midsummer. The symptoms only appear at a late stage of infection, which often makes it difficult to distinguish them from the normal symptoms of aging (15). In glasshouses, where seasonal variation is less evident, the disease occurs throughout the year (13). A potential danger is that infected plants may tolerate the pathogen when growing conditions are optimal. Moreover, after irrigation or a fall in temperature, the plants can recover. In these plants, *Verticillium* can be isolated from infected tissue even if there are no symptoms. With a return to drier conditions, wilting can occur again (10,12–15,25). Infected but symptomless plants delivered by a supplier may show symptoms after being planted by the grower. Therefore, it is important to apply effective detection methods before planting or grafting (13,26).

A serological test based on MAbs would enable the detection of *V. dahliae* and *V. albo-atrum* in propagation material at an early stage of infection. MAb-based immunoassays have been developed to detect *Botrytis cinerea* (2,19), *Ophiostoma ulmi* (7,8), *Phytophthora* spp. (11,16), and *Pythium ultimum* (28). For detection of *Verticillium,* only polyclonal antisera have been raised. These antisera were used to compare isolates of *V. albo-atrum* and *V. nigrescens* (27), to determine the antigenic similarities between different *Verticillium* spp. (9,20), or to investigate common antigens among cultivars of cotton and isolates of *Fusarium oxysporum* f. sp. *vasinfectum, F. solani* f. sp. *phaseoli, V. albo-atrum,* and *V. nigrescens* (3). Our aim was to develop a specific serological assay to detect *V. dahliae* and *V. albo-atrum* and that would not react with common antigens of other fungi or plant material.

This paper reports the selection of MAbs and the development of a monoclonal double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to detect *V. dahliae* and *V. albo-atrum* and differentiate between these two *Verticillium* spp. and other fungi in rose and chrysanthemum. To develop the antibodies, we used water-soluble antigens, likely extracellular polysaccharides (EPS) (5) obtained from surface washings and liquid cultures of *V. dahliae*, for immunization. The MAbs were tested for cross-reactions with plant material, a number of different *Verticillium* spp., and pathogenic and nonpathogenic fungi isolated from roses and other plants. The applicability of the monoclonal DAS-ELISA was evaluated with inoculated, naturally infected, and healthy roses and with inoculated chrysanthemums.

MATERIALS AND METHODS

Fungal cultures. Isolates of *Verticillium* spp. and other fungi obtained from roses and other sources (Table 1) were kept on Czapek Dox agar (CDA) or potato-dextrose agar (PDA) at 4°C in

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the dark. The fungi were cultured for surface washings on PDA slants at 20°C in the dark. Liquid cultures in Czapek Dox medium were grown at 21°C in the dark.

Antigen preparation. Extracellular antigens to immunize mice, screen hybridoma supernatants, or test for cross-reactions were obtained from surface washings, exudates in liquid cultures, and plant extracts.

i) Surface washings were obtained by washing mycelium on PDA slants with 2 ml of ultra pure water and gently scraping the surface with a Pasteur pipette. The resulting spore suspension was centrifuged for 10 min at 1,500 × *g*, and the supernatant was concentrated in an Amicon Ultrafiltration cell (Amicon Corporation, Scientific Systems Division, Danvers, MA), using a filter with a cut off of 10 kDa until a protein concentration of about 100 µg/ml was reached.

ii) Liquid cultures were obtained after removal of the mycelium by filtering through cheesecloth, followed by removal of the conidia by centrifuging for 10 min at 1,500 × *g*. The remaining particles were removed from the supernatant using a 0.65-µm filter. The culture liquid was concentrated in a Filtron Ultrafilter (Filtron Technology Corporation, Northborough, MA) with a 5-kDa cut off until a concentration of 100 µg/ml was reached.

iii) Plant extracts were obtained by grinding stems of infected or healthy roses in a blender with extraction buffer (0.01 M phosphate buffered saline/0.1% Tween [PBST] containing 4% polyvinylpyrrolidone, pH 7.2). The stem segments were disinfested with 70% ethanol. The sample liquid was centrifuged for 10 min at 1,500 × *g* to remove any cell debris and stored at –20°C until use. For more concentrated extracts, the stems were cut into small pieces and crushed with a hammer in plastic bags after extraction buffer was added in a ratio of 1:2 (wt/vol, stem/buffer). The sample liquid was centrifuged for 10 min at 9,500 × *g* in an Eppendorf centrifuge and stored at –20°C until use.

TABLE 1. Cross-reactivity of monoclonal antibody 7E12 in double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with extracellular antigens from surface washings (SW) and liquid cultures (LC) of *Verticillium dahliae* isolates, other *Verticillium* spp., and pathogenic and nonpathogenic fungal species isolated from roses

Species	Isolate source	No. of isolates	SW[a]	LC[a]
Verticillium dahliae	Potato	2	++	++
V. dahliae	Rose	1	++	++
V. dahliae	Rose	12	++	nd
V. dahliae	Rose	1	+	nd
V. dahliae	Chrysanthemum	1	++	nd
V. dahliae	Chrysanthemum	4	+	nd
V. dahliae	Maple	1	+	nd
V. albo-atrum	Potato	1	+	++
V. albo-atrum	Tomato	1	++	nd
V. tricorpus	Potato	1	+	++
V. tricorpus	Apple	1	+	+
V. tricorpus	Celery	1	+	nd
V. tricorpus	Pelargonium	1	+	nd
V. nigrescens	Celery	1	–	++
V. nigrescens	Carthamus	1	+/–	nd
V. nubilum	Protozo	1	–	++
V. nubilum	Potato	1	+	nd
V. chlamydosporum var. *chlamydosporum*	Nematode	1	–	–
V. chlamydosporum var. *catenulatum*	Forest ground	1	–	–
Botrytis cinerea	Rose	2	+/–	nd
B. cinerea	Rose	4	–	nd
Fusarium sp.	Rose	2	–	nd
Alternaria sp.	Rose	3	–	nd
Penicillium sp.	Rose	3	+/–	nd
Penicillium sp.	Rose	1	–	nd
Phomopsis sp.	Rose	1	+/–	nd
Coniothyrium fuckelii	Rose	1	–	nd

[a] DAS-ELISA reactions: – = no reaction above background; +/– = weak reaction slightly above background; + = strong reaction; ++ = very strong reaction; and nd = not done.

Immunization. Mice were injected three times at 4-week intervals with about 10 µg of antigens from surface washings or liquid cultures of *V. dahliae* isolate B2. For the first two injections, Freund's incomplete adjuvant was added to the antigens (FIA; Sigma Chemical Co., St. Louis). FIA was omitted for the last immunization (21).

Fusion. Three days after the last injection, the mice splenocytes were fused with SP2/0-Ag-14 myeloma cells using polyethylene glycol 4000 (21). The cell suspension was plated in 96-well microtiter plates with selective medium at 10^4 cells per well (100 µl). About 15 days after the fusion, the cell-culture supernatants were screened for the presence of specific antibodies by ELISA as described below. The hybridomas secreting antibodies that reacted with extracts of rose stems infected with *V. dahliae* were cloned in microtiter plates by limiting dilution (one cell per well) until stability (21). The stable clones were cultured in spinner flasks to 1 liter for large-scale production of MAbs (21).

ELISA. A DAS-ELISA was used to screen for specific antibody-secreting hybridoma cells, according to standard ELISA procedures (4,23). Briefly, the wells of the plates were coated with a polyclonal rabbit serum (0.12 µg per well) against conidia of *V. dahliae* (provided by J. van der Wolf, IPO-DLO, Wageningen, the Netherlands). After blocking with PBST containing 0.5% bovine serum albumin (BSA) and incubating with undiluted plant extracts, hybridoma supernatant was added to the wells. The reaction was visualized with a rat anti-mouse alkaline phosphatase-conjugate (RaAM-AP, Jackson Immuno-Research Laboratories, Inc., West Grove, PA) with *p*-nitrophenyl phosphate as substrate and was measured by determining the optical density at 405 nm.

In a second screening, the positive cultures were retested in the same ELISA for their reactivity with extracts of healthy roses and antigens from surface washings or liquid cultures (1.5 µg per well) of *V. dahliae* B2.

Two protocols were used with the purified and conjugated MAbs for the development of a rapid and specific ELISA.

i) *Antigen-coated plate-ELISA (ACP-ELISA).* Plant extracts, were coated directly on the wells of the plate, and the ELISA was continued according to standard procedures (4,23). Briefly, the plate was blocked with PBST/0.5% BSA and incubated with an alkaline phosphatase (AP)-conjugated MAb using 15 ng per well. The reaction was visualized with *p*-nitrophenyl phosphate and measured at 405 nm.

ii) *DAS-ELISA.* The plates were coated with 100 µl of polyclonal antibodies or purified MAb in different dilutions. The ELISA was continued according to standard procedures (4,23). Briefly, after blocking with PBST/0.5% BSA, the plate was incubated with plant extracts of infected or healthy roses and with an AP-conjugated MAb (15 ng per well). The reaction was visualized with *p*-nitrophenyl phosphate and measured at OD_{405}.

Isotype determination. The subclass of the MAbs was determined as described by Schots et al. (21). The hybridomas were tested in an ELISA with monoclonal rat anti-mouse immunoglobulin (Ig) IgA, IgM, IgG1, IgG2a, IgG2b, IgG3, IgG, and kappa antibodies. The reaction of the antibodies with rat anti-mouse isotype antibodies was visualized with a RaAM-AP conjugate.

Purification and conjugation of MAbs. The antibodies were purified from the large-scale cultures by chromatography (21). The IgG2 MAbs were purified using a Protein-A column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Bound antibodies were eluted with a 0.1 M citric acid-NaOH buffer, pH 4.0. The IgG1 MAbs were purified using thiophilic agarose (Affi-T, Biozym, Copenhagen), in which bound antibodies were eluted with a 0.05 M Tris-HCl buffer, pH 9.0. Further purification was carried out by size-exclusion chromatography on a Superdex 200 column (Pharmacia). The purified antibodies were conjugated with AP as described by Tijssen (23). The immunoglobulins were conjugated with AP in potassium phosphate buffer using glutaraldehyde. After incubation, the antibodies were dialyzed against 0.15 M Tris-HCl buffer, pH 8.0.

Plant material. The monoclonal DAS-ELISA was evaluated with samples taken at different heights of artificially infected roses and chrysanthemums and naturally infected roses. Chrysanthemums were inoculated with *V. dahliae* or *V. albo-atrum*; roses with a *V. dahliae* isolate (from potato or rose). For inoculation, 5 ml of a suspension of conidia (10^7/ml) was added to soil in 13-cm pots. For some experiments with roses the roots were slightly damaged to increase the chance of infection. In one experiment, roses were infected through an incision in the stem. A drop of a spore suspension (10^7/ml) was placed on the incision and allowed to be taken up by the plant. Stem parts with a length of 1 cm were taken, disinfested with 70% ethanol, and plated on PDA for chrysanthemums and on CDA for roses.

RESULTS

Selection of hybridoma cell lines. The fusions with splenocytes from mice immunized with extracellular antigens obtained from liquid cultures resulted in eight clones that produced antibodies that reacted with extracts from rose stems infected with *V. dahliae*. The eight clones and their isotypes are 2D6 (IgG1), 7E12 (IgG2a), 8D10 (IgG1), 10B11 (IgM), 11B6(IgG1), 12A3 (IgG1), 13F6 (IgG1), and 14C9 (IgG2b). They also reacted with extracellular antigens from surface washings and liquid cultures of *V. dahliae* and did not react with extracts of healthy roses. The isotypes are listed in Table 2. The fusions for which mice were injected with extracellular antigens from surface washings only led to positive clones that also reacted with extracts of healthy plants. These clones were all of the IgM isotype, which often reacts nonspecifically. The eight specific clones were all cultured for large-scale production of antibodies, purified, and conjugated to AP.

ELISA. When tested in different ELISA formats, MAb 7E12, an IgG2a, gave the strongest reaction and the lowest background with extracts of healthy plants. This antibody, therefore, was characterized in detail, both for cross-reactions and applicability in a routine test.

The polyclonal and monoclonal DAS-ELISA gave better results than the ACP-ELISA. Moreover, little background was observed with the DAS-ELISA. In contrast, the optical density values of the ACP-ELISA were very low, and the background was almost as high as the reaction with the infected plants. The optical density values of the DAS-ELISA, using 7E12 as capture antibody, were

TABLE 2. Comparison of results of monoclonal double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with monoclonal antibody 7E12, using extracts of stem segments, and results of plating *Verticillium* from the corresponding segments on agar[a]

Experiment	Plant species	Fungal species	Inoculation method[b]	No. of plants	ELISA result	Plating result[c]	Ratio plating/ ELISA
1	Chrysanthemum	*V. dahliae*	Roots	21	7	9	1.29
		V. albo-atrum	Roots	3	1	1	1
		Control	...	5	0	0	1
2	Rose	*V. dahliae*	Roots	10	10	10	1
		V. dahliae	Incision	2	2	2	1
		Control	...	3	0	0	1
3	Rose	*Verticillium* sp.	Natural	43	20	13[d]	0.65
4	Rose	*Verticillium* sp.	Natural	31	25	11[d]	0.44

[a] The plants were grown on rock wool, clay grains, and soil. Homogenates of stem segments for ELISA were made with a blender (experiments 1 and 3) or through crushing with a hammer (experiments 2 and 4). The number of positive plants, in the ELISA or plating experiment, of the total number of plants in each experiment is indicated. The last column shows the correspondence of the ELISA results to the plating results and is calculated as the ratio of plating versus ELISA

[b] Inoculation methods include: roots = inoculation by placing a plant with slightly damaged roots in 13-cm pots with soil to which 5 ml of a suspension of conidia (10^7/ml) had been added; incision = inoculation by making an incision in the stem and adding a drop of a suspension of conidia (10^7/ml); natural = plants obtained from growers that possibly were infected.

[c] In the plating experiments, potato-dextrose agar was used for chrysanthemums, and Czapek Dox agar was used for roses.

[d] The numbers indicate plating results that were clearly *Verticillium*, on other plates overgrowth of *Verticillium* may have been masked by other fungi, such as *Penicillium* sp. and *Botrytis cinerea*.

TABLE 3. Examples of results of monoclonal double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with monoclonal antibody 7E12, using extracts of stem segments at different heights (centimeters), and results of plating *Verticillium dahliae* from the corresponding segments on agar, to study the distribution pattern of *V. dahliae* in the stem and its effect on DAS-ELISA

			ELISA[b,c]			Plating[c,d]			
Plant species	Cultivar	Symptoms[a]	0–10	10–25	25–50	0	10	25	50
Chrysanthemum	Reyellow	+	+	+	–	+	+	+	+
Chrysanthemum	Funshine	+	+	+	–	–	+	+	+
Chrysanthemum	Funshine	+/–	–	+	–	+	+	–	–
Chrysanthemum	Regoltime	+/–	+	–	–	+	+	+	–
Chrysanthemum	Yellow Reagan	+	+	–	+	+	+	+	–
Rose	Tineke	+/–	–	+	+	+	+	+	+
Rose	Tineke	+	+	+	+	+	+	+	+
Rose[e]	Yellow River	+	+	+	nd[f]	+	+	nd	nd
Rose[e]	Yellow River	+/–	+	+	nd	+	+	nd	nd
Rose	Baronesse	–	–	+	–	–	+	+	nd
Rose	Unknown	+	+ (0–20)[c]	+ (20–40)[c]	nd	–	nd	+ (20)[c]	nd
Rose	Unknown	+	+ (0–20)[c]	+ (20–40)[c]	+ (40–60)[c]	– (0)[c]	nd	– (20)[c]	nd

[a] + = Wilt symptoms; +/– = few wilt symptoms; – = no wilt symptoms.

[b] + = Positive ELISA results; – = no reaction above background level.

[c] Samples were taken at stem heights indicated. For ELISA, stem segments of indicated length were taken; for plating, stem segments of approximately 1 cm were taken.

[d] + = Growth of *V. dahliae* on agar; – = no *V. dahliae* on agar. Stem segments of chrysanthemum were plated on potato dextrose agar; stem segments of rose were plated on Czapek Dox agar.

[e] Results of two stems from the same plant.

[f] nd = not done because the plant was less than 50 cm in height.

about five times higher than those of the polyclonal rabbit antiserum. We determined that the optimal concentrations of 7E12 to carry out the DAS-ELISA were 1.3 µg/ml for coating and 150 ng/ml for the AP-conjugated second-step antibody.

Extracellular antigens from surface washings of various fungi (Table 1) were tested in the DAS-ELISA with MAb 7E12 for cross-reactions. Every *V. dahliae* isolate reacted in the ELISA. Reactions with the *V. albo-atrum* isolates and one *V. tricorpus* isolate were about as high as the reaction with *V. dahliae*. The reaction with *V. tricorpus* PD 90/2043 and *V. nubilum* PD 76/379 was lower. Isolates of the other *Verticillium* spp. did not react, except for *V. nigrescens* PD 88/348, which showed a reaction slightly above the background. The ELISA showed a reaction a little above the background (optical densities between 0.1 and 0.2) with some of the isolates of *Penicillium* sp., *B. cinerea*, and *Phomopsis* sp., whereas the optical densities with most *V. dahliae* isolates were over 1.0. No reaction was found with the other fungi tested.

Extracellular antigens from liquid cultures of all *V. dahliae* and *V. albo-atrum* isolates reacted in the ELISA, and with *V. tricorpus, V. nigrescens,* and *V. nubilum* isolates as well. *V. chlamydosporum* var. *chlamydosporum* and *V. chlamydosporum* var. *catenulatum* did not react with 7E12 (Table 1).

Evaluation of DAS-ELISA with MAb 7E12. ELISA carried out with stems homogenized in the blender resulted in low optical density readings and some false negatives compared with the plating results. Better ELISA results, and no false negatives, were obtained by crushing the stems with a hammer. Using this method, the antigen was less diluted because the extracts were made from a small part of the stem in a small amount of buffer.

The results of the ELISA with inoculated chrysanthemums corresponded with the symptoms of the plants and the growth of *Verticillium* on agar (Table 2, experiment 1). No false positives were found in the ELISA. A few plants that showed putative symptoms were negative, both in the ELISA and on agar. Using the ELISA, we were able to detect *V. dahliae* in plants that had only minor symptoms. Lower stems of the plants reacted most strongly.

The experiment with roses inoculated through an incision in the stem (Table 2, experiment 2) showed positive reactions in the ELISA in every inoculated stem and at almost every height. The optical density readings were higher with samples from the top parts than with samples from the lower parts of the plants. *V. dahliae* was isolated on agar from all samples. Roses inoculated by placing plants with damaged roots in infested soil reacted in the ELISA, but a reaction was not observed in all stems. However, if a stem was infected, a positive reaction was recorded with samples taken at every height. In some stems the optical density values were higher in the lower parts. The healthy plants did not react. Other fungi, such as *Penicillium* spp. and *B. cinerea*, were isolated on agar from one of the noninoculated plants. Although we found some reaction with the surface washings of these fungi, this plant never reacted in the ELISA, so these fungi did not give rise to false positive results.

The experiments with naturally infected roses from growers (Table 2, experiments 3 and 4) showed that every plant with clear symptoms reacted in the ELISA. *V. dahliae* was isolated from each of these plants and from almost every stem part. The plants with fewer symptoms also showed lower reactions in the ELISA, and they reacted only when *V. dahliae* also was isolated on agar. Only the lower parts of the weak plants, without any symptoms of Verticillium wilt, reacted; the optical densities were low; and we were never able to isolate *Verticillium* on agar. In a few cases, the latter may have been a consequence of overgrowth by other fungi. The healthy plants did not react. False positive results were not observed. Healthy plants that were severely infected with other fungi and bacteria had optical density readings that were not above the background level where *V. dahliae* could not be isolated. The plants from which *B. cinerea* or *Penicillium* spp., but not *V. dahliae,* was isolated were always negative in the ELISA, so again these fungi did not give rise to false negative results. Some plants reacted more strongly in the upper parts (Table 3). Therefore, it is difficult to draw conclusions on the distribution pattern of *V. dahliae* in the plant.

DISCUSSION

A monoclonal DAS-ELISA was developed to detect *V. dahliae* and *V. albo-atrum* in roses and chrysanthemums based on Mab 7E12. This antibody was selected from a panel of eight hybridoma clones that were obtained from fusions screened with extracts of roses infected with *V. dahliae*. They also reacted with surface washings and culture supernatant of *V. dahliae* and did not react with sap from healthy roses.

Several ELISA formats have been tried. An ACP-ELISA with MAb 7E12, in which plant extracts were coated directly to the plate, gave low optical density readings. Apparently the number of antigen molecules bound to the well of the microtiter plate was too low. The number can be increased using DAS-ELISA (6,8). The bound antibody specifically captures the relevant antigen, while irrelevant material is removed by washing. Similar problems were encountered by Sundaram et al. (22). They prepared polyclonal antisera to detect *V. dahliae* in potatoes with DAS-ELISA. The results were satisfactory, and the antisera did not cross-react with the other tested fungi. They also reported that polyclonal antisera can be more specific when the antigen used for immunizing mice is obtained by polyacrylamide gel electrophoresis fractionation. We did not need to isolate a more specific antigen. Using extracellular molecules of *V. dahliae* as immunogen, we were able to develop a MAb with sufficient specificity.

The DAS-ELISA wherein MAb 7E12 was used as the capturing and detecting antibody resulted in optical density values about five times higher than those of the DAS-ELISA wherein the polyclonal antiserum was used as capturing antibody. Furthermore, the reaction with healthy roses was lower. Priestly and Dewey (17) also coated plates with MAbs, but they used a polyclonal antiserum as the detecting antibody. With our monoclonal DAS-ELISA, however, sufficient antigen-binding sites are left after capture of the antigen by the coated MAbs for binding using the same MAbs as detecting antibodies. The putative character of the antigen is that of EPS (5) and is not proteinaceous according to former blotting experiments (data not shown).

The cross-reaction experiments with extracellular antigens from surface washings resulted in a reaction with all 22 tested *V. dahliae* isolates, not only those isolated from roses, but also those isolated from plants such as chrysanthemum, potato, and maple. This indicates that when using this test it is likely that any *V. dahliae* isolate will be detected. Within the genus *Verticillium*, *V. albo-atrum* and *V. tricorpus* reacted in the DAS-ELISA with MAb 7E12 and one each of the *V. nigrescens* and *V. nubilum* isolates reacted as well, whereas the *V. chlamydosporum* var. *chlamydosporum* and *V. chlamydosporum* var. *catenulatum* isolates did not.

The ELISA signal obtained with surface washings of the *V. albo-atrum* isolates is as strong as the signal obtained with the surface washings of *V. dahliae*, which is an advantage because both fungi are pathogenic. In the experiments with artificially inoculated chrysanthemums, *V. albo-atrum* also was detected in the ELISA. *V. tricorpus* reacted almost as strongly as *V. dahliae*, but until now, this fungus has never been isolated from roses and chrysanthemums. The surface washings of the isolates of *Fusarium* spp., *Alternaria* spp., *Phomopsis* spp., and most isolates of *Penicillium* spp. and *B. cinerea* did not cross-react. A reaction somewhat above the background level was found with the extracellular antigens from surface washings of some isolates of *Penicillium* spp. and *B. cinerea*. In the experiments with naturally and artificially infected roses, however, we showed that if *B. cinerea* and *Penicillium* spp. were isolated on agar and *V. dahliae* was not, we never found a positive reaction in the ELISA. These fungi do not systemically infect roses and chrysanthemums. The absence of a reaction with the surface washings of *Coniothyrium fuckelii*

is important because this fungus does systemically infect roses.

In initial experiments, *V. dahliae* was not detected in every infected plant. We reasoned that the antigens were diluted too much, because a large amount of buffer is necessary to grind the plants in a blender. This is likely a consequence of the fact that *V. dahliae* often does not spread throughout the entire stem. Using the hammer method, we were able to make more concentrated extracts, leading to ELISA readings that correlated with the plating results. Testing extracts from different small parts of the stem separately increases the chance of detecting *V. dahliae* in one of the stem parts and allows the determination of the distribution of *V. dahliae* throughout the plant. Therefore, the artificially and naturally infected plants were tested at different heights.

In contrast to chrysanthemums, roses in artificially infested soil did not become infected. When the plants were inoculated through an incision in the stem, every plant reacted with ELISA. However, this method of inoculation is different from the natural infection means. Inoculation through infested soil after the roots had been slightly damaged also was effective. Using DAS-ELISA with MAb 7E12, we were able to detect *V. dahliae* in every plant that was inoculated through damaged roots and crushed with a hammer. The results of the DAS-ELISA correlated with those of the platings for almost every height of the plant. There were no false positives in the ELISA.

It is possible to detect *V. dahliae* in slow-growing plants without any symptoms of Verticillium wilt. This is shown in the experiments with samples from infected roses from growers using DAS-ELISA with Mab 7E12.

It is obvious that the distribution of *Verticillium* in the plant has implications for the performance of the tests. It is difficult to determine how *V. dahliae* actually spreads through the plant (Table 3). Generally, reactions were highest in the lower parts of the plants. However, there were also plants in which reactions were highest in the upper parts. Other plants showed even reactions at every height, but not every stem reacted. This concurs with the findings of Benken and Khakimov (1) and Rijkers et al. (18) who reported the same findings for the distribution of microsclerotia in petioles of cotton and ash trees. Therefore, several samples of one plant must always be included in the test.

From some plants that reacted positively with ELISA, *V. dahliae* was only occasionally isolated on agar. Here the stems were thick and woody, which made isolation cumbersome. Similar problems were mentioned by Woolliams (26).

In almost 100% of the artificially and naturally infected roses, using the hammer method of crushing, *Verticillium* could be detected without any false positive reactions, even when no symptoms of wilt were visible yet. The test is now in use by the Inspection Service for Floriculture and Arboriculture for Ornamental Flowers, Roelofarendsveen, the Netherlands, to screen for *V. dahliae* on roses and chrysanthemums to be used for propagation.

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