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Detection of tomato spotted wilt virus using monoclonal antibodies and riboprobes

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Summary. The immunoreactivity of a panel of monoclonal antibodies raised to tomato spotted wilt virus (TSWV) was examined in enzyme-linked immunosorbent assays (ELISA) and dot immunobinding assays (DIBA) procedures. MAbs 6.12.15 and 2.9 were specific for the nucleocapsid protein of TSWV. The sensitivity of the two immunoassays was compared with that of a dot-blot hybridization technique using riboprobes (RNA transcripts) to TSWV M RNA. Using deproteinized plant extracts or purified virus preparations, as little as 1 pg RNA could be detected. Although an ELISA using MAb 6.12.15, a DIBA procedure using MAb 3.22.6 and the dot-blot hybridization, detected several TSWV isolates in different host species equally well, the ELISA was most precise and most suitable for routine diagnosis in the field.

Introduction

Tomato spotted wilt virus (TSWV) can cause serious yield losses in a large number of economically important crops all over the world. The virus has an extremely broad host range. Up to 350 plant species belonging to at least 50 families, both monocotyledons and dicotyledons, can be infected [D. Peters, pers. commun.]. TSWV is exclusively transmitted by thrips species and at least nine species of this insect have so far been reported as vector [9, 11]. Recent data on the molecular structure of the virus indicate that TSWV should be considered a member of the *Bunyaviridae* [3, 4].

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TSWV occurs mainly in the subtropical climate zones, causing considerable losses to tomato, tobacco, sweet pepper, lettuce, peanut and papaya crops. Although the virus has been virtually absent in Western Europe, the recent introduction of one of its vectors: *Frankliniella occidentalis* has made TSWV a potential threat to greenhouse cultivation of tomatoes, peppers and several ornamental plants like chrysanthemum, begonia, impatients, and ageratum. The indexing of crops grown for seed production and the testing of plants used in breeding require fast, cheap, reliable and sensitive virus detection assays. Serological tests used in the past for detecting TSWV have lacked reliability and sensitivity, mainly because of the variable and low quality of the antiserum preparations that were used [9, 10, 15].

To improve detection of TSWV, polyclonal and monoclonal antibodies were raised to TSWV and used to develop two sensitive serological detection assays: an enzyme-linked immunosorbent assay (ELISA) and a dot-immunobinding assay (DIBA). In the present paper, these two assays are compared with a method of TSWV detection based on molecular hybridization using RNA transcripts called "riboprobes". This system is very sensitive due to the formation of stable RNA-RNA hybrids and the high labeling efficiency of uniform and specific RNA probes. Recently a riboprobe detection assay has been described for detection of potato spindle tuber viroid (PSTV) in potato [12] or plum pox virus in apricot trees [16]. In the case of PSTV detection, the assay was able to detect less than 1 pg of RNA [12].

Materials and methods

Virus

Two isolates of TSWV, one (S) from the Netherlands and the other (CNPH1) from Brazil, were maintained in tomato by grafting. Virus was purified from mechanically inoculated *Nicotiana rustica* leaves [15].

Monoclonal antibodies

Three 10 weeks old BALB/c mice, were immunized by a series of 6 intraperitoneal injections of 30 µg purified TSWV-S, emulsified with an equal volume of complete Freund's adjuvant at 2 weeks intervals. One month after the last injection, the mouse giving the highest specific antibody response in indirect double sandwich ELISA was boosted by two intraperitoneal injections of 30 µg virus in phosphate buffered saline (PBS), pH 7.4 (0.14 M NaCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.5 mM KCl). Three days after the second booster injection, spleens were excised and used for cell fusion. The fusion experiment was performed as described earlier [7].

Monoclonal antibodies (MAbs) were obtained in large amounts in ascitic fluids from pristane-primed BALB/c mice which had been injected with 10^6 hybridoma cells.

Immunoassay used for screening hybridomas and MAb characterization

Supernatants of growing hybridoma cultures were screened for the presence of specific antibodies to TSWV by a double antibody sandwich ELISA (DAS-ELISA). The microtiter plates (Nunc, Maxisorp F96) were coated with 2 µg/ml anti-TSWV-S rabbit immunoglob-

ulin (anti-TSWV-S Ab^R) diluted in carbonate buffer, pH 9.6, for 2 h at 37 °C. After 30 min incubation with 1% bovine serum albumin (BSA) at 37 °C, the plates were incubated with TSWV infected plant extract diluted 1/10 and 1/100 in PBS containing 0.05% Tween 20 (PBS-T). After washing with PBS-T, the plates were incubated with hybridoma culture supernatant (twofold diluted) followed by alkaline phosphatase-labeled goat anti-mouse globulins (GAM-PAL) diluted 1/2,000 (Sigma, St. Louis). The enzyme conjugate was detected by addition of substrate (p-nitrophenyl-phosphate 1 mg/ml in 0.1 M diethanolamine buffer, pH 9.8) and incubation at room temperature. A control consisting of healthy plant extracts was included with each supernatant tested. The absorbance was read at 405 nm using a "Titertek Multiscan" photometer (Flow Laboratories) and the values were considered positive if they exceeded the buffer control by a factor two.

Isotyping of MAbs was done by an agar-diffusion test [8], using subclass specific rabbit anti-mouse sera.

The specificity of the MAbs for different virus preparations was determined in ELISA procedure 2 (Table 1). The microtiter plates were coated with anti-TSWV-S Ab^R at a concentration of 2 µg/ml. The antigen concentration was 1 µg/ml when purified virus and purified nucleocapsid preparations were used while plant extracts were diluted 1/100. The MAbs were applied at an ascitic fluid dilution of 1/1,000, and the GAM-PAL at a dilution of 1/2,000.

Biotinylation of MAbs and preparation of enzyme-labelled streptavidin

For biotinylation of MAbs, N-hydroxysuccimidobiotin (E. Y. Labs, San Mateo) was diluted to 5 mg/ml in 0.01 M $NaHCO_3$ and added to ascitic fluids [diluted 1/10 (v/v) in 0.01 M $NaCO_3$], at a 1/25 (v/v) ratio. The mixture was incubated for 4 h at 25 °C and the reaction was stopped by addition of 1 M NH_4Cl [17].

Conjugated streptavidin was obtained by mixing streptavidin (1 mg in PBS) (Sigma, St. Louis) with 100 µl alkaline phosphatase (10 mg/ml, Boehringer) and dialysing the mixture for 5 h at room temperature against PBS containing 0.06% glutaraldehyde (v/v) and subsequently against several changes of PBS [1].

Polyclonal antibodies

Rabbits received a series of injections of 300 µg of virus (TSWV-S and TSWV-CNPH 1), emulsified in incomplete Freund's adjuvant at two-weeks intervals. The rabbits were bled 2 weeks after the third injection.

ELISA used for TSWV diagnosis

Different ELISA procedures (Table 1) were evaluated for their ability to detect virus in purified preparations or in sap from infected plants. Plant extracts were prepared by grinding leaf material in PBS-T (1 g/10 ml buffer) and filtering through miracloth. The dilutions of ascitic fluids used in these assays are indicated in Table 2. The plates were coated with anti-TSWV-S Ab^R at a concentration of 2 µg/ml. The polyclonal antibodies anti-TSWV-CNPH 1 alkaline phosphatase conjugate (Ab^R-PAL) was used at a dilution of 1/1,000. The streptavidin alkaline phosphatase (Stre-PAL) and the anti-mouse globulin conjugates (GAM-PAL) were used at a dilution of 1/2,000. A negative control consisting of crude sap from healthy plants was included in all assays. The assays were performed as described above.

Table 1. ELISA procedures used for TSWV detection

Procedure	Successive steps of the assay			
1	Ab^R	Ag	Ab^R-PAL	
2	Ab^R	Ag	MAb	GAM-PAL
3	MAb	Ag	Ab^R-PAL	
4	MAb	Ag	MAb-B	Stre-PAL

Ab^R Anti-TSWV rabbit antibodies; *Ag* antigen (purified virus, purified nucleocapsid or plant extract from infected leaves); *PAL* alkaline phosphatase label; *MAb* monoclonal antibodies (ascitic fluid); *GAM* goat anti-mouse globulins; *B* biotin label; *Stre* streptavidin

Dot-immunobinding assays (DIBA)

Plant extracts were prepared as described in the previous paragraph. Dilutions of purified virus preparations and plant extracts were spotted on nitrocellulose sheets (3 µl/spot) and allowed to air dry. The sheets were then soaked in 3% BSA in PBS for 30 min, followed by an incubation of 1 h with Ab^R-PAL (anti-TSWV-CNPH 1) (1/500 dilution) in procedure A, or with MAb (1/1,000 dilution) followed with GAM-PAL (1/1,000 dilution) in procedure B, or with biotinylated MAb (MAb-B) (1/1,000 dilution) followed with Stre-PAL (1/1,000 dilution) in procedure C (Table 4). These dilutions were made in 0.3% PBS-BSA. After rinsing 3 times for 10 min with 0.3% BSA in PBS, and twice for 5 min in 0.1 M Tris-HCl, 5 mM $MgCl_2$, pH 9.5 (TM buffer), the sheets were incubated in a substrate solution consisting of 0.33 mg/ml nitroblue tetrazolium (NBT) and 0.16 mg/ml 5-bromo,4-chloro,3-indoleyl phosphate (BCIP) in TM buffer. The reaction was stopped by soaking the sheets in 20 mM Tris-HCl buffer, pH 7.5, with 5 mM EDTA.

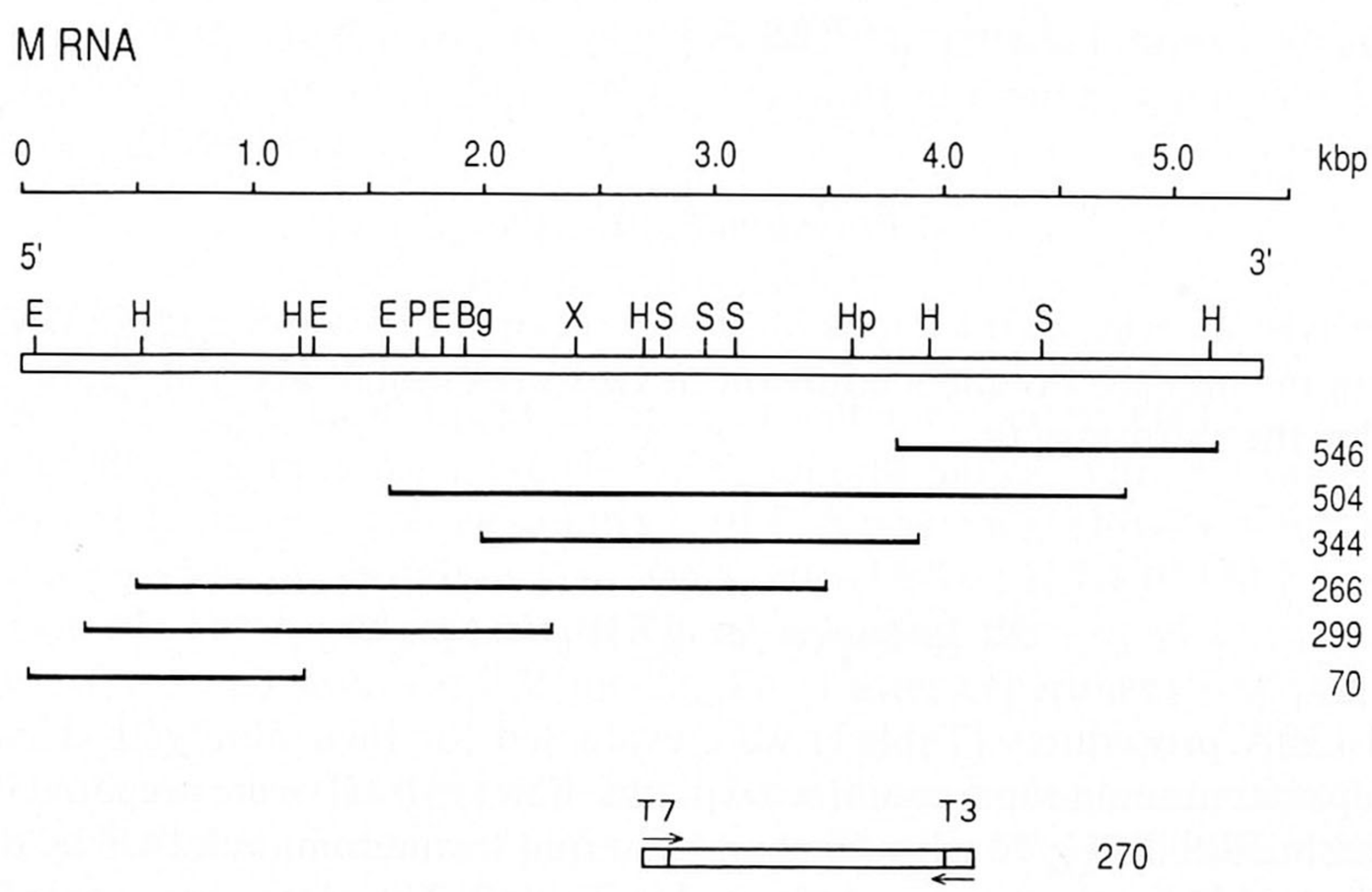

Fig. 1. Restriction endonuclease map of aligned cDNA clones of TSWV M RNA and the location of cDNA 270, used as template for synthesis of ^{32}P-labeled RNA probes. The numbers correspond to the depicted cDNA clones. T3/T7 represent the promoter sequence of the phage T3/T7 RNA polymerase. The restriction enzymes are abbreviated as follows: *Bg* BglII; *E* EcoRI; *H* HindIII; *Hp* HpaI; *P* PstI; *S* SphI; *X* Xbal

Synthesis of riboprobes

Complementary DNA to TSWV-CNPH1 RNA was synthesized and cloned as described previously [4]. cDNA clone 270 (1.2 kb), corresponding to the central part of the M RNA was subcloned in the vector pSK$^+$ (Stratagene, San Diego). ^{32}P-labeled, "run off", strand specific RNA transcripts were synthesized using T3 or T7 RNA polymerase after linearisation of the template according to the manufacturer's conditions (Fig. 1). The specific radioactivity was usually 10^9 cpm/µg template RNA.

Dot-blot hybridization

Crude RNA samples were prepared by grinding 1 g leaf material in 5 ml extraction buffer (10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 5 mM EDTA; 0.1% Triton X100, 0.1% 2-mercaptoethanol), followed by phenol extraction. Dilutions of purified virus and crude RNA preparations were spotted on Hybond N membranes (Amersham), 3 µl/spot. The RNA was UV-cross-linked, prehybridized for 4 h in a buffer consisting of 5 × SSC (standard sodium citrate) (20 × SSC is 3 M NaCl, 0.3 $C_6H_5Na_3O_7$, pH 7.0), 5 × Denhardt's (50 × Denhardt's is 10 g/l ficoll, 10 g/l polyvinylpyrrolidone, and 10 g/l BSA), 50 mM NaH_2PO_4 (pH 7.0), 50 mM EDTA and 100 µg/ml denaturated herring sperm DNA at 60 °C and subsequently hybridized in the same buffer after adding 10^5 cpm/ml probe RNA, for 18 h at 60 °C. The filters were washed twice with 2 × SSC containing 0.1% SDS for 5 min at room temperature and once with 0.1 × SSC with 0.1% SDS at 50 °C and exposed to X-ray films.

Results

Characterization of TSWV monoclonal antibodies

Six MAbs were obtained from one fusion experiment, and their isotypes were determined (Table 2). Each hybridoma clone was injected into pristane-primed

Table 2. Reactivity of MAbs with purified TSWV, purified nucleocapsids, and extracts from infected *N. rustica* plant, in DAS-ELISA procedure 2

MAb	Subclass type	Working dilution of ascitic fluid			Reactivity in ELISA procedure 2 with		
		MAb used in proc. 2	Coating MAb used in proc. 3 & 4[a]	MAb-B used in proc. 4[a]	PV	PN	IPE
6.12.15	IgG1	10^{-4}	10^{-3}	10^{-3}	1.548[b]	1.813	1.389
3.22.6	IgG2a	10^{-4}	10^{-3}	10^{-3}	0.024	0.089	1.648
7.22.6	IgG1	10^{-3}	10^{-3}	10^{-3}	0.020	0.051	1.229
2.9	IgG2a	10^{-4}	10^{-3}	5.10^{-2}	0.783	1.715	1.347
8.11	IgM	10^{-2}	10^{-2}	–	0.099	0.093	0.759
6.7	IgG1	10^{-3}	10^{-2}	5.10^{-2}	0.350	0.337	0.592

PV Purified virus (1 µg/ml); *PN* purified nucleocapsid; *IPE* infected plant extract (diluted 1/10); *MAb* monoclonal antibodies (ascitic fluid); *B* biotin label

– Not done

[a] These dilution figures are given for comparison only

[b] OD 405 nm, after 1 h substrate incubation

BALB/c mice for ascitic fluid production. The MAbs were biotinylated for use in ELISA procedure 4. The working dilutions of ascitic fluid, used as coating antibody, detecting antibody or labeled antibody in the different ELISA procedures (Table 1), were chosen such that an absorbance of about 1.0 was obtained after one hour substrate incubation (Table 2).

In the case of MAbs 6.7 and 8.11, working dilutions of about 10^{-2} had to be used in most ELISA procedures in order to reach an OD of 1.0. In contrast, polyclonal antiserum could be used at a dilution of 10^{-3} to detect the same amount of virus. In ELISA procedure 2, in which MAbs are used at pH 7.4, most ascitic fluids could be used at dilutions of 10^{-3} or 10^{-4}. In ELISA procedures 3 and 4, MAbs are adsorbed to the plastic at pH 9.6, which seems to decrease their reactivity. As a result, the working dilutions are lower, i.e., 10^{-2} or 10^{-3} (Table 2). ELISA procedure 4 also decreased antibody reactivity, and lower working dilutions of MAb-B had to be used. One exception is MAb 7.22.6, which was not affected by adsorption to plastic or biotinylation, and which could be used at the same dilution in the various ELISA procedures.

TSWV is a very labile virus; recent studies showed that proteolytic degradation occurred during purification on the envelope glycoproteins (results not shown). Antigenically, the virus is behaving differently as the native form in plant sap, or as purified virus. In order to be able to use MAb for diagnostic purposes, it was important to know whether their reactivity depended on the form of TSWV antigen used in the assays. The MAbs were tested against purified virus preparations, purified nucleocapsid preparations, and extracts from infected plants in ELISA procedure 2. The results are presented in Table 2. Since the MAbs were screened initially against plant extracts, this may explain why they react better with the virus in plant extracts than with the other antigen preparations. MAb 6.12.15 and MAb 2.9 also reacted strongly with purified virus and purified nucleocapsid preparations. These antibodies are therefore directed to the nucleocapsid protein. The other MAbs reacted with virus only in plant extracts, presumably because they are directed to glycoproteins that are not present in their native form in purified virus preparations.

Because of their good reactivity with TSWV in plant extracts, all of the MAbs, except MAb 8.11, were evaluated for their suitability for diagnozing TSWV.

Optimization of ELISA tests

Using polyclonal and monoclonal antibodies, the four ELISA procedures described in Table 1 were tested for their ability to detect TSWV in plant extracts or in purified virus preparations. Figure 2 shows the reactivity of extracts from infected plants with 5 MAbs in ELISA procedures 2, 3, and 4 and with polyclonal serum in ELISA procedure 1. The values given in this figure are the differences between OD readings of sap from infected and from healthy plants. MAbs 6.12.15, 3.22.6, and 2.9 showed a high reactivity in all three procedures. In contrast, MAb 7.22.6 reacted strongly only in procedure 3, whereas MAb 6.7 reacted satisfactorily only in procedure 2.

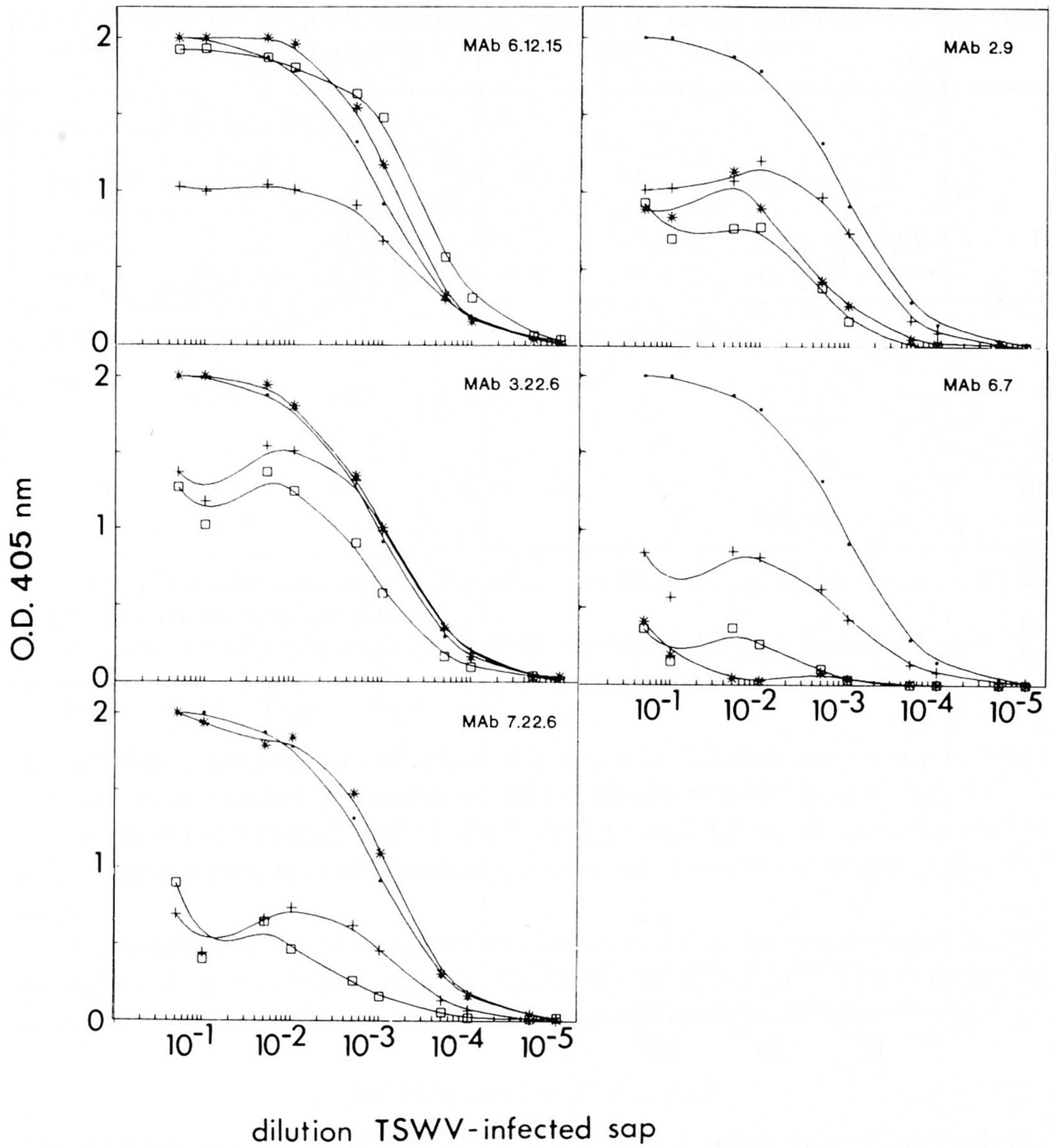

Fig. 2. Reactivity of 5 MAbs to TSWV-infected plant extracts in ELISA procedures 1, 2, 3, and 4. ● ELISA procedure 1; + ELISA procedure 2; * ELISA procedure 3; □ ELISA procedure 4

The results obtained when purified virus preparations were tested with MAbs and polyclonal antibodies in the different ELISA procedures are presented in Fig. 3. Whereas MAbs 6.12.15 and 2.9 reacted well in all three procedures and were able to detect a lower concentration of virus than polyclonal antiserum (Table 3), the other MAbs were less suitable for detecting purified virus than polyclonal antibodies (Fig. 3).

An OD $>$ 0.5 after 1 h substrate incubation was arbitrarily set as threshold to determine the limit dilution of plant extracts or the concentration of antigen at which each MAb could detect TSWV. The data summarized in Table 3

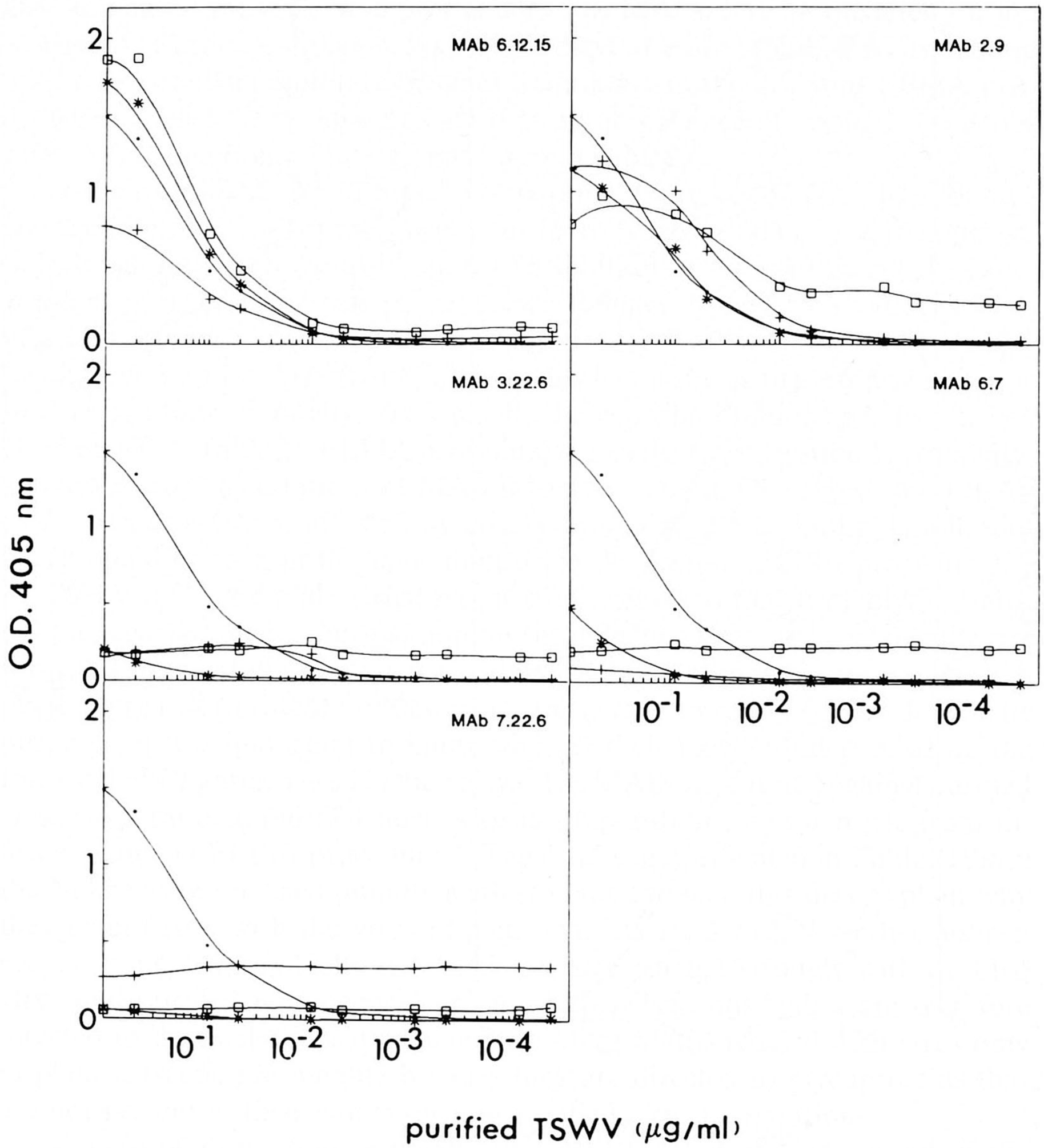

Fig. 3. Reactivity of 5 MAbs to purified virus preparations in ELISA procedures 1, 2, 3, and 4. ● ELISA procedure 1; + ELISA procedure 2; * ELISA procedure 3; □ ELISA procedure 4

demonstrate that the smallest quantity of purified virus was detected with MAbs 6.12.15 and 2.9 in procedure 4. Since biotinylated MAb 6.12.15 was used at a working dilution of 10^{-3} (Table 2), this antibody is the most suitable one for detecting purified virus. MAb 6.12.15 also detected the lowest concentration of virus in plant extracts (Table 3).

MAb 3.22.6 and 7.22.6, which were used at working dilutions of 10^{-3}–10^{-4} were also satisfactory for detecting virus in plant extracts by procedures 2 and 3. MAb 2.9 used at a 10^{-4} dilution was satisfactory in procedure 2. In view

Table 3. Sensitivity of TSWV detection in infected *N. rustica* plants and purified virus preparations by ELISA procedures 1, 2, 3, and 4

Antibody	Amount of antigen detected*							
	Purified virus (μg/ml)				Plant extract dilution			
	ELISA proc. 1	ELISA proc. 2	ELISA proc. 3	ELISA proc. 4	ELISA proc. 1	ELISA proc. 2	ELISA proc. 3	ELISA proc. 4
Ab^R	0.10				1/1,200			
MAb								
6.12.15		0.50	0.09	0.08		1/1,150	1/1,280	1/1,600
3.22.6		–	–	–		1/1,300	1/1,300	1/1,050
7.22.6		–	–	–		1/1,010	1/1,250	1/100
2.9		0.08	0.09	0.03		1/1,090	1/500	1/450
6.7		–	1.00	–		1/1,000	–	–

* Estimated virus concentration (μg/ml) or sap dilution necessary to obtain an OD_{405} of 0.5 after 1 h substrate incubation

Ab^R Anti-TSWV rabbit antibodies; *MAb* monoclonal antibodies (ascitic fluid)

of the high concentration of MAb 6.7 required for detecting virus in plant extracts or in purified preparations, this antibody was the least useful.

As shown in Tables 2 and 3, MAbs 6.12.15 and 2.9 which were directed to nucleocapsids were the only antibodies that could be used for detecting purified virus.

When different MAbs were used as coating and detecting antibody in ELISA procedure 4, it was found that the sensitivity of detection was not increased compared to results obtained when the same MAb was used in both positions.

Optimization of DIBA tests

The 3 DIBA procedures shown in Table 4 were tested for their ability to detect TSWV in plant extracts and in purified virus preparations. Table 5 shows the sensitivity of procedures A and B for detecting virus with the different MAbs. Procedure C using biotinylated MAb gave high background staining on nitrocellulose membranes. This procedure was therefore not used in subsequent experiments.

The best results were obtained with MAb 3.22.6 which was able to detect TSWV in plant extracts when diluted up to 1/5,000 (Fig. 4). MAb 6.12.15 detected the lowest concentration of TSWV (33 μg/ml) in purified virus preparations (0.1 μg of virus in the 3 μl tested). Procedure A, in which polyclonal antibodies were used, was even more sensitive since an amount of 3 μg/ml of virus could still be detected (0.01 μg of virus in the 3 μl tested). Procedures A and B, using MAb 6.12.15 and 3.22.6, were therefore used for detecting TSWV with the DIBA test.

Table 4. DIBA procedures used for TSWV detection

Procedure	Successive steps of the assay		
A	Ag	Ab^R-PAL	
B	Ag	MAb	GAM-PAL
C	Ag	MAb-B	Stre-PAL

Ab^R Anti-TSWV rabbit antibodies; *Ag* antigen; *PAL* alkaline phosphatase label; *MAb* monoclonal antibody 6.12.15 (ascitic fluid); *B* biotin label; *Stre* streptavidin

Table 5. Sensitivity of TSWV detection in infected *N. rustica* plants and purified virus preparation in DIBA procedures A and B

Antibody	Working dilution of ascitic fluid	Amount of antigen detected*	
		Purified virus (µg/ml)	Plant extract dilution
Ab^R (DIBA proc. A)	5.10^{-2}	3	1/500
MAb (DIBA proc. B)			
6.12.15	10^{-3}	33	1/50
3.22.6	10^{-3}	166	1/5,000
7.22.1	10^{-2}	166	1/1,000
2.9	10^{-3}	166	1/1,000
6.7	10^{-2}	166	1/1,000

* Virus concentration (µg/ml) or sap dilution necessary to obtain a purple colored spot after 20 min substrate incubation

Ab^R Anti-TSWV rabbit antibodies; *MAb* monoclonal antibodies (ascitic fluid)

Sensitivity of dot-blot hybridization assay with riboprobes for TSWV detection

A number of methods can be used for preparing the labeled probes to be used in the detection of viral RNA by molecular hybridization. The method using labeled synthetic RNA transcripts [6] is particularly sensitive [12]. A 1.2 kb cDNA fragment corresponding to the central part of the TSWV M RNA segment [4], was subcloned in the plasmid pSK^+, creating a template for synthesis of strand specific RNA probes.

As a next step in the development of a sensitive detection assay, experiments were performed to optimize the conditions (results not shown). The optimal membrane type, hybridization and washing conditions as well as sample preparation are described under Materials and methods. To prepare samples from infected plants a number of different grinding buffers were tried. Buffers that

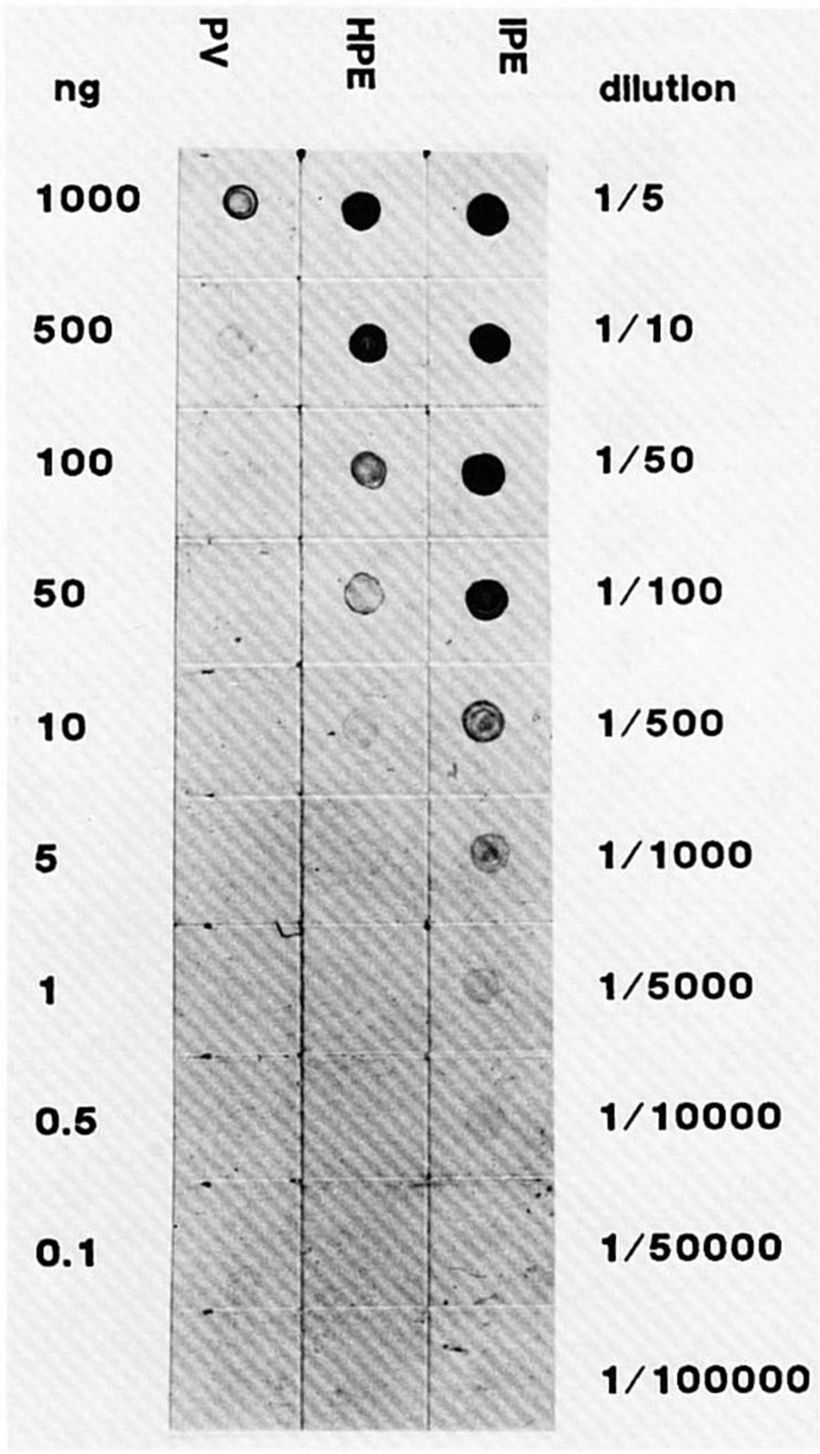

Fig. 4. Reactivity of MAb 3.22.6 to infected plant extract dilutions and purified virus dilutions in DIBA procedure B. *IPE* Infected plant extract; *HPE* healthy plant extract; *PV* purified virus (10 ng of virus corresponding to a concentration of 3 µg/ml)

gave the best results contained 0.1% Triton X100 and 0.1% 2-mercaptoethanol. An extraction step with phenol was used to remove proteins that caused non-specific background readings.

The polyphenols present in some test samples such as tomato could be removed by adding 1% L-polyclar (BDH Dorset, England) during extraction. Polysaccharides present in many ornamental plant leaves were removed by adding 1% cetyl-trimethyl-ammonium-bromide (CTAB) to the buffer. Samples of purified viruses could be spotted directly on the membrane without any treatment.

As can be seen in Fig. 5, approximately 0.033 µg/ml of protein (0.1 ng of protein in the 3 µl tested) could be readily detected in dot-blot hybridization, corresponding to 0.33 ng/ml of viral RNA (1 pg of RNA in the 3 µl tested). TSWV could still be detected in infected *Nicotiana rustica* plants after diluting the deproteinized samples 5,000 times. In order to detect TSWV in other plant species, deproteinized samples were normally diluted only twofold.

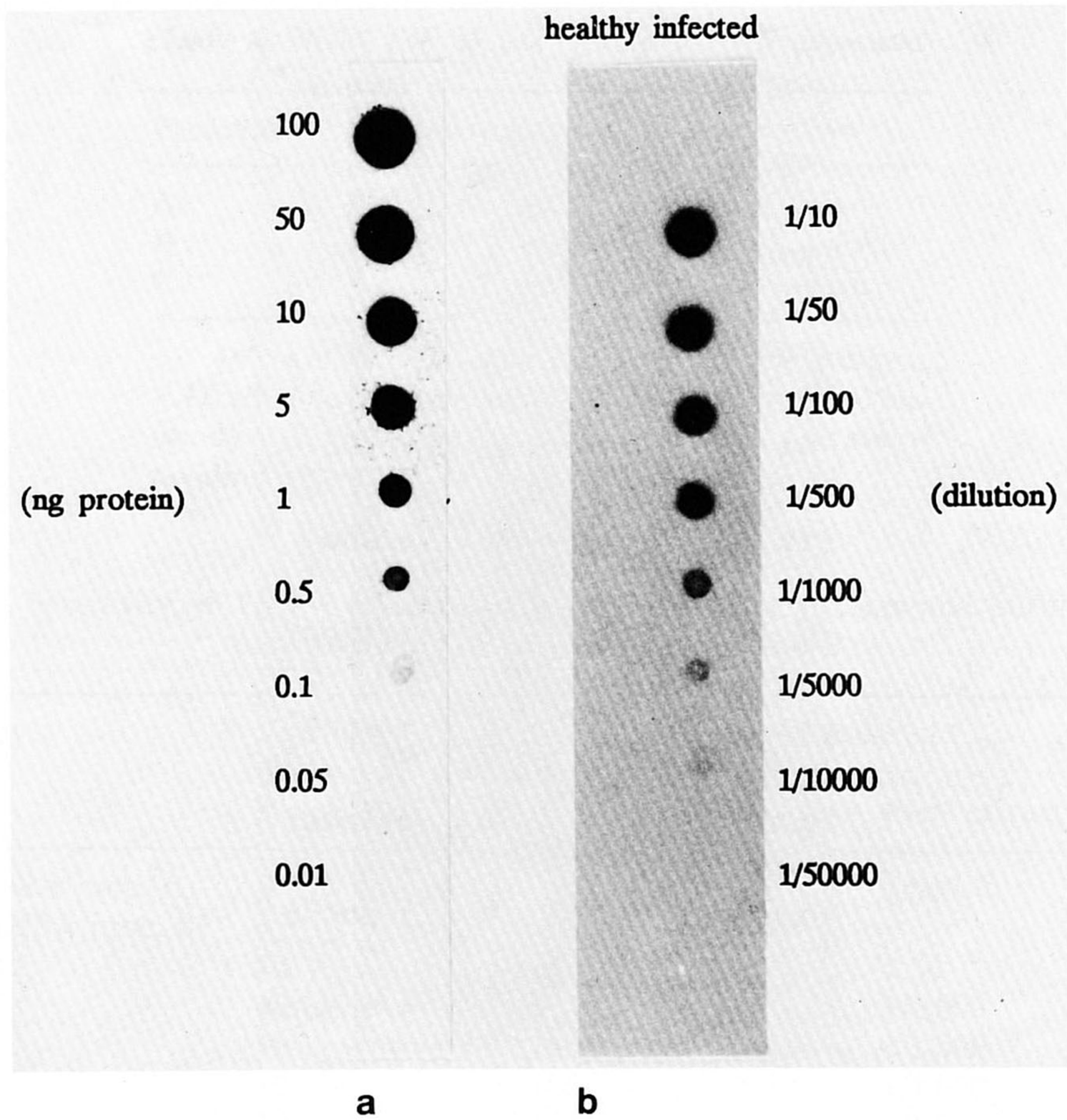

Fig. 5. Detection of TSWV in dilutions of a purified virus (**a**) preparation, and extracts of infected plant (**b**) by dot-blot hybridization with riboprobes

Comparison of the different assays in TSWV diagnosis

Seven plants of the species *Nicotiana rustica, Lycopersicum esculentum, Datura stramonium, Capsicum annuum, Chrysanthemum morifolium*, and *Impatiens* sp. no. 1 and no. 2, suspected to be infected with TSWV were tested by the different optimized assays. The results are summarized in Table 6.

ELISA procedures 1 and 4 detected virus in four and six samples respectively, if an OD > 0.5 was used as threshold. The OD values were higher with procedure 4 than with procedure 1. In the case of procedure 4, the diagnosis could be carried out by eye without the use of an ELISA reader.

The DIBA assay also gave a good discrimination between healthy and infected plants, especially when MAb 3.22.6 was used in procedure B (Fig. 6) and allowed virus to be detected virus in six plants.

The dot blot hybridization assay detected virus in the same six infected plants (Fig. 7).

Table 6. Detection of TSWV in several plants species with the different techniques

Plant sampled	ELISA*		DIBA			Dot-blot hybridization
	Proc. 1	Proc. 4 MAb 6.12.15	Proc. A	Proc. B MAb 6.12.15	Proc. B MAb 3.22.6	
N. rustica	1.316	1.590	+	+	+	+
L. esculentum	1.133	1.561	+	±	+	+
D. stramonium	0.875	1.626	+	+	+	+
C. annuum	0.030	0.625	+	+	+	+
C. morifolium	0.296	1.178	+	±	+	+
Impatients sp. no. 1	1.228	1.633	+	+	+	+
Impatients sp. no. 2	0	0.003	−	−	−	−

* OD_{405} after 1 h substrate incubation
\+ Positive reaction; − negative reaction; ± low reaction

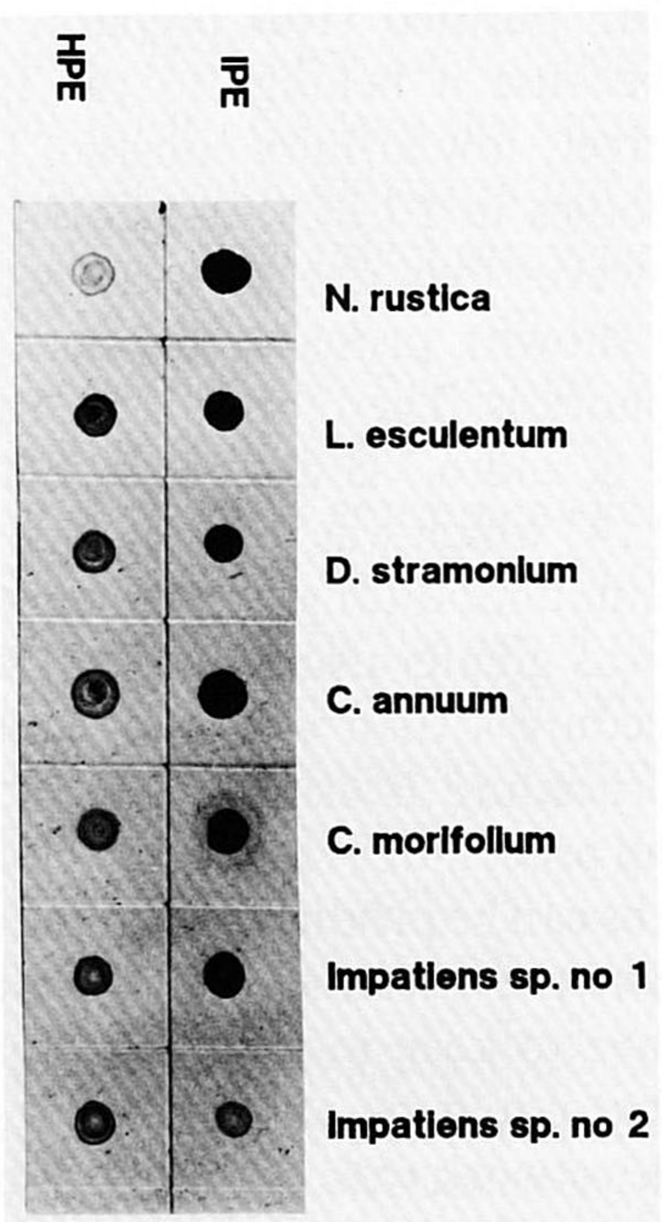

Fig. 6

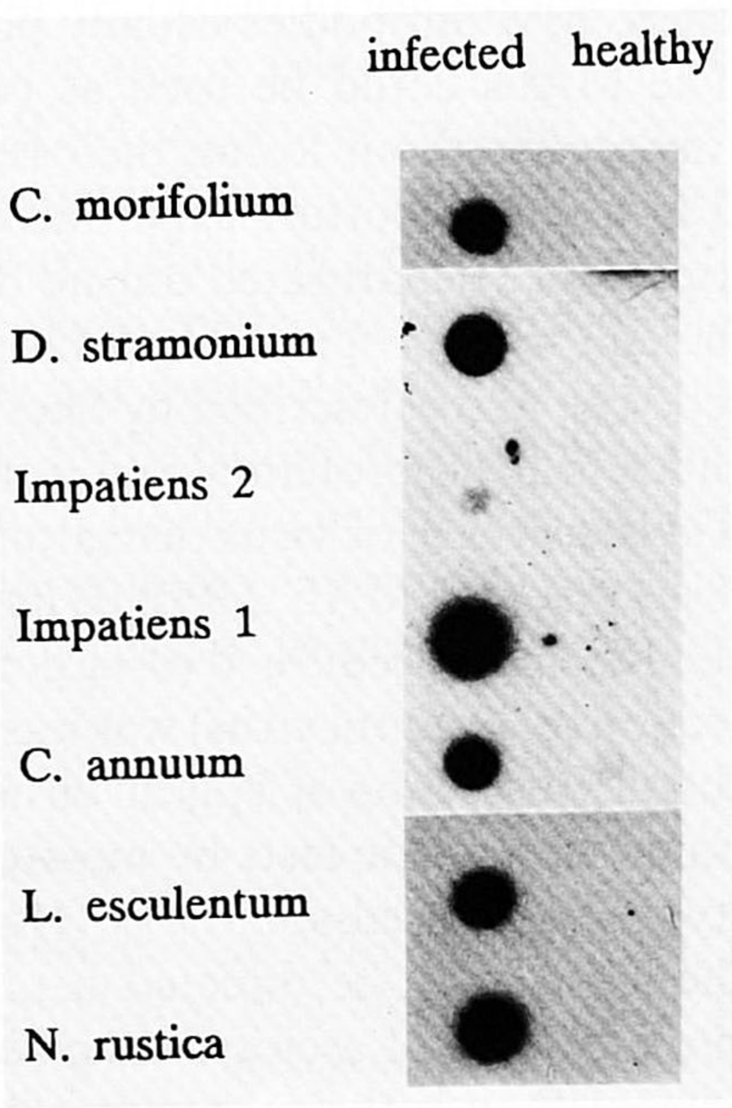

Fig. 7

Fig. 6. DIBA test used as diagnosis on different plant species. *IPE* Infected plant extract diluted 1/50; *HPE* healthy plant extract diluted 1/50

Fig. 7. Dot-blot hybridization test used as diagnosis on different plant species. Deproteinized infected plant extract diluted 1/2; deproteinized healthy plant extract diluted 1/2

Discussion

The six MAbs produced against TSWV reacted with different preparations of the antigen. MAbs 6.12.15 and 2.9 reacted strongly with both purified virus and purified nucleocapsid preparations and are directed to epitopes on the nucleocapsid protein. The nucleocapsid protein of TSWV is probably conserved between isolates of the virus as has been reported for the other nucleocapsid proteins of other enveloped viruses. Nucleocapsid proteins are either type specific as in the influenza viruses [13] or group specific as in the bunyaviruses [2]. MAb 6.12.15 reacted in ELISA with three isolates of TSWV, obtained from geographically different areas (data not shown). The MAb obtained by Sherwood et al. [14], also recognized five TSWV isolates from different hosts and geographical areas.

MAbs 3.22.6 and 7.22.6 recognized TSWV in plant extracts only and did not react with purified virus. These antibodies seem to react with epitopes that are degraded or at least damaged during purification and are no longer present in purified TSWV preparations. These epitopes are presumably located on the envelope proteins of the virus which during purification are more exposed to proteolytic damage than the internal nucleocapsid protein.

MAb 8.11 showed only a weak reaction with purified virus preparations and extracts from infected plants, probably because it belongs to the IgM subclass. IgM antibodies usually possess a relatively low affinity constant [4].

The MAbs could be used as coating antibodies in ELISA and could be biotinylated without losing their reactivity. However they did not react when used in immunoblotting experiments (data not shown), probably because the epitopes were denaturated during SDS-electrophoresis. Therefore the location of the epitopes recognized by MAb 3.22.6, 7.22.6, and 6.7 could not be determined. The MAb described by Sherwood et al. [14] which was directed against the nucleocapsid protein also did not react in immunoblotting experiments.

The sensitivity of virus detection by ELISA was greater using MAb 6.12.15 (0.08 µg/ml) and MAb 2.9 (0.03 µg/ml) in procedure 4 than using polyclonal antibodies in procedure 1 (0.1 µg/ml). DIBA procedure B using MAb 3.22.6 (detection of 33 µg/ml virus) was less sensitive than procedure A using polyclonal antibodies (detection of 3 µg/ml virus). Since MAbs can be produced in unlimited amounts, diagnostic tests by means of MAbs have the advantage of reproducibility and increased specificity. However we have to keep in mind that some of the MAb may be directed to an epitope that could be absent in certain isolates. The use of ascitic fluid makes it possible to work with higher dilutions of reagent than is the case with polyclonal antiserum. In our assays, however, ascites could not be diluted more than 10^{-4} (i.e., about 1 µg/ml since ascites usually contain about 10 mg/ml of IgG) while polyclonal antisera was also used at a concentration of 1 µg/ml. This may be due to the fact that the polyclonal antisera recognized several structural proteins of the virus, while the MAbs were directed to a single epitope which could have been precisely degraded in virus preparations. Although MAbs have normally higher affinity for their

antigen, they are also more specific. In our case, with very labile proteins, a high specificity for regions exposed to degradations will decrease the sensitivity of detection.

When the sensitivity of virus detection in purified preparations was compared for the three assays, dot-blot hybridization (with a maximal detection of 0.03 µg/ml) and ELISA (with a maximal detection of 0.03 µg/ml) were equally sensitive, DIBA was 100 times less sensitive (maximal detection of 3 µg/ml). In theory, the dot-blot hybridization assay has the highest sensitivity, when speaking in absolute quantities, with a detection of 0.1 ng of purified virus against 0.3 µg in case of ELISA. For the diagnosis of TSWV-infected plants, ELISA procedure 4 using MAb 6.12.15, DIBA procedure A, DIBA procedure B using MAb 3.22.6, and dot-blot hybridization gave similar results.

ELISA tests have the advantage that they give numeric data and clearly discriminate between healthy and infected plants. In addition, for field applications they can also be utilized without an ELISA reader. They require 9.5 h to be performed, are rather inexpensive, and can be carried out in any laboratory.

The DIBA test can be performed in only 4 h. The discrimination between healthy and infected plants is usually good, but depends on the duration of substrate incubation and on a sometimes delicate assessment of the degree of staining. It is also an inexpensive assay, but the low sensitivity could be a problem for detecting the virus when the concentration in the sample is low.

The dot-blot hybridization assay is sensitive and is able to detect TSWV at an early stage of infection. When conserved RNA sequences are used as probe, the likelihood that certain isolates are not identified is negligible. However, methods based on molecular hybridization are relatively expensive and time consuming (the assay requires 2 days to be performed). Working with radio-isotopes requires equipment which is usually not available in plant pathology laboratories that carry out routine diagnosis. In the future, this problem might be circumvented by the use of non-radio-isotope labelling techniques. Although this method is valuable for virus identification under laboratory conditions, additional refinement and simplification are necessary to make it suitable for diagnostic purposes.

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