Immunodiffusion Tests for Potato Y and Tobacco Etch Viruses

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Accepted for publication 2 February 1970.

ABSTRACT

Immunodiffusion tests were developed for serodiagnosis of tobacco etch (TEV) and potato Y (PVY) viruses in tobacco. Alkaline-treated extracts from plants infected with either TEV or PVY gave strong precipitin lines against homologous antisera, but untreated extracts reacted weakly or not at all. Evidence was obtained that the alkali-derived antigens of TEV and PVY are serologically related. Antigens of both viruses reacted after 7 months' storage in either frozen or dried tobacco leaf tissue.

Phytopathology 60:1036-1039.

Immunodiffusion tests in agar gels have proved extremely useful in plant virology, especially for diagnosis and studies of relationships among plant viruses (11, 15). Because the longer rod-shaped viruses diffuse poorly in agar gels, immunodiffusion tests have been used mostly for the isometric and shorter, rod-shaped viruses. Alkali (6, 7, 8, 9, 14), detergents (5, 9), and ultrasonics (10) have been used to degrade several purified filamentous viruses into smaller components that can diffuse into agar and retain the ability to react with antisera obtained by injecting virus preparations into rabbits. While immunodiffusion tests using appropriately treated crude extracts have been developed for some filamentous viruses (5, 8, 9), reliable immunodiffusion tests have not been developed for most viruses 700-800 mμ long. The present report describes simple and reliable procedures for serodiagnosis of tobacco etch and potato Y viruses in agar gels using alkaline treatment of crude tobacco leaf extracts.

MATERIALS AND METHODS.—The work with tobacco etch virus (TEV) was done with an isolate used previously (7), designated TEV-F, an isolate from North Carolina designated TEV-NC, or field isolates. Stock cultures were maintained in *Datura stramonium* L. The tests with potato Y virus (PVY) were done with a North Carolina isolate that was maintained in tobacco (*Nicotiana tabacum* L. 'Turkish NN') or field isolates. The viruses were maintained in separate greenhouses during this study.

TEV was purified from tobacco as previously described (7), or by a slight modification of a procedure developed for PVY (Fig. 1). The TEV antisera were prepared by injecting partially purified preparations into rabbits. Each rabbit was injected intramuscularly three times with 10 mg of virus emulsified with Freund's incomplete adjuvant. The second and third injections were made 3 and 6 weeks, respectively, after the initial injection. The rabbits were bled periodically for 2 months, starting 2 weeks after the final injection, and sera from the individual bleedings were pooled and stored frozen. TEV-F was injected into two rabbits; three were injected with TEV-NC. The sera from each rabbit were maintained and tested separately.

The PVY antisera were prepared by injecting rabbits with virus partially purified by the procedure outlined in Fig. 1. Each rabbit was twice injected intramuscularly with about 10 mg of virus emulsified in Freund's incomplete adjuvant. The second injection was made 1 month after the first injection. Antisera were collected, starting 1 week after the final injection, and sera from the two rabbits were pooled, lyophilized, and reconstituted just prior to use.

The medium for immunodiffusion tests consisted of 0.85% Noble agar, 0.85% sodium chloride, and 0.1% sodium azide, in distilled water. Twelve ml of the medium were added to each standard 100- × 15-mm plastic petri dish. The pattern consisted of a center well encircled by six peripheral wells 7 mm in diam with the peripheral wells 5 mm from the center well. The plates were incubated in a moist chamber at 24 C.

Antigens for immunodiffusion were prepared in 0.5 M ethanolamine-hydrochloride buffer (ETA) at pH 10.5 in two ways, termed (Methods A and B). All procedures were done at room temp.

Method A.—Infected tissue was triturated in ETA (1 g/2 ml). The juice was squeezed through cheesecloth and allowed to stand up to 2 hr prior to use.

Method B.—Infected tissue was triturated in distilled H_2O (1 g/2 ml), and the juice was expressed through cheesecloth. The juice was centrifuged for 30 min at 2,500 *g*, and the pellets were resuspended in ETA (0.5 ml/g tissue) with the aid of a Pasteur pipette and used after standing 15 min to 2 hr. Preliminary tests indicated that enough antigen sedimented under these conditions to give a good reaction, so the supernatants were discarded.

RESULTS.—*Immunodiffusion tests with extracts from tobacco plants infected with PVY or TEV.*—ETA-treated extracts from tobacco plants infected with either PVY or TEV gave precipitin lines against their respective antisera in immunodiffusion tests, but untreated extracts usually failed to form precipitin lines.

TEV prepared by either Method A or B gave straight precipitin lines midway between serum and antigen wells within 24 hr when tested against undiluted TEV antiserum. The precipitates were much stronger, however, with antigen prepared by Method B (Fig. 2-A, well 1), than with antigen prepared by Method A (Fig. 2-A, well 4). The zone of precipitate could usually be resolved into two closely spaced lines. The titers of representative TEV-F and TEV-NC antisera were one-sixteenth when tested against TEV-F pre-

INFECTED TOBACCO LEAVES
—Refrigerate 5 C overnight
—Homogenize 5 min in 0.5 M Na citrate (1 ml/g tissue) containing 1% 2-mercaptoethanol
—Strain through cheesecloth

Pulp*

FILTRATE
—Add butanol (to 6.5-7%)
—Stir 30 min
—Centrifuge 30 min at 12,000 g

Pellet*

SUPERNATANT
—Filter through glass wool
—Incubate overnight at 5 C
—Centrifuge 15 min at 12,000 g

Pellet*

SUPERNATANT
—Centrifuge 60 min at 75,000 g

Supernatant*

PELLET
—Resuspend in 0.02 M borate buffer, pH 7.8 (8.2 for TEV)
—Centrifuge 10 min at 12,000 g

Pellet*

SUPERNATANT
—Centrifuge 60 min at 75,000 g

Supernatant*

PELLET
—Resuspend in borate buffer
—Centrifuge 10 min at 12,000 g

Pellet*

SUPERNATANT
—Adjust virus concentration to 1 mg/ml
—Add NaCl (to make 0.5%) and acetic acid (0.2%) until virus precipitates (TEV, pH 4.8; PVY, pH 5.6)
—Centrifuge 15 min at 12,000 g

Supernatant*

PELLET
—Resuspend in borate buffer
—Centrifuge 10 min at 12,000 g

Pellet*

SUPERNATANT
(Suspension of partially purified virus)

*Discard

Fig. 1. Flow sheet depicting procedure used for purifying potato Y virus for use in serology with modifications indicated for purification of tobacco etch virus.

pared by Method B. Each of the five sera gave similar qualitative results. Zones of granular precipitation were often observed at the edge of antigen wells after 2 days' incubation. These were considered to be nonspecific, since they also occurred with normal serum. Extracts from TEV-infected tissue were more apt to form this nonspecific precipitation than extracts from healthy plants. None of the TEV sera reacted with ETA-treated extracts from healthy plants in these tests except for the nonspecific granular precipitates mentioned previously.

PVY also gave strong precipitin lines against PVY antiserum (Fig. 2-B). As with TEV, stronger reactions were obtained with PVY antigen prepared by Method B (Fig. 2-B, well 2) than by Method A (Fig. 2-B, well 5). The dilution end point of the PVY antiserum was one-sixteenth against PVY antigen prepared by Method B. The PVY antiserum apparently also contained antibodies to normal plant proteins, and reacted weakly with healthy antigens to give zones of precipitation around the antiserum well.

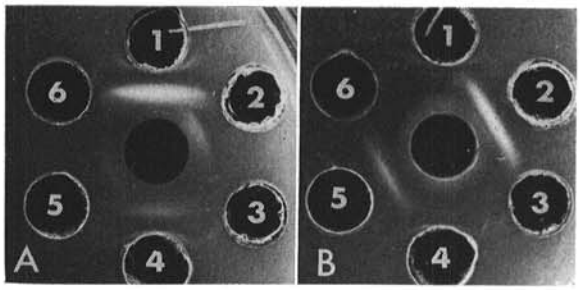

Fig. 2. Photographs of immunodiffusion plates showing precipitin reactions of alkaline-treated TEV or PVY and their antisera. The center wells contain **A)** antiserum to TEV-NC; **B)** antiserum to PVY. Peripheral wells 1, 2, and 3 contain TEV-F, PVY, and healthy tobacco antigens, respectively, each derived from ethanolamine-treated pellets obtained by centrifuging aqueous homogenates of leaf tissue at 2,500 *g* for 30 min; wells 4, 5, and 6 contain TEV-F, PVY, and healthy tobacco antigens, respectively, each prepared by homogenizing tissue directly in ethanolamine.

Weak heterologous reactions were observed in some of the reciprocal tests with TEV and PVY. Each of the five antisera to TEV reacted weakly with ETA-treated PVY. The PVY antiserum reacted with purified, concentrated, alkaline-treated TEV. This antiserum occasionally reacted with TEV antigen prepared by Method B, but never with antigen prepared by Method A. None of the heterologous reactions were as strong as the homologous reactions.

Intragel absorption tests (12) were set up to study cross reactions and provide information concerning specificity of the immunoprecipitates. Antigens from TEV-infected, PVY-infected, and control plants were prepared by Method B, and each was added to separate center wells of the patterns. The residues were removed after 24-36 hr, and undiluted sera were added to the center wells. The peripheral wells were filled with freshly prepared antigen, and the plates were incubated 2 days. The results of a typical experiment are given in Table 1. Intragel absorption of the TEV antisera with PVY antigens eliminated cross reactive antibody and made the sera specific for TEV. Intragel absorption with healthy antigens removed host-specific antibody present in the PVY antiserum, but otherwise did not appear to influence other precipitin reactions with PVY or TEV antigens. It appears, therefore, that the heterologous reactions observed are evidence of a serological relationship between alkaline-derived antigens of TEV and PVY. No attempts were made to determine effects of intragel absorption on serum titers to the various antigens.

As further evidence of the specificity of the reactions, TEV and PVY prepared by Method B did not form precipitin lines when tested against antisera to clover yellow mosaic, papaya mosaic, potato X, southern bean mosaic, or tobacco mosaic viruses. Likewise, alkaline-treated extracts from tobacco plants infected with tobacco mosaic, cucumber mosaic, potato X, or tobacco ringspot viruses did not react with the TEV antisera,

TABLE 1. Effects of intragel absorption on immunodiffusion precipitin reactions with alkaline-treated TEV and PVY antigens[a]

Antiserum in center well	Antigen used for absorption[b] in center well	Reaction against antigens in peripheral wells[c]		
		TEV-F	PVY	Healthy
TEV-F	TEV-F	—[d]	—	—
	PVY	+	—	—
	Healthy	+	+	—
	None	+	+	—
PVY	TEV-F	—	+	—
	PVY	—	—	—
	Healthy	—	+	—
	None	+	+	+
Normal	TEV-F	—	—	—
	PVY	—	—	—
	Healthy	—	—	—
	None	—	—	—

[a] All antigens prepared by Method B (see MATERIALS AND METHODS).

[b] Antigens added to center wells; 24-36 hr later the residues were removed, undiluted serum was added to center wells, and freshly prepared antigens were added to peripheral wells.

[c] + = Presence of precipitin line; — = no perceptible precipitation other than precipitation in ring around center well due to intragel absorption or nonspecific precipitation at edge of antigen well that was observed in some cases.

[d] In 3 of 5 experiments, this reaction was negative; in two others, a faint precipitate was detected in addition to the ring around the center well, indicating incomplete absorption of antibody.

and reacted similarly to extracts from healthy plants in tests with the PVY antiserum.

Although systemically infected leaves were used for most of the tests, inoculated leaves which have been reported to reach highest titers with PVY (3) and TEV (4) about 10 days after inoculation were also used, and proved to be good sources of antigen.

Tobacco plants which have been infected by TEV for 3 weeks or more tend to show chlorotic mottling at the tips of the youngest leaves, whereas the basal portions of these leaves are green. Comparative immunodiffusion tests showed that the tips of such leaves were good sources of antigen, but extracts from the green basal portions gave no reaction.

Several tests indicated that leaf tissue desiccated over $CaSO_4$ at 4 or 24 C was a good source of either TEV or PVY antigens. In one experiment, it was determined that both TEV and PVY antigens survived 7 months' storage in frozen or vacuum-dried tissue and in frozen crude sap, but antigens prepared in ETA and then frozen were not well-preserved.

Immunodiffusion tests with PVY and TEV antigens from other hosts.—Preliminary tests indicated that the procedures used for serodiagnosis of TEV and PVY in tobacco may also be useful for detection of these viruses in certain other hosts. Positive and specific reactions were obtained in limited immunodiffusion tests with TEV antigens prepared from *Capsicum annuum* L. 'Calwonder', *Cassia tora* L., *Datura stramonium* L., *Nicotiana glutinosa* L., and *Trigonella foenum-graecum* L. PVY reacted positively from *C. annuum.*

Neither TEV nor PVY gave reliable results in three tests with infected tomato (*Lycopersicon esculentum* Mill. 'Homestead'). Antigen concentration seemed to be low as judged by weakness of precipitin lines and spurious precipitates that resembled the virus-specific reactions occurred with normal serum, thereby making the results difficult to interpret. No efforts were made to determine why tomato failed to give satisfactory results.

DISCUSSION.—The simple, reliable procedures described herein for serological identification of PVY and TEV in extracts from tobacco leaves by immunodiffusion tests have considerable potential for routine diagnostic work. The sedimentation of antigen from aqueous homogenates by slow speed centrifugation may have resulted from aggregation due to the acidic nature (pH 5.5-6.0) of tobacco sap. At any rate, antigen prepared in this way and resuspended in ethanolamine at pH 10.5 gave stronger reactions than antigen in tissue ground directly in the ethanolamine. The rapidly diffusing antigens are probably derived from degraded virus particles, since alkaline treatment of partially purified virus gave similar reactions, but this has not been proven.

The sera used in our study were prepared by injecting partially purified virus into rabbits, and these sera reacted with alkaline-treated extracts of plants infected with homologous virus. It remains to be demonstrated what the relationship is between the antigens derived by alkaline treatment to the antigens of the intact PVY or TEV. Von Wechmar & van Regenmortel (13) found that bromegrass mosaic virus has antigenic determinants that are specific for the capsid surface, and determinants specific for the subunit surface. Whatever the origin of the antigens involved in the immunodiffusion precipitates with TEV and PVY antisera, it seems that virus purification methods, suspending media, immunization procedures or schedules, and the particular animals injected might all influence the production of antibody specific for these antigens.

Bawden & Kassanis (2) reported that TEV showed no serological cross-reaction with PVY. Bartels (1), however, using liquid precipitin tests, reported that PVY and TEV are distantly related serologically. Although our study indicates there may be serological relatedness between the alkali-derived antigens of TEV and PVY, our results are not comparable to those of the other workers because they used different virus isolates, other methods of serological testing, and different methods of preparing the antigens.

Since dried or frozen tobacco leaf tissue served as a good source of antigen for both TEV and PVY, large numbers of samples could be collected, stored, and processed later. The use of dried tissue would also greatly facilitate shipment of antigens to other investigators for comparative serological tests involving identification of these viruses.

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