Serological relationships among potyviruses

by

Michael Ray McLaughlin

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INTRODUCTION

Review of Literature

Sixteen groups of plant viruses have been described on the basis of host relations, vector relations, and particle characteristics (21). The largest group, the potyviruses, whose type member is potato virus Y (PVY), is probably the least well described. Viruses in this group are long flexuous rod-shaped particles ranging in size from 720 to 780 nm in length and about 15 nm in diameter. They have relatively narrow host ranges and induce mosaic and mottle symptoms in their hosts. In nature they are transmitted by aphids in a nonpersistent manner.

The biochemical properties of three members of the potyvirus group, tobacco etch virus (TEV), and turnip mosaic virus (TurMV) and maize dwarf mosaic virus-B strain (MDMV-B), have been elucidated (16,17,23,24,25,26), and a fourth, PVY, has been partially investigated (33,55). All contain RNA encapsidated by a single species of coat protein. TEV and TurMN contain 5% RNA (17,23) while MDMV-B contains 6% RNA (26,28,38). Amino acid analyses show similarities, with the exception of the relatively high glycine and serine content in MDMV-B (26). These data provide support for the relationships of these viruses.

Brandes and co-workers have proposed classifying elongated plant viruses on the basis of morphology and serology (9,10).

Although morphologically similar, members of the potyvirus group exhibit distant or no serological relatedness (2,3,5, 6,7,8,31,34,37,49,50,56). Many of the tests to elucidate potyvirus serological relationships employ antisera prepared against only partially purified viruses which generally have not been extensively characterized. Serological relationships between MDMV-B, TurMV, and TEV have not been well established. Recent studies used degraded virus proteins (34,40,41) or virus-induced host inclusion body proteins (22,42) in attempts to discern relationships within this group.

The usefulness of degraded virus coat proteins in serological studies for both taxonomic and diagnostic purposes has been clearly demonstrated (19,41,48,49,50,51,54). The reliability of such studies as suggested by the work of Shepard and co-workers (46,47,51) is limited if serological tests utilize virus proteins as antigens and antisera prepared to virus particles. Only when antiserum is prepared against depolymerized virus protein (D-protein) are tests using D-protein antigens reliable. Presumably, due to conformational changes, D-protein of potato virus X (PVX) is sufficiently different antigenically from intact PVX to be considered serologically distinct but related to the PVX particle (46, 52,53). A reasonable hypothesis suggests that potyviruses and their D-proteins exhibit similar serological behavior.

Statement of Problem

This study explores the area of potyvirus serology using a model system composed of the only thoroughly characterized members of the group, namely MDMV-B, TurMV, and TEV. The data reported define more clearly the serological relationships within the potyvirus group and suggest possibilities for improved methods of serodiagnosis of diseases caused by potyviruses.

MATERIALS AND METHODS

Viruses Used

Virus isolates used were TEV (ATCC-PV69) described by Purcifull (39) and characterized by Damirdagh and Shepherd (16), TurMV (ATCC-PV134) isolated and characterized by Hill and Shepherd (23), and MDMV-B (ATCC-PV53) isolated by Ford et al. (18) and characterized by Hill et al. (25,26).

Virus Propagation and Purification

TEV was maintained in tobacco (Nicotiana tabacum L. 'Havana 425') grown in autoclaved soil and purified according to Damirdagh and Shepherd (16) except that polyethylene glycol (PEG) precipitation was accomplished using 8% (w/v) PEG (MW 6,000-7,500), instead of 4%, and the suspension was incubated overnight (instead of 60-90 minutes) at 4 C before precipitated virus was collected by low speed centrifugation. Yields averaged 15 mg purified virus per kilogram of infected tissue.

TurMV was maintained in the greenhouse in Tendergreen mustard (Brassica campestris L.) and purified according to Hill and Shepherd (23). Yields averaged 17 mg purified virus per kilogram of infected tissue.

MDMV-B was maintained in the greenhouse in sweetcorn (Zea mays L. 'Golden Bantam') and purified as described by Hill et al. (26). Yields ranged from 0.5-3.5 mg purified

virus per kilogram of infected tissue.

Virus purifications were monitored periodically via stream birefringence and quality and quantity of purified virus was checked spectrophotometrically. Virus concentrations were estimated using the extinction coefficient for TEV of 2.4 cm^2 mg^{-1} at 261 nm (39).

Preparation of Depolymerized Virus Coat Proteins

D-proteins from all three viruses were prepared by the warm formic acid method (32). Three X volumes of 88% formic acid were added to the virus suspensions and the mixtures were incubated at 37 C for 20-22 hours. Virus proteins were precipitated from the formic acid solution with a 2X volume of saturated ammonium sulfate, incubated at room temperature 2 hours and centrifuged at 10,000 X g for 15 minutes. Pellets were resuspended in 8.0 M deionized urea containing 0.1% (v/v) 2-mercaptoethanol. Protein preparations were precipitated a second time and the pellets washed twice with 0.1 M ammonium acetate in 95% ethanol to remove contaminating nucleotides (45). The final pellet was resuspended in 8.0 M urea containing 0.1% (v/v) 2-mercaptoethanol and centrifuged at 100,000 X g for 1½ hours to remove undegraded virus or aggregated protein. Protein preparations were dialyzed exhaustively against distilled deionized water, lyophylized, and stored at -10 C.

To insure homogeneous protein preparations and to prevent

changes in the antigenic determinant of the virus proteins resulting from polymerization of the subunits (43,44,60) all protein antigens were carboxymethylated (14,23,26), lyophylized again, and stabilized with 0.2% formaldehyde in 0.05 M borate buffer, pH 8.2, for use as immunogens (52,60). Unreacted formaldehyde was removed by exhaustive dialysis against several changes of borate buffer over a 3-day period. Final protein concentrations were determined spectrophotometrically using extinction coefficients of 1.07 cm^2 mg^{-1} at 280 nm for TurMV protein (23), 1.015 cm^2 mg^{-1} at 280 nm for MDMV-B protein (26), and 0.95 cm^2 mg^{-1} at 280 nm for TEV protein (17).

Antisera Production

Intact virus antigens were dialyzed overnight at 4 C against 0.05 M borate buffer, pH 8.2, and emulsified 1:1 (v/v) with Difco Freund's incomplete adjuvant. Adult rabbits were immunized over a 21-day period by successive intramuscular injections of 1.5, 1.0, 1.0, and 1.5 mg of virus at 0, 7, 14, and 21 days with a total of 5.0 mg virus injected per animal. Serum was collected by cardiac puncture 7 and 14 days after the final injection.

Rabbits were immunized against D-proteins over a 14-day period by injections of 0.15-0.3, 0.075-0.15, and 0.075-0.15 mg protein at 0, 7, and 14 days. Initial injections were made in 0.05 M borate-buffered saline, pH 7.4, administered intravenously. Subsequent injections of protein emulsified

1:1 (v/v) with Freund's incomplete adjuvant were administered intramuscularly. Twenty-four days after the initial injection, serum was collected by terminal bleeding. All sera, including normal serum obtained by cardiac puncture 8 weeks before the immunization schedules were begun, were processed as described by Campbell et al. (11) and stored at -60 C.

Two rabbits were immunized against each intact virus and one rabbit was immunized against each D-protein. Serum from each animal, as well as different bleedings of the same animal, was processed and tested separately.

Serological Tests

The microprecipitin test described by Van Slogteren (59) as modified by Ball (1) was used in all tests involving only intact virus antigens. Serial twofold dilutions of sera were made in 0.05 M borate-buffered saline, pH 7.4, and of purified virus antigens in 0.05 M sodium - potassium phosphate buffer, pH 7.4, containing 0.5 M deionized urea and 1% (v/v) 2-mercaptoethanol for MDMV-B, and 0.025 M sodium - potassium phosphate buffer, pH 7.4, containing 0.5 M deionized urea and 1% (v/v) 2-mercaptoethanol for TEV and TurMV. Viral antigens were adjusted to initial concentrations of 1.0 mg per ml. Tobacco mosaic virus (TMV) and healthy sap in 0.01 M potassium-phosphate buffer, pH 7.0, were used as controls. Healthy sap antigens were prepared by grinding leaf tissue from healthy Zea mays L. 'Golden Bantam' sweetcorn, Nicotiana tabacum L.

'Havana 425' tobacco, or Brassica campestris L. Tendergreen mustard, in 0.01 M potassium phosphate buffer, pH 7.0, and clarifying by centrifugation at 8,000 X g for 10 min. Microprecipitin plates were incubated for 2 hours at 25 C followed by 21 hours at 4 C. Plates were examined and homologous and heterologous titers determined. Only sera from the first collection date were used in these tests which were conducted twice. Where results differed, a range was reported.

In order to minimize variation and to maximize detection of distant serological relationships, the higher titered antisera of the two produced for each virus was used to prepare cross-absorbed antisera according to Chester (13). Antisera were fully cross-absorbed by addition of a 4X volume of heterologous antigen (0.5 mg per ml) as determined by the absence of precipitate in the microprecipitin test after cross-absorption. Tests were as described previously.

Ouchterlony double immunodiffusion tests (30,36) were conducted, using the higher titered of each virus antisera, in small (50 mm diameter) tight-lid Petri dishes (29). Six ml of 1% (w/v) Difco Bacto-agar, containing 0.85% (w/v) NaCl and 0.2% (w/v) NaN_3, were pipetted into each dish and allowed to solidify and cool to room temperature. Wells (30 mm diameter) were cut using an LKB (LKB Produkter AB; Stockholm, Sweden) template and charged immediately. Plates were incubated at room temperature in a moist chamber and observed daily for several days.

D-protein antisera titers were determined in agar-gel

double immunodiffusion tests with the homologous protein antigens (0.1 mg per ml) in the center well and serially diluted antisera (1 through 1/128) in eight peripheral wells.

Infected host sap used in agar-gel double immunodiffusion tests was prepared by grinding systemically infected leaves of each host plant in 5.0 ml 0.5 M sodium - potassium phosphate buffer, pH 7.1, and clarifying by centrifugation at 10,000 X g for 10 min. Healthy host sap was prepared as described for the microprecipitin tests.

For all agar-gel tests, intact virus antigens in buffers previously described for the microprecipitin tests were used at concentrations of 1.0 mg per ml. Formaldehyde stabilized D-protein antigens were used at concentrations of 0.1 mg per ml. Antisera and healthy and infected host sap antigens were used undiluted as prepared by procedures previously described. Tests were repeated ten to twelve times and results were recorded photographically using a Leitz-Wetzler Macro Dia photocopy stand equipped with a Leica M-2 camera back, and lighted indirectly (35) by a dark field illuminator specially constructed for this purpose according to Crowle (15).

RESULTS

Microprecipitin Tests

Homologous and heterologous titers of antisera prepared to intact virus (Table 1) were sufficiently high to suggest serological relationships. It is significant that in each case where two rabbits were immunized against the same intact virus, highly consistent results were obtained between the two antisera produced.

Titers of cross-absorbed antisera (Table 2) showed the presence of definite but distant serological relationships. In all cases, cross-absorption resulted in reduction of titers by one or two dilution steps. Results shown in Tables 1 and 2 are directly comparable, as the titers reported are adjusted for antiserum dilution due to the cross-absorption process.

Control tests showed no reactions between normal serum and any of the intact virus antigens. Sap from healthy host plants showed no reactions when tested against antisera to their respective viruses. Similarly, buffer and saline controls in each microprecipitin plate were negative. In addition, when TMV was used as a control antigen in tests with each antisera, no specific reactions were observed.

Agar-gel Immunodiffusion Tests

Antisera produced against D-proteins of MDMV-B and TEV showed homologous titers of $\frac{1}{2}$ while TurMV D-protein antisera

Table 1. Homologous and heterologous titers of intact virus antisera as determined by microprecipitin tests (titers are expressed as the reciprocal of the highest dilution giving a positive reaction)

Antiserum	Rabbit no.	Antigen		
		MDMV-B	TEV	TurMV
MDMV-B	1	256-512	32	32
	2	512-1024	64	64
TurMV	1	4-8	64	1024-2048
	2	8-16	32	1024
TEV	1	8-16	1024	64
	2	16-32	1024	16-32

Table 2. Homologous and heterologous titers of cross-absorbed intact virus antisera as determined by microprecipitin tests (titers are expressed as the reciprocal of the highest dilution giving a positive reaction)

Antiserum	Cross absorbed with	Antigen		
		MDMV-B	TEV	TurMV
MDMV-B (2)[a]	TurMV	128	16-32	0
	TEV	128-256	0	32
TurMV (1)[a]	MDMV-B	0	16-32	1024
	TEV	2	0	1024
TEV (1)[a]	MDMV-B	0	512	16
	TurMV	16-32	512-1024	0

[a]Refers to rabbit no. in Table 1.

showed no reaction to its homologous antigen.

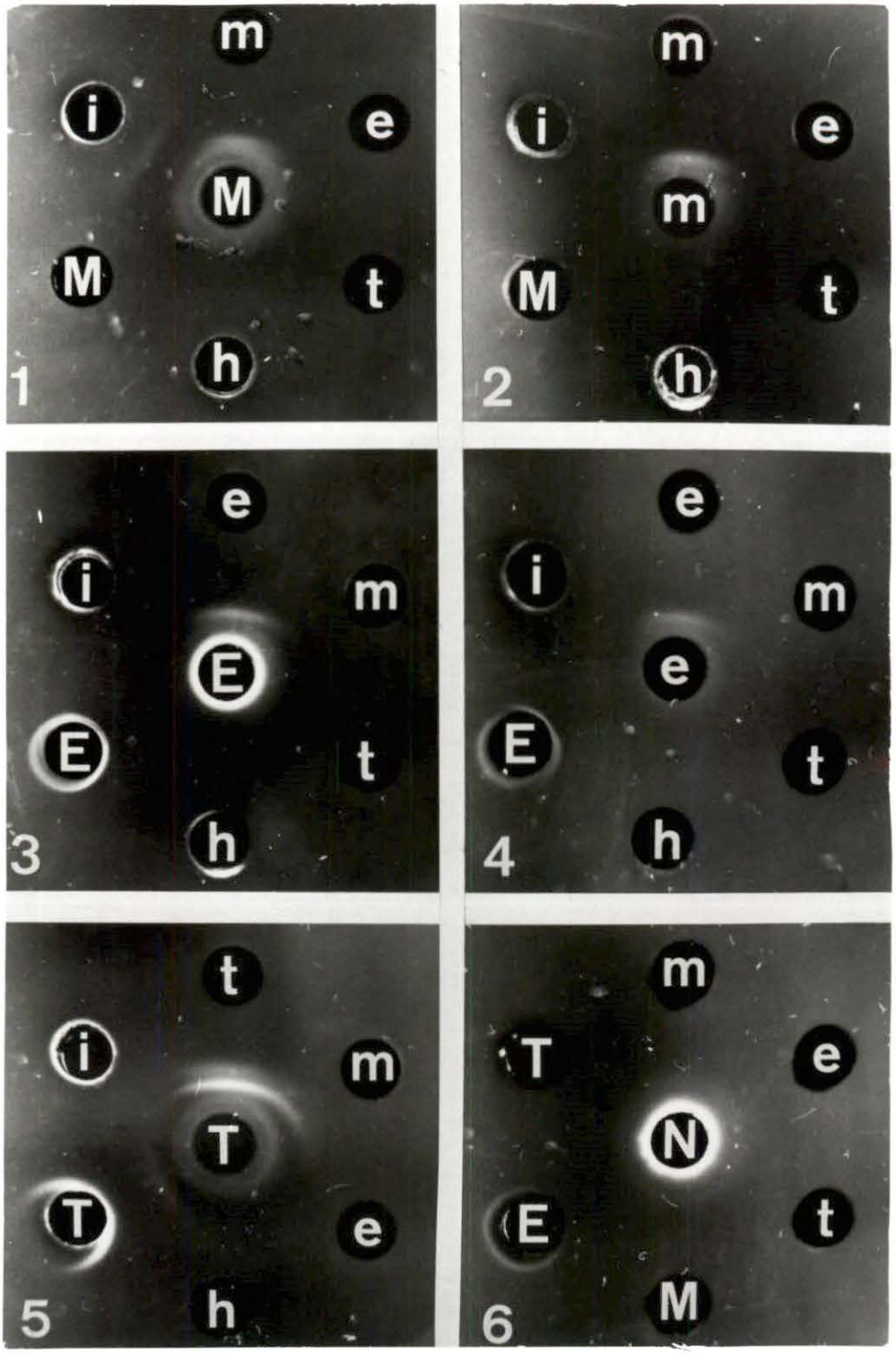
Results of double-diffusion tests in agar-gel are shown in Figure 1. MDMV-B D-protein antigen produced a precipitin line very near the antiserum well in tests with antisera to MDMV-B and MDMV-B protein (Fig. 1-1, 1-2). The same tests often showed precipitin formation immediately around the antigen wells containing purified MDMV-B. Similar results were observed with antisera to TEV and TEV D-protein (Fig. 1-3, 1-4). Although no reactions were obtained in any tests involving TurMV D-protein antisera, TurMV antisera reacted strongly with TurMV D-protein antigen to form intense precipitin lines (Fig. 1-5). Only local precipitin formation around the antigen wells was observed in tests involving purified TurMV and TEV with their homologous antisera (Fig. 1-3, 1-5).

The only heterologous reaction observed was the formation of a faint precipitin zone midway between TurMV antisera and MDMV-B D-protein antigen wells (Fig. 1-5). This reaction occurred consistently in all tests conducted.

Although sometimes difficult to distinguish, line-splitting was often observed in precipitin lines formed against TurMV and TEV D-protein antigens at the concentrations cited.

No reactions were observed between D-protein antisera and infected sap from their respective host plants. Intact virus antisera, on the other hand, showed definite reactions to their respective infected host sap antigens (Fig. 1-1, 1-3,

Figure 1. Agar-gel double immunodiffusion tests; center wells contain antisera and peripheral wells contain antigen (purified virus at 1.0 mg/ml and D-protein at 0.1 mg/ml); M = MDMV-B, E = TEV, T = TurMV, N = normal serum, m = MDMV-B D-protein, e = TEV D-protein, t = TurMV D-protein, h and i = healthy and infected sap, respectively, of corn (1 & 2), tobacco (3 & 4), and mustard (5)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1-5). MDMV-B-infected corn sap produced precipitin lines midway between antigen and antisera wells when reacted with MDMV-B antisera (Fig. 1-1). A similar but very faint and inconsistent reaction was observed between TEV-infected tobacco sap and TEV antisera. TurMV antisera often produced intense precipitin halos around antigen wells containing TurMV-infected mustard sap (Fig. 1-5).

Normal serum did not react with either intact virus or D-protein antigens (Fig. 1-6). Similarly, no reactions were observed in tests involving antisera against their respective healthy host sap.

The serological relationships between intact virus and D-protein antigens of MDMV-B, TurMV, and TEV revealed by this study may be summarized as follows: whole virus and its D-protein antigen are serologically related, as evidenced by the reactivity of intact virus and D-protein antisera with D-protein antigen in agar-gel tests (Fig. 1).

DISCUSSION

Relatively low heterologous titers from reciprocal microprecipitin tests of cross-absorbed (Table 2) and noncross-absorbed antisera (Table 1), indicates that MDMV-B is distantly related to TurMV and TEV. Slightly higher reciprocal heterologous titers indicate that TurMV and TEV are more closely related to each other than either is to MDMV-B. This degree of relatedness might be expected based on biochemical data which shows TurMV and TEV possess 5% RNA (17,23) while MDMV-B contains 6% RNA (26). Similarly, the amino acid content of TurMV and TEV, although similar to MDMV-B diverges widely in the relative amounts of glycine and serine (17, 23, 26). These observations when considered with the data reported herein provide further support for placing MDMV-B in the potyvirus group.

The use of chloroform and urea in MDMV-B purification was reported by Langenberg to be unsuitable for serology due to contamination of preparations by host proteins (28). It is significant that a high degree of specificity was observed in all antisera tested in this study. Intact virus antisera of moderately high titers were produced which showed no reactions to healthy host sap in either microprecipitin or agar-gel tests.

The use of borate buffer in serological tests has sometimes produced spurious results (51), but presented no

problems in the present study.

Heterologous reactions were never observed between D-protein antigens when tested against D-protein antisera. The low homologous titers of the D-protein antisera, however, may have precluded heterologous reactions. Similar titers have been reported for antisera against PVX D-protein (51,52) and against inclusion body proteins of Bidens mottle virus, pepper mottle virus, PVY, TEV, and TurMV (42).

Although this study suggests that potyvirus D-protein antigens are not closely related serologically, the conclusion that they are not related cannot be made. As pointed out by Kleczkowski (27) it is necessary to use high-titered antisera when attempting to discern distant serological relationships within a group of antigens. If future investigations result in high titered D-protein antisera to study viruses in this group, the distant relationships that may exist between D-protein antigens will perhaps become apparent. Some evidence to support this exists in the faint but consistent heterologous reaction observed between TurMV antisera (highest titered of all antiserums used in this study) and MDMV-B D-protein (Fig. 1-5).

I have no complete explanation for the apparent lack of reactivity observed in TurMV D-protein antisera. However, there is no doubt that TurMV D-protein is antigenic as demonstrated by its reaction with TurMV antisera (Fig. 1-5). Line-splitting observed in this reaction at antigen concentrations

of only 0.1 mg/ml, suggesting antigen excess (11,48), is further evidence that this protein is highly antigenic even in small quantities. This suggests TurMV D-protein is probably also immunogenic.

The use of D-protein antisera to detect MDMV-B-, TurMV-, and TEV-infected plants should be further explored. The prime limitation of such a study, the difficulty of obtaining sufficiently high titered antisera, may be traced to difficulties in obtaining sufficient quantities of homogeneous virus and subsequent preparation of D-protein. Although the quantities of D-protein necessary for suitable immunization schedules are difficult to obtain, the results of this study show that relatively low titered but highly specific antisera can be produced by immunization schedules using less than 0.5 mg total protein. If increased quantities of these immunogens can be prepared, the production of high titered antisera may be possible.

Agar-gel tests involving virus-infected host sap and homologous intact virus antisera demonstrates that this approach to diagnosis may have potential. Tests with MDMV-B-infected corn sap yielded unexpected results. A highly specific antiserum of moderately high titer, produced against a highly purified and homogeneous preparation of MDMV-B, reacted with antigen present in MDMV-B-infected sap to produce very distinct precipitin lines in agar-gels midway between antigen and antisera wells (Fig. 1-1). The antigen involved is

specific to MDMV-B-infected sap because no reactions occurred with healthy sap. The antigen is probably not intact MDMV-B particles, because these do not readily diffuse through agar gel as shown in tests using purified MDMV-B antigens. Furthermore, the antigen involved is not characteristic of MDMV-B D-protein which migrates rapidly in agar-gel diffusion tests and forms precipitin lines near the antiserum well (Fig. 1-1, 1-2). It is also not characteristic of purified MDMV-B which under conditions of prolonged storage begins to break down and produces multiple precipitin lines (often 4-5) when reacted with antiserum specific to intact MDMV-B (M. R. McLaughlin, J. H. Hill, and H. I. Benner, Iowa State University, Ames, Iowa, 1973, unpublished results). D-protein antisera also does not react with MDMV-B-infected sap containing the active antigen.

The serologically active agent in MDMV-B-infected corn sap may be a virus specific soluble antigen (58), possibly a low molecular weight protein antigenically related to the capsid protein, but antigenically different from D-protein. Soluble antigens have been detected in plants infected with TMV (4,57), clover yellow mosaic virus (41), barley stripe mosaic virus (20), PVX (47), and apple chlorotic leaf spot virus (12).

The possibility exists that the antigenically active agent in MDMV-B-infected corn sap is inclusion body protein. Recent work by Purcifull, Hiebert, and co-workers has shown

the presence of antigenically active virus-induced inclusion body proteins in plants infected with Bidens mottle virus, pepper mottle virus, PVY, TEV, and TurMV (22,40). However, if this is the case, similar results might be expected in tests of TurMV- and TEV-infected sap. No such reaction was ever observed in tests involving TurMV-infected mustard sap. Although such a reaction was observed with TEV-infected tobacco sap, precipitin lines formed inconsistently and were very faint.

These findings suggest potential for adaptation to routine disease diagnosis procedures. In the instances of MDMV-B and TEV the possibly presence of soluble antigen in infected tissue provides a good starting point toward development of agar-gel double diffusion systems for rapid diagnosis of diseases caused by these viruses. The strong precipitin halos observed around antigen wells containing TurMV-infected mustard sap (Fig. 1-5) reacted with TurMV antisera suggests radial diffusion systems in agar-gel may be a promising diagnostic technique.

Further research in the area of potyvirus serology should yield valuable and interesting information in the areas of potyvirus taxonomy and disease diagnosis.

SUMMARY

Serological relationships of maize dwarf mosaic virus-strain B (MDMV-B), turnip mosaic virus (TurMV), and tobacco etch virus (TEV) were studied through the use of antisera produced against the intact viruses and their depolymerized coat proteins (D-proteins). Microprecipitin tests showed that MDMV-B, TurMV, and TEV are distantly related serologically. This relationship provides further evidence for placing MDMV-B in the potyvirus group of plant viruses. Immunodiffusion tests showed homologous reactions of MDMV-B, TurMV, and TEV with their respective D-proteins. The D-proteins exhibited a high degree of antigenic specificity and appeared not to be related serologically, with the possible exception of a distant relationship between MDMV-B and TurMV D-proteins when reacted with TurMV antiserum. Tests using intact virus antisera indicated the possible presence of virus-induced soluble antigens in MDMV-B- and TEV-infected plant sap. These and other preliminary tests indicate potential for serodiagnosis of plant diseases caused by potyviruses.

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