Virus Infection of *Trifolium* Species in Cell Suspension Cultures

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ABSTRACT

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Cell suspensions derived from callus tissue cultures of *Trifolium repens* L., *T. ambiguum* Bieb., *T. hybridum* L., and the hybrid *T. ambiguum* × *T. hybridum* were inoculated with clover yellow mosaic virus (CYMV) or clover yellow vein virus (CYVV) and analyzed for ability to support virus multiplication. Inoculations with purified viruses were made 24 hr after cell subculture. Samples of inoculated cells, taken 6 and 12 hr later and thereafter at 24-hr intervals through 144 hr, were examined for the presence of inclusion bodies, and virus concentration was determined by latex serology. CYMV multiplication occurred in cultures of all species, but concentrations were lower in *T. ambiguum* and the hybrid. When cultures were inoculated with CYVV, *T. ambiguum* and the hybrid did not support virus multiplication, while *T. repens* and *T. hybridum* produced serologically detectable virus. These in vitro responses to both viruses closely approximated responses to in situ plant inoculations.

Additional key words: white clover, Kura clover, forage disease.

White clover (*Trifolium repens* L.) and alsike clover (*T. hybridum* L.) are widely planted perennial forage legumes. Because of their broad environmental tolerance, nutritional balance, and efficient nitrogen fixation, cultivation of these species has spread from the Mediterranean to the temperate regions of both hemispheres. The persistence of the clovers in pasture populations, however, is affected by their susceptibility to three major viral pathogens (2), clover yellow vein virus (CYVV), alfalfa mosaic virus (AMV), and peanut stunt virus (PSV). A related species, *T. ambiguum* Bieb., is resistant to the above pathogens (2), but has fewer desirable agronomic characteristics (5). All white and alsike clover plants tested were susceptible to clover yellow mosaic virus (CYMV) but only 11% of the *T. ambiguum* were susceptible. Hybrids among these species have been proposed as a means of obtaining cultivars with virus resistance.

Cell and tissue culture systems are receiving increasing attention as means of propagation, hybridization, and selection of improved cultivars. Generation of interspecific hybrids from either somatic fusions or from culture of proembryonic tissue reasonably may be expected to include cycles wherein the initially small amount of tissue available for plant regeneration is increased through callus culture. Use of in vitro procedures at these early stages for detection of heritable characteristics such as virus disease resistance could save time, space, and labor. Few investigations have been reported of virus infection and replication in cell and callus cultures of legumes. Cowpea leaf mesophyll protoplasts, however, have been inoculated successfully with cowpea mosaic virus (CPMV) (4) and CYMV (11). Replication of southern bean mosaic virus was found to occur in inoculated callus cultures of soybean (13).

In the present investigation CYMV and CYVV were used to inoculate cell suspension cultures of *T. repens, T. hybridum, T. ambiguum,* and an interspecific *T. ambiguum* × *T. hybridum* hybrid to determine: whether clover viruses can multiply in vitro in cultures of *Trifolium* species and hybrids; whether virus inclusion bodies can be detected in cultured cells by light microscopy; and whether in vitro results are comparable to those involving whole plants.

MATERIALS AND METHODS

Plants of *T. repens, T. hybridum, T. ambiguum,* and *T. ambiguum* × *T. hybridum* were maintained in the greenhouse. The *T. ambiguum* and *T. hybridum* plants were the parents of the hybrid. Because genetic variability would be expected among seedlings of these self-incompatible species, replicates of the genotypes were made from stolon or rhizome cuttings.

Callus cultures were initiated from explants of young leaf tissue. The solid medium for callus induction and maintenance consisted of Murashige and Skoog (10) salt formulation, 4.30 g/L; *i*-inositol, 0.1 g/L; casein hydrolysate, 0.1 g/L; Staba (12) vitamin concentrate, 20 ml/L; sucrose, 30 g/L; and Difco Bacto-Agar, 10 g/L. The growth regulators, 4-amino-3,5,6-trichloropicolinic acid (Picloram) and N^6-isopentenyladenine (2-iP), were added at the rate of 1.0 mg/L each. These concentrations had been found to induce callus growth while inhibiting differentiation. The pH was adjusted to 6.0 with 0.1 N NaOH. Liquid media were prepared in the same manner except for omission of the agar. Cultures were maintained on lighted shelves, at approximately 4,000 lux and 22–24C.

The CYMV and CYVV isolates were the same as those used by Barnett and Gibson (2). Partially purified preparations were obtained by extraction from *Pisum sativum* L. in 0.03 M phosphate buffer, pH 7.5, and 0.05 M urea with chloroform clarification (1:1, v/v), polyethylene glycol (4% w/v) precipitation, and differential centrifugation. Concentrations of the viruses in the final preparations were obtained by UV absorption using extinction coefficients ($E_{1\text{ cm}}^{0.1\%}$) at 2.60 nm, corrected for light scattering of 2.97 and 2.4 for CYMV and CYVV, respectively.

Before viral inoculations, approximately 700 mg of friable callus tissue was incubated in 2.0 ml of liquid medium in 10-ml vials for 24 hr on a rotary shaker. Cells were present singly and in small aggregates. Purified CYMV or CYVV was added to each vial to give a final virus concentration of approximately 200 μg/ml. Vials were then vibrated at 800 rpm for 20 sec on a vortex mixer and incubated for 30 min. The material contained in each vial was emptied into a 50-mm funnel lined with autoclaved filter paper, washed with 15 ml of fresh liquid medium to remove unattached virus, and transferred to clean vials containing 2.0 ml of medium (9). The inoculated cells were again incubated on a rotary shaker at 120 rpm and 22–24C.

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To detect viral infection, a portion of each vial's contents (approximately 100 mg) was removed after each of eight sequential time intervals (6, 12, 24, 48, 72, 96, 120, 144 hr after inoculation) for testing by latex serology and inclusion body staining. The remainder from each vial, approximately 500 mg, was frozen for later use in a local lesion assay on an indicator plant. Three complete replications of eight vials per replication were performed separately for each of the four taxa.

For detection of CYMV inclusion bodies (7), the tissue was stained 5–15 min in a solution of Azure A (0.1 g dye in 100 ml 2-methoxyethanol) and 0.2 M disodium phosphate (9:1, v/v), rinsed in absolute ethanol for 30 sec, mounted in euparal, and examined immediately. The number of cells containing inclusions among a total of 340–360 cells in five microscope fields was recorded.

Antibody-sensitized latex was prepared by the procedures of Abu Salih et al (1) and used to detect the presence of viral antigens in cultured tissues. Approximately 100 mg of tissue was homogenized in 0.2 M tris-HCl buffer, pH 7.2, containing .02% polyvinyl pyrrolidone. A series of two-fold dilutions was made, and one drop of each dilution was added to two drops of CYMV or CYVV antibody-latex conjugate. The plates were rocked gently for 5 min before reaction results were recorded. A fine, evenly dispersed clumping indicated a positive reaction.

Local lesion assays for CYMV were made in the greenhouse with *Gomphrena globosa* L. as the indicator plant. At the time these tests were made, our *Chenopodium quinoa* Willd. indicators, the preferred local lesion host for CYMV, were not responding to inoculations. Thawed samples from inoculated cultures were ground and mechanically inoculated to leaves of *G. globosa* according to a 3×3 lattice square design (6). After 7 days the lesions on each leaf pair were recorded. Each of the nine samples, representing eight time periods and one uninoculated control, was inoculated to two leaf pairs in the two-square design, which utilized three leaf-pair positions per plant, three plants per square. Each replication thus contained six *G. globosa* indicator plants for each of the four *Trifolium* taxa.

RESULTS

Preliminary experiments utilizing inoculation procedures described by Kassanis et al (8) and Murakishi et al (9) were tried, but virus replication could not be confirmed by latex serology. The procedure used for our investigation was a modification of those used by Murakishi et al (9) and Wu and Murakishi (13); the major

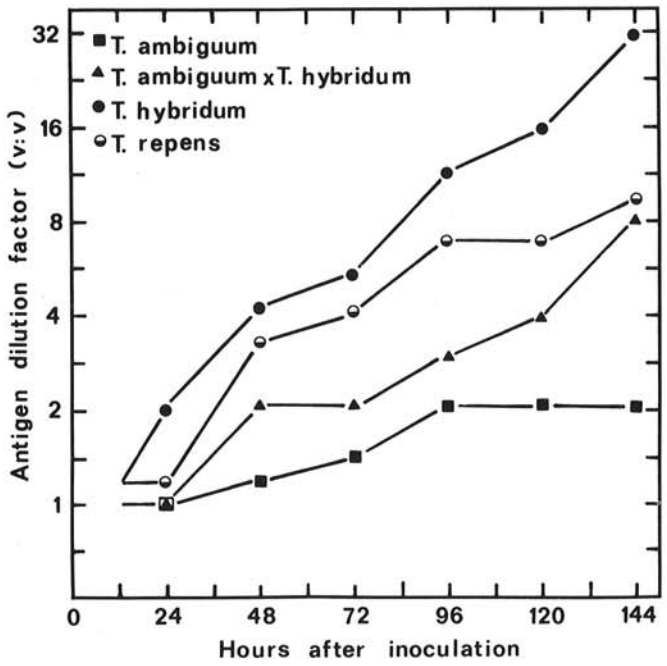

Fig. 1. Detection of clover yellow mosaic virus in extracts of cell cultures. Antigens were applied in a series of twofold dilutions (1 = undiluted). Data points represent the highest reacting dilutions for the time periods and are averages of three samples, with the exception of *T. ambiguum,* of which only one sample reacted at the 6-hr period.

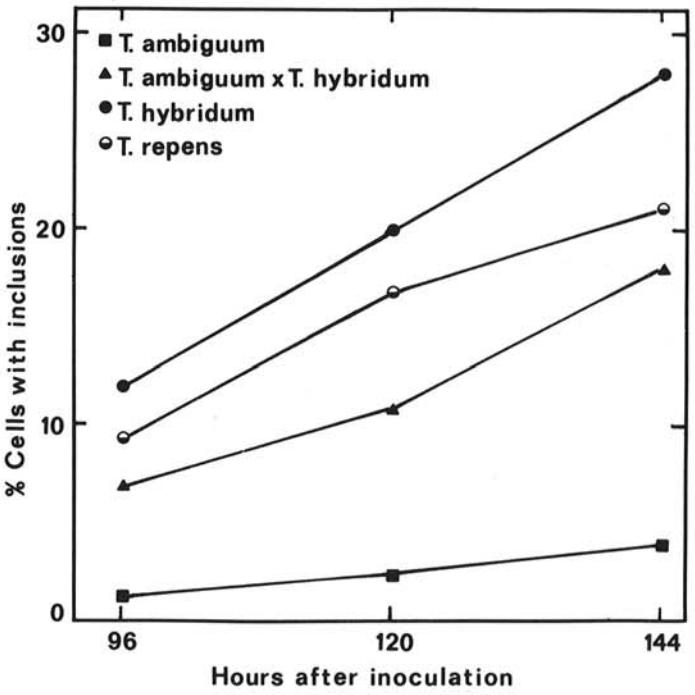

Fig. 2. Percentage of cells containing inclusion bodies in clover yellow mosaic virus-inoculated suspension cultures of *Trifolium* species and a *T. ambiguum* × *T. hybridum* hybrid. Percentages of cells with one or more inclusions per cell were averaged for three replications. At each sampling period, a total of 340–360 cells, viewed from five random microscope fields, were examined after being stained with Azure A.

TABLE 1. Comparison of intact plant and cultured tissue susceptibility to clover yellow mosaic virus (CYMV) and clover yellow vein virus (CYVV)

	CYMV				CYVV	
	Intact plants		Cell suspension		Intact plant	Cell suspension
Species	Systemic symptoms[a]	Latex[b]	Percent cells with inclusions[c]	Latex[b,c]	Systemic symptoms[a,d]	Latex[b,c]
T. repens	S	8	21	10	S	2
T. ambiguum	n	–	3	2	n	–
T. hybridum	S	8	28	32	S	4
T. ambiguum × *T. hybridum*	(s)	2	19	8	n	–

[a] S = systemic symptoms, (s) = symptomless systemic infection, and n = no infection.
[b] Reciprocal of antigen dilution reacting with antibody sensitized latex; – = no reaction.
[c] Data obtained from samples collected 144 hr postinoculation.
[d] Ratings for CYVV on plants are from Barnett and Gibson (2), excepting that for the hybrid.

changes, which were suggested by H. H. Murakishi, were an increase in the concentration of inoculum and subsequent incubation of the cells in liquid medium.

Our CYMV antibody-latex conjugate reacted with suspensions containing 10 μg/ml, but not with those containing 5 μg/ml of purified virus; the CYVV antibody-latex conjugate reacted with suspensions containing 10 μg/ml, but not with those containing 1 μg/ml of purified virus. Neither latex preparation reacted with uninoculated cultured tissues nor with extracts from uninfected plants. Stained inclusions were not found in uninoculated cultures or intact plants.

Results obtained from in vitro and in vivo inoculation of the three *Trifolium* species and the interspecific hybrid with CYMV are shown in Table 1, Figs. 1 and 2. Although neither inclusion bodies nor latex reactions were found in samples taken 6 hr after inoculation, infection occurred in indicator plants of *G. globosa* when these were inoculated with suspension-cultured samples of all species except *T. hybridum*. Both the susceptibility of *G. globosa* plants, and the lesion numbers among leaf pairs on a plant, varied greatly; therefore, reliable quantitative data were not obtained. No infectivity assay of CYVV was performed.

After 12 hr, cultures of all species gave detectable latex reactions in varying amounts (Fig. 1). Inclusion bodies were first observed after 96 hr and the percentage of cells containing these bodies rose consistently thereafter (Fig. 2). Although the results from inoculations of *G. globosa* leaves showed no consistent correlation with the length of incubation time, larger numbers of lesions generally were formed after inoculation with samples taken between 72 and 144 hr.

When propagules of the four parent plants used to obtain the initial callus cultures were mechanically inoculated with purified CYMV, visible mosaic symptoms occurred only on *T. repens* and *T. hybridum*. However, when propagules were tested by latex serology, *T. ambiguum* × *T. hybridum* also contained a low concentration of virus, indicating a symptomless infection. *T. ambiguum* plants showed no visible symptoms, and no virus was detected by latex serology.

Suspension cultures of the species and hybrid were inoculated subsequently with CYVV using the same procedure as for CYMV. However, only two time periods, 6 and 144 hr after inoculation, were analyzed for latex reactions. As with CYMV, the 6-hr samples gave no positive reactions. The 144-hr samples, however, showed the presence of the virus in *T. repens* and *T. hybridum*, but not in *T. ambiguum* or the interspecific hybrid (Table 1).

DISCUSSION

Our results indicated that both CYMV and CYVV caused infection and replicated in *Trifolium* cells in suspension culture, and that the reaction of cultured cells generally agrees with known resistance or susceptibility of the source plants. Evidence of virus infection was demonstrated by a progressive increase in the amount of antigen precipitated by virus-specific antibody and by an increase in percentage of cells containing inclusions. Since the inclusions found in cultured clover cells show the banded pattern typical of CYMV infection (7), we conclude that the increase in number of inclusion-containing cells with time is evidence that CYMV is multiplying in the cultures.

These observations are in general accord with those obtained by Rao and Hiruki (11) from fluorescent antibody staining of CYMV in inoculated cowpea protoplasts. After incubation of the virus with 0.4 μg poly-L-ornithine, followed by incubation of the inoculated protoplasts for 48 hr, 38% of the cowpea protoplasts contained viral antigen. At the 48-hr callus suspension sampling time, we could not yet identify inclusions, although latex reactions were occurring in two- to four-fold dilutions of the antigen from susceptible taxa. After 144 hr, *T. hybridum*, the most susceptible clover species, contained inclusions in only 28% of the cells. The apparently lower rates of infection in susceptible cultures in our experiments may reflect physiological variation among taxa, differences in culture environment and additives, or an effect of the barriers presented by more or less intact callus cell walls.

Barnett and Gibson (2) mechanically inoculated plants of *T. hybridum, T. repens,* and *T. ambiguum* (among others) with CYMV in order to assess resistance to the virus. Only *T. ambiguum* showed any resistance; approximately 11% of the plants could be infected. *T. ambiguum* × *T. hybridum* was later found to show no visible symptoms upon infection with this virus although the virus could be detected serologically (*unpublished*). Our in vitro observations are in general agreement with those obtained from mechanically inoculated whole plants (Table 1). Both plants and cultured tissue of *T. repens* and *T. hybridum* were susceptible to CYMV. Although the *T. ambiguum* parent plant could not be infected, low levels of virus were found in cultured tissue. Plants of the hybrid were infected but showed no symptoms, and intermediate levels of virus were found in cultured tissue (Figs. 1, 2).

Somewhat comparable differences between CPMV infection in culture and in intact plants were reported by Beier et al (4) who observed virus replication in protoplasts of 54 field-resistant lines of cowpeas. Protoplasts of only one line were resistant to infection, and in further studies (3) even this line was found to support a low level of replication. This suggests the presence of more than one level of cytoplasmic resistance in addition to any resistance derived from the multicellular structure of the whole plant. Since our *T. ambiguum* cell cultures had not been treated with enzymes to remove cell walls, or with additives to increase virus penetration, we suggest that they provide additional evidence that resistance to infection and resistance to replication are controlled by separate genetic mechanisms, with resistance to infection being overridden in suspension cultures. The intermediate levels of virus produced in suspension cultures of the hybrid also suggest that the genetic controls of resistance to CYMV in the *T. ambiguum* parent are incompletely dominant.

When whole plants were inoculated with CYVV, *T. repens* and *T. hybridum* were highly susceptible, while *T. ambiguum* and the interspecific hybrid showed no infection. Comparable reactions were observed in cultured tissue, indicating that genetic resistance to CYVV in *T. ambiguum* is under stronger control than is resistance to CYMV.

Approximately 2 mo on callus induction medium were required before sufficient tissue was available for serological and inclusion studies. Similar indications of resistance can be obtained from protoplasts or intact plants within 48 hr. However, when intact plants are not available, as in tissue culture systems for development of resistant hybrids or mutants, procedures for inoculation of small amounts of callus should be useful. The number of culture lines to be regenerated could be reduced at an early stage of the investigation. Similar procedures should be effective in detection of resistance to other virus diseases.

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