Pathogenesis of Tomato Spotted Wilt Virus in Peanut Plants Dually Infected with Peanut Mottle Virus

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ABSTRACT

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Peanut (*Arachis hypogaea*) plants dually infected with tomato spotted wilt tospovirus (TSWV) and peanut mottle potyvirus (PMV) exhibited a wide variety of symptoms, ranging from PMV-like symptoms of transient mild leaf mottle to TSWV-like symptoms of severe leaf distortion and stunting of the plant. Dual infection did not cause greater symptom severity than infection with either virus alone. In the early stages of disease development, PMV symptoms were similar to the first leaf symptoms of TSWV infection, suggesting that identification of TSWV in field-grown peanuts should not depend on visual observation. The virus titer, determined by enzyme-linked immunosorbent assay, indicated a delay in TSWV disease progress in double-infected plants, compared to plants infected with TSWV alone. In the later phase of disease progress, the virus titer in dually infected plants was not significantly different from that of singly infected plants. Infection with TSWV and PMV alone and with both viruses in combination was consistent among commercially grown peanut cultivars. In plants inoculated with TSWV or PMV alone or with both viruses in combination, the length of the latent period and final disease incidence, as measured by the number of plants showing symptoms, did not depend on the cultivar.

Tospoviruses cause major diseases of peanut worldwide (38). Tomato spotted wilt tospovirus (TSWV) has become increasingly important in the production of peanut (*Arachis hypogaea*) in the southeastern United States. Severe damage to peanut caused yield reductions of up to 95% in Texas in 1986, 1990, 1991, and 1992 (5,31,32). Although economic losses in peanut production are less pronounced in other peanut-growing areas, reports of field surveys and anecdotal information indicate an increasing incidence of TSWV in Georgia in 1989–1990 (13), Alabama (25,27), Florida, Mississippi, and North Carolina.

Programs to prevent further spread of TSWV by insecticidal control of its thrips vector did not sufficiently reduce TSWV incidence (32,44,45). Several management tools have been recommended for minimizing crop damage due to this virus (6,10,23). Other investigations have focused on the use of less susceptible varieties (4,15,26) and breeding for disease resistance (14,16,37).

Another widespread virus on peanut, peanut mottle potyvirus (PMV), was first described in 1965 (29). Its transmission by seeds is assumed to be the reason for its worldwide distribution in peanut (7) and in

other important crops, such as soybean (*Glycine max*), pea (*Pisum sativum*), and bean (*Phaseolus vulgaris*) (11). The predominant strain of PMV in the United States is a mild strain (19,34), which causes a faint mottling of the youngest leaves and little or no reduction in vegetative plant growth (29). Field surveys indicate the presence of several other strains causing more severe symptoms, including the necrosis (N), necrosis/chlorosis (NC), chlorotic stunt (CS), and chlorosis (C) strains (3,30,33,43). The symptoms caused by PMV strains N, C, and CS in peanut are sometimes similar to those due to TSWV (30,42). In addition to the similarity of symptoms caused by these viruses, mixed infections by PMV and TSWV have been reported in naturally infected peanuts in Georgia (24,42).

Infection by two unrelated viruses is common in higher plants and sometimes leads to a synergistic disease reaction. In particular, combinations of a potyvirus and an unrelated virus are known to cause synergistic disease reactions, the best-studied example being the interaction between potato virus Y and potato virus X (8,17,22,39). Mixed infections often result in a pronounced increase in disease severity over that induced by each virus separately (2,12), sometimes leading to a severe reduction in plant growth and yield losses (35,40). Frequently the titer of the potyvirus in dually infected plants is similar to that in singly infected plants, while the titer of the unrelated virus is significantly higher than in singly infected plants (2,8,12,21,36,41).

The role of PMV in the disease complex varies, depending on the co-infecting virus. PMV has a synergistic reaction with cucumber mosaic virus in cowpea (18) but no effect in combination with bean pod mottle virus in soybean (2).

In this study we determined the potential role of PMV in the pathogenesis of TSWV in peanut plants dually infected with these two viruses. Experiments were conducted with six peanut cultivars to reveal the influence of host genetic background on the interaction.

MATERIALS AND METHODS

Viruses. A TSWV isolate collected in 1991 from peanut in North Carolina and a PMV isolate that originated from infected peanut collected in 1994 in Georgia were used in this study. The authenticity of the isolates was confirmed by enzyme-linked immunosorbent assay (ELISA) and Western blot using specific antiserum and by host range studies. TSWV was maintained in peanut (*A. hypogaea* 'NC7'), *Nicotiana tabacum* 'Burley 21,' and *Emilia sonchifolia*. PMV was maintained in peanut only. Both viruses were transferred to *N. benthamiana* for one passage to obtain highly concentrated inoculum for mechanical inoculation of peanut.

Virus inocula were prepared from 7- to 8-week-old *N. benthamiana* plants infected with TSWV or PMV. Systemically infected leaves of these plants were triturated 1:5 (wt/vol) in 0.01 M Tris, pH 7.8, containing 0.01 M Na₂SO₃ and 0.1% cysteine hydrochloride. All inoculations were made by rubbing Carborundum-dusted leaves with inoculum-soaked cotton wool applicator sticks. For inoculations with both viruses in combination, inocula of the two viruses were mixed 1:1 (vol/vol) before inoculation. In all experiments a control of mock-inoculated plants was included.

Peanut cultivars. The peanut genotypes GK7, NC7, VC1, Florunner, Southern Runner, and Spanco were propagated from seeds. The seeds were pregerminated for 2 days in plastic trays containing Metro-Mix (Scotts-Sierra Horticultural Products, Marysville, Ohio) and then were transferred to 4-in. clay pots. The plants were grown, two plants per pot, in a 1:2 mixture of Metro-Mix and soil, containing fertilizer (8–8–8). The plants were kept under greenhouse conditions with natural light only, at temperatures between 20 and 30°C.

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Experimental design. The experiments were conducted in a split-plot design with 10–12 plants of each cultivar. All experiments were repeated three times at different seasons of the year. For the split-plot design, each experiment was divided into three blocks, and in each block the cultivars were randomly assigned to different rows. In the analysis of the data, samples of each cultivar and treatment combination in each block were combined. The blocks are considered replicates in each experiment. With one exception, there was no interaction between the cultivar and the virus or viruses used for inoculation. Therefore, comparisons of treatments were averaged over the cultivars.

Synergism experiments. Symptoms induced by TSWV and PMV in dually infected plants were compared with those caused by these viruses individually. When plants had developed two or three leaves, 10–14 days after planting, all leaflets of a single quadrifoliate leaf of each seedling were inoculated. The same inoculum source was used for dual and single inoculations. The titer of TSWV and PMV in the two youngest fully or nearly fully developed quadrifoliate leaves of singly and dually inoculated plants was monitored by ELISA at various intervals from 8 to 47 days after inoculation.

Evaluation of the latent period. The reactions of the peanut cultivars to TSWV and PMV were monitored after inoculation with either virus singly or with both viruses simultaneously. The number of plants showing TSWV symptoms was determined at 2-day intervals after observation of the first symptoms. Plants showing symptoms on at least one leaf were designated symptomatic. Disease progress curves were constructed for each cultivar, using the number of plants with virus symptoms. Symptom expression and severity in singly and dually infected plants were compared.

ELISA. To monitor the antigen titer of TSWV and PMV in singly and dually in-

fecting peanut plants, an indirect ELISA was performed. Leaf samples were prepared in 1:100 coating buffer (0.05 M carbonate buffer, pH 9.6), applied directly to the microtiter plate, and incubated overnight at 4°C. The plates were then incubated with blocking buffer (0.01 M phosphate buffer, containing 0.1 M NaCl and 1% bovine serum albumin, pH 7.4) overnight at 4°C. TSWV- or PMV-specific antibodies, diluted in wash buffer (0.05 M Tris-HCl buffer, containing 0.1 M NaCl and 0.1% bovine serum albumin, pH 7.3), were then applied for 2 h at 37°C. The TSWV antiserum was obtained in our laboratory. The PMV antiserum was provided by John Sherwood, University of Georgia, Athens. For the detection of TSWV, purified immunoglobulin G was used at a concentration of 0.2 mg/ml. PMV antiserum was applied at a dilution of 2×10^{-5} . Subsequently, the plates were incubated for 2 h at 37°C with alkaline phosphatase-labeled goat anti-rabbit antibodies at 1:3,000 in wash buffer. Absorbance readings were determined after incubation of the substrate (1 mg of *p*-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, per ml) at 37°C for 1 h (PMV) or 2 h (TSWV). Between each step, the plates were washed at least three times with wash buffer.

RESULTS

Evaluation of the TSWV and PMV latent period. The first symptoms induced by TSWV were observed in all cultivars 7–9 days after mechanical inoculation (Fig. 1, TSWV), followed by a linear progression of disease incidence over time. There was no significant difference in the latent period in different peanut genotypes.

Disease progress after mechanical inoculation with PMV was uniform in all peanut cultivars, with the first symptoms being expressed 5 days after inoculation (Fig. 1, PMV). There was no evidence of different reactions to PMV in any of the germ plasm.

Dually infected plants developed leaf symptoms 5–9 days after inoculation (Fig. 1, TSWV/PMV). All peanut cultivars reacted uniformly following inoculation with mixed inoculum.

Identification of symptomatic plants by visual diagnosis was confirmed by ELISA.

In most cultivars, the final level of disease incidence was reached 3 weeks after inoculation, with 70–100% of the inoculated plants exhibiting visible symptoms of TSWV or PMV infection and detectable virus concentrations.

Symptom expression in singly and dually infected plants. Greenhouse-grown peanut plants inoculated with TSWV alone at the two- to three-leaf stage developed typical TSWV symptoms on all systemically infected leaves, but not always on all leaflets of the quadrifoliate leaves. The symptoms began with chlorotic spots, which developed into concentric rings, sometimes accompanied by chlorosis and bronzing of the leaves (Fig. 2B). Symptoms in later stages of disease development included stunting and distortion of leaves in the terminal bud and reduced plant growth. The expression and severity of TSWV symptoms varied, but there was no correlation between symptom types and peanut cultivars.

Plants inoculated with PMV alone exhibited leaf mottle and a slight reduction in leaf size. Leaves were generally narrower than healthy leaves and had pointed tips. The first symptoms were usually expressed on the first leaves to emerge following inoculation. Particularly in the early stages of disease development, plants singly infected with PMV exhibited symptoms very similar to initial symptoms of TSWV infection (Fig. 2A). With further disease development the similarity in symptoms was less pronounced. The leaf mottle faded as the plants aged and often was completely masked approximately 1 month after inoculation. After recovery from PMV infection, the plants grew in a

Fig. 1. Percentage of plants of peanut cultivars GK7, NC7, VC1, Florunner (FR), Southern Runner (SR), and Spanco exhibiting visual symptoms over time following mechanical inoculation with tomato spotted wilt virus (TSWV), peanut mottle virus (PMV), or a mixture of both viruses (TSWV/PMV).

manner similar to that of the uninoculated control.

Plants inoculated with both TSWV and PMV exhibited a wide range of symptoms, including typical TSWV and PMV symptoms (Fig. 2C and D). New symptom types, differing from those of either virus, were not observed. Most of the dually infected plants exhibited TSWV-like symptoms (Fig. 2C). These plants were severely stunted, and all systemically infected leaves were chlorotic, distorted, and reduced in size. A few doubly infected plants expressed a PMV-like leaf mottle, which later faded as the plants recovered (Fig. 2D).

TSWV antigen titer in singly and dually infected plants. The TSWV titer in the youngest fully or almost fully expanded quadrifoliate leaf of singly and doubly infected peanut plants varied over time (Table 1). In plants singly infected with TSWV, the virus titer reached a detectable level 2 weeks after inoculation. The high-

est concentration of antigen was detected approximately 3 weeks after inoculation. In dually infected plants TSWV could be detected after 7–10 days. For both treatments, the peak of TSWV antigen was followed by a decrease in virus concentration. Up to 3 weeks after inoculation, there were significant differences between the virus titer in plants singly infected with TSWV and that in dually infected plants. During this period, the TSWV titer also varied among the peanut genotypes. In later stages of disease development, neither the inoculated virus or viruses nor the cultivar induced significant differences in virus titer.

Besides the nonuniform distribution of the virus in a single plant at a specific time and over the course of disease development, there were considerable differences in the TSWV and PMV titers in plants of the same cultivar subjected to the same treatment. The wide variation in titer in individual plants is the reason for the high

standard deviation of the experimental treatments (Tables 1 and 2).

In similar experiments conducted with mechanically inoculated *N. benthamiana*, the same delay in building up a detectable TSWV titer occurred in dually infected plants, compared with singly infected plants. TSWV was detected by ELISA 5 days after inoculation with TSWV alone and 10 days after inoculation in dually infected plants. In later stages of infection of *N. benthamiana*, singly and dually infected plants developed the same TSWV titer (data not shown).

PMV antigen titer in singly and dually infected plants. At the beginning of infection, the PMV titer was significantly higher in singly than in dually infected plants (Table 2). In later stages of disease development, no significant differences were observed between the PMV antigen titer in quadrifoliate leaves of singly infected plants and that in corresponding leaves of dually infected plants. The PMV titer varied strongly over time, peaking twice in both singly and dually infected plants, 13 and 33 days after inoculation. Since no cultivar $\times$ treatment interactions occurred, comparisons of treatments were averaged over the cultivars.

DISCUSSION

In greenhouse studies, no significant differences in disease progress were observed in the peanut cultivars GK7, NC7, VC1, Florunner, Southern Runner, and Spanco after mechanical inoculation with TSWV, PMV, or both viruses. The period of time required for symptom expression and the percentage infected plants, as measured by visual assessment of symptoms and ELISA, were similar in all cultivars. With respect to TSWV, these results differ from previous observations. In field-grown peanuts subjected to the natural spread of the virus transmitted by its vector, the final incidence of spotted wilt was lower and disease progress was slower in Southern Runner than in Florunner (15). From our results it is concluded that the resistance observed in the field is not due to specific resistance against TSWV. Therefore, this resistance may be effective only at low inoculum dosage, as has been shown for peanut bud necrosis tospovirus (20), or it may be vector dependent.

No synergistic disease reaction was observed in plants dually infected with TSWV and PMV. Dually infected plants may exhibit symptoms of either virus, with a wide range of symptom severity. Peanut plants inoculated with TSWV and PMV mainly expressed symptoms similar to those induced by TSWV alone. In general, symptom severity did not increase by co-inoculation with TSWV and PMV. Up to 3 weeks after inoculation, a delay in disease progress in the presence of PMV was detected. In dually infected plants the TSWV titer reached a detectable level later than in

Fig. 2. Systemically infected leaves of the peanut cultivar GK7, 14 days after inoculation with peanut mottle virus (A) and tomato spotted wilt virus (B), showing similar symptoms, and 14 days after inoculation with a mixture of the two viruses (C and D), indicating a wide range of symptom types and severity.

plants infected with TSWV only. During disease development, PMV did not induce an increase in the TSWV titer, nor did it increase symptom severity in dually infected plants.

In many cases of infection by a potyvirus and an unrelated virus, the effect on the host plant is different from that observed in this experiment. For example, dual infections with soybean mosaic potyvirus and different comoviruses caused a synergistic disease reaction in soybean plants, including increases in symptom severity and in the virus titer of the comovirus (2,12,40). Similar effects occur in cowpea stunt disease, caused by a synergistic reaction of cucumber mosaic cucumovirus (CMV) and blackeye cowpea mosaic potyvirus (1,35), as well as in combinations of CMV with zucchini yellow mosaic potyvirus (36) and with turnip mosaic potyvirus (41).

For the interaction of potato virus X and potato virus Y (17,22,39), the molecular mechanisms causing plant viral synergism have been investigated in detail (8), indicating that 5' proximal potyviral sequences mediate the synergistic disease reaction in tobacco (9).

As shown previously by Kresta et al. (28) for naturally infected peanuts, TSWV was not distributed uniformly throughout the plant, with the highest virus concentration in young, developing leaf tissue. The expression of TSWV symptoms was positively correlated with the detection of the virus by ELISA. Symptomless leaves of infected plants and even symptomless leaflets of leaves with typical TSWV symptoms did not give positive ELISA readings (28). We found no correlation between symptom severity and ELISA readings, in contrast to the findings of Kresta et al. (28).

After infection with TSWV and PMV, peanut plants exhibited a wide range of symptoms, including typical symptoms of either virus. In addition, particularly in the early stages of disease development, plants infected with PMV exhibited symptoms very similar to the initial leaf symptoms of TSWV infection. These results confirm earlier observations of naturally infected peanuts in the field (42). Identification of TSWV in field-grown peanuts should therefore not rely solely on visual detection of virus symptoms. Laboratory tests are required for reliable virus identification as a basis for adequate control measures.

Table 1. Tomato spotted wilt virus (TSWV) antigen titer in peanut plants infected with TSWV or with TSWV and peanut mottle virus (PMV), as determined by enzyme-linked immunosorbent assay (ELISA)

Days after inoculation ^a	ELISA values ^b		Standard error	Treatment difference
	TSWV	TSWV+ PMV ^c		
8	0.014	0.017	± 0.001	$P \leq 0.05$
13	0.083	0.017	± 0.008	$P \leq 0.05$
19	0.268	0.064	± 0.014	$P \leq 0.05$
26	0.284	0.252	± 0.017	...
33	0.222	0.183	± 0.013	$P \leq 0.001$
40	0.098	0.114	± 0.012	...
47	0.115	0.099	± 0.011	...

^a The youngest quadrifoliate leaf was assayed at the times indicated. Leaf extracts were prepared at 1:100 (wt/vol) in carbonate coating buffer (pH 9.6).

^b Optical density ($A_{405\text{nm}}$), mean value of 72 plants. No cultivar × treatment interaction occurred. Therefore, comparisons of treatments were made with data averaged over the cultivars. Optical density values of the uninoculated control ranged between 0.01 and 0.02.

^c Inocula of the viruses were mixed 1:1 prior to inoculation.

^d Not significant.

Table 2. Peanut mottle virus (PMV) antigen titer in peanut plants infected with PMV or with PMV and tomato spotted wilt virus (TSWV), as determined by enzyme-linked immunosorbent assay (ELISA)

Days after inoculation ^a	ELISA values ^b		Standard error	Treatment difference
	PMV	PMV+ TSWV ^c		
8	0.777	0.385	± 0.058	$P \leq 0.05$
13	1.847	1.498	± 0.044	$P \leq 0.05$
19	1.158	1.373	± 0.084	...
26	0.288	0.331	± 0.037	...
33	0.672	0.882	± 0.048	$P \leq 0.001$
40	0.611	0.551	± 0.049	...
47	0.255	0.303	± 0.032	...

^a The youngest quadrifoliate leaf was assayed at the times indicated. Leaf extracts were prepared at 1:100 (wt/vol) in carbonate coating buffer (pH 9.6).

^b Optical density ($A_{405\text{nm}}$), mean value of 72 plants. No cultivar × treatment interaction occurred. Therefore, comparisons of treatments were made with data averaged over the cultivars. Optical density values of the uninoculated control ranged between 0.01 and 0.03.

^c Inocula of the viruses were mixed 1:1 prior to inoculation.

^d Not significant.

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