Annals of Applied Biology **151:67-75 (2007)**

RESEARCH ARTICLE

Symptom development and distribution of *Tomato spotted wilt virus* in flue-cured tobacco

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Keywords
TSWV; flue-cured tobacco; symptoms, distribution in plant, ELISA, mature plant resistance.

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Abstract

Tomato spotted wilt virus (TSWV) is an economically important viral pathogen of flue-cured tobacco (*Nicotiana tabacum* L.). Disease development and *in planta* distribution of TSWV were studied following mechanical inoculation of cv K326 at various stages of growth. The effect of plant age on the disease development, distribution of symptoms and TSWV were studied by inoculating plants in five age-groups, 40, 60, 75, 95 and 100 days after sowing (DAS). The plant age at the time of infection had no significant influence on the incidence of localized infection; however it had a significant effect on the development of systemic symptoms and distribution of TSWV in the plant. In a higher proportion of plants (89.2%), no systemic symptoms developed when plants were inoculated at 60 to 100 DAS. However, 90% of plants became systemically infected when plants were inoculated at 40 DAS. The systemic symptom expression was severe and distributed in all the leaves in 40 DAS plants, whereas in 60 to 100 DAS plants, it was erratic and restricted only to a few upper leaves. Results show that plant age is an important factor for TSWV infection of tobacco and mature tobacco plants significantly reduced the systemic development of the disease.

Introduction

Tomato spotted wilt virus (TSWV), of genus *Tospovirus* and family *Bunyaviridae*, is one of the most economically important plant viruses, and causes serious losses in numerous crops worldwide (Moyer, 1999, Mumford et al., 1996, Pappu, 2007, Sherwood *et al*., 2000). TSWV has a very broad host range and is known to infect as many as 900 plant species (Peters, 1998). Several species of thrips are reported to transmit TSWV (Whitfield *et al*., 2005).

In the southern United States, TSWV continues to be a production constraint in peanut (*Arachis hypogaea* L.), pepper (*Capsicum annuum* L.), tobacco (*Nicotiana tabacum* L.) and tomato (*Lycopersicon esculentum* Mill.). In Georgia, the first epidemic of TSWV in flue-cured tobacco was recorded during 1988-1989 (Culbreath *et al*., 1991), and TSWV is now considered the most serious pathogen of flue-cured tobacco in this state. In tobacco, TSWV causes necrosis, blighting and withering of leaves, directly damaging the economically important plant part (Mandal *et al*., 2006; Pappu, 1999). In recent years, the incidence of TSWV in flue-cured tobacco has increased and the cultivation was seriously affected, causing an average stand loss of 41% at an estimated loss of more than $19.4 million annually (Williams-Woodward, 2000). Control of TSWV in tobacco by insecticides and cultural practices is not satisfactory (McPherson *et al*., 1992 & 1995). Resistant cultivars are generally considered to be the most effective tool for managing TSWV, but so far no resistant source has been found in tobacco. Seasonal abundance of vector thrips in flue-cured tobacco has been recorded and it was found that *Frankliniella fusca* and *F. occidentalis* were the major vectors of TSWV (McPherson *et al*., 1992). Abundance

of thrips vectors, a broad range of alternate sources of infection, and lack of host resistance make it difficult to control TSWV epidemics in flue-cured tobacco.

Susceptibility of plant parts to virus infection changes with age (Lobenstein, 1972). This phenomenon, referred to as 'age-related resistance' or 'mature plant resistance'. Although TSWV has been recognized as a serious pathogen of tobacco, age related resistance in flue-cured tobacco to TSWV has not been studied. Response of pepper, potato (*Solanum tuberosum* L.) and tomato against TSWV has been studied as it relates to time of infection (Bald, 1937; Moriones *et al*., 1998; Soler *et al*., 1998; Wilson, 2001). Under field conditions, symptoms of TSWV in flue-cured tobacco are known to occur very soon after transplanting and the disease incidence increases between 120 to 160 Julian days. The disease incidence decreases later and very few infections are observed after flowering (Csinos *et al*., 2001). It has been observed that tobacco plants infected 1-2 weeks after transplanting in the field suffered serious losses in yield due to spotted wilt disease, whereas plants infected at later stages suffered relatively less yield loss (A. S. Csinos, unpublished). In the initial trials of mechanical inoculation of flue-cured tobacco with TSWV under greenhouse conditions, we noticed that inoculation of the upper most leaf that was halfway expanded did not result in a high level of transmission, whereas inoculation of the upper expanded leaves resulted in a higher transmission rates. These observations led us to conduct a systematic study with the objective to understand the pattern of symptom development and distribution of TSWV in flue-cured tobacco plant following infection at various stages of plant age.

Materials and Methods

Source of inoculum, test plants and mechanical inoculation

An isolate of TSWV collected from Colquitt County, Georgia (Mandal et al., 2001) that causes severe necrosis and death of tobacco cultivar (cv) K326, was used in this study. The isolate was maintained by mechanical inoculation of cv K326 in the greenhouse. Newly infected leaves with systemic symptoms 15 days post-inoculation (DPI) were used as the source of inoculum for all the experiments described below. A single source of TSWV-infected plant was used in all experiments and repeated passages of the virus was avoided to prevent the potential accumulation of defective interfering RNAs,

Seeds of cv K326 were sown in the greenhouse in seed-pans containing media mix consisting of Canadian sphagnum peat moss (75-85%), perlite (15-20%) and vermiculite (5-10%) (Berger Peat Moss-Lee Berger Itee, Saint-Modeste, Quebec, Canada). Twenty five days after seeding (DAS), seedlings were transplanted into Styrofoam float-tray (Speedling Inc., Sun City, Florida, USA) with 6.45 cm^2 cell size containing media mix. Eight to 10 days after first transplanting, seedlings were transplanted individually into plastic pots (20.5 cm diameter).

The mechanical inoculation procedure for TSWV was as described by Mandal *et al.* (2001). Inoculum was prepared by grinding systemically infected leaves in a 1:10 ratio (w/v) of 0.1M phosphate buffer pH 7.0, containing 0.2 % Na_2SO_3 and 0.01M mercaptoethanol. Debris was removed by filtering the extract through a layer of non-absorbent cotton. To this extract, 2% Carborundum 320 grit and 1% Celite 545 (Fisher Scientific, Fair Lawn, NJ, USA) were added and the inoculum was maintained

on ice until inoculations were completed. Plants were inoculated by gently rubbing the leaves with a cotton swab soaked in the inoculum. Inoculations were performed in a greenhouse after sunset when temperatures were 25 to 30^{o}C. After inoculation, plants were briefly misted with water.

Leaf position and TSWV susceptibility

All the leaves from the top to bottom of young (40 DAS) and old (80 DAS) tobacco plants were mechanically inoculated. The position of the leaf was assigned a number in descending order from the top of the plant. Approximately, 1 ml of inoculum was applied to each leaf. In 40 DAS plants, there were three leaves: leaf number one (leaf-1) was the top most emerging leaf, leaf-2 was a middle leaf, which was newly expanded and the leaf-3 was the lower most leaf that expanded prior to the leaf-2. For the age group 40 DAS, one hundred eight leaves of 36 plants were inoculated. In 80 DAS old plants, there were six leaves: leaf-1 was an emerging leaf, leaf-2 was a partially expanding leaf, leaves-3 and -4 were completely expanded young leaves, leaf-5 and -6 were expanded old leaves. For the age group 80 DAS, one hundred forty four leaves of 24 plants were inoculated. The numbers of local lesions were counted at 6 DPI for 40 DAS plants and 8 DPI for 80 DAS plants. Data from the replicated experiments were analyzed by SAS (SAS version 7, SAS Institute, Cary, NC). All experiments were repeated at least two times.

Age of plant and TSWV susceptibility

Tobacco seeds were sown every 15 to 20 days over a period of 60 days to produce

plants of different ages for TSWV inoculation. The different age groups included for evaluating their susceptibility to TSWV were 40, 60, 75, 95 and 100 DAS. One ml of inoculum was used to inoculate two youngest and fully expanded leaves of each plant in different age groups. A similar sized area was inoculated among plants of different age groups. For the age group 40 DAS, the entire surface area of leaf number two and three was inoculated. In age groups 60 to 100 DAS, the inoculation area was estimated by placing an excised leaf from a 40 DAS plant and two newly expanded leaves (leaves-3 and -4) were inoculated. The number of lesions that developed on the inoculated leaf was counted at 7 DPI. The proportion of plants with local infection was determined by testing a portion of the inoculated leaf by enzyme linked immunosorbent assay (ELISA) using a TSWV specific kit (Agdia Inc., Elkhart, IN, USA). The following controls were used in ELISA: the extract of the TSWV infected tobacco leaf as positive control, non-inoculated tobacco leaf as negative control and only coating buffer without any leaf extract as buffer control. The ELISA value greater than twice the healthy control was considered as positive reaction.

The relative levels of TSWV in the inoculated leaves of plants under each age group were analyzed by ELISA at 1:100 dilution. A total of 150 plants were assayed for TSWV (30 plant samples for each age group). The proportion of plants with systemic symptoms was recorded at 10, 15, 20 and 25 DPI. The final incidence of systemic infection was based on ELISA of root and leaf samples from all the plants under each age group at 25 DPI. The design of experiment was a randomized complete block consisting of three replications and five different ages of plant with 10 plants in each replication. Data on local and systemic infection, and TSWV titer was

analyzed by analysis of variance using SAS. Area under disease progress curve (AUDPC) was calculated using the following formula (Culbreath *et al*., 1993):

$$AUDPC = \Sigma_{i=2} \{(L_{i-1} + L_i)/2\} \{t_i - t_{i-1}\}$$

Where, t = days post-inoculation, L_i = the proportion of symptomatic plants on dates i = 2,3,4.

AUDPC was analyzed using Proc MIXED in SAS. Preliminary analysis of the disease incidence showed interaction between age of plants at the time of inoculation and date of observation as DPI. The following linear model was used to show the interaction:

$$Y = a + bx\text{Age} + c \times \text{Age} \times \text{Age} + d \times \text{DPI} + e \times \text{DPI} \times \text{DPI} + f \times \text{Age} \times \text{DPI}$$

Where Y = percent symptomatic plants; a, b, ...,f = coefficient determined from the regression; age is expressed as actual age at the time of inoculation -75; and DPI expressed as actual DPI-17.5 (Draper & Smith, 1981).

Systemic distribution of symptoms and TSWV in tobacco

The inoculated plants under different age group mentioned in the previous section were used to study the *in planta* distribution of symptoms and TSWV. The distribution of systemic symptoms in the foliage of all the inoculated plants under each age group was recorded based on visual observation at 25 DPI. To determine the distribution of TSWV in plants in each symptom category, four to five plants were selected for ELISA. A plant had a minimum of six and a maximum of 15 leaves and all the leaves of each plant were tested for the presence of TSWV. To determine the

distribution of TSWV in roots, three root samples were taken from each selected plant, namely upper roots from one side of a plant, upper roots from the opposite side of the same plant, and roots from the bottom portion of the taproot. Altogether, 217 samples were analyzed by ELISA to determine the distribution of TSWV in plants.

Results

Leaf position and TSWV susceptibility

Inoculation of all the leaves of 40 DAS plants with TSWV resulted, on an average, in 23.7 local lesions on the leaf-2, which was a newly expanded leaf at the time of inoculation. However, leaf-1 and -3, which were the top emerging leaves and the lower most leaf, respectively at the time of inoculation produced 1.3 and 5.9 lesions, respectively (Fig. 1A). In 80 DAS plants, 0.4 to 2.0 local lesions developed on the leaves-1 and -2, which were the top emerging and partially expanding leaves at the time of inoculation, respectively; and 0.2 to 2.4 local lesions developed on the leaf-5 and -6, which were fully expanded but older leaves at the time of inoculation. Leaves-3 and -4, which were fully expanded youngest leaves at the time of inoculation, had 10.8 and 5.8 local lesions, respectively (Fig.1B). The results showed that expanded young leaves irrespective of plant age produced significantly higher number of local lesions compared to any other leaves.

Age of plant and TSWV susceptibility

A large number of local lesions (ranging from 38.8 to 68.1 per plant) developed following mechanical inoculation of two newly expanded leaves of the plants in the age groups 40 to 75 DAS (Table 1). By 10 to 15 DPI, lesions coalesced and lamina

withered in plants under age group 40 to 60 DAS. Plants in the age group 75 DAS produced lesions with dark brown necrotic zones around the blighted center and the lamina became chlorotic. Plants in the age groups 95 to 100 DAS produced scattered necrotic lesions with chlorotic tissues surrounding the lesions. Later, necrotic dots developed in the chlorotic tissue surrounding the lesion. All the plants in age groups 40, 60, and 75 DAS developed local infections as determined by ELISA, whereas only 76.7 to 86.7% plants in the age group 95 to 100 DAS developed local infections (Table 1). ELISA readings of the inoculated leaves of 95 to 100 DAS plants was lower (A_{405} values, 2.36 to 2.55) than those of 40 and 75 DAS plants (A_{405} values, 3.49 to 3.94).

Systemic infection was the highest among the plants of the youngest age 40 DAS, where TSWV was detected in leaf and root samples of 93.3% and 100% plants, respectively. Plants inoculated 60 to 75 DAS had a lower percentage of systemic infection (23.3 to 32.8% plant) compared to the plants of the youngest age (40 DAS). Percent systemic infection further declined in the age groups 95 to 100 DAS, where TSWV was detected in leaves and roots of only 3.3% and 10 to 16.7% plants, respectively. Statistical analysis of data showed that there was a significant effect of plant age on the progression of systemic symptoms (Fig. 2) and there was an interaction between age and time (days) post inoculation (Fig.3). The AUDPC values for the 40 DAS plants were 350 to 450, whereas the values for the 60 to 100 DAS plants were 0 to 91.6 at all DPI. This showed that the 40 DAS plants were most susceptible compared to plants of all the other age groups. The disease progression in older age groups was similar and slower. The rate of increase in disease incidence was

similar among the plants in age groups 40, 60 and 75 DAS; and was different in the plants under age groups 95 and 100 DAS (Fig. 3). The apparent rate of change (slope) in disease incidence, calculated by differentiation of the regression equation, was -3.284 for 10 DPI and -3.692 for 25 DPI. The rate of change of disease incidence decreased 0.38% for every five-day increase in plant age ($P<0.01$). The apparent age at which the minimum disease incidence occurred at 10 to 25 DPI was about 83 to 88 days after seeding.

Systemic distribution of symptoms and TSWV in tobacco

At 25 DPI, the distribution of systemic symptoms in the plants inoculated at different ages were observed to be of four major categories: (i) 76.6% of 40 DAS plants with systemic symptoms in all the leaves and plants became severely stunted (Fig. 4A); (ii) 13.3% of 40 DAS plants, which had systemic symptoms in all the lower leaves and developed new growth of 4 to 5 asymptomatic leaves (Fig. 4B); (iii) 10.8 % of 60 to 100 DAS plants with systemic symptoms several leaves (5 to 7 leaves) away from the inoculated leaves. Symptomatic leaves were often observed to be positioned on one side of the plant and the emerging and expanding leaves were either asymptomatic or symptomatic (Fig. 4 C); and (iv) 89.2% of 60 to 100 DAS plants with no systemic symptoms in any leaves (Fig. 4 D).

The distribution of TSWV as detected by ELISA was seen in all leaves that were symptomatic (Fig.4). In 60 to 100 DAS plants, TSWV was not detected in the asymptomatic leaves located between the symptomatic and inoculated leaves. However, TSWV was detected in the upper asymptomatic leaves adjacent to the

symptomatic leaves. In the asymptomatic plants, TSWV was not detected in any non-inoculated leaves with the exception of two plants out of a total of five tested, where only the asymptomatic leaf closest to the inoculated leaf showed weak positive ELISA value of 0.1 and 0.3 compared to the positive control value of 2.3 to 3.0. In 40 DAS plants, those showed recovery of symptoms in the newly developed leaves contained TSWV in all the symptomatic and asymptomatic leaves (Fig. 4B) In these plants, the ELISA readings were lower in the asymptomatic leaves (0.1 to 0.5) compared to those in symptomatic leaves (2.3 to 3.7). The root system of all symptomatic plants, and 6.7 to 23.3% asymptomatic plants in the different age groups were positive by ELISA.

Discussion

TSWV infection in flue-cured tobacco has two distinct phases of symptom expression: (i) the local phase when necrotic lesions were produced on the inoculated leaves and (ii) the systemic phase when necrosis develops on the foliage distal to the site of inoculation. This study demonstrated that the susceptibility of flue-cured tobacco to TSWV differed based on both the maturity of the leaf and the age of the plant, and mature plants are tolerant to systemic development of spotted wilt disease. Our results showed that in 40 DAS plants, fully expanded youngest leaf at the time of inoculation was most susceptible to local infection compared to the small emerging and the older leaves. This observation was also valid in 80 DAS plants, where fully expanded young leaves positioned in the upper middle portion of the plant were most susceptible to the formation of local lesions. The emerging leaves, irrespective of the

age of the plant, were less susceptible to mechanical inoculation with TSWV. Similar results were described in 'Samsun NN' tobacco against *Tobacco mosaic virus* (TMV), where small young and older leaves were less susceptible for formation of local lesions compared to the well-expanded middle leaves (Takahashi, 1972). Based on this study, Takahashi (1972) concluded that the susceptibility for local lesion formation was strictly based on the leaf age at the time of inoculation.

As there is variability in the susceptibility among the leaves of a plant, it is important to identify the most susceptible leaf so that it can be inoculated for true differentiation of disease progression among plants under different treatments. In the present study, inoculation of leaves at varying developmental stages resulted in the identification of the fully expanded young leaves of a flue-cured tobacco plant as the most susceptible site of inoculation of TSWV. The effect of plant age on the development of TSWV infection in flue-cured tobacco was evaluated by inoculating the well-expanded young leaves in the plants under age groups 40, 60, 75 and 100 DAS. The plant age showed no significant difference on the incidence of locally infected plants and there was high incidence (75.7 to 100%) of such plants across all the age groups. However, the severity of local symptoms was higher only in the 40 to 60 DAS plants, where the inoculated leaves wilted and withered rapidly. Therefore, plant age influenced the severity of local infection, but not the incidence of local infection.

Plant age at the time of infection had a profound effect on the development and distribution of systemic infection. In older plants (60 to 100 DAS), local infection did not always lead to the development of systemic symptoms. Expression of

systemic symptoms in the youngest plants (40 DAS) was most severe and was distributed in all the leaves. In the older plants (60 to 100 DAS), the symptoms were restricted to the upper leaves and the distribution of symptoms varied from plant to plant. The symptom distribution is a transitional phenomenon in the growing plant and is based on the time of infection and the time of observation. The various patterns of distribution of systemic symptoms were observed in the present study (Fig. 4) were based on the age of the plant at 25 DPI. The symptom pattern, category-ii, described at a time point in 40 DAS inoculated plants (Fig. 4 B) is expected to change as these groups of plants were only 65 DAS old at the time of observation and had the potentiality to grow further. The upper asymptomatic leaves in this group of plants may develop symptoms at a later time point as TSWV was detected in these asymptomatic leaves. The 60 to 100 DAS-inoculated plants attained the age of 85 to 125 DAS at the time of observation, which is the mature stage of growth period. Therefore, the two types of symptom categories described in these groups of plants (Fig. 4 C, D) are expected to be of final distribution and characteristic of symptoms expected for plants infected with TSWV at later stage of the plant growth.

Symptoms were always associated with the presence of the virus. However, the presence of virus was not always associated with symptoms in a systemically infected plant. TSWV was distributed in all the symptomatic and asymptomatic leaves when plants were infected at the youngest age (40 DAS). In the older plants (60 to 100 DAS), TSWV was distributed in the root, symptomatic leaves and in the upper asymptomatic leaves adjacent to the symptomatic leaf but not in the asymptomatic leaves positioned below from the symptomatic leaf (Fig. 4 D). The uneven

distribution of TSWV in the foliage is apparently due to dissemination of TSWV from the inoculated leaves through the section of stem without infecting the adjacent leaves. Successful systemic disease development is primarily based on the rapid movement of virus within plants through the phloem (Leisner & Turgeon, 1993). The pattern of systemic distribution of virus within the plant is influenced by transport of photoassimilates and phyllotaxis (Leisner & Turgeon, 1993). This could be the probable reason for the erratic distribution of symptoms and TSWV in the older plants.

Although, the systemic distribution of symptoms and the virus was erratic in the foliage of the plants infected at older ages, the distribution of TSWV was uniformly detected in the root system. TSWV was always detected in the roots samples collected from the three locations of root system of each plant where systemic infection established, irrespective of the plant being symptomatic or asymptomatic. Comparison of TSWV detected in root and leaf samples of plants under various age groups showed that up to 13.4% plants had TSWV only in roots but not in the foliage (Table 1). Asymptomatic plants with TSWV only in the root systems were encountered more frequently when plants were inoculated at 75 to 100 DAS. Analysis of spotted wilt progress with reference to ages of flue-cured tobacco plants showed that as plants' age increased the expression of systemic symptoms decreased. There was a very high percentage (89.2%) of older plants (60 to 100 DAS) without any systemic symptoms. This showed mature flue-cured tobacco plants has resistance to systemic development of spotted wilt. Resistance in mature plants has been attributed to restriction of long distance movement of virus within plants

(Garcia-Ruiz & Murphy, 2001; Goodrick *et al*., 1991; Nono-Womdun *et al*., 1991). This study showed that TSWV movement in flue-cured tobacco was restricted to the inoculated leaves or the roots in asymptomatic older plants. The incidence of TSWV in flue-cured tobacco is often determined based on ELISA of leaf samples (Csinos *et al*., 2001; Pappu *et al*., 2000). In the case of asymptomatic plants, it is difficult to select the leaf sample for ELISA since the virus may not be present in the foliage. Root samples may be the best sample for estimation of TSWV incidence as TSWV was frequently detected in the roots when it was not detected in leaves. The value of testing roots would be better understood if the effect of root infection on yield losses in asymptomatic plants was known. In a situation where it is not possible or practical to collect root samples, leaf samples should be collected from multiple locations of the upper foliage of plants, as both symptoms and TSWV are distributed in the upper foliage during the development of systemic infection.

The pattern of susceptibility of flue-cured tobacco to TSWV using thrips-mediated inoculation was not investigated. The basic difference between inoculation by thrips and sap would be in the amount of virus in the inoculum. The amount of virus used during the inoculation likely will have little impact on the development of systemic symptoms since this study showed that in spite of a high level of initiating local infections, the final incidence of systemic symptoms was low in the older plants. The pattern of infection, systemic invasion and symptom expression that were observed may be affected if other isolates with greater or lesser virulence were used. From a management perspective, the mere presence of TSWV in plant or local lesions is not of major concern, but it is the systemic foliar symptoms that finally cause the

most economic damage in tobacco. Transplanting flue-cured tobacco at the time of emergence of viruliferous thrips is expected to result in high disease incidence. In peanut, planting dates that avoid synchronization of young peanut plants with peak thrips populations appear to significantly reduce TSWV infection levels (Brown *et al*., 2005).

Delay of infection to post flowering or fruit setting has been shown to reduce the impact of virus diseases on yield of several crops (Agrios *et al*., 1985; Avilla et al., 1997; Blua & Perring, 1998; Rosenkranz & Scott, 1978). In the case of TSWV, Moriones et al., (1998) assessed the impact of natural infection of TSWV on tomato yield at various times after transplanting in the field. The yield of tomato was dramatically affected in plants expressing symptoms at the early developmental stage (up to 45 days post transplanting) than that was at a more mature stage (more than 60 days post transplanting). However, the quality of the marketable fruit was affected by TSWV infection irrespective of ages of symptoms expression. Wilson (2001) reported significant effect of plant age at inoculation with TSWV on foliar and tuber infection in potato. Inoculation after 38 days of emergence failed to produce tuber infection.

Our previous studies showed that treatment of flue-cured tobacco plants with acibenzolar-S-methyl (ASM) could significantly reduce spotted wilt incidence under field conditions (Csinos *et al*., 2001; Mandal *et al*., 2002; Pappu *et al*., 2000). Treatment of flue-cured tobacco with ASM at early stage of plant growth (before transplanting into the field) was effective to induce resistance against TSWV (Csinos *et al*., 2001). The present study provides information on the effect of age on the susceptibility of flue-cured tobacco to TSWV and underlines the management strategy

for TSWV based on ASM-induced resistance and mature plant resistance that would protect the plant from early and late infections of TSWV, respectively.

Acknowledgments

We are thankful to Wanda Tillery for technical help, Ben Mullinix for statistical analysis, and Drs. D. Gent, S.K. Mohan and H.F. Schwartz for critical reading of the manuscript. This work was supported in part by funding from the Georgia Agricultural Commodity Commission for Tobacco, and USDA-CSREES Special Grant (99-34412-7415). B. Mandal was supported by a post-doctoral fellowship from the USDA-CSREES Special Grant (99-34412-7415). PPNS No. 0455, Department of Plant Pathology, College of Agricultural, Human, and Natural Resource Sciences Agricultural Research Center, Project No. WNP0 0545, Washington State University, Pullman, WA 99164-6430, USA.

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Table 1 Susceptibility of tobacco cultivar K326 at different days after seeding (DAS) to *Tomato spotted wilt virus* (TSWV), as indicated by number of local lesions, TSWV titer and incidence of local and systemic infection.

Age[a] (DAS)	ELISA absorbance[b]	Local infection		Systemic infection[c] (%plants)	
		Lesions/plant	% plants	Leaf	Root
40	3.94 x	38.8 xy	100.0 x	93.3 x	100.0 x
60	3.49 xy	68.1 x	100.0 x	23.3 yz	33.3 yz
75	3.62 x	61.0 x	100.0 x	32.8 y	43.3 y
95	2.55 yz	25.1 y	86.7 x	3.3 z	16.7 y
100	2.36 z	24.1 y	76.7 x	3.3 z	10.0 z
LSD[d]	1.04	31.7	29.2	27.0	30.9

[a] Age: days after seeding (DAS), two fully expanded youngest leaves (leaf number two and three and leaf number three and four, counted from the top of the plant, for 40 DAS and 60 to 100 DAS plants, respectively) of each plant were mechanically inoculated with TSWV.

[b] Absorbance values were determined by enzyme linked immunosorbent assay (ELISA) at 1:100 dilution of sap obtained from inoculated leaves. All the absorbance values shown were indicative of TSWV infection.

[c] Local and systemic infection based on ELISA at 7 and 25 days post inoculation, respectively.

[d] LSD calculated at $P = 0.05$. Data are mean of 10 sub samples (plants) from each of 3 replications ($n = 30$).The values marked with the same letter are not significantly different.

Figure legends

Figure 1 Susceptibility of leaves of flue-cured tobacco plants, cultivar K326 to *Tomato spotted wilt virus* in (A) plants inoculated 40 days after seeding (DAS) and (B) plants inoculated 80 DAS under greenhouse conditions. The positions of leaves are numbered from top to bottom of a plant. The bars are mean of 12 plants in each of three replicates (n = 36). The bars marked with the same letter are not significantly different at $P = 0.05$ according to least significant difference 4.28 and 2.16 for 40 and 80 DAS plants, respectively.

Figure 2 Progress of spotted wilt disease in flue-cured tobacco cultivar K326 mechanically inoculated with *Tomato spotted wilt virus* at different plant ages as measured by the area under disease progress curve (AUDPC) under greenhouse conditions. The least significant difference (LSD) between days post inoculation (DPI) for the same age = 37.4, and LSD between ages for the same DPI = 71.2 ($P = 0.05$).

Figure 3 Relationship of age of flue-cured tobacco plants cultivar K326 (days after seeding) and spotted wilt incidence following mechanical inoculation of *Tomato spotted wilt virus* under greenhouse conditions at different times of observations (days post inoculation [DPI]) based on the predicted values from the regression equation: $Y = a + b \times Age + c \times Age \times Age + d \times DPI + e \times DPI \times DPI + f \times Age \times DPI$, where Y

= percent symptomatic plants, the coefficient a = 13.2, b = -0.8203, c = 0.03811, d = 0.9194, e = -0.04667 and f = 0.02723 ($P<0.01$).

Figure 4 Diagram of flue-cured tobacco plants (cultivar K326) showing distribution of spotted wilt symptoms and *Tomato spotted wilt virus* (TSWV) at 25 days post inoculation following mechanical inoculation of the plants at 40, 60, 75,95 and 100 days after seeding (DAS) under greenhouse conditions. The lower most leaves drawn with lighter shade are the inoculated leaves, which were fully expanded young leaves at the time of inoculation. The elliptical shaded areas indicate the sites of inoculation in older plants (60 to 100 DAS). The symptomatic leaf is indicated by black fill and asymptomatic leaf is indicated by no-fill. TSWV in the leaves and three sections of roots was detected by enzyme linked immunosorbent assay (ELISA). +: indicates moderate to strong ELISA readings (A_{405} values 0.3 to 3.5), +/- : indicates weak ELISA readings (A_{405} values <0.3) and – : indicates negative ELISA reading. ELISA values for the positive control ranged from 2.3 to 3.0 and negative controls ranged from 0.008 to 0.01. Symptom and TSWV distribution patterns in plants inoculated at 40 DAS and 60 to 100 DAS are shown in A & B and C & D, respectively.

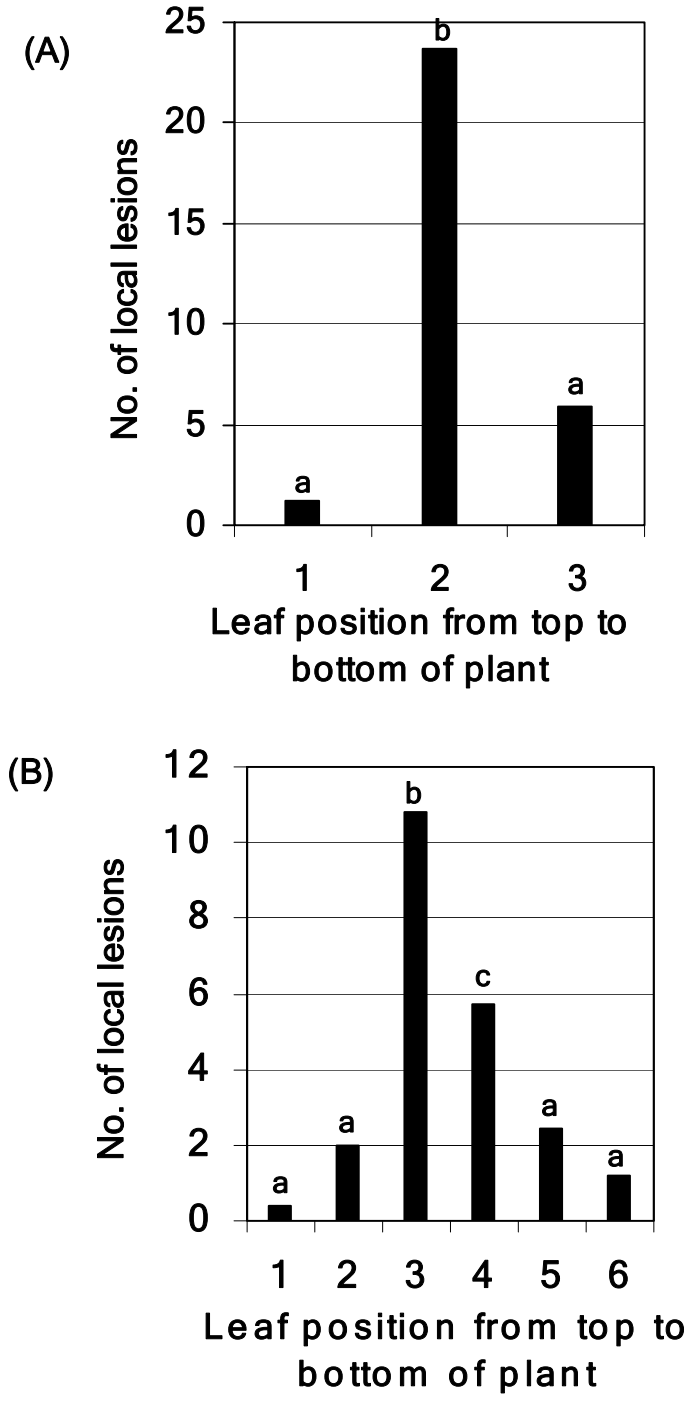
(A)
No. of local lesions
25
20
15
10
5
0
a
b
a
1
2
3
Leaf position from top to bottom of plant
(B)
No. of local lesions
12
10
8
6
4
2
0
a
a
b
c
a
a
1
2
3
4
5
6
Leaf position from top to bottom of plant

Figure 1

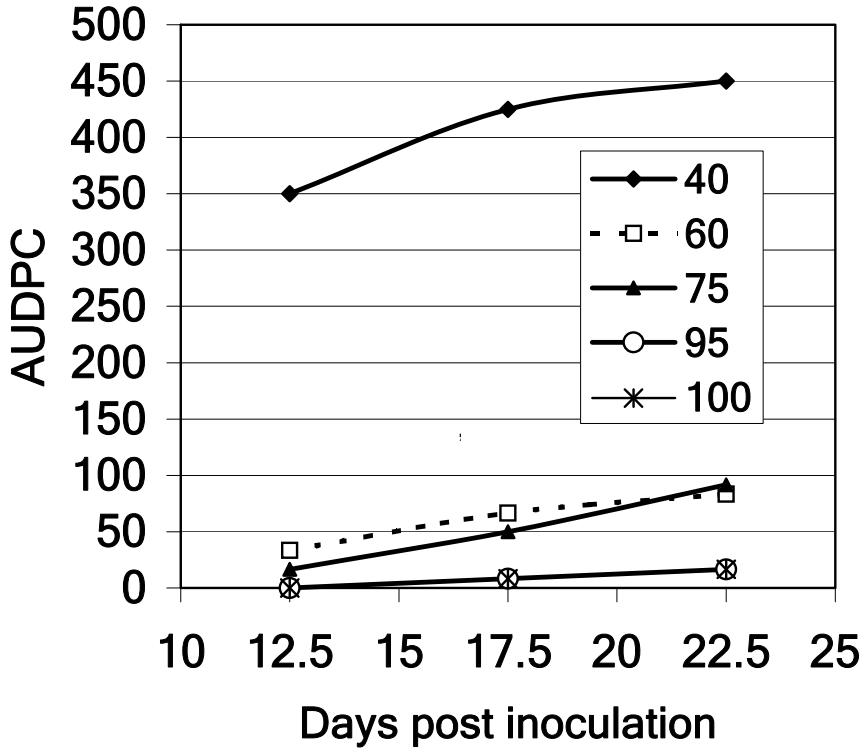
500
450
400
350
300
250
200
150
100
50
0
AUDPC
40
60
75
95
100
10
12.5
15
17.5
20
22.5
25
Days post inoculation

Figure 2

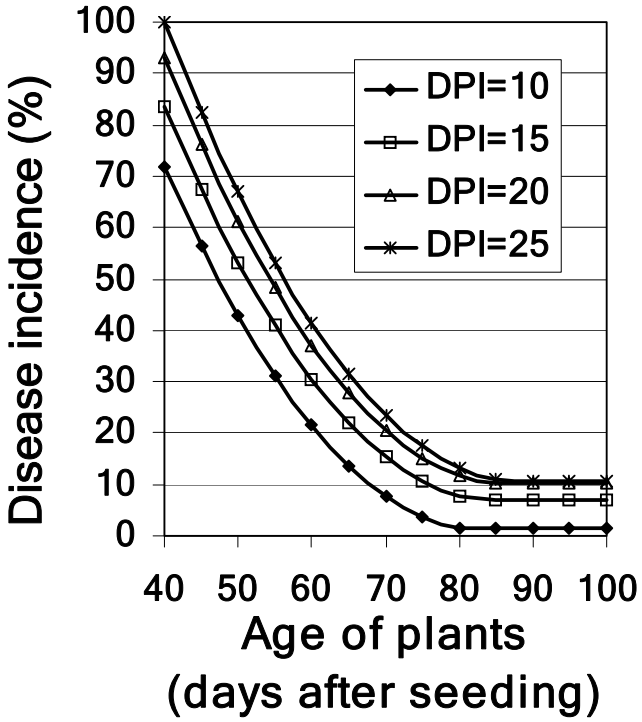
Disease incidence (%)
100
90
80
70
60
50
40
30
20
10
0
DPI=10
DPI=15
DPI=20
DPI=25
40 50 60 70 80 90 100
Age of plants
(days after seeding)

Figure 3

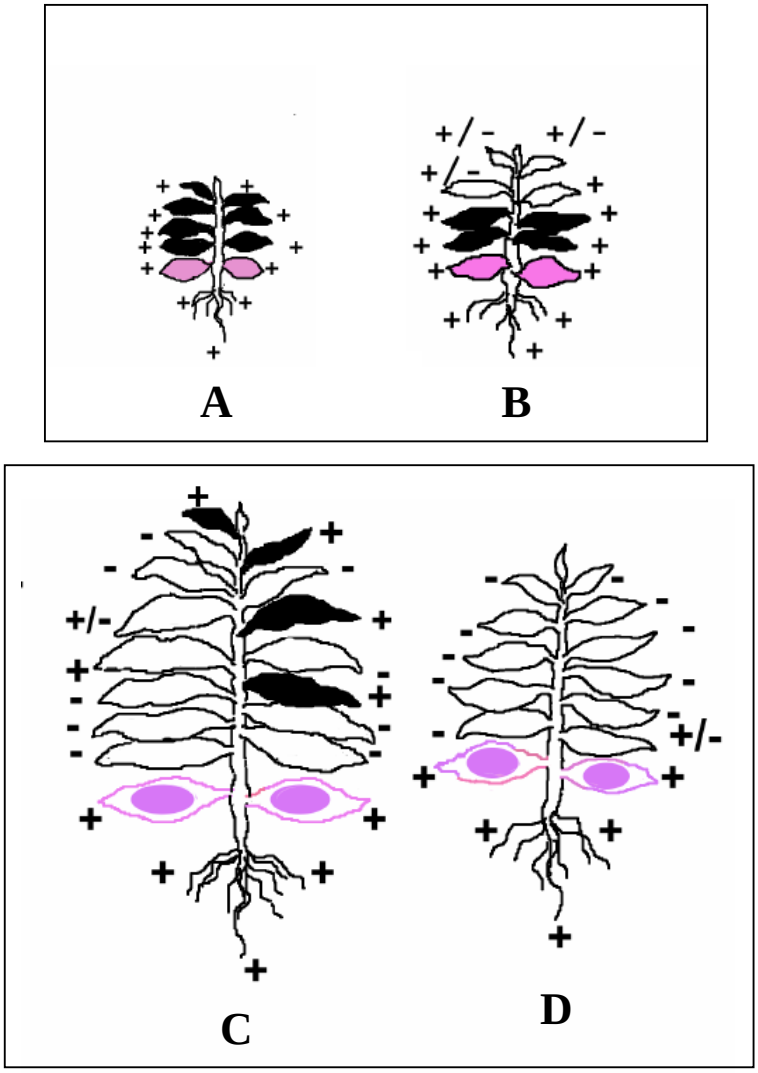

Figure 4