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Eliminating Tobacco Rattle Virus from Viruliferous *Paratrichodorus allius* and Establishing a New Virus-Vector Combination[1]

Hassan Mojtahedi,[2] G. S. Santo,[3] P. E. Thomas,[4] J. M. Crosslin,[2] and R. A. Boydston[4]

Abstract: A reliable method to eliminate tobacco rattle virus (TRV) from viruliferous *Paratrichodorus allius* populations was developed. This virus is vectored by *P. allius* in the Pacific Northwest and causes corky ringspot disease (CRS) of potato. The viruliferous nematodes that were reared on 'Vernema' alfalfa or '770' scotch spearmint for at least 3 months did not transmit TRV to 'Samsun NN' tobacco, a suitable indicator plant, and did not cause CRS symptoms on 'Russet Norkotah' tubers. A new isolate of TRV was introduced into a nonviruliferous population of *P. allius*. First, tobacco plants were inoculated with a field population of *P. allius* that transmitted an isolate of TRV that caused severe symptoms on potato. The tobacco roots were then washed free from soil and dipped in 0.525% sodium hypochlorite to remove the initial nematode inoculum. After the disinfected tobacco plants recovered and began to grow, the virus-free population of *P. allius* was introduced around the root system to acquire the new virus isolate from tobacco roots. The newly established virus-vector combination caused CRS symptoms on 'Russet Norkotah' that were characteristic of the more virulent virus isolate, indicating that the virus-free *P. allius* population had reacquired virus.

Key words: alfalfa, corky ringspot disease, potato, scotch spearmint, tobacco, tobacco rattle virus.

Corky ringspot disease (CRS) is a major disease of potato (*Solanum tuberosum* L.) in the Pacific Northwest (Thomas et al., 1993). It is caused by tobacco rattle virus (TRV) (Robinson and Harrison, 1989), which is transmitted by the nematode *Paratrichodorus allius* (Jensen) Siddiqi in Washington, Oregon, and Idaho (Mojtahedi et al., 2000). Symptoms of CRS in potato are characterized by necrotic arcs, concentric rings, or diffuse extensive browning of tuber flesh that later dries into cork-like tissue. The blemished tubers are considered unmarketable and, thus, are culled. Currently, costly soil fumigation with 1,3-dichloropropene is the only tool available to growers for control of CRS in potato (Santo et al., 1997). However, extensive breeding programs (Brown et al., 2000; Crosslin et al., 1999) are under way to develop resistant and(or) tolerant (= symptomless) cultivars that can be used to alleviate CRS and reduce production costs.

To study the epidemiology of the disease, the pathogenic potential of TRV isolates, and the efficiency of different vector populations to transmit TRV, we have eliminated naturally associated virus from vectors and (or) introduced a new virus isolate into virus-free vector populations. Like other plant-parasitic nematodes, *P. allius* molts several times during its life cycle and sheds the virus with each molt (Robinson and Harrison, 1989). Thus, if young nematodes are denied access to a virus source, the population will cleanse itself of virus after several generations. The key to success of this process is to identify a plant that serves as a suitable host for *P. allius* without being a host for TRV. Jensen et al. (1974) indicated that peppermint and onion were not infected with TRV in fields that were infested with viruliferous *P. allius*. Rowe (1993) suggested that incidence of CRS on potato is reduced if it follows alfalfa, although no specific data were presented. The latter observations implied that alfalfa did not serve as host for the virus and(or) vector; therefore, the inoculum potential was reduced in alfalfa fields.

We have studied the host status of numerous crops for *P. allius* (Mojtahedi and Santo, 1999) and TRV (Crosslin, unpubl.). Alfalfa, peppermint, and spearmint were particularly intriguing because of their indeterminant growth and because they can be trimmed and kept in the greenhouse as long-term maintenance hosts of *P. allius*. The steps that were taken to produce a virus-free population of *P. allius* and also the procedure used to introduce TRV to a nonviruliferous population of the nematode are described in this study.

Materials and Methods

Two populations of *P. allius* were obtained from fields known to have a high incidence of CRS in Pasco, Washington, and Umatilla, Oregon, and increased on 'Samsun NN' tobacco (*Nicotiana tabacum* L.). Tobacco is a suitable host for the nematode and shows typical foliar symptoms after infection with TRV (Mojtahedi and Santo, 1999). The CRS symptoms were more severe at the Pasco site than in the Umatilla field across 25 potato cultivars, breeding lines, and clones tested over a 2-year period (Brown et al., 2000). Nematodes were extracted using sieving and sugar-centrifugal flotation (Jenkins, 1964). The identity of the virus in tobacco and other plant root tissue was confirmed by enzyme-linked immunosorbent assay (ELISA) and(or) reverse transcription-polymerase chain reaction (RT-PCR), as previously described (Crosslin and Thomas, 1995; Crosslin et al., 1999).

Alfalfa (*Medicago sativa* L.) was grown from seeds, and peppermint (*Mentha piperita* L.) and scotch spearmint (*Mentha cardiaca* Baker) plants were grown from cuttings 3 weeks before transplanting into a loamy sand

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[1] PPNS Paper 0320, Department of Plant Pathology, College of Agriculture and Home Economics Research Center, Project No. 0650, Washington State University, Pullman, WA 99164-6430.

[2] Research Associates and [3]Professor, Irrigated Agriculture Research and Extension Center, 24106 Bunn Rd., Prosser, WA 99350-8694.

[4] Research Plant Pathologist and Plant Physiologist, respectively, USDA-ARS, Prosser, WA 99350.

E-mail: mojtahed@tricity.wsu.edu

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soil (83.1% sand, 15% silt, and 1.9% clay), which was previously fumigated with methyl bromide (0.3 kg/m^3). A total of 100 nematodes of mixed stages were pipeted to five replicate pots of each test plant species. Tobacco plants served as positive controls. Pots were fertilized with 5 g Osmocote slow-release fertilizer (N = 14, P = 14, K = 14% from Scott-Sierra Horticultural Products Co., Marysville, OH). The pots were arranged in randomized complete blocks on greenhouse benches (20–28 °C) and maintained at high moisture levels to increase the *P. allius* population (Mojtahedi and Santo, 1999).

In the first experiment, the suitability of tobacco, alfalfa, peppermint, and scotch spearmint as hosts for *P. allius* from the Pasco field was determined in 15-cm-diam. clay pots containing 1,500 cm^3 soil. The nematodes were extracted from the entire pot 55 days after inoculation, and the reproductive factor ($RF = P_f \div P_i$) (O ostenbrink, 1966) was determined. The experiment was performed twice. Differences in nematode reproduction among the test plants were determined by analysis of variance followed by Duncan's multiple-range test.

In the second experiment, the seedlings were transplanted into 4-liter plastic pots and inoculated with viruliferous *P. allius* from Pasco. On a monthly basis for 5 months, 250-cm^3 soil samples were obtained by sampling each pot with a soil tube. Care was taken in sampling and extraction to avoid cross contamination by dipping the equipment in 5.25% sodium hypochlorite. The nematodes were extracted from soil samples, counted, and added to 1-month-old tobacco seedlings. After 1 month, nematodes on tobacco were counted and roots were tested for TRV using ELISA and (or) RT-PCR.

Because there was evidence that the Pasco isolate of TRV caused more severe symptoms than the Umatilla isolate (Brown et al., 2000; Mojtahedi et al., 2001), differences in virus isolates and nematode vectors from different locations were examined. Roots of five symptomatic tobacco plants infected with the Pasco or Umatilla isolates of TRV were rigorously washed free from soil and dipped in 0.525% sodium hypochlorite for 5 minutes before rinsing in running tap water for 10 minutes. Plants were repotted and allowed to recover for 2 weeks. To obtain new virus-vector combinations, a virus-free Pasco population of *P. allius* (reared on alfalfa for 3 months) was extracted and added to tobacco plants infected with the Umatilla isolate of TRV. Experiments with the Umatilla *P. allius* population and Pasco TRV isolate were conducted similarly. A set of bleached tobacco plants were kept uninoculated to ascertain that they were truly free of nematodes after bleaching and washing procedures. The number of nematodes in soil was determined at the end of each experiment. Mechanical inoculation of tobacco foliage, commonly done with other viruses to establish a virus-infected plant (Matthews, 1991), was avoided with TRV because it may lead to loss of nematode transmissiblity (Hernandez et al., 1996).

In the third experiment, six nematode populations with or without virus were tested on 'Russet Norkotah' potato to compare CRS severity caused by new virus-vector combinations. Potato seedlings were grown from certified seed pieces and transplanted into half-filled 4-liter plastic pots before 100 *P. allius* in 5 ml water were pipeted onto the root system. Soil was added as plants grew to keep tubers subterraneous and exposed to nematodes. The tubers were collected from the pots 100 days after inoculation and evaluated for CRS symptoms (Mojtahedi and Santo, 1999). Soil in each pot was thoroughly mixed, and a 250-cm^3 sample was taken for nematode extraction.

Results and Discussion

Paratrichodorus allius reproduced (RF > 1) on the alfalfa, scotch spearmit, and two peppermint cultivars as well as the 'Samsun NN' tobacco plants, indicating that all test plants were suitable hosts for the nematode (Table 1). No foliar symptoms developed on any of the test plants except tobacco. The symptoms on tobacco were similar to those described previously (Mojtahedi and Santo, 1999). Tobacco rattle virus was detected by RT-PCR in root tissue of 'Vernema' alfalfa and 'Black Micham' peppermint. The virus was not detected in root tissue of 'Murry Micham' peppermint and '770' scotch spearmint 55 days after exposure to viruliferous *P. allius*.

Viruliferous *P. allius* populations that reproduced ef-

Table 1. Reproductive factor (RF) of *Paratrichodorus allius* on alfalfa and mint cultivars, detection of tobacco rattle virus (TRV) in their root system, and ability of extracted nematodes to transmit TRV to tobacco seedlings.[a]

Host plants	RF[b]	Detection of TRV in root system by		Transmission of TRV to tobacco by nematodes extracted from different plants[c]
		ELISA	RT-PCR	
Scotch spearmint '770'	34.4 a[d]	–[e]	–	–
Peppermint 'Murray Mitcham'	15.3 a	–	–	–
Peppermint 'Black Mitcham'	23.4 a	–	+	+
Alfalfa 'Vernema'	21.5 a	–	+	–
Tobacco 'Samsun NN'	41.0 a	+	+	+

[a] Nematodes were collected from a corky ringspot field in Pasco, Washington, and increased on 'Samsun NN' tobacco. Each host plant was infested with 100 nematodes, and the experiment terminated 55 days later. Nematodes were extracted from pots and added to 'Samsun NN' tobacco.

[b] $RF = P_f \div P_i$.

[c] Transmission was determined by appearance of foliar symptoms on tobacco plants typical of TRV.

[d] RF values are means of 10 replications, and those followed by the same letter are not different at $P > 0.05$ according to Duncan's multiple-range test.

[e] + and – represent the presence and absence of TRV, respectively.

ficiently on 'Vernema' alfalfa and '770' scotch spearmint lost their ability to transmit TRV to tobacco within 3 months (Table 2). In contrast, the populations reared on tobacco remained viruliferous even after 5 months (Table 2). Although TRV was detected in two alfalfa root systems by RT-PCR, *P. allius* did not acquire the virus from these infected plants. This could be due to either low concentration of virus or the presence of an NM-type of TRV. This type of infection, in which only RNA1 of TRV is present, is probably not transmitted by nematodes (Robinson and Harrison, 1989).

The bleached tobacco plants wilted after transplanting, and the larger leaves became necrotic and eventually dropped. However, within 2 to 3 weeks, new leaves developed and normal growth resumed. No nematodes were recovered from the soil around these plants 3 months after incubation, indicating that the washing and bleaching procedures successfully eliminated *P. allius*.

The Pasco and Umatilla populations of nematodes obtained from alfalfa reproduced successfully on potato without causing any CRS symptoms on tubers (Table 3). This confirms the previous observations that *P. allius* loses TRV if maintained on alfalfa for several generations. As observed in the field (Brown et al., 2000) and greenhouse tests (Mojtahedi et al., 2001), the virus from Pasco caused more severe symptoms on tubers than the Umatilla isolate (Table 3). Similarly, Umatilla nematodes raised on tobacco infected with the Pasco isolate of the virus caused more severe symptoms on tubers than the Pasco nematode raised on tobacco with the Umatilla virus. These data demonstrate that different isolates of TRV can be introduced into a *P. allius* population and that CRS severity on potato is determined by the virus isolate and not the nematode population. This conclusion supports the view of Brown et al. (2000) that CRS resistance is based on the reaction of potato genotypes to the virus and not the vector.

Our data clearly indicate that alfalfa and scotch spearmint can be used to eliminate TRV from *P. allius* populations. In more than 5 years of greenhouse testing, *P. allius* reared on 'Vernema' alfalfa for more than 3 months has never transmitted TRV to tobacco or potato. Interestingly, greenhouse trials revealed the presence of RT-PCR-detectable TRV in the roots of seven of eight cultivars grown in soil infested with viruliferous *P. allius* (Crosslin, unpubl.). Yet, by planting four of those alfalfa cultivars in heavily infested Pasco field, the impact of CRS on potato was reduced after 1 year (Thomas et al., 1999).

In the current experiment, TRV was detected in, and transmitted from, roots of 'Black Mitcham' peppermint but not from 'Murray Mitcham'. This may indicate a cultivar response for TRV infection similar to that reported for potato (Brown et al., 2000).

Complete elimination of CRS from an alfalfa or scotch spearmint may depend on management of weeds that are hosts for TRV and the nematode (Cooper and Harrison, 1973). Currently, we are testing the host status of common weeds found in alfalfa and spearmint fields for TRV and *P. allius*.

TABLE 2. Ability of *Paratrichodorus allius* populations extracted from different sources to transmit tobacco rattle virus (TRV) to ''Samsun NN' tobacco.[a]

	P. allius raised on and extracted from					
	Tobacco 'Samsun NN'		Alfalfa 'Vernema'		Scotch spearmint '770'	
Extracted after: (months)	*P. allius*/250 cm^3 soil	TRV in tobacco	*P allius*/250 cm^3 soil	TRV in tobacco	*P. allius*/250 cm^3 soil	TRV in tobacco
1	21 ± 7	+[b]	112 ± 60	+	10 ± 3	+
2	172 ± 63	+	206 ± 75	+	80 ± 18	–
3	300 ± 140	+	1837 ± 250	–	165 ± 60	–
4	448 ± 225	+	862 ± 220	–	240 ± 100	–
5	310 ± 120	+	410 ± 115	–	350 ± 135	–

[a] Nematodes were collected from a corky ringspot field in Pasco, Washington, and increased on 'Samsun NN' tobacco. Each source plant was initially inoculated with 100 *P. allius* and replicated five times. The nematodes were extracted and added to tobacco to transmit TRV. The transmission was determined 1 month after inoculation by observing foliar symptoms and by testing tobacco root samples using ELISA and(or) RT-PCR. A test was recorded positive if at least one of five replicates showed foliar symptoms or if TRV was detected in its root system.

[b] + and – represent the presence and absence of TRV, respectively.

TABLE 3. Transmission of two tobacco rattle virus (TRV) isolates by two *Paratrichodorus allius* populations to 'Russet Norkotah' potato and subsequent development of corky ringspot disease in tubers.

Source of viruliferous *P. allius*[a]	Host sequence prior to potato	*P. allius*/250 cm^3 at potato harvest	Corky ringspot disease[b]	
			Severity (0–8)[c]	Incidence (%)
Pasco	TRV-free alfalfa	100 ± 35	0	0
Pasco	Tobacco infected with Pasco TRV	80 ± 27	4.0 ± 1.2	45.0 ± 15.3
Pasco	Alfalfa, then tobacco infected with Umatilla TRV	120 ± 44	1.6 ± 0.8	12.5 ± 2.1
Umatilla	TRV-free alfalfa	85 ± 43	0	0
Umatilla	Tobacco infected with Umatilla TRV	160 ± 85	1.2 ± 0.9	14.0 ± 3.1
Umatilla	Alfalfa, then tobacco infected with Pasco TRV	20 ± 7	4.4 ± 1.5	63.8 ± 21.9

[a] Nematodes were collected from fields with a history of CRS in Pasco, Washington, and Umatilla, Oregon, and increased on 'Samsun NN' tobacco.

[b] Potato plants were grown in 4-liter plastic pots and were infested with 100 *P. allius* vectoring different TRV isolates. Tubers were harvested 100 days after the experiment was initiated.

[c] Tubers were cut into four wedges, and the cut surfaces were evaluated for CRS symptoms on a scale of 0 to 8 where 0 = no symptoms and 8 = all surfaces blemished.

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