Stolbur Phytoplasma Transmission to Maize by *Reptalus panzeri* and the Disease Cycle of Maize Redness in Serbia

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ABSTRACT

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Maize redness (MR), induced by stolbur phytoplasma ('*Candidatus* Phytoplasma solani', subgroup 16SrXII-A), is characterized by midrib, leaf, and stalk reddening and abnormal ear development. MR has been reported from Serbia, Romania, and Bulgaria for 50 years, and recent epiphytotics reduced yields by 40 to 90% in South Banat District, Serbia. Potential vectors including leafhoppers and planthoppers in the order Hemiptera, suborder Auchenorrhyncha, were surveyed in MR-affected and low-MR-incidence fields, and 33 different species were identified. Only *Reptalus panzeri* populations displayed characteristics of a major MR vector. More *R. panzeri* individuals were present in MR-affected versus low-MR fields, higher populations were observed in maize plots than in field border areas, and peak population levels preceded the appearance of MR in late July. Stolbur phytoplasma was detected in 17% of *R. panzeri* adults using nested polymerase chain reaction but not in any other insects tested. Higher populations of *R. panzeri* nymphs were found on maize, Johnsongrass (*Sorghum halepense*), and wheat (*Triticum aestivum*) roots. Stolbur phytoplasma was detected in roots of these three plant species, as well as in *R. panzeri* L_3 and L_5 nymphs. When stolbur phytoplasma-infected *R. panzeri* L_3 nymphs were introduced into insect-free mesh cages containing healthy maize and wheat plants, 89 and 7%, respectively, became infected. These results suggest that the MR disease cycle in South Banat involves mid-July transmission of stolbur phytoplasma to maize by infected adult *R. panzeri*. The adult *R. panzeri* lay eggs on infected maize roots, and nymphs living on these roots acquire the phytoplasma from infected maize. The nymphs overwinter on the roots of wheat planted into maize fields in the autumn, allowing emergence of phytoplasma-infected vectors the following July.

Additional keywords: epidemiological cycle, hemipteran vectors, *Mollicutes*, *Zea mays*.

In 2002 and 2003, maize redness (MR) or corn reddening was linked to 10 to 90% yield reduction in maize (*Zea mays* L.) in and around the South Banat District of Serbia (5). The disease has been reported periodically in the Banat regions of Serbia, Romania, and Bulgaria over the past 50 years (33), but the incidence and intensity of the disease has increased over the past several years. Symptoms of the disease begin to appear in late July and continue to intensify through the beginning of September. Midrib reddening is the first symptom to appear, followed by reddening of leaves and stalks and then whole-plant desiccation. MR is also associated with abnormal ear development and reduced seed numbers, leading to yield reduction. Environmental factors play a role in both the intensity and incidence of the disease, with more severe disease being associated with early-planted fields and hot, dry summers.

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figure 3 appears in color online.

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Abiotic factors, including soil fertility, and biotic factors, including *Fusarium* spp. and fastidious bacteria, were proposed as causal agents of MR (5,33). In 2006, stolbur phytoplasma (subgroup 16SrXII-A, '*Candidatus* Phytoplasma solani') was shown to be associated with MR in maize plants collected from South Banat (13). The 16S rDNA sequence of the MR phytoplasma is most closely related to a stolbur phytoplasma isolated from peppers (*Capsicum annuum* L.) in Serbia. Jović and coworkers (20) showed that stolbur phytoplasma was detected in 85% of symptomatic maize plants. The cixiid *Reptalus panzeri* was shown to be a vector of MR in Serbia. Large populations of *R. panzeri* were present in MR-affected fields and 20% of these insects were positive for the phytoplasma. Stolbur phytoplasma-infected *R. panzeri* transmitted the phytoplasma to healthy maize, producing midrib and leaf reddening.

Phytoplasmas are fastidious prokaryotic, phloem-limited plant pathogens that are members of the class *Mollicutes* (22). These pathogens cannot be cultured and must be transmitted to plant hosts by insects, grafting, or parasitic plants (39). Members of the stolbur phytoplasma group can infect a number of dicotyledonous crop hosts, including tomato (*Lycopersicon esculentum* L.), potato (*Solanum tuberosum* L.), pepper (*C. annuum* L.), celery (*Apium graveolens* L.), strawberry (*Fragaria* spp.), carrot (*Daucas carota* L.), parsley (*Petroselinum crispum* L.), grape (*Vitis vinifera* L.), sugar beet (*Beta vulgaris* L.), and rape (*Brassica rapa* L.) (14,38). Weedy hosts of stolbur phytoplasma include several solanaceous

plants (e.g., *S. nigrum*), bindweed (*Convolvus arvensis*), periwinkle (*Catharanthus roseus*), nettle (*Urtica dioica*), jimsonweed (*Datura stramonium*), dandelion (*Taraxacum officinale*), bladder campion (*Silene vulgaris*), and coltsfoot (*Tussilago farfara*) (14,21,31,38).

Phytoplasmas are transmitted by leafhoppers, planthoppers, and psyllids belonging to the suborders Auchenorrhyncha and Stenorrhyncha, order Hemiptera. The pathogens are transmitted in a persistent, replicative manner. Thus, insects are also hosts of phytoplasmas. Stolbur phytoplasma can be transmitted by the cixiid planthoppers *Hyalesthes obsoletus, Pentastiridius beieri*, and *R. panzeri* (14,15,20,32) and the leafhoppers *Macrosteles quadripunctulatus* and *Anaceratagallia ribauti* (3,30). Stolbur phytoplasma has also been detected in several other cixiid and leafhopper species (4,14,28,37). *H. obsoletus* is important for the spread of 'bois noir' caused by stolbur phytoplasma in grapevines (9,18), while *R. panzeri* is implicated in the spread of MR in maize (20).

R. panzeri was not considered to be of agronomic importance before the discovery that it transmits stolbur phytoplasma to maize. Little is known about the life cycle of *R. panzeri* and it is unclear why populations of the insect have increased in Serbia in recent years (19,20). Polyphagous adults occur from mid-June to the beginning of August in England, south-central and southeastern Europe, the Mediterranean region, Asia Minor, and the Caucasus region (17). The insect is generally found on shrubs and herbaceous plants in hot and dry areas, especially sunny hillsides or on plateaus. Although not previously described for *R. panzeri*, some cixiid species are known to lay their eggs on the roots of various grasses, with the nymphs overwintering on these species (17). Newly emerged adults crawl up to the surface of the soil in search of new plant hosts.

Development of an understanding of the etiology and epidemiology of MR requires knowledge about the disease cycle, including identification of major insect vectors and reservoir hosts. In addition, it is important to characterize the life cycle of *R. panzeri* and its interactions with maize and stolbur phytoplasma. We carried out surveys of insect species from the suborder Auchenorrhyncha and of weedy plants in and around MR-affected fields to identify other potential vectors of MR and plant reservoirs of stolbur phytoplasma. Because a maize–wheat crop rotation is common in South Banat District, interactions among maize, wheat, *R. panzeri*, and stolbur phytoplasma were characterized.

MATERIALS AND METHODS

Qualitative and quantitative analyses of potential insect vectors. A survey of insects in the suborder Auchenorrhyncha, including species in the families Cixiidae, Delphacidae, Dictyopharidae, Issidae, Cercopidae, and Cicadellidae (subfamilies Cicadellinae, Typhlocybinae, and Deltocephalinae), was carried out at five sites in 2005 and 2006. The survey sites included three MR-affected maize fields in the South Banat District of Serbia, one each in Kovaćica, Uzdin, and Samoš; and two fields with little or no MR (hereafter referred to as "low MR") near Belgrade, one each in Zemun and Dobanovci. In South Banat, the usual crop rotation is maize–wheat; therefore, the selected maize fields were adjacent to wheat fields. For each location, insects were collected every 15 days from 1 May until 1 September from five plots (10 by 10 m) spaced 5 m apart along a transect within the maize field and five plots (10 by 2 m) spaced 5 m apart along the weed-covered field border adjacent to the wheat field. For plots within maize fields, insects were collected by mouth aspirator directly from plants by two individuals for 15 min per plot. In border plots, one individual swept the entire surface of the plot with a sweep net, then collected Auchenorrhyncha species with a mouth aspirator.

Specimens were kept in vials containing 80% ethanol and subsequently identified to the species level using taxonomic keys provided by Holzinger et al. (17) and Biedermann and Niedringhaus (6). Proc GLM of SAS (SAS Institute, Cary, NC) was used for analysis of variance and means comparisons of the frequency of each identified species within plots.

Phytoplasma detection in adult insects. To identify phytoplasmas in insects, specimens were separately and randomly collected from each of the five plots in the South Banat maize fields and each of the five border plots. Specimens were stored in 80% ethanol at –20°C prior to DNA extraction from individual insects using a modified cetyltrimethylammonium bromide method (15). Phytoplasmas were detected in insect DNA samples by nested polymerase chain reaction (PCR) amplification of ribosomal DNA using the universal phytoplasma primer pairs P1/P7 and F2n/R2 followed by digestion with *Tru1I* (23).

***R. panzeri* nymph populations on plants in MR-affected fields in South Banat.** *R. panzeri* nymph populations on the roots of maize, wheat, *Solanum nigrum*, *D. stramonium*, *Convolvulus arvensis*, *Sorghum halepense*, and *Setaria viridis* were assessed. Nymphs were collected from all species except wheat in fields at Samoš and Uzdin on 15 October 2006 and 2007. Wheat samples were collected from the same fields on 15 May 2007. Thirty maize plants were collected along a transect from the corner of each field. Thirty plants of each weed species were collected randomly depending on the species' distribution within the field. For each plant, a 30-by-30-by-30-cm cube of soil containing the plant's root system was dug, and nymphs were collected from the roots and soil using an aspirator and moved to a box containing maize roots for counting. For wheat, 10 30-cm^3 blocks of soil containing roots of ≈30 plants were similarly screened for the presence of nymphs. The presence of white wax along roots in the soil was used as an indication of the presence of *R. panzeri* nymphs.

Stolbur detection in *R. panzeri* nymphs. On 15 August 2006, a maize field in which ≈40% of the plants had MR symptoms was identified in Samoš. *R. panzeri* nymphs were collected from soil cubes from this field in Autumn 2006 and Spring 2007 as outlined above. The collected live nymphs were counted in the laboratory, then placed in vials containing 80% ethanol and stored at –20°C. DNA was isolated from individual nymphs using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and tested for the presence of stolbur phytoplasma using nested PCR using the species-specific primers Stol11 primers F2/R1 and F3/R2 (11).

Detection of stolbur phytoplasma in weedy plants from MR-affected field borders. Weedy plants in field border areas surrounding the three MR-affected maize fields in Kovačica, Samoš, and Uzdin were collected randomly. Because none of the weedy species in these fields had symptoms associated with phytoplasma infection, species were selected for collection on the basis of their abundance in the field or previously reported association with stolbur phytoplasma. Genomic DNA was extracted from roots of individual plants (2) and assayed for the presence of stolbur phytoplasma by nested PCR using the Stol11 primers.

Detection of stolbur phytoplasma in maize and wheat roots. Wheat and maize roots from the Samoš maize field with 40% MR infection in 2006 were tested for the presence of stolbur phytoplasma. The phytoplasma was confirmed in 15 symptomatic maize plants by nested PCR with the Stol11 primers. Maize roots were collected from the field on 23 October 2006, just after the field was harvested. Vigorous remnant roots from 60 plants were sampled randomly along three 20-m transects forming a "Z" pattern in the field. DNA was isolated from individual roots and tested for stolbur phytoplasma using nested PCR with the Stol11 primers. This field was planted with winter wheat on 15 November 2006.

Wheat plants were collected from the field using the same pattern in March through May 2007. A few wheat plants had

symptoms, especially reddening of upper leaves, and both symptomatic and asymptomatic plants were collected. DNA was isolated from individual wheat roots using the DNeasy Plant Mini Kit (Qiagen) and tested for the presence of stolbur phytoplasma using nested PCR with the Stol11 primers.

Acquisition and transmission of stolbur phytoplasma by *R. panzeri* nymphs. Stolbur phytoplasma was transmitted to periwinkle (*Catharanthus roseus*) plants using naturally infected *R. panzeri* collected from an MR-affected corn field in South Banat. The presence of the phytoplasma in periwinkle leaves and roots was confirmed by nested PCR with the Stol11 primers prior to the acquisition experiments.

R. panzeri nymphs hatched from two pools of eggs oviposited by two field-collected females on 28 July 2006. Eggs were kept in outdoor insectariums, out of direct sunlight and with temperatures between 16 and 26°C, until L_1 nymphs hatched 32 days later. In total, 82 L_1 *R. panzeri* nymphs were reared to the L_3 instar on maize and wheat roots in pots (10 by 15 cm) of sterile soil (Klasmann TS 1; Klassman-Dielmann, Antalya, Turkey) in a growth chamber maintained at 24 ± 1°C with 16 h of light and 8 h of darkness. On 21 October, 73 L_3 nymphs were transferred to the stolbur phytoplasma-infected periwinkle for an 8-day acquisition access period.

The nymphs were then transferred to a sweet maize cultivar (ZP 231 *su*; Maize Institute, Zemun Polje) at the three-leaf stage and 3-week-old wheat seedlings (NS 40S; Institute of Field and Vegetable Crops, Novi Sad) to test for transmission of the phytoplasma. Three test plant formats were used: four plants each of maize and wheat grown in a 20-by-30-by-10-cm container, eight plants each of maize and wheat grown in a 20-by-30-by-10-cm container, and a single maize plant with four wheat plants grown in 15-cm pots. L_3 nymphs were allowed to feed on the root systems of these plants for 45 days (13 December 2006). Eight maize and wheat plants grown in pots that were not exposed to *R. panzeri* nymphs were used as controls. During the inoculation access period, plants were kept in a growth chamber at 24 ± 1°C and a period of 16 h of light and 8 h of darkness. Of the 73 nymphs applied to plants, 59 were recovered after the inoculation access period and transferred to 10-by-15-cm pots of eight wheat seedlings and moved to outdoor insectariums for overwintering.

Maize and wheat plants were sampled on 15 February 2007, and the 59 surviving *R. panzeri* nymphs were collected on 5 April 2007 to test for the presence of phytoplasma. DNA was extracted from individual plants or larvae using the DNeasy Plant Mini Kit or DNeasy Blood & Tissue Kit (Qiagen) and tested for the presence of stolbur phytoplasma using nested PCR with the Stol11 primers.

RESULTS

Survey of Auchenorrhyncha spp. in MR-affected and low-MR plots. Because the majority of phytoplasma vectors are hemipteran insects in the suborder Auchenorrhyncha, populations

TABLE 1. Hemipteran insects in and around fields affected by maize redness (MR)

Species	Family	Total[a]	MR affected[b]		Low MR[c]		MRA/LMR[d]	M/B[e]
			Maize	Border	Maize	Border		
Hyalesthes obsoletus	Cixiidae	5	3	1	1	0	–	–
Reptalus panzeri	Cixiidae	1,733	1,692	20	20	1	MRA	M
R. quinquecostatus	Cixiidae	134	15	106	6	7	MRA	B
Delphacodes capnodes	Delphacidae	1	1	0	0	0	–	–
Dicranotropis hamata	Delphacidae	535	90	86	177	182	LMR	N
Laodelphax striatella	Delphacidae	1,457	350	281	589	237	LMR	M
Toya propinqua	Delphacidae	117	16	2	50	49	LMR	N
Dictyophara europaea	Dictyopharidae	45	4	10	16	15	LMR	N
Agalmatium bilobum	Issidae	19	0	0	8	11	LMR	N
Issus coleoptratus	Issidae	83	13	4	39	27	LMR	M
Neophilaenus campestris	Cercopidae	4	2	2	0	0	–	–
Philaenus spumarius	Cercopidae	105	16	81	5	3	MRA	B
Arocephalus languidus	Cicadellidae	1	0	1	0	0	–	–
Athysanus argentarius	Cicadellidae	1	0	0	0	1	–	–
Cicadella viridis	Cicadellidae	28	8	9	1	10	N	N
Emelyanoviana mollicula	Cicadellidae	1	0	0	0	1	–	–
Errastunus ocellaris	Cicadellidae	80	6	49	8	17	N	B
Eupteryx atropunctata	Cicadellidae	52	21	15	6	10	N	N
E. filicum	Cicadellidae	2	0	0	1	1	–	–
Euscelis distinguendus	Cicadellidae	1	0	1	0	0	–	–
E. incisus	Cicadellidae	154	22	53	43	36	LMR	B
Graphocraerus ventralis	Cicadellidae	43	2	5	0	36	LMR	B
Jassargus obtusivalvis	Cicadellidae	289	30	89	64	106	LMR	B
Macrosteles lividus	Cicadellidae	22	0	0	15	7	LMR	N
M. ossianilssoni	Cicadellidae	29	7	4	12	6	LMR	N
M. quadripunctulatus	Cicadellidae	17	1	1	5	10	LMR	N
Metalimnus steini	Cicadellidae	3	3	0	0	0	–	–
Mocydia crocea	Cicadellidae	156	22	24	49	61	LMR	N
Mocydiopsis parvicauda	Cicadellidae	5	4	0	0	1	–	–
Ophiola decumana	Cicadellidae	74	5	1	45	23	LMR	M
Psammotettix alienus	Cicadellidae	6,572	1,819	2,499	771	1,483	MRA	B
Recilia schmidtgeni	Cicadellidae	6	1	0	1	4	–	–
Zyginidia pullula	Cicadellidae	4,272	1,867	1,173	1,110	122	MRA	M

[a] Number of specimens of the species collected from plots in all fields on all dates.
[b] Number of specimens of the species collected in maize or border plots from fields Kovačica, Uzdin, and Samoš (MR-affected) on all dates.
[c] Number of specimens of the species collected in maize or border plots from two fields near Belgrade (low-MR) on all dates.
[d] Distribution of species between MR-affected and control fields. MRA and LMR indicate higher populations of the species on MR-affected or low-MR fields, respectively (least significant difference [LSD], $P < 0.05$). N = no difference between field types; – = not reported because <10 specimens were collected.
[e] Distribution of species between maize and border plots. M and B indicate higher populations of the species in maize or border plots, respectively (LSD, $P < 0.05$). N = no difference between plot types; – = not reported because <10 specimens were collected.

of species in this suborder were monitored in three MR-affected maize fields in South Banat and at two low-MR fields near Belgrade during 2005 and 2006. The 900 samples collected from all plots contained 16,046 specimens belonging to six families, 28 genera, and 33 species (Table 1). For 11 species, including *H. obsoletus*, <10 total specimens were identified from the 900 samples during the 2-year survey. Among the species for which >10 specimens were collected, 5 were more abundant in MR-affected fields, including *Philaenus spumarius, Psammottetix alienus*, *Zyginidia pullula*, *R. panzeri*, and *R. quinquecostatus.* In contrast, 14 species were more abundant on low-MR fields and three were not differentially distributed. Five species, including *R. panzeri* and *Z. pullula*, were more abundant in maize plots, while seven were more abundant in border plots. Thus, of the 33 species identified, *R. panzeri* and *Z. pullula* were significantly more abundant in maize plots of the MR-affected fields.

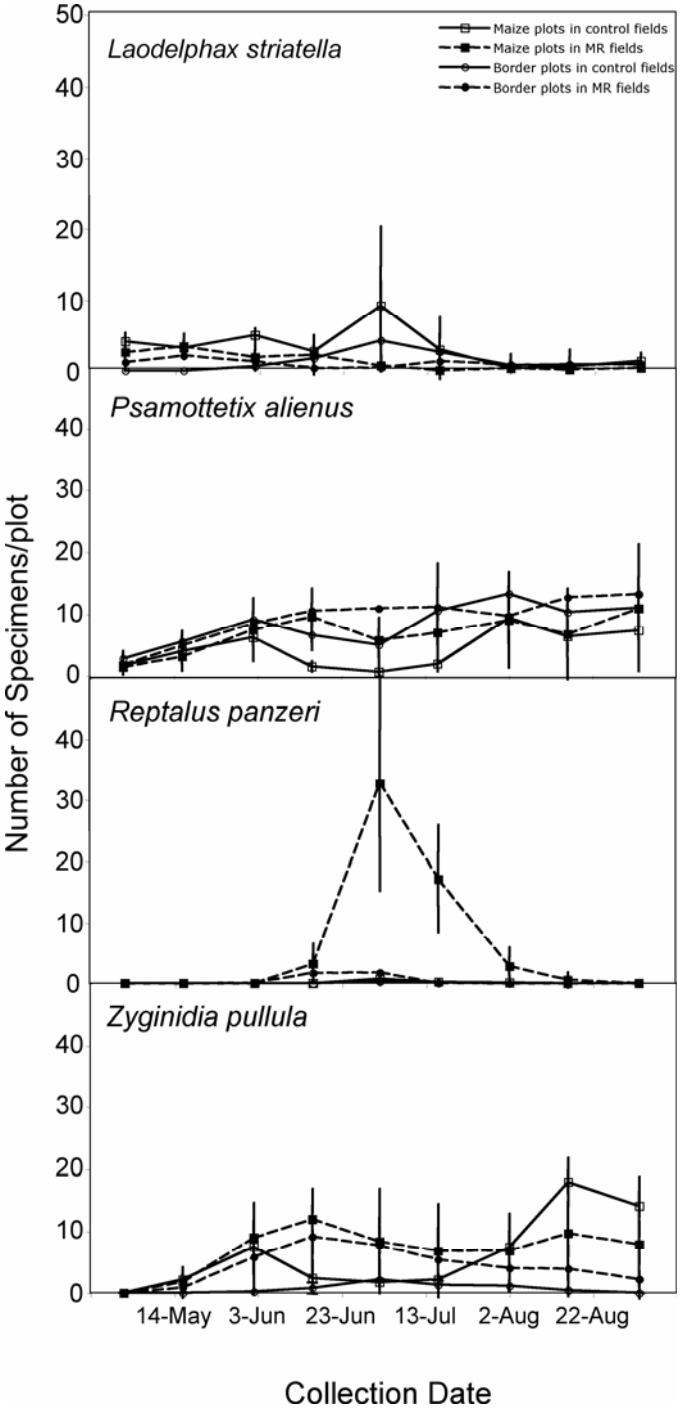

Fig. 1. Distribution of the four most prevalent Auchenorrhyncha spp. in maize redness (MR)-affected and control fields. Data presented are the mean number of specimens ± standard deviation on each date (n = 30 for MR fields and 20 for control fields).

Seasonal population levels differed for the four most abundant species (Fig. 1). *R. panzeri* populations were highest on maize in MR-affected fields between 1 and 15 July but were relatively low otherwise. *Z. pullula* populations were higher in MR-affected fields on 15 June and 1 July and were negligible in low-MR fields during this time. *Laodelphax striatella* and *P. alienus* populations were relatively constant over the season and were not different between MR-affected and low-MR fields or between maize and border plots ($P < 0.05$). For *R. quinquecostatus* and *Philaenus spumarius*, two species that were also more abundant in MR-affected fields, higher populations were found in border plots, and populations averaging <2 specimens per plot peaked in early to mid-July and mid- to late August, respectively (data not shown). These data indicate that peak population levels of the relatively abundant *R. panzeri* and *Z. pullula* in MR-infected maize fields occur a few weeks prior to the appearance of MR symptoms on maize in late July and early August.

Phytoplasmas in Auchenorrhyncha spp. PCR-based analysis for phytoplasmas using DNA from collected adult insects (23) indicated that 3 of the 13 species collected from MR-affected fields were positive for the presence of phytoplasmas (Table 2). Phytoplasmas were detected in 17, 17, and 7% of *R. panzeri*, *Mocydia crocea*, and *Psammottetix alienus* individuals tested, respectively (Table 2). However, digestion of the PCR products with *TruI* indicated that only the *R. panzeri* individuals were infected with stolbur phytoplasma (group 16SrXII-A) and that all phytoplasma-infected *R. panzeri* carried stolbur phytoplasma. *M. crocea* and *P. alienus* were positive for phytoplasmas from the aster yellows group (16SrI-C and I-B). None of the 139 *Z. pullula* individuals tested was positive for phytoplasmas.

***R. panzeri* life cycle and infection with stolbur phytoplasma.** Although *R. panzeri* adults were infected with stolbur phytoplasma, it was not clear when or where these insects acquired the phytoplasma. Because most cixiids lay eggs in soil surrounding plant hosts and nymphs develop on host plant roots (17), *R. panzeri* nymph populations in MR-affected fields were examined in 2.7×10^4 cm^3 soil samples surrounding plants (Table 3). Soil cubes contained roots primarily from a single plant, except for those containing wheat plants. These cubes were estimated to contain roots from ≈30 plants. The highest numbers of nymphs were detected in the autumn on maize roots, with almost 14 nymphs per plant. Significant numbers of nymphs were detected on Johnsongrass (*Sorghum halepense*) in the spring but not in the autumn. Nymphs were present in lower numbers on jimsonweed (*D. stramonium*), bindweed (*Convolvulus arvense*), and creeping thistle (*Cirsium arvense*). Soil samples obtained from wheat stands contained high numbers of *R. panzeri* nymphs. We attributed the relatively low number of nymphs per plant to the fact that wheat is planted at high density relative to other crop species. Stolbur phytoplasma was detected by nested PCR with the Stol11 primers in 4 and 5% of the L_3 and L_5 nymphs, respectively, but was not detected in 48 L_2 nymphs collected from maize roots in the MR-affected field (Table 4). These results indicated that *R. panzeri* nymphs were concentrated on wheat, maize, and Johnsongrass roots, and that a proportion of the L_3 and L_5 nymphs carried stolbur phytoplasma.

Stolbur phytoplasma in weedy plants from MR-affected field borders. To determine whether *R. panzeri* might be able to acquire stolbur phytoplasma from weeds present in and around MR-affected maize fields, roots of seven common weed species found growing in the areas bordering the MR-affected fields in Kovačica, Samoš, and Uzdin were collected and tested for the presence of stolbur phytoplasma (Table 5). None of the weeds had symptoms commonly associated with phytoplasma infection.

However, ≈20% of the 33 Johnsongrass (*S. halepense*) plants tested were positive for stolbur phytoplasma. No stolbur phytoplasma was detected in bindweed, alfalfa (*Medicago sativa*), jimsonweed, black nightshade, black bindweed (*Bilderdykia convolvulus*) or wild mustard (*Sinapis arvensis*). These data suggest that Johnsongrass cannot only serve as a host for *R. panzeri* but is also a possible source of stolbur phytoplasma infection.

Stolbur phytoplasma in plant roots from MR-affected fields. Large numbers of *R. panzeri* nymphs were also found on maize and wheat roots (Table 3), raising the possibility that nymphs could acquire stolbur phytoplasma from infected roots of these plants. To test this, vigorous maize roots remaining after harvest of maize from an MR-affected field in Samoš in which ≈40% of maize plants had MR symptoms were collected and assayed for the presence of stolbur phytoplasma. PCR analysis of DNA from the maize roots indicated that 13 of 60 roots (22%) were positive for stolbur phytoplasma (Fig. 2). Wheat was planted into this field on 15 November 2006 and wheat roots were similarly harvested and tested for the presence of phytoplasma in spring 2007. For roots harvested on 19 March, 15 April, and 5 May 2007, 1 of 30, 1 of 61, and 1 of 27 samples were positive for stolbur phytoplasma, respectively. No correlation between the presence of stolbur phytoplasma and upper leaf reddening was seen in the sampled wheat plants, because phytoplasma was detected in only 1 of >30 wheat plants with leaf reddening (data not shown). No amplification products were detected using DNA isolated from 18 samples each of maize and wheat roots from healthy, greenhouse-grown maize and wheat. These data indicate that wheat is also a host of the stolbur phytoplasma and that the phytoplasma is present in maize and wheat root tissues during the time when *R. panzeri* nymphs are developing in the field.

Stolbur phytoplasma transmission to wheat and maize by *R. panzeri* nymphs. To determine whether the *R. panzeri* nymphs can transmit stolbur phytoplasma to maize and wheat, L_3 nymphs

TABLE 4. Stolbur phytoplasma infection of *Reptalus panzeri* nymphs in maize redness affected fields

Instar	Collection date	No. of nymphs tested	No. stolbur positive[a]
L_2	13 October 2006	47	0
	23 October 2006	19	0
L_3	13 October 2006	35	1
	23 October 2006	35	2
L_5	9 June 2007	39	2

[a] Stolbur phytoplasma was detected in nymph DNA using polymerase chain reaction with the Stol11 primers.

TABLE 2. Phytoplasmas in Auchenorrhyncha spp. collected in and around maize redness affected fields in South Banat

		No. of specimens tested[a]			Phytoplasma infection[b]	
Species	Year	Total	Maize	Border	No. of positives	16Sr group
Reptalus panzeri[c]	2005	188	188	0	30	XII-A
	2006	216	216	0	40	XII-A
R. quinquecostatus	2005	5	5	0	0	…
	2006	4	3	1	0	…
Mocydia crocea	2005	6	…	6	1	I-C
	2006	0	…	…	…	…
Hyalesthes obsoletus	2005	0	…	…	…	…
	2006	3	3	0	0	…
Psammotettix alienus	2005	156	76	80	10	I-C (9), I-B (1)
	2006	31	10	21	3	I-C
Zyginidia pullula	2005	95	41	54	0	…
	2006	44	44	0	0	…
Laodelphax striatella	2005	38	34	4	0	…
	2006	3	3	0	0	…
Macrosteles lividus	2005	0	…	…	…	…
	2006	2	2	0	0	…
M. ossianilssoni	2005	1	1	0	0	…
	2006	0	…	…	…	…
M. quadripunctulatus	2005	1	1	0	0	…
	2006	0	…	…	…	…
Neophilenus campestris	2005	1	0	1	0	…
	2006	0	…	…	…	…
Eupteryx atropunctata	2005	6	6	0	0	…
	2006	0	…	…	…	…
Cicadella viridis	2005	1	0	1	0	…
	2006	1	1	0	0	…

[a] Number of specimens collected from a maize redness-affected field and their distribution between the maize plot and field border.
[b] Number of specimens that tested positive for phytoplasma using the nested polymerase chain reaction assay and their classification into 16Sr groups by digestion with *Tru*1I (23).
[c] Data from Jović (20) and are included for comparison.

TABLE 3. *Reptalus panzeri* nymph populations on plant roots in and around maize redness affected fields in South Banat

Species	Common name	Collection date	No. of plants	No. of nymphs	Nymphs/plant
Zea mays	Maize, corn	October 06/07	30	413	13.8
Solanum nigrum	Black nightshade	October 06/07	30	0	0
Datura stramonium	Jimson weed	October 06/07	30	13	0.4
Convolvulus arvense	Bindweed	October 06/07	30	5	0.2
Sorghum halepense	Johnsongrass	October 06/07	30	0	0
Setaria viridis	Green bristle grass	October 06/07	30	0	0
Triticum aestivum	Wheat	May 07	>300	86	0.3
Convolvulus arvense	Bindweed	May 07	30	9	0.3
Sorghum halepense	Johnsongrass	May 07	30	67	2.2
Cirsium arvense	Creeping thistle	May 07	30	5	0.2

hatched from eggs under controlled conditions were allowed to acquire stolbur phytoplasma from infected periwinkle for 8 days, and were then transferred to maize and wheat seedlings for 45 days (Table 6). Subsequent analysis of plant DNA using nested PCR with the Stol11 primers indicated that >89% of maize plants became infected with stolbur phytoplasma, as did ≈7% of wheat plants. Of the 59 nymphs remaining at the end of the experiment, 47% were infected with stolbur phytoplasma. No phytoplasma was detected in plants not exposed to *R. panzeri*. These data indicate that *R. panzeri* nymphs can transmit stolbur phytoplasma to maize and, with lower efficiency, to wheat.

DISCUSSION

Although recent work indicated that MR is caused by stolbur phytoplasma and that *R. panzeri* is a vector of MR (13,19,20), the possible involvement of other vectors and plant hosts in the disease cycle had not yet been examined. Four characteristics are expected of potential MR vectors: (i) vector populations should be higher in areas with MR than in areas without MR, (ii) vector populations should be high in or around affected fields, (iii) peaks in vector populations should be consistent with the appearance of disease symptoms, and (iv) vectors should carry the stolbur phytoplasma. For *R. panzeri*, all four criteria are met. The insect was ≈50-fold more abundant in MR-affected than in low-MR fields, was 20- to 80-fold more abundant in maize than in border plots, had populations that peaked in early to mid-July in advance of symptom appearance on maize (late July to early August), and adult insects capable of transmission were infected (15 to 20%) with stolbur phytoplasma.

Of the other 32 hemipteran species identified in the surveys, none met the four criteria for being a major vector. In particular, only five specimens of the known stolbur phytoplasma vector *H. obsoletus* (8,32) were collected in this 2-year survey, indicating that this vector is of little or no importance in the epidemiology of MR in South Banat. Low populations of other insects known to harbor stolbur phytoplasma, including *Macrosteles quadripunctulatus* (3), *Mocydia crocea* (32), and *R. quinquecostatus* (37), were found in the MR-affected fields, and none of the individuals tested for these species was positive for stolbur phytoplasma (Table 2). However, fairly small numbers of individuals were tested for the presence of phytoplasmas and there is some potential for insect-specific interference with the PCR assay; therefore, it is possible that these species could transmit MR but it is unlikely that this transmission would be epidemiologically important. Substantial populations of *L. striatella, P. alienus*, and *Z. pullula* were identified in the survey. *L. striatella* is a vector of virus genera and *P. alienus* is known to harbor several phytoplasmas, including stolbur (36). *P. alienus* from the MR-affected fields carried aster yellows and clover phyllody phytoplasmas, but stolbur phytoplasma was not detected in any individuals of these three species. Populations of *P. alienus* and *Z. pullula* were low in MR-affected fields from late June to mid-July, the time in which stolbur phytoplasma transmission to maize would be likely. Thus, although it is possible that some other species are capable to transmitting stolbur phytoplasma to maize, *R. panzeri* is likely to be the major vector of MR in South Banat.

Phytoplasma diseases require a reservoir of the bacteria from which vectors acquire the pathogen. We detected stolbur phytoplasma in two new plant hosts: Johnsongrass and wheat. Johnsongrass has long been known as a reservoir of maize-infecting viruses (29), and our results suggest that this perennial weed might also serve as a reservoir for stolbur phytoplasma. The identification of wheat as a stolbur phytoplasma host provides both an alternative pathogen reservoir and an overwintering food source for *R. panzeri* nymphs as outlined below. Interestingly, stolbur phytoplasma was not detected in other weeds collected from the MR-affected fields, particularly the known hosts *C. arvensis*, *Medicago sativa*, *D. stramonium*, and *Solanum nigrum* (24,25,38). Although the numbers of samples tested for the latter three species were limited, no positive samples of *C. arvensis* were identified from among >100 samples collected on three different dates. It is not likely that there was interference with PCR in these samples, because we have detected stolbur phytoplasma in *C. arvensis* collected from other regions of Serbia (data not shown). Thus, the stolbur phytoplasma associated with MR in South Banat was not found in known dicotyledonous hosts in MR-affected fields. *R. panzeri* nymphs were found feeding on *C. arvensis* under field conditions (Table 3) and adults were occasionally found on these weedy dicots in the field (data not shown), suggesting that the lack of infection is not associated with a lack of *R. panzeri* feeding. In addition, preliminary results indicate that *H. obsoletus* naturally infected with stolbur phytoplasma could transmit the pathogen to periwinkle but not to maize (*unpublished results*). These results suggest a strong interrelationship among vector, plant host, and pathogen, conditions that could favor diversification of the pathogen.

Identification of the source of stolbur-infected *R. panzeri* and the conditions leading to high populations of *R. panzeri* in South Banat are two important factors in understanding MR epidemiology. Based on our results, we propose a model for the interactions

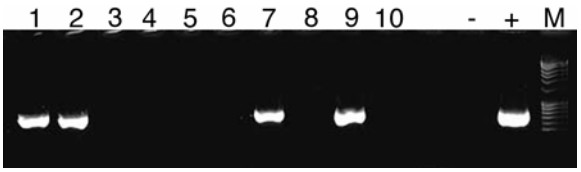

Fig. 2. Maize roots are infected with stolbur phytoplasma in the field. Maize roots were collected from maize redness (MR)-affected fields after harvest and tested for the presence of stolbur phytoplasma as outlined in Materials and Methods. Lanes 1 to 10, DNA from roots of 10 different maize plants; – = no DNA control; + = DNA from Bois noir-infected grapevine; M = molecular weight markers.

TABLE 5. Stolbur phytoplasma in weeds collected near maize redness infected fields in South Banat

Species	Common name	Known stolbur host[a]	Sample date	No. of plants tested	No. stolbur positive[b]
Convolvulus arvensis	Bindweed	Yes	6 July 06	36	0
	…	…	18 July 2006	10	0
	…	…	18 November 2006	60	0
Medicago sativa	Alfalfa	Yes	23 July 2006	9	0
Datura stramonium	Jimson weed	Yes	23 July 2006	9	0
Solanum nigrum	Black nightshade	Yes	23 July 2006	9	0
Bilderdykia convolvulus	Black bindweed	No	18 July 2006	9	0
Sinapis arvensis	Wild mustard	No	18 November 2006	2	0
Sorghum halepense	Johnsongrass	No	13 August 2006	15	5
	…	…	5 July 2007	18	2

[a] Yes = species was previously shown to be a host of stolbur phytoplasma (24,25,38) and No = to our knowledge, the species was not previously demonstrated to be a host.

[b] DNA isolated from root tissue of all species was analyzed for stolbur phytoplasma using the Stol11 primers (11) as outlined in Materials and Methods.

between *R. panzeri*, maize, and wheat that explains the source of infected vectors as well as their relatively high population frequencies (Fig. 3). As previously determined (20), stolbur phytoplasma-infected *R. panzeri* appear on maize in MR-affected areas in mid-June, and these insects are capable of transmitting MR to healthy maize. Toward the beginning of August, the adult *R. panzeri* lay eggs in the soil surrounding maize plants. No stolbur phytoplasma was detected in early-stage (L_2) nymphs collected from MR-affected fields (Table 4), consistent with the notion that MR is not generally transovarially transmitted. However, transovarial transmission of phytoplasmas by *Scaphoideus titanus* (1,7), *Matsumuratettix hiroglyphicus* (16), and *Cacopsylla* sp. (35) has been reported, suggesting that further work is needed to rule out this possible mode of transmission for MR.

Stolbur phytoplasma-infected *R. panzeri* L_3 nymphs were detected by mid-October in these studies (Table 5). These nymphs were associated with maize roots remaining in the field after harvest of the crop in mid-October. A significant portion (>20%) of these remaining maize roots was infected with stolbur phytoplasma, and *R. panzeri* nymphs were capable of acquiring stolbur phytoplasma from periwinkle roots under controlled conditions (Table 6). These results, coupled with the relatively high nymph populations on maize roots (Table 3), suggest that acquisition of stolbur phytoplasma from maize by *R. panzeri* nymphs in the fall influences the population of infected vectors observed the following summer.

The acquisition of stolbur phytoplasma from maize roots by *R. panzeri* nymphs explains the source of infected vectors but does

TABLE 6. Transmission of stolbur phytoplasma by *Reptalus panzeri* nymphs

		No. of plants[b]		No. of stolbur-positive plants[c]		No. surviving	
Trial	No. of nymphs applied[a]	Maize	Wheat	Maize	Wheat	Nymphs	Stolbur-positive nymphs[d]
1	25	4	4	4	1	18	8
2	25	8	8	7	0	21	12
3	8	4	4	4	1	6	3
4	5	1	4	1	0	5	3
5	5	1	4	1	0	5	2
6	5	1	4	0	0	4	0
7	0	8	8	0	0	…	…

[a] L_3 nymphs were allowed an 8-day acquisition access period on stolbur phytoplasma-infected periwinkle. The indicated number of nymphs was transferred to pots with maize and wheat seedlings as indicated.
[b] Each pot contained the indicated numbers of maize and wheat seedlings.
[c] Nymphs were allowed to feed on the maize and wheat seedlings for 45 days. Two months after removing the *R. panzeri*, plant DNA was isolated and tested for stolbur phytoplasma using polymerase chain reaction (PCR) with the Stol11 primers.
[d] After feeding on the maize and wheat seedlings noted above for 45 days, the surviving nymphs were moved to wheat. Two months later, the nymph DNA was isolated and tested for stolbur phytoplasma using PCR with the Stol11 primers.

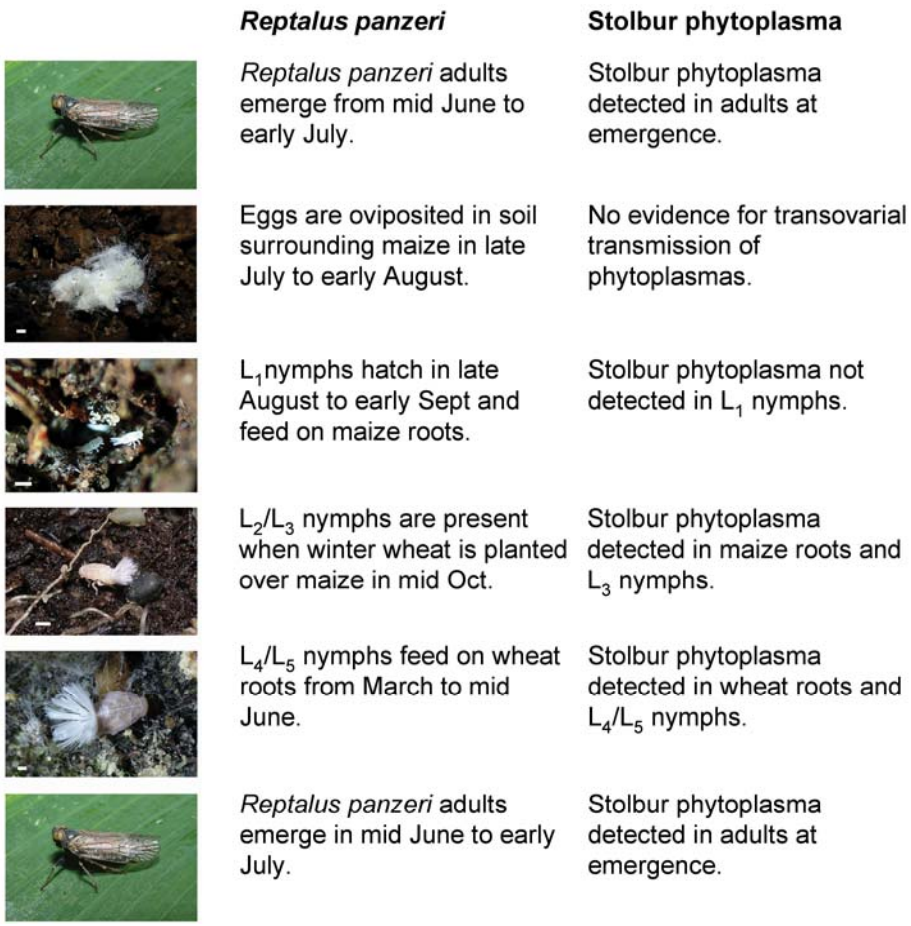

Fig. 3. *Reptalus panzeri* life cycle and disease cycle for stolbur phytoplasma infection of maize. Adults are 6 to 9 mm long. In images of nymphs, the bar is 0.5 mm.

not explain their relatively high numbers in MR-affected areas in recent years. One would expect that survival and development of nymphs into adults would be limited for insects in fields containing only perennial weeds and senescing maize roots. New stands of winter wheat planted following maize provide a fresh overwintering food source for vulnerable L_2 and L_3 *R. panzeri* nymphs. By spring, fairly high populations of *R. panzeri* were detected on wheat roots (Table 3). In total, ≈5% of the L_5 nymphs collected from these roots were positive for stolbur phytoplasma (Table 4). The *R. panzeri* nymphs are also capable of transmitting the phytoplasma to wheat (Table 6), allowing for infection of wheat with subsequent spread of the phytoplasma in the *R. panzeri* population and emergence of large populations of infected adults from wheat fields in July (Fig. 3).

There are several implications of this model. One is that maize itself is an important source of primary infection of *R. panzeri* and that high levels of MR infection in 1 year would lead to a high percentage of infection in the *R. panzeri* population the following year. A second important implication is that the current maize–wheat rotation may be exacerbating MR disease problems by fostering high *R. panzeri* population levels. Without winter wheat, the *R. panzeri* nymphs would be limited to weedy perennial roots, which do not sustain high numbers of nymphs (Table 3). It is possible that insertion of another crop species in the rotation, between maize and wheat, would result in diminished incidence of MR; however, additional research to examine this possibility is needed.

Even though *R. panzeri* is widely distributed in Europe (17), there was no reported association between maize and this insect prior to the discovery that it transmitted stolbur phytoplasma to maize (20). Dense populations of *R. panzeri* on maize in South Banat were previously reported by Tanasijević (34), but this cixiid was never correlated with epiphytotic outbreaks of MR. The few earlier studies available indicated that *R. panzeri* is primarily an arborous species that inhabits scattered shrubs, and is often present on *Rosa* spp. and *Prunus spinosa*, as well as on woody plants such as *Salix*, *Crataegus*, *Pinus*, and *Clematis* spp. (26). *R. panzeri* is considered to be similar to most cixiids, having nymphs that are considered to be polyphagous subterranean feeders.

The current study indicates substantially different behavior for *R. panzeri* populations that inhabit maize fields in South Banat. Here, *R. panzeri* showed obvious feeding preference for and aggregation of both adults and early instar nymphs on maize (Tables 1 and 3). It is possible that intensive fertilization practices used in the maize–wheat rotation increase concentrations of total nitrogen, amino acids, and organic compounds in both the crops and weeds, leading to a nutritional balance that facilitates planthopper fecundity and survival of young nymphs (10,27). Thus, the high populations of *R. panzeri* found on maize in South Banat are associated with a host shift for the cixiid. This is in contrast to previous studies that suggested that planthoppers do not easily adapt to novel host species (12). Additional taxonomic and population genetics research would aid in understanding the rapid adaptation of *R. panzeri* to maize and its expansion into Serbian regions outside of South Banat (J. Jović, *unpublished data*).

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