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SHORT COMMUNICATION

Roles of stolbur phytoplasma and *Reptalus panzeri* (Cixiinae, Auchenorrhyncha) in the epidemiology of Maize redness in Serbia

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Abstract Maize redness (MR), a disease causing midrib, leaf and stalk reddening and abnormal ear development in maize, has been reported from Serbia, Romania and Bulgaria for 50 years. Recent epiphytotics reduced yields by 40%–90% in southern Banat, Serbia. MR was recently associated with the presence of the stolbur phytoplasma, although the epidemiology of the disease remained unknown. Diseased fields in southern Banat were surveyed for potential vectors of the phytoplasma during 2005 and 2006, and high populations of *Reptalus panzeri* were found. In affected fields, 20% of the *R. panzeri* individuals and 85% of symptomatic maize plants carried the stolbur phytoplasma. When stolbur phytoplasma-infected *R. panzeri* were introduced into insect-free mesh cages containing healthy maize plants, midrib and leaf reddening developed on 48% of plants and stolbur phytoplasma was detected in 90% of the symptomatic plants. No symptoms or phytoplasma-positive plants were found in cages without insects. These data indicate that MR symptoms are associated with the stolbur phytoplasma. *Reptalus panzeri* is both abundant in affected fields and can transmit the stolbur phytoplasma, indicating the insect is likely to be a major vector of MR.

Keywords Class Mollicutes · Banat region · *Candidatus* Phytoplasma solani · Vector

Maize redness (MR) has been reported from Serbia, Romania and Bulgaria for the past 50 years (Šutić, Tošić, Starović, Stanković, & Tomić, 2002). MR symptoms appear late in July

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and include reddening of the leaf midrib, followed by reddening of leaves and stalks. Ear development is abnormal and seed set is greatly reduced. No dwarfing or phyllody is associated with MR. Recently, MR was linked to 40%–90% yield reduction in southern Banat, Serbia. Previously, it was suggested that MR symptoms were caused by *Fusarium spp.* or fastidious bacteria (Šutić et al., 2002); however, Duduk and Bertaccini (2006) reported the detection of stolbur phytoplasma (subgroup 16SrXII-A, *Candidatus* Phytoplasma solani) in symptomatic maize plants collected from southern Banat in 2005. The phytoplasma from maize had the highest sequence identity with a stolbur phytoplasma from Serbian pepper (*Capsicum annum*) plants. Although the epidemiology of MR is unknown, maize hybrids showed differential sensitivity to MR and insecticide-treated plots had reduced MR incidence (Šutić et al., 2002).

Phytoplasmas are members of the class *Mollicutes* and are prokaryotic, phloem-limited plant pathogens that, as yet, cannot be cultured (Lee, Davis, & Gundersen-Rindal, 2000). They are obligately transmitted by insects, grafting or parasitic plants (Weintraub & Beanland, 2006). Insect vectors of phytoplasmas include leafhoppers, planthoppers and psyllids. The stolbur phytoplasma is transmitted by the cixiids *Hyalesthes obsoletus* and *Pentastiridius beieri* (Fos, Danet, Zreik, Garnier, & Bové, 1992; Gatineau et al., 2001), and it has been detected in *R. panzeri* from infected vineyards in Hungary (Palermo et al., 2004). This phytoplasma was also detected in several leafhopper species in Israel (Orenstein et al., 2003). Stolbur phytoplasma has a broad plant host range. Bindweed (*Convolvus arvensis*) and several other perennial weeds are hosts that might provide a pathogen reservoir in southern Banat (Fos et al., 1992; Langer & Maixner, 2004).

Identification of potential vectors of MR to maize in Serbia is a key to understanding the etiology and epidemiology of the disease, and will be critical for development of effective crop management. We hypothesize that at least one vector of MR is likely to be newly or only periodically associated with maize in southern Banat, because of the recent and cyclic emergence of MR.

Hemipteran vectors including leafhoppers, planthoppers and cixiids were collected from 15 May to 1 October during 2005 and 2006 in three MR-affected fields near Pančevo in southern Banat about 15 km ENE of Belgrade. Two control fields near Dobanovci and Zemun (about 16 west and 6 km WNW of Belgrade, respectively) did not have MR. Insects were collected using sweep nets and mouth-aspirators from marked plots (10×10 m each) along a transect in each field. Collected insects were placed in vials containing 80% ethanol, and stored at 8°C. *Reptalus panzeri* was identified as described by Holzinger, Kammerlander, and Nickel (2003). Among the hemipteran insects identified, populations of *R. panzeri* were particularly high in the MR-affected fields, with 239 ± 81 (mean ± s.d., $n = 10$) and 281 ± 108 ($n = 10$) individuals per plot in July 2005 and 2006, respectively. In contrast, *R. panzeri* populations in control plots were 16 ± 8 ($n = 10$) in 2005 and 29 ± 17 ($n = 10$) in 2006. Populations of the known stolbur phytoplasma vector *Hyalesthes obsoletus* were negligible in affected fields, with none and three collected in 2005 and 2006, respectively.

To determine whether *R. panzeri* present in southern Banat harboured the stolbur phytoplasma (Palermo et al., 2004), DNA was isolated from individual insects and amplified using a modification of the stolbur phytoplasma-specific nested PCR protocol (Clair et al., 2003; Daire, Clair, Reinert, & Boudon-Padieu, 1997). A 0.72 kb PCR product was detected for stolbur phytoplasma-positive grapevine with Bois Noir, as well as in *R. panzeri* from an MR affected maize field (Fig. 1A). In 2005 and 2006, respectively, 30 of 188 and 40 of 216 *R. panzeri* from the affected fields were PCR-positive for the phytoplasma, but just two of 167 collected from the control fields in 2006 were positive (data not shown). Restriction fragment length polymorphism (RFLP) analysis was used to confirm the identity of the stolbur phytoplasma (Lee, Gundersen, Davis, & Bartoszyk, 1998). The *Tru*I digestion patterns from amplified rDNA were identical to those obtained for DNA from the stolbur phytoplasma-infected grapevine (Fig. 1B). Thus, 17% of *R. panzeri* collected from

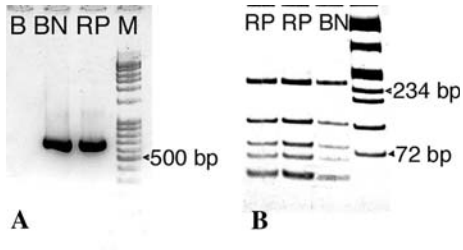

Fig. 1 PCR and RFLP analysis of stolbur phytoplasma in *Reptalus panzeri* from southern Banat. (**A**) Nested PCR with DNA isolated from *R. panzeri* (RP) and leaves of stolbur phytoplasma-infected grapevine showing Bois Noir (BN). DNA was isolated according to Daire et al. (1997). The initial round of PCR was carried out with the STOL11 f2 and r1 primers, and the second round with the STOL11 f3 and r2 primers using conditions outlined by Clair et al. (2003), except the primer concentration was 0.75 μM, and 0.02 μl of the first round PCR reaction was for the second round of PCR. PCR products were visualized after electrophoresis on 1% agarose gels. Lane B, no DNA control; lane M, 100 bp ladder (Serva). (**B**) RFLP analysis of phytoplasma rDNA. rDNA was amplified from RP and stolbur phytoplasma-infected grapevine leaves (BN) according to Lee et al. (1998) and digested with *Tru*I (Fermentas). PCR products were visualized after electrophoresis on 13% polyacrylamide gels. Lane M, ϕX174 ladder (Fermentas)

MR-affected fields were infected with the stolbur phytoplasma across 2 years.

In 2005, four of ten *R. panzeri* adults collected on 5 July tested positive for the phytoplasma using the nested PCR assay. The first appearance of *R. panzeri* adults in 2006 was on 28 June, and 19 of 65 adults collected on that date tested positive for the phytoplasma. These data indicated the stolbur phytoplasma is present in the insects several weeks prior to the appearance of MR symptoms in maize.

To determine whether the stolbur phytoplasma was associated with MR in maize, plants with midrib and leaf reddening (Fig. 2) were collected from the plots surveyed for insects in southern Banat in July 2006 and analyzed using the nested PCR assay. More than 85% of symptomatic plants (26 of 30 samples) were positive for the phytoplasma (Fig. 3). No symptomatic plants were found in the control plots, and no asymptomatic plants (0 of 20) were PCR-positive for the phytoplasma. These data demonstrate the association of the stolbur phytoplasma with MR

Fig. 2 Maize redness symptoms in field grown maize from southern Banat (August 13, 2006). The plant shown was positive for stolbur phytoplasma

symptoms in maize, confirming the results of Duduk and Bertaccini (2006).

A field experiment was conducted to determine the association between *R. panzeri*, reddening symptoms in maize and the presence of stolbur phytoplasma. Mesh cages (2.2 × 2.2 × 2.5 m) were set up on 26 April 2006 on plots in control fields at the Plant Protection Institute in Zemun, Serbia. The cages were treated on 28 April and 19 May with bifenthrin (Talstar-10 EC, FMC, Philadelphia, PA, USA) and deltamethrin (Decis 2.5 EC, AgrEvo Hoechst Schering, Berlin, Germany) according to the labels. Weeds were controlled manually. Sweet maize hybrid 'ZP 231 su' was seeded into the cages on 26 May. Between 4 and 7 July, 120 *R. panzeri* collected from the southern Banat fields were released into each of seven cages (Table 1). Although the presence of stolbur phytoplasma in these insects was not assessed directly, more than 20% of *R. panzeri* collected during this time from the same fields were positive for the phytoplasma. Plants in four cages that did not receive *R. panzeri* served as controls. Maize plants were evaluated for symptoms weekly after the introduction of insects. At the time that symptoms began to appear (8–9 August), the cages were

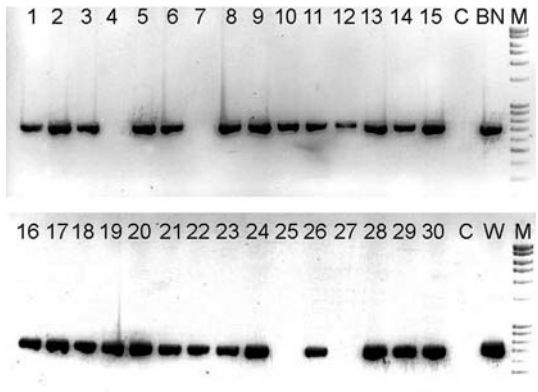

Fig. 3 PCR products from symptomatic plants collected from the MR-affected fields in southern Banat. An agarose gel with the 0.72 kb product amplified from DNA using the nested PCR protocol (Fig. 1) is shown. Lanes 1–30: DNA from symptomatic maize; BN, stolbur phytoplasma-infected grapevine; W, stolbur phytoplasma infested *R. panzeri*; M, 100 bp marker; C, no DNA control

treated with propargite (Omite 57 E, Chemtura Corp., Middlebury, CT, USA) and deltamethrin. From 5 August to 10 September, samples were collected from individual plants in test cages and from pools of three plants from control cages and tested using nested PCR.

Four weeks after release of the *R. panzeri*, leaf and midrib reddening began to appear on plants in test cages, and symptoms continued to develop and intensify similarly to MR in the field over the next 4 weeks (Fig. 4). Ultimately, 75 of 132 plants from the 7 test cages were symptomatic, with 44%–68% of the plants in each cage having symptoms (Table 1). Stolbur phytoplasma was confirmed in 61 of 75 symptomatic plants using nested PCR. No symptoms were seen on 83 plants in the four control cages and 0 of 24 control plants were positive for the phytoplasma. These data strongly suggest *R. panzeri* is a transmitter of the stolbur phytoplasma that is associated with MR.

Reptalus panzeri was abundant in the MR affected region, but was much less abundant in an adjacent area with no MR. Two independent tests showed that *R. panzeri* from MR affected fields harboured the stolbur phytoplasma. We also confirmed the presence of the stolbur phytoplasma in maize with reddening symptoms in Serbia, and showed that *R. panzeri* is capable of transmitting stolbur phytoplasma to maize.

The populations phytoplasma infected *R. panzeri* in the southern Banat fields in 2005 and 2006 were likely to be sufficient to account for the spread of MR, as phytoplasmas replicate in their insect hosts and are transmitted in a persistent manner (Weintraub & Beanland, 2006). Duduk and Bertaccini (2006) hypothesized that *H. obsoletus*, a known vector of stolbur phytoplasma, could be important in the spread of MR in Serbia; however, populations of this species were negligible in this study, suggesting it did not play a major role in the epidemiology of MR in the fields we studied.

MR symptoms appeared only in maize exposed to insects in the test for stolbur phytoplasma transmission by *R. panzeri*. Since phytoplasmas are not seed-transmitted, the presence of insects in the field cages was controlled and no

Fig. 4 Development of MR symptoms in maize grown in mesh field cages after exposure to *R. panzeri*. Development of symptoms on a single plant shown at 29 (**A**), 34 (**B**) and 38 (**C**) days after releasing insects. The plant was positive for stolbur phytoplasma as indicated by the nested PCR protocol

Table 1 Stolbur phytoplasma transmission by *R. panzeri*

Cage	Plants[a]	*R. panzeri*[b]	Symp.[c]	PCR pos.[d]
1	18	120	11	9
2	21	120	14	11
3	19	120	9	8
4	19	120	11	8
5	20	120	10	7
6	19	120	13	13
7	16	120	7	5
8	13	0	0	0
9	22	0	0	0
10	24	0	0	0
11	24	0	0	0

[a] The number of maize plants germinated per cage

[b] The number of *R. panzeri* released per cage

[c] The number of plants showing MR symptoms

[d] The number of symptomatic (cages 1–7) plants that were positive for stolbur phytoplasma using the nested PCR assay. For cages 8–11, two pools of three randomly selected plants per cage were tested

symptomatic plants were found in control experiments, it is not likely that the symptoms observed were independent of *R. panzeri*. The insect is not a known vector of viruses and no other phytoplasmas were detected in the RFLP assay, so the symptoms observed are not likely to be associated with another pathogen. Because about 20% of the insects introduced into each cage were likely to be infected with the phytoplasma and up to 50% of the plants became infected, *R. panzeri* may be an efficient vector of the phytoplasma.

While further experiments are required to complete Koch's postulates for stolbur phytoplasma as the MR pathogen, our data strongly suggest that *R. panzeri* not only harbours the phytoplasma, but is capable of transmitting it. These data provide information useful for the development of approaches to manage MR in Serbia. Additional current research is directed at identification of the natural reservoir(s) of phytoplasma in Serbia, and the stage(s) at which *R. panzeri* acquires the phytoplasma.

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