Ćurčić Ž., Stepanović J., Zübert C., Taški-Ajduković K., Kosovac A., Rekanović E., Kube M., Duduk B. Rubbery taproot disease of sugar beet in Serbia associated with *'Candidatus* phytoplasma solani'. Plant Disease 2021, 105 (2), 255 – 263. https://doi.org/10.1094/PDIS-07-20-1602-RE.

Rubbery Taproot Disease of Sugar Beet in Serbia Associated with '*Candidatus* Phytoplasma solani'

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Keywords: Stolbur, *Beta vulgaris*, Etiology

Funding: Ministry of Education and Science, Republic of Serbia 451-03-68/2020-14/200214 and 451-03-68/2020-14/200032

ABSTRACT

Ćurčić, Ž., Stepanović, J., Zuebert, C., Taški-Ajduković, K., Kosovac, A., Rekanović, E., Kube, M., and Duduk, B. 2020. Rubbery Taproot Disease of Sugar Beet in Serbia Associated with '*Candidatus* Phytoplasma solani'. Plant Disease 104:xxx-xxx.

Rubbery taproot disease (RTD) of sugar beet was observed in Serbia for the first time in the 1960s. The disease was already described in neighbouring Bulgaria and Romania at the time, but it was associated with abiotic factors. In this study on RTD of sugar beet in its main growing area of Serbia, we provide evidence of the association between '*Ca*. P. solani' (stolbur phytoplasma) infection and the occurrence of typical RTD symptomatology. '*Ca*. P. solani' was identified by PCR and the sequence analyses of 16S *rRNA*, *tuf*, *secY* and *stamp* genes. In contrast, the causative agent of the syndrome "basses richesses" of sugar beet, namely, '*Ca.* A. phytopathogenicus', was not detected. Sequence analysis of the stolbur strain's *tuf* gene confirmed a previously reported and a new, distinct tuf stolbur genotype (named 'tuf d') that is prevalent in sugar beet. The sequence signature of the *tuf* gene, as well as the one of *stamp* both correlate with the epidemiological cycle and reservoir plant host. This study provides knowledge that enables for the first time the differentiation of stolbur strains associated with RTD of sugar beet from closely related strains, thereby providing necessary information for further epidemiological work seeking to identify insect vectors and reservoir plant hosts. The results of this study indicate that there are differences in hybrid susceptibility. Clarifying the etiology of RTD as a long-known and economically important disease is certainly the first step towards disease management in Serbia and neighboring countries.

Keywords: Stolbur, *Beta vulgaris*, Etiology

INTRODUCTION

Rubbery taproot disease (RTD) of sugar beet (*Beta vulgaris*) was observed in Serbia for the first time in the Central Banat and Northern Bačka regions in the 1960s (Marić 1974; Marić et al. 1970). The disease was already known at the time in neighboring Bulgaria and Romania, but it was associated with abiotic factors (Christova 1950; Marić 1974; Racovita 1959). After its epidemic phase during the late 1960s, the disease abated but remained present in the 1970s, when it was sporadically observed across the region, showing higher prevalence in dry seasons (Marić 1974). In 2018, the disease entered a new epidemic phase in Serbia, suggesting that it may be related to current outbreaks in other European sugar beet-growing regions. A condition known in sugar beet crops as low sugar content disease, and also as the syndrome “basses richesses” (SBR), has been reported in France repeatedly since the 1990s, while it has been observed in Germany and Switzerland since 2009 and 2017, respectively (Fankhauser 2019; Richard-Molard et al. 1995; Schröder et al. 2012). The SBR is associated with a phloem-limited prokaryote, '*Candidatus* Arsenophonus phytopathogenicus', whose vector is *Pentastiridius leporinus* (Auchenorrhyncha: Cixiidae) (Bressan et al. 2008, 2009, 2012; Gatineau et al. 2002; Sémétey et al. 2007b). However, another phloem-limited prokaryote, '*Candidatus* Phytoplasma solani' (stolbur phytoplasma), is vectored on sugar beet by the same insect, and it has been sporadically detected in SBR-affected plants, albeit it does not play a significant role in SBR etiology (Bressan et al. 2008; Gatineau et al. 2001; Sémétey et al. 2007b). The most apparent symptoms of SBR are the brownish discoloration of taproot vascular tissue, and the deformation and discoloration of young and old leaves. The disease causes a reduction in taproot sugar content as well as losses as high as those exceeding 50% in France in 1991 (Richard-Molard et al. 1995). In

South America, a sugar beet disease known as ”yellow wilt” was observed for the first time in Argentina in the 1930s and later in Chile in the 1940s (Bennett and Munck 1946; Vallejo 1970). Eventually, it was associated with a phytoplasma belonging to the ribosomal group 16SrIII-J (Castro et al. 2000; Fiore et al. 2015). Yet another disease of unknown etiology with similar symptoms was reported in Arizona, USA (Ruppel 1969).

Presence of the SBR agent '*Ca.* A. phytopathogenicus' has never been recorded in Serbia, while stolbur as a disease of pepper and other Solanaceae, had been known in Serbia since 1949 (Martinović and Bjegović, 1950). The research on the epidemiology of stolbur induced pepper yellow wilt in Serbia, conducted as early as in the 60s, has confirmed the data on *Convolvulus arvensis* as the stolbur inoculum source and planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae) as its vector, corresponding to the previous findings in the former USSR (Aleksić et al. 1967; Suhov and Vovk 1949). Although recent researches of stolbur diseases in Serbia have become mainly oriented toward Bois noir of the grapevine and corn reddening disease (Cvrković et al. 2014; Duduk et al. 2004; Duduk and Bertaccini 2006; Jović et al. 2009; Mori et al. 2013; Kosovac et al. 2019), numerous other annual and perennial crops have also been reported as infected and economically affected by '*Ca.* P. solani' such as potato, carrot, parsley etc. (Mitrović et al. 2013, Mitrović et al. 2016).

Molecular characterisation of the '*Ca.* P. solani' strains can give an insight into the pathogen’s epidemiological cycle that permeates natural and cultivated vegetation. Conserved house-keeping *tuf* gene has been so far demonstrated as the epidemiologically most informative genetic marker indicating the inoculum source plant (Aryan et al. 2014; Atanasova et al. 2015; Kosovac et al. 2016; Langer and Maixner 2004). Another house-keeping gene, *secY* has also been frequently used in epidemiological studies, while more elaborate tracking of the ‘*Ca*. P. solani’ pathways is

feasible when combined with more divergent *stamp* gene. Variability of the *stamp* gene follows a specific *tuf* epidemiological cycle and in some disease outbreaks correlates with specific transmission pathway and vector population (Fabre et al. 2011b; Kosovac et al. 2016; Murolo and Romanazzi 2015).

Sugar beet RTD symptoms in Serbia resemble those of sugar beet yellow wilt in South America (leaf yellowing and wilting), more than the SBR in Europe, while the disease pattern (its recurrent epidemic outbreaks and distinctive development, i.e. temporal and spatial distribution), to some extent, is similar to other phytoplasma-induced diseases of annual crops in Serbia (Duduk and Bertaccini 2006). This resemblance indicates a possible association of the disease with phytoplasmas, even though 16SrIII-J phytoplasmas are not present in Europe and the '*Ca.* P. solani' found in France was not associated with symptoms similar to those of the RTD.

The latest outbreak of this important disease, alongside its economic impact on sugar beet in Serbia, has enabled trials to be carried out in naturally infected experimental fields, aiming to: (i) clarify the etiology of sugar beet rubbery taproot disease in the main sugar beet growing area in Serbia, (ii) molecularly characterise the causal organism and (iii) evaluate the disease prevalence.

MATERIAL AND METHODS

The etiology of RTD, as well as hybrid sensitivity and disease prevalence, was examined in a long-term stationary trial based at Rimski Šančevi (N 45°20′; E 19°51′) at the Institute of Field and Vegetable Crops, Novi Sad. The location is situated in South Bačka district, the Region of Vojvodina, claiming 27% and 97%, of total 50,000 ha sugar beet-growing area in Serbia (for the district and the region, respectively). Disease prevalence and hybrid sensitivity were assessed in 2018 and 2019, while the etiology of the disease was examined in 2019. The long-term stationary

trial was set up in 1965 as a four-field crop rotation scheme for sugar beet, corn, sunflower and wheat. The soil type in the field is chernozem, subtype on loess, variety carbonate, form medium deep.

Experimental design and data analysis. The experiment was organised in a randomised complete block design observing eight hybrids in 80 replications. The hybrids used in the experiment in 2018 were Tesla, Eduarda, Original, Tajfun, Nansen, Terranova, Nora and Leopolda, while in 2019 the latter two were replaced with Sioux and Sixtus. The hybrids used in the experiment, are widely grown sugar beet hybrids in Serbia. Each sugar beet hybrid was considered a plot treatment. The experimental plot size was 24 m^2 (four 12 m long rows), and wheat was the preceding crop. Field size was 2 ha. Sowing was performed on April 11, 2018 and March 22, 2019, at 0.09 m distance within rows and 0.5 m between rows. After the second pair of leaves developed, seedlings were thinned to a final, recommended crop density of 100,000 plants ha^{-1}. Standard agricultural practice for sugar beet crops, including herbicides and fungicides, were applied during the vegetation period. Beside insecticide (containing two active ingredients thiamethoxam and tefluthrin) that was used for the seed pretreatment in both 2018 and 2019, the treatments in 2018 included an additional insecticide (containing two active ingredients chlorpyrifos and cypermethrin) foliar treatment against beet weevil on May 4.

In October of both years, the prevalence of sugar beets expressing RTD symptoms was determined within each sampling plot by subjecting all samples to symptom observation and counting. The prevalence was calculated using the following formula: prevalence (%) = number of plants with RTD symptoms/total number of plants x100.

One-way ANOVA (analysis of variance) was performed to analyse the collected data separately for each year, using the *Statistica 13* software package (2013, StatSoft, Tulsa, OK,

USA) and Tukey's range test for detecting of statistically significant differences between sugar beet hybrids. Only the six hybrids which were used in both years of the experiment were included in the statistical analyses.

Symptom observation and sample collection. The experimental production field served for monitoring the occurrence and development of disease and sampling for etiological research. Field observation included monitoring symptoms on leaves and roots at weekly intervals over the vegetation period, starting from 1 May and running until harvest in November.

At harvest during the first half of November 2019, 30 symptomatic and 20 asymptomatic samples of the Original sugar beet hybrid were randomly collected from the experimental field for etiological research. Simultaneously, 30 symptomatic and 20 asymptomatic samples of the other seven hybrids tested in the experiment were randomly collected and also subjected to etiological research.

Two sugar beet plants with typical symptoms of SBR (HN1220/5 and HN1220/6) were collected from an area with natural SBR infection in Biberach bei Heilbronn, Germany, and they served as SBR positive controls for '*Ca.* A. phytopathogenicus'.

Additionally, three symptomatic and one asymptomatic samples of sugar beet were collected from a commercial field at Bačko Dobro Polje 40 km north-east of the experimental field (N 45°30'49"; E 19°42'59"), another area with natural RTD infection.

Molecular detection. Nucleic acid extraction from sugar beet samples was performed from 0.5 g of taproot or leaf midrib tissues, following the CTAB protocol (Doyle and Doyle 1990). Total nucleic acids were precipitated with isopropanol, re-suspended in a TE buffer (10 mM Tris pH 8 and 1 mM EDTA) and stored at -20˚C.

For phytoplasma assessment in the collected samples, direct PCR assays with the universal phytoplasma primer pair P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995), and nested PCR with the primer pair R16F2n/R2 (Lee et al. 1995), were carried out. Each 25 µL PCR mix contained 20 ng of template DNA, 1× PCR Master Mix (Thermo Scientific, Vilnius, Lithuania) and 0.4 µM of each primer. Samples lacking DNA were employed as negative controls. In total, 1 µL of direct PCR amplicon diluted 30× in sterile water was used as a template for the nested PCR. Thirty-five PCR cycles were performed, for both amplifications, under previously described conditions (Deng and Hiruki 1991). Six microliters of PCR products were separated in 1% agarose gel, stained with ethidium bromide and visualized with a UV transilluminator. The detected phytoplasmas were identified by RFLP analysis, using the *Tru1*I (Thermo Scientific, Vilnius, Lithuania) restriction enzyme on R16F2n/R2 amplicons. Restriction products were separated in 8% polyacrylamide gel, stained and visualized as described above. The P1/P7 PCR products of 12 randomly selected samples were sequenced in both directions, with primers applied for amplification (Macrogen Inc., Seoul, Korea). The obtained sequences were assembled using Pregap4 from the Staden Package (Staden et al. 2000), manually inspected and compared. A 1,688 nt-long nucleotide sequence of the almost complete 16S ribosomal RNA gene, the internal spacer region and a 5' portion of the 23S ribosomal RNA gene of '*Ca.* P. solani' strain 429/19, was deposited in GenBank under accession number MT157232. For identification purposes, the sequence was compared with sequences available in NCBI's GenBank database.

For the '*Ca.* A. phytopathogenicus' assessment, two separate PCRs were applied to all sugar beet samples analysed in this work. The first one employed the Alb1/Oliv1 primer pair specific to the internal transcriber spacer (ITS) region between the 16S and 23S ribosomal genes of '*Ca.* A. phytopathogenicus', while the other one used the Fra5/Fra4 primer pair, which allows for the

amplification of the 16S rRNA gene of '*Ca.* A. phytopathogenicus'. The conditions for amplification and reaction mix compositions were the same as described previously (Sémétey et al. 2007a; Zreik et al. 1998). The PCR products in both analyses were visualised as described above. The Fra5/Fra4 PCR products of the two SBR sugar beet samples from Germany were sequenced in both directions with primers applied for amplification. The obtained sequences were assembled, manually inspected and compared as described above. A 515 nt-long nucleotide sequence of the 16S ribosomal RNA gene of '*Ca.* A. phytopathogenicus' strain HN1220/5 was deposited in GenBank under accession number MT139648. The sequence was compared with available sequences as described above.

Multi locus sequence typing analyses. The *secY*, *tuf* and *stamp* genes were additionally analyzed in the six samples from the experimental field selected for phytoplasma 16S rDNA sequence analysis, an additional three randomly selected from among the positive samples from the experimental field and three from Bačko Dobro Polje. In order to amplify the *tuf* gene, the fTufAy/rTufAy primer pair was used in direct PCR assays (Schneider and Gibb 1997), while direct PCR with an AYsecYF1/AYsecYR1 primer pair was performed for amplifying the *secY* gene (Lee et al. 2006). For amplification of *stamp* gene nested PCR assays were performed using Stamp-F/R0 primer pair in direct followed by Stamp-F1/R1 in nested reactions (Fabre et al. 2011b). The PCR mix composition for all reactions was as described previously, as well as PCR conditions (Fabre et al. 2011b; Lee et al. 2006; Schneider and Gibb 1997). The fTufAy/rTufAy, AYsecYF1/AYsecYR1 and Stamp-F1/R1 PCR products were sequenced in both directions with the primers applied for amplification (Macrogen Inc., Seoul, Korea). The obtained sequences were assembled and compared as described earlier. A 906 nt-long nucleotide sequence of the partial *tuf* gene, a 1,234 nt-long complete sequence of the *secY* gene and a 474 nt-long nucleotide

sequence of the partial *stamp* gene of the phytoplasma strain 429/19 from sugar beet were deposited in GenBank under accession numbers MT157234, MT157233 and MT783826, respectively. The obtained sequences were compared with available ones as reported before.

The *tuf* sequences were aligned with those of the strains representing three previously described *tuf* types, using ClustalX (Thompson et al. 1997), under MEGA version 7 (Kumar et al. 2016). The *tuf* gene sequences of strains CrHo13_1183, CrHo12_601 and CrHo12_650 (acc. numbers KJ469707, KJ469708 and KJ469709, respectively), representing those previously described as the '*Ca.* P. solani' *tuf* types tuf a, tuf b1 and tuf b2, respectively (Aryan et al., 2014), were added to the alignment, and putative restriction site maps were generated using the software pDRAW32 (http://www.acaclone.com/). In order to confirm virtual RFLP, the obtained fTufAy/rTufAy amplicons were subjected to RFLP analyses with the *Tai*I restriction enzyme (Thermo Scientific, Vilnius, Lithuania). Restriction products were separated, stained and visualised as described above. To obtain better visualization of the *stamp* gene divergence among detected '*Ca*. P. solani' isolates, genealogical network was calculated in software TCS v.1.21 (Clement et al. 2000) using statistical parsimony with a confidence level of 93% (Templeton et al. 1992). All *stamp* genotypes belonging to the *stamp* genetic clusters II tuf-b and III tuf-b that had been detected in Serbia were added to the analysis (Fabre et al. 2011; Cvrković et al. 2014).

The *stamp* sequences were aligned with those of the strains representing previously described *stamp* genotypes in Serbia. The *stamp* gene sequences of the isolates STOL Rqg50, Rpm35, Rqg31, Vv24 and HoC68 (genotype M5) (acc.nos. FN813261, KC703019, KC703015, KC703017, KC703022 and KP337316, respectively) were used for the genotyping of the obtained '*Ca.* P. solani' isolates (Atanasova et al. 2015; Cvrković et al. 2014; Fabre et al. 2011b; Mitrović et al. 2016).

In order to check over '*Ca.* P. solani' *tuf* and *stamp* genotypes in different hosts and geographic areas we selected 40 '*Ca.* P. solani' strains from phytoplasma collection at the Laboratory of Phytopathology, Institute of Pesticides and Environmental Protection, Belgrade, Serbia, from different hosts and areas over the period 2009-2019, and included them in the *tuf* and *stamp* analyses (Table 1). Among the 40 '*Ca.* P. solani' strains, two were obtained from 10 and 20 sugar beet roots randomly collected from the locations Srem and Kačarevo (one per location), respectively, from those prepared for industrial procession and without symptom assessment. In strains that had not been identified earlier, '*Ca.* P. solani' identification was confirmed as described above, and the *tuf* and *stamp* genes were determined for all samples as described above.

RESULTS

Disease symptoms. The first symptom of sugar beet rubbery taproot, usually appearing in the second half of July, is a loss of turgor in leaves during the hottest part of the day. It is followed by yellowing and, later, necrosis of the oldest leaves, starting from their margins (Fig. 1A). Eventually, all leaves become necrotic, which leads to the complete decline of the plant (Fig. 1B). Symptoms become obvious in August and stay visible until harvesting. Taproots of diseased plants wilt, become rubbery and stay without any rot symptoms until after plant decline (Fig. 1C). Sections of symptomatic taproots reveal no visual difference from those of healthy taproots, or any discoloration (Fig. 1D). However, symptomatic taproots in the field are prone to rotting after plant decline, due to the activity of saprobes. As a consequence, some of infected taproots, especially those infected in the early season, do not last until harvest. When diseased sugar beets

are harvested, their rubbery taproots cannot be used for industry, as they cannot be sliced into cossettes, which is why they have to be discarded.

Molecular analysis reveals '*Ca.* P. solani' infection. Nested PCR reactions with the phytoplasma-specific R16F2n/R2 primer pair resulted in an expected length fragment amplification for phytoplasmas (1.2 kb) in all symptomatic samples of the sugar beet hybrid Original, in all but one (29 out of 30) symptomatic samples of the other tested sugar beet hybrids and in all three symptomatic samples from Bačko Dobro Polje. No amplification was obtained from 20 asymptomatic samples of the hybrid Original, 20 asymptomatic or one symptomatic sugar beet samples randomly collected from the other hybrids, one asymptomatic sample from Bačko Dobro Polje, two SBR symptomatic samples from Germany and from the negative controls.

All 62 positive samples, when R16F2n/R2 amplicons were subjected to RFLP analysis with the *Tru*I restriction enzyme, showed restriction profiles identical (data not shown) and referable to the profile of '*Ca.* P. solani' (stolbur phytoplasma, 16SrXII-A ribosomal group) according to the previously published phytoplasma classification system (Lee et al. 1998).

Sequences of the almost complete 16S ribosomal RNA gene, the internal spacer region and a 5’ portion of the 23S ribosomal RNA gene were determined for 12 representative samples amplified in direct PCR with a P1/P7 primer pair. Multiple sequence alignment showed that the sequences of all Serbian '*Ca.* P. solani' samples were identical, and, when compared to the available sequences, they were also identical with several other sequences of '*Ca.* P. solani', from corn, grapevine, tobacco (from Serbia) and potato (from Russia), i.e. JQ730739, JQ730745, JQ730746, JQ730750, JQ868436 and EU344884.

'*Ca.* A. phytopathogenicus' assessment PCRs, using both Alb1/Oliv1 and Fra5/Fra4 primer pairs, yielded amplicons of expected sizes (four amplicons ranging between 732 and 927bp with Alb1/Oliv1, and 550bp with Fra5/Fra4 primer pairs), but only for the two SBR-positive controls from Germany, while no amplification was obtained from any sugar beet sample from Serbia. For the two SBR positive controls from Germany, amplified in direct PCR with the Fra5/Fra4 primer pair, sequences of the partial 16S ribosomal RNA gene were determined. Multiple sequence alignment showed that the obtained sequences for the two SBR strains from Germany were mutually identical, and compared to available sequences they were identical to the sequences of '*Ca.* A. phytopathogenicus' from the SBR-affected sugar beet from France (AY057392) and those of four closely related proteobacteria from strawberry affected with strawberry marginal chlorosis from Italy (DQ538379, DQ538378, DQ538377, DQ538375). The analyses confirmed the identification of '*Ca.* A. phytopathogenicus' in positive controls obtained from the SBR-affected sugar beet from Germany.

Non-ribosomal genes. With the AYsecYF1/R1 primer pair, expected amplicons of approximately 1.2 kb in length were obtained for all 12 selected symptomatic sugar beet samples in which '*Ca.* P. solani' presence was confirmed with P1/P7 primers and a 16S rDNA sequence was determined. Multiple sequence alignment showed that the determined *secY* gene sequences for all 12 Serbian '*Ca.* P. solani' samples were mutually identical, and compared to publicly available sequences they were identical with four other sequences of '*Ca*. P. solani': three strains from Serbia (strains 284/09, FO393427 and 142/09, JQ730747 both from tobacco, and strain 204/10, JX645766 from periwinkle) and one from Bosnia and Herzegovina (strain M7, KU374896 from corn).

Using the fTufAy/rTufAy and Stamp-F1/R1 primer pairs, the expected amplicons of approximately 950 bp and 480 bp, respectively in length were obtained for 52 selected '*Ca.* P. solani' infected sugar beets and other samples (Table 1). Sequences of a partial *tuf* and *stamp* genes were determined for all samples.

Multiple sequence alignment showed that the determined *tuf* gene sequences can be discriminated in two genotypes with a single nucleotide polymorphism (SNP) difference. One genotype was identical to a previously described '*Ca.* P. solani' *tuf* genotype tuf b1 (Aryan et al. 2014; Langer and Maixner 2004), but the other represents a new *tuf* genotype, since the SNP on position 174 after the *tuf* start codon ((G/A), which is synonymous (Glu) when translated) differentiates it from all genotypes described so far and those publicly available. Considering that the unique SNP affects a restriction site for the *Tai*I, *Set*I and *Hpy*CH4IV restriction enzymes, and that the tuf c genotype was described previously for a genotype differentiated with the *Hpa*II restriction enzyme (Langer and Maixner 2004), we named the new *tuf* genotype "tuf d". When compared to the sequences of the previously described '*Ca.* P. solani' *tuf* genotypes tuf b1, tuf b2 and tuf a (Aryan et al., 2014), the new genotype tuf d revealed one, two and three SNPs, respectively (Table 2). Virtual RFLP analysis demonstrated that the three restriction enzymes can differentiate the tuf d strains, the one prevalent in sugar beet, from all other publicly available '*Ca.* P. solani' strains, specifically genotypes tuf a, tuf b1 and tuf b2. RFLP analysis of the selected '*Ca.* P. solani' tuf b and tuf d strains with the *Tai*I restriction enzyme confirmed virtual RFLP results, allowing for rapid differentiation between the new tuf d and other tuf types. The difference shown in virtual RFLP analysis is clearly visible in the representative polyacrylamide gel run after RFLP reaction (Fig. 2).

316 The *stamp* gene sequences obtained from 52 samples in this work can be discriminated into
317 six genotypes (Table 1, Fig. 3). Most of the sugar beets (12/14) bear STOL genotype. Per one
318 sugar beet sample correspond to the Rqg50 and M5 genotypes. Genotype Rqg50 has shown
319 widest host range being detected in as many as 9/12 plant species analysed in this study: carrot,
320 parsley, tobacco, *C. arvensis*, grapevine, parsnip, celery, valerian and naturally infected
321 periwinkle (Table 1, Fig. 3). Genotypes Rpm35 and Rqg31 were sporadically detected in celery,
322 parsley and *C. arvensis* with no specific host preference or geographic pattern. In our study two
323 Vv24 isolates were detected in pepper and parsley (Table 1, Fig. 3). All sugar beet isolates
324 determined on the *stamp* gene as the STOL genotype are correlated to the newly detected tuf d
325 type, while the two samples bearing tuf b1 genotype corresponded to Rqg50 and M5. The same
326 situation was with parsnip and parsley. Unlike these samples, another four STOL isolates from
327 corn and pepper as well as other five *stamp* genotypes (Rqg50, Rpm35, Rqg31, Vv24 and M5)
328 are characterized on the *tuf* gene as the tuf b1 strains

329 **Disease prevalence, susceptibility of sugar beet cultivars to RTD.** Field evaluation in both
330 years indicated significant differences amongst the hybrids in terms of mean disease prevalence
331 (Fig. 4). In the both years Terranova was the most susceptible, Tesla, Eduarda and Original were
332 moderately susceptible, while Tajfun and Nansen were the group of the least susceptible
333 cultivars. Cultivars Nora and Leopolda were the most susceptible in 2018, with Nora showing a
334 poor germination in addition. Therefore the two were replaced with Sioux and Sixtus which
335 showed to be among the least susceptible in 2019. However, due to fact that both cultivar pairs
336 were analysed for only one year, they were excluded from the statistical analyses.

337 Average RTD prevalences in the plots were 23.7% and 3.32%, with maximal prevalences of
338 77.1% and 27.2%, respectively for 2018 and 2019. Although there is a difference between

prevalences in 2018 and 2019, given the occurrences per plot (Fig. 5), a clear pattern was discerned in the distribution of high RTD prevalence plots, which were for both years most prevalent at the borders of the experimental field.

DISCUSSION

Sugar beet RTD in Serbia was associated in the present study with '*Ca*. P. solani'. '*Ca.* A. phytopathogenicus' was not detected in any of the 134 symptomatic and asymptomatic sugar beets collected in Serbia, which at the very least excludes it as the main etiological agent of RTD, if present at all. Previously, RTD had been documented in literature in Serbia and neighboring countries as a destructive, yield-reducing disease which appears periodically in epidemic scales, with unknown etiology or associated to the abiotic factors, due to fact that no phytopathogenic fungi or bacteria could be isolated (Marić 1974; Marić et al. 1970). Almost strict correlation between the presence of '*Ca*. P. solani' in plants and their typical RTD symptomatology provides strong evidence that '*Ca*. P. solani' is the causal agent of this economically damaging disease in Serbia. While all 41 asymptomatic sugar beets tested negative, and 62 out of 63 symptomatic sugar beets tested positive, it is possible that the one symptomatic sugar beet tested negative due to its low phytoplasma titer, uneven distribution, the presence of PCR-inhibiting compounds or other technical challenges. '*Ca*. P. solani' was identified by using PCR amplification and sequence analysis of 16S rDNA, as well as the housekeeping gene *tuf* and a more variable single copy *secY* and *stamp* genes.

'*Ca*. P. solani' has long been known as a widespread problem on other host plants (grown and wild) in Serbia, but it has never been reported on sugar beet. It has been described on sugar beet

362 in France, but not yet in terms of causing significant disease. Actually, '*Ca*. P. solani' has been
363 occasionally (usually in fewer than 10% of SBR-affected samples) detected in SBR-affected
364 sugar beets in France (but not in Germany and Switzerland), besides its main pathogen '*Ca.* A.
365 phytopathogenicus', and it was not found to play a significant role in SBR epidemics (Gatineau et
366 al. 2002; Sémétey et al. 2007b). Remarkably, the symptoms of '*Ca*. P. solani' on sugar beet
367 described in this work in Serbia are strikingly different from those reported in France (Bressan et
368 al. 2008).

369 The symptoms observed on RTD-affected sugar beets in Serbia differ in relation to the most
370 characteristic symptoms for the two diseases, namely rubbery taproot and no vascular
371 discoloration in RTD, in contrast to firm taproot and brownish vascular discoloration in SBR.
372 RTD affects the yield and physical property of the roots, i.e. their ”sliceability”. Although there
373 are no quantitative criteria to assess the physical properties of sugar beet roots, such as
374 sliceability, in case of high prevalence (more than 5%), the RTD often results in a situation
375 whereby root quality does not match manufacturing requirements and therefore leads to the
376 rejection of a complete yield by the processing industry.

377 Bearing in mind that symptoms of RTD caused by '*Ca*. P. solani' on sugar beet in Serbia are
378 closer in similarity to those of sugar beet yellow wilt disease, caused by another phytoplasma
379 (16SrIII-J) in South America, than to those of SBR (Bennett and Munck 1946; Fernández et al.
380 2020; Vallejo 1970), notion of other diseases of unknown etiology with similar symptoms in
381 other parts of the world having a common causal agent-phytoplasma (e.g. the one reported in
382 Arizona, USA by Ruppel 1969) should be reconsidered. In terms of temporal (time of symptom
383 appearance and recurrent epidemics) and in-field spatial distribution (more severe nearer to the
384 field borders), RTD is reminiscent of other '*Ca*. P. solani'-induced diseases of annual crops in

Serbia, such as corn reddening and stolbur on pepper (Duduk and Bertaccini 2006). Since the RTD, similarly to other diseases of annual crops associated with *'Ca*. P. solani', appears with higher intensity in dry and hot years, and in temperate regions of the Pannonian Plain, it is expected to be strongly affected by climate changes, such as more frequent and intense summer heatwaves and droughts (Spinoni et al. 2015), and it raises the question as to whether the disease should be expected to reemerge more often and with greater severity in the future. Moreover, it raises concerns over its spreading to other regions of Europe under an enduring trend of climate change, both where *'Ca*. P. solani' is already present on sugar beet or on other hosts. The results of this study suggest that differences in hybrid susceptibility constitute a factor that should be taken into account when developing a strategy to control sugar beet RTD.

Sequence analysis of *secY* gene revealed no variability among 12 Serbian *'Ca*. P. solani' strains from sugar beet and was therefore not applied for the analyses of other samples. The *tuf* gene analysis revealed two stolbur tuf genotypes in Serbia. Beside the already reported one tuf b (b1) type, so far associated in Serbia with *C. arvensis* and *Crepis foetida* as phytoplasma infection sources (Kosovac et al. 2019), a new and distinct *tuf* genotype (named tuf d) was prevalent in sugar beet and present in *Apiaceae*. As *tuf* gene sequence variability has been reported to correlate with the epidemiological cycle and reservoir plant host (Aryan et al. 2014; Kosovac et al. 2016; Langer and Maixner 2004), the new *tuf* genotype raises the question as to whether the tuf d strains share biology with the tuf a/b2 (nettle), tuf b(b1) or with the one reported in France and associated with sugar beet/*P. leporinus*.

The most prevalent *stamp* genotype in analyzed sugar beet samples, STOL, had been initially isolated from *Capsicum annuum* from Serbia and associated with *H. obsoletus ex C. arvensis* (Aleksić et al. 1967; Fabre et al. 2011b). More recently it was reported in Serbia within the

epidemiologies of Bois noir, related to cixiids *Reptalus panzeri* and *H. obsoletus ex C. foetida*, and potato stolbur (Cvrković et al. 2014; Kosovac et al., 2019; Mitrović et al. 2016). Other two genotypes detected in per one sugar beet sample Rqg50 and M5, were both previously associated with the same hosts, grapevine and potato, and corresponding vector *H. obsoletus ex C. arvensis* (Atanasova et al., 2015; Mitrović et al., 2016). Additionally, genotype Rqg50 had been linked with two other vectors, *R. panzeri* and *R. quiniqueostatus* (Cvrković et al. 2014). In this study this genotype showed the widest host range, being detected in as many as 9/12 plant species analysed: carrot, parsley, tobacco, bindweed, grapevine, parsnip, celery, valerian and naturally infected periwinkle (Table 1). Genotypes Rpm35 and Rqg31 were in this work sporadically detected in celery, parsley and *C. arvensis* with no specific host preference or geographic pattern, while both *stamp* genotypes had also been associated with Bois noir and potato stolbur and *H. obsoletus ex C. arvensis* and *R. panzeri* as vectors (Cvrković et al. 2014; Mitrović et al. 2016). *Stamp* genotype Vv24 had been in the region mainly associated with grapevine (Atanasova et al. 2015; Cvrkovic et al. 2014; Kosovac et al. 2016), while in our study two Vv24 isolates were detected in pepper and parsley (Table 1). The six *stamp* genotypes obtained in this work (i.e. STOL, Rqg50, Rpm35, Rqg31, Vv24 and M5) (Table 1, Fig. 3), had been all described previously in Serbia (Atanasova et al. 2015; Cvrković et al. 2014; Fabre et al. 2011b; Mitrović et al. 2014). Typing of the *stamp* gene revealed affiliation of all analysed samples to the tuf b(b1) epidemiological pathway (Atanasova et al. 2015; Cvrković et al. 2014), whether they are belonging to the tuf b(b1) or the new tuf d genotype. However, differentiation of strains from sugar beet and the ones from Apiaceae based of *tuf* gene sequence analyses was corroborated by analyses based on *stamp* gene. Therefore, the main tasks in the future epidemiological research should be tracing of the insect vectors employed in different '*Ca*. P. solani' epidemiological routs in Serbia and

confirmation of their role by transmission trials, along with the survey on the presence of *P. leporinus*, the vector of both, SBR and '*Ca*. P. solani' on sugar beet in France.

Clarification of the etiology of RTD as a long-known and economically important disease in Serbia and neighbouring countries as a result of this work is certainly the first step towards disease management. Bearing in mind complex ecology of '*Ca*. P. solani', the acquired knowledge about the genetic relationship between the '*Ca*. P. solani' strain prevalent in sugar beet in Serbia and other strains on different plant species is crucial for further epidemiological work to identify insect vectors responsible for the disease, and possible plant reservoir hosts in the area. This is even more important, since the observed frequency of high prevalence plots at field borders suggests infections through vectors migrating from outside the field. Although the significance of finding a distinct genotype of the *tuf* gene is still to be completely evaluated, the unique property of the *tuf* gene of the strain prevalent in sugar beet in Serbia, differentiable by RFLP, can certainly help in further epidemiological work seeking to identify insect vectors and possible plant reservoir hosts of this prokaryote with an extremely wide host range.

ACKNOWLEDGMENTS

We thank agronomist Živko Vasin for helpful suggestions, sharing important experience and technical assistance in the field.

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598 Table 1. '*Ca.* P. solani' tuf and stamp genotypes detected in RTD affected sugar beet and strains
599 from other hosts

	Host	Year	Locality	tuf type	Stamp genotype*	Reference
9X	sugar beet	2019	Rimski Šančevi	d	STOL	This paper
2X	sugar beet	2019	Bačko Dobro Polje	d	STOL	This paper
	sugar beet	2019	Bačko Dobro Polje	b1	M5	This paper
	sugar beet	2019	Kačarevo	d	STOL	This paper
	sugar beet	2019	Srem	b1	Rqg50	This paper
	carrot	2010	Begeč	b1	Rqg50	Mitrović et al. 2013
	parsley	2009	Pančevo	d	STOL	Mitrović et al. 2013
8X	parsley	2009	Pančevo	b1	Rqg50	Mitrović et al. 2013
	parsley	2009	Pančevo	b1	Vv24	Mitrović et al. 2013
	parsley	2009	Pančevo	b1	284/09 (Rpm35)	Mitrović et al. 2013
	pepper	2010	Begeč	b1	Vv24	Mitrović et al. 2013
	pepper	2011	Pirot	b1	STOL	This paper
	tobacco	2010	Inđija	b1	Rqg50	Mitrović et al. 2013
2X	tobacco	2010	Bavanište	b1	Rqg50	Mitrović et al. 2013
	convolvulus	2017	Montenegro	b1	Rqg50	This paper
	convolvulus	2018	Pančevo	b1	231/09 (Rqg31)	This paper
	convolvulus	2018	Leskovac	b1	Rqg50	This paper
	grapevine	2010	Aleksandrovac	b1	Rqg50	Mitrović et al. 2013
	grapevine	2010	Bela Crkva	b1	Rqg50	Mitrović et al. 2013
3X	parsnip	2016	Rimski Šančevi	d	STOL	Medić Pap et al. 2017
3X	parsnip	2016	Rimski Šančevi	b1	Rqg50	Medić Pap et al. 2017

	corn	2009	Perlez	b1	STOL	Mitrović et al. 2013
	corn	2013	Bulgaria	b1	STOL	Genov et al. 2014
	corn	2013	Debeljača	b1	STOL	This paper
2X	celery	2013	Svilajnac	b1	Rqg50	This paper
	celery	2013	Svilajnac	b1	284/09 (Rpm35)	This paper
	celery	2013	Paraćin	b1	231/09 (Rqg31)	This paper
	valerian	2009	Pančevo	b1	Rqg50	Mitrović et al. 2013
2X	periwinkle	2019	Belgrade	b1	Rqg50	This paper

600 * previously published genotypes (Fabre, Danet, et al. 2011; Cvrković et al. 2014; Atanasova et

601 al. 2015)

602 Table 2. Differential nucleotides of the four *tuf* types and SNP positions

Strain (Acc. No.)	Tuf genotype	174 - *Tai*I (aa)	666 - *Hpa*II (aa)	727 (aa)
CrHo13_1183 (KJ469707)	a	A (Glu)	C (Thr)	G (Val)
CrHo12_601 (KJ469708)	b1	A (Glu)	T (Thr)	A (Ale)
CrHo12_650 (KJ469709)	b2	A (Glu)	T (Thr)	G (Val)
429/19 (MT157234)	d	G (Glu)	T (Thr)	A (Ale)

603 Fig. 1. Symptoms of sugar beet rubbery taproot disease. A, Yellowing of the oldest leaves. B, complete plant decline
604 (left). C, rubbery taproot. D, Sections of symptomatic taproots (left) show no difference to those of healthy taproots
605 (right) or discoloration.

606

607 Fig. 2. Analysis of the fTufAy/rTufAy amplicon. A, Restriction map showing positions of restriction enzyme
608 recognition sites in the tuf gene of '*Ca.* P. solani' tuf types a, b and d. Differential recognition sites are indicated by
609 arrows, while all other recognition sites are present in all tuf types assessed. Numbers are nucleotide positions
610 counted from the beginning of the fTufAy/rTufAy amplicon. B, Representative virtual and actual gels showing
611 patterns of 'Ca. P. solani' tuf types a, b and d digested with the *Tai*I restriction enzyme. Fragment sizes of marker and
612 the obtained fragments are indicated in bp. Reference strain sequences for tuf types in virtual RFLP are indicated in
613 Table 2.

614

615 Fig. 3. Genealogical network based on parsimony analysis obtained for six 'Ca. P. solani' stamp genotypes detected
616 in this study: STOL, Rqg50, Rpm35, Rqg31, Vv24 and M5, supplemented with additional genotype BG4560 (acc.
617 no. FN813252) previously detected in Serbia (Cvrković et al., 2014), but not found during this study. Each of the
618 seven *stamp* genotypes is represented with a circle proportional in size to the number of belonging isolates. Colors of
619 the circle sections correspond to the specific host plant as given in the legend. Red dashed line indicates on the
620 isolate group that corresponds to the newly detected tuf d genotype. Interconnecting dots represent nucleotide
621 differences between genotypes; more than 1 nucleotide difference is shown with corresponding number and
622 abbreviation “nt”.

623

624 Fig 4. Mean prevalence of RTD on sugar beet hybrids. Different lowercase letters indicate significant ($P < 0.05$)
625 differences in RTD prevalence. Vertical bars represent standard errors.

626

627 Fig 5. Schematic representation of the map of the experimental field. Each rectangle represents a plot, while numbers
628 and coloured rectangles represent prevalence (in %) in each plot. Empty rectangles in 2018 represent plots with poor
629 germination, excluded from analyses.

630

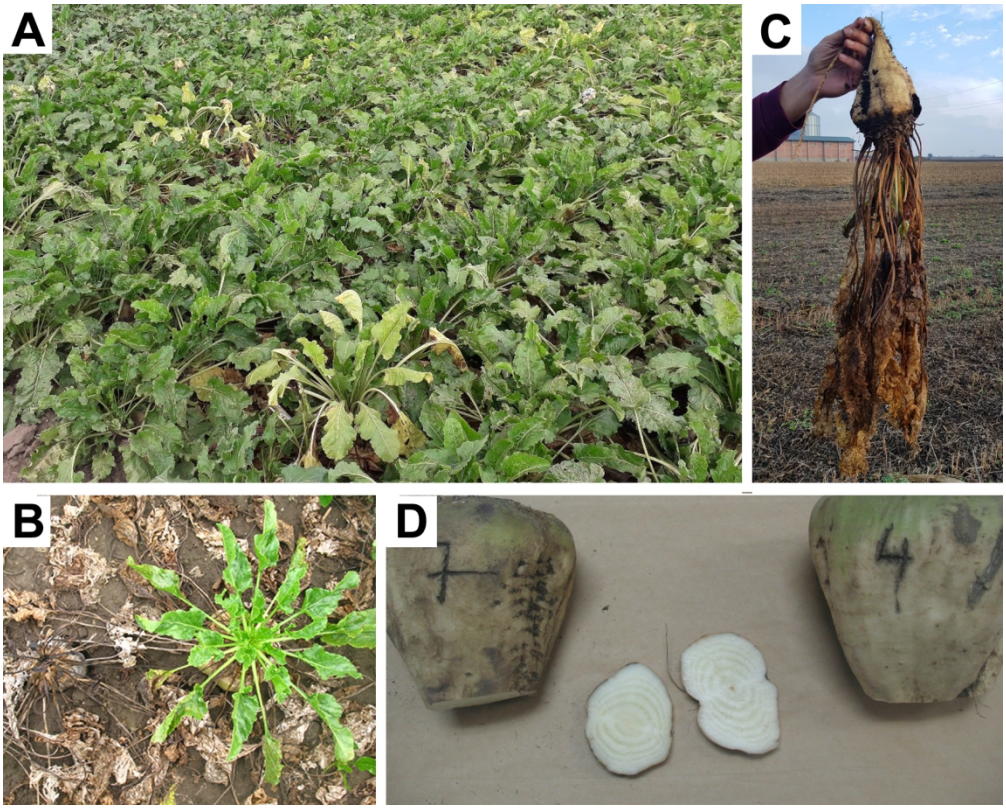

Fig. 1. Symptoms of sugar beet rubbery taproot disease. A, Yellowing of the oldest leaves. B, complete plant decline (left). C, rubbery taproot. D, Sections of symptomatic taproots (left) show no difference to those of healthy taproots (right) or discoloration.

74x59mm (600 x 600 DPI)

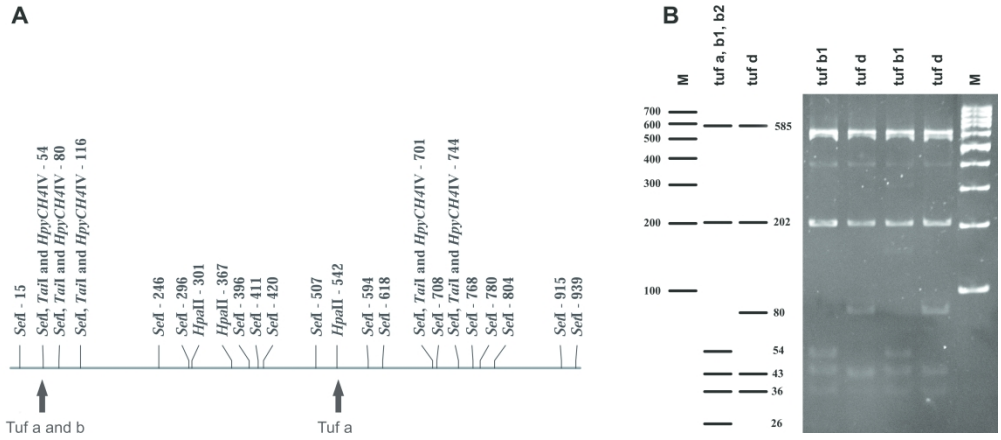

Fig. 2. Analysis of the fTufAy/rTufAy amplicon. A, Restriction map showing positions of restriction enzyme recognition sites in the tuf gene of 'Ca. P. solani' tuf types a, b and d. Differential recognition sites are indicated by arrows, while all other recognition sites are present in all tuf types assessed. Numbers are nucleotide positions counted from the beginning of the fTufAy/rTufAy amplicon. B, Representative virtual and actual gels showing patterns of 'Ca. P. solani' tuf types a, b and d digested with the TaiI restriction enzyme. Fragment sizes of marker and the obtained fragments are indicated in bp. Reference strain sequences for tuf types in virtual RFLP are indicated in Table 2.

268x116mm (600 x 600 DPI)

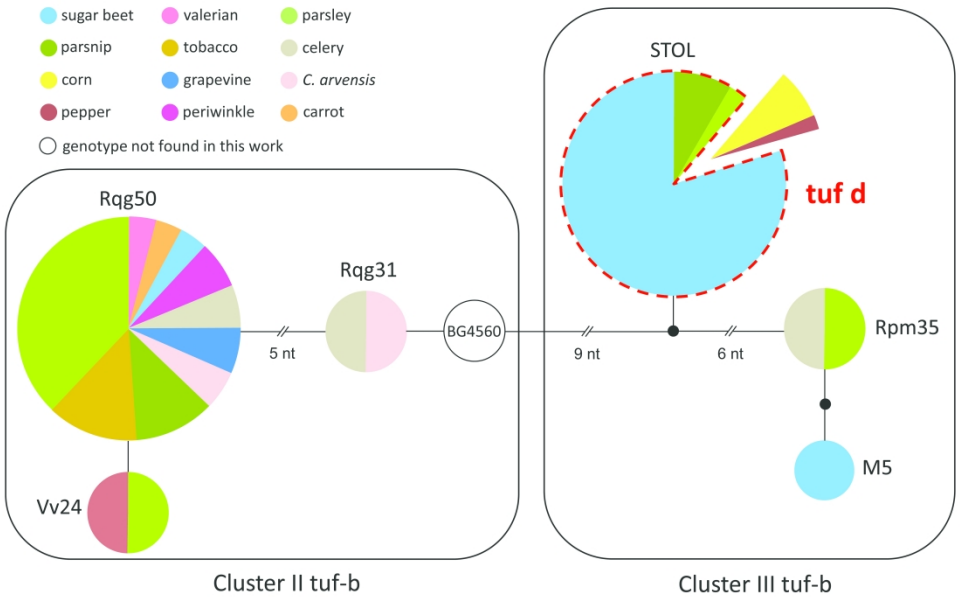

Fig. 3. Genealogical network based on parsimony analysis obtained for six 'Ca. P. solani' stamp genotypes detected in this study: STOL, Rqg50, Rpm35, Rqg31, Vv24 and M5, supplemented with additional genotype BG4560 (acc. no. FN813252) previously detected in Serbia (Cvrković et al., 2014), but not found during this study. Each of the seven stamp genotypes is represented with a circle proportional in size to the number of belonging isolates. Colors of the circle sections correspond to the specific host plant as given in the legend. Red dashed line indicates on the isolate group that corresponds to the newly detected tuf d genotype. Interconnecting dots represent nucleotide differences between genotypes; more than 1 nucleotide difference is shown with corresponding number and abbreviation "nt".

249x160mm (600 x 600 DPI)

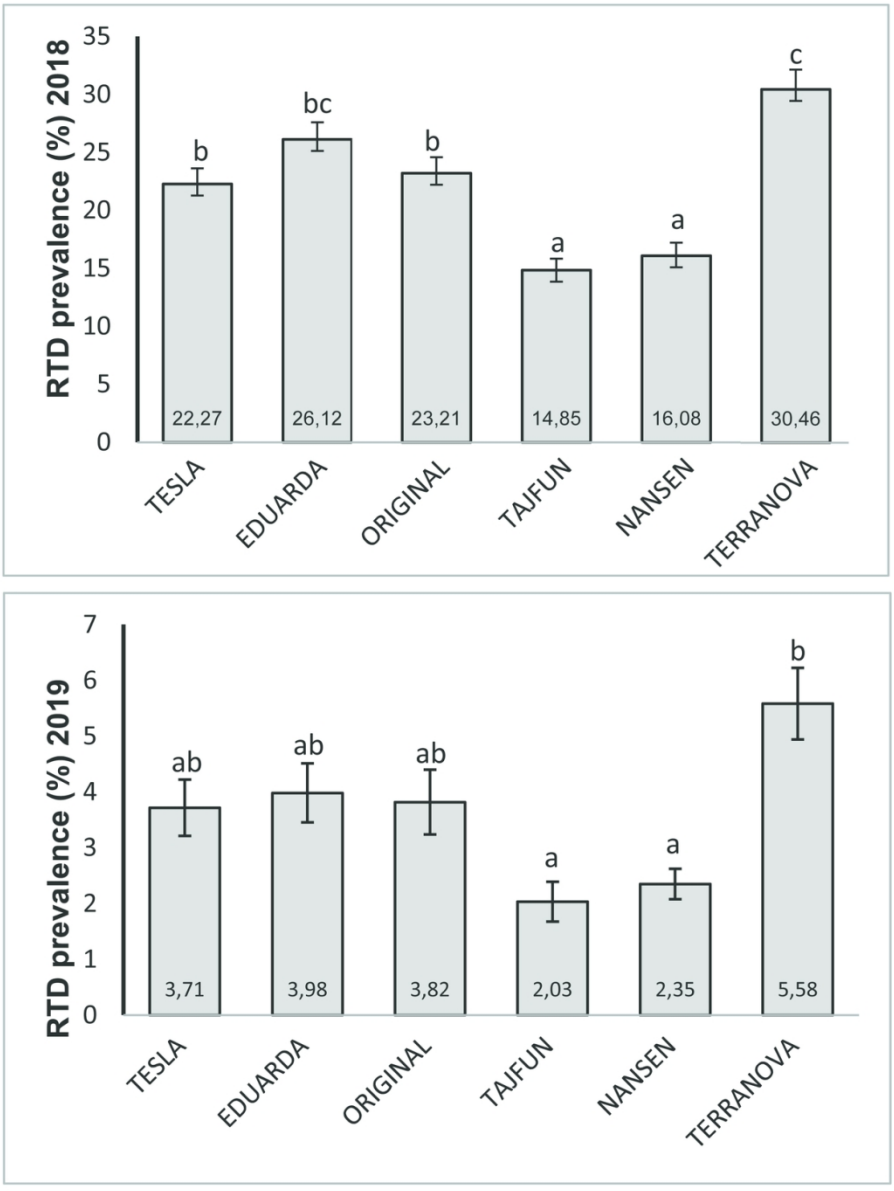

Fig 4. Mean prevalence of RTD on sugar beet hybrids. Different lowercase letters indicate significant ($P < 0.05$) differences in RTD prevalence. Vertical bars represent standard errors.

107x141mm (300 x 300 DPI)

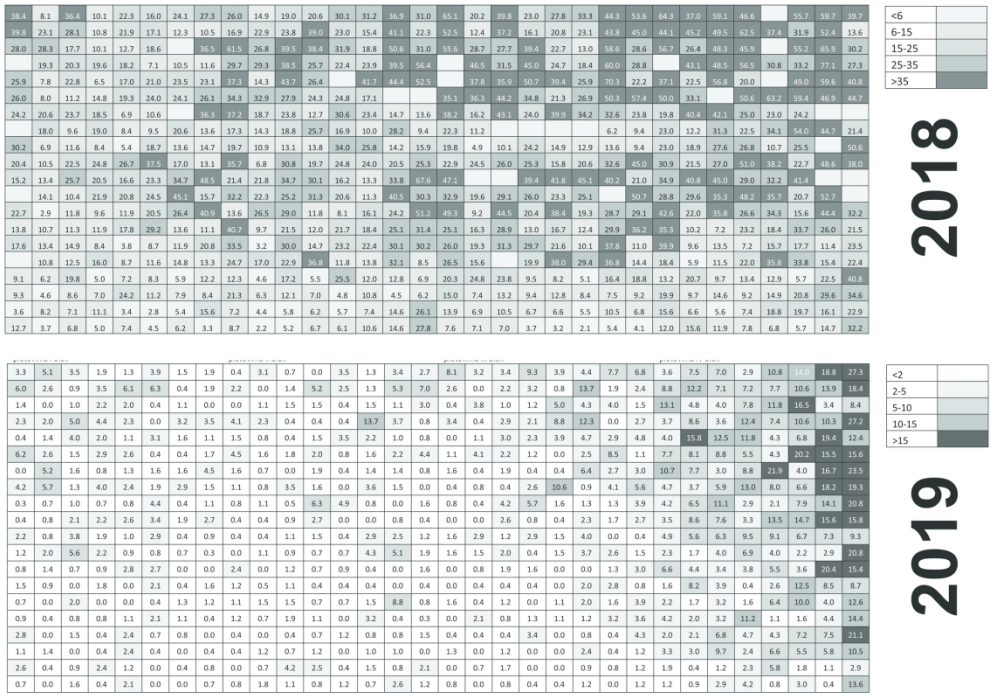

Fig 5. Schematic representation of the map of the experimental field. Each rectangle represents a plot, while numbers and coloured rectangles represent prevalence (in %) in each plot. Empty rectangles in 2018 represent plots with poor germination, excluded from analyses.

332x233mm (200 x 200 DPI)