CHARACTERIZATION OF *XANTHOMONAS ARBORICOLA* pv. *JUGLANDIS* ISOLATED FROM WALNUTS IN LITHUANIA

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SUMMARY

This study records the presence of bacterial walnut blight, caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*), on walnuts (*Juglans* spp.) in Lithuania and confirms that its geographic distribution has been underestimated. The pathogen caused the most serious symptoms on *J. cinerea* and *J. mandshurica* trees. DNA typing methods (rep-PCR and MLST) used for characterization of *Xaj* isolates disclosed a high similarity among them. Strains from Lithuania, however, showed a still not fully understood difference from *Xaj* strains from Poland, a neighbouring country, that needs further investigation.

Key words: Juglans spp., bacterial walnut blight, rep-PCR, MLST, diagnosis.

Lithuania is the northernmost country in Europe where walnuts are successfully overwintering, fruits ripen and can be harvested. Walnuts are grown mainly individually in private and botanical gardens and parks and in a limited number of nurseries (Navasaitis, 2004). Thus, walnuts have no economic importance in Lithuania, although the interest for the crop is growing and new species and varieties are introduced. At the moment, five walnut species and a few interspecific hybrids are grown in the country and the nuts harvested.

Bacterial walnut blight caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*) is one of the most serious and economically important diseases of walnut, occurring in all major walnuts-growing areas of the world (CABI-EPPO, 2001). There are no effective control methods, thus bacterial walnut blight may cause considerable yield losses (up to 70%) under favourable conditions for the disease (Gironde *et al*., 2009; Lang and Evans, 2010; Mulrean and Schroth, 1981). Till now, walnut bacterial diseases have not been investigated in Lithuania.

In the years 2008-2009, different disorders were observed on leaves and nuts of *Juglans* spp. trees. Leaves showed necrotic leaf veins and irregularly shaped, small, dark brown spots, gradually increasing in size (Fig. 1). Characteristic sunken brown to black spots with a chlorotic margin were found on fruits and exudates were observed under humid environmental conditions (Vasinauskiene *et al*., 2007). These symptoms corresponded to those of walnut blight caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*) as described in literature (Mulrean and Schroth, 1982) and bacteria, forming yellow colonies on semi-selective brilliant cresyl blue starch agar were consistently isolated from them.

The aim of this study was to identify and to characterize genetically the causal agent of bacterial walnut blight in Lithuania using genetic diversity analysis of the pathogen with nucleic acid-based techniques (rep-PCR and MLST).

Five walnut species, *Juglans cinerea* L., *J. mandshurica* Maxim., *J. regia* L., *J. nigra* L., *J. ailantifolia* Carr. and two hybrids, *J.* x *bixbyi* Rehd. (*J. cinerea* x *J. ailantifolia*) and *J.* x *quadrangulata* (Carr.) Rehd. (*J. cinerea* x *J. regia*) were inspected. Thirty plant samples showing typical blight symptoms were collected and analysed. A total of 59 *Xanthomonas*-like isolates were recovered from sample homogenized with mortar and pestle and streaked on brilliant cresyl blue starch agar (Mulrean and Schroth, 1981). When DNA extracts from all isolates were submitted to PCR using the *Xanthomonas* genus-specific primers X1 and X2 (Maes, 1993), 8 isolates yielded the *Xanthomonas*-specific PCR amplification product of *ca*. 480 bp. Five strains out of 8 were identified as *Xaj* after PCR with the pathovar-specific primers XajF and XajR (Gironde *et al*., 2009), showing the expected DNA product of 216 bp.

The five *Xaj*-specific PCR-positive isolates were ultimately identified through pathogenicity, biochemical and physiological tests. Strain *Xaj* LMG 746 and data from the literature served as reference (Klement *et al*., 1990; Lelliott and Stead, 1987; Schaad *et al*., 2001). *Xaj*-like isolates grew on potato dextrose agar, yeast extract-glucose-calcium carbonate and yeast extract-dextrose-calcium carbonate agar as slimy yellow colonies (Lelliott and Stead, 1987). All isolates were Gram-negative with oxidative metabolism of glucose only, did not produce oxidase and catalase, grew at 37°C, hydrolysed starch

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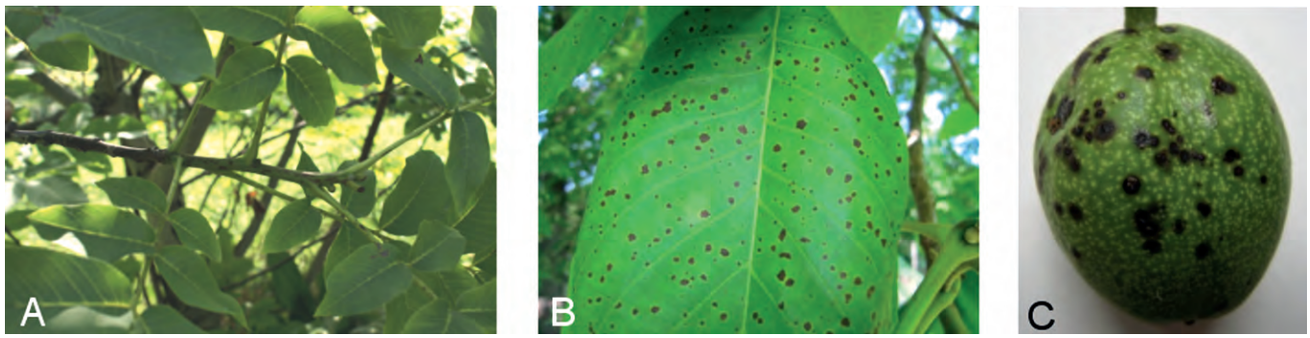

Fig. 1. Symptoms of bacterial blight on walnut shoots (A), leaves (B) and fruit (C) observed in Lithuania in 2009.

and esculin, induced hypersensitive reactions on tomato leaves (Schaad *et al*., 2001; Lelliott and Stead, 1987), caused necrosis on immature walnut fruits when tested according to Aletà *et al*. (2001) and grew on succinate quinate medium (Lee *et al*., 1992). All these features were comparable to those of the reference strain LMG 746. Thus, based on disease symptoms and the characteristics they were tested for, the five isolates in question were identified as *X. arboricola* pv. *juglandis*, and denoted NRCIB X1, NRCIB X2, NRCIB X3, NRCIB X4 and NRCIB X5.

To determine their genetic diversity multilocus sequence typing (MLST) and repetitive sequence-based polymerase chain reaction (rep-PCR) were conducted on total DNA prepared according to Aljanabi and Martinez (1997). As reference, 4 known *Xaj* strains from Poland (RIPF GH1, RIPF X04, RIPF X06, RIPF X07) and 3 strains from other European culture collections, LMG 746 (UK), CFBP 7179 (France), I-391 (Portugal) were used.

In our MLST study partial sequences of three genes were investigated, i.e. *gyrB* (DNA gyrase subunit B), *fyuA* (tonB-dependent receptor) and *rpoD* (RNA polymerase sigma factor). The fragment of *gyrB* gene was amplified according to Parkinson *et al*. (2007). PCR products from *fyuA* and *rpoD* genes were obtained using the primers described by Young *et al*. (2008). Sequences of *Xaj* strains from Lithuania were deposited in GenBank with accession numbers HE610436 to HE610450. Concatenated sequences of sequenced loci with a total length of 1,939 bp were submitted to phylogenetical analysis using the MEGA 5 software package

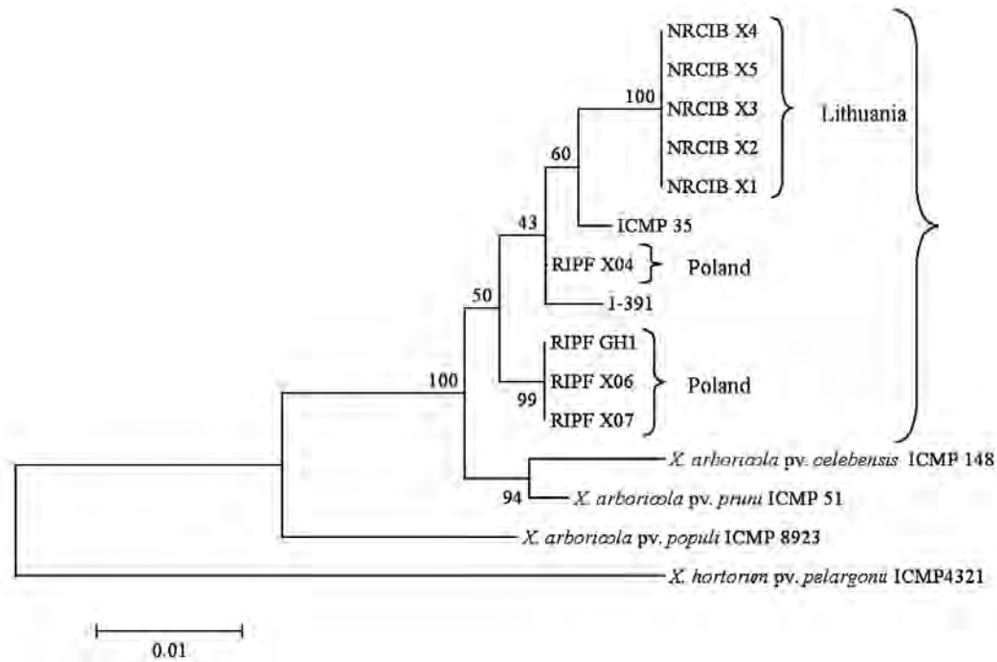

Fig. 2. Maximum likelihood tree showing the phylogenetic relationship between strains *Xaj* strains from Lithuania, Poland and other reference strains based on concatenated sequences of genes *fyuA*, *gyrB* and *rpoD*. Bootstrap values (expressed as percentages of 1000 replications) are given at the nodes. Bar – estimated nucleotide substitutions per site is 0.01.

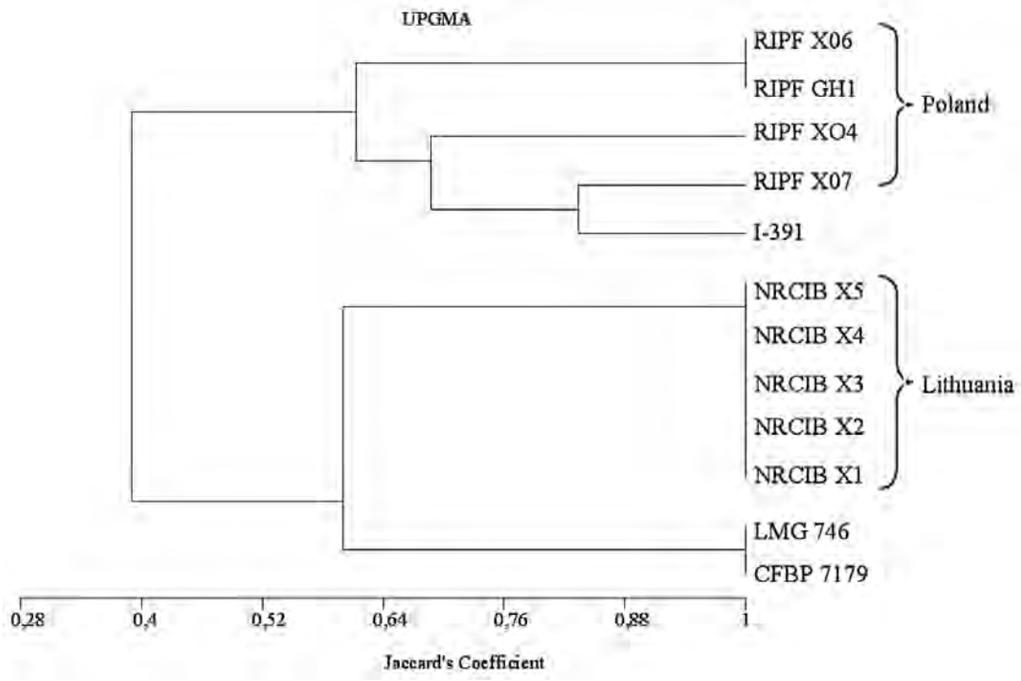

Fig. 3. Dendrogram of relationships between *Xanthomonas arboricola* pv. *juglandis* strains analysed by rep-PCR using primers BOX, ERIC and REP. The results per strain were clustered by an unweighted average pair group method (UPGMA) using Jaccard's coefficient.

with the Maximum likelihood method and the Tamura-Nei model (Tamura *et al.,* 2011). Bootstrap analysis with 500 replicate data sets was performed to assess the validity of the clusters found.

All *Xaj* strains were grouped in one monophyletic cluster the similarity of their concatenated sequences ranging from 98.2 to 100%. The Lithuanian strains had identical sequences for all tested genes. There was a difference with sequences of strains from other countries, but the level of similarity was still high, e.g. for Polish strains it was 98.3-100%, 97.3% and 100% in the case of genes *fyuA*, *gyrB* and *rpoD*, respectively (Fig. 2).

For rep-PCR, carried out according to Louws *et al.* (1994) and Versalovic *et al.* (1991, 1994), the primers targeting the conserved repetitive sequences REP, ERIC and BOX were used. Rep-PCR-amplified bands were scored as 1 (present) or 0 (absent) for all strains. Only reproducible, unambiguous, and clear-cut genomic fingerprints were taken into account. Reproducibility of the rep-PCR results was tested by repeating the reactions at least twice under the same conditions. No changes in DNA fingerprints were observed in any of the replicated experiments. Cluster analysis was performed using Free Tree software (Hampl *et al.*, 2001) and dendrograms were constructed from Jaccard's similarity coefficient data by UPGMA.

Rep-PCR was suitable for the molecular characterization of *Xaj* strains (Fig. 3). Fingerprints obtained in our assays disclosed a substantial genetic diversity among the few *Xaj* strains studied, coming from two different geographical areas (Lithuania and Poland). Fingerprint profiles generated with BOX, REP or ERIC primers, were different for Lithuanian and Polish strains of *Xaj* and two clusters were observed. REP PCR primers were the best for discrimination of *Xaj* (Fig. 4). No differences were

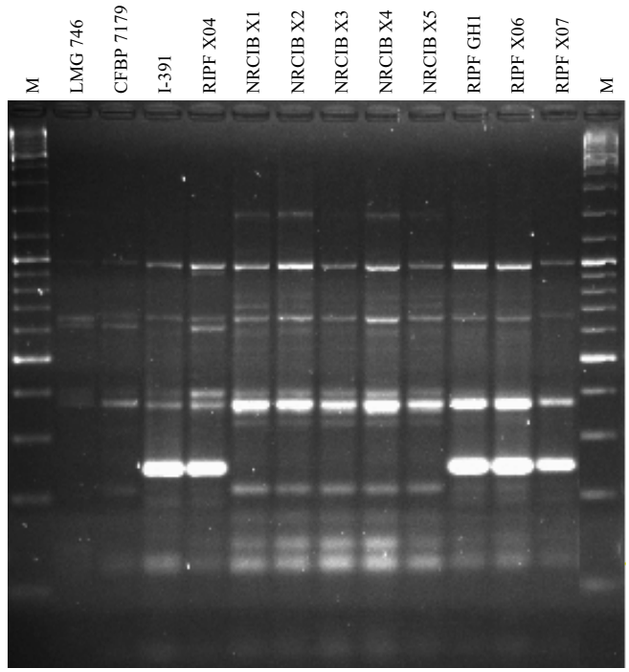

Fig. 4. Characteristic rep-PCR fingerprint patterns of *Xaj* using REP PCR primers.

observed among the 5 Lithuanian strains, even though they were isolated from different *Juglans* species, i.e. *Juglans cinerea* (NRCIB X1 and NRCIB X2) and *J. mandshurica* (NRCIB X3, NRCIB X4 and NRCIB X5).

In the present study, *Xaj* was isolated from *J. cinerea* and *J. mandshurica* in Lithuania but not from other *Juglans* species that showed symptoms resembling those of walnut blight but were infected by *Melanconium oblongum* Berk. and *Gnomonia leptostyla* (Fr.) Ces. et De Not., as shown by the successful recovery of these fungi.

In our hands, both MLST and rep-PCR proved useful for the characterisation of bacterial isolates at the pathovar level and for discriminating strains within the pathovar. The genetic diversity between *Xaj* strains had already been studied using AFLP, rep-PCR, MLST (Loreti *et al.*, 2001; Marcelletti *et al.*, 2010; Rademaker *et al.*, 2005) and some differences between populations were found. Some relation between genetic characteristics geographical origin and virulence of the pathogen was found in one study (Scortichini *et al.*, 2001). Marcelletti *et al.* (2010) after analysis of four housekeeping genes of 45 *Xaj* strains did not support the notion that the genetic diversity of the strains is associated with geographical isolation, in contrast with our finding that Lithuanian *Xaj* isolates could be discriminated from some Polish ones. However, to what extent the observed difference is to be attributed to geographical separation remains to be elucidated with further studies using more strains.

Our study shows that in Lithuania, as in many other European countries, walnut trees are vulnerable to walnut blight caused by *Xaj*. Since walnut blight have not been investigated in Lithuania so far, the present investigation is epidemiologically important and provides new information on the spread of this disease and pathogen in Europe. A better understanding of the structure and dynamics of pathogenic bacteria populations may stimulate the development of more efficient control measures and of more advanced and specific diagnostic protocols.

ACKNOWLEDGEMENTS

This work was conducted within the framework of COST Action 873 and was supported by the Science Council of Lithuania and the Polish Ministry of Science and Higher Education Grant 118/N-COST/2008/0.

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