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Assessment of Bacterial Blight Tolerance of Persian Walnut Based on Immature Nut Test

Attila BANDI[1, 2*], Mária HEVESI[1], Zsolt SZANI[3], Magdolna TÓTH[1]

[1]Corvinus University of Budapest, Faculty of Horticultural Science, Department of Pomology, Villányi str. 29-43, Budapest, Hungary; attila.bandi@uni-corvinus.hu; maria.hevesi@uni-corvinus.hu; magdolna.toth@uni-corvinus.hu
[2]Sapientia University, Faculty of Technical and Human Science, Department of Horticulture, Sighişoara str. 1/C, Tîrgu-Mureş, Romania; bandiattila@ms.sapientia.ro (*corresponding author)
[3]National Food Chain Safety Office, Variety Testing Department for Horticultural Crops, Keleti K. str. 24, Budapest, Hungary; SzaniZs@nebih.gov.hu

Abstract

Bacterial blight is one of the most serious diseases affecting Persian walnut (*Juglans regia* L.). Susceptibility to this disease was evaluated by artificial inoculation in an *in vitro* experiment for cultivars developed in Hungary and for selections from Transylvania. Thirty pieces of immature fruit of 11 walnut cultivars and 10 selections were inoculated by punction using a suspension containing a mixture of *Xaj*-isolates of controlled virulence. As control, a moderate resistant (mR) 'Pedro' and a highly susceptible (hS) 'Milotai intenzív' cultivars were used. After ten days the diameter of the necrotic area around the inoculation points was measured and the disease rate (DR) was noted. For the calculation of indexes it was taken the structure of the lesions (diffuse or defined margin) into consideration. None of the 21 cultivars/selections analysed were found to be hardly resistant, although all of them showed a higher degree of resistance than the susceptible control ('Milotai intenzív'). The majority of the analysed cultivars fit into the moderate susceptible (mS) and moderate resistant (mR) group. 'Milotai kései' and SZEN-10, had a notable reaction as considered to be moderate resistant (mR), showed a similar degree of resistance compared to the control cultivar 'Pedro'. It was concluded that these cultivars, based on their resistance to *Xanthomonas arboricola* pv. *juglandis,* could be proposed as resistance gene sources, as well as for production purposes in the environmental conditions of the Carpathian Basin.

Keywords: artificial inoculation*, in vitro,* isolates, *Juglans regia* L, stress*, Xanthomonas*

Introduction

Walnut trees may suffer stress from abiotic or biotic environmental factors, which can lead to crop failure. The bacterial blight disease, *Xaj* (*Xanthomonas arboricola* pv. *juglandis* (Pierce)) (Vauterin *et al*.,1995) is one of the most serious diseases affecting Persian (English) walnut *(Juglans regia* L.) (Loreti *et al*., 2001). The disease has been known since the end of the 19th century (Ferraris, 1927) and is widespread in walnut growing areas. It causes severe damage to leaves, twigs, buds, petioles, rachides, male and female catkins, nutlets and kernels, and is considered to be a major cause of reduction in fruit yield and tree vigour (Belisario *et al*., 1999). The damage produced by this pathogen is predisposed by wet springs. Rainy springs, dew and continual high humidity conditions are favourable to the development of severe blight, resulting in significant crop loss (Belisario, 1997). If this happens just before and after the flowering time, it may cause losses of 50% to 80% of the crop (Charlot and Radix, 1993). The bacteria enter the walnut tissues through natural openings, such as stomata and damaged tissues (Sharma and Sharma, 1999). All succulent new growths are reported to be very susceptible to the disease, becoming more resistant when maturing (Belisario *et al*., 1997).

Susceptible cultivars may have an increased level of damage on new plantings. Soon after the walnut blight was first described (Pierce, 1901), there were many reports of resistance to the disease (Soltani and Aliabadi, 2010). Resistance of trees has been evaluated in orchard condition, so it has not been clarified whether the absence of blight was due to disease escape or to an unknown form of resistance. A different susceptibility to walnut blight within walnut cultivars has been reported. Several authors, Aletà and Ninot (1993), Germain (1997), Woeste *et al*. (1992) have discovered no resistant cultivars; however 'Franquette' and 'Hartley' were reported as resistant cultivars (Belisario, 1997; Belisario *et al*., 1999).

Using the *in vitro* method, assessment of the resistance of walnut genotypes to bacterial blight has been carried out in several studies (Belisario, 1997; Belisario *et al*., 1999; Martins, 1997; Soltani and Aliabadi, 2010; Özaktan *et al*., 2007; Tsiantos *et al*., 2008). Detailed cultivar examination offers the potential of reducing the disadvantageous influence of the main stress factors (Szani, 2009). Searching for the sources of resistance is important, since large walnut trees are not easy to treat, and furthermore, increasingly copper-resistant *Xaj* strains are developing (Solar *et al*., 2007).

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Table 1. Examined walnut cultivars and selections

Cultivar or selection denomination	Parentage	Origin
Hartley	Franquette x Mayette[a]	USA
Pedro	Conway Mayette x Payne[a]	USA
Milotai 10	Selection	HU
Alsószentiváni 117	Selection	HU
Alsószentiváni 118	Selection	HU
Milotai intenzív	Milotai 10 x Pedro[b]	HU
Milotai kései	Milotai 10 x Pedro[b]	HU
Milotai bőtermő	Milotai 10 x Pedro[b]	HU
Alsószentiváni kései	Alsószentiváni 117 x Pedro[b]	HU
Bonifác	Alsószentiváni 117 x Pedro[b]	HU
M-10-25	Milotai 10 x Pedro[b]	HU
SZEN-10	selection	RO
ALB-22	selection	RO
FFA-11	selection	RO
OZSD-37	selection	RO
SAR-33	selection	RO
SOM-50	selection	RO
SOM-90	selection	RO
SOM -101	selection	RO
SOM-120	selection	RO
SOM-274	selection	RO

[a]Tulecke and McGranahan, 1994
[b]Szentiványi, 1990

Table 2. The evaluation of disease severity based on the infection of immature walnut fruits

Disease appearance/diameter of spots (mm)	Scale of susceptibility
No symptoms	0
Less than 1.00 mm, superficial and diffuse spots on the inoculation point	1
Slight blackening on the inoculation point of nut by 1.01 mm to 2.00 mm	2
Blackening on the inoculation point of nut by 2.01 mm to 5.00 mm of delved spot	3
Blackening on the inoculation point of nut more than 5.01 mm of crater- type spot	4

Based on the results of previous evaluations and open field examinations (Thiesz *et al.,* 2007, 2009; Szani, 2009), there was identified a different degree of susceptibility/resistance in the analysed populations and registered cultivars. However, in most open field assessments, the disease was detected by means of the leaf. The degree of fruit infection has been investigated to a lesser extent. Therefore, it was considered necessary to carry out artificial inoculation in controlled circumstances. The aim of the study was to evaluate the susceptibility to *Xaj* of the cultivars improved in Hungary, and of the selections from Transylvania, by means of an artificial inoculation technique in an *in vitro* experiment.

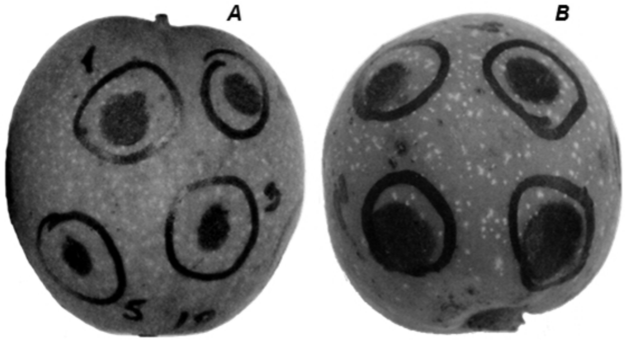

Fig. 1. The results of pathogenicity test on immature walnut fruit (A: SOM-120; B: ALB-22)

Materials and methods

Plant material

During the research between 2010 to 2013, it was evaluated the degree of susceptibility of the cultivars listed in Table 1, in comparison to 'Pedro', moderate resistant (mR) and 'Milotai intenzív' highly susceptible (hS), used as control cultivars. The samples were collected before shell hardening in June, from the Trial Station of the National Food Chain Safety Office at Pölöske (HU) and from farmer orchards in Transylvania (RO).

Bacteria

For the artificial inoculation there were collected 61 isolates from different walnut-growing areas of Hungary and Transylvania. The ability to induce hypersensitive reaction was examined on the tobacco leaf - *Nicotiana tabacum* L. (Klement, 1963) - and bean pod (*Phaseolus vulgaris* L.). The pathogenicity test was controlled on immature walnut fruits (Fig. 1). For the artificial inoculation, it was used a mixture of isolates from a homogeneous bacteria population (based on the phenotype) from the green husk of two walnut fruits, one originating from Hungary B02489 (HU), and the other from Transylvania B02490 (RO). They showed the same degree of virulence. The biochemical identification of the two isolates (henceforth, 'strain') was already carried out in previous studies (Bandi *et al.*, 2014).

Inoculation

For the artificial inoculation it was adjusted a suspension of 10^8 cell.mL^{-1} concentration utilising a spectrophotometer (570 nm wavelength) from the respective strains. The susceptibility

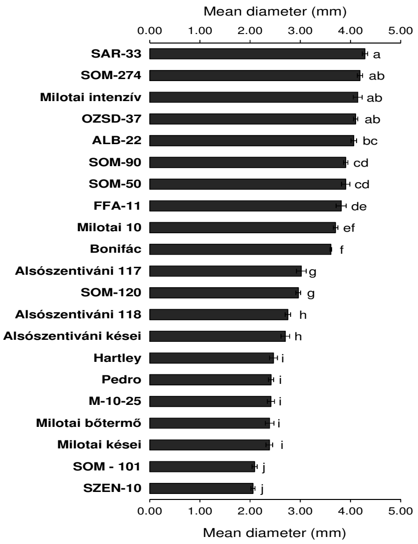

Fig. 2. Mean diameter of spots produced by two *Xaj* isolates around the inoculation (2010-2013)

Values with the different letter indicate cultivars that differ significantly (Duncan's test, p≤0.05); The error bars show SE of the mean

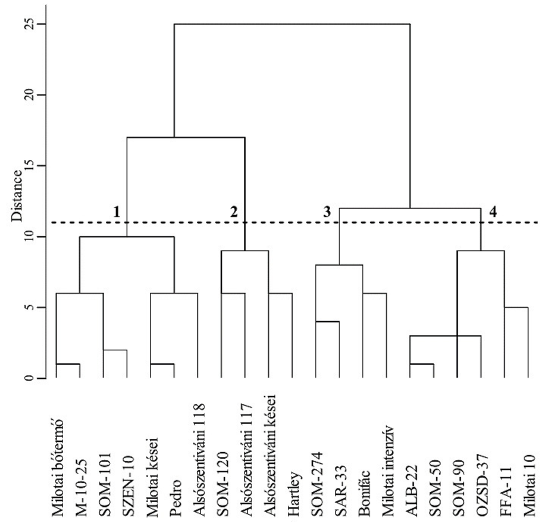

Fig. 3. Dendogram based on the average linkage (between groups) method of cluster analysis by means of necrotic spot diameter and disease rate of the walnut blight infection for walnut cultivars (2010–2013)

Numbers on the dendogram indicate different groups: 1: moderate resistant (mR), 2: moderate susceptible (mS), 3: susceptible (S), 4: highly susceptible (hS)

tests were conducted based on the method of Özaktan *et al.* (2008).

During the investigation, 30 crops (20 for artificial inoculation and 10 for control treatment) were collected. Each of the 20 fruit was injected in five (0.5 cm^2) places with a 20 μl bacterial suspension. The susceptibility of each cultivar was evaluated for 100 instances of infection. The control fruits were inoculated with sterile distilled water. The inoculated fruits were then placed in closed transparent plastic boxes in order to assure an adequate humidity (85%) and temperature (26-28 °C).

Assessment of the susceptibility of fruits

After inoculation, the fruits were monitored continuously, and on the tenth day the symptoms were summarised. In order to determine the mean diameter of the necrotic area, a digital slide-calliper attached to a computer (providing accuracy to the millimetre) was used. To determine the degree of the susceptibility, there were taken into consideration all the developed symptoms, as well as those that did not appear. After this, the dimensions of the necrotic areas were evaluated based on the five-stage scale developed by Özaktan *et al.* (2008) (Table 2).

Determination of cultivar susceptibility

Using data from the infection scale and the formulas found in the related literature, different indexes were produced. The disease rating (as an index showing the incidence of the disease) was determined by using data from the infection scale and the following formula by Bertrand and Gottwald (1986):

Disease rate=$\sum(a_i x f_i)/n$

where a_i- scale value (index of infection), f_i- frequency referring to the disease rate; *n*- the number of analysed fruit per cultivar.

Statistical evaluation

The data were evaluated using the SPSS software (IBM SPSS 22.0, Chicago, IL). The statistical analyses were determined based on the sample size, distribution analysis (Kolmogorov-Smirnov test) and equivalence of deviation (Levene test). Where the condition of normality was fulfilled, and means proved to be homogeneous, Tukey's multiple range test was used. If homogeneity of distribution was not fulfilled, differences were analysed using the Games-Howell test. In case of an abnormal distribution, the Kruskal-Wallis non-parametric method was applied; then, for the comparison of the pairs, the Mann-Whitney test was used.

It was accepted the p≤95% confidence level. The results were given as average ± standard error (SE). It was conducted a hierarchical cluster analysis based on four-year data regarding the diameter and disease rate, in order to classify the cultivars into susceptibility groups. The results were represented on a dendogram.

Results and discussions

The susceptibility of the analysed cultivars was evaluated in different groups according to the experimental year. Table 3 contains annual mean values regarding the disease rate. In the case of several cultivars, major differences between the annual results were noted. Therefore, further research will be required to clarify the susceptibility of 'Alsószentiváni 118' and 'Milotai kései'.

Based on the results of the artificial inoculation from 2010, compared to other cultivars, the mean disease rate value of SZEN-10 was significantly lower ($p<0.05$), which means it can be designated to the moderate susceptible class. OZSD-37, SOM-90, ALB-22 and SOM-50 cultivars proved to be highly susceptible. In the case of data from 2011, the Mann-Whitney test classified the cultivars with the average disease rate value under 2 into the homogeneous group (moderate susceptible), being significantly different ($p\leq0.05$). Considering the 2012 disease rate results, the susceptibility of cultivars showed a better differentiation. Based on the statistical evaluation of data, SOM-101 and SZEN-10 proved

Table 3. Disease rate of the analysed walnut cultivars in the individual years[a]

Cultivar or selection	Disease rate (0-4)			
	2010[b]	2011[c]	2012[b]	2013[c]
Hartley		2.91±0.03[a-c]	2.19±0.04[g]	
Pedro		1.64±0.21[gh]	2.10±0.03[gh]	
Milotai 10		2.48±0.24[de]	2.90±0.05[a-d]	
Alsószentiváni 117		2.39±0.16[d-f]	2.64±0.05[de]	2.88±0.10[d]
Alsószentiváni 118		1.52±0.38[gh]	2.53±0.02[ef]	
Milotai intenzív	2.55±0.20[a-c]	2.57±0.24[b-e]	2.80±0.08[b-e]	
Milotai bőtermő		2.31±0.06[ef]	2.18±0.08[g]	
Milotai kései		1.29±0.29[h]	2.16±0.01[g]	
M-10-25		1.91±0.04[gh]	2.21±0.01[fg]	
Alsószentiváni kései		2.72±0.03[a-c]	2.21±0.05[fg]	
Bonifác	2.13±0.40[bc]	2.50±0.08[c-e]	2.95±0.01[a-d]	
ALB-22	3.09±0.08[a]	3.09±0.03[a]	2.96±0.02[a-d]	3.06±0.00[a]
FFA-11	2.58±0.12[a-c]	2.95±0.03[a-c]	2.93±0.03[a-d]	3.00±0.00[bc]
OZSD-37	3.01±0.01[a]	3.08±0.01[a]	2.98±0.03[a-c]	3.01±0.01[b]
SAR-33	2.73±0.23[ab]	2.73±0.23[a-c]	3.06±0.07[ab]	3.10±0.05[a]
SZEN-10	1.85±0.10[c]	2.24±0.09[fg]	1.89±0.13[gh]	2.48±0.21[e]
SOM-50	3.15±0.06[a]	2.91±0.08[a-c]	3.10±0.06[ab]	2.99±0.01[c]
SOM-90	3.08±0.01[a]	3.00±0.02[ab]	3.20±0.06[a]	3.00±0.00[bc]
SOM -101	2.37±0.05[a-c]	1.61±0.09[gh]	1.81±0.09[h]	2.30±0.03[f]
SOM-120	2.41±0.12[a-c]	2.79±0.06[a-d]	2.69±0.06[c-e]	2.88±0.03[d]
SOM-274	2.36±0.09[a-c]	3.00±0.12[ab]	2.95±0.11[a-d]	3.00±0.00[bc]

[a]The mean values±SE of the cultivars in a given year
[b]The different letters indicate the significant difference between the cultivars ($p \leq 0.05$; Tukey's multiple range test)
[c]The different letters indicate the significant difference between the cultivars ($p \leq 0.05$; Mann-Whitney test)

to be moderate resistant (mR), while moderate susceptibility (mS) was detected for 'Pedro', 'Milotai kései', 'Milotai bőtermő', 'Hartley', M-10-25 and 'Alsószentiváni kései' cultivars. Based on the average data from 2013, similar to the results of previous years, it was determined a significantly low susceptibility in the case of SOM-101 and SZEN-10.

Fig. 2 contains the mean data for the diameter of the necrotic spots. 'Milotai intenzív', included in the experiment as the susceptible control, proved to be the third most susceptible. Similar, highly susceptible cultivars were SAR-33, SOM-274, OZSD-37 and ALB-22. Compared to the moderate resistant (mR) 'Pedro' control cultivar, SZEN-10 and SOM-101 showed a major resistance and these differences were statistically significant ($p < 0.05$).

The dendogram (Fig. 3), containing the annual diameter of the necrotic spots and the disease rate values, differentiated the cultivars belonging to certain susceptibility classes – from the susceptible group to less susceptible ones. The results from different years regarding the degree of susceptibility/resistance of cultivars showed a correspondence for the majority of cultivars; in the case of those cultivars, which showed a major difference between the annual results, it was accepted the classification from the dendogram.

Rovira *et al.* (2007) considered the artificial inoculation applied to be important because open field recordings of cultivar susceptibility are not sufficient.

The reaction of the studied cultivars to the *Xaj* bacteria was measured for the first time using *in vitro* experiment with an inoculation method; therefore, only the control cultivar data can be compared to previous results. Several studies provide information regarding the degree of susceptibility to bacterial blight of 'Hartley' and 'Pedro'. Özaktan *et al.* (2007, 2008) evaluated the susceptibility of several cultivars with artificial inoculation, and based on data from 2007, it was determined a low susceptibility for 'Pedro' (blight severity=14.1%) and a moderate susceptibility according to data from 2008 (blight severity=55%). In the current experimental data, in 2011 'Pedro' showed a moderate resistance, while in 2012 it was determined moderate susceptible. Information about the susceptibility of 'Hartley' and 'Milotai 10' cultivars can be obtained from the results of Tsiantos *et al.* (2008). Based on the 2007 and 2008 Varietal Susceptibility Index (VSI) values and the susceptibility classes, 'Hartley' proved to be moderate susceptible (VSI = 25; Class of Susceptibility=B), while 'Milotai 10' was found susceptible (VSI = 80, Class of Susceptibility = D). Solar *et al.* (2008, 2009) also determined a moderate susceptibility in case of 'Hartley' cultivar. According to the current results 'Hartley' turned out to be susceptible in 2011, yet moderate susceptible in 2012.

Thiesz *et al.* (2007) considered the Transylvanian cultivars to have a low susceptibility against *Xaj*. According to the seven year results of Szani (2009), cultivars improved in Hungary showed a low or moderate infection rate. Based on the annual measurements it can be concluded that the majority of analysed cultivars were moderate susceptible (mS) and susceptible (S). Among the cultivars improved in Hungary, 'Milotai kései', 'Alsószentiváni 118', M-10-25 and 'Milotai bőtermő' showed a low degree of susceptibility. Among the Transylvanian selections, SZEN-10 and SOM-101 showed a notable reaction, since according to the annual results they were determined with moderate resistance (mR) and a moderate susceptibility (mS).

Conclusions

The present research provided comprehensive results regarding the degree of susceptibility/resistance of the studied cultivars. Previous open field *Xaj* assessments based on the leaf quality proved that the cultivars have a good resistance to the disease. However, during the current experiment regarding artificial inoculation of immature fruits, it was proved that several cultivars have *Xaj* susceptibility. Therefore, the evaluation based on the leaf was found not reliable. Thus it may be concluded that the 'Pedro' transmitted the *Xaj* resistance into certain progeny. This could prove effective as a gene source in further improvements and for production in the environmental conditions of the Carpathian Basin. The results of these analyses show that, with adequate results regarding cultivar susceptibility, the losses caused by *Xaj* bacteria can be reduced.

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