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Comparison of *Xanthomonas arboricola* pv. *juglandis* isolates from walnut trees grown in Romania and Hungary

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Summary: Among diseases that affect walnut, bacterial blight is considered the most important one in all walnut growing areas both from Hungary and Romania. For the determination of susceptibility/resistance of walnut cultivars in our posterior work planned, 61 bacterial isolates were collected from walnuts showing symptoms of blight, with the purpose of isolating *Xanthomonas arboricola* pv. *juglandis* (*Xaj*). The characteristic *Xaj* colonies, frequently present as saprophytes in the infected plant tissues, were separated from other bacteria according to their morphology, yellow colour, hydrolysis of starch, and oxidation of glucose. All isolates were tested for pathogenicity by hypersensitive reaction on tobacco leaves (*Nicotiana tabacum* L.), bean pods (*Phaseolus vulgaris* L) and unripe nuts (*Juglans regia* L.). Determination of taxonomy of the selected isolates denotes a possible subdivision (races, biotypes) of *Xaj* occurring in different geographical areas, API 20NE and API 50CH kits were used. Hungarian and Romanian strains showed a high degree of similarity of carbohydrate utilization but slightly differed from the type strain. All were Cu-sensitive.

Keywords: *biochemical comparison, copper resistance, walnut, Xanthomonas arboricola pv. juglandis*

Introduction

The Persian (English) walnut (*Juglans regia* L.) is very sensitive to a number of abiotic and biotic factors. Two of the most important abiotic factors are the autumn frost, sometimes leading to tree death, and the late spring frost, having an effect on stem form. The main biotic damage factor is *Xanthomonas arboricola* pv. *juglandis* (Pierce 1901) Vauterin *et al.,* 1995 (*Xaj*), damages leaves and young shoots in humid and mild climate and after several rainy summers some trees might even die (Fernandez-Lopez and Pereira, 1997).

The bacterial blight disease is one of the most important diseases of Persian walnut (Loreti, 2001) and is widespread in walnut growing areas. The disease severity depends on the weather, which may be different in each year. It causes severe damage to leaves, twigs, buds, petioles, rachides, male and female catkins, nutlets and kernels, and it is considered a major cause of reduction in fruit yield and tree vigour (Belisario *et al*., 1999) *(Figure 1.).*

The inoculum is spread by the action of wind and rain, and infection requires the presence of moisture (Miller and Bollen, 1946, Olson *et al*., 1997). The damage produced by this pathogen is favoured by wet springs, with high humidity. Rainy springs, dew, and continual high humidity conditions are favourable for the development of severe blight, resulting in significant crop loss (Belisario, 1997). Especially if this happens just before and after the flowering time, it may cause losses up to 80% of the crop (Charlot and Radix, 1993; Miller, 1934).

The control of the disease is difficult, since large walnut trees are not easy to treat and because more and more copper-resistant Xaj strains are developing (Solar *et al*., 2007; Giovanardi *et al.,* 2010). To date, no *Xaj* resistance has been found in walnut. Differences have been detected only in the severity of symptoms shown by cultivars planted in the same environment (Frutos and López, 2012).

One of the main objectives of breeding and production improvement activities is to produce resistant cultivars. The production and introduction into production of resistant cultivars is one of the possibilities for preventive measures against the walnuts xanthomonas infection.

In several walnut producing areas of the world plant breeding is practiced, although the resistance of resistant cultivars improved in distant places is questionable against bacterias from walnut population of other production lands.

Many bacterial species show considerable variability in biochemical characters.

Hayward has distinguished isolates of *Pseudomonas (Ralstonia) solanacearum* on the basis of utilization of three hexose alcohols and three sugars and proposed subdivision of the species to biovars (biotypes) (Hayward, 1964).

Scortichini has concluded that genetic diversity exists among *Xaj* strains from different geographical areas of the

world,for instance from Europa and California (Scortichini et al., 2001). Consequently a breeding programme for a long-lasting resistance should take into account different or similar potential virulence of the pathogen according to their geographical areas.

We considered the comparative analyses of the *Xaj* strains isolated in Hungary and Romania by means of biochemical methods necessary because supposedly walnut varieties may differ in the susceptibility to the bacterial pathogen in Hungary and Romania, and the potential of virulence of *Xaj* could also be different according to the biotype and race.

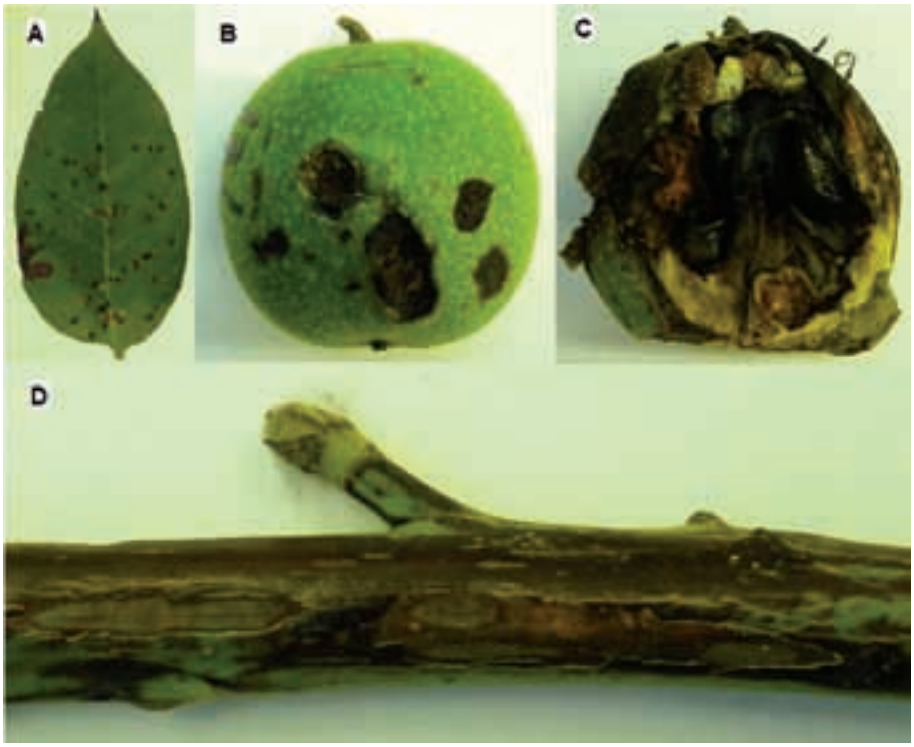

Figure 1. Bacterial blight symptoms on walnut *leaves, immature fruits, kernels* and young shoots

Materials and methods

Parts of the plants showing symptoms of xanthomonas infection have been collected from different walnut producing areas of Hungary and Transylvania.

In order to carry out the identification of the pathogenic agent, the examination of its morphological, biochemical and physiological characteristics, pathogenicity tests were conducted in the bacteriology laboratory of the Department of Pomology of Corvinus University of Budapest. 61 *Xaj* isolates were examined. These were chosen based on the colony growth characteristics (yellow pigment production, smooth, mucoid, convex colony type, oxidative breakdown of glucose, hypersensitive reaction on tobacco leaves)

Once they were identified, they were placed in the DNA database of the NATIONAL COLLECTION OF AGRICULTURAL AND INDUSTRIAL Microorganisms, Corvinus University of Budapest Hungary.

The ability to induce hypersensitive reaction was examined on tobacco leave (*Nicotiana tabacum* L.) (Klement, 1963) and bean pod (*Phaseolus vulgaris* L), the pathogenicity test was controlled on unripe walnut fruits, similarly we used an isolate from the husk of the walnut fruit from Hungary B02489 (HU) and one from Transylvania B02490 (RO) showing a similar degree of infection (virulence).

As a reference to the biochemical analyses of the two isolates (henceforth strains) we used the NCPPB 411 strain, brought from the National Collection of Plant Pathogenic Bacteria, United Kingdom (NCPPB), deposited also in the NCAIM, Budapest, Hungary (http://ncaim.uni-corvinus.hu).

During the biochemical evaluations, the exploitation of the substrates were made on API rapid diagnostic kits (API 20NE és API 50CH – bioMérieux, France), following the instructions of the manufacturer. The evaluations were made after 24 and 48 hours *(Figure 2).*

The API 50CH kit is based on observing the colour change: according to their ability to utilize or oxidize disaccharides and hexose alcohols, if that particular bacteria utilizes the carbohydrate, the original red coloured solution is changing to yellow (acid production), while in the case of positive test result, during gelatin breakdown the gelatin is liquefied (hydrolysis of gelatin) and a black colour reaction appears.

For inoculation we prepared a bacteria suspension with a concentration of 6×10^8 cells/ml of 24 h-old cultures grown on Nutrient agar substrate. The incubation temperature was 28 °C.

Evaluations, based on the colour changes were realised daily for six days, following the degree of carbohydrate utilization *(Figure 3).*

The resistancy tests of the isolates were conducted and the starch hydrolysis capacity was checked.

Figure 2. The 48 hours result of API 20NE diagnostic kit for B02489 (HU) isolate

Figure 3. Carbohydrates utilized by B02489 (HU) isolates in different times (A: 0 h; B: 144 h) (0: control; 1-49 different carbohydrates; colour changes indicate the decomposition)

Results

Neither of the collected 61 *Xaj* strain proved to be Copper-resistant. All of the strains hydrolysed starch. The results of API 20NE system showed that B01395 (USA) strain, and B02490 (HU), B02489 (RO) *Xaj* strains, analysed in details assimilated substrates in identical ways. Some difference could be noticed only in the case of trisodium citrate.

The B02490 (HU) strain gave a negative reaction, while the B01395 (USA) and the B02489 (RO) strain gave a positive reaction.

Hevesi *et al.* (2004) determined the utilization of 49 types of carbohydrate in the case of *Erwinia amylovora* and grouped them according to the speed of carbohydrate breakdown.

We used this classification in our present study with API 50CH test (Biomérieux, Marcy l'Etoile, France).

Shami *et al.* (2013) found a difference in the utilization of cellobiose, maltose, mannose, fructose, citrate, dulcitol, adonitole, oxalate, surbose and sorbitol in the case of 14 *Xaj* isolates isolated in the provinces Lorestan, Kordestan and Alborz from Iran.

According to our results, until the end of the 24-48 h time of reaction all the three analysed *Xaj* strains utilized three carbohydrates (Esculin ferric citrate, D-Lactose, Starch) from the 49 quickly and completely, while other four carbohydrates (D-Fucose, az L-Fucose, a D-Saccharose, D-Lactose) were utilized differently *(Table 2).*

Until the end of 72-96h, and 144 h time of reaction three (D-Glucose, D-Melibiose, D-Lyxose) and four (Amygdalin, D-Cellobiose) types of carbohydrates were utilized in equal proportion. The B02490 and B02489 strains utilized totally identical carbohydrates, with the difference that B02489 utilized them more quickly, while B02490 – more slowly. The control strain from the USA utilized five carbohydrates (N-Acetyl Glucosamine, D-Saccharose, D-melezitose, D-raffinose, Glycogen) less, than the B02490 (HU) and B02489 (RO) strains.

The difference of the carbohydrate utilization between the USA and EU strains could be important in the resistance breeding, if in the case of cultivars is combined with different virulence (specific host change). According to this difference we have to choose cultivars resistant to the races present on the particular growing area, or during the breeding it is advisable to take those phenotypes from the population, which are resistant against the local varieties, using them as parents in cross-breeding.

Considering the identical carbohydrate-utilization of the isolates from Hungary and Transylvania, we can draw the conclusion that during the resistance/susceptibility test it is sufficient to make the evaluation of cultivar with a mix of isolates. As a result we consider it is possible to accomplish the breeding of a cultivar which is intended to be xanthomonas-resistant.

Table 1. Results from API 20NE system

Carbohydrate		Ref. Strain	Isolates	
		B01395 (USA)	**B02490 (HU)**	**B02489 (RO)**
potassium nitrate	NO_2	–		
	N_2	–		
L-tryptophane		–	–	–
D-glucose		–	–	–
L-arginine		–	–	–
urea		–	–	–
esculin ferric citrate		+	+	+
gelatine (bovine origin)		+	+	+
4-nitrophenil-βD-galactopiranoside		+	+	+
D-glucose		+	+	+
L-arabinose		–	–	–
D-mannose		+	+	+
D-mannitol		–	–	–
N-acetyl-glucosamine		+	+	+
D-maltose		+	+	+
potassium gluconate		–	–	–
capric acid		–	–	–
adipic acid		–	–	–
malic acid		+	+	+
trisodium citrate		+	–	+
phenylacetic acid		–	–	–

Table 2. Carbohydrates utilized by *Xaj* isolates using API 50CH system

Ref. Strain		Isolates			
B01395 (USA)		**B02490 (HU)**		**B02489 (RO)**	
Utilized quickly, completly (24-48 h)					
Nr.	*Carbohydrates*	*Nr.*	*Carbohydrates*	*Nr.*	*Carbohydrates*
25	Esculin ferric citrate	25	Esculin ferric citrate	25	Esculin ferric citrate
29	D-Lactose	29	D-Lactose	29	D-Lactose
36	Amidon (Starch)	36	Amidon (Starch)	31	D-Saccharose
44	L-Fucose			36	Amidon (Starch)
Utilized slowly, completly (72-96 h)					
Nr.	Carbohydrates	Nr.	Carbohydrates	Nr.	Carbohydrates
3	D-Arabinose	11	D-Glucose	3	D-Arabinose
10	D-Galactose	30	D-Melibiose	10	D-Galactose
11	D-Glucose	31	D-Saccharose	11	D-Glucose
12	D-Fructose	32	D-Trehalose	12	D-Fructose
13	D-Mannose	35	D-Raffinose	13	D-Mannose
28	D-Maltose	41	D-Lyxose	28	D-Maltose
30	D-Melibiose	43	D-Fucose	30	D-Melibiose
41	D-Lyxose	44	L-Fucose	32	D-Trehalose
43	D-Fucose			35	D-Raffinose
Utilized slowly, weakly (144 h)					
Nr.	Carbohydrates	*Nr.*	*Carbohydrates*	*Nr.*	*Carbohydrates*
23	Amygdalin	3	D-Arabinose	22	N-AcetylGlu-cosamine
27	D-Cellobiose	10	D-Galactose	23	Amygdalin
32	D-Trehalose	12	D-Fructose	27	D-Cellobiose
		13	D-Mannose	34	D-Melezitoze
		22	N-AcetylGlu-cosamine	37	Glycogen
		23	Amygdalin		
		27	D-Cellobiose		
		28	D-Maltose		
		34	D-Melezitoze		
		37	Glycogen		
Not utilized					
Nr.	*Carbohydrates*	*Nr.*	*Carbohydrates*	*Nr.*	*Carbohydrates*
1	Glycerol	1	Glycerol	1	Glycerol
2	Erythritol	2	Erythritol	2	Erythritol
4	L-arabinose	4	L-arabinose	4	L-arabinose
5	D-Ribose	5	D-Ribose	5	D-Ribose
6	D-xylose	6	D-xylose	6	D-xylose
7	L-xylose	7	L-xylose	7	L-xylose
8	D-adonitol	8	D-adonitol	8	D-adonitol
9	Methyl-βD-xylopyranoside	9	Methyl-βD-xylopyranoside	9	Methyl-βD-xylopyranoside
14	L-sorbose	14	L-sorbose	14	L-sorbose
15	L-rhamnose	15	L-rhamnose	15	L-rhamnose
16	Dulcitol	16	Dulcitol	16	Dulcitol
17	Inositol	17	Inositol	17	Inositol
18	D-manitol	18	D-manitol	18	D-manitol
19	D-sorbitol	19	D-sorbitol	19	D-sorbitol
20	Methyl-βl-mannopyranoside	20	Methyl-αd-mannopyranoside	20	Methyl-αd-mannopyranoside
21	Methyl-βD-glucopyranoside	21	Methyl-αD-glucopyranoside	21	Methyl-αD-glucopyranoside
22	N-AcetylGlu-cosamine	24	Arbutin	24	Arbutin
24	Arbutin	26	Salicin	26	Salicin
26	Salicin	33	Inulin	33	Inulin
31	D-Saccharose	38	Xylitol	38	Xylitol
33	Inulin	39	Gentiobiose	39	Gentiobiose
34	D-melezitose	40	D-turanose	40	D-turanose
35	D-raffinose	42	D-tagatose	42	D-tagatose
37	Glycogen	45	D-arabitol	45	D-arabitol
38	Xylitol	46	L-arabitol	46	L-arabitol
39	Gentiobiose	47	Potassium gluconate	47	Potassium gluconate
40	D-turanose	48	Potassium 2-ketogluconate	48	Potassium 2-ketogluconate
42	D-tagatose	49	Potassium 5-ketogluconate	49	Potassium 5-ketogluconate
45	D-arabitol				
46	L-arabitol				
47	Potassium gluconate				
48	Potassium 2-ketogluconate				
49	Potassium 5-ketogluconate				

Discussion

The present study provides comprehensive information regarding the carbohydrate utilization of *Xaj* bacteria. Based on our results we determined the groups of carbohydrates utilized in a various degrees by the pathogen: carbohydrates utilized quickly and completely, carbohydrates utilized slowly and completely, carbohydrates utilized weakly and to a lesser extent, and non-utilized carbohydrates.

We suppose that carbohydrates utilized quickly and completely, and the ones utilized slowly and completely influence the extent and spread of the infection.

There wasn't a difference between the types of carbohydrates in the case of B02490 (HU) and B02489 (RO) isolates, only in the speed of breakdown.

The B01395 (USA) strain utilized less carbohydrate (see 22, 31, 35, 37 carbohydrates), than the EU isolate.

Our results support that widespread opinion, according to which the walnut (*Juglans regia* L.) has a weak ability to adapt. Therefore in the case of the production in a remote area from the breeding place, not only the manifestation of the biological potential of the cultivar is low, but, as the consequence of the different biotic and abiotic stress factors, it can also suffer damages. Our walnut production has to be based on locally bred cultivars, which are adapted to this specific environment.

Due to the identical carbohydrate utilization of the two strains from Transylvania B02489 (RO) and Hungary B02490 (HU), it is assumed that these belong to the same biotype, and they have been subjected to the same stress factors. At the same time the strain originated from the USA (B01395), because of its different carbohydrate utilizing makes us reach the conclusion that we have to be precautious with the naturalization of cultivars from remote places, and resistance against local varieties has to be analysed.

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