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Genetic resistance to bacterial blight disease in Persian walnut

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Abstract Bacterial blight disease of Persian walnut (*Juglans regia,* L.), caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*), leads to significant nut losses in northern, central and western areas of Iran. To identify the natural sources of resistance to disease in the endemic walnut genotypes of Iran, sixteen walnut genotypes, collected from different areas of Hamedan province, were inoculated with *Xaj* in a randomized complete block design with five replicates for each genotype. Two-year old genotypes were gently sprayed with a suspension of bacteria adjusted to approximately 2×10^9 cfu ml^{-1} of distilled water in May. Infected leaves were rated for disease 28 and 42 days after inoculation, using a 0 to 5 severity scale, based on the number, size and distribution of lesions on the leaves. Data analyses showed that there were variations among genotypes in response to pathogen. Upon inoculation by bacterial suspension genotype 94 showed the highest resistance to both disease incidence and its progress after 4–6 weeks of infection. Genotype 65 showed high susceptibility to disease and genotype 69 showed high susceptibilities both to disease incidence and its progress after 4–6 weeks of infection.

Keywords *Juglans regia* · *Xanthomonas arboricola* pv. *juglandis* · Genetic variation · Disease resistance

Abbreviations
Xaj *Xanthomonas arboricola* pv. *Juglandis*
Cfu Colony forming units

Introduction

Bacterial blight disease of Persian walnut (*Juglans regia,* L.), caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*), is one of the most destructive diseases of walnuts worldwide. This disease is distributed across Iran, and every year leads to significant nut losses in northern, central and western areas of Iran (Golmohammadi 1999). The causative bacterium attacks buds, leaves, petioles, catkins, twigs, pistil, ovary, kernel and nuts. The bacteria enter the walnut tissues from natural openings, such as stomata and from wounded tissues (Verma and Sharma 1999). Fresh succulent tissues are sensitive to disease but become resistant over time. The presence

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of succulent tissues and presence of free water on the plant surfaces are the main factors in the infection process (Miller and Bollen 1946). 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 of 50% to 80% of the crop (Charlot and Radix 1993; Miller 1934). The inoculum disseminates by rain drops, pollen, the mite *Aceria tristeriata* var. *erinaea* (Nal.), the large walnut aphid *Chromaphis juglandicola*, and human activities (Ark 1944; Rudolph 1943). The primary inoculum overwinters in dormant buds, twig cankers, and infected nuts that are abscissed or remain on the trees. However, the secondary inoculum primarily originates from diseased leaves and twigs (Martins 1997). The epiphytic and endophytic bacteria on seemingly healthy buds and leaves also can overwinter, and serve as the sources of primary and secondary inoculum (Mulrean and Schroth 1982). The bacterium can also survive on weeds (Swing and Civerolo 1993). Soon after the first report on walnut blight (Pierce 1901), artificial inoculations for evaluating of disease resistance were applied (Pierce 1904). Then, late leafing was recognized as a trait of disease escape (Ramsey 1908), and several reports on disease resistance were published (Rudolph 1943; Smith 1922; Ware 1904), that were discredited later (Miller and Bollen 1946). However, reports on natural resistance of walnut genotypes continued to appear (Adhikari et al. 1988; Tamponi and Donati 1990; Verma and Sharma 1999). In 1992, variation among walnut genotypes to bacterial blight was shown by artificial inoculations (Woest et al. 1992). Using this method, assessing of resistance of walnut genotypes to bacterial blight was carried out in several countries (Belisario 1997; Belisario et al. 1999; Martins 1997). None of the *Juglans* species were immune to this disease, but the Persian walnut, *J.regia*, was the most sensitive species (Belisario et al. 1999). In *J.regia*, Franquette and Hartley cultivars are reported as resistant (Belisario 1997; Belisario et al. 1999). Iran is one the main countries for walnut growing. There are unique walnut genotypes in Hamedan province of the country. In this research, we aimed at initial evaluation of Iranian walnut genotypes from Hamedan province for their resistance to bacterial blight disease.

Materials and methods

Walnut genotypes

Walnut genotypes, used in this study, were provided by the Walnut Research Station of Farasfaj, Hamedan. Eighty two-year old plants, maintained in pots, were transferred to an open field at the Alborz Research Center, Forests and Pastures Organization, Karaj (Tehran, Iran) and were arranged in a randomized complete block design with five replicates of sixteen genotypes. *c*. 300 g animal manure was added to each pot, and the plants were placed outdoor at 75 cm distance from each other. A list of genotypes is presented in Table 1. The plants were irrigated once weekly.

Bacterial strain

The Iranian strain of *Xanthomonas arboricola* pv. *juglandis Xaj* 35 (Golmohammadi 1999), isolated from diseased walnuts in Iran, was used for inoculations. The lyophilized bacteria were re-suspended in sterile distilled water and grown on Nutrient Agar (NA) medium. After overnight incubation at 25°C,

Table 1 Details of walnut genotypes used in this study

Leafing date	Location of collecting seeds[a]	Genotype code[b]
13th April	Tuyserkan, Sarabi	13
13th April	Simin, Chaleh Baghiha	45
11th April	Nahavand-Kuhani	53
13th April	Shams abad	65
13th April	Shams abad	66
22nd April	Varkaneh	69
13th April	Varkaneh	71
1st May	Tuyserkan, Faghan	73
13th April	Tuyserkan, Sarabi	78
13th April	Tuyserkan, Gazandar	80
13th April	Tuyserkan, Gazandar	81
22nd April	Tuyserkan, Vardavard	83
13th April	Tuyserkan, Dareh Pahane	92
13th April	Tuyserkan, Dareh Pahane	93
22nd April	Tuyserkan, Gazandar Jonubi	94
13th April	Tuyserkan, Gazandar	95

[a] All locations are within Hamedan province

[b] The code is the same as is in the collection of Walnut Research Station of Farasfaj, Hamedan

the emerged colonies were re-streaked on fresh NA for 48 h growth at 25°C for further use. In the short term, the bacterial suspensions were stored in distilled water at 4°C.

Bacterial virulence test

To test the virulence of *Xaj* 35, the bacteria were grown on NA for 24 h at 25°C. The cells were scraped and suspended in sterile distilled water to an OD600 of 0.05. An aliquot of bacterial suspension was injected under the epidermal layer of walnut leaves to watersoak *c.* 0.5–1 mm of the leaf tissue. Virulence was assessed after 3 days.

Inoculation procedure

Three inoculation procedures were tested for their effectiveness. In the first procedure, the detached walnut leaves were placed in glass tubes, and a suspension of 10^5 cfu ml^{-1} bacteria was injected under the epidermal layer to soak *c.* 0.5–1 mm of the leaf tissue. Disease symptoms were assessed 7 days after inoculation. In the second procedure, the detached walnut leaves were placed in glass tubes, and a suspension of 10^5 cfu ml^{-1} bacteria was sprayed on the leaves, and the leaves were maintained under nylon plastics until they lost their freshness. In the third procedure, the whole plant was inoculated with two different suspensions of bacteria (2×10^7 & 2×10^9 cfu ml^{-1}) by spraying, maintained one night under nylon plastics, and placed in the open field until the symptoms appeared.

Inoculation of genotypes

Plants were inoculated 2 weeks after leafing date (27th April), and once more, 10 days after the first inoculation (6th May). To provide optimum conditions for bacterial infection, the day before and just after inoculations, plants were irrigated. On the evenings of the inoculation dates, plants were first sprayed with water, and then a bacterial suspension (*Xaj* 35) of 2×10^9 cfu ml^{-1} in distilled water was sprayed on the leaves of the whole plant till run off, using a sprayer according to Woest et al. 1992. The spraying was performed on the both sides of leaves. The control plants were treated in such a way, but by sterile distilled water only. The plants with their pots were enclosed in wet nylon plastics overnight (Mulrean and Schroth 1982). The day after, the nylon plastics were removed in the morning and replaced in the evening after distilled water was sprayed on the plant's leaves. This was repeated for 7 days to provide suitable conditions for bacteria. From the 2nd week the plants were sprayed in the evenings (without enclosing), 3–4 times a week by distilled water.

Symptom emergence and validation of bacterial infection

In order to record the first emergence of disease symptoms, plants were checked regularly after the first day, and the suspected infections were verified under the light microscope by observation of ooze secretion from the tissues. Upon the emergence of disease symptoms, from 1 to 2 infected leaves of each genotype the typical pale yellow bacteria were re-isolated as described by Mulrean and Schroth 1981.

Assessment of disease progress

The incidence of bacterial blight infection, and its progress, from the 28th to 42nd day after inoculation, were assessed. To this end, based on the number, size and distribution of lesions on each leaf, a 0 (healthy

Table 2 Scoring of walnut bacterial blight disease based on the number, size and distribution of lesions on the leaf

Foliar disease symptom	Disease scale[a]
Healthy uninfected leaf	0
Necrotic spots or lesions on the 1–25% area of the leaf	1
Necrotic spots or lesions on the 25–50% area of the leaf	2
Necrotic spots or lesions on the 50–75% area of the leaf	3
Necrotic spots or lesions on the 75–90% area of the leaf	4
Necrotic and detached leaf	5

[a] The average of the whole leaves of all shoots, in five replicates, was considered as disease scale for each genotype

Fig. 1 Advanced watersoaked symptoms of walnut bacterial blight on a leaf inoculated with *Xanthomonas arboricola* pv. *juglandis* strain 35

uninfected leaf) to 5 (necrotic and detached leaf) severity scale was used (Table 2). The average of the whole leaves of all shoots, in five replicates, was considered as disease scale for each genotype. The data were evaluated by MSTATC software for analyses of variance (ANOVA), and Duncan's multiple range test was applied for rank mean separation of the genotypes.

Results and discussion

Bacterial virulence test

Three days after inoculation of walnut leaves, the watersoaked lesions were observed that developed gradually, and after 3–4 weeks turned to dark brown (necrotic) areas with a translucent watersoaked zone. From the watersoaked zones the bacteria were re-isolated.

Inoculation procedure

The inoculation of detached leaves, even by injection or spraying of the bacterial suspension, did not result in disease symptoms. The leaves lost their freshness after 1 week, and except for some small watersoaked spots, no other symptom was seen. However, spraying of the bacterial suspension on the whole plant yielded the watersoaked symptoms after 10 days, and during the month after the symptoms were developed. Hence, for bacterial inoculation of the genotypes this procedure was followed.

Symptom emergence and validation of bacterial infection

During the 10 days after the first inoculation (27th April) no symptom was detected. However, after 10 days of the second inoculation (6th May) the disease symptoms emerged as watersoaked leaf spots that developed in 1 week to visible translucent spots (Fig. 1). During the 45 days after the second inoculation, the leaf spots further developed but eventually stopped progressing in all cases. Leaf drop was not observed until 4 months after inoculation (September). Under the light microscope the oozing of bacteria was seen from 1 to 2 infected leaves of

Table 4 Analysis of variance of bacterial blight disease scores in walnut genotypes (inoculated by *X. a. juglandis* strain 35) on the 42nd day after inoculation

Source of variance.	Degrees of freedom	Sum of squar	Mean square	*F*-value
Replicate	4	1.925	0.481	2.5163ns
Treat	15	33.697	2.264	11.7462[a]
Error	60	11.475	0.191	
Total	79	47.097		

ns: Not significant

[a] Significant at 1% level

Table 3 Analysis of variance of bacterial blight disease scores in walnut genotypes (inoculated by *X. a. juglandis* strain 35) on the 28th day after inoculation

Source of variance.	Degrees of freedom	Sum of squares	Mean square	*F*-value
Replicate	4	0.281	0.07	1.234ns
Treat	15	11.8	0.787	1.8026[a]
Error	60	3.419	0.057	
Total	79	15.5		

ns: Not significant

[a] Significant at 1% level

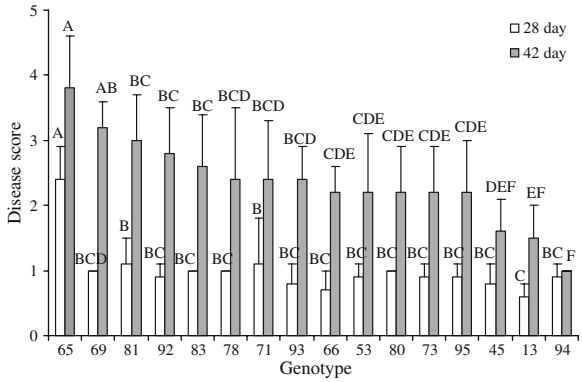

Fig. 2 Walnut bacterial blight disease scale on 28th and 42nd day, after inoculation of a suspention of 2×10^9 Cfu ml^{-1} *X. a. juglandis* strain 35 on walnut genotypes used in this study. Data (significant at $P\leq0.01$) are averages of five replicates. Error bars indicate standard errors. Similar letters indicate no significant difference

each genotype. Bacteria were re-isolated from such tissues on nutrient agar, and after 2–3 days, pale yellow colonies of bacteria were grown.

Assessment of disease progress

Walnut genotypes varied significantly ($P\leq0.01$) in incidence and severity of the bacterial blight infection, and its progress from the 28th to 42nd day after the second inoculation (Tables 3 and 4). Based on the 0 to 5 disease scoring, genotype 65 showed highest susceptibility on both dates, and genotype 69 on the 42nd day (Fig. 2). On the other hand, genotypes 13 and 94 scored as least susceptible (Fig. 2). This scoring indicates the ability of bacteria to enter the leaf tissues and establish the disease. Hence, genotypes 13 and 94 might resist bacterial infection or disease establishment.

However, upon infection the genotypes show different phenotypes when disease progress was evaluated. As it can be seen from Table 5 and Fig. 2, disease progress in genotypes 69, 66, 92, and 93 were highest, whereas in genotypes 94 it was the least. It might be noted that in both 69 and 94 genotypes, the leafing date was on the same day and almost 1 week later than the average. However, regarding the late leafing dates of genotypes 73 and 83, it seems that the leafing date has no direct effect on the disease incidence and its progress in artificial inoculation. In genotype 65, although the disease scored highest, the progress of disease from 28th to 42nd day was very low compared to other genotypes (2nd after genotype 94). This may indicate susceptibilty of pre-existing defence structures of this genotype, but biochemical resistance against disease progress. In conclusion, genotype 94 shows low degrees of disease incidence and its progress. This high degree of resistance against disease progress might indicate a genetic/biochemical resistance of this genotype against walnut bacterial blight and might be of special interest for molecular investigation of the basis of this bacterial blight resistance.

Table 5 Progress of severity of disease scores from date 28 to date 42 on the leaves of infected walnut genotypes

Leafing date	Genotype code	Rate of disease progress on 42nd vs. 28th day (fold)
11th April	53	2.4
13th April	13	2.5
13th April	45	2.0
13th April	65	1.6
13th April	66	3.1
13th April	71	2.2
13th April	78	2.4
13th April	80	2.2
13th April	81	2.7
13th April	92	3.1
13th April	93	3.0
13th April	95	2.4
22nd April	69	3.2
22nd April	83	2.6
22nd April	94	1.1
1st May	73	2.4

Conclusion

There are excellent walnut genotypes in the Iranian walnut growing areas, especially in Hamedan province, with a wide range of resistance to plant diseases. The artificial inoculation method of spraying the bacterial suspensions on the fresh, young plants is a good means to evaluate such walnut plants for their resistance to bacterial blight disease. In this study, Iranian walnut genotypes from Hamedan province showed variation in their resistance to bacterial blight

caused by an Iranian strain of *Xaj* 35. The genotype 65 shows high susceptibility to disease incidence, but genotype 69 shows high susceptibilities both to disease incidence and its progress. Interestingly, the genotype 94 shows a high resistance to both disease incidence and its progress. Hence, genotypes 94, 66, and 65 could serve for investigation of molecular/genetic basis of walnut resistance/susceptibilty to bacterial blight.disease.

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