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Les mûriers et les framboisiers comme sources alternatives de résistance à la brûlure bactérienne

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Résumé de l'article

Erwinia amylovora est responsable de la brûlure bactérienne principalement chez la poire, la pomme et le coing. Cet agent pathogène bactérien infecte également d'autres plants de *Rosaceous*, comme les mûriers et les framboisiers. Une structure de race a été établie entre un isolat de *E. amylovora* et ces baies en utilisant 40 génotypes de mûrier sauvage et 7 de framboisier sauvage. Lors de tests de pathogénicité, les génotypes de mûrier et framboisier sauvages ont montré trois réactions génotypiques, soit une sensibilité accrue, une sensibilité intermédiaire et de la résistance. Nous avons noté une plus grande croissance bactérienne de plus de 300×10^9 cfu mL^{-1} chez les plants avec une sensibilité accrue, alors que les génotypes résistants montraient une croissance bactérienne d'environ 150×10^9 cfu mL^{-1}. Ces résultats sont également liés aux symptômes observés 29 jours après l'inoculation. Cette résistance fait l'objet d'évaluation pour la lutte à la brûlure bactérienne.

Blackberry and raspberry are alternative resistance sources to fire blight

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Erwinia amylovora causes fire blight mainly on pear, apple and quince trees. This bacterial pathogen also infects other *Rosaceous* plants, such as blackberry and raspberry. A race structure was established between an isolate of *E. amylovora* and berries using 40 wild blackberry and 7 wild raspberry genotypes. In pathogenicity tests, wild blackberry and raspberry genotypes had three phenotypic reactions: enhanced susceptibility, moderate susceptibility and resistance. We noted a higher bacterial growth of over 300×10^9 cfu mL^{-1} in plants with enhanced susceptibility, with resistant genotypes showing a bacterial growth of around 150×10^9 cfu mL^{-1}. These results are also associated with symptoms observed at 29 days post-inoculation. This resistance is being evaluated to control fire blight.

Keywords: fire blight, resistance, wild blackberry, wild raspberry

[Les mûriers et les framboisiers comme sources alternatives de résistance à la brûlure bactérienne]

Erwinia amylovora est responsable de la brûlure bactérienne principalement chez la poire, la pomme et le coing. Cet agent pathogène bactérien infecte également d'autres plants de *Rosaceous*, comme les mûriers et les framboisiers. Une structure de race a été établie entre un isolat de *E. amylovora* et ces baies en utilisant 40 génotypes de mûrier sauvage et 7 de framboisier sauvage. Lors de tests de pathogénicité, les génotypes de mûrier et framboisier sauvages ont montré trois réactions génotypiques, soit une sensibilité accrue, une sensibilité intermédiaire et de la résistance. Nous avons noté une plus grande croissance bactérienne de plus de 300×10^9 cfu mL^{-1} chez les plants avec une sensibilité accrue, alors que les génotypes résistants montraient une croissance bactérienne d'environ 150×10^9 cfu mL^{-1}. Ces résultats sont également liés aux symptômes observés 29 jours après l'inoculation. Cette résistance fait l'objet d'évaluation pour la lutte à la brûlure bactérienne.

Mots-clés : brûlure bactérienne, framboisier sauvage, mûrier sauvage, résistance

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INTRODUCTION

Bacterial fire blight causes damage on pear (*Pyrus* spp.), apple (*Malus* spp.) and quince (*Cydonia* spp.) trees that amounts to millions of Euros in Turkey and around the world. Fire blight occurrence is erratic, causing more frequent and more devastating outbreaks in the presence of several factors such as plant susceptibility, growing conditions and cultural practices. Blossom blight can be seen in warm, humid weather, which is conducive to the development of a blight epidemic, but rainfall, surface moisture and plant varietal differences should also be taken into consideration (Momol *et al.* 1992). Fire blight is a serious disease in pear, apple and quince trees because the disease results in yield loss, and its control requires the removal of trees (Agrios 1997). The bacteria also affect other plants in the *Rosaceae* family, including loquat (*Eriobotrya* spp.), cherry (*Prunus avium*), raspberry and blackberry (*Rubus* spp.) (Agrios 1997; Bonn and Van der Zwet 2000). Fire blight causes significant loss in pear, apple and quince orchards, which are commonly treated with copper containing chemical pesticides (Johnson and Stockwell 1998; Norelli *et al.* 2003). Management strategies in pear and quince trees focus on timely applications of copper with a systemic fungicide since copper is not a systemic chemical, nor is it carried internally through the plant to kill the pathogen. Economic pressures, environmental regulations and chemical resistance to fire blight are forcing scientists and growers to identify alternative measures to overcome these challenges (Rosaceae Whitepaper 2011). Control measures for fire blight are expensive, labour-intensive and difficult, though still manageable (Norelli *et al.* 2003; Van der Zwet and Beer 1991). The exploitation of resistance genes introduced from other crop relatives is a novel tactic that will reduce environmental and human health risks, while safeguarding agronomic characters, such as fruit yield and quality. A long-term approach is necessary to characterize, maintain, and increase the availability of these beneficial genetic resources (Rosaceae Whitepaper 2011).

Fire blight caused by *Erwinia amylovora* (Burrill) Winslow *et al.* affects the cane tips of blackberry and raspberry. The infected berry tips are blackened and take on a "shepherd's crook" appearance with typical discoloration. Entire berry leaves and fruit clusters may turn black, and young berries become hard and dry. The infected canes may produce a milky to clear ooze that can be observed on the blackened stems. The berry necrosis and tip dieback of primocanes can reach 15 to 30 cm in length (Braun *et al.* 1999, 2004; Evans 1996). Although outbreaks are less common in berry species, these lesions can reach economically damaging levels under certain conditions (Braun *et al.* 1999).

Molecular studies in pear, apple and quince trees have been essential for dissecting and understanding their molecular structures for horticultural and biotechnological purposes (Lu *et al.* 2010). However, the development of disease-resistant varieties has been limited because rosaceous plants have long generation times and complex polyploid genomes (Norelli *et al.* 2003). New genomics approaches to produce genetically-resistant plants hold great promise to accelerate the pace and increase the odds of successfully achieving this goal (Venisse *et al.* 2002; Rico *et al.* 2004). Blackberry and raspberry are able to use alternative resistance sources to control fire blight. A wide range of resistance to fire blight is found among the genotypes of berries, and this genetic richness may have potential for developing broad-based genetic resistance (Steward *et al.* 2005). Additionally, the berries are self-fertile, have good fruit production, a short generation time, and can be grown in all soil conditions. These advantages make berries model plant systems for developing high-throughput approaches to test and confirm their genomes' resistance functions. Integrated research offers new opportunities for characterizing germplasm, streamline the incorporation of resistance genes into commercial plants, and identify new sources of resistance (Rosaceae Whitepaper 2011).

Fire blight in berries is not well understood, and much research remains to be done. Genetic resistance is more likely to be used for control than preventive sprays or predictive models. Therefore, the objectives of this study were to determine the level of resistance of blackberry and raspberry genotypes against a virulent strain of *E. amylovora*, and to identify the berries' sources of resistance.

MATERIAL AND METHODS

Plant material

Wild plants are naturally present in the Black Sea region. Wild blackberries grow naturally from sea level to an altitude of 2000 m all over the Middle and Eastern Black Sea regions in Turkey. Wild raspberries have been located at higher altitudes, from 1300 m to 2000 m above sea level, in small areas of the Eastern Black Sea region. A total of 40 blackberry and 7 raspberry plants were collected from eight provinces in the Black Sea region. The collected plants were transplanted into 25-cm-diam plastic pots filled with soil. The pots were placed in a greenhouse located at Gaziosmanpasa University (Tokat, Turkey) at 22±2 °C with a 16 h light:8 h dark cycle. The pots were watered regularly throughout the growing season.

Bacterial strain and culture conditions

The causal agent of fire blight, *E. amylovora* strain I2, is commonly isolated in Turkey (Unlu and Basim 2001). The bacterial culture was obtained from Prof. Dr. Huseyin Basim (Akdeniz University, Antalya, Turkey). The pathogenic *E. amylovora* strain I2 was quadrant-streaked on King's B medium (King *et al.* 1954) and incubated at 28 °C for 1 to 3 d to obtain individual colonies. Yellow, circular, individual bacterial colonies were later streaked on King's B medium and incubated at 28 °C overnight. Bacterial suspensions were prepared from 24-h fresh bacterial culture in King's B broth and were centrifuged at 3000 rpm before being diluted in sterile 25 mM $MgCl_2$ buffer.

Bacterial inoculation

The bacterial suspension was measured with a UV spectrophotometer (Biomate 3, Thermo Electron, USA) and adjusted at an absorbance of 0.20 at 600 nm wavelength. The absorbance represents 1×10^8 colony-

forming units (cfu) bacteria per milliliter (mL), based on viable replicate measurements. The prepared liquid 1×10^8 cfu mL^{-1} bacterial suspensions were injected by inserting a 5 mL hypodermic needle completely through 30-cm-long primocanes. The suspension of 5 mL liquid was injected to completely fill several wounds until visible drops became visible in the wounds. The inoculated 30-cm-long primocanes were labelled with flagging tape for measurement (Norelli *et al.* 1986). The control plants were similarly inoculated with distilled sterile water. Plants were then incubated in a glasshouse at 20 to 22 °C, 16 h light: 8 h dark cycle. Blackberry and raspberry inoculations were done in three replicates. Each presented value is the average of three replicates.

Bacterial isolation and cell counts

Inoculated canes were sampled every 7 d. Plant tissues (1 cm long) were cut from the inoculated primocanes and immediately placed in an ice-filled cooler. Samples were later triturated in a sterile mortar containing 1 mL $MgCl_2$ (25 mM). Then, 1 mL of bacterial suspension was diluted 10-fold, with up to seven 10-fold dilutions; 10 µL of each dilution were plated onto King's B medium. Plates were incubated at 28 °C for 24 h to count individual colonies. From the visible colonies of *E. amylovora*, an actual bacterial concentration was determined using the plate count.

Disease scoring

Infected plants were assessed for disease development at 7, 14, 21 and 29 days post-inoculation (dpi). To determine host range on wild blackberry and rasp-berry genotypes, plants were rated on a scale of 0 to 5 based on the severity of their symptoms, with 0 meaning no visible symptoms, and 5 meaning complete necrosis (dieback) from the point of inoculation to the bottom of the shoot (Steward *et al.* 2005). An analysis of variance was used to determine the effect of replication and trial on disease severity ratings. Average bacterial ratings for wild berry genotypes were computed using Duncan's multiple range test.

Table 1. Distribution of 40 blackberry genotypes inoculated with *E. amylovora* and their phenotypes as determined by Duncan's tests and their bacterial growth on King's B agar.

Plant genotype	Average number of bacteria in three plant replicates	Bacteria per cm at 29 dpi (x 100 million)	Resistant	Moderately resistant	Susceptible
B31	23.80^{a}*	40	X		
B14	45.60^{ab}	101	X		
B45	47.40^{abc}	95	X		
B39	48.20^{abc}	102	X		
B07	49.30^{abcd}	110	X		
B17	49.70^{abcd}	140	X		
B49	56.10^{bcde}	170	X		
B18	62.70^{bcdef}	127	X		
B48	68.10^{bcdef}	108	X		
B53	68.30^{bcdef}	135	X		
B12	68.90^{bcdef}	115	X		
B34	74.30^{bcdef}	145	X		
B21	75.50^{cdef}	145	X		
B41	78.30^{defi}	101	X		
B09	79.00^{defi}	121	X		
B15	79.70^{def}	160	X		
B51	88.90^{dfhi-}	145	X		
B55	89.60^{dfhi}	180	X		
B02	91.00^{fghi}	150	X		
B43	92.80^{ghi}	130	X		
B40	93.20^{ghi}	185		X	
B42	95.70^{ghij}	160		X	
B35	99.10^{hijkl}	148		X	
B23	99.20^{hijkl}	188		X	
B28	99.90^{hijkl}	225		X	
B36	103.50^{hijkl}	222		X	
B46	106.40^{ijklm}	160		X	
B33	115.80^{jklmn}	167		X	
B52	117.80^{jklmn}	160		X	
B06	122.80^{jklmn}	196		X	
B37	126.80^{mno}	225		X	
B08	132.10^{mnop}	235		X	
B54	137.00^{nopq}	198		X	
B32	144.90^{opqr}	217			X
B30	157.20^{opqr}	400			X
B50	161.20^{qr}	213			X
B13	167.80^{rs}	205			X
B25	168.10^{rs}	240			X
B47	191.70st	314			X
B19	198.10st	294			X
Control	0^{a}	0			

*Means followed by a different letter(s) in the same column differ significantly ($P < 0.05$) according to Duncan's multiple range test.

Table 2. Bacterial growth of *E. amylovora* isolate on raspberry genotypes during pathogenicity tests.

Plant genotype	Average number of bacteria in three plant replicates	Bacteria per cm at 29 dpi (x 100 million)	Resistant	Moderately resistant	Susceptible
A08	57.40[a]*	111	X		
A05	60.50[a]	126	X		
A07	84.70[ab]	130	X		
A13	118.70[b]	168		X	
A22	122.60[b]	200		X	
A04	123.90[b]	199		X	
A14	224.00[c]	550			X

*Means with the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

RESULTS

Fire blight resistance in wild berries

Blackberry and raspberry genotypes were the main sources of variance in this study. Our inoculation method resulted in typical fire blight symptoms, which began to appear 3 to 6 dpi. On some wild berry genotypes, blackened areas around the wound sites were the first visible symptoms; these subsequently spread to the veins. We assessed bacterial population growth in different berry genotypes that have an effect on the rate of disease spread. While most plants showed at least some symptoms of disease development and produced bacterial ooze at 7 dpi, mean bacterial growth strongly correlated with the final number of bacteria at 29 dpi. Therefore, mean bacterial growth values were grouped into three categories: resistant ($80 \times 10^9 \pm 10\%$ cfu mL^{-1}), moderately resistant ($80\text{-}160 \times 10^9 \pm 10\%$ cfu mL^{-1}) and susceptible ($160 \times 10^9 \pm 10\%$ cfu mL^{-1}) berries. No control plants treated with sterile water showed any symptoms in our assessments. Significant differences were found among the 40 wild blackberry genotypes. Inoculation results were evaluated in the three categories, and 20 wild blackberry genotypes did not sustain the growth of *E. amylovora*; their numbers were less than 180×10^9 cfu mL^{-1} in plant tissues (Table 1). However, in the second category, 13 wild blackberry genotypes partially sustained bacterial growth, and their numbers were less than 235×10^9 cfu mL^{-1} (Table 1). In the third group, 7 wild blackberry genotypes sustained bacterial growth, and their numbers were more than 235×10^9 cfu mL^{-1} (Table 1). These counts are referring to the resistance or susceptibility to *E. amylovora*: bacterial counts could decrease significantly in resistant berries, but the bacterial population is too high for this to happen in susceptible blackberries.

Blackberry genotype B30 was the most susceptible (bacterial concentration increased to 400×10^9 cfu mL^{-1} at 29 dpi (Table 1)), while B31 was the most resistant (bacterial growth was 40×10^9 cfu mL^{-1} at 29 dpi (Table 1)). Hence, B30 sustained at least 10 times higher bacterial concentration than B31 at 29 dpi (Table 1). In order to quantify berry genotypes, we considered the means of bacterial values from three plant replicates; in most blackberries, mean values are associated with bacterial concentration per cm of plant tissue at 29 dpi. However, the results obtained could not be fitted with the moderately resistant and susceptible blackberry genotypes (Table 1).

Raspberry genotypes sustained more bacterial growth than blackberry genotypes (Table 2). *Erwinia amylovora* bacterial concentration reached 111×10^9 cfu mL^{-1} in resistant genotype A08 (Table 2). Moderately resistant raspberry genotypes sustained around 190×10^9 cfu mL^{-1} concentration in their tissues (Table 2). The highest bacterial concentration was found in genotype A14, with 550×10^9 cfu mL^{-1} (Table 2). Raspberry susceptible genotype A14 contained a bacterial concentration five times higher than that of genotype A8, indicating that raspberry genotypes are more susceptible to *E. amylovora* (Table 2).

Resistance is the most effective way to control fire blight. As seen in Tables 1 and 2, 40 blackberry and 7 raspberry genotypes had various degrees of resistance to the *E. amylovora* isolate.

DISCUSSION

Several plant species originate from the Black Sea region of Turkey, including blackberry and raspberry. In recent years, several projects have been conducted to describe the region's plant richness. Naturally, while wild blackberry and raspberry plants were being collected, typical disease symptoms were observed on the wild berries. Therefore, a known fire blight isolate was tested to establish a race structure between the bacterium and the wild berries.

The wild blackberry and raspberry genotypes collected have high antioxidant activity (Cekic and Özgen 2010), genetic differences in resistance/susceptibility and short life cycles, which are handy molecular tools that should enable us to discover new ways of controlling fire blight not only in berries, but also in pear, apple and quince trees. Much of the variability found in this study has to do with either limiting or promoting bacterial growth. Pathogenicity test results show that the blackberry and raspberry genotypes make up three distinct groups, in which the resistant berry genotypes did not sustain *E. amylovora* growth, and on which no or very limited dieback symptoms were observed. The blackberry genotypes are more resistant than the raspberry ones, as shown by their respective average number of bacteria (Tables 1 and 2, respectively). Additionally, the resistant genotypes contain higher phenolic substances (Cekic and Özgen 2010), which could be due to their antimicrobial activity against fire blight.

In this paper, we used wild blackberry and raspberry genotypes as experimental host plants to identify possible resistance sources in berries. Pathogenicity test results, along with disease symptoms, confirmed that both wild berry genotypes have various degrees of resistance. A race structure was established in this study between berry genotypes and a known isolate of *E. amylovora*. Resistance mechanisms should be further studied in order to identify resistance sources and their signaling pathways. Once the resistance sources are identified, they could be used in pear, apple and quince trees.

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