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SHORT NOTES

Immature walnut fruit inoculation for evaluation of *Brenneria nigrifluens* pathogenicity

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Summary. A reliable, reproducible, rapid and specific test for the pathogenicity of *Brenneria nigrifluens*, the causal agent of shallow bark canker of Persian walnut, was developed. When the mesocarp of immature walnut fruits was infiltrated by syringe with bacterial suspensions (10^8 cells mL^{-1}) of *B. nigrifluens*, necrosis and reddish brown exudates started to appear at the inoculation sites, as early as 2 days after inoculation. No symptoms were detected in control fruits or in fruits inoculated with other bacteria frequently associated with walnut cankers. This pathogenicity test saves time and space when compared with the plant stem inoculation technique, in which the canker symptoms do not appear until at least 1 month after inoculation.

Key words: *Brenneria nigrifluens*, diagnosis, *Juglans regia*.

Introduction

Shallow bark canker caused by *Brenneria nigrifluens* (Wilson *et al*.) Hauben *et al*. (synonym *Erwinia nigrifluens*) is one of the most dangerous diseases of Persian walnut (*Juglans regia* L.) trees. First reported in California (Wilson *et al*., 1957), the disease was subsequently recorded in Spain (López *et al*., 1994), Iran (Harighi and Rahimian, 1997), Italy (Morone *et al*., 1998; Saccardi *et al*., 1998) and France (Ménard *et al*., 2004). The disease is mainly characterized by shallow, irregularly shaped cankers in the bark of the trunk and branches, from which a dark-coloured watery exudate oozes through small cracks in the bark. The disease causes severe damage to young nursery plants (Saccardi *et al*., 1998) and to adult trees (Piccirillo, 2003). A number of other bacterial species with a similar colony morphology are frequently isolated from walnut cankers, whose identification requires pathogenicity tests as well as phenotypic and molecular tests to distinguish them (Moretti *et al*., 2007; Loreti *et al*., 2008). Since the pathogenicity test for *B. nigrifluens* now in use is usually carried out on the tree by stem inoculation, and requires at least 1 month to give a result, we developed an alternative test using immature walnut fruits, a method already applied for testing the pathogenicity of *Xanthomonas arboricola* pv. *juglandis* (Pierce) Vauterin *et al*., the causal agent of walnut bacterial blight (Aletà *et al*., 2000).

Materials and methods

Immature fruits were collected before shell hardening from 25–30-year-old walnut (*Juglans regia*) trees located near Perugia (Italy) in June of 2008 and 2009. For inoculum preparation, the

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bacteria listed in Table 1 were grown on nutrient agar at 27°C for 48 h and the cultures suspended in deionised sterile water. The suspensions were spectrophotometrically adjusted to 10^8 cells mL^{-1} (OD_{660}=0.06). Inoculations were performed using two techniques: i) infiltration of the bacterial suspensions into the mesocarp of walnut fruits by a syringe with a needle; ii) infiltration by syringe without a needle. With the former technique, the needle was inserted at about a 30-degree angle to the fruit surface and an area about 1 cm in diameter was infiltrated with the bacterial suspension. With the technique set up not using a needle the fruit mesocarp was pricked (three punctures per fruit) and the inoculation sites were infiltrated with the bacterial suspensions by a syringe without a needle (infiltration areas 0.5–0.8 cm in diameter). Control fruits were infiltrated with water. Inoculated and control fruits were placed on wet filter paper in hermetically closed transparent plastic boxes, which were kept for 8 days in a growth chamber set at 20°C, 240 μE m^{-2} s^{-1} illumination and a 12 h day.

Results and discussion

When walnut fruits were inoculated with bacterial suspensions of *B. nigrifluens* DAPP-PG 509 from 10^2 to 10^8 cells mL^{-1}, only fruits inoculated with the highest concentration developed bark canker symptoms at the inoculation sites, which became a necrotic area from which a reddish brown exudate oozed (Figure 1a-e). Symptoms started to appear 2 days after inoculation and became more pronounced in following days (Figure 1f-l). In cross-section, inoculated fruits clearly showed a necrotic area in the mesocarp around the inoculation site, and this necrotic area expanded in the endocarp (Figure 1m, n).

All the three *B. nigrifluens* strains produced necrotic symptoms and exudates when inoculated in immature walnut fruits (Figure 2). No symptoms were detected in fruits inoculated with any of the other bacteria isolated from walnut cankers by Moretti *et al.* (2007): *Dickeya chrysanthemi* (Burkholder *et al.*) Samson *et al.*; *Erwinia rhapontici* (Millard) Hauben *et al.*; *Ralstonia pickettii* (Ralston *et al.*) Yabuuchi *et al.*; *Spingobacterium spiritovorum* (Holmes *et al.*) Yabuuchi *et al.*; and an unknown enterobacteriacea (Figure 2). Inoculation with the related phytopathogenic bacterium *Brenneria alni* (Surico *et al.*) Hauben *et al.* also did not produce any symptoms (Figure 2). The experiments on the fruits collected in 2008 and those in 2009 gave the same results. From diseased walnut fruits inoculated with *B. nigrifluens*, bacteria were re-isolated, whose colony morphology was identical to that of the isolates. In addition, REP-PCR, performed as described by Moretti *et al.* (2007), revealed that re-isolates generated the same fingerprints as the respective original isolates (data not shown).

The pathogenicity test we developed is both specific and reproducible since: i) disease symp-

Table 1. List of bacterial strains used in the study.

Bacterial species	Strain [b]
Brenneria nigrifluens	LMG 2694^T
B. nigrifluens[a]	DAPP-PG 509
B. nigrifluens[a]	DAPP-PG 621
B. alni	CFBP 3923
Dickeya chrysanthemi[a]	DAPP-PG 637
Erwinia rhapontici[a]	DAPP-PG 636
Ralstonia pickettii[a]	DAPP-PG 640
Spingobacterium spiritovorum[a]	DAPP-PG 639
Unknown enterobacteriacea[a]	DAPP-PG 638

[a] Bacteria isolated from walnut bark cankers and identified by Moretti *et al.* (2007).
[b] CFBP, Collection Française de Bactéries Phytopathogènes, France; DAPP-PG, Phytopathogenic bacteria collection of the Arboriculture and Plant Protection Section, Department of Agricultural and Environmental Sciences, University of Perugia, Perugia, Italy.
LMG, Culture Collection Laboratorium voor Microbiologie, University of Gent, Gent, Belgium.

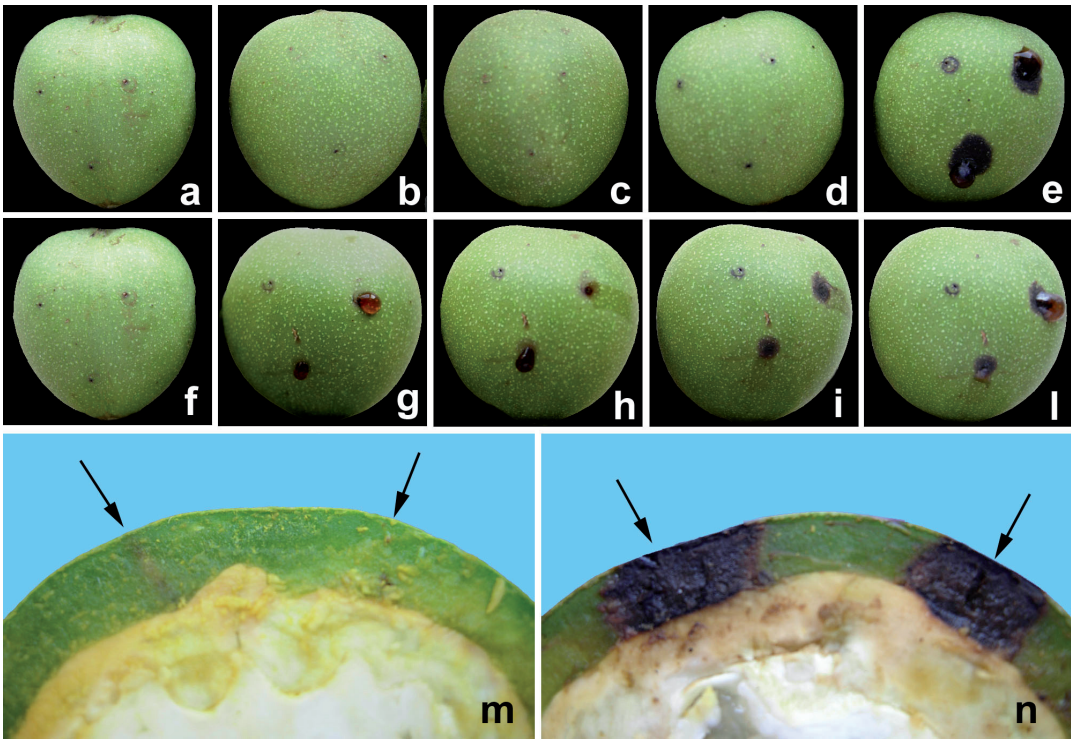

Figure 1. Symptoms on immature walnut fruits inoculated with *Brenneria nigrifluens* DAPP-PG 509 using the syringe without needle technique. Fruits infiltrated with water (a) or bacterial suspension at 10^5 (b), 10^6 (c), 10^7 (d) and 10^8 (e) cells mL^{-1}, 8 days after inoculation. Fruits infiltrated with water (f) or the bacterium (10^8 cells mL^{-1}) after 2 (g), 4 (h), 6 (i) and 8 (l) days after inoculation. Control (m) and inoculated (n) fruits sectioned at the inoculation sites (arrows), 8 days after inoculation. Necrotic symptoms occur in the walnut mesocarp, around the inoculation sites, and expand in the endocarp.

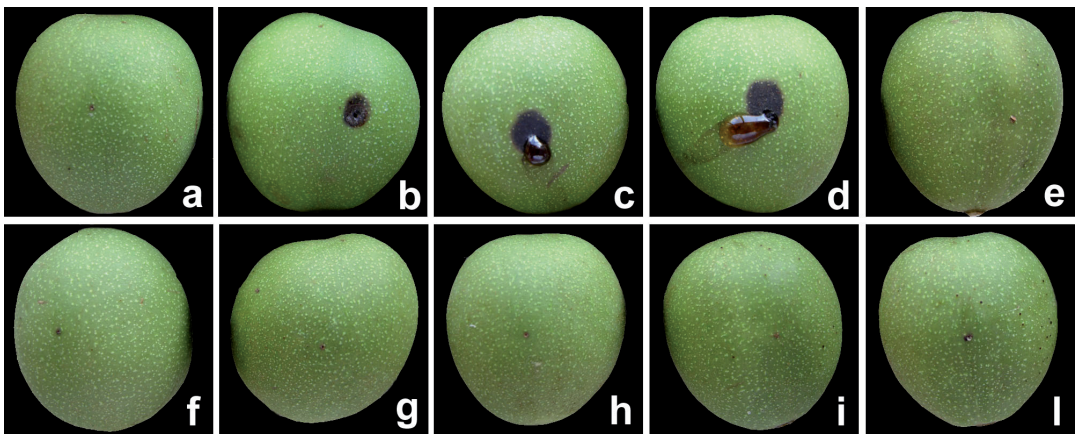

Figure 2. Symptoms on immature walnut fruits inoculated with bacterial suspensions (10^8 cells mL^{-1}) of *Brenneria nigrifluens* DAPP-PG 509 (b), *B. nigrifluens* DAPP-PG 621 (c), *B. nigrifluens* LMG 2694^T (d), *Dickeya chrysanthemi* DAPP-PG 637 (e), *Erwinia rhapontici* DAPP-PG 636 (f), *Ralstonia pickettii* DAPP-PG 640 (g), *Spingobacterium spiritovorum* DAPP-PG 639 (h), an unknown enterobacteriacea DAPP-PG 638 (i), and *B. alni* CFBP 3923 (l) using the syringe with needle technique, 8 days after inoculation. (a) Control fruit infiltrated with water.

toms developed only on fruits inoculated with *B. nigrifluens* strains and not on fruits inoculated with any other bacterial species frequently isolated from Persian walnut bark cankers, ii) the same results were obtained when the pathogenicity test was carried out on fruits in 2008 and in 2009. The test is also very rapid, compared with the plant stem inoculation technique, in which the canker symptoms do not appear until at least 1 month after inoculation. Its rapidity is ensured by a high inoculum dose (10^8 cells mL^{-1}) (see Figure 1), which is probably also required in natural infections, as a long time (3 months) was necessary before the first disease symptoms were seen in open field conditions on adult plants inoculated with $1–2\times10^8$ cells mL^{-1} (Scortichini, 1999).

In conclusion, the inoculation of immature walnut fruits with the newly developed technique is a specific, reproducible and rapid test to evaluate *B. nigrifluens* pathogenicity and can be used to select this bacterial species from among other, morphologically similar bacteria isolated from bark canker of Persian walnut.

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