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First Report of *Brenneria nigrifluens* as the Causal Agent of Shallow-Bark Canker on Walnut Trees (*Juglans regia*) in Serbia

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Abstract

In late summer 2011, shallow, irregular cankers were observed on trunks and branches of non-chemically-treated walnut trees (*Juglans regia* L.) on a 30-year-old orchard in the region of Fruška Gora (Vojvodina, Serbia). Disease incidence was ~80% and yield loss was ~50%. For pathogen isolation, small pieces (~5 mm diameter) of wood tissue collected at the edge of the cankers were macerated in sterile distilled water and streaked onto nutrient agar with 5% sucrose. Plates were then incubated at 28°C for 2 days. The prevalent bacterial colonies and those similar in appearance to *Brenneria nigrifluens* (Wilson et al.) Hauben et al. were purified on nutrient agar (NA). Eight gram-negative, oxidasenegative, catalase-positive strains, showing oxidative and fermentative metabolism, were selected for further characterization. To identify the bacteria on a molecular basis, we analyzed the 16S rDNA and *gyr* B gene sequences. The 16S rDNA partial sequences of analyzed strains were amplified using the primers P0 (5′-GAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′) (3).

Additionally, the *gyr* B gene sequences were generated with primers GyrB-F (5′-MGGCGGYAAGTTCGATGACAAYTC-3′) and GyrB-R (5′-TRATBKCAGTCARACCTTCRCGSGC-3′) (2). All amplicons were purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions and sequenced by Macrogen Inc. (Seoul, South Korea) using the same primers used for amplification. The sequences were edited using FinchTV v.1.4.0, assembled using the *Clustal* W program integrated into MEGA5 software (4), and deposited in NCBI GenBank under accessions JX484738 to 40 for the 16S rDNA gene and KC571240 to 47 for the *gyr* B gene. The 1,359-bp 16S rDNA sequences obtained for the eight strains were compared to the reference 16S rDNA sequences retrieved from GenBank. BLAST analysis revealed 100% homology of Serbian strains with sequences of *B. nigrifluens* (Z96095 and FJ611884). The *gyr* B gene sequences of our strains were 100% homologous to the sequences of *B. nigrifluens* deposited in GenBank (JF311612 to 15). Pathogenicity of all strains was confirmed on young fruits by infiltration of bacterial suspensions (10^8 CFU ml^{-1} from a 48 h NA culture) with syringe into the mesocarp of walnut fruits and by stem infiltration with syringes without needles into branch wounds (1). Inoculated fruits were incubated in plastic boxes for 8 days at 20°C, 80 to 100% RH, with a 12-h photoperiod. Inoculated plants were maintained for 3 months at 22 to 28°C with continuous light and at 70 to 80% RH in plastic tunnels. Inoculated fruits developed bark canker symptoms at the inoculation sites, which became necrotic and released a reddish brown exudate. Necrotic lesions were observed on inoculated branches. *B. nigrifluens* was reisolated from the margins of necrotic fruit and stem tissue. Physiological and biochemical tests showed that strains grew at 36°C and did not produce arginine dihydrolase, H_2S, indole, nitrate, nor a fluorescent pigment on King's B medium. They did not induce a hypersensitive reaction on tobacco leaves and did not hydrolyse gelatin and starch. They produced acid without gas from glucose, inositol, sorbitol, arabinose, and sucrose, but not from maltose and lactose (1). Results of pathogenicity and biochemical tests were also the same for reisolated strains. This is the first report of *B. nigrifluens* as the causal agent of shallow-bark canker on walnut trees in Serbia.

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