A PROTOCOL FOR RAPID IDENTIFICATION OF *BRENNERIA NIGRIFLUENS* AMONG BACTERIA ISOLATED FROM BARK CANKERS IN PERSIAN WALNUT PLANTS

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SUMMARY

A protocol based on API 20E, REP-PCR or Biolog analyses is described for rapid and reliable identification of *Brenneria nigrifluens* among the bacteria isolated from bark cankers in walnut plants. Bacteria that are soluble in 3% KOH solution (Gram negative), oxidase negative and with oxidative (O) and fermentative (F) metabolism are subjected to API 20E, REP-PCR or Biolog analyses. Isolates that generate a 7-digit code = 0005773 in the API 20E system, a REP-PCR fingerprint or Biolog profile very similar (87-100%) to those of the *B. nigrifluens* reference strains are assigned to this bacterial species. Using the protocol, we identified 4 isolates of *B. nigrifluens* among the 28 gram negative isolates (14 with O/F and 14 with O metabolism) obtained from bark cankers in walnut plantations on 10 farms in Central Italy. Among the other ten O/F isolates, only 2, assigned to *Pectobacterium chrysanthemi*, were identified with reliability by Biolog analysis. This analysis also permits identification of isolates with O metabolism. A pathogenicity test performed on walnut plants revealed that only the 4 *B. nigrifluens* isolates provoked bark canker symptoms 3 months after the inoculation. We also found that one isolate of four and the type strain (LMG 2694^T) of *B. nigrifluens* are urease negative, when determined in Dye's medium. Since our data are in contrast with those previously reported, this phenotypic character needs further verification for *B. nigrifluens* identification.

Key words: API 20E, Biolog, *Juglans regia*, REP-PCR, *Brenneria nigrifluens*.

INTRODUCTION

A number of biotic diseases affect plants of Persian walnut (*Juglans regia* L.) and can provoke significant reduction in walnut and timber production. Among them,

shallow bark canker incited by *Brenneria nigrifluens* (Wilson *et al.*) Hauben *et al.* (synonym *Erwinia nigrifluens*) is considered one of the most dangerous for timber production. First reported in California (Wilson *et al.*, 1957), the disease was also recorded from Spain (López *et al.*, 1994), Iran (Harighi and Rahimian, 1997) and several locations in Italy (Saccardi *et al.*, 1998; Morone *et al.*, 1998; Scortichini, 1999; Carella *et al.*, 2003) 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 can ooze through small cracks in the bark. It can provoke severe damage in young nursery plants (Saccardi *et al.*, 1998) and on adult trees (Piccirillo, 2003).

Since a number of bacterial species were frequently isolated from cankers present in walnut stands which we surveyed in farms of North and Central Italy, we developed a rapid and reliable protocol, based on the API 20E system, REP-PCR and Biolog analyses, to verify whether *B. nigrifluens* was present among the bacteria obtained. Preliminary results of this study were reported earlier (Moretti *et al.*, 2004).

MATERIALS AND METHODS

Surveys, isolations and reference bacteria. Surveys for bark canker were done in 2001-2002 on walnut (*Juglans regia* L.) trees grown for timber on 10 farms in North and Central Italy (Table 1). For bacterial isolation, the bark immediately surrounding the canker was removed with a flame-sterilized knife. Small pieces of tissue, collected with a scalpel at the edge of the cankers, were immersed in a few drops of sterile 0.85% NaCl solution contained in Eppendorf tubes, which were kept in ice until the isolation. Within 6 h after sampling, affected tissues were ground in the tubes using steel pestles. A loopful of the homogenate was streaked onto nutrient agar (NA) and incubated at 27±1°C. The prevalent bacterial colonies and those similar in appearance to *Brenneria nigrifluens* were selected and purified on NA amended with 5% sucrose. Twenty-eight isolates were obtained (Table 1), and stored in 15% glycerol solution at –80°C.

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Table 1. Bacterial isolates from walnut and reference strains of *Brenneria nigrifluens, Brenneria rubrifaciens* and *Xanthomonas arboricola* pv. *juglandis*, farm locations where walnut stands were surveyed and from which bacterial isolates were obtained, and basic microbiological tests.

Bacteria		Farm no. and location	Oxidative (O)/ Fermentative (F) metabolism	Oxidase	Urease
Isolate no.	6	5 - Passaggio di Bettona (PG)*	O/F	-	+
	7	6 - Torgiano (PG)	O/F	-	-
	10	9 - Montefiore dell'Aso (AP)	O/F	-	-
	12	9 - Montefiore dell'Aso (AP)	O/F	-	+
	13	9 - Montefiore dell'Aso (AP)	O/F	-	+
	36	7 - Ostra Vetere (AN)	O/F	-	-
	37	7 - Ostra Vetere (AN)	O/F	-	+
	38	7 - Ostra Vetere (AN)	O/F	-	-
	39	10 - S. Matteo (MN)	O/F	-	-
	40	10 - S. Matteo (MN)	O/F	-	-
	41	10 - S. Matteo (MN)	O/F	-	-
	42	10 - S. Matteo (MN)	O/F	-	-
	43	10 - S. Matteo (MN)	O/F	-	-
	44	7 - Ostra Vetere (AN)	O/F	-	-
	4	3 - Passaggio di Bettona (PG)	O	-	+
	9	8 - Fossombrone (PU)	O	-	-
	11	8 - Fossombrone (PU)	O	-	+
	15	1 - Ponte Valleceppi (PG)	O	+	+
	21	1 - Ponte Valleceppi (PG)	O	+	+
	25	1 - Ponte Valleceppi (PG)	O	+	+
	26	2 - Orvieto (TR)	O	+	-
	27	6 - Torgiano (PG)	O	-	-
	28	6 - Torgiano (PG)	O	-	-
	29	5 - Passaggio di Bettona (PG)	O	-	+
	30	6 - Torgiano (PG)	O	-	-
	32	6 - Torgiano (PG)	O	-	-
	33	5 - Passaggio di Bettona (PG)	O	-	-
	34	6 - Torgiano (PG)	O	-	+
Strains	LMG 694[T a]		O/F	-	-
	LMG 5107[a]		O/F	-	+
	LMG 5953[a]		O/F	-	+
	LMG 709[Tb]		O/F	-	-
	ISF 174 [c]		O	-	-

All isolates and strains are soluble in KOH.
* Province initials are in brackets. AP = Ascoli Piceno; AN = Ancona; MN = Mantova; PG = Perugia; PU = Pesaro-Urbino; TR = Terni.
[a] *Brenneria nigrifluens*; [b] *Brenneria rubrifaciens*; [c] *Xanthomonas arboricola* pv. *juglandis*.

The strains LMG 2694[T], LMG 5107 and LMG 5953 of *B. nigrifluens*, the strain LMG 2709[T] of *Brenneria rubrifaciens* (Wilson *et al.*; Hauben *et al.*), obtained from the BCCM™ (The Belgian Coordinated Collections of Microorganisms, Gent, Belgium), as well as the strain ISF 174 of *Xanthomonas arboricola* pv. *juglandis* (Pierce) Vauterin *et al.*, kindly provided by Dr. M. Scortichini (CRA, Istituto Sperimentale per la Frutticoltura, Rome, Italy), were used as reference strains in identification tests.

Biochemical, physiological and nutritional tests. All isolates were tested as follows: solubility of bacterial cells in 3% KOH, oxidative and/or fermentative glucose metabolism, presence of cytochrome *c* oxidase and presence of urease determined in Dye's medium (Klement *et al.*, 1990; Schaad *et al.*, 2001). Isolates that were KOH-soluble (Gram negative), oxidase-negative and with oxidative and fermentative metabolism were submitted to the API 20E procedure (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's in-

structions. All isolates were tested twice for their metabolism of 95 organic substrates using Biolog GN2 microplates (Biolog Inc., Hayward, CA, USA). They were grown on Tryptic soy agar (AES Laboratoire, Rennes, France) for 24 h at 27±1°C. Bacterial cells, washed twice with 25 ml of 0.85% NaCl to remove extracellular polysaccharides, were suspended in the GN/GP inoculating fluid (Biolog Inc., Hayward, CA, USA) and the absorbance at 595 nm adjusted to between 0.3 and 0.5 according to Toth *et al.* (1999). Metabolism of substrates by bacterial isolates was visually evaluated 24 h after incubation of the microplates at 27±1°C. Data, expressed in 3 categories (positive, negative and doubtful), were analysed using the Biolog Microlog™ 4.1 software for bacterial identification and the GN database, version 6.01.

REP-PCR. Bacterial isolates were subjected to rep-PCR analysis using the primers REP 1R and REP 2I, according to the procedure of Rademaker and De Bruijn (1997). This analysis was repeated twice. DNA was extracted from bacterial cells grown on Luria-Bertani broth for 16 h at 27±1°C in an orbital shaker at 200 rpm, according to Ausubel *et al.* (1988). Amplicons were separated by electrophoresis on 1.5% agarose gels in 0.5x TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.0) at 50 V and 4°C for 14 h. DNA fingerprints were visualised with a UV transilluminator (EuroClone, Milan, Italy) and their images captured with the EuroClone Photoprint camera system.

The molecular sizes of fragments generated were estimated by comparison with simultaneously run Ladder Mix (MBI Fermentas, Burlington, ON, Canada). Lanes were compared by reading horizontally across the gel image, from bottom to top; if a band was present, it was assigned a value of 1 at that location, whereas if absent, it was assigned a value of 0. The presence/absence of bands was collated into a binary data matrix. Cluster analysis was performed on similarity matrices, which were produced using Dice's coefficient (Dice, 1945) or Jaccard's similarity index (Jaccard, 1908) and subjected to the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm, using NTSYSpc software (Exeter Software, New York, NY, USA), version 2.1. To establish robustness of clusters, the cophenetic value was obtained using the NTSYSpc software.

Pathogenicity test. Bacterial isolates were tested for pathogenicity on 2-year-old Persian walnut seedlings, about 120 cm high. To prepare the inoculum, bacteria were grown onto NA at 27±1°C for 48 h, suspended in sterile deionized water and spectrophotometrically adjusted to 10^8 cfu ml^{-1}. Twenty µl of the suspensions were placed in wounds (about 1 cm long) made in the bark of walnut stems with a scalpel. The wounds were protect-

ed with Parafilm M (American National Can, Chicago, IL, USA). Sterile water, instead of bacterial suspension, was used for control plants. Plants were kept in a heated greenhouse at 22-28°C under natural light conditions and 70-85% RH. Three months after the inoculation, symptoms were recorded.

RESULTS

As reported in Table 1, bacteria of all isolates were soluble in KOH and therefore were Gram-negative. Fourteen isolates showed oxidative (O) and fermentative (F) metabolism and were oxidase-negative; 4 of them (6, 12, 13 and 37) were urease positive. Fourteen isolates showed O metabolism; among them, 4 were oxidase-positive and 7 urease-positive (Table 1).

Among the isolates with O/F metabolism assayed with the API 20E system, no. 6, 10, 12 and 13 gave the same results of those obtained with the *Brenneria nigrifluens* reference strains LMG 2694^T, 5107 and 5953 (Table 2). They generated the 7-digit code 0005773, identical to that reported by Mergaert *et al.* (1984) for the type strain (NCPPB 564) and the reference strains (NCPPB 565, 566) of *B. nigrifluens*. When compared with the 7-digit codes reported by the same authors, isolate no. 7 generated a code identical to those of *Pectobacterium cypripedii* (Hori) Hauben *et al.* (NCPPB 2636), *Pectobacterium carotovorum* subsp. *carotovorum* (Jones) Hauben *et al.* (NCPPB 1625) and *Pantoea agglomerans* (Ewing and Fife) Gavini *et al.* (van Vuuren 117 strain), while nos. 36, 40, 41 and 43 proved identical to *Pectobacterium rhapontici* (Millard) Hauben *et al.* (NCPPB 1578, 2560, 2559, 2548, 2606 and 2549). According to the API computer service, isolates no. 39 and 42 were assigned to *Pantoea* spp. (similarity index = 0.95) and *Raoultella terrigena* (Izard *et al.*) Drancourt *et al.* (similarity index = 0.92), respectively. Neither the comparison with the 7-digit codes reported by Mergaert *et al.* (1984) nor the API computer service permitted identification of isolates no. 37, 38 and 44.

Biolog analysis showed that isolates no. 6, 10, 12 and 13 generated physiological profiles very similar (similarity index = 0.87-1.00) to those of *B. nigrifluens* reference strains LMG 2694^T, 5107 and 5953. Since *B. nigrifluens* profiles are not present in the Biolog GN database, we report the data obtained in Table 3. In addition, the same analysis did not permit clear identification of the others isolates with O/F metabolism not belonging to *B. nigrifluens* and characterized by API 20E (no. 37, 39, 40, 41, 42 and 44), except for isolates no. 36 and 38, which were assigned to *Pectobacterium chrysanthemi* (Burkholder *et al.*) Brenner *et al.* (similarity index = 0.93). The Biolog system identified 12 isolates (no. 4, 15, 21, 25, 26, 27, 28, 29, 30, 32, 33 and 34) as *Ralstonia pickettii* (Ralston *et al.*) Yabuuchi *et al.* (similarity index

Table 2. API 20E system data of oxidase negative bacterial isolates with oxidative and fermentative metabolism. Data were compared with those obtained with the strains LMG 2694^{T}, LMG 5107 and LMG 5953 of *Brenneria nigrifluens*.

Test	Isolate number and strains							
	6, 10, 12, 13, LMG 2694^{T}, LMG 5107, LMG 5953	7	36, 43	37, 38	39	40, 41	42	44
β-galactosidase	-	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	+
Lysine decarboxylase	-	-	-	-	-	-	+	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-
Citrate utilization	-	+	+	+	-	+	-	-
H$_2$S production	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-
Tryptophane deaminase	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-
Acetoin production	+	+	+	-	+	+	+	+
Gelatinase	-	-	-	-	-	-	-	-
Assimilation of :								
Glucose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Inositol	+	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	-	-	+	-
Rhamnose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-
NO$_2$ production	-	-	-	+	-	-	+	+
Reduction to N$_2$	-	-	+	-	+	+	-	+
7-digit code	0005773	1205573	1205773	1204773	1005373	1205373	5005773	3005173
Bacterial identification according to Mergaert *et al.* (1984) codes or API computer service	*Brenneria nigrifluens*	*Pectobacterium cypripedii, Pectobacterium carotovorum* subsp. *carotovorum, Pantoea agglomerans*	*Pectobacterium rhapontici*	Not identified	*Pantoea* spp.	*Pectobacterium rhapontici*	*Raoultella terrigena*	Not identified

Table 3. Carbon source utilisation of *Brenneria nigrifluens* and walnut isolates no. 6, 10, 12 and 13, determined by Biolog analysis.

Carbon sources	*Brenneria nigrifluens* strains			Isolate no.			
	LMG 2694[T]	LMG 5107	LMG 5953	6	10	12	13
Dextrin	+	+	d	+	+	+	+
Tween 80	+	+	+	+	d	+	+
Tween 40	+	+	d	+	d	+	+
Acetic Acid	+	d	+	+	d	+	+
Formic Acid	-	+	-	-	+	+	-
Glucuronamide	-	-	-	d	d	-	+
Adonitol, L-alanine	d	-	-	-	-	-	-
i-Erythritol, glycyl-L-aspartic acid, urocanic acid	-	-	-	d	-	-	-
Maltose, xylitol, β-hydroxy butyric acid, *p*-hydroxy phenylacetic acid, L-alanylglycine	-	-	-	-	d	-	-
D-Galacturonic acid	-	-	d	-	-	-	-
D-Glucosaminic acid, L-histidine	-	d	-	-	-	-	-
α-Hydroxy butyric acid	-	-	-	d	d	-	-

+ = carbon source utilised; - = carbon source not utilised; d = doubtful reaction.
All strains and isolates tested utilised the following carbon sources: N-acetyl-D-glucosamine, L-arabinose, D-arabitol, D-fructose, D-galactose, gentiobiose, α-D-glucose, m-inositol, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, methyl pyruvate, mono-methyl-succinate, D-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, succinic acid, bromo succinic acid, succinamic acid, L-asparagine, L-aspartic acid, L-glutamic acid, L-serine, uridine, thymidine, glycerol, D,L-α-glycerol phosphate, glucose-1-phosphate, glucose-6-phosphate.

= 0.99), and the others 2 (no. 9 and 11) as *Sphingobacterium spiritovorum* (Holmes *et al.*) Yabuuchi *et al.* (similarity index = 0.99). This identification, however, needs further confirmation.

REP-PCR analysis, carried out on all bacterial isolates, produced the dendrogram shown in Fig. 1. Identical results were obtained on repeating the analysis. The genomic REP-PCR profiles consisted of bands ranging in size from 300 to 2000 bp. Seventeen reproducible, clearly resolved bands were scored for UPGMA analysis. Dice's coefficient and Jaccard's similarity index yielded very similar dendrograms (data not shown). The results obtained with Dice's coefficient are reported in Fig. 1. A cophenetic value of 0.89 was determined for the matrix, indicating a high goodness-of-fit for the cluster analysis. Isolates no. 6, 10, 12 and 13 generated the same fingerprints, which were very similar to those of *B. nigrifluens* reference strains. In particular, they had a similarity index 0.88 with the type strain LMG2694[T] and 0.93 with the strains LMG 5107 and LMG 5953 (0.93). *Brenneria rubrifaciens* (strain LMG 2709[T]) and *Xanthomonas arboricola* pv. *juglandis* (strain ISF 174), the causal agents of two important walnut diseases characterized by the canker symptom, generated fingerprints different and with low similarity (0.31 and 0.45, respectively) in comparison with those of *B. nigrifluens* strains. It is worth noting that isolates belonging to *R. pickettii*, according to Biolog analysis, formed a cluster (Fig. 1).

Three months after inoculation on walnut plants, only isolates no. 6, 10, 12 and 13 produced bark cankers (Fig. 2), from which bacteria with REP-PCR profiles identical to those of the respective isolates were re-isolated (data not shown). No symptoms were observed in control plants.

DISCUSSION

Since the enactment of ECC regulation no. 2080/92, which aims at promoting reforestation of rural lands, a significant increase in area devoted to timber production has been recorded in European countries. In Italy, from 1994 to 2000, more than 104,000 ha of new stands were planted mainly with broadleaf species, Persian walnut included (Colletti, 2001). During this period, outbreaks of shallow bark canker of walnut provoked by *Brenneria nigrifluens* have been recorded in Italy, both in new and old stands. The widespread occurrence in Italy of this disease, whose epidemiological aspects are unknown, have suggested that the bacterium is part of the resident microflora and that it behaves as a pathogen when the plant is stressed (Morone *et al.*, 1998). In order to verify this hypothesis and to investigate the epidemiology of this disease, systematic studies are necessary, for which rapid protocols for the identification of the bacterium are essential. The traditional diagnostic tests proposed by Schaad *et al.* (2001) and those used by Morone *et al.* (1998), Saccardi *et al.* (1998) and Ménard *et al.* (2004) for identification of *B. nigrifluens* are time-consuming. Here, we have shown
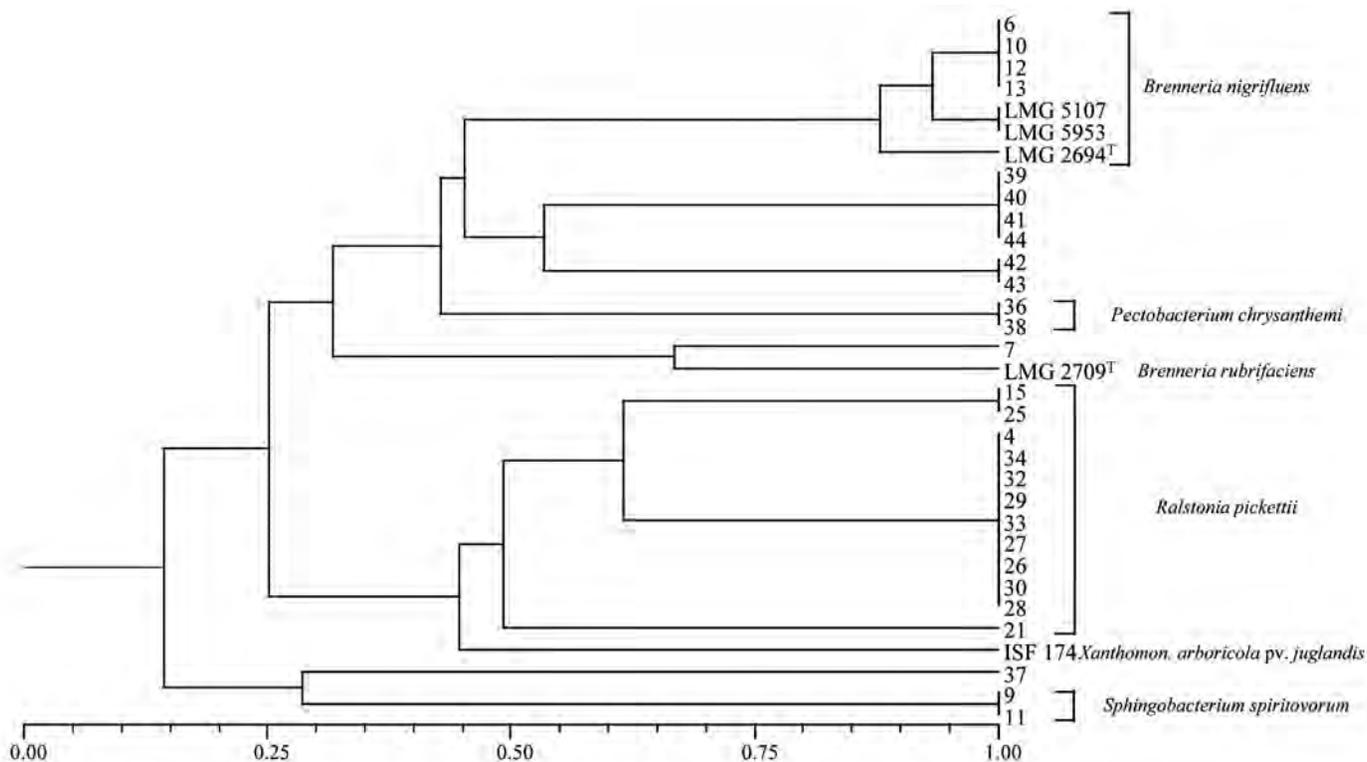
Fig. 1. Dendrogram derived from REP-PCR fingerprint data, obtained by cluster analysis (UPGMA) and Dice's coefficient.

that API 20E, REP-PCR and Biolog analyses can rapidly and reliably identify *B. nigrifluens* and we propose a protocol for quick identification of the bacterium, schematized in Fig. 3. The identity of the *B. nigrifluens* isolates determined with one of the analyses, can be confirmed with the pathogenicity test on walnut plants.

We also show that the presence of urease is unreli-

Fig. 2. Pathogenicity test of the isolate no. 6 of *Brenneria nigrifluens* on Persian walnut plants. Symptoms on control (**A**) and inoculated plants (**B**) 3 months after inoculation. Browning of the internal wood is visible under the bark tissue in correspondence of the site inoculated with the bacterial isolate.

able as a key test for identification of *B. nigrifluens* among the *Erwinia amylovora* group as reported by Schaad *et al.* (2001). In our hands, we found the type strain (LMG 2694[T]) and the isolate no. 10 of *B. nigrifluens* to be urease-negative (Table 1).

B. nigrifluens cannot be confused with *Brenneria rubrifaciens*, the causal agent of deep bark canker of walnut, which generates different 7-digit codes (0004022 or 0004122; Mergaert *et al.*, 1984) with the API 20E system and a REP-PCR fingerprint dissimilar to *B. nigrifluens* (Fig. 1).

We isolated *B. nigrifluens* only from 2 of the 10 farms surveyed, where bark canker symptoms were recorded. In the other 8 farms, a number of gram-negative bacteria non-pathogenic on walnut plants, with some O/F and others with O metabolism were isolated from the cankers. These may be saprophytic organisms which may compete for nutrients and hamper the isolation of *B. nigrifluens*. However, we cannot exclude that the symptoms observed were provoked by other pathogens.

Among the other bacteria with O/F metabolism we found associated with bark cankers and which were not definitively identified by API 20E system, isolates were assigned by Biolog analysis to *Pectobacterium chrysanthemi* and *Ralstonia pickettii*. The former bacterium can attack several plant species (Bradbury, 1986), but these strains were not pathogenic to walnut.

We demonstrated that bacteria that resemble *Ralstonia pickettii* in the Biolog system apparently are fre-
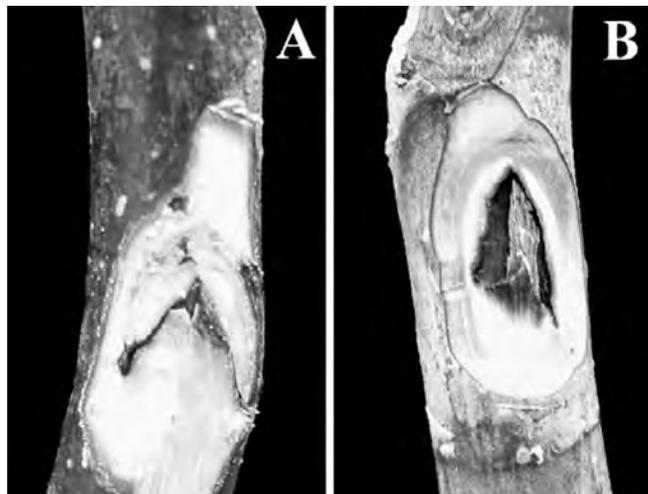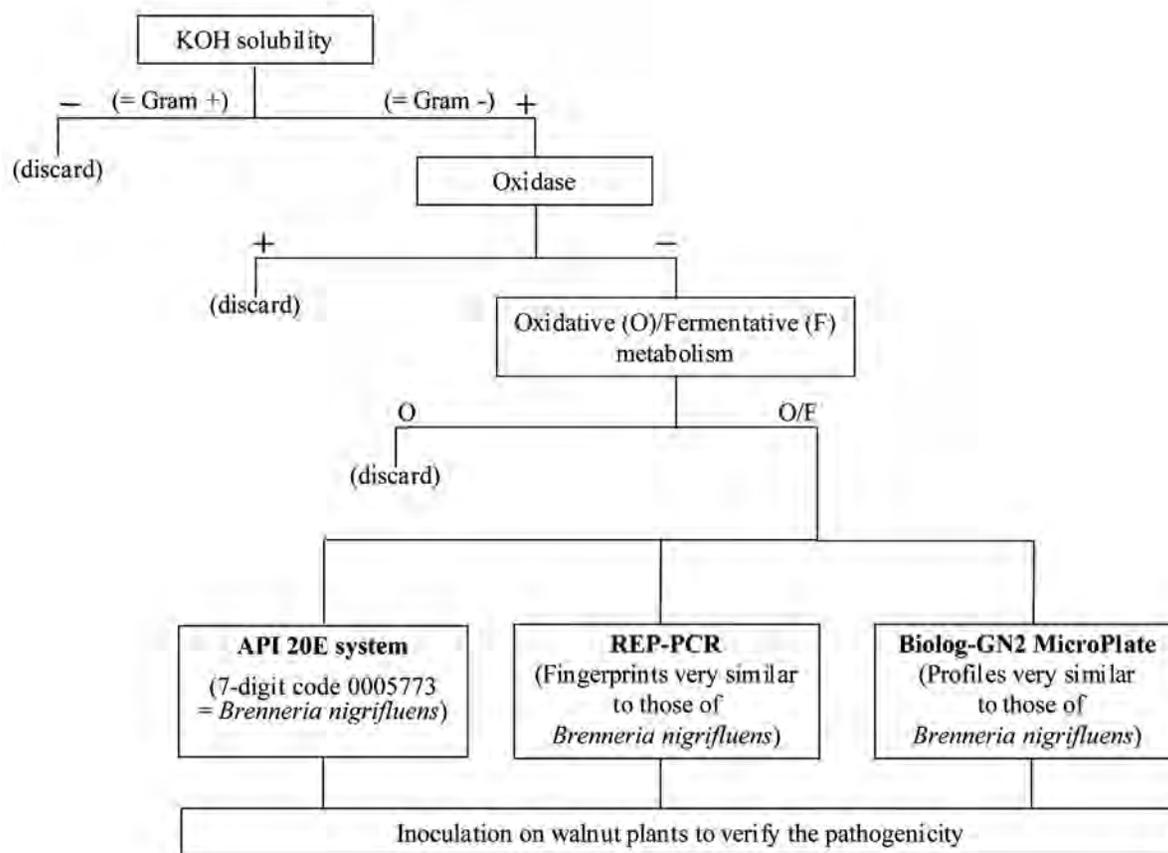
Fig. 3. Flow diagram of the protocol for the rapid and reliable identification of *Brenneria nigrifluens* based on API 20E, REP-PCR or Biolog analyses.

quently present as endophytes in walnut plants. Similar bacterial were recovered as endophytes from soybean plants (Kuklinsky-Sobral *et al.*, 2005) and maize roots (McInroy and Kloepper, 1995). Further studies are necessary to clearly identify these bacteria and to understand their role, if any, in disease development.

Further work is in progress to develop on the basis of REP-PCR specific amplicon and 16S rDNA sequences, primers for a PCR-based method to detect *B. nigrifluens* in walnut samples.

REFERENCES

Ausubel F.M., Brent R, Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., Struhl K., 1988. Current Protocols of Molecular Biology. John Wiley and Sons, New York, NY, USA.

Bradbury J.F., 1986. Guide to plant pathogenic bacteria. CAB International, Wallingford, UK.

Carella D., Spigno P., De Vita N., 2003. Un caso di cancro corticale superficiale del noce in Campania. *Informatore Fitopatologico* **53 (7-8)**: 32-33.

Colletti L., 2001. Risultati dell'applicazione del Regolamento CEE 2080/92 in Italia. *Sherwood* **70**: 23-31.

Dice L., 1945. Measurement of the amount of ecological association between species. *Ecology* **26**: 297-302.

Harighi B., Rahimian H., 1997. Widespread occurrence of the bark canker of walnut trees in Mazandaran Province. *Iranian Journal of Plant Pathology* **33**: 48-50.

Jaccard P., 1908. Nouvelles recherches sur la distribution florale. *Bulletin de la Societé Vandoise de Science Naturelle* **44**: 223-270.

Klement Z., Rudolph K., Sands D.C., 1990. Methods in Phytobacteriology. Akadémiai Kiado´, Budapest, Hungary.

Kuklinsky-Sobral H.L., Araujo W.L., Mendes R., Pizzirani-Kleiner A.A., Azevedo J.L., 2005. Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. *Plant and Soil* **273**: 91-99.

López M.M., Marti R., Morente C., Oreliana N., Ninot T., Aleta N., 1994. Phytopathogenic bacteria identified in walnut in Spain. *Investigación Agraria, Producción y Protección Vegetale* Fuera de Serie **2**: 307-314.

McInroy J.A., Kloepper J.W., 1995. Development of delivery systems for introducing endophytic bacteria into cotton. *Biocontrol Science and Technology* **5**: 407-416.

Mergaert J., Verdonk L., Kersters K., Swings J., Boeufgras J.M., De Ley J., 1984. Numerical taxonomy of Erwinia species using API systems. *Journal of General Microbiology* **130**: 1893-1910.

Ménard M., Delort F., Baudry A., Le Saux M., 2004. First report of bacterial canker of walnut caused by *Brenneria nigrifluens* in France. *Plant Disease* **88**: 220.

Moretti C., Buonaurio R., 2003. Metodi molecolari nella diagnosi fitobatteriologica. *Petria* **13**: 47-75.

Moretti C., Silvestri F.M., Rossini E., Natalini G., Buonaurio R., 2004. Diagnostic tool for the identification of *Brenneria nigrifluens*, the casual agent of Persian walnut bark canker. *Journal of Plant Pathology* **86**: 300-301.

Morone C., Janse J.D., Scortichini M., 1998. Bark canker of Persian walnut (*Juglans regia*) tree incited by *Erwinia nigrifluens* in Italy. *Journal of Phytopathology* **146**: 637-639.

Piccirillo P., 2003. Il quadro fitopatologico del noce (*Juglans regia* L.) attraverso le osservazioni dell'ISF di Caserta. *Frutticoltura* **10**: 39-42.

Rademaker J.L.W., De Bruijn F.J., 1997. Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. In: Caetano-Anolles G. and Gressfoff P. (eds.). Protocols, application and overviews, pp. 151-171. J. Wiley and Sons, New York, NY, USA.

Saccardi A., Bonetti V., Melegatti A., Cristanini M., 1998. Occurrence of *Erwinia nigrifluens* on English walnut (*Juglans regia*) tree in the Veneto region (Northern Italy). *Journal of Plant Pathology* **80**: 63-65.

Schaad N.W., Jones J.B., Chun W., 2001. Laboratory guide for identification of plant pathogenic bacteria, third edition. APS Press, St. Paul, MN, USA.

Scortichini M., 1999. Rinvenimento di *Erwinia nigrifluens* su noce da legno nel Lazio. *Informatore Fitopatologico* **49 (9)**: 52-54.

Toth I.K., Bertheau Y., Hyman L.J., Laplaze L., López M.M., McNicol J., Niepold F., Persson P., Salmond G.P.C., Sletten A., van der Wolf J.M., Pérombelon M.C.M., 1999. Evaluation of phenotypic and molecular typing techniques for determining diversity in *Erwinia carotovora* subspp. *atroseptica*. *Journal of Applied Microbiology* **87**: 770-781.

Wilson E.E., Starr M.P., Berger J.A., 1957. Bark canker, a bacterial disease of the Persian walnut tree. *Phytopathology* **47**: 669-673.