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Identification of a genetic lineage within *Xanthomonas arboricola* pv. *juglandis* as the causal agent of vertical oozing canker of Persian (English) walnut in France

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A new bacterial disease of Persian (English) walnut (*Juglans regia*) has been observed in France. This disease, called vertical oozing canker (VOC), is characterized by vertical cankers on trunks and branches of affected walnut trees with oozing exudates. To determine the aetiology of the disease, a study was carried out in 79 walnut orchards and nurseries located in southeastern and southwestern France. Bacterial analysis from diseased samples yielded 36 strains identified as *Xanthomonas arboricola* and 32 strains identified as *Brenneria nigrifluens* on the basis of biochemical tests. The causal agent of VOC was identified as *X. arboricola* by pathogenicity tests on walnut. Fluorescent amplified fragment length polymorphism (F-AFLP) was carried out on 36 strains of *X. arboricola* collected in this study, 24 strains of *X. arboricola* pv. *juglandis* isolated from walnut blight symptoms and one strain of *X. arboricola* pv. *corylina* included as an outgroup. Based on cluster analysis of F-AFLP data, most *X. arboricola* strains responsible for main VOC outbreaks showed a high degree of similarity, forming a cluster clearly separate from strains of *X. arboricola* pv. *juglandis* isolated from walnut blight symptoms. It is suggested that VOC is caused by a distinct genetic lineage within the pathovar *juglandis* of *X. arboricola* that is also able to cause classical bacterial blight symptoms on walnut leaves and fruits.

Keywords: *Brenneria nigrifluens*, fluorescent-AFLP, *Juglans regia*, molecular typing, symptomatology, vertical oozing canker

Introduction

Walnut species (*Juglans* sp.) are important nut and timber producers in temperate regions of Europe, Asia, North America and South America. The Persian (English) walnut (*J. regia*) is the most horticulturally developed and widely cultivated and is easily the leading producer of commercial nuts. Among biotic diseases that affect walnut, bacterial blight is considered as the most important one in all walnut-growing areas (Leslie *et al.*, 2006). *Xanthomonas arboricola* pv. *juglandis* (*X. a.* pv. *juglandis*) (Vauterin *et al.*, 1995), also known as *X. campestris* pv. *juglandis*, is the causal agent of the disease. It causes necrosis on leaves, catkins, twigs and fruits, and can induce important crop losses. In addition to bacterial blight, three other necrotic syndromes have been observed on walnut. One affects the fruit and causes brown apical necrosis (BAN). Aetiology of BAN has been discussed and has revealed that *Fusarium* is the most common genus associated with this complex disease (Belisario *et al.*, 2002). However, *X. a.* pv. *juglandis* is also associated with the BAN syndrome and could be the true causal agent of the disease (C. Moragrega, Institute of Food and Agricultural Technology, University of Girona, Spain, personal communication). The others affect trunks and branches and are due to two bacteria belonging to the genus *Brenneria* (formerly *Erwinia*). *Brenneria nigrifluens*, the causal agent of shallow bark canker, was first reported in California (Wilson *et al.*, 1957). This disease was also recorded in Spain (López *et al.*, 1994), Iran (Harighi & Rahimian, 1997) and Italy (Saccardi *et al.*, 1998). It affects the bark of trunk and scaffold branches. At first, the necrotic areas appear as small circular spots that later enlarge and coalesce to form extensive irregular-shaped cankers, from which a dark-coloured watery exudate appears through small cracks in the bark. Generally, the cankers are described as relatively shallow, extending only approximately a quarter to a third the depth of the bark. In more severe cases, the lesions may reach the cambium (Wilson *et al.*, 1957). A closely related bacterium, *B. rubrifaciens*, which produces a red pigment (rubrifacine), causes a similar disease known as deep bark canker. After its occurrence in California (Wilson *et al.*, 1967), this bacterium was also reported in Europe (González *et al.*, 2002). Deep bark canker symptoms typically appear in mature trees and are characterized by development of deep longitudinal

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cracks on the trunk, scaffolds or larger branches which exude a dark sap (Wilson *et al.*, 1967).

During intensive surveys aimed at ascertaining the sanitary status of the crop in France, unusual symptoms were often noticed on old and young trees in orchards and on young trees in nurseries. These symptoms are characteristics of a new disease termed vertical oozing canker (VOC). VOC was first reported in France in 2004 but the isolation and identification of the causal agent were not achieved (Ménard *et al.*, 2004). In fact, attempts to reproduce typical VOC symptoms using *B. nigrifluens* isolates recovered from diseased samples have failed. According to Ménard *et al.* (2004), 2 and 5 months after inoculations, no external cankers similar to those observed in the field were recorded and only necrotic lesions were observed in the inner bark with dark lines in internal wood. The status of VOC in France has remained unclear, with no recent substantiated reports.

The present study reports for the first time on the association of *X. a.* pv. *juglandis* with VOC in France. Fluorescent amplified fragment length polymorphism (F-AFLP) was used for identification and characterization purposes. This technique, based on the selective PCR amplification of a subset of DNA restriction fragments generated by restriction enzyme digestion, is a highly discriminatory and reproducible genotyping method (Vos *et al.*, 1995; Janssen *et al.*, 1996; Cirvilleri *et al.*, 2006; Alavi *et al.*, 2008; Shaik *et al.*, 2009). The pathogenicity of a set of isolates responsible for main VOC outbreaks was also tested on walnut. The results of this research can help to further elucidate the disease aetiology and epidemiology.

Materials and methods

Bacterial isolations and biochemical tests

Field surveys were carried out in the two main walnut-growing areas of France. A total of 455 samples with typical VOC symptoms were collected from 79 orchards and nurseries located in southeastern and southwestern France. For bacterial isolations, the bark immediately surrounding the cankers was removed aseptically with a flame-sterilized knife. Small pieces of tissue, collected with a scalpel at the edge of cankers, were immersed in sterile distilled water (SDW). Samples were also collected from liquid exuded by cortical lesions on diseased trunks. Samples were stored at 4°C and processed for isolation within 48 h of collection. Bacterial isolations were done by targeting the main bacteria associated with cankers (*Brenneria* sp. and *Xanthomonas* sp.) and also other bacteria present in high frequency. Aliquots of the resulting suspensions were streaked onto King's medium B (KB; King *et al.*, 1954) and incubated at 28°C for 3 days. Yellowish and white-grey bacterial colonies growing from the suspensions were re-streaked onto KB and YPGA (yeast extract, 5 g; Bactopeptone, 5 g; glucose, 10 g; agar, 15 g; distilled water, 1 L; pH 7·2) to obtain single colonies. The identification of *B. nigrifluens* isolates was carried out on the basis of biochemical and physiological characteristics according to Ménard *et al.* (2004). With all *Xanthomonas* sp. isolates and the reference strain of *X. a.* pv. *juglandis* (CFBP 2528), the following biochemical tests were performed following the techniques of Lelliott & Stead (1987): Gram reaction, oxidation and fermentative metabolism of glucose, oxidase reaction, catalase reaction, hydrolysis of gelatin, starch and aesculin, Tween 80 lipolysis, milk proteolysis, urease activity, indole production and nitrate reduction.

Pathogenicity tests

To fulfil Koch's postulates, bacterial suspensions of one strain of *Xanthomonas* sp. (12714) and one strain of *B. nigrifluens* (CFBP 6756) were inoculated on a trunk of a 4-year-old tree of *J. regia*. Then, 16 strains isolated from VOC (marked with an asterisk (*) in Table 1) were randomly selected for pathogenicity tests done in the open field. Four-year-old trees of *J. regia* cultivars Fernor RA 1156 and Franquette RA 311 were used for pathogenicity tests. To prepare the inoculum, bacteria were grown onto YPGA at 28°C for 48 h, suspended in SDW and adjusted to 1×10^9 CFU mL^{-1}. Three transverse incisions were made along the trunk and drops of the suspension were deposited with a micropipette to fill the wound. Each strain was inoculated on 15 scions per cultivar (three inoculation points per scion). Sterile distilled water and two reference strains: strain CFBP 2564 of *X. a.* pv. *juglandis* and the avirulent strain CFBP 1022 of *X. arboricola* isolated from walnut, were inoculated in the same way and used as controls. Symptoms were checked 3 months after inoculation. Re-isolations were performed after symptom appearance to confirm the presence of the bacterium using the same techniques previously described.

The pathogenicity of four strains (CFBP 1022, CFBP 2528, 12763 and 12785) representative of the bacterial collection was also tested on immature fruits following the technique described by Aletà *et al.* (2001). In addition, two strains (CFBP 2528 and 12785) were inoculated onto the foliage of walnut plantlets. Walnut seedlings cv. Lara were raised in a greenhouse until there were four to six young leaves. Plantlets were then placed in a growth chamber with cycles of 16 h daylight and 8 h night at 25°C. Bacterial suspensions (adjusted to 1×10^9 CFU mL^{-1} in SDW) were sprayed onto the foliage and plants were maintained for 2 days under plastic bags. The plastic bags were then removed and the plants were maintained in the growth chamber under the same climatic conditions. Typical necrotic spots were observed on leaves 12 days after inoculation of *X. a.* pv. *juglandis* strains.

F-AFLP analysis

Analysis was carried out on 36 *X. arboricola* strains collected in this study, including 29 directly isolated from VOC symptoms on trunks and branches and seven from

Table 1 Bacterial strains used in this study

Strain[a]	Host plant/cultivar	Origin of isolation[b]	Geographical region	Year of isolation	Pathogenicity tests
Xanthomonas arboricola (avirulent strain)					
CFBP 1022*	*Juglans regia*	WB	France	1967	–
X. arboricola pv. *juglandis*					
CFBP 878	*J. regia*	WB	France	1966	
CFBP 2528^T=ATCC 49083=LMG 747=NCPPB 411	*J. regia*	WB	New Zealand	1956	
CFBP 2564*	*J. regia*	WB	Italy	1985	–
CFBP 2568	*J. regia*	WB	Italy	1985	
12572	*J. regia*	WB	Gironde, France	2001	
12573	*J. regia*	WB	Gironde, France	2001	
12574	*J. regia*	WB	Gironde, France	2001	
12575	*J. regia*	WB	Gironde, France	2001	
12576	*J. regia*	WB	Gironde, France	2001	
12577	*J. regia*	WB	Gironde, France	2001	
12578	*J. regia*	WB	Gironde, France	2001	
12579	*J. regia*	WB	Gironde, France	2001	
12580	*J. regia*	WB	Gironde, France	2001	
12581	*J. regia*	WB	Gironde, France	2001	
12582	*J. regia*	WB	Gironde, France	2001	
12583	*J. regia*	WB	Gironde, France	2001	
12584	*J. regia*	WB	Gironde, France	2001	
12585	*J. regia*	WB	Gironde, France	2001	
12586	*J. regia*	WB	Gironde, France	2001	
12587	*J. regia*	WB	Gironde, France	2001	
12588	*J. regia*	WB	Gironde, France	2001	
12589	*J. regia*	WB	Gironde, France	2001	
12590	*J. regia*	WB	Gironde, France	2001	
12591*	*J. regia*	VOC	Dordogne, France	2001	+
12592	*J. regia*	VOC	Dordogne, France	2001	
12680	*J. regia*	VOC	Gironde, France	2002	
12681	*J. regia*	VOCt	Gironde, France	2002	
12707	*J. regia*/Fernor	VOCt	Isère, France	2002	
12708	*J. regia*/Fernor	VOCt	Isère, France	2002	
12709	*J. regia*/Fernor	VOCt	Isère, France	2002	
12710	*J. regia*/Franquette	VOCt	Isère, France	2002	
12711	*J. regia*/Fernor	VOC	Lot et Garonne, France	2002	
12712	*J. regia*/Lara	VOCt	Lot et Garonne, France	2002	
12713	*J. regia*/Fernor	VOCt	Gironde, France	2002	
12714*	*J. regia*/Fernor	VOC	Isère, France	2002	+
12762	*J. regia*/Fernor	VOC	Isère, France	2002	
12763	*J. regia*/Fernor	VOC	Lot et Garonne, France	2002	
12764	*J. regia*	VOC	Gironde, France	2002	
12765*	*J. regia*/Fernor	VOC	Lot, France	2003	+
12766*	*J. regia*/Fernor	VOC	Lot, France	2003	+
12767*	*J. regia*/Fernor	VOC	Corrèze, France	2003	+
12768*	*J. regia*/Fernor	VOC	Lot, France	2003	+
12769*	*J. regia*/Fernor	VOC	Dordogne, France	2003	+
12770	*J. regia*/Fernor	VOC	Lot, France	2003	
12771	*J. regia*/Chandler	VOC	Corrèze, France	2003	
12772	*J. regia*/Fernor	VOC	Dordogne, France	2003	
12773	*J. regia*/Fernor	VOC	Corrèze, France	2003	
12774*	*J. regia*/Lara	VOC	Isère, France	2003	+
12775	*J. regia*/Lara	VOC	Drôme, France	2003	
12776*	*J. regia*/Lara	VOC	Drôme, France	2003	+
12777*	*J. regia*/Fernor	VOC	Isère, France	2003	+
12778*	*J. regia*/Fernor	VOC	Isère, France	2003	+
12779*	*J. regia*/Fernor	VOC	Isère, France	2003	+
12780*	*J. regia*/Franquette	VOC	Isère, France	2003	+
12781	*J. regia*/Lara	VOC	Isère, France	2003	

Table 1 *Continued*

Strain[a]	Host plant/cultivar	Origin of isolation[b]	Geographical region	Year of isolation	Pathogenicity tests
12782	*J. regia*/Franquette	VOC	Lot, France	2003	
12783*	*J. regia*/Hartley	VOC	Lot, France	2003	–
12784*	*J. regia*/Vina	VOC	Corrèze, France	2003	+
12785*	*J. regia*/Franquette	VOC	Lot, France	2003	+
X. arboricola pv. *corylina*					
CFBP 1159^{Pt}=ATCC 19313= LMG 689= NCPPB 935	*Corylus maxima*		USA	1939	
Brenneria nigrifluens					
CFBP 6756	*J. regia*/Franquette	VOC	Dordogne, France	2002	

[a]CFBP, Collection Française des Bactéries Phytopathogènes, Angers, France; ATCC, American Type Culture Collection, Manassas, VA, USA; LMG, BCCM/LMG Bacteria Collection, University of Gent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK.
[b]WB, strains isolated from blight symptoms on leaves, twigs and fruits of *Juglans regia* (Persian walnut); VOC, strains isolated from vertical oozing canker symptoms on trunks and branches; VOCt, strains isolated from walnut blight symptoms on walnut trees displaying VOC symptoms.
^{T}type strain; ^{Pt}pathotype strain.
*Strains with which pathogenicity tests on walnut were performed: + indicates the formation of VOC symptoms and – indicates the absence of VOC symptoms.

walnut blight symptoms on trees displaying VOC symptoms. In addition, 24 strains of *X. a.* pv. *juglandis* isolated from walnut blight symptoms (five reference strains under CFBP accessions and 19 isolated during a previous survey under accessions 12572–12590) and one strain of *X. a.* pv. *corylina*, were included for comparative purposes, making a total working collection of 61 strains (Table 1). Total genomic DNA was extracted from all bacterial strains grown overnight at 28°C on YPGA medium following the standard cetyltrimethylammonium bromide (CTAB) method described by Ausubel *et al.* (1992). Concentration and purity of the extracted DNA was evaluated with a micro-spectrophotometer (Nanodrop ND-1000, Nanodrop Technologies). DNA preparations were then stored at 5 ng μL^{-1} in SDW at 4°C until use.

The F-AFLP procedure was performed as described by Boudon *et al.* (2005). The amplification products were separated by capillary electrophoresis for 35 min (15 kV) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with performance-optimized polymers POP-4. Fragments were sized by using ABI Genescan version 2.1 software (Applied Biosystems) and all the electropherograms were superimposed, visually inspected and compared for polymorphisms using the same software. The threshold for assigning a peak was set to 100 relative fluorescence and any peak less than this value was not included in the analysis.

F-AFLP patterns were then transformed into a tabular binary matrix reflecting the presence (coded as '1') or the absence (coded as '0') of fragments obtained by the analysis from the different bacterial strains. The program DistAFLP (Mougel *et al.*, 2002) was used to calculate similarities with the Dice coefficient (Dice, 1945). A similarity matrix was then exported and used to evaluate phylogenetic relationships among tested strains. For this, a dendrogram, rooted with *X. a.* pv. *corylina* strain CFBP 1159, was built using the neighbour-joining method (Saitou & Nei, 1987) with the NJplot software (http://pbil.univ-lyon1.fr/software/njplot.html). The robustness of the dendrogram was assessed by bootstrap analysis with the DistAFLP program (1000 replications) (Felsenstein, 1985).

Results

Description of the disease

Initial VOC symptoms include longitudinal deformations of affected trunks (Fig. 1a). Then, vertical cankers develop on trunks and branches of diseased trees with brown to black exudates staining the bark, which appear mainly in summer months (Fig. 1b,c). The final stage of the disease is characterized by severe distortion of affected trunks. The symptoms of this new disease progressed rapidly in French walnut orchards and nurseries, suggesting a rapid spread of the pathogen. Indeed, the disease affects all cultivars of walnut especially cvs Fernor, Chandler, Mayette and Hartley, affecting hundreds of hectares devoted to walnut cultivation. Cultivars Lara and Franquette are also affected with less severity.

Isolations and biochemical tests

From samples collected in the surveyed orchards and nurseries displaying VOC, the KB medium consistently allowed the recovery of 68 bacterial isolates. These isolates were divided into two groups including 32 identified as *B. nigrifluens* following the methodology described by Ménard *et al.* (2004), and 36 as *Xanthomonas* sp. according to their colony morphology. The 36 *Xanthomonas* sp. isolates formed yellow-coloured mucoid and convex colonies on YPGA medium after 3 days of incubation at 28°C. All *Xanthomonas* sp. isolates were characterized

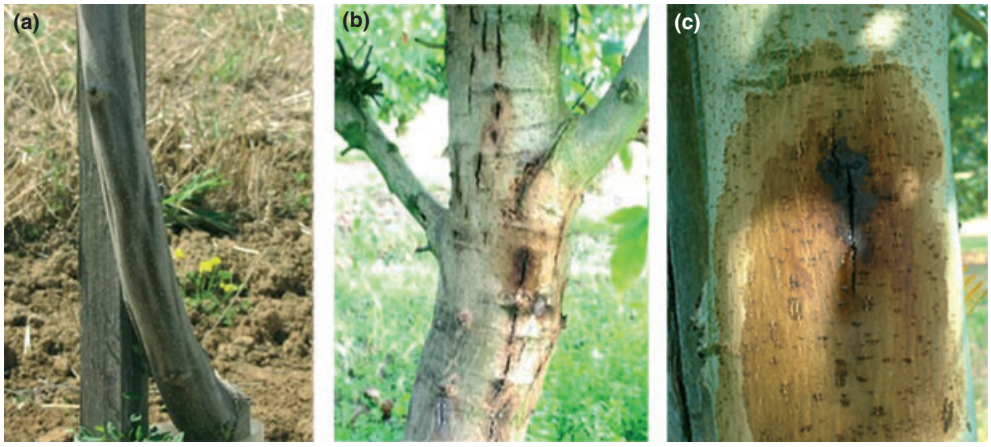

Figure 1 Natural vertical oozing canker (VOC) symptoms induced by *Xanthomonas arboricola* pv. *juglandis*. (a) Longitudinal deformations on a trunk of *Juglans regia* (Persian walnut tree); (b) vertical cankers on a trunk of *Juglans regia*. Traces of exudates can be seen; (c) during summer, diseased *Juglans regia* trees exhibit black oozing exudates staining the bark.

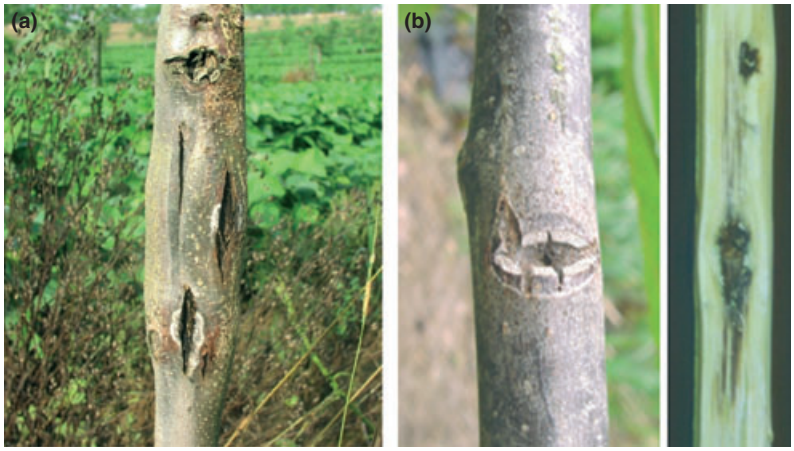

Figure 2 Symptoms observed after artificial inoculation of (a) *Xanthomonas arboricola* pv. *juglandis* vertical oozing canker (VOC) strain 12714 (typical VOC symptoms were observed); (b) *Brenneria nigrifluens* strain CFBP 6756 (a leaf scar and limited necrosis were observed).

as Gram-negative rods and glucose was utilized oxidatively. Negative oxidase reaction and positive reactions for presence of catalase, hydrolysis of gelatin, starch, esculin, lipolysis of Tween 80 and proteolysis of milk were observed. Urease was not detected. Indole was not produced and nitrates were not reduced. This biochemical pattern is representative of the *X. arboricola* strains, since similar results were obtained with the reference strain of *X. a.* pv. *juglandis* (CFBP 2528).

Pathogenicity tests

First, inoculation of bacteria initially isolated from VOC symptoms and then purified led to the identification of the VOC causal agent. The inoculation of *X. arboricola* strain 12714 isolated from black juice collected on an oozing trunk led to the reproduction of typical VOC symptoms on walnut trees (Fig. 2a). Then, re-isolations performed on YPGA medium from trees with symptoms consistently yielded typical yellowish bacterial colonies that were identical in appearance to those used for the inoculations, fulfilling Koch's postulates. In contrast, the inoculation of *B. nigrifluens* strain CFBP 6756 isolated from cankers induced a scar and local necrosis (Fig. 2b) of inoculated tissues but no cankers were observed on trunks (Koch's postulates not completed), confirming the findings of Ménard *et al.* (2004). These data lead to the conclusion that the causal agent of VOC is a *Xanthomonas* sp. and not *B. nigrifluens*.

Secondly, the ability of strains to cause VOC on trunks was tested in orchards. Three months after inoculation, all tested strains of *X. arboricola* isolated from VOC (with the exception of strain 12783) were pathogenic and

caused typical VOC symptoms similar to those observed on diseased samples collected in orchards (Fig. 1). Symptoms consisted of vertical cankers on trunks with appearance of dark exudates around the inoculation site. *Xanthomonas arboricola* was re-isolated from the inoculated trees and was found to be identical to the isolates used as inoculum in all characteristics. In contrast, inoculations with *X. arboricola* strains CFBP 2564 and CFBP 1022 did not develop VOC symptoms. Control plants inoculated with SDW also showed no symptoms.

In addition, two strains (12763 and 12785) isolated from VOC induced the same necrotic symptoms as the strain CFBP 2528 isolated from walnut blight when they were inoculated in immature fruit according to the technique described by Aletà *et al.* (2001). When inoculated by spraying bacterial suspensions on the leaf surface of walnut plants in growth chamber, the strain 12785 isolated from VOC and the strain CFBP 2528 isolated from walnut blight induced necrotic spots on leaves similar to symptoms observed in orchards. The pathogenic response of VOC strains on walnut supports their belonging to *X. a.* pv. *juglandis* as they also cause typical bacterial blight symptoms on walnut growing parts.

F-AFLP analysis

The reproducibility of F-AFLP patterns of tested strains was assessed using three strains (CFBP 1159, CFBP 1022 and CFBP 2564) which were processed three times. In replicated experiments, the banding pattern of these strains remained constant. F-AFLP analysis of *X. arboricola* strains generated a total of 15–40 scorable fragments, ranging from 150 to 600 bp. In total, the F-AFLP analysis generated 164 fragments for the 61 tested strains, of which 39 were common to all samples; thus, 76% of these fragments were polymorphic (data not shown).

The F-AFLP analysis clearly distinguished three main groups among *X. a.* pv. *juglandis* tested strains (Fig. 3). The most numerous one was represented by 40 strains and was called VOC cluster (indicated by a dotted box in Fig. 3). Most strains isolated from VOC (31 out of 36) belonged to this cluster. Clustering of these strains was not correlated to their geographical origins since the VOC cluster contained strains collected in both southeastern and southwestern areas (Table 1). The pathogenicity of strain 12783 has not been demonstrated. Further testing of the pathogenic behaviour of strains 12775, 12710, 12707 and 12680 could provide a better understanding of their unexpected position outside the VOC cluster. The bootstrap value (97) of the VOC cluster depicted the robustness of this lineage formed by the F-AFLP analysis within *X. a.* pv. *juglandis*. Consequently, VOC strains can be considered as forming a distinct genetic lineage within the pathovar *juglandis* of *X. arboricola*.

In addition, nine strains of *X. a.* pv. *juglandis* (12574, 12580, 12582, 12583, 12584, 12585, 12587, 12589 and 12590) previously isolated from walnut blight symptoms were assigned to the VOC cluster (Fig. 3). Two hypotheses can be formulated to explain this finding: either VOC strains and those causing walnut blight are closely related phylogenetically or they might coexist in the same tree. The second hypothesis was previously reported in the case of *X. a.* pv. *juglandis* strains causing BAN and walnut blight that could be present at the same time on the same trees in Italy (Belisario *et al.*, 2002).

The second group consists of strains (with the exception of strain 12775) isolated on the basis of walnut blight symptoms from different trees in the same orchard at the same time. This grouping is supported by a high bootstrap value (96). The remaining strains form a diffuse third group that contains reference strains of *X. a.* pv. *juglandis* causing walnut blight taken as controls in this study (CFBP 878, CFBP 2528, CFBP 2564 and CFBP 2568) and one avirulent strain of *X. arboricola* (CFBP 1022) that appeared weakly related to each other (Fig. 3). F-AFLP analysis also revealed that pathovars *juglandis* and *corylina* are weakly related to each other. This observation, which is supported by the absence of a high bootstrap value (Fig. 3), is congruent with a previous grouping confirming that *X. arboricola* pathovars are different genetic entities (Scortichini & Rossi, 2003).

Discussion

In France, vertical oozing canker is a potentially damaging disease that remains a threat for walnut cultivation, especially in nurseries. The present work constitutes a first step towards a better understanding of the aetiology of the disease. On the basis of bacterial isolations, biochemical tests, pathogenicity tests and analysis performed using F-AFLP, this study ascertained that *X. a.* pv. *juglandis* is the causal agent of the disease. The only indication of VOC in France was provided by Ménard *et al.* (2004). In that study, the authors reported for the first time the presence of *B. nigrifluens* in France. Although *B. nigrifluens* was frequently isolated from diseased walnut trees, this bacterium was often present on healthy walnut trees as well, and results with inoculations performed with isolates of *B. nigrifluens* did not result in typical VOC symptoms, although it caused limited bark necrosis. Such findings suggest that *B. nigrifluens* might be part of the resident microflora of *Juglans* and behaves as a pathogen until particular conditions such as a stress factor occur, as hypothesized in Italy (Morone *et al.*, 1998). This is not too surprising, however, since a heterogeneous microflora, including different bacterial and fungal species, has been associated with walnut cankers (Mazzaglia *et al.*, 2005; Moretti *et al.*, 2007).

Fluorescent AFLP analysis confirmed its highly discriminative resolution since it has been successfully used in this study for the investigation of the genetic diversity of the aggressive *X. a.* pv. *juglandis* strains associated with VOC in France. The data showed that *X. a.* pv. *juglandis* strains responsible for VOC outbreaks in France form a very tight, homogeneous cluster, readily distinguishable from other *X. a.* pv. *juglandis* strains causing walnut blight. In addition, a good correlation

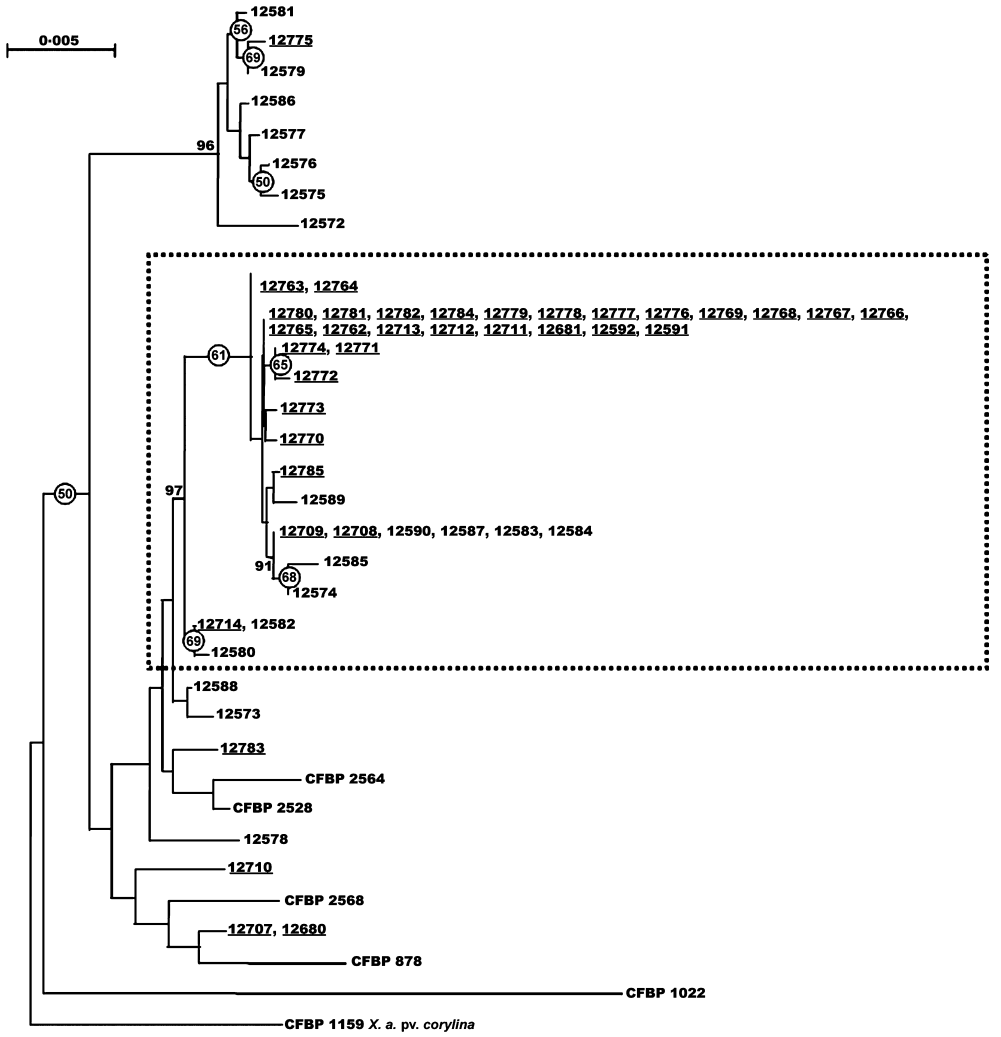

Figure 3 Dendrogram of 61 *Xanthomonas arboricola* strains analysed by fluorescent AFLP. Similarity between fingerprints was calculated by the neighbour-joining method using Dice's coefficient. The VOC cluster is indicated by a dotted box. Strains isolated from VOC are underlined. Numbers at the branch points represent bootstrap values generated from 1000 replications (only values >50 are shown). *Xanthomonas arboricola* pv. *corylina* strain CFBP 1159 was used as an outgroup.

between this F-AFLP data and the pathogenicity tests was found and provided sufficient evidence for considering this bacterial population as a distinct pathogenic genetic lineage within *X. a.* pv. *juglandis*. Linkage of different genotypes to geographical origin was previously reported by Scortichini *et al.* (2001) in the case of *X. a.* pv. *juglandis* strains causing walnut blight. In the case of VOC strains, the genomic heterogeneity is not correlated with the geographic origins of strains, so one can speculate that the remarkable homogeneity of the genetic lineage formed by VOC strains could be explained, at least in part, by the adaptation of these strains to particular habitats. The results presented here raise interesting questions concerning the origin of this newly established disease in France. In fact, the F-AFLP data point out a remarkable homogeneity among VOC strains that is coherent with the occurrence of a unique bacterial population. This aggressive bacterial population could have been either introduced with imported walnut material or selected within endemic populations of *X. a.* pv. *juglandis* and propagated when environmental conditions were favourable.

'Emerging diseases' is a term commonly used to describe diseases caused by known pathogens that appear in new areas. Within the complex species *X. arboricola* (Vauterin *et al.*, 1995), this was the case of bacterial spot of stone fruit trees caused by *X. a.* pv. *pruni*, that emerged in orchards of the Rhône valley in France in 1995 (Boudon *et al.*, 2005). The present study provides evidence of another emergence (Anderson *et al.*, 2004), since it reveals the development of a previously unknown disease (VOC) caused by particular strains belonging to a pathovar (*X. a.* pv. *juglandis*) already identified as the causal agent of a known disease on walnut (bacterial blight). Although VOC strains are able to cause a new type of symptom on walnut, these strains must not be considered

as a novel pathovar of *Xanthomonas* different from *X. a.* pv. *juglandis,* since they cause indistinguishable symptoms of bacterial blight on leaves and fruits. Furthermore, they form a distinct genetic lineage that fits well into the phylogenetic branch that groups all strains of *X. a. pv. juglandis* (Vauterin *et al.*, 1995).

The present study not only clarifies the taxonomic position of VOC strains as belonging to the pathovar *juglandis* of *X. arboricola*, but also supports the relevant genetic variability of *X. a.* pv. *juglandis* strains causing walnut blight as indicated previously by other authors (Du Plessis & Van der Westhuizen, 1995; Loreti *et al.*, 2001; Scortichini *et al.*, 2001; Barionovi & Scortichini, 2008). Such heterogeneity can be explained by its broad geographical distribution, and long association with walnut, which is endemic in Europe. This heterogeneity differentiates this pathogen from *X. a.* pv. *pruni*, pathogenic on peach, where almost all varieties in Europe are imported. The findings are also interesting from a phylogenetic point of view since this technique revealed that there are probably different genetic lineages within *X. a.* pv. *juglandis*. Selective pressure on the host plant and environmental conditions might play a central role in selecting different populations of this pathogen. The sequencing of seven housekeeping genes is currently being undertaken and a multi-locus sequence analysis (MLSA) might help to clarify the relationships within the strains of this pathogen.

The knowledge of the genetic diversity of the pathogen is also important for breeding programmes aimed at selecting walnut genotypes tolerant and/or resistant against VOC. Such a perspective will be possible by setting up a reliable test to screen many walnut cultivars for susceptibility to the disease. Differences in susceptibility to walnut blight due to *X. a.* pv. *juglandis* within *J. regia* cultivars were previously reported (Woeste *et al.*, 1992; Tsiantos *et al.*, 2009). Because of the difficulty of inoculating a large collection of isolates on walnut, it is proposed to use F-AFLP based markers to select isolates of *X. a.* pv. *juglandis* showing the highest genetic diversity within *X. a.* pv. *juglandis* in order to screen cultivar susceptibility. The pathogenicity of VOC strains for inducing necrosis on fruits deserves further investigation since the involvement of these strains in the fruit fall is still unknown.

Currently, nothing is known about pathogenicity determinants that can account for the emerging character of *X. a.* pv. *juglandis* strains responsible for main VOC outbreaks in France. The fact that VOC strains are genetically distinct from those causing walnut blight suggests that these strains may possess different pathogenicity determinants, e.g. type III effectors (T3Es) and integron driven genes. T3Es are candidate determinants of host specificity of pathogenic bacteria since it has been shown that many T3Es can act as molecular double agents that betray the pathogen to plant defences in some interactions and suppress host defences in others (Alfano & Collmer, 2004). Recently, it has been reported that the distribution of T3Es within *Xanthomonas* strains may suggest a basic role in aggressiveness and host specificity (Hajri *et al.*, 2009). VOC strains would thus be a good model to test whether a modification in T3E repertoire would lead to changes in the pathogenic behaviour of the pathogen. The study of Gillings *et al.* (2005) pointed out that integrons can be considered as a major source of diversity in *Xanthomonas* species and might be strictly linked to host-specific pathogenicity. Recently, it has been shown that variability can exist in the integron gene cassette arrays of *X. a.* pv. *juglandis* strains causing walnut blight (Barionovi & Scortichini, 2008). Assuming the possibility that the genetic diversity within the pathovar *juglandis* is higher than in most other *X. arboricola* pathovars, a certain level of diversity in integron array profiling might be found among VOC strains.

In France, VOC is an emerging disease that may have an increasing economic impact in the future. The extent and severity of the disease in French orchards requires careful investigation. When VOC was first discovered in France, it was not intensively controlled and as a consequence has been allowed to spread. A rapid, specific and sensitive PCR based detection of VOC strains in orchards and nurseries prior to symptom development will facilitate setting up of control strategies against the disease. This study clearly confirms the discriminative power of the F-AFLP technique. Cloning specific F-AFLP fingerprints obtained in the frame of this work could provide useful markers for the detection of the VOC causal agent. The specificity of such a tool could then be tested on a larger collection of strains including new collected VOC isolates, walnut-associated bacteria, bacteria from other taxons and epiphytic bacterial isolates from walnut leaves.

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