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Characterization of *Pseudomonas syringae* Strains Isolated from Diseased Horse-chestnut Trees in Belgium

A. Bultreys[1], I. Gheysen[1], and V. Planchon[2]

Abstract *Pseudomonas syringae* was isolated from lesions found in cortical tissues of Belgian horse-chestnut (*Aesculus hippocastanum*) trunks. A collection of about 50 strains was established from 6 sites in Brussels and 11 sites in Wallonia, but the pathogen was not found in the South-East of Belgium. The strains were identified by pyoverdin tests, induction of potato rot, the *cfl* PCR test, and REP-PCR. The investigated strains were highly virulent on horse-chestnut detached twigs collected in winter and summer, although the propagation outside the inoculated zones was reduced in summer. The accentuated propagation during winter was confirmed by inoculation of 20 young horse-chestnut trunks: in the end of winter and early spring, a mean progression in the cortical tissues of 3.45 mm in 48 days was observed (0.05 mm for the controls), whereas the progression during spring and summer was reduced by a factor seven. In this latter period, canker formations were observed; also, *P. syringae* was still isolable from naturally infected older trunks. Comparison of REP-PCR profiles were carried out with 60 pathovars of *P. syringae* and with *Pseudomonas viridiflava*, *Pseudomonas meliae*, *Pseudomonas ficuserectae*, *Pseudomonas cannabina* and *Pseudomonas tremae*. REP- and ERIC-PCR analyses indicated the relatedness of the horse-chestnut strains, although site-related genetic groups were observed, as well as genetic similarities and differences with *P. syringae* pv. *morsprunorum* race 1, pv. *aesculi*, pv. *cunninghamiae* and pv. *daphniphylli*; both the pathovars *morsprunorum* race 1 and *aesculi* possessed the *cfl* gene and all the investigated horse-chestnut strains produced coronatine. Only strains from horse-chestnut and pathovar *aesculi* were similarly virulent on *A. hippocastanum*. The data indicate that *P. syringae* strains are living on *A. hippocastanum* for a long time and would agree with their grouping with *P. syringae* pv. *aesculi* inducing leaf spots on *Aesculus indica*, but this pathovar would then be genetically heterogeneous.

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Keywords Horse-chestnut, canker, *Pseudomonas syringae*, morsprunorum, aesculi, emerging disease

1 Introduction

Since December 2004, horse-chestnut trees (*Aesculus hippocastanum*, originating from the Balkans) showing cankers and/or lesions in the cortical tissues with sometimes tarry exudations have been studied by the Walloon Agricultural Research Centre in Brussels and in Wallonia (Southern part of Belgium). The same syndrome has been reported in the Netherlands and United Kingdom (Janse et al., 2006; Webber et al., 2006). The disease can induce dead of branches and trees. It is of great concern for cities like Brussels and for regional administrations in charge of plantations. Indeed, horse-chestnut is an attractive tree that is frequently planted alongside roadways and avenues, as well as in parks. Bacterial analyses were initiated in January 2006 because of the non-confirmation of the involvement of an initially suspected *Phytophthora* (Bultreys et al., 2006). *Pseudomonas syringae* was readily isolated from lesions in the cortical tissues of horse-chestnut.

2 Materials and Methods

2.1 Isolation and Identification

Strain isolations were performed onto King B medium (King et al., 1954) from the margins of fresh or dry lesions found on horse-chestnut trees located in 6 sites in Brussels and 11 sites in Wallonia. These sites had been detected during a survey in Brussels and Wallonia (Bultreys et al., 2006). Identification was through pyoverdin-based tests, including visual, spectrophotometric and high performance liquid chromatography (Bultreys et al., 2001, 2003), the induction of potato rot, and repetitive extragenic palindromic (REP)-PCR. REP-PCR performed on lysed cells (5×10^5 lysed cells per reaction) prepared as previously described (Bultreys and Gheysen, 1999). The PCR conditions were modified from Pooler et al. (1996) and Kingsley et al. (2002); in 25-µl total volume: 1 U *Taq* DNA polymerase (Qiagen) used with its buffer, 200 µM of each dNTP, 2.5 mM $MgCl_2$, and 2 µM of the REP primers (Versalovic et al., 1991). The program was: an initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 1 min, 44°C for 1 min, and 72°C for 2 min; and a final elongation at 72°C for 10 min. The electrophoresis conditions were those used by Cubero and Graham (2002). The REP-PCR profiles were compared with the profiles of the first strains isolated, but each strain was conserved independently of its profile. The modified PCR test detecting the *cfl* gene involved in coronatine

synthesis was also used in identification (Bereswill et al., 1994; Bultreys and Gheysen, 1999)

2.2 Strain Characterization

Coronatine production was detected using a biological test on Spunta potato slides (Bultreys, 2001). Analysis of the *cfl* gene by PCR and comparison of REP-PCR profiles were carried out for 60 pathovars of *P. syringae*, including the pathovar *aesculi* inducing leaf spots on *Aesculus indica* in India (Durgapal and Singh, 1980), and the pathotype strains of the related *Pseudomonas* species *P. viridiflava*, *P. meliae*, *P. ficuserectae*, *P. cannabina* and *P. tremae*. Enterobacterial repetitive intergenic consensus (ERIC)-PCR analyses were also carried out on the pathovars that appeared related by REP-PCR. The PCR conditions were those used for REP-PCR but the Tm temperature was 52°C and the ERIC primers (Hulton et al., 1991) were used.

2.3 Pathogenicity Tests

Pathogenicity tests were conducted on horse-chestnut and sweet cherry 1-year-old detached twigs and on trunks of young horse-chestnut trees. Bacteria grown in King B agar medium for 24 h were used to produce bacterial suspensions in water of about 2.6×10^8 CFU per ml. The tests on 1-year-old horse chestnut twigs were performed in winter and summer. The twigs of the winter test were collected on young *A. hippocastanum* trees grown in pots; those of the summer tests on young trees (trunk diameter: 10–12 cm) of *A. hippocastanum* Baumanii transplanted in Gembloux in March 2006. The test on 1-year-old sweet cherry twigs was performed in winter; the twigs of the Napoleon variety were taken from an orchard in Gembloux. The twigs were superficially washed and disinfected, cut to an appropriate length and sealed with paraffin jelly. Cortical tissues were exposed in an about 1.5×0.5 cm area by removing superficial bark and 20 µl of either sterile water or bacterial suspension were delicately spread on the exposed tissues. The twigs were put independently in sterile glass tubes containing absorbent cotton-wool saturated with water. The tubes were sealed with cellophane and incubated at 15°C in the dark for 20–30 days. The reading was performed by visual estimation of the area presenting a browning resembling that observed during isolations from naturally infected trees; the twigs were distributed in classes of damage: 0, no damage, up to 4 all the area damaged. The strains investigated on either horse-chestnut or cherry were MY2-1, MY2-2, MY2-3, MY3-1, MY3-2, MY3-3, CH3, N2 and SB5-1, which are *P. syringae* strains from horse-chestnut, MY3-4, an unidentified white-colored bacterium isolated from horse-chestnut together with *P. syringae*, *P. syringae* pv. *morsprunorum* race 1 PmC36, pv. *aesculi* CFBP 2894, pv. *cunninghamiae*

CFBP 4218 and pv. *daphniphylli* CFBP 4219. Five or ten twigs were used per test. Anova analysis (SAS software version 9.1.3) was used for strain comparisons based on classes of virulence. Levene's test was applied for variance comparisons, and the test of Tukey for mean comparisons between strains.

Twenty trunks of young horse-chestnut trees were inoculated in March 2006: in two places on the same trunk, the superficial bark was lifted up in a 0.5 × 0.5 mm area to expose cortical tissues; a 20-µl drop of either sterile water or bacterial suspension of *P. syringae* strain MY3-1 were delicately spread on the exposed tissues; then, the superficial bark was replaced and fixed with grafting rubber. Disease symptoms were measured after 48 and 186 days. In both types of test, isolations were performed from progression outside the inoculated zones and the purified bacteria were tested for pyoverdin produced to confirm that they belonged to the *P. syringae* group.

3 Results and Discussion

3.1 Isolation and Identification

Sites and cities in the Walloon and Brussels-Capital Regions were contacted or visited to detect diseased horse-chestnut trees (Fig. 1A–1C). Fluorescent *Pseudomonas* were easily isolated, often in pure culture, from the margins of freshly attacked tissues found in 6 isolation sites in Brussels and 11 isolation sites in Wallonia (Fig. 1D). A collection of about 50 strains was established, but despite contacts with cities and active research, the disease was not found in the South-East of Belgium. Whether the disease is present in this region will be further investigated in the future.

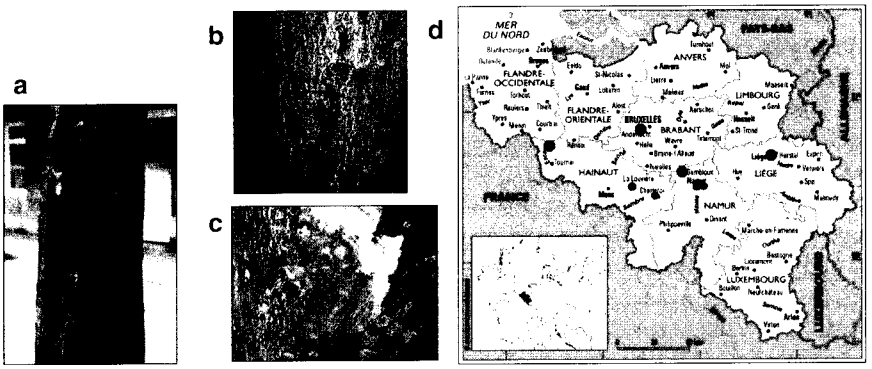

Fig. 1 The horse-chestnut disease in Belgium. **a** Bleeding cankers on trunk. **b** Exudations on trunks. **c** Lesions in cortical tissues. **d** Belgian sites (large dots) where *P. syringae* was isolated from diseased trees. The larger dots indicate more than one isolation site at the location

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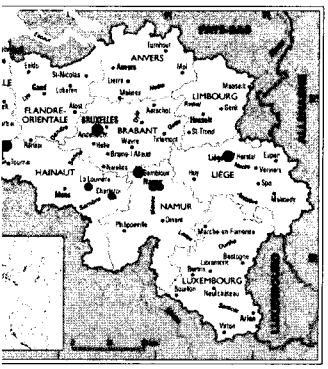

cankers on trunk. **b** Exudations on
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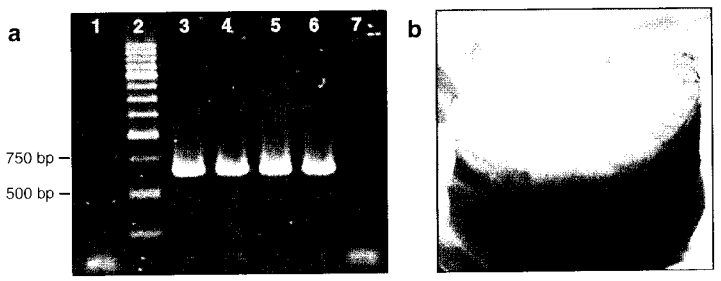


Fig. 2 Coronatine tests. **a** Detection of a 655-bp fragment of the *cfl* gene involved in coronatine synthesis by PCR: lane 1, water; lane 2 DNA-marker XVI; lanes 3–6, *P. syringae* strains from horse-chestnut MY3-2, MY3-1, MY2-2, MY2-1; lane 7, coronatine-non-producing *P. tremae* CFBP 3229. **b** Visualization of coronatine production on a potato slice: induction of callus production by a coronatine producing *P. syringae* strain

Fluorescent *Pseudomonas* were easily isolated, often in pure culture, from freshly attacked tissues. This was apparently true independent of the season since isolations were successful in winter, spring and summer. Also, similar strains were isolated from leaves. The isolates were initially identified as *P. syringae* by the HPLC detection of pyoverdin and by their inability to induce potato rot. The REP-PCR profiles of the first isolated strains were then used as references in REP-PCR identification. No isolated *P. syringae* strain produced a REP-PCR fingerprint radically different from the references. It was also noticed that the isolated strains gave positive responses in the *cfl* PCR test (Fig. 2A) and this test was then also used in identification. It was then confirmed that all the isolated *P. syringae* strains from horse-chestnut possessed the *cfl* gene. Only a simplified visual pyoverdin test, REP-PCR and the *cfl* PCR test were finally used in identification.

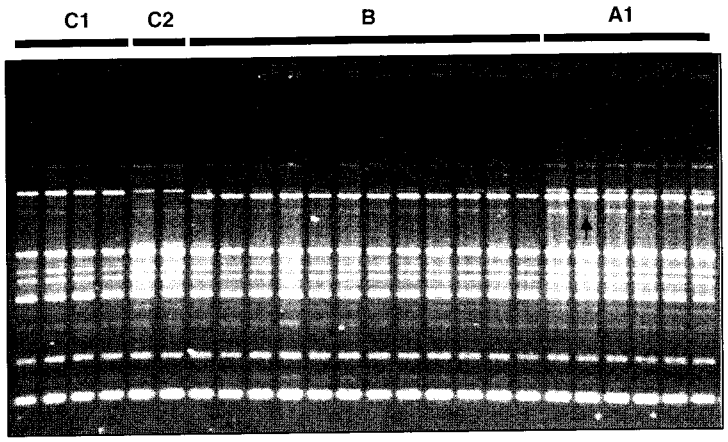
3.2 Strain Characterization

All horse-chestnut strains induced callus formation on potato slices (Fig. 2B), a phenotype which is related to the production of coronatine (Sakai et al., 1979); in a previous study (Bultreys, 2001), this biological test showed a good correlation with the possession of the *cfl* gene by *P. syringae* strains.

The ERIC-PCR analyses indicated the relatedness of the *P. syringae* strains isolated from horse-chestnut, although site-related small variations were observed (Fig. 3). At least three and maybe five genetic groups were apparent based on the variation found in one particular place of the profiles. Interestingly, in one isolation site one genetic group was most generally dominant.

The REP-PCR analyses also indicated similarities and differences between the horse-chestnut strains and strains of *P. syringae* pv. *morsprunorum* race 1, pv. *aesculi*, pv. *cunninghamiae* and pv. *daphniphylli* (Fig. 4A). ERIC-PCR analyses did not enabled to distinguish between the different genetic groups detected by REP-PCR

a



b

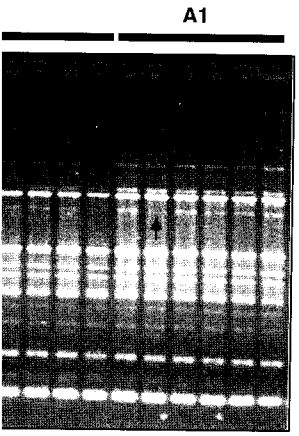
	S1	S2	S3	S4	S5	S6	S7	S8
A	16	11		1				
B	1		6	2	3	2		2
C			3				4	

Fig. 3 REP-PCR analyses among horse-chestnut strains. **a** Genetic similarities and differences among *P. syringae* strains isolated from horse-chestnut trees in Belgium. **b** Distribution of Belgian strains isolated from eight sites (S1–S8) among the three principal genetic groups (A–C) showing relations between the genetic groups and the isolation sites

among the Belgian horse-chestnut strains, and they confirmed the similarities and differences with the pathovars *morsprunorum* race 1, *aesculi*, *cunninghamiae* and *daphniphylli* (Fig. 4B). The presence of the *cfl* gene and the probably linked production of coronatine, was observed in different pathovars not previously known to possess this gene: the pathovars *aesculi*, *alisalensis*, *berberidis*, *ulmi*, *porri*, *spinaceae*, and *zizaniae*; as well as in the species *P. cannabina*. However, among the pathovars showing similarities with the horse-chestnut strains, only the pathovars *morsprunorum* race 1 and *aesculi* possessed the *cfl* gene, the production of coronatine by *P. syringae* pv. *morsprunorum* race 1 being well known (Bereswill et al., 1994).

3.3 Pathogenicity Tests

The first six isolated *P. syringae* strains MY2-1, MY2-2, MY2-3, MY3-1, MY3-2 and MY3-3 were investigated in January and February 2006 for their virulence on horse-chestnut detached twigs, in comparison with the unidentified white-colored bacterium MY3-4 isolated together with *P. syringae* from diseased cortical tissues. All the *P. syringae* strains proved highly virulent and statistically similarly virulent,



5	S6	S7	S8
	2		2
		4	

Genetic similarities and differences
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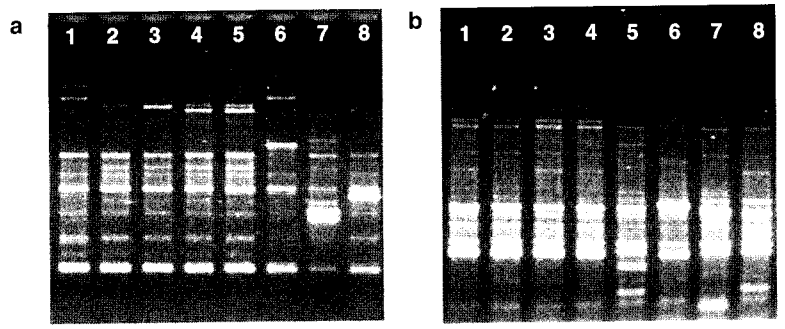

Fig. 4 REP- and ERIC-PCR analyses among pathovars and species. **a** REP-PCR analyses for the *P. syringae* strains from horse-chestnut SB5-1, N2, CH3, Bxl4 from different genetic groups (lanes 2–5) and for *P. syringae* pv. *morsprunorum* race 1 LMG 2222 (lane 1), pv. *cunninghamiae* CFBP 4218 (lane 6), pv. *daphniphylli* CFBP 4219 (lane 7), and pv. *aesculi* CFBP 2894 (lane 8). **b** ERIC-PCR analyses for *P. syringae* strains from horse-chestnut SB5-1, N2, CH3, Bxl4 from different genetic groups (lanes 1–4) and for *P. syringae* pv. *morsprunorum* race 1 LMG 2222 (lane 5), pv. *aesculi* CFBP 2894 (lane 6), pv. *cunninghamiae* CFBP 4218 (lane 7), and pv. *daphniphylli* CFBP 4219 (lane 8)

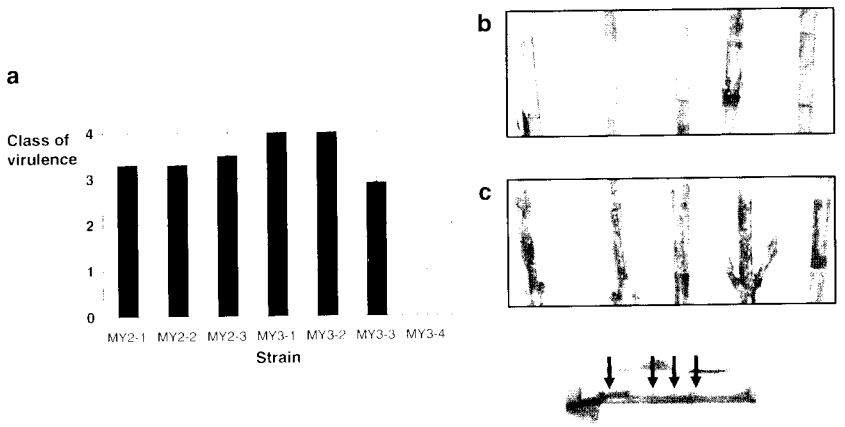

Fig. 5 Pathogenicity test on horse-chestnut detached twigs. **a** Mean and standard deviation for six *P. syringae* strains (black bars) and one unidentified white-colored bacterium (white bar). **b** Few symptoms were induced by the non pathogenic strain MY3-4: the inoculated zone most generally remained totally green. **c** Lesions induced by a *P. syringae* strain: the inoculated zones turned brown and expansion was noted outside the inoculated zone

whereas MY3-4 induced little damage (Fig. 5). Only in the case of the *P. syringae* strains was a progression of the damages outside the inoculated zone systematically observed. Also, *P. syringae* could be systematically re-isolated from these progression zones, validating the Koch's postulate for the strain abilities to induce damages in horse-chestnut cortical tissues.

The ability to cause disease during summer was confirmed by the virulence of the strain MY3-1 on horse-chestnut twigs collected in Augustus 2006 (mean virulence class of 3.75 ± 0.75 compared to 0.5 ± 0.35 for the water control), but no progression outside the inoculated zone was noticed in that test. Increased susceptibility during winter was confirmed by inoculation of 20 horse-chestnut trunks in March 2006: in the end of winter and early spring, a mean progression of 3.45 ± 1.21 mm in the cortical tissues of ten inoculated trunks was observed after 48 days, compared to 0.05 ± 0.15 mm for the water controls on these trunks, whereas the progression noted after 186 days on the ten other inoculated trunks was only 4.8 ± 1.21 mm. The observed progression during spring and summer was therefore reduced by a factor seven when compared to winter (Fig. 6A). In summer, higher frequency of canker formation was observed at inoculation sites on trunks than in the water controls (Fig. 6B and 6C) and, interestingly, typical longitudinal canker formation was sometime noticed (Fig. 6D). This result confirmed that the presence of virulent *P. syringae* strains in the cortical tissues of horse-chestnut in trunks can result in canker formation similar to that observed under natural conditions. However, *P. syringae* was only re-isolated once from cankers in the end of summer, whereas it was systematically re-isolated from the damaged cortical tissues in early spring. This would mean that canker formation is a reaction of the tree and that the survival of *P. syringae* in summer in young trees is limited. The high difficulty of isolating *P. syringae* from cankers was also noticed on naturally infected older trees. Also, the successful isolations of *P. syringae* in summer from older trees were not obtained from cankers, but from damaged cortical tissues that externally presented no other symptom than small exudations on trunk resembling those presented in Fig. 1B. This would mean that, at least in older trees, *P. syringae* can be present in horse-chestnut cortical tissues in summer without canker presence and, apparently, without canker formation.

Given the high similarity of the REP-PCR profiles of the horse-chestnut strains and *P. syringae* pv. *morsprunorum* race 1 (Fig. 4A), the virulence of the strains MY3-1, MY3-2 and MY2-3 on sweet cherry detached twigs was investigated.

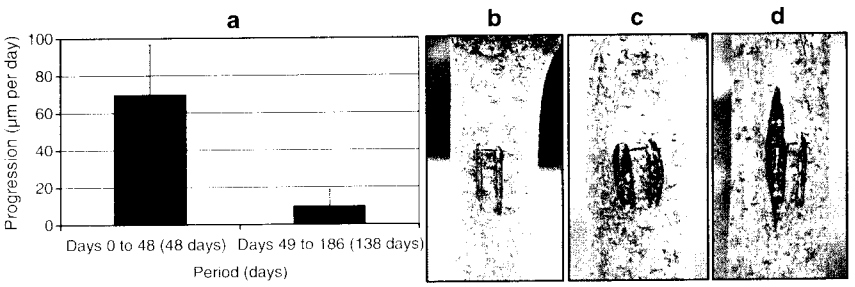

Fig. 6 Pathogenicity tests on horse-chestnut trunks. **a** Difference in progression in cortical tissues in the periods 'end of winter/early spring' (days 0–48) and 'spring/summer' (days 49–186). **b–d** Cankers observed on trunks in the end of summer, 186 days after inoculation, for a water inoculated site **b** and for *P. syringae* inoculated sites **c** and **d**

confirmed by the virulence of
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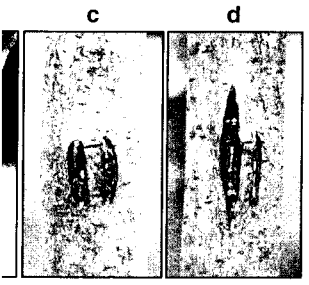

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However, these strains proved to be non-virulent when compared to water and the virulent strain *P. syringae* pv. *morsprunorum* race 1 PmC36 (not shown). Horse-chestnut strains representative of different genetic groups and the pathotypes strains of *P. syringae* pv. *aesculi*, pv. *cunninghamiae* and pv. *daphniphylli* were investigated for their virulence on horse-chestnut twigs in September 2006. Only representative of the different horse-chestnut groups and of pathovar *aesculi* were similarly virulent on detached *A. hippocastanum* twigs (Fig. 7).

The genetic and pathogenicity data, as well as the production of coronatine and the fact that both type of strains induce disease in the *Aesculus* genus would agree with the grouping of the Belgian isolates of *P. syringae* from *A. hippocastanum* with *P. syringae* pv. *aesculi*, known to induce leaf spots on *A. indica* (Durgapal and Singh 1980). However, the genetic analyses presented here indicate clearly that this pathovar would then be genetically heterogeneous since all the Belgian strains can be grouped in an ERIC group 2 whereas the pathotype strain of *P. syringae* pv. *aesculi* showed a profile than can be named ERIC group 1 (Fig. 4b); also, it should also be confirmed that the Belgian *A. hippocastanum* isolates can induce leaf spot on *A. indica*. The conclusions are rather in agreement with published results obtained in United Kingdom where it was noticed that *P. syringae* strains isolated from *A. hippocastanum* had the same gyrase B gene (*gyrB*) sequence as *P. syringae* pv. *aesculi* (Webber et al., 2006). Whether *gyrB* sequence data would be less informative than the REP- and ERIC-PCR analyses presented here (Fig. 4) to detect differences between the *P. syringae* isolates from *A. hippocastanum* and *A. indica* is a possibility, but a comparison of strains from different countries should confirm that the *A. hippocastanum* pathogens are identical in all European countries; indeed closest similarities of isolates with *P. tremae* and *P. syringae* pv. *ulmi* have also been reported (Janse et al., 2006).

The REP-PCR (Fig. 3) data indicate that a genetic diversity already exists within the Belgian strains from *A. hippocastanum*, which indicates that these strains have

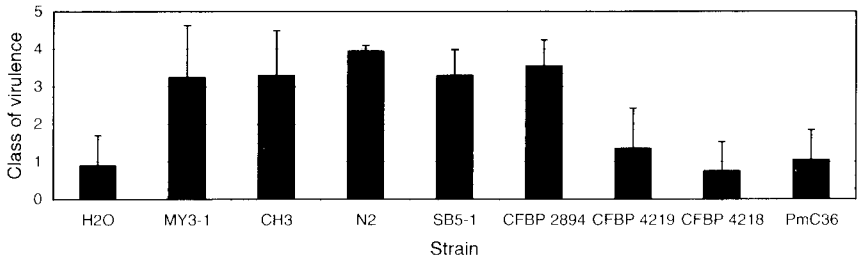

Fig. 7 Pathogenicity on horse-chestnut detached twigs of representatives of genetic groups of *P. syringae* from *A. hippocastanum* (MY3-1, CH3, N2 and SB5-1) and the genetically related strains *P. syringae* pv. *aesculi* CFBP 2894, pv. *daphniphylli* CFBP 4219, pv. *cunninghamiae* CFBP 4218 and pv. *morsprunorum* race 1 PmC36. Statistical analysis indicated no difference of variance for the different strains. On the other hand, significant differences between means were observed ($p < 0.0001$) and the test of Tukey showed clearly two homogeneous groups of strains differing by their virulence: N2, CFBP 2894, CH3, SB5-1 and MY3-1on one side, and CFBP 4219, PmC36, H20 and CFBP 4218 on the other side

already evolved on this host. The REP- and ERIC-PCR genetic data (Fig. 4) also indicate that all the Belgian strains differ more markedly from the Indian strain of *P. syringae* pv. *aesculi* isolated from *A. indica* in India than from each other, but also that there is a clear similarity of both types of strain with the pathovar *morsprunorum* race 1, which is also similar at the phytotoxin production level. Globally, these observations are rather in agreement with an evolution of the strains from a common ancestor by specialization on their respective hosts during evolution. This is in disaccord with the common feeling encountered among Belgian administrators that the horse-chestnut canker disease could be due to the recent apparition of a new pathogen; this opinion being justified by the apparent sudden outbreak of the disease reported these last years. By contrast, it seems probable that *P. syringae* strains live for a long time on *A. hippocastanum*. This highlights the need to determine the factors that could have resulted, these last years, in either a stronger propagation of the disease or an aggravation of the symptoms.

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