Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz
Journal of Plant Diseases and Protection
111 (1), 39–51, 2004, ISSN 0340-8159

Nested PCR (polymerase chain reaction) for detection of *Xanthomonas fragariae* in symptomless strawberry plants

Nested PCR zum Nachweis von *Xanthomonas fragariae* an symptomlosen Erdbeerpflanzen

Carolin Zimmermann[1], J. Hinrichs-Berger[1], Esther Moltmann[2], H. Buchenauer[1]
[1] Institute of Phytomedicine (360), University of Hohenheim, Otto-Sander-Str. 5, D-70599 Stuttgart, Germany
[2] Landesanstalt für Pflanzenschutz, Reinsburgstr. 107, D-70197 Stuttgart, Germany

Received 21 October 2003; accepted 4 December 2003

Summary

For rapid and sensitive detection of *Xanthomonas fragariae,* the causal agent of angular leaf spot of strawberry, a nested polymerase chain reaction (PCR) was developed. A specific fragment was amplified from *X. fragariae* DNA using primers 245A and 245B, described previously (Pooler et al. 1996). The fragment was sequenced and internal primers suitable for nested PCR were selected. Using this internal pair of primers, a specific fragment was amplified from all of the 14 *X. fragariae* isolates tested but no fragment was amplified from *X. campestris* isolates or from unidentified bacteria, which were isolated from strawberry plants. Whereas detection limit of simple PCR was 20 pg DNA per reaction, nested PCR was up to a hundred times more sensitive and even from 200 fg DNA per reaction, a fragment was amplified. In addition, fragments amplified by nested PCR were always clearly detectable on agarose gels, whereas fragments amplified by simple PCR were only visible as faint bands when template concentrations decreased. Applicability of nested PCR was tested on samples from naturally infected fields and from nursery plants showing no symptoms by visible inspection. In symptomatic plants, *X. fragariae* was regularly detected in leaves, especially in old leaves. In the crown, it was only occasionally detected. Simple PCR was sufficient for confirmation of symptomatic infection and sometimes for detection of *X. fragariae* in symptomless parts of symptom-bearing plants and in latently infected nursery plants, respectively. However, using more sensitive nested PCR, latent infections were more frequently detected and PCR fragments were more clearly visible on agarose gels.

Key words: strawberry; *Xanthomonas fragariae;* latent infection; detection; nested PCR

Zusammenfassung

Für einen möglichst schnellen und sensitiven Nachweis von *Xanthomonas fragariae,* dem Erreger der Eckigen Blattfleckenkrankheit an Erdbeere, wurde eine verschachtelte Polymerase-Kettenreaktion (nested PCR) entwickelt. Mit den Primern 245A und 245B (Pooler et al. 1996) wurde ein spezifisches Fragment von *X. fragariae* DNA amplifiziert. Das Fragment wurde sequenziert und innere Primer, die sich für eine nested PCR eignen, wurden ausgewählt. Mit diesem inneren Primer-Paar wurde an allen 14 getesteten *X. fragariae* Isolaten ein spezifisches Fragment amplifiziert, an *X. campestris* Pathovaren und an weiteren nicht identifizierten Bakterien, die von Erdbeerpflanzen isoliert worden waren, wurde dagegen kein Fragment amplifiziert. Während die Nachweisgrenze der einfachen PCR bei 20 pg DNA pro PCR-Ansatz lag, wurde mit der neu entwickelten nested PCR eine bis zu 100 × höhere Sensitivität erzielt und auch mit 200 fg DNA/Ansatz ein nachweisbares Fragment amplifiziert. Darüber hinaus war

das Fragment, das mit der nested PCR amplifiziert wurde stets deutlich auf dem Agarosegel sichtbar, während bei der einfachen PCR die Bandenstärke des amplifizierten Fragments mit abnehmender Matritzen-Konzentration abnahm. Die Praxistauglichkeit der nested PCR wurde an Proben aus natürlich befallenen Feldbeständen und an äußerlich gesund aussehenden Jungpflanzen geprüft. *X. fragariae* wurde regelmäßig in Blättern, insbesondere in älteren Blättern, nachgewiesen. Im Rhizom wurde das Bakterium nur gelegentlich nachgewiesen. Die einfache PCR war für den Nachweis von Befallssymptomen ausreichend und teilweise wurde auch an symptomlosen Pflanzenteilen von Symptome aufweisenden Pflanzen bzw. an latent infizierten Jungpflanzen *X. fragariae* nachgewiesen. Mit der sensitiveren nested PCR wurde latenter Befall häufiger nachgewiesen und die PCR Fragmente waren auf dem Agarosegel deutlicher sichtbar.

Stichwörter: Erdbeere; *Xanthomonas fragariae;* latente Infektion; Nachweis; nested PCR

1 Introduction

Angular leaf spot disease of strawberry, caused by the Gram-negative bacterium *Xanthomonas fragariae* (Kennedy & King), was first reported in 1960 in Minnesota, USA (Kennedy and King 1962). Since then, it was observed in other parts of the USA and in several other strawberry-growing countries around the world (CABI/EPPO 1998), probably having been spread in imported planting material (Maas 1998). In Europe, *X. fragariae* was first reported in Italy (Sicily) in 1972, probably introduced from California (Mazzucchi et al. 1973). Then it was observed in France (Rat 1974), Greece (Panagopoulos et al. 1978), Portugal (Fernandes and Pinto-Ganhao 1984), Spain (Lopez et al. 1985), Romania (Severin et al. 1985), 1992 in Switzerland (Grimm et al. 1993), 1994 in Germany (Billen 1995) and in the following years in the Netherlands and in Belgium (Bultreys et al. 2000). In the European Union, *X. fragariae* is listed as quarantine organism (OEPP/EPPO 1986; EU-Richtlinie 2000/29/EG).

Typical symptoms of angular leaf spot initially appear as minute, water-soaked lesions on the lower leaf surface. They enlarge and are translucent when viewed with transmitted light but dark green when viewed with reflected light. Under moist conditions, bacterial exudate is visible on the lower leaf surface. Later, lesions may become visible on the upper leaf surface as irregular, reddish brown spots. Occasionally, infection follows the major veins and heavily infected leaves may die. Under favorable conditions, the calyx may be infected (Maas 1995). In addition, vascular collapse of the plant from systemic colonization by the bacterium has been observed (Hildebrand et al. 1967; Moltmann 1997). Only little is known on yield losses caused by *X. fragariae*. Experimentally, *X. fragariae* reduced marketable yield about 8 % (Roberts et al. 1997). Yield losses up to 30 % due to infection of sepals were reported from Germany in 1995 (Litterst 1996) and in Wisconsin yield losses of 75–80 % were attributed to *X. fragariae* (Epstein 1966). Direct control is difficult, since antibiotics are not permitted in most countries and copper-containing pesticides, which have been shown to be effective protectants in some instances, are phytotoxic and cause yield reductions (Roberts et al. 1997). Although resistance to *X. fragariae* exists in breeding material (Maas et al. 2000; Lewers et al. 2003), it is not available in important commercial cultivars. As *X. fragariae* probably cannot survive free in the soil, crop rotation in combination with healthy planting material, being tested for infection with *X. fragariae,* is the most effective control measure. The European and Mediterranean Plant Protection Organization (EPPO) recommends a certification scheme for strawberry propagation (OEPP/EPPO 1994) with zero tolerance for *X. fragariae* in visual inspection of nuclear stocks, propagation stocks and certified stocks. Symptoms can easily be determined when many fresh lesions are visible. However, low numbers of spots or latent infections cannot be detected by visual inspection. For confirmation of visible infections, direct isolation or indirect immunofluorescence antibody staining (IFAS) is recommended (OEPP/EPPO 1994). Isolation is very difficult since *X. fragariae* is growing very slowly and associated micro-organisms, which are growing faster, often mask its presence in culture plates. Besides, isolation is very time consuming, because identity has to be confirmed by additional physiological and pathogenicity tests. Furthermore, an enzyme-linked immunosorbent assay (ELISA) was described (Rowhani et al. 1994) for detection of *X. fragariae* in leaves with visible symptoms. Unfortunately, cross-reactions with several *X. campestris* pathovars were observed (Opgenorth et al. 1996).

PCR-based detection methods, which are considered to be highly sensitive, have been described. A rep-PCR with primers that anneal to dispersed repetitive bacterial sequences is useful for the identification of pure cultures by their genomic fingerprints, but it is not directly applicable to plant extracts (Opgenorth et al. 1996). Pooler et al. (1996) developed three pairs of primers (specifically extended RAPD-primer) that can be used for multiplex PCR. This multiplex PCR was also combined with an immunocapture technique (Hartung and Pooler 1997). Roberts et al. (1996) developed PCR assays including a semi-nested PCR, amplifying a fragment in the *hrp*-gen region. Using this semi-nested PCR, *X. fragariae* could be detected in samples of symptomatic and asymptomatic tissue of spray-inoculated plants and was also detected in crowns of nursery plants (Mahuku and Goodwin 1997). Increase of sensitivity by nested techniques has been reported in PCR-based diagnostic protocols for several phytopathogenic bacteria (Hartung et al. 1996; Lee et al. 1997; Poussier and Luisetti 2000). In this study, we describe the development and application of a nested PCR for sensitive and rapid detection of *X. fragariae* in samples of symptomatic and symptomless strawberry plants collected from fruit production fields and from nursery plants.

2 Material and methods

2.1 Bacterial strains and culture conditions

Bacteria used in this study are listed in Table 1. *X. fragariae* and *X. campestris* pathovars were isolated at the Landesanstalt für Pflanzenschutz (LfP), Stuttgart, from naturally infected host plants and provided as pure cultures. *X. fragariae* was grown on sucrose peptone agar (SPA) (Gillings et al. 1998) or in liquid sucrose peptone media. Unidentified bacteria, which were isolated from strawberry plants, were cultured on SPA or in nutrient broth. For *X. campestris* pathovars, yeast extract dextrose chalk agar (YDC) (Schaad 1988) or nutrient broth were used. All isolates were grown at 26 °C. For long-term storage, liquid cultures were amended with glycerol to a final concentration of 30 % and stored at –20 °C.

2.2 Pathogenicity test

For inoculum preparation, isolates were streaked onto SPA. After 5 to 7 days incubation at 26 °C, bacteria were suspended in sterile distilled water and the suspensions were adjusted to an optical density (OD_{600nm}) of 0.1, corresponding to approximately 10^8 colony-forming units per milliliter (CFU/ml). Just prior to inoculation, density and viability of bacteria were checked by serial dilution plating on SPA. Young fully expanded strawberry leaves (cultivar 'Kent') were cut just above the crown and their petioles were placed in small flasks (50 ml) filled with tap water. Bacterial suspensions were infiltrated into the lower leaf surface using a syringe without a needle (Civerolo et al. 1997; Maas et al. 2000). Leaves were kept in a greenhouse at approximately 24 °C/18 °C with a 16 h photoperiod and were covered with a plastic hood to maintain a humid environment. Inoculation sites were examined for angular lesions at weekly intervals. For re-isolation of *X. fragariae,* leaves were surface sterilized with 70 % ethanol and lesions were homogenized in sodium phosphate buffer (0.01 mol/l, pH 7.4). The homogenate was streaked onto SPA and incubated at 26 °C.

2.3 DNA extraction

Two loops of bacteria grown for 2 to 3 days on solid media were suspended in 400 µl of extraction buffer (50 mmol/l Tris, 10 mmol/l EDTA, 2 % sodium dodecyl sulfate [SDS]; pH 8.0) followed by addition of 10 µl lysozym (20 mg/ml) and 10 µl RNase A (10 mg/ml). After 10 min incubation 420 µl phenol/chloroform (1 : 1) were added and the suspension was shaken for 10 min. After centrifugation at 15.300 × *g* for 20 min at 4 °C, the supernatant was transfered to a new tube and extracted with an equal volume of phenol/chloroform (1 : 1) for another two times. Then 0.1 volumes of ammonium acetate (5 mol/l) and 2.5 volumes ethanol (99 %) were added to the supernatant and DNA was precipitated overnight at –20 °C. After centrifugation (15.300 × *g,* 20 min, 4 °C), the pellet was washed with 70 % ethanol and vacuum-dried before re-dissolving in sterile bi-distilled water. DNA solutions

were stored at –20 °C. DNA quantity was estimated photometrically and by comparison with known standards in ethidium bromide-stained 1 % agarose gels.

DNA from plant extracts was prepared by total nucleic acid extraction using the NucleoSpin Plant Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

2.4 PCR amplification

PCR amplification was performed in 25 µl reaction mixtures, containing PCR buffer (10 mmol/l Tris-HCl [pH 8.8], 50 mmol/l KCl, 0.08 % Nonidet P40; supplied with *Taq* polymerase by MBI Fermentas), 2.5 mmol/l $MgCl_2$, 250 µmol/l deoxynucleoside triphosphate (dNTP), 0.2 µmol/l each primer and 1 U *Taq* DNA polymerase (MBI Fermentas). Primers were manufactured by Thermo Hybaid (Ulm, Germany). Primer sequences and their binding sites are listed in Table 2. Positive controls (20 ng DNA from *X. fragariae* 3.9.m per reaction) and negative controls (water) were included in each PCR amplification experiment. DNA amplification was carried out in a thermal cycler (Mastercycler® gradient, Eppendorff-Netheler-Hinz GmbH, Hamburg, Germany) with an initial denaturing step at 94 °C for 4 min, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 68 °C (primers 245A-245B) or 62 °C (primers 245.5-245.267) for 1 min and extension at 72 °C for 1 min. It was completed by a final extension step at 72 °C for 5 min. For PCR amplification of the bacterial 16S-23S rDNA spacer region with universal primers 16S-2 and 23S-10 (Table 2), the initial denaturing step was followed by 30 cycles of 30 s at 94 °C, 1 min at 50 °C, 1 min at 72 °C and a final extension step of 5 min at 72 °C.

For nested PCR, DNA was amplified in a first PCR with primers 245A and 245B. After amplification, 1 µl of the reaction mixture was used as template in a second PCR with the internal primers 245.5 and 245.267. For comparison of the sensitivity of the PCR assays, tenfold dilution series ranging from 20 pg to 2 fg *X. fragariae* DNA per reaction were used as template.

PCR products (8 µl reaction mixture) were analyzed by electrophoresis on 1.2 % agarose-TAE (40 mmol/l Tris-acetate, 2 mmol/l EDTA; pH 8.5) gels, followed by ethidium bromid staining and visualization under UV light. As molecular weight marker, a 100 bp DNA Ladder (GeneRuler™ 100 bp DNA Ladder Plus; MBI Fermentas, Lithuania) was used.

2.5 Design of PCR primers for specific nested PCR

The PCR product amplified by primers 245A and 245B (Table 2) was purified using a PCR Purification Kit (Böhringer, Mannheim, Germany) and sequenced from both sides to the end of the fragment. Sequencing was performed by SeqLab (Sequence Laboratories Göttingen GmbH, Göttingen, Germany) using primers 245A and 245B, respectively. For selection of the internal primer pair, primer analysis software 'GeneFisher' (Giegerich et al. 1996) was used to avoid the formation of primer dimers, self-priming or hairpins. For determination of suitable annealing conditions, amplification was done at annealing temperatures from 48 °C to 68 °C (ΔT = 2 °C). Specificity was tested by performing PCR with DNA (50 ng/reaction) extracted from pure cultures of *X. fragariae* (14 isolates), *X. campestris* (30 isolates, representing 14 pathovars) and unidentified bacteria (17 isolates) isolated from strawberry plants (Table 1). The bacterial 16S-23S rDNA spacer region was amplified with universal primers (Table 2) to demonstrate that DNA preparations were suitable for PCR amplification.

2.6 Detection of *X. fragariae* in strawberry plants

Plant material was washed with distilled water and dried with paper towels. Then it was cut into small pieces with a scalpel or a usual household mincing-knife. Plant pieces were shaken in sodium phosphate buffer (0.01 mol/l, pH 7.4; at a ratio of 20 ml buffer per 1 g fresh weight) in flasks placed on a rotary shaker at 150 rpm and room temperature for about 16 h to allow the bacteria to diffuse into the buffer. One ml of the liquid phase was centrifuged (15.300 × *g,* 4 °C, 20 min) and DNA was extracted from the pellet with the NucleoSpin Plant Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). To avoid contamination, filter tips were used and all tools were treated with NaOCl (2 %). One µl of DNA solution was analyzed by PCR (primers 245A-245B) and nested PCR (first round: primers 245A-245B, second round: primers 245.5-245.267).

For pathogenicity tests, aliquots of the plant extracts, which were prepared for DNA extraction, were infiltrated into strawberry leaves. Inoculation sites were examined daily during the first week after inoculation and then every fourth day for a period of 4 weeks.

3 Results

3.1 Specific and sensitive amplification of *X. fragariae*-DNA

The 16S-23S rDNA spacer region of all bacteria tested (Table 1) was amplified with eubacterial universal primers (Table 2). With primer pair 245A-245B (Table 2; Pooler et al. 1996) no fragment was amplified from different *X. campestris* pathovars or from unidentified bacteria, which were associated with strawberry plants. On the other hand, a specific PCR product was obtained with the same primer pair from all of the 14 *X. fragariae* isolates tested indicating a high specificity of primer set 245A-245B.

Sequencing of this PCR fragment resulted in a sequence of 308 bp. On the basis of the sequence data, primer pair 245.5 and 245.267 (Table 2) was selected for nested PCR. With these primers, a fragment of the expected size (286 bp) was amplified from *X. fragariae* (isolate 3.9.m) at all annealing temperatures from 48 °C to 68 °C (ΔT = 2 °C). Since rising annealing temperature increases specificity and reduces sensitivity, a moderate annealing temperature (62 °C) was used in subsequent experiments, because both specificity and sensitivity are required. The primer pair 245.5-245.267 was specific for

Table 1. Bacterial strains used to test specificity of PCR primer pair 245A-245B and the internal primer pair 245.5-245.267

Bacterial species (No. of isolates)	Culture code[a]	PCR amplification with primer pair		
		16S-2[b] 23S-10	245A[c] 245B	245.5[d] 245.267
X. fragariae (14)	3.9.a, 3.9.b, 3.9.c, 3.9.d, 3.9.f, 3.9.g, 3.9.h, 3.9.i, 3.9.j, 3.9.k, 3.9.l, 3.9.m, 3.9.n, 3.9.o	+	+	+
X. campestris pv. *pelargonii* (2)	3.1.l, 3.1.n	+	–	–
X. campestris pv. *campestris* (4)	3.2.a, 3.2.d, 3.2.e, 3.2.h	+	–	–
X. campestris pv. *lobeliae* (1)	3.3.e	+	–	–
X. campestris pv. *vesicatoria* (3)	3.4.a, 3.4.c, 3.4.d	+	–	–
X. campestris pv. *phaseoli* (2)	3.6.f, 3.6.h	+	–	–
X. campestris pv. *phaseoli* var. *fuscans* (1)	3.6.g	+	–	–
X. campestris pv. *hederae* (2)	3.7.c, 3.7.d	+	–	–
X. campestris pv. *juglandis* (2)	3.8.b, 3.8.c	+	–	
X. campestris pv. *zinniae* (1)	3.10.b	+	–	–
X. campestris pv. *cucurbitae* (1)	3.11.a	+	–	–
X. campestris pv. *carotae* (2)	3.12.a, 3.12.b	+	–	–
X. campestris pv. *begoniae* (3)	3.13.c, 3.13.d, 3.13.e	+	–	–
X. campestris pv. *vitians* (3)	3.14.a, 3.14.b, 3.14.c	+	–	–
X. campestris pv. *glycines* (2)	3.18.a, 3.18.b	+	–	–
X. campestris pv. *dieffenbachiae* (1)	3.19.a	+	–	–
Bacteria isolated from strawberry plants (17)[e]	none	+	–	–

[a] LfP (Landesanstalt für Pflanzenschutz, Stuttgart) culture collection code
[b] eubacterial universal primers amplifying the 16S-23S rDNA spacer region
[c] primer pair specific for *X. fragariae* developed by Pooler et al. (1996)
[d] pair of primers for nested PCR in combination with primer pair 245A-245B
[e] isolates were not identified

X. fragariae, since a single PCR product of the expected size (286 bp) was obtained with all *X. fragariae* isolates tested (Table 1, Fig. 1), and no products of any size were obtained using this pair of primers and DNA of *X. campestris* pathovars as template. In addition, the 17 unidentified bacteria isolated from strawberry were negative in PCR, too (Table 1).

3.2 Detection limit of PCR and nested PCR

Detection limit of simple PCR with primer pair 245A-245B was 20 pg DNA per reaction (Fig. 2a). Using the internal primer pair 245.5-245.267 in simple PCR, at least 2 ng DNA per reaction were necessary to obtain positive results (data not shown). For nested PCR, DNA amplified in a first round of PCR with primer pair 245A-245B was used as template in a second round of PCR with primer pair

Table 2. Primer sequences and binding sites

Primer	Sequence 5'-3'	Binding site	Reference
16S-2	TTGTACACACCGCCCGTC	16S rDNA	Gürtler and
23S-10	CCTTTCCCTCACGGTACT	23S rDNA	Stanisich (1996)
245A	CGCGTGCCAGTGGAGATCC	unknown (extended RAPD primer)	Pooler et al.
245B	CGCGTGCCAGAACTAGCAG	unknown (extended RAPD primer)	(1996)
245.5	GGTCCAGTGGAGATCCTGTG	245A-245B fragment	this study
245.267	GTTTTCGTTACGCTGAGTACTG	245A-245B fragment	

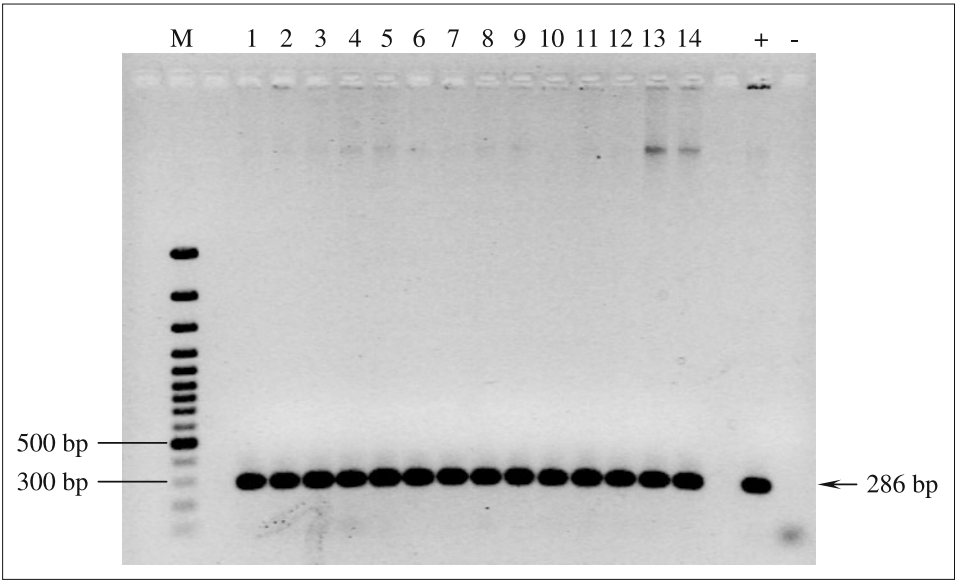

Fig. 1. Polymerase chain reaction products amplified from DNA of *Xanthomonas fragariae* isolates 3.9.a (lane 1), 3.9.b (lane 2), 3.9.c (lane 3), 3.9.d (lane 4), 3.9.f (lane 5), 3.9.g (lane 6), 3.9.h (lane 7), 3.9.i (lane 8), 3.9.j (lane 9), 3.9.k (lane 10), 3.9.l (lane 11), 3.9.m (lane 12), 3.9.n (lane 13), 3.9.o (lane 14) using primer pair 245.5 and 245.267. DNA from *X. fragariae* isolate 3.9.m was used as positive control (+), H_2O served as negative control (–). PCR products were separated by TAE agarose gel electrophoresis and stained with ethidium bromid. As molecular size marker a 100 bp DNA ladder (MBI Fermentas, Lithuania) was included (M).

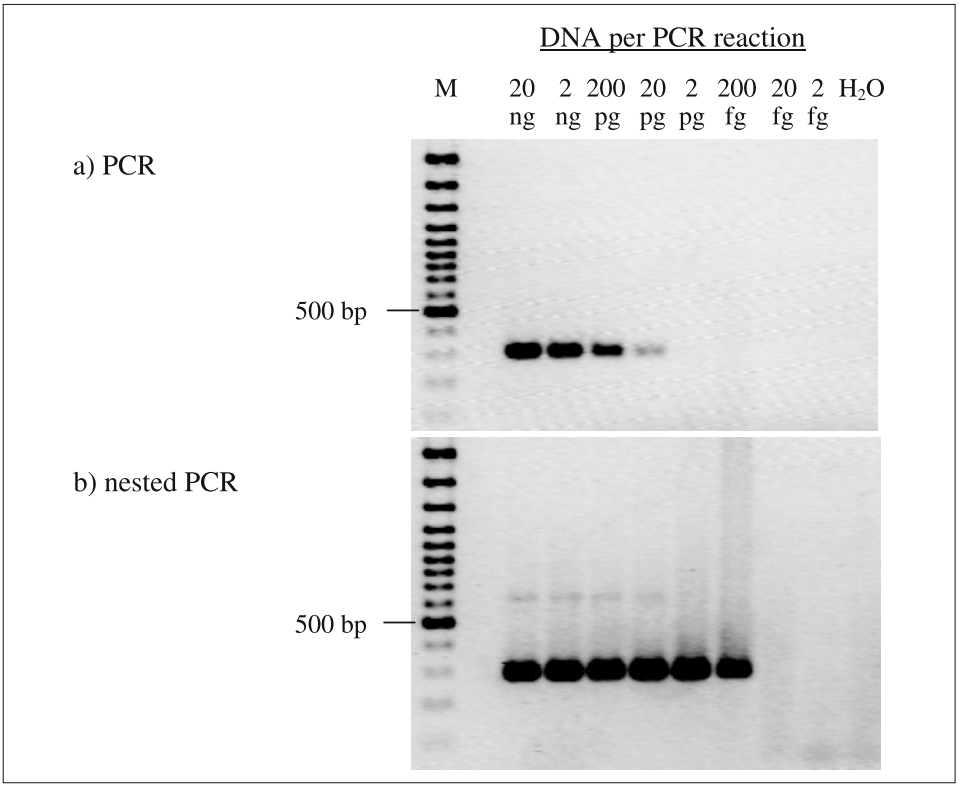

Fig. 2. Amplification of *Xanthomonas fragariae*-DNA by PCR (a) using primer pair 245A-245B and by nested PCR (b) using primer pair 245A-245B in a first round of PCR and the internal primer pair 245.5-245.267 in a second round of PCR. Tenfold dilution series of total genomic DNA of *X. fragariae* (isolate 3.9.m) from 20 ng to 2 fg per PCR reaction were used as template. As negative control, H_2O was used. PCR products were analyzed after TAE-agarose gel electrophoresis and ethidium bromid staining. A 100 bp DNA ladder (MBI Fermentas, Lithuania) was used (M) as molecular size marker.

245.5-245.267. With high template concentrations besides the 286 bp fragment, a second fragment of approx. 650 bp, which was hardly visible on agarose gels, was amplified in some cases. Nested PCR was up to a hundred times more sensitive than simple PCR, since even 200 fg DNA per reaction were detected (Fig. 2b). In addition, fragments amplified by simple PCR with primers 245A-245B were often only detectable as very faint bands on agarose gels, especially with decreasing amounts of template DNA. In contrast, after nested PCR, the 286 bp fragment was always clearly visible on agarose gels, even with low template concentration, and positive results were unambiguously determined (Fig. 2b).

3.3 Detection and distribution of *X. fragariae* in naturally infected strawberry plants

Symptomatic and asymptomatic leaves and runner plants as well as crowns and roots of naturally infected nursery plants were tested separately by simple PCR with primer pair 245A-245B (Table 3). *X. fragariae* was detected in all leaf samples with or without symptoms, in all symptomatic and in most asymptomatic runner plants. Besides *X. fragariae,* DNA was amplified in four of ten crown samples but in none of the root samples (Table 3). Consequently, roots can be excluded from the assay.

Similar results were obtained with strawberry plants, which were collected from naturally infected fruit production fields. From each plant, two old but not senescent leaves, two young and fully

expanded leaves and the crown were examined by visual inspection, PCR and nested PCR (Table 4). By visual inspection, symptoms were observed frequently on old leaves, only one plant showed symptoms on a young leaf, and no symptoms were observed on any of the crowns. In all symptomatic leaf samples,

Table 3. Occurrence of *Xanthomonas fragariae* in different parts of naturally infected nursery plants detected by simple PCR with primer pair 245A-245B

Plant No.	Leaves		Runner plants		Crown	Root
	with symptoms	without symptoms	with symptoms	without symptoms		
1	1/1[a]	2/2	–	–	0/1	0/1
2	1/1	1/1	1/1	1/1	0/1	0/1
3	1/1	2/2	–	1/1	0/1	0/1
4	1/1	3/3	–	0/1	1/1	0/1
5	1/1	1/1	1/1	1/1	0/1	0/1
6	1/1	2/2	–	1/1	0/1	0/1
7	–	3/3	1/1	–	1/1	0/1
8	2/2	–	1/1	1/1	1/1	0/1
9	1/1	2/2	1/1	–	0/1	0/1
10	1/1	2/2	1/1	–	1/1	0/1
total (in %)	10/10 (100 %)	18/18 (100 %)	6/6 (100 %)	5/6 (83 %)	4/10 (40 %)	0/10 (0 %)

[a] number of positive samples/samples tested; – = not tested as not available

Table 4. Detection of *Xanthomonas fragariae* in old leaves, young leaves and crowns of plants collected from naturally infected fruit production fields using PCR and nested PCR

Plant[a] No.	Old leaves[b]			Young leaves[c]			Crown[d]		
	symptoms	PCR	nested[e] PCR	symptoms	PCR	nested[e] PCR	symptoms	PCR	nested[e] PCR
1	2	2	2	0	0	1	–	–	–
2	2	2	2	0	0	2	–	–	–
3	1	2	2	0	0	2	–	–	+
4	2	2	2	1	1	2	–	–	–
5	2	2	2	0	1	2	–	–	–
6	2	2	2	0	1	2	–	–	–
7	2	2	2	0	1	2	–	–	+
8	2	2	2	0	1	1	–	+	+
9	1	2	2	0	1	1	–	+	+
10	2	2	2	0	1	2	–	+	+
11	0	0	0	0	0	1	–	–	–
12	0	0	1	0	0	1	–	–	+
13	1	1	2	0	0	0	–	+	+
14	1	2	2	0	0	2	–	+	+
15	2	2	2	0	0	2	–	–	+
total[f] (in %)	22/30 (73 %)	25/30 (83 %)	27/30 (90 %)	1/30 (3 %)	7/30 (23 %)	23/30 (77 %)	0/15 (0 %)	5/15 (33 %)	9/15 (60 %)

[a] plants were collected from two fields (field 1: plant 1–10; field 2: plant 11–15)
[b] from each plant, two old but not senescent leaves were tested; the number of positive samples is indicated
[c] from each plant, two young, fully expanded leaves were tested; the number of positive samples is indicated
[d] the crown was cut lengthwise into quarters, one quarter was used for the assay; + = positive, – = negative
[e] nested PCR was performed if PCR was negative; samples being positive by PCR or nested PCR are indicated in this column
[f] number of positive samples/samples tested

infection with *X. fragariae* was confirmed by PCR. In addition, *X. fragariae* was detected in symptomless leaves (three old leaves and six young leaves) and in five of the 15 crowns tested. Using nested PCR, another sample was positive. Altogether *X. fragariae* was detected in almost every old leaf (27 of 30), in 23 of the 30 young leaves and in 9 of the 15 crowns (Table 4). Since *X. fragariae* was most frequently detected in leaves, especially in old leaves, in subsequent studies the applicability of pooled leaf samples was tested. From fruit production fields, 20 old but not senescent leaves and 20 young, fully expanded leaves were collected randomly and pooled into samples of old leaves and young leaves, respectively. Obvious symptoms were observed especially on old leaves (Table 5). In all samples exhibiting symptoms, infection of *X. fragariae* was confirmed by simple PCR. With primer pair 245A-245B, an amplified fragment of the expected size was clearly visible on the ethidium bromide-stained agarose gel. Additionally, *X. fragariae* was detected in samples showing no symptoms, but PCR products were often only visible as faint bands. Using nested PCR, presence of *X. fragariae* was clearly detected in these samples. Furthermore, one sample with no symptoms, which was negative in simple PCR, was positive in nested PCR.

3.4 Detection of *X. fragariae* in symptomless nursery plants

PCR and nested PCR were also applied to check nursery plants. *X. fragariae* was detected by simple PCR in two of seventeen batches of nursery plants, which had appeared healthy by visible inspection, and in six additional batches by nested PCR. Furthermore, PCR products, which were amplified by simple PCR, were only visible as very faint bands on the agarose gel, while fragments, which were

Table 5. Comparison of visual inspection, PCR and nested PCR for detection of *Xanthomonas fragariae* in strawberry plants from fruit production fields

Sample[a]			Results using different detection methods		
Sample No.	Origin (field No.)	Age of the tested leaves	Symptoms[b]	PCR[c]	nested PCR[c]
1	1	old	–	–	–
2	1	young	–	–	–
3	2	old	+	++	++
4	2	young	–	–	–
5	3	old	++	++	++
6	3	young	–	–	++
7	4	old	++	+	++
8	4	young	–	+	++
9	5	old	++	++	++
10	5	young	–	+	++
11	6	old	–	+	++
12	6	young	–	–	–
13	7	old	–	–	–
14	7	young	–	–	++
15	8	old	++	++	nt
16	8	young	+	+	++
17	9	old	++	++	nt
18	9	young	–	+	++
19	10	old	++	++	nt
20	10	young	–	+	++
21	11	old	++	++	nt
22	11	young	–	+	++

[a] From each field, a sample of old leaves and a sample of young leaves, consisting of 20 leaves each, were tested separately
[b] – = no smptoms, + = individual leaves showing slight symptoms, ++ = at least 50 % of the leaves show typical angular leaf spot symptoms
[c] The intensity of the 308 bp (PCR) or the 286 bp fragment (nested PCR) in ethidium bromide-stained 1.2 % agarose gels; – = no band; + = weak band or moderate band; ++ = strong band; nt = not tested by nested PCR because PCR produced already a strong band.

amplified by nested PCR, were visible as strong bands (Fig. 3). Ten samples, including seven being positive by nested PCR, were also subjected to pathogenicity tests (Fig. 3). Leaf extracts were infiltrated into strawberry leaves and symptom expression was evaluated over a period of 5 weeks. For five of the seven samples, which were positive in nested PCR, infection with *X. fragariae* was confirmed by pathogenicity assay. The extracts of samples, which were positive by simple PCR (Fig. 3 sample no. 3 and 8), caused earlier and more severe symptom expression than the extracts of samples, which were tested positive only by nested PCR. Extracts of the latter samples caused only single and very small but typical angular leaf spots. However, *X. fragariae* was isolated from inoculation sites and pure cultures were identified as *X. fragariae* by PCR. Samples being negative by nested PCR were also negative by pathogenicity test.

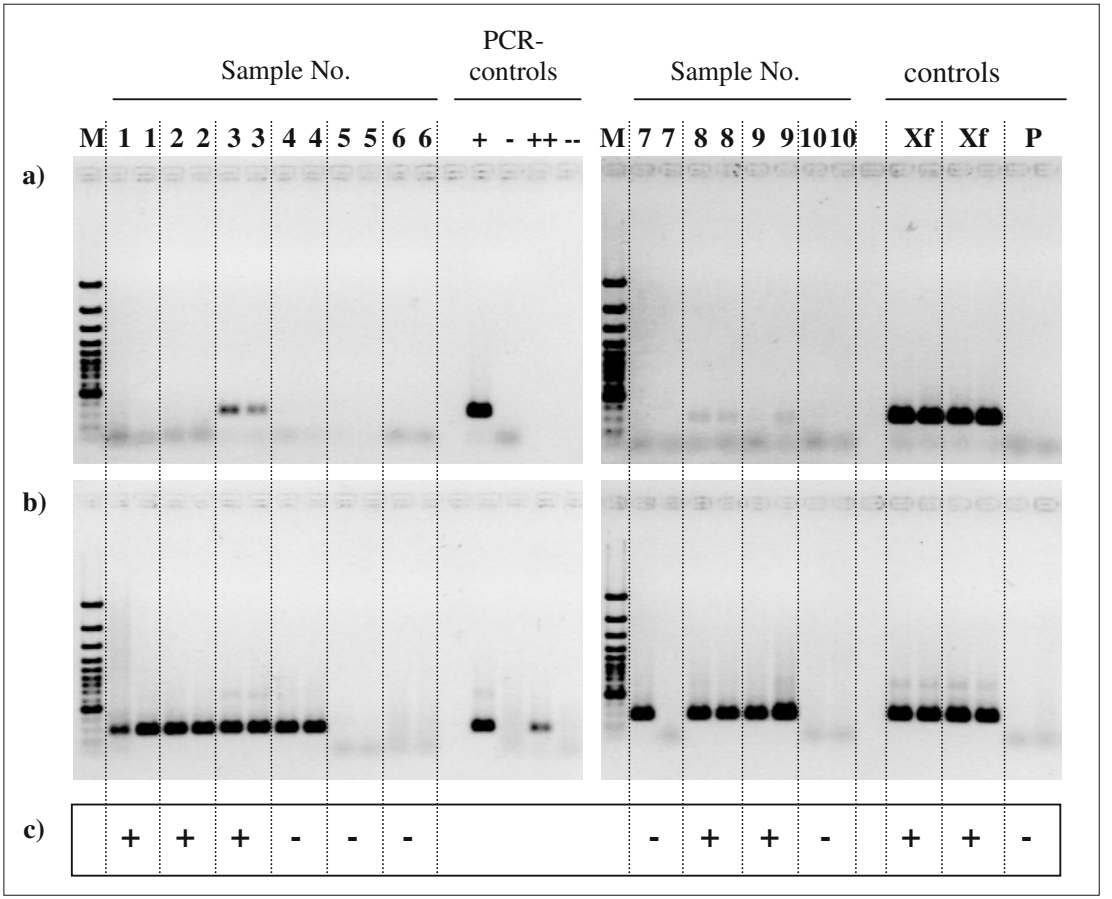

Fig. 3. Comparison of PCR (a) (primers 245A-245B), nested PCR (b) (first round of PCR: primer pair 245A-245B, second round of PCR: primer pair 245.5-245.267) and pathogenicity assay (c) for detection of *Xanthomonas fragariae* in symptomless nursery plants. Ten samples were tested (1–10). DNA was extracted from leaf extracts of 15 to 20 leaves per sample and amplified by PCR and nested PCR (two PCR-replicates per sample). As positive controls, 20 ng *X. fragariae* DNA (+) or healthy leaves mixed with a pure culture of *X. fragariae (Xf)* were used. H_2O (–) or healthy leaves (P) served as negative controls. PCR products were separated by TAE-agarose gel electrophoresis and stained with ethidium bromide. A 100 bp marker (M) was used as molecular weight marker. Pathogenicity assays were performed with plant extracts. Leaf extracts were infiltrated into strawberry leaves and symptom expression was evaluated (+ = typical angular leaf spots; – = no symptoms).

Besides the specific fragments, no PCR product of any size was amplified, neither in positive nor in negative samples of field-grown strawberry plants. Therefore, amplification of DNA from micro-organisms associated with strawberry plants, which could compete with or disturb amplification of the specific fragments from *X. fragariae,* is not expected.

4 Discussion

The use of healthy planting material is the most effective way to fight angular leaf spot, caused by *X. fragariae.* Thorough inspection of planting material would have prevented long distance distribution of *X. fragariae,* which probably spread to many countries all over the world by import of contaminated nursery plants (Maas 1998). For effective control of spreading of the disease, it is essential that low levels of infection and even latent infection can be detected. Therefore, it is necessary to develop highly sensitive assays.

Such assay might be the nested PCR presented in this study. By nested PCR, *X. fragariae* was detected in leaves of asymptomatic nursery plants, confirming that *X. fragariae* can actually be present on symptomless plants, with which it can be spread in spite of visual inspection.

Nested PCR was chosen, because it improves sensitivity and specificity of DNA amplification. With primer set 245A-245B (Pooler et al. 1996), DNA of *X. fragariae* was reliably amplified and the PCR was very robust to changing amplification conditions. A specific PCR product was obtained at annealing temperatures from 50 to 70 °C (data not shown). At annealing temperature of 68 °C, we reached a detection limit of 20 pg DNA of *X. fragariae* per reaction. For nested PCR, a set of internal primers (245.5 and 245.267) was selected within the fragment amplified by primers 245A and 245B and sensitivity was increased up to 100 times.

Specificity was confirmed for both the internal and the external set of primers. Specific products were amplified from all 14 *X. fragariae* isolates tested, but not from *X. campestris* isolates (30 isolates representing 14 pathovars) or from unidentified bacteria associated with strawberry leaves (17 isolates). In addition, the specificity of the external primer set was shown previously by Pooler et al. (1996), who tested 62 epiphytic bacterial strains isolated from strawberry plants and 40 isolates of *X. campestris.*

Sensitivity of simple PCR is sufficient to confirm symptomatic infections, since all samples of naturally infected strawberry plants which showed symptoms were tested positive. For the detection of *X. fragariae* on plants without symptoms or on asymptomatic parts of symptom bearing plants, nested PCR was often necessary. Although PCR is considered to be highly sensitive, it is not unusual that it can confirm only severe infections, whereas nested PCR is needed for the detection of slight or latent infections (Bonants et al. 1997; Poussier and Luisetti 2000). Consequently, if sensitive detection of latent infections is required, nested PCR is advisable. However, some problems have to be considered with the application of nested PCR, which are valid for all sensitive detection assays. First, with increasing sensitivity of the detection assay, the risk of contamination is increasing, too. Therefore, strict laboratory hygiene is recommended. To our experience, contamination was prevented successfully by treating all tools with NaOCl (2 %) and the usage of filter tips. Second, the confirmation of positive results is difficult, as independent assays (based on different properties of the bacterium) with comparable sensitivity are hardly available. However, since specificity was confirmed for both the internal and the external set of primers and since nested PCR itself improves specificity in comparison to simple PCR, nested PCR should be a useful tool to screen breeding and planting material without any additional test.

However, in some instances, it might be required that results are confirmed by a second test. In phytobacteriology, isolation and pathogenicity tests are generally desired for confirmation of positive results. Isolation of *X. fragariae* from latently infected samples is almost impossible due to its slow growth and the lack of selective media. Pathogenicity tests, using plant extracts, might confirm some latent infections, as shown in this study (Fig. 3). Unfortunately, such pathogenicity assays are very time consuming and seem to be less sensitive than nested PCR. Therefore, it would be wise to use nested PCR for rapid screening and to perform an additional pathogenicity test only with samples, which have been tested positive by nested PCR. However, if confirmation by pathogenicity test is required, more samples will be tested false negative as with nested PCR alone. Alternatively, a semi-nested PCR described by

Roberts et al. (1996) could be taken into account to confirm positive results, obtained by nested PCR. This semi-nested PCR amplifies products from the *hrp* gene region (Roberts et al. 1996) and was reported to detect *X. fragariae* on symptomless strawberry plants (Mahuku and Goodwin 1996). As this semi-nested PCR assay amplifies products from a different region of the genome than the nested PCR described in this study, both nested techniques can be considered as independent assays.

EPPO recommends the investigation of symptom-bearing parts of the plant or the crowns from asymptomatic plants (OEPP/EPPO 1994). However, in our experiments, *X. fragariae* was detected less frequently in crowns than in leaves and pooled leaf samples were sufficient to detect *X. fragariae* in symptomless nursery plants. Therefore, leaves and especially old leaves should be tested preferably. Only if leaves are not available (e. g., frigo plants), crowns should be used instead.

Acknowledgements

This work was funded in part by the Bundesministerium für Verbraucherschutz, Ernährung und Landwirtschaft (BMVEL), research assignment no. 98HS019.

Literature

Billen, W.: Die Eckige Blattfleckenkrankheit. – Eine neue Gefahr für den Erdbeeranbau? – Obstbau, **20** (**2**), 74–75, 1995.

Bonants, P., M. Hagenaar-De Weerdt, M. van Gent-Pelzer, I. Lacourt, D. Cooke, J. Duncan: Detection and identification of *Phytophthora fragariae* Hickman by the polymerase chain reaction. – Eur. J. Pl. Pathol. **103,** 345–355, 1997.

Bultreys, A., A. Robbe, J.-C. van Schingen: Detection de *Xanthomonas fragariae* en culture de fraisier en Belgique. – Fruit Belge **68,** 202–206, 2000.

CABI/EPPO: *Xanthomonas fragariae.* – Distribution maps of Quarantine Pests for Europe No. 284. CAB INTERNATIONAL, Wallingford, UK, 1998.

Civerolo, E. L., A. J. Feliciano, J. A. Melvin, W. D. Gubler: A detached strawberry leaf bioassay for *Xanthomonas fragariae.* – In: Proceedings 9th International Conference of Plant Pathogenic Bacteria, p. 89–94. Ed. Mahadevan A. University of Madras, India, 1997.

Epstein, A. H.: Angular leaf spot of strawberry. – Pl. Dis. Rep. **50,** 167, 1966.

Fernandes, A. M. M., J. F. Pinto-Ganhao: *Xanthomonas fragariae* Kennedy & King, a bacteriosis new to Portugal. – Rev. Pl. Pathol. **63,** 3469 (Abstract), 1984.

Giegerich, R., F. Meyer, C. Schleiermacher: GeneFischer – Software Support for the detection of postulated genes. – In: Proceedings of the Fourth International Conference on Intelligent Systems for Molecular Biology, AAAI Press, 1996.

Gillings, M. R., P. C. Fahy, J. Bradley: Identification of *Xanthomonas fragariae,* the cause of an outbreak of angular leaf spot on strawberry in South Australia, and comparison with the cause of previous outbreaks in New South Wales and New Zealand. – Australas. Pl. Path. **27,** 97–103, 1998.

Grimm, R., T. Lips, J. Vogelsanger: Eckige Blattfleckenkrankheit der Erdbeere. – Schweiz. Z. Obst-Weinbau **129,** 130–131, 1993.

Gürtler, V., V. A. Stanisich: New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. – Microbiology **142,** 3–16, 1996.

Hartung, J. S., M. R. Pooler: Immunocapture and multiplexed-PCR assay for *Xanthomonas fragariae,* causal agent of angular leafspot disease. – Acta Hort. **439,** 821–828, 1997.

Hartung, J. S., O. P. Pruvost, I. Villemot, A. Alvarez: Rapid and sensitive colorimetric detection of *Xanthomonas axonopodis* pv. *citri* by immunocapture and a nested-polymerase chain reaction assay. – Phytopathology **86,** 95–101, 1996.

Hildebrand, D. C., M. N. Schroth, S. Wilhelm: Systemic invasion of strawberry by *Xanthomonas fragariae* causing vascular collapse. – Phytopathology **57,** 1260–1261, 1967.

Kennedy, B. W., T. H. King: Angular leaf spot of strawberry caused by *Xanthomonas fragariae* sp. nov. – Phytopathology **52,** 873–875, 1962.

Lee, I.-M., I. M. Bartoszyk, D. E. Gundersen, B. Mogen, R. E. Davis: Nested PCR for ultrasensitive detection of the potato ring rot bacterium *Clavibacter michiganensis* subsp. *sepedonicus.* – Appl. Environ. Microb. **63,** 2625–2630, 1997.

Lewers, K. S., J. L. Maas, S. C. Hokanson, C. Gouin, J. S. Hartung: Inheritance of resistance in strawberry to bacterial angular leafspot disease caused by *Xanthomonas fragariae.* – J. Am. Soc. Hortic. Sci. **128,** 209–212, 2003.

Litterst, M.: Neue Krankheit im Erdbeeranbau. – Badische Bauernzeitung **49** (**13**), 28, 1996.

Lopez, M. M., J. M. Aramburu, M. Cambra, V. Borras: Deteccion e identificacion de *Xanthomonas fragariae* en Espana. – An. INIA/ Ser. Agric. **28,** 245–259, 1985.

Maas, J. L.: Compendium of strawberry diseases. 2nd edition, APS Press, St. Paul, Minnesota, USA, 1998.

Maas, J. L., M. R. Pooler, G. J. Galletta: Bacterial angular leafspot disease of strawberry: present status and prospects for control. – Adv. Strawberry Res. **14,** 18–24, 1995.

Maas, J. L., C. Gouin-Behe, J. S. Hartung, S. C. Hokanson: Sources of resistance for two differentially pathogenic strains of *Xanthomonas fragariae* in *Fragaria* genotypes. – HortScience **35,** 128–131, 2000.

Mahuku, G. S., P. H. Goodwin: Presence of *Xanthomonas fragariae* in symptomless strawberry crowns in Ontario detected using a nested polymerase chain reaction (PCR). – Can. J. Plant Pathol. **19,** 366–370, 1997.

Mazzucchi, U., A. Alberghina, A. Dalli: Occurrence of *Xanthomonas fragariae* Kennedy & King in Italy. – Phytopathol. Z. **76,** 367–370, 1973.

Moltmann, E.: Die Eckige Blattfleckenkrankheit *(Xanthomonas fragariae)* in Baden-Württemberg. – Phytomedizin **27**(**1**), 56–57, 1997.

OEPP/EPPO: EPPO Data sheets on quarantine organisms No. 135. *Xanthomonas fragariae* Kennedy & King. – Bull. OEPP/EPPO Bull. **16,** 17–20, 1986.

OEPP/EPPO: Phytosanitary procedure. *Xanthomonas fragariae.* Detection methods in strawberry plants. – Bull. OEPP/EPPO Bull. **24,** 343–346, 1994.

OEPP/EPPO: Certification Scheme No. 11: Pathogen-tested strawberry. – Bull. OEPP/EPPO Bull. **24,** 875–889, 1994.

Opgenorth, D. C., C. D. Smart, F. J. Louws, F. J. De Bruijn, B. C. Kirkpatrick: Identification of *Xanthomonas fragariae* field isolates by rep-PCR genomic fingerprinting. – Pl. Dis. **80,** 868–873, 1996.

Panagopoulos, C. G., P. G. Psallidas, A. S. Alivizatos: A bacterial leafspot of strawberry in Greece caused by *Xanthomonas fragariae.* – Phytopathol. Z. **91,** 33–38, 1978.

Pooler, M. R., D. F. Ritchie, J. S. Hartung: Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. – Appl. Environ. Microb. **62,** 3121–3127, 1996.

Poussier, S., J. Luisetti: Specific detection of biovars of *Ralstonia solanacearum* in plant tissues by nested-PCR-RFLP. – Eur. J. Pl. Pathol. **106,** 255–265, 2000.

Rat, B.: Présence en France de la maladie des taches angulaires du fraisier. – Ann. Phytopathol. **6**(**2**), 223, 1974.

Roberts, P. D., R. D. Berger, J. B. Jones, C. K. Chandler, R. E. Stall: Disease progress, yield loss, and control of *Xanthomonas fragariae* on strawberry plants. – Pl. Dis. **81,** 917–921, 1997.

Roberts, P. D., J. B. Jones, C. K. Chandler, R. E. Stall, R. D. Berger: Survival of *Xanthomonas fragariae* on strawberry in summer nurseries in Florida detected by specific primers and nested polymerase chain reaction. – Pl. Dis. **80,** 1283–1288, 1996.

Rowhani A., A. J. Feliciano, T. Lips, W. D. Gubler: Rapid identification of *Xanthomonas fragariae* in infected strawberry leaves by enzyme-linked immunosorbent assay. – Pl. Dis. **78,** 248–250, 1994.

Schaad, N. W.: Laboratory guide for identification of plant pathogenic bacteria, 2nd edition, APS Press, St. Paul, Minnesota, USA, 1988.

Severin, V., C. Stăncescu, E. Zambrowicz: Angular leaf spot, a new bacterial disease of strawberry in Romania. – Bul. Prot. Plant. **1–2,** 21–23, 1985.