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Tracing latent infection of *Colletotrichum acutatum* on strawberry by PCR

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Abstract

Colletotrichum acutatum, a quarantine organism on strawberries in the EU, was found in Finland for the first time in 2000. Concern about rapid, unnoticeable spread of this pathogen has necessitated studies to find methods with which the quiescent fungus infection can be detected in imported, cold-stored strawberry plant material. Successful detection of *C. acutatum* in strawberry tissues by polymerase chain reaction (PCR) is dependent on the method of DNA extraction used. Good-quality nucleic acid, free of PCR inhibitors, was successfully prepared by slightly modifying the DNA extraction method of a commercially available kit. Species-specific primers, previously described in the literature, were successfully used in the PCR reaction. *C. acutatum* was detected by PCR both on symptomatic and asymptomatic plant parts and in artificially and naturally infected strawberry tissues. Positive PCR results were obtained from ripe and unripe berries, runners, petioles and different parts of crowns. The data demonstrate that the PCR technique can be used to detect *C. acutatum* in strawberry tissue even in plant parts that do not show visible symptoms.

Introduction

Colletotrichum acutatum causes black spot disease on strawberry petioles, crowns, leaves, flowers and fruits (Howard et al., 1992). The fungus was first detected as a strawberry pathogen in Australia in 1954 and Simmonds (1965) identified it as a *Colletotrichum* species. In the USA, *C. acutatum* was found in 1983 (Smith and Black, 1986). The fungus is one of the major diseases affecting strawberries in France, especially in south-western areas (Denoyes and Baudry, 1996). In southern Europe, it was discovered in the main strawberry production region in Huelva, Spain in 1998 (De los Santos et al., 1999). *C. acutatum* has also already been detected in northern Europe: in Norway in 1999 (Stensvand et al., 2001) and in Finland in 2000 (Parikka and Kokkola, 2001). *C. acutatum* has severely infected nurseries in Israel (Freeman and Katan, 1997) and California, USA (Eastburn and Gubler, 1990).

The *Colletotrichum* species *C. acutatum*, *C. fragariae* and *C. gloeosporioides* have been distinguished and identified according to morphology and growth (Smith, 1990) and sensitivity to benomyl (Brown et al., 1996). However, the morphological characters and symptoms vary and do not provide a reliable basis for identification (Freeman et al., 1998). Various molecular techniques, e.g. arbitrarily primed polymerase chain reaction (PCR) and species-specific PCR, have been used to accurately differentiate and identify genotypes and species of *Colletotrichum* (Sreenivasaprasad et al., 1992; 1996; Freeman et al., 1993; 1998; Brown et al., 1996; Freeman and Katan, 1997). Freeman et al. (2000) have also investigated whether the species relationships based on various molecular methods are consistent with the morphotaxonomic criteria of the genus *Colletotrichum*. *C. acutatum*-specific primers have also been used in detection of infection in several plant species (Sreenivasaprasad et al., 1996; Freeman et al., 2001).

Colletotrichum acutatum is a quarantine organism on strawberries in the EU (Anon., 1997). The fungus can survive in plants as epiphytic and endophytic

infections (Freeman et al., 2001; Leandro et al., 2001). Transplants with latent infection are the most important source of primary inoculum in fruit production fields (Legard, 2000). In Finland, the only approved method for quarantine testing is the 'bio-amplification' ELISA (Hughes et al., 1997). This method, however, is time consuming. The aim of this study was to test the PCR method to determine the reliability of molecular identification in detecting *C. acutatum* in different plant parts, especially when the fungus is in a quiescent stage.

Materials and methods

Inoculation of strawberry plants

Strawberry runner plants (cv. Jonsok, Cavendish, Marlate, Mara des Bois, Rita, Florence, Symphony, Rosie and Kimberly) produced from healthy mother plants originating from micropropagated material were rooted in a peat substrate and grown at 20–24 °C, with a 16-h light period in a greenhouse. Six-week-old plants from each cultivar were inoculated with a *C. acutatum* spore suspension ($1.6–2.4 \times 10^6$ conidia ml $^{-1}$) using a hand sprayer. The fungus originally isolated in Finland from symptomatic strawberry fruits (CA 2000-1) was grown on potato dextrose agar (PDA) 20 g l^{-1} (Biokar Diagnostics, France) at 24 °C, and the mycelium with conidia was removed from 2-week-old cultures by adding 20 ml of distilled water and scraping the agar surface with a glass rod. The suspension with spores and mycelial fragments was mixed with distilled water. The plants were sprayed until runoff. The humidity in the greenhouse was maintained at 100% immediately after the inoculation and during the nights for 1 week after inoculation with a humidifier. The temperature was 24–26 °C (day) and 18 °C (night). The humidity and temperatures were controlled and registered by the greenhouse control device (ITU Multistation 10, Hortimic Inc, Finland).

Uninoculated control plants were grown in separate greenhouse compartments in normal conditions (20–24 °C day and 18 °C night) without water spray and high humidity.

Plants with natural infection

Naturally infected plants with visible black spot symptoms on berries were collected from a cultivar field trial at MTT Agrifood Research Finland/Horticulture in Piikkiö. Samples from cultivars L'Autenthique d'Orleans and Nadine, as well as some strawberry clones (SJ9327-17, SJ973-1 and SJ8302-2) were investigated. Samples were also taken from 28 commercially imported plant lots.

Sampling and sample preservation

Different plant parts were sampled for analysis 2–8 weeks after artificial inoculation. Symptomatic berries and stolons were sampled as well as asymptomatic petiole bases, crowns, folded young leaves, full-grown leaves and upper part of crowns. Symptomatic berries, petiole bases and crowns were sampled from naturally infected plants in a field trial. The samples from commercial plant lots consisted of asymptomatic petioles.

A sample consisted of 100 mg of plant tissue frozen at −20 °C in an 1.5 ml micro test tube. Control samples were taken from uninoculated plants grown in a greenhouse.

At sampling, surface sterilised pieces of the inoculated plant parts were also placed on PDA plates (20 g l^{-1}) to isolate *C. acutatum*. No antibiotics were added to the medium.

Extraction of DNA

DNA was isolated with a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions with minor modifications, which included increasing the amount of lysis buffer from 400 to 600 µl and addition of 15 mg of insoluble polyvinylpolypyrrolidone (PVPP, Sigma Chemical) to the lysis buffer in each sample at the second step. The optional centrifugation step was also used to remove precipitates of plant material and PVPP in the fourth step. The DNA was eluted with 60 µl of preheated (65 °C) AE buffer. The samples were used directly for PCR amplifications or were stored at −80 °C.

Oligonucleotide primers

Previously described primers, the specific primer for *C. acutatum* (CaInt2) and the internal transcribed spacer primer (ITS4) from the conserved region of ribosomal DNA (Brown et al., 1996; Sreenivasaprasad et al., 1996), were used.

Oligonucleotides were purchased from Med Probe, Norway.

PCR amplification

Polymerase chain reaction amplification and cycling parameters for PCR were performed and calculated according to the instructions of Ready-To-Go PCR beads and puReTaq Ready-To-Go PCR beads (Amersham Biosciences). The final volume of the PCR mixture was 25 µl. For each reaction, 1 µl of each primer (10 pmol μl^{-1}), 18 µl of sterile water and 5 µl of template DNA were added to a tube containing a PCR bead. PCR amplification was performed using a PTC-200 DNA engine (MJ Research, Inc. Watertown, USA). After an initial denaturation step (95 °C for 5 min), PCR was performed for 35 cycles, each at 95 °C for 30 s, at 58 °C for 30 s for annealing primer and at 72 °C for 1 min, followed by an extension step at 72 °C for 5 min. To determine if the correct sized PCR product was amplified, aliquots (16.7 µl) of the PCR products were electrophoresed at 90 V for 1 h in 1 × Tris–acetate–EDTA buffer (TAE). Gels were stained with ethidium bromide and viewed under UV light.

Purification of amplified PCR products

The PCR products were separated on a 0.8% low melting temperature agarose, Sea Plaque GTG (FMC), at 50 V for 1.5–2.0 h in 1 × TAE buffer. After running the gel, the PCR products were isolated with a QIAquick Gel Extraction Kit (Qiagen) using a microcentrifuge. The DNA was eluted with 100 µl of sterile water.

Sequencing and sequence analysis

Sequencing of the PCR products was performed in both directions by means of a Mega BACE 500 DNA analysis system (Amersham Biosciences). Sequencing reactions contained the same primers (CaInt2 and ITS4) that were used in the PCR. Multiple sequence alignments and comparisons of nucleotide sequences were performed with the PC/GENE programs (release 6.85; IntelliGenetics). Nucleotide homology searches were performed with the nucleotide program BLAST (http://www.ncbi.nlm.nih.gov).

Results

Extraction of DNA from the samples and PCR amplification

The slight modifications to the commercial DNA extraction kit sufficiently facilitated the extraction of *C. acutatum* DNA from plant tissues. The primers CaInt2 and ITS4 amplified a 490 bp product when used to assay the *Colletotrichum* species that had earlier been identified as *C. acutatum* by conidia and cultural

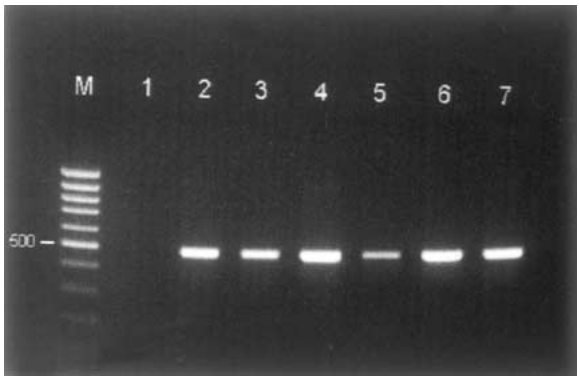

Figure 1. Detection of the *C. acutatum*-specific 490 bp fragment amplified by PCR from the artificially (lanes 2–4) or naturally (lanes 5 and 6) infected strawberry plants. Lane 1: uninoculated control; lane 2: young runners; lane 3: petioles, lane 4: crown; lane 5: asymptomatic petioles from imported plant; lane 6: symptomatic berry; lane 7: *C. acutatum* mycelium; lane M: 100 bp DNA ladder (Gene Ruler).

Figure 2. Detection of the *C. acutatum*-specific 490 bp fragment amplified from artificially inoculated strawberries, cultivar Mara des Bois 2 months after inoculation (lanes 2–5) and cv. Jonsok 1 month after inoculation (lanes 6–9). The following plant parts were used: folded young leaves (lanes 2 and 6), petioles (lanes 3 and 7), tops of crowns (lanes 4 and 8), crowns (lanes 5 and 9). Lane 1: uninoculated control; lane 10: *C. acutatum* mycelium; lane M: 100 bp DNA ladder (Gene Ruler).

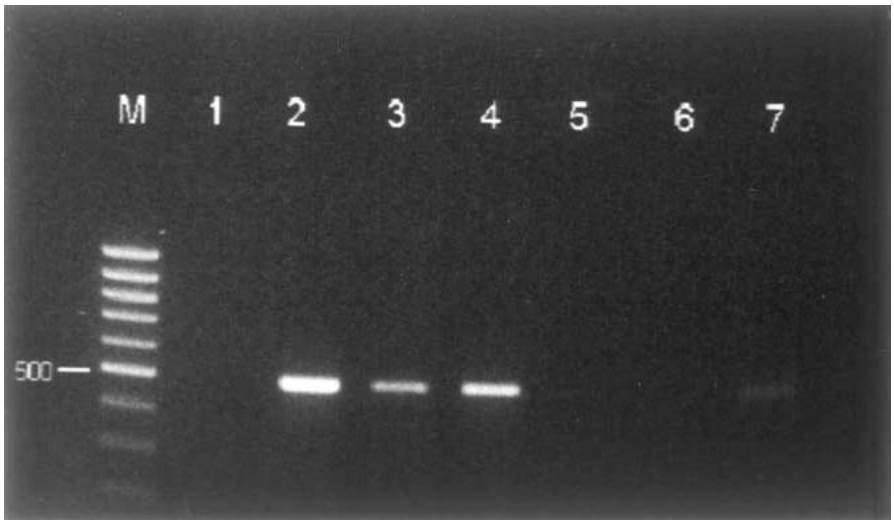

Figure 3. Detection of the *C. acutatum*-specific 490 bp fragment amplified from naturally infected, imported strawberries from a trial field. All samples were from petioles, but from different breeding lines and cultivars. All samples were positive although two of them were very weak (lanes 5 and 6). Lane 1: uninoculated control; lane 7: *C. acutatum* mycelium; lane M: 100 bp DNA ladder (Gene Ruler).

characteristics. The same product was amplified by PCR from different parts of artificially and naturally infected strawberry plants. Results were obtained from ripe and unripe berries, runners, petioles and different parts of crowns. The correct size PCR product (490 bp) was also amplified from randomly taken samples of imported strawberry plants (Figure 1). The presence of *C. acutatum* was also revealed by PCR in strawberry plant parts in which clear symptoms could not yet be observed with the naked eye. Samples from petioles proved to work well in the PCR test. However, negative results were repeatedly obtained from folded young leaves of inoculated plants (Figure 2).

The *C. acutatum*-specific band (490 bp) was also amplified from strawberry petioles collected from cultivars (L'Autenthique d'Orleans, Nadine) and selected clones in the field trial of MTT Horticulture in Piikkiö (Figure 3). Previously, berries of these plants had been tested by PCR and the results had been positive. *C. acutatum* was also isolated on PDA from these plants.

Homology comparison of the sequences

Multiple sequence alignment of the amplified PCR product (490 bp) of three isolates of *C. acutatum* (Figure 1) revealed that all the sequences from strawberry samples were identical to each other and to the sequences of *C. acutatum* in the GenBank database. Homology comparison of the sequence of the original *C. acutatum* isolate found in Finland (CA 2000-1) with sequences in the GenBank revealed that the highest nucleotide sequence identity (100%) in the sequenced fragment (383 bp) was shared with nine *C. acutatum* isolates, e.g. isolate TUT-5954 with accession number AF 207794. Sequences of two isolates, of which one was an imported sample and another was a sample from Piikkiö (CA 2002-5), were identical to each other, but there were differences in six nucleotides among the sequenced area of 383 bp compared to the sequence of the original *C. acutatum* isolate CA 2000-1. The degree of identity of all the three sequenced isolates of *C. acutatum* was 98.4%.

The non-specific band

Although the *C. acutatum*-specific band (490 bp) was easy to distinguish, there sometimes appeared a non-specific band measuring about 650 bp. This band was sometimes amplified in healthy samples, too (Figure 2). The amplified PCR product was sequenced, and homology comparison with sequences in the GenBank revealed that the most identical sequence was that of *Fragaria* sp. CFRA 538 18S, which shared 94% identity in the sequenced region (549 bp).

Symptoms on the plants and isolation of C. acutatum *on PDA medium*

The artificially inoculated plants showed no visible symptoms of *C. acutatum* in the petiole bases, leaves or upper part of crowns during the test period of 2 months in the greenhouse. In the lower crown tissues, a slight brown discolouration in the vascular tissues could be detected on some cultivars. One and 2 months after inoculation, *C. acutatum* was isolated from the upper part of crowns and from the lower crown tissues. The fungus was also isolated from samples of naturally infected plants collected from a field trial (CA 2002-4, CA 2002-5, CA 2002-6). These isolates differed in morphology from the *C. acutatum* isolate (CA 2000-1) used in artificial inoculation.

Discussion

The first detection of strawberry black spot (*C. acutatum*) in Finland in 2000 (Parikka and Kokkola, 2001) and concern about rapid latent spread of this pathogen have necessitated studies to find tools with which this quiescent fungus infection can be detected in cold-stored strawberry material. Molecular tools have already been successfully utilised to differentiate between the different species of *Colletotrichum* and to identify 'atypical' strains (Sreenivasaprasad et al., 1992; 1996; Freeman et al., 1993; Brown et al., 1996). However, in many reports, the fungus had been first isolated from plants on nutrient medium, and molecular methods were used to idetify the fungal cultures (Brown et al., 1996; Freeman and Katan, 1997). Our initial results using the PCR method confirmed that fungal isolate CA 2000-1 was *C. acutatum* (Parikka and Kokkola, 2001).

The chief limiting factor when using the PCR technique in routine diagnosis is the preparation of a good-quality nucleic acid, free of PCR inhibitors. In the current study, DNA samples were purified by using a slightly modified DNA extraction method based on the commercially available DNeasy Kit (Qiagen) and by adding PVPP to the lysis buffer. The intensity of the band with the correct size varies, but the species-specific primer analysis was accurate in detecting *C. acutatum* in strawberry by amplification of the specific band of 490 bp. Although there existed a non-specific band ~650 bp, it appeared only randomly and it was shown to be amplified from *Fragaria* DNA. This band did not disturb the interpretation of results of *C. acutatum* and it could possibly be eliminated by optimising the PCR conditions.

We succeeded in detecting *C. acutatum* in asymptomatic as well as symptomatic plant parts. Sreenivasaprasad et al. (1996) also reported PCR detection of *C. acutatum* in samples from black-spotted strawberry fruits and leaves. We detected *C. acutatum* in fruits too, but we were also able to identify this fungus in petioles and crowns of naturally infected strawberry plants.

Colletotrichum acutatum can survive and be transported in asymptomatic plants as secondary conidia and appressoria (Leandro et al., 2001). Freeman et al. (2001) reported that the fungus is present in asymptomatic artificially infected plants as epiphytic and endophytic fungal growth and that it can be reisolated from the tissues. We inoculated strawberry plants artificially in greenhouse conditions and the fungus was often reisolated on nutrient medium from petioles and upper crown tissues. This may be due to the survival of the original inoculum on plant surfaces over 2 months as Freeman et al. (2001) have observed or the fungus had formed secondary conidia on plant surfaces as Leandro et al. (2001) have reported. Isolation of *C. acutatum* from inner parts of lower crown tissues in asymptomatic plants after 1 or 2 months of inoculation shows, however, that infection has taken place. Negative PCR results from young folded leaves may be due to that they had escaped infection of *C. acutatum* while they had developed after inoculation and the fungus had not grown from the upper parts of the crowns into new, developing plant parts.

Isolation of *C. acutatum* on nutrient media (PDA containing no antibiotics) was not successful from the samples (petiole) taken from commercial plant lots showing positive PCR. This could be due to cold storage of the sampled plants and possible fungicide applications during the growing season which had weakened or killed the fungus. The PCR products could have been amplified from non-viable pathogen propagules.

The results were verified by sequencing the PCR product of the *C. acutatum* isolate used in artificial infection and the PCR product from the naturally infected symptomatic and asymptomatic plants. The two fungal isolates which were sequenced (CA 2000-1 and CA 2002-5) differed from each other in morphology and growth, but they had high similarity (98.4%) in the sequenced region (383 bp).

Our data demonstrate that PCR with species-specific primers can be used to detect *C. acutatum* on strawberry material from asymptomatic plant parts. Petioles

are used in quarantine tests, and they were very suitable samples for PCR. In addition, the PCR technique does not require paraquat treatment, as does the 'bio-amplification' ELISA (Hughes et al., 1997). The only weakness is that the CaInt2 primer may detect nonviable as well as viable propagules of *C. acutatum* as Sreenivasaprasad et al. (1996) have already observed. However, *C. acutatum* is a quarantine pathogen on strawberry and it must not be present in the plant material. PCR can be used in quarantine inspections to screen material to detect *C. acutatum*. The material that gives only a weak reaction could be examined more thoroughly. The possibility of detecting quiescent infections is important in order to control the spread of the pathogen.

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