Phytophthora cactorum and *Colletotrichum acutatum*: Survival and Detection

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Summary

Phytophthora cactorum and *Colletotrichum acutatum* are pathogens which are transported with plant material as latent infections and can also survive in soil and plant debris. Since the beginning of 1990's *P. cactorum* caused losses in strawberries in Finland and increased culling of silver birch seedlings in forest nurseries because of stem lesions. In this study primers specific for the pathogen were designed, and in a simple PCR they gave an amplification product from pure cultures only when *P. cactorum* was used as a template. No cross reactions were found with other *Phytophthora*s in group I or other microbes. Inoculated strawberry plants gave also a clear band in PCR-analyses when the template concentration was diluted. However, amplification was not always reproducible with birch seedlings. With soil samples the best result was gained by a combination of baiting and isolation.

C. acutatum is a quarantine pathogen on strawberry in the European Union and thus the infected plants are destroyed in Finland to avoid further spread of the pathogen. The pathogen has earlier been found to survive over one winter in infected plant debris and soil. In the survival test (2003-2005) done in this study, specific amplification products were obtained from test plants inoculated with artificially infected plant residues after 20 months of storage outdoors on soil surface. More positive results were achieved from bait plants grown in soil collected from the field where infected plants had been destroyed two years before, than from samples collected a year after the plant destruction.

Key words

Fragaria x ananassa, Betula pendula, crown rot, leather rot, soil, plant debris

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Introduction

Phytophthora cactorum (Lebert and Cohn) J. Schröt and *Colletotrichum acutatum* Simmonds are pathogens which are transported with plant material as latent infections and can also survive in soil and plant debris. *P. cactorum* is an economically important soil-borne pathogen of many herbaceous and woody species (Nienhaus, 1960). On cultivated strawberry (*Fragaria* x *ananassa* Duch.) it causes both crown rot and leather rot of fruits (Rose, 1924). Susceptible strawberry plants affected by this oomycetous pathogen in class Pseudofungi (Baldauff et al., 2000) wilt and the base of petiotiles and the upper part of the crowns turn dark brown. Infected fruits do not ripen and they turn leathery in texture. In forestry *P. cactorum* is known to cause root rot and stem cankers on many tree species (Niehaus, 1960; Erwin and Ribeiro, 1996). In Sweden it was present in soil together with other *Phytophthora* species in stands where oaks (*Quercus robur* L.) showed tree crown defoliation (Jönsson et al., 2003). In inoculations it caused significant dieback of fine root and necrotic lesions on coarser root of oak seedlings (Jönsson, 2003). In Finland *P. cactorum* was isolated for the first time in 1990 from strawberry plants suffering from crown rot (Parikka, 1991). A year later *P. cactorum* was isolated from necrotic stem lesions on silver birch (*Betula pendula* Roth.) seedlings growing in forest nurseries (Lilja et al., 1996; Hantula et al., 1997, 2000). Since then this imported pathogen has caused crop losses in strawberry fields mainly as an agent of crown rot and increased culling of seedlings in forest nurseries.

Anthracnose of strawberry, caused by *Colletotrichum acutatum* is another fungal disease that is imported into Finland in plant material and might be in future a high risk for strawberry plantations. According to Wilson et al. (1992) the fungus can overwinter in diseased berry residues in cool conditions. *C. acutatum* can survive in plants as epiphytic and endophytic infections (Freeman et al., 2001), and several weed species can serve as alternative hosts (Berrie and Burgess, 2003). The fungus was detected for the first time in Finland in 2000 in imported strawberry plants (Parikka and Kokkola, 2001). It is a quarantine pathogen on strawberry in the European Union (Anon., 1997). In Finland, whenever a *C. acutatum* infection is detected, the infected plants are destroyed to avoid further spread of the pathogen.

Usage of different types of PCR-analyses have become very popular in molecular diagnostics (Sreenivasaprasad et al., 1996; Schubert et al., 1999; Nechwatal et al., 2001; Grote et al., 2000, 2002; Martinez-Culebras et al., 2003; Martin et al., 2004; Ippolito et al., 2004; Parikka and Lemmetty, 2004; Causin et al., 2005). Causin et al (2005) reported that it is possible to detect *P. cactorum* in plants with a nested PCR. *C. acutatum* specific primers have also been used in detection of infection in several plant species (Sreenivasaprasad et al., 1996; Martinez-Culebras et al., 2003; Parikka and Lemmetty, 2004).

The aim of this study was to find out for how long *C. acutatum* can survive in plant debris and in soil under Finnish conditions. One target was also to develop primers specific for *P. cactorum* and test their reliability in a simple PCR.

Materials and methods

Phytophthora cactorum

Isolates

Phytophthora cactorum isolates, *Phytophthora* isolates from group I (Waterhouse, 1963), other used microbes and their origin are presented in Table 1.

DNA techniques

DNA extractions. DNA from mycelial cultures was isolated as described by Vainio et al. (1998). However, previously extracted DNA (Hantula et al., 1997) was used when possible (isolates S10, EM294, 9/88/92, 1557, Ph3 and Ph20).

DNA from plant material was isolated using Ultraclean Soil DNA Isolation Kit (MO Bio Laboratories Inc.) in combination with the method of Vainio et al. (1998) as described by Pennanen et al. (2001). The DNA extractions from the strawberry plants were done 3-4 days after inoculation at the stage when the disease symptoms were not visible. On birch, small lesions were already present on the stem when DNA was isolated.

Primer design. Amplification product GT750 (Hantula et al., 1997) was cloned from isolate S9 as described previously (Vainio and Hantula, 2000). The selection of fragments was based on standard agarose gel electrophoresis. The cloned inserts were sequenced by A.L.F. DNA Sequencer™ (Pharmacia Biotech, Uppsala, Sweden) using M13 reverse and forward primers and the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, England). Primers *Pcact*SF (5′CCCGTACTACTCGCGACTCT), and *Pcact*SR (5′TGACGGGAAAGATGGCG) were designed in order to amplify a 153 bp fragment from isolates belonging to *P. cactorum*.

PCR-analyses. In all PCR-analyses the buffer conditions were the same as suggested by the manufacturer of the Dynazyme polymerase (Finnzymes Ltd, Espoo, Finland). The amplification conditions for primer pair *Pcact*SF + *Pcact*SR were optimised. The amplification was carried out using a "hot start" protocol where DNA samples and primers *Pcact*SF and *Pcact*SR (each

Table 1.
Origin of *Phytophthora cactorum* isolates

Collection code	Host	Source	Country
S3	Strawberry	P. Parikka	FI
S5	Strawberry	P. Parikka	FI
S6	Strawberry	P. Parikka	FI
S7	Strawberry	P. Parikka	FI
S9	Strawberry	P. Parikka	FI
S10	Strawberry	P. Parikka	FI
S13	Strawberry	P. Parikka	FI
S14	Strawberry	P. Parikka	FI
S15	Strawberry	P. Parikka	FI
S19	Strawberry	P. Parikka	FI
Ph1	Scots pine	A. Lilja	FI
Ph2	Silver birch	A. Lilja	FI
Ph3	Silver birch	A. Lilja	FI
Ph4	Silver birch	A. Lilja	FI
Ph5	Silver birch	A. Lilja	FI
Ph8	Silver birch	A. Lilja	FI
Ph10	Silver birch	A. Lilja	FI
Ph11	Silver birch	A. Lilja	FI
Ph14	Silver birch	A. Lilja	FI
Ph15	Silver birch	A. Lilja	FI
Ph17	Strawberry	A. Lilja	FI
Ph18	Silver birch	A. Lilja	FI
Ph20	Silver birch	A. Lilja	FI
Ph33	Alder	A. Lilja	FI
Ph35	Pond water	A. Lilja	FI
A1	Strawberry	S. Werres	DE
2/94/3	Horse chestnut	S. Werres	DE
5/94	Horse chestnut	S. Werres	DE
CH09	Strawberry	S. Werres	DE
9/88/92	Rhododendron	S. Werres	DE
1557	Rhododendron	S. Werres	DE
B1	soil	S. Werres	DE
CH12	Strawberry	C. Olsson	SE
CH15	Strawberry	C. Olsson	SE
CH17	Strawberry	C. Olsson	SE
CH19	Strawberry	C. Olsson	SE
EM294	Apple	D. Harris	UK
PE	Apple	D. Harris	UK
R12	Red raspberry	D. Harris	UK
H145	Strawberry	D. Harris	UK
TAM1	Strawberry	D. Kennedy	UK
P. idaei R66	Red raspberry	D. Kennedy	UK
P. undulata	Norway spruce	A. Lilja	FI
P. cladestina		CBS 347 86	
P. iranica		CBS 374 72	
P. pseudotsugae		CBS 446 84	
P. citrophora	Pointsettia	P. Parikka	NL
Pythium anandrum	Scots pine	A. Lilja	FI

in a concentration of 0.5 µM) were denaturated at 95°C for 10 minutes, after which a dNTP-mix (each deoxynucleotide in a concentration of 0.2 mM) and 2 U of Dynazyme thermostable DNA-polymerase (Finnzymes Ltd, Espoo, Finland) were added. Then 45 cycles of amplification (30 s denaturation at 95°C, 45 s annealing at 63°C, 1 min extension at 72°C) and a final extension of 7 minutes were carried out.

Electrophoresis. Amplification products were separated by electrophoresis in gels containing 1.0% agarose (FMC BioProducts) and 1.0% SynerGel (Diversified Biotech). The electrophoresis was run in TAE-buffer (40 mM Tris-Acetate pH 8.0, 1 mM EDTA), and the amplification products were visualized by ethidium bromide in UV-light. The lengths of the amplification products were estimated by comparing them to a 100 bp DNA ladder (Gibco BRL).

Inoculation of plants

Strawberry

Test 1. Strawberry plants, cv 'Jonsok' (which is known to be susceptible to this oomycete; Parikka, 2003) were inoculated with *P. cactorum* isolate from strawberry. Runner plants were cut and rooted in limed, low-humified Sphagnum peat (800 g dolomite limestone, 50 g fine ground limestone, 50 g super phosphate, and 130 g peat fertiliser l^{-1} (Turpeen Y-lannos, Kemira Agro Oy). After 40 days, the plants were removed from the rooting substrate and the roots were washed with tapwater. Before inoculation the crown of each plant was wounded 0.5 cm to the ground level with a sharp stick. The wound (2 mm deep and 2 mm wide) was covered with mycelium from 3-week-old culture on potato dextrose agar (PDA 39 g, Difco l^{-1}). The inocula were secured with parafilm (Parafilm M, Amer. Nat. Can.) for two days. Each plant received one inoculation. After inoculation the plants were planted in pots (500 ml) containing the same pot mix as used above. Plants were placed on a laboratory table under two fluorescent lights with an intensity of 10 000 lx and 8-h photoperiod with a temperature range of 22-24 °C for 4 days before DNA extraction.

Test 2. Strawberry plants were produced for tests by rooting 1-leaf cuttings in 5-cm rockwool cubes for three weeks and inoculating the rooted plants in NFT system with *P. cactorum* sporangia (Parikka, 2006). Strawberry cultivars with different susceptibility to *P. cactorum*, 'Jonsok', 'Oka', 'Gyda', 'Korona' and 'Bounty' were used in the test. Plants with and without symptoms were collected after 4-week period for testing.

Birch seedlings

Test 3 and *Test 4.* In both tests the one-year-old, silver birch seedlings have been produced according to normal nursery practice in hard plastic containers (Plantek 25, Lännen Plant Systems, Finland) filled with low humified, coarse-textured Sphagnum peat (M6, Kekkilä Corp., Finland). Seedlings were inoculated with each *P. cactorum* strain with a 9 mm^2 agar block from a 1-week-old culture on PDA. The inocula were secured with parafilm for 2 days. Seedlings were placed on a laboratory table under two fluorescent lights with an intensity of 10 000 lx and 8-h photoperiod with a temperature range of 22-

24 °C for 7 days before DNA extraction. In the first test one strain, Ph20 was used and in the second one the number of *P. cactorum* strains was five.

Soil samples

Test 5. Soil samples were collected from strawberry fields in Suonenjoki. Samples were taken from fields where leather rot and crown rot symptoms had been observed. Two kilograms of soil was sampled from each site, both from planting rows and between rows. The presence of *P. cactorum* was tested from the samples with *Rhododendron* leaf baits according to Themann and Werres (1997).

Colletotrichum acutatum

Survival test of *C. acutatum* and soil sampling

Test 6. The survival of *C. acutatum* in infected plant material was studied in 2003-2005 with artificially infected strawberry plant parts (Parikka et al., 2006) The nylon-mesh bags with plant debris were placed in plastic buckets filled with sandy soil in Autumn 2003: on the soil surface and 10 cm deep. The buckets were placed outdoors and covered with a net.

The nylon-mesh bags were removed from the buckets in August 2004, then June 2005 and September 2005. The material from the bags and soil samples were used to inoculate young strawberry plants (cv Jonsok) in a greenhouse as described by Parikka et al. (2006).

Test 7. Soil samples were taken in May 2004 from a field (MTT Horticulture, Piikkiö, Finland), where naturally infected strawberry plants had been destroyed by burning in July 2002. Sampling was conducted from soil surface and 5 cm below surface as described by Parikka et al. (2006). PCR-analysis were conducted of bait plants grown in soil samples collected from the field.

PCR detection of *C. acutatum*

Samples for PCR (100 mg) were taken from petiole bases and crowns and stored at -20°C in micro test tubes. DNA extraction was performed as described by Parikka and Lemmetty (2004). For PCR reactions, the primers and reaction conditions described by Martinez-Culebras et al. (2003) were used.

Results

***Phytophthora cactorum* specific primers**

Amplification was conducted with primer pair *Pcact*SF and *Pcact*SR and DNA from pure cultures of oomycetes. An amplification product of approximately 150 bp was obtained from all *P. cactorum* isolates from strawberry, and from 12 out of 13 isolates from birch (Table 2).

Table 2.
The presence of amplification products obtained with the primer pair *Pcact*SF + *Pcact*SR using mycelial cultures

Strain	Host	Amplification of the ca 150 bp fragment
S3	Strawberry	+
S5	Strawberry	+
S6	Strawberry	+
S7	Strawberry	+
S9	Strawberry	+
S10	Strawberry	+
S13	Strawberry	+
S14	Strawberry	+
S15	Strawberry	+
S19	Strawberry	+
Ph1	Scots pine	+
Ph2	Silver birch	+
Ph3	Silver birch	+
Ph4	Silver birch	+
Ph5	Silver birch	+
Ph8	Silver birch	+
Ph10	Silver birch	+
Ph11	Silver birch	–
Ph14	Silver birch	+
Ph15	Silver birch	+
Ph17	Strawberry	+
Ph18	Silver birch	+
Ph20	Silver birch	+
A1	Strawberry	+
2/94/3	Horse chestnut	+
5/94	Horse chestnut	+
CH09	Strawberry	+
9/88/92	Rhododendron	+
1557	Rhododendron	+
B1	soil	+
CH12	Strawberry	+
CH15	Strawberry	+
CH17	Strawberry	+
CH19	Strawberry	+
EM294	Apple	+
PE	Apple	+
R12	Red raspberry	+
H145	Strawberry	+
TAM1	Strawberry	+
P. idaei R66	Red raspberry	–
P. undulata	Norway spruce	–
P. cladestina		–
P. iranica		–
P. pseudotsugae		–
P. citrophora	Pointsettia	–
Pythium anandrum	Scots pine	–

A product of similar size was also amplified from all other *P. cactorum* isolates from other plants including horse chestnut, rhododendron, apple, red raspberry and Norway spruce, as well as from an isolate originating from a soil sample (Table 2). However, no amplification products were obtained from isolates of *P. undulata*, *P. cladestina*, *P. iranica*, *P. pseudotsugae* or *Pythium anan-*

Table 3.
Test 1. Detection of *Phytophthora cactorum* from inoculated, healthy looking strawberry plants representing cv. Jonsok)

Sample	PCR
0	–
1	+
2	+
3	++
4	++
5	+
6	++

+ = band, ++ = clear band, 0= negative control,
1–6 inoculated test plants

drum. Using *P. cladestina* DNA as template an extremely faint product of similar size was reproducibly amplified, but its intensity was considerably lower compared to the amplification product from *P. cactorum*. Thus, the primer sequences allowed preferential, although not completely specific amplification of *P. cactorum* from pure cultures.

Test 1. We also tested the applicability of the two primers in amplifying DNA from plants. In this test where strawberry plants susceptible to *P. cactorum* (cv Jonsok) were inoculated, we observed bands in all six test plants although they looked healthy except one, which had a small lesion on crown (Table 3). No amplification was observed in the negative control.

Test 2. In another experiment (Table 4) we used five cultivars of strawberry inoculated with *P. cactorum*. In this experiment we used five different concentrations of strawberry template DNA, and the optimal concentration was obtained when 1-2 µl of sample was used (the DNA concentrations were not determined, as plant and oomycete DNA would not be distinguished). In one of the varieties no bands were observed in any of the dilutions.

Test 3. When silver birch seedlings were inoculated with *P. cactorum*, an amplification product was observed from four out of the five samples. In one of the seedlings amplification products were observed in all dilutions, but also in this case the most effective amount of template DNA was one µl (Table 5).

Test 4. We also conducted experiments (Table 6), where silver birch seedlings were inoculated with different strains of *P. cactorum* and the PCR-analysis was repeated four times. Only two of the strains were detected in all four experiments, and two of the strains were observed in only half of the experiments.

Presence of *Phytophthora cactorum* in soil

Test 5. Only *P. cactorum* was isolated from lesions that developed on *Rhodondendron* leaf baits.

Table 4.
Test 2. Detection of inoculated *Phytophthora cactorum* from healthy looking strawberry plants representing different varieties

Sample	Dilution of DNA				
	10 µl	5 µl	2 µl	1 µl	0.2 µl
0	–	–	–	–	–
Jonsok[1)]	–	–	+	+	++
Jonsok	–	–	+	+	++
Oka	–	–	+	+	–
Gyda	–	–	–	–	–
Korona	–	–	+	+	–
Bounty	–	–	+	+	+

[1)] small lesion on crown, (+ = band, ++ = clear band,
0 = negative control

Table 5.
Test 3. Detection of *Phytophthora cactorum* from lesions on inoculated silver birch seedlings

Sample	Dilution of DNA				
	1:3	1:10	10µl	3 µl	1 µl
0	–	–	–	–	–
Seedling 1	–	–	–	–	–
Seedling 2	–	–	–	+	+
Seedling 3	–	–	–	(+)	(+)
Seedling 4	(+)	(+)	+	+	(+)
Seedling 5	(+)	–	(+)	+	+
Mycelium	–	+	–	–	–

(+) = faint band, + = band, ++ = clear band,
0 = negative contro)

Table 6.
Test 4. Detection of *Phytophthora cactorum* from lesions on silver birch seedlings inoculated with different strains. The samples were analysed four times with PCR.

Sample	PCR1	PCR2	PCR3	PCR4
0	–	–	–	–
Ph18	(+)	(+)	–	
Ph20	++	+	+	+
Ph24	–	+	–	(+)
Ph33	+	(+)	(+)	–
Ph35	+	+	+	++

(+) = faint band, + = band, ++ = clear band,
0 = negative control

Survival of *Colletotrichum acutatum* in plant residues and soil

Test 6. The pathogen survived two winters in artificially infected plant residues on the soil surface from autumn 2003 until June 2005. Survival was tested with bait plants in a greenhouse, and samples of test plants were analysed by PCR (Table 7). *C. acutatum*-specific band (318 bp) was amplified from strawberry bait plants

Table 7.
Test 6. Survival of *Colletotrichum acutatum* in plant residues in 2003 (7–9 months), 2004 and 2005 (12–24 months). Bait plants were inoculated with the residues and analysed with PCR

Residue baits	Analysed samples of bait plants total	Positive samples/ analysed	Positive reactions of bait plants			
			From plant parts		Inoculum from different depths	
			Petiole	Crown	Soil surface	9–15 cm
2003	108	48	18	30	22	26
2004	2	0	0	0	0	0
2005	8	1	1	0	1	0

inoculated with infected plant debris preserved on soil surface for 20 months, but not from controls. Symptoms on bait plants were inconspicuous after the 5-week test period.

Test 7. Positive PCR results were also obtained from bait plants grown in soil collected from the field where infected plants had been destroyed nearly two years before sampling. *C. acutatum*-specific band (318 bp) was amplified from bait plants in soil sampled both from the soil surface and from the depth of 5 cm. No symptoms of *C. acutatum* developed on the plants during the 8-week test period.

Discussion

Survival of *Colletotrichum acutatum*

In this study according to the result of PCR-test, *C. acutatum* survived two winters (2003-2005) in artificially infected plant residues on the soil surface, although the plant debris did not cause clear, visual symptoms on the bait plants during the 5-week test period. In our previous work *C. acutatum* was, however, found to cause symptoms on strawberry plants when debris was collected after the first winter. In that case the fungus was also successfully isolated on PDA medium from petioles and runners of bait plants cv Jonsok and Rita (Parikka et al., 2006). *C. acutatum* can survive on plants without symptoms and latent infections without visible symptoms have been detected by PCR on artificially infected plants two months after inoculation (Parikka and Lemmetty, 2004). According to Wilson et al. (1992) fluctuating temperatures alone have no apparent effect on recovery of *C. acutatum* from fruit residues and under field conditions the fungus can recover after six months under cold winter conditions both on the soil surface and buried in the soil in depths of 5-8 cm. During the winter periods 2003-2004 and 2004-2005 the minimum temperatures at soil level fluctuated between +5°C and –27°C, being below –20°C only a few days. In summer 2004 the precipitation was exceptionally high. According to the literature temperature and soil moisture have a strong influence on the survival of *C. acutatum* and the fungus survives best under cool and dry conditions where competing microflora is not very active since *C. acutatum* itself uses the colonized substrate slowly (Easburn and Gubler, 1992). Thus high soil moisture and higher autumn and spring temperatures in general would indicate reduced *C. acutatum* survival. However, *C. acutatum* seemed to survive in plant debris on soil surface over two winters under Finnish conditions.

PCR detection (with CaInt2 primers) from strawberry petioles and crowns had earlier been made according to Parikka and Lemmetty (2004) and *C. acutatum*-specific band (490bp) was amplified from strawberry bait plants inoculated with the plant debris preserved over one winter on soil surface or buried in soil (Parikka et al., 2006). Besides the correct size band (490 bp), non-specific bands were also amplified when the samples were taken later in summer (Parikka et al., 2006). Supposedly there were more infections of bait plants with other fungi competing with *C. acutatum* in the plant debris and this might be one reason for the weaker intensity of *C. acutatum* -specific bands and the number of non-specific bands. When primers Acut1 and Col2 by Martinez-Culebras et al. (2003) were used here, the *C. acutatum*-specific band (318 bp) was obtained from a petiole sample of a test plant inoculated with plant debris stored on soil surface for 20 months.

Eastburn and Gubler (1990) have found that the viable fungal population in soil gradually declined during 11 months. Here more positive PCR results were obtained from bait plants grown in soil collected from the field where infected plants had been destroyed two years before, than from samples collected a year after the plant destruction. *C. acutatum*-specific band (318 bp) was amplified from bait plants grown in soil sampled both from the surface and from the depth of 5 cm. The low soil temperatures during winter might have enhanced the survival of the fungus even without debris (Eastburn and Gubler, 1992). This result of two-year survival of *C. acutatum* in soil may be caused by *C. acutatum* spores or latent infections have been present in alternate hosts. Many weed species are known to have *C. acutatum* infection with

inconspicuous symptoms (Berrie and Burgess 2003). Among the species was *Ranunculus repens* L., which is a common weed on strawberry fields in Finland.

Phytophthora cactorum

The *P. cactorum* specific primers *Pcact*SF and *Pcact*SR developed here were derived from RAMS genemarkers. They worked well with *P. cactorum* pure cultures and the results were excellent and in accordance with the recently published study by Causin et al. (2005). In that work the primers for *P. cactorum* detection were based on the sequence of RAPD generated fragment (Causin et al., 2005). The primer pair developed amplified in a simple PCR a clear band and had a good degree of specificity without cross reactions with other related *Phytopthora* species from group I, which might be a problem, or other microbes (Lacourt et al., 1997; Tooley et al., 1997; Trout et al., 1997). However, when we tried to utilize the methodology on diseased plants, problems were arising. In general, inoculated strawberry plants gave a clear band with diluted DNA as a template. In the case where no band was observed, the strawberry variety was known to be highly resistant. The main backlash in our system was that amplification was not always reproducible when DNA from birch seedlings was used. Naturally the problems we observed in this study may be due to our primer system. We however developed also another primer pair, with very similar results (unpublished). Therefore, we consider it is more probable that the problems described here were not due to our system, but more generally indicate problems associated with e.g. interactions among genotypes of cultivar and pathogen or substances inhibiting the PCR-amplification process.

Problems with PCR

One of the limiting factors when using PCR technique in routine diagnosis is the preparation of a good quality nucleic acid, free of PCR inhibitors. The failure of PCR has been suggested to be a result from the presence of phenols, polysaccharides or salts (Wilson, 1997). With *C. acutatum* a new system for the DNA extraction was used and dilution of DNA product was not necessary (Parikka and Lemmetty, 2004). With *P. cactorum* the template concentrations were mostly quite narrow. In fact, birch, as also other trees, are known to contain high amount of secondary metabolites (Tahvanainen et al., 1985; Rousi, 1990). Many of those metabolites easily co-purify with DNA, and therefore high template concentrations may inhibit PCR-reactions. This was obvious also in work by Causin et al. (2005): the detection limit of *P. cactorum* DNA decreased greatly when it was mixed with the DNA of tomato or downy oak and the negative effect of the oak DNA was greater than that of the DNA of tomato.

As molecular diagnostics are used in restricting the dispersal of alien pathogens, our results strongly underline the importance of proper testing of the detection systems. If that is not conducted, the outcome will result in poor plant health. To control pathogens like *P. cactorum* and *C. acutatum* that can be present as latent infections in plants, efficient detection systems are needed. Disease resistance is also of great importance in avoiding spread of pathogens. Our results indicate also that *C. acutatum* can survive in fallowed soil at least until the next growing season, possibly even for two years. If strawberry cultivars susceptible to *C. acutatum* are grown in a contaminated field, the plants can be infected. To ensure that there is no viable *C. acutatum* present, proper fallowing with weed control is essential during the quarantine period.

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