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Quantification of downy mildew (*Peronospora sparsa*) in *Rubus* species using real-time PCR

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Abstract

Downy mildew disease caused by *Peronospora sparsa*, also known as 'dryberry' disease, is a serious threat to the cultivation of arctic bramble (*Rubus arcticus*) and boysenberry (*Rubus* spp. hybrid). A quantitative and sensitive screening method is necessary for the breeding of downy mildew resistant cultivars and for determining efficient disease control methods. A quantitative real-time PCR method using SYBR® Green I fluorescent dye was developed for the analysis of *P. sparsa* in arctic bramble, other *Rubus* species and roses. Primers were designed to amplify a *P. sparsa* specific 94-bp fragment from the internal transcribed spacer region (ITS1) and a 140-bp fragment from a conserved region of plant 5.8S ribosomal DNA, which served as an internal control in the samples. Linear amplification from genomic DNA and control plasmids was achieved with both primers, and even 37 fg of *P. sparsa* conidial DNA was detected. In the samples collected from the field, quantities as low as 0.2 ppm of *P. sparsa* DNA in plant DNA were detected, thus enabling the diagnosis of weak and latent infections. Arctic bramble cvs Pima, Mespi and Mesma, all showing distinct foliar symptoms, were tested to assess the relative amount of downy mildew DNA present. The symptoms and the amount of *P. sparsa* DNA detected correlated only in cv. Pima, indicating that visual inspection of symptoms is not a reliable method for assessing the extent of tissue infection. The number of conidiophores of *P. sparsa* on *in vitro* inoculated leaves of two arctic bramble cultivars correlated with the results obtained by real-time PCR.

Abbreviations: bp – base pair; Ct – threshold cycle; ITS – internal transcribed spacer; rDNA – ribosomal DNA

Introduction

Oomycete plant pathogens, including genera such as *Albugo*, *Peronospora*, *Phytophthora* and *Pythium*, cause great economic losses in plant production worldwide. Downy mildews (e.g. *Bremia, Peronospora, Plasmopara, Sclerospora*) form a specialized group of oomycetes that are obligate biotrophs and usually have a narrow host range. *Peronospora sparsa* (syn. *P. rubi*) causes downy mildew disease in *Rubus* and *Rosa* species (Hall, 1989; Breese et al., 1994), resulting in the 'dryberry' disease and severe yield losses in *Rubus* plants such as blackberry (*Rubus fruticosus*), boysenberry (*Rubus* spp. hybrid) and arctic bramble (*Rubus arcticus* subspecies *arcticus*) (Tate, 1981; McKeown, 1988; Hall, 1989; Lindqvist et al., 1998).

Arctic bramble is a northern *Rubus* species cultivated mainly in Finland for its valuable berries, which are used e.g. by the liqueur industry because of their unique aroma. However, the cultivation

areas have remained small because of *P. sparsa* infections, which cause high annual fluctuations in the yield (Lindqvist et al., 1998; Kokko et al., 1999). The symptoms are the reddish, angular and interveinal lesions on the leaves, conidiophores on the abaxial surface of the leaves and, later during the season, dry berries, which occasionally become malformed. However, the visible symptoms emerge at the later stages of infection and even then different cultivars may display variable symptoms or no symptoms at all, particularly in the leaves. Downy mildew spreads in the field via asexual conidia, which are easily carried by wind or free water onto the foliage or berries where they develop secondary infections, spreading the disease rapidly over the whole field particularly under wet and cool conditions. Therefore, disease control methods need to be applied before the emergence of the infection or at the early stages when the external symptoms may not yet be visible. Some success has been achieved in rose and blackberry (*Rubus fruticosus*) (O'Neill et al., 2002), but further efforts are needed to find efficient control methods for downy mildew in *Rubus* and *Rosa* species (Aegerter et al., 2002; Walter et al., 2004), since the regular use of fungicides may lead to the development of resistant *P. sparsa* strains. Resistant plant cultivars would be the best solution to the problem.

Downy mildew is widely distributed in the wild populations of arctic bramble and cloudberry (*Rubus chamaemorus*) in Finland and Sweden (Lindqvist et al., 1998; Koponen et al., 2000). Some resistance to the disease has been observed among arctic bramble breeding lines in field studies at the University of Kuopio (unpublished), in another subspecies of arctic bramble (*R. arcticus* subsp. s*tellatus*) and in the hybrid of these two (Koponen et al., 2000). These lines might serve as starting materials in the breeding for resistance or tolerance, which is the most urgently needed trait in new arctic bramble cultivars. Sensitive quantification of *P. sparsa* is useful for the evaluation of breeding material, where quantitative differences in tolerance are likely to be found. Further, tolerance of arctic bramble clones to different *P. sparsa* isolates should be assessed, because various isolates have shown genetic variability, possibly affecting their virulence (Lindqvist-Kreuze et al., 2002). Quantification of the pathogen is also needed when its epidemiology is pursued or the efficacy of plant protection methods is being evaluated. Finally, for testing seedlings for the absence of the disease before multiplication and release to industry a sensitive method would be useful. The method would be potentially valuable in the horticultural industry for monitoring the health of stocks of roses, raspberries and blackberries especially prior to import and export with a Plant Passport.

Quantitative real-time PCR was introduced in the monitoring of plant pathogens directly from plant tissues at the end of 1990s, and has been adapted to many plant-pathogen systems since then (reviewed by e.g. Gachon et al., 2004; Schena et al., 2004). The aim of this work was to develop a specific and sensitive real-time PCR method for the relative quantification of *P. sparsa* in arctic bramble as well as in other *Rubus* and *Rosa* species. The second aim was to use the real-time PCR for the quantification of infection in arctic bramble cultivars expressing variable symptoms and in inoculated *in vitro* grown plantlets.

Materials and methods

Plant material

Arctic bramble cvs Mespi, Mesma and Pima (Mespi × Mesma) showing downy mildew symptoms, *Rubus arcticus* subsp. *stellatus, Rubus odoratus*, cloudberry clones Cloudy and Ruby, and strawberry (*Fragaria* × *ananassa*) cv. Jonsok samples were collected from the germplasm of the University of Kuopio during 2003 to 2005. Arctic bramble cvs Pima, Muuruska and Elpee were grown *in vitro*. A sample of rose (*Rosa* × *hybrida*) cv. Circus was obtained from a commercial rose garden (Tarina, Siilinjärvi, Finland) in 2005.

Isolation and maintenance of Peronospora sparsa

Peronospora sparsa used in this study was isolated from arctic bramble cv. Pima grown at the University of Kuopio in August 2005. The isolate was maintained on detached leaves of *in vitro* grown arctic bramble (Pima, Elpee) at room temperature on 0.8% water agar plates sealed with plastic film. The plates were kept on a window sill under prevailing light conditions, where new conidiophores developed within 3 to 5 days after inoculation. The

culture was refreshed at least once a month by suspending the conidia in a small amount of sterile water and applying a drop of the suspension onto the fresh leaves. The conidia were harvested for DNA extraction by centrifuging the suspension at $15{,}000 \times g$ for 10 min.

Other fungal material

Phytophthora fragariae var. *rubi* isolate 9246 was obtained from the Norwegian Crop Research Institute, and DNA isolated from *Phytophthora cactorum* isolates PH15 (from *Betula pendula*) and S7 (from strawberry) from the Finnish Forest Research Institute. *Arabidopsis thaliana* (Columbia) leaves infected with compatible and incompatible strains of *Hyaloperonospora parasitica* were obtained from the University of Fribourg (Switzerland).

Inoculation experiment

Arctic bramble cvs Pima and Muuruska were grown *in vitro* in MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg l^{-1} 6-benzylaminopurine, 0.25 mg l^{-1} indole−3-butyric acid and 2 ml l^{-1} plant preservative mixture (PPM^{TM}). The leaves were detached under sterile conditions and placed abaxial leaf surface uppermost on water agar plates. Three plates with 15 leaves on each plate were prepared from both cultivars. Fresh suspension of newly formed *P. sparsa* conidia (100 μl^{-1}) was prepared in water and 1 µl was applied on each leaflet. Conidiophores were counted under a stereomicroscope 3 and 6 days after inoculation, and 5 leaves per plate were combined, weighed and stored at −20 °C prior to DNA extraction.

DNA extraction

Larger leaf samples (0.1–2 g) were homogenized with a mortar and pestle in liquid nitrogen. Smaller samples (< 0.1 g) were homogenized with a disposable plastic pestle and sterilized sea sand (Merck) in extraction buffer in 2 ml tubes. DNA was extracted from the *in vitro* grown plantlets of arctic bramble, freeze-dried leaves of *Arabidopsis*, harvested *P. sparsa* conidia and *P. fragariae* mycelium using DNeasy Plant Minikit (Qiagen). From the plants grown outdoors or in the greenhouse, DNA was extracted using the method described by Doyle and Doyle (1990). As a modification to the method, 1% polyvinyl polypyrrolidone (PVPP) was added in the extraction buffer. Reagent volumes were adjusted according to the amount of starting material, 10 ml of extraction buffer being used per gram of leaves. Precipitated DNA was dissolved in sterile water. DNA concentration and purity were measured with a NanoDrop® ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Primer design and construction of control plasmids

Forward primer P1 (5′-CAC GTG AAC CGT ATC AAC C-3′) and reverse primer P2 (5′-GAT AGG GCT TGC CCA GTA G-3′) were designed to amplify a 94 bp fragment from the ITS1 region of *P. sparsa* (GenBank accession Y15816) showing specificity for Peronosporales. Forward primer R1 (5′-CAA ACG ACT CTC GGC AAC-3′) and reverse primer R2 (5′-CCT CGG CCT AAT GGC TT-3′) were designed to amplify a 140 bp fragment from the highly conserved region of 5.8S plant ribosomal DNA on the basis of sequences of arctic bramble and the Rosaceae. The primers, which amplify a short fragment, were designed to fulfil the requirements of successful real-time PCR better than the previously designed primers by Lindqvist et al. (1998). Sequences used for primer design were derived from GenBank (NCBI, USA) and aligned and compared using ClustalW and BLAST programmes. Applicability of the plant primers was confirmed by comparing sequences from several plant species, particularly from the Rosaceae. Specificity of *Peronospora* primers was evaluated by comparing sequences from oomycete species as well as from pathogenic and non-pathogenic fungi commonly present in *Rubus* species. The comparison performed with EMBoss Pairwise alignment tool (www.ebi.ac.uk/emboss/align/index.html) is shown in Table 1.

The ITS and 5.8S fragments were amplified for the construction of control plasmids, which were used as standards in real-time PCR to control the plate-to-plate variation. Genomic DNA from arctic bramble cv. Pima with or without *P. sparsa* was used as a template for P- and R-primers, respectively. The PCR reaction mixture consisted of 1.5 mM $MgCl_2$, 2.5 µl $10 \times$ PCR buffer, 5 mM

Table 1. The number of DNA base pairs of the P1 and P2 primers and their PCR amplicon that exactly match a range of *P. sparsa* and other fungal species found from *Rubus* plants

Species/Host	GenBank accession	Identity[a] in primer		Identity in amplicon	Gaps in amplicon
		P1	P2		
Peronospora sparsa/*Rubus arcticus*	Y15816	19/19	19/19	94/94	0
P. sparsa/*Rubus arcticus*	Y15815	19/19	19/19	93/94	0
P. sparsa/*Rosa multiflora*	AY608610	19/19	19/19	93/94	0
P. sparsa/*Rosa* spp.	AF266783	19/19	19/19	92/94	0
P. alchemillae/*Alchemilla vulgaris*	AY198303	19/19	19/19	93/94	0
Phytophthora cactorum/*Rubus idaeus*	AF266772	17/19	16/19	80/95	3
P. cactorum/*Fragaria* × *ananassa*	DQ338531	17/19	16/19	80/95	3
P. fragariae var. *rub*/*Rubus idaeus*	AF242804	19/19	15/22	72/100	9
Hyaloperonospora parasitica/*Arabidopsis thaliana*	AF241746	18/19	11/19	65/94	3
Botrytis cinerea/*Fragaria* × *ananassa*	Z73765	12/25	9/19	54/107	18
Didymella applanata/*Rubus idaeus*	AJ428534	9/22	13/25	52/118	35
Phoma spp./*Rubus arcticus*	AJ493582	9/24	13/25	55/134	40

[a] Expressed as the number of identical nucleotides in the pathogen sequence compared with the corresponding sequence of *P. sparsa*; parameters in the alignment: gap open penalty 10.0, gap extension penalty 0.5, matrix Blosum62.

dNTPs, 0.3 μM primers, 0.625 U Taq DNA polymerase (MBI Fermentas) and 1 μl of template DNA in a final volume of 25 μl. PCR was performed in a PTC-100 PCR cycler (MJ Research, Inc., Watertown, USA) with the following programme: initial denaturation at 94 °C for 2 min, followed by 35 cycles with denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s and extension at 72 °C for 45 s. The final extension was at 72 °C for 5 min. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen) and cloned into the multiple cloning site of pT-Adv plasmid with AdvanTAge^{TM} PCR Cloning Kit (Clontech Laboratories Inc.). The purified (QIAprep Spin Miniprep Kit, Qiagen) plasmids were transferred into *Escherichia coli* DH5α. The correct PCR amplification and cloning was confirmed by sequencing the inserts using Thermo Sequenase CY5 Dye Terminator Kit (Amersham Biosciences) and an automated A.L.F. express DNA sequencer (Amersham Biosciences). The plasmids were named after the primers and DNA used in their construction, i.e. P-plasmid according to P. *sparsa* and R-plasmid according to R. *arcticus*.

Real-time PCR protocol

Real-time PCR reactions were performed using FailSafe^{TM} Green Real-time PCR Kit from Epicentre Technologies. The kit contains an enzyme blend and a premix of other reagents. The PreMix K was selected among 12 available mixes for further reactions by using a special selection and optimization kit. The reaction mixture consisted of 10 μl of PreMix K, 0.4 μl of enzyme blend, 0.6 μM P or R forward and reverse primers, and 3 μl of DNA in a final volume of 20 μl. Reactions were carried out in duplicate or triplicate in separate wells of iCycler iQ^{TM} 96-well PCR plates (Bio-Rad Laboratories) with iCycler^{TM} (Bio-Rad, USA). Negative (water) and positive (plasmids or DNA known to be positive) controls were included in all analyses. The following PCR protocol was used: initial denaturation at 94 °C for 2 min followed by 38 cycles at 94 °C for 20 s, 57 °C for 20 s and 72 °C for 20 s. The final extension was at 72 °C for 5 min. The fluorescence of amplified products was measured after each extension step at 72 °C. After the final amplification cycle, melting temperature curves were obtained by decreasing the temperature stepwise (0.5 °C intervals) from 95 °C to 50 °C and recording the fluorescence at each step. The amount of fungal DNA was normalized to plant DNA using the following equation: *P. sparsa* DNA% $= 2^{\text{Ct Plant–Ct P. sparsa}} \times 100$.

The efficiency and sensitivity of the real-time PCR detection were determined for both primer pairs using calibration curves. The curves were obtained by analysing a series of 10-fold diluted genomic DNA (stock concentration 1 ng μl^{-1}) from arctic bramble cv. Elpee and *P. sparsa* conidia. Alternatively, standard curves were

constructed from the 10-fold diluted control P- and R-plasmids (stock concentration 10 ng μl^{-1}). For standard plasmids, matrix DNA was added in the reaction mixture when low concentrations were used because, in the absence of non-target DNA, not only does the formation of primer-dimers lower the sensitivity and skews the standard curves but DNA may also adhere to the plastic ware. Matrix DNA for P-plasmid was from *in vitro* grown arctic bramble (3 ng per reaction), and for R-plasmid the pT-Adv plasmid DNA without the insert (1.5 ng per reaction). Within-plate variation was tested by preparing 10 parallel reactions from one genomic DNA stock solution. Plate-to-plate variation was tested by analysing three replicates in four plates. Variation tests were performed both with arctic bramble and *P. sparsa* conidial DNA.

Quantification of symptoms

Downy mildew symptoms were measured from photographs using Assess software (The American Phytopathological Society, Minnesota, USA). The shoots were photographed from a constant distance with a digital camera before the leaves were detached and stored at −80 °C for DNA extraction. The amount of symptoms was correlated with the amount of *Peronospora sparsa* DNA in cv. Pima by analysing the Pearson correlation coefficient (SPSS 11.5, SPSS Inc., USA). *Peronospora sparsa* quantities in different cultivars and leaves were tested for statistical differences with the independent samples *t*-test (95% confidence interval).

Results

Assay quality

Plant primers were designed on the highly conserved region of 5.8S gene. Primers were tested for several *Rubus* species (*R. arcticus, R. arcticus* × *Rubus arcticus* subsp. *stellatus, R. chamaemorus, R. odoratus*), strawberry, rose and *Arabidopsis*. The primers detected all plant species tested, although *Arabidopsis* and strawberry DNA gave clearly higher Ct values (Ct 25–26) compared with *Rubus* and rose DNA (Ct 15–18). This can be explained by the difference of 2 to 3 nucleotides in the primer sequence of strawberry and *Arabidopsis*. The primers should be further tested and adjusted if they were to be used for other plants than those in the *Rubus* and *Rosa* genera, which were the primary species of interest in this work.

The specificity of *Peronospora* primers was evaluated by comparing sequences retrieved from GenBank. Primer P1 had an almost complete match to a large number of oomycete species, mainly from *Phytophthora* and *Peronospora* genera, whereas the second primer P2 was highly specific to *P. sparsa* (Table 1). All *P. sparsa* isolates had identical ITS1 sequences, where differences of only one or two nucleotides were found outside the annealing site of the primers. In addition to *P. sparsa*, various *Phytophthora* species, i.e. *P. fragariae* var. *rubi, P. cactorum* and *P. idaei*, causing root rot, have been found in *Rubus* plants, although not in arctic bramble. *Phytophthora fragariae* var. *rubi* and two isolates of *P. cactorum* gave negative results in real-time PCR under the conditions described. The primer sites of *P. idaei* and *P. fragariae* var. *fragariae* are identical to those of *P. cactorum* and *P. fragariae* var. *rubi*, respectively, and were not tested separately. True fungi have ITS sequences clearly distinct from those of oomycetes, and cannot thus be amplified with these primers. Only fungal species hosted by *Rubus* plants were evaluated here. *Hyaloperonospora parasitica* was tested with negative results as an example of other oomycete species.

Linear amplification was achieved both with R- and P-primers by using the corresponding plasmids or genomic DNA from arctic bramble and *P. sparsa* conidia as templates (Figure 1). As expected, the amplification products of arctic bramble DNA and R-plasmid and of *P. sparsa* DNA and P-plasmid were similar, as indicated by the melting curves and bands in the agarose gel (Figure 2a). The slopes of the regression lines were all close to each other and to the slope of −3.3 for optimal theoretical amplification, indicating equal and sufficient amplification efficiencies (Figure 1). Further, R^2 values above 0.980 were obtained. The within-plate variation of replicate samples was 1.2% and plate-to-plate variation was 2.5%.

DNA extracted in different days gave slightly different efficiencies (the greatest difference observed between the slopes was 0.7), which would make the use of external calibration curves difficult. Also the difference in the Ct values (6 cycles) between the same concentrations of plant and

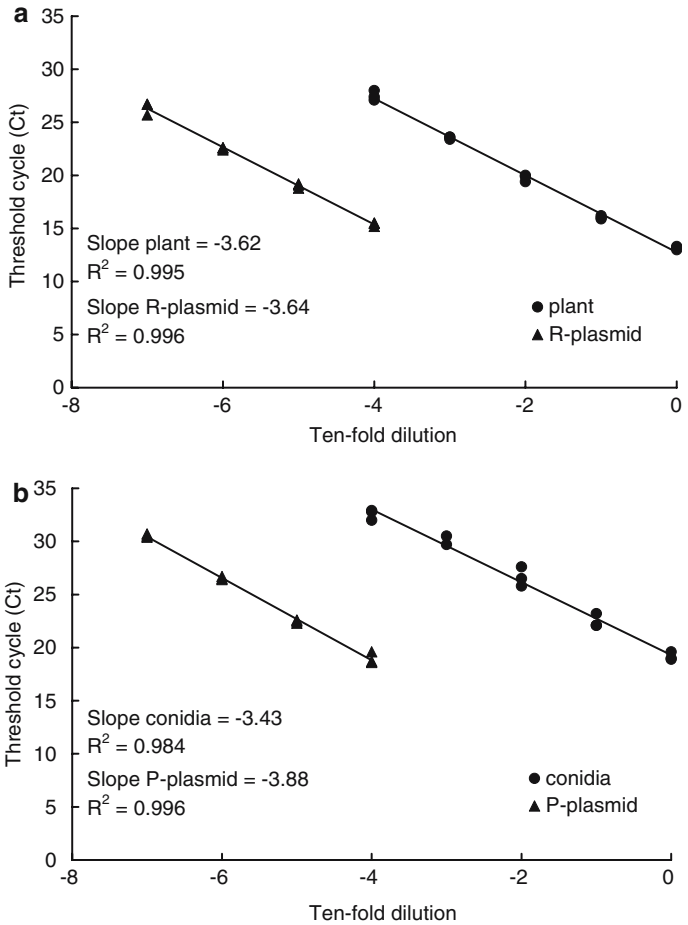

Figure 1. Comparison of the amplification of genomic DNA and the corresponding plasmid DNA. (a) DNA from arctic bramble cv. Elpee and R-plasmid amplified with R-primers and (b) DNA from *P. sparsa* conidia and P-plasmid amplified with P-primers.

conidial DNA may be due to the variation in DNA quality, or due to the fact that conidial DNA stock solution was very dilute and prone to errors in e.g. determination of the accurate DNA content (Figure 1).

Specific amplification was achieved even at the lowest dilution of *P. sparsa* DNA, i.e. 37 fg, giving Ct value of 33 with 200 pg (Ct 16-17) of arctic bramble DNA as the matrix (Figure 2b). The maximal load of total DNA was 10 ng, above which the Ct values could not be determined reliably. Among the infected arctic bramble samples, the lowest detected quantities were 0.2 ppm (0.00002%) of *P. sparsa* in plant DNA. With plasmid DNA, amounts below 1 fg (corresponding to 230 copies of ITS sequence) were easily detected. It might thus be possible to detect just a few conidia, if the ITS region has tens of repeats. Blank samples often also showed a faint, small-size band in the gel, but in the melting analysis weak fluorescence was seen only around 67 °C instead of 83 °C, indicating that the bands represent primer-dimers (Figure 2).

Quantification of P. sparsa *in arctic brambles with distinct symptoms*

Arctic bramble cultivars display different foliar symptoms, as illustrated in Figure 3. Old leaves of cv. Mesma were widely covered with reddish lesions restricted to leaf veins, whereas the youngest leaves were clean. No lesions were seen in cv. Mespi. Cultivar Pima, unlike cv. Mesma, had the strongest, typical downy mildew

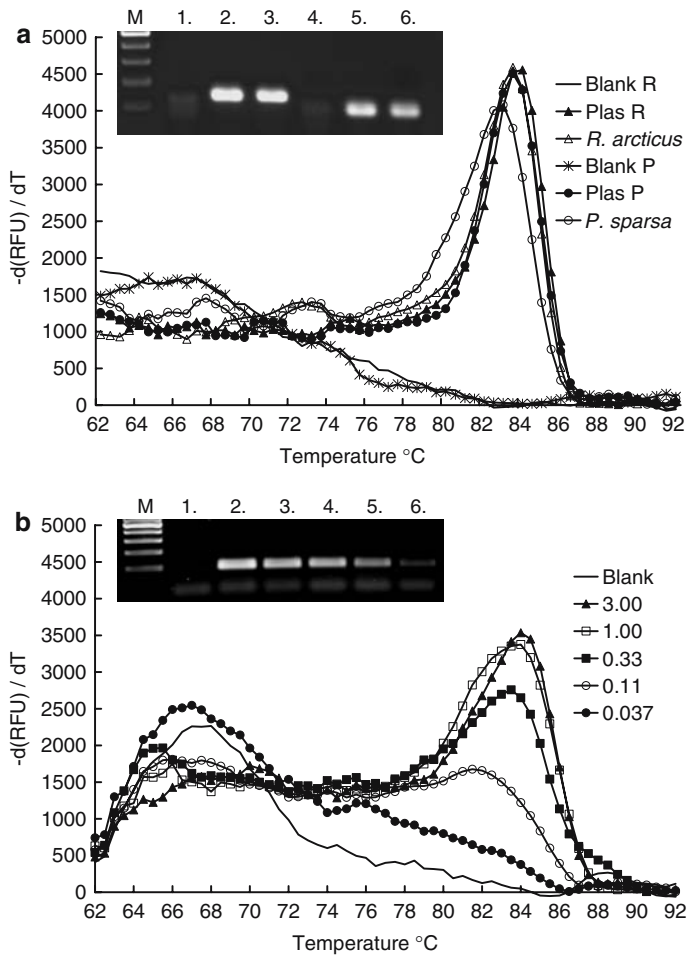

Figure 2. Agarose gel electrophoresis and melting curve analysis. (a) 1 and 4, blank; 2, control R-plasmid; 3, DNA from *R. arcticus* amplified with R-primers; 5, control P-plasmid; 6, DNA from *P. sparsa* conidia amplified with P-primers; M, molecular weight marker 100 bp. Melting curves are from the same samples. (b) 1, blank (no template); 2, 3 pg; 3, 1 pg; 4, 0.33 pg; 5, 0.11 pg; 6, 0.037 pg DNA from *P. sparsa* conidia in 200 pg of arctic bramble DNA matrix amplified with P-primers. Note the shift in the melting temperature to the left with the decreasing template concentration. Fluorescence around 67 °C is due to primer-dimers.

symptoms on the young leaves, some lesions also being found on the older leaves. The amount of *P. sparsa* was determined in the youngest and in approximately 3-week older leaves to study the correlation between the symptoms and the amount of *P. sparsa* DNA (Table 2). All samples of Mesma and Mespi gave positive reaction in PCR, but the level of *P. sparsa* DNA was very low both in young and old leaves. No significant difference was observed between young and old leaves of Mesma or Mespi. In contrast, Pima had significantly more *P. sparsa* DNA in the youngest leaves compared with the old leaves or any leaves of Mesma and Mespi. The correlation between symptom expression and *P. sparsa* DNA was significant (Pearson correlation coefficient 0.582) in Pima.

Quantification of P. sparsa *in in vitro inoculated leaves*

Table 3 shows the number of conidiophores and the amount of *P. sparsa* DNA in the inoculated leaves of cv. Pima and Muuruska 3 and 6 days after inoculation. There was practically no production of conidiophores in Muuruska, while they developed rapidly in all inoculated leaves of Pima. There was a good correlation between the number

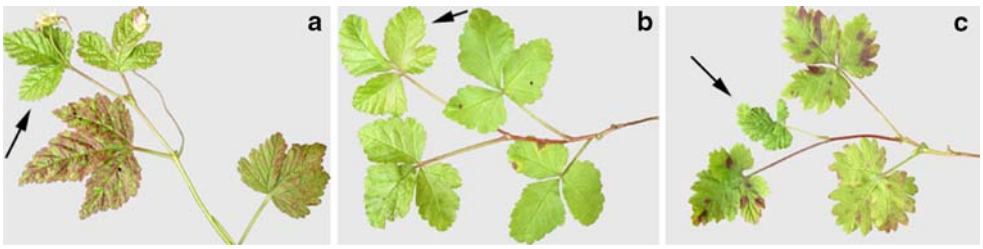

Figure 3. Symptoms on arctic bramble cvs (a) Mesma, (b) Mespi and (c) Pima. These cultivars express heavy, absent and moderate symptoms, respectively. Arrows point to the youngest leaves in the shoots.

of conidiophores and the amount of *P. sparsa* DNA quantified with real-time PCR. Three days after inoculation, the first conidiophores had developed in Pima, and the quantity of *P. sparsa* was already higher than in Muuruska after 6 days. In Muuruska, the amount of *P. sparsa* was only slightly increased from day three to day six, whereas more than 10-fold increase was seen in Pima. Based on the short cultivation experience, cv. Muuruska has shown some resistance to downy mildew in the field while cv. Pima is highly susceptible to the disease. The *in vitro* results were thus in accordance with the previous field observations.

Discussion

PCR methods based on ITS between ribosomal DNA have been developed to detect downy mildews of *Rubus* and *Rosa* species, but the published methods are not quantitative (Lindqvist et al., 1998; Williamson et al., 1998; Aegerter et al., 2002). In this study, a sensitive real-time PCR method was developed to quantify downy mildew infections in arctic bramble and other rosaceous host plants of *P. sparsa* using the relative quantification approach. In real-time PCR, Ct values can be directly related to the copy number of a target gene using an external standard curve (absolute

Table 2. The extent of visible symptoms in relation to the amount of *P. sparsa* DNA in young and old leaves of cvs Mesma, Mespi and Pima

Cultivar	Symptomatic area in young leaves[a] (%)	*P. sparsa* DNA in plant DNA (%)	Symptomatic area in old leaves[a] (%)	*P. sparsa* DNA in plant DNA (%)
Mesma[b]	0	0.0003	61.4	0.0002
	0	0.0002	63.5	0.0002
	0	0.0000	64.2	0.0001
	0	0.0001	59.7	0.0003
	0	0.0002 ± 0.0001, a[c,d]	62.2 ± 2.1	0.0002 ± 0.0001, a
Mespi	0	0.0018	0	0.0005
	0	0.0081	0	0.0187
	0	0.0004	0	0.0006
	0	0.0001	0	0.0016
	0	0.0009	0	0.0002
	0	0.0023 ± 0.0033, a[c,d]	0	0.0043 ± 0.0081, a
Pima	8.8	0.9184	7.6	0.0106
	16.9	0.7289	2.5	0.0047
	4.2	0.4187	0.7	0.0058
	4.3	0.3100	0.7	0.0258
	10.6	1.1842	4.3	0.0466
	9.0 ± 5.2	0.7120 ± 0.3583, b	3.2 ± 2.9	0.0187 ± 0.0177, a

[a] Young leaves are the youngest, top leaves; old leaves are the third leaves from the top.
[b] Young and old leaves are not necessarily from the same plant in each row in cv. Mesma.
[c] Mean ± standard deviation.
[d] Means followed by different letters were significantly different at $P < 0.05$ (T-test).

Table 3. Quantification of *P. sparsa* in the leaves of Pima and Muuruska grown *in vitro*

Sample	Number of conidiophores per leaf	*P. sparsa* DNA in plant DNA (%)
Pima 3 days[a]	0.3 ± 0.5	0.31 ± 0.26[b]
Pima 6 days	45.0 ± 7.9	4.89 ± 1.46
Muuruska 3 days	0	0.05 ± 0.03
Muuruska 6 days	0.1 ± 0.2	0.08 ± 0.03

[a] Days after inoculation.
[b] Mean ± standard deviation of three replicates.

quantification) or, alternatively, related to another gene present in the sample by measuring the difference between the threshold cycles of the two genes (relative quantification) (Livak and Schmittgen, 2001). In plant-pathogen systems, it is convenient to derive the ratio of fungal DNA to host plant DNA in each sample (Winton et al., 2001; Gachon and Saindrenan, 2004).

Plant R-primers designed in this study amplified the 5.8S sequence from several *Rubus* plants and rose, and a weaker amplification was seen in strawberry and *Arabidopsis*. Our focus was on the known hosts of *P. sparsa*, but the primers are likely to be applicable to a much wider variety of plants, since the target sequence is highly conserved among plants. The P-primers designed to amplify the ITS1 fragment of *P. sparsa* were found specific to this pathogen. Based on sequence comparison, *P. alchemillae* is highly similar to *P. sparsa* and these species, presently classified separately (Voglmayr, 2003), may actually be a single species as was previously discovered for *P. sparsa* and *P. rubi* (Breese et al., 1994). Among other fungal species found in *Rubus*, only *Phytophthora idaei*, *P. cactorum* and *P. fragariae* were considered putative targets for amplification based on sequence analysis. However, no amplification was observed with these *Phytophthora* species tested.

Sensitive, reproducible, and efficient amplification was achieved both with *Peronospora* and plant primers, enabling the quantification of low amounts of fungal DNA together with host plant DNA. The sensitivity of the method is high enough to detect 37 fg of conidial DNA, which is approximately one-tenth of what was detected with conventional PCR (250 fg) previously developed for *P. sparsa* by Lindqvist et al. (1998). However, with low amounts of target DNA (Ct above 30), it is advisable to check the melting curve, since primer-dimers may sometimes give Ct values around 33 cycles in blank samples. It should also be recognized that the melting temperature and, particularly, intensity slightly change with the dilution of the template (see also Ririe et al., 1997). Similarly, poor quality of template DNA may change the melting curves to resemble those obtained with a low amount of target DNA due to ineffective amplification. This may be a problem if the DNA is extracted from old leaves, since high amounts of secondary metabolites, such as tannins, interfere with the PCR. Day-to-day variation in the DNA quality was found to affect amplification efficiencies, but the copy number of rDNA repeats may also vary in different organisms, resulting in different Ct values even with equal loading and quality of the DNA sample. The variation among sample replicates was acceptable (1–3%) but seemed to increase near the detection limit, which has also been observed by others (Filion et al., 2003). For the reasons mentioned above, the relative quantification method was found preferable over external calibration, since the variation in amplification efficiency, DNA quality and day-to-day performance are all eliminated in the normalization process. Additionally, variation in DNA loading is less critical and there is no need to run complete calibration curves for each plate, thus saving time and reagents.

Commercial cultivation of arctic bramble is based on two main cultivars, Pima and Mespi, the latter being planted mainly for cross-pollination purposes of this self-incompatible species. Large-scale cultivation has been hampered by the fact that both cultivars are susceptible to downy mildew, producing dry berries in rainy seasons. However, typical foliar symptoms such as those detected in boysenberry and blackberry, are only expressed in cv. Pima (McKeown, 1988; Hall, 1989; Lindqvist et al., 1998; Kokko et al., 1999). In this work, more information was obtained about the correlation between symptom expression and the extent of infection using the real-time PCR method developed.

Despite their distinctive foliar symptoms, cvs Pima, Mespi and Mesma were all positive in PCR, indicating the presence of *P. sparsa*. A clear correlation between symptom expression and *P. sparsa* content was only found in cv. Pima; the

level of infection did not differ between cvs Mesma and Mespi regardless of their different foliar symptoms. The disease pressure was similar for both Pima and Mespi growing in a mixed culture, suggesting that Mespi may be more resistant to downy mildew than Pima. The faint positive PCR results in cvs Mespi and Mesma may be, at least partly, caused by secondary conidia dispersed from neighbouring Pima plants even though the sampled plant would otherwise be uninfected. In Pima, the strongest symptoms and largest quantities of *P. sparsa* DNA were found in the youngest leaves, which may be more susceptible to the secondary conidia. According to these results, the youngest leaves are the most reliable targets for screening of downy mildew to reveal potentially infected plants at the early stages of the disease in the field. Similarly, young leaves have been found more susceptible to downy mildew in grapevine (Reuveni, 1998). On the other hand, the reddish lesions detected on cv. Mesma may not be caused solely by *P. sparsa* but also by another stress, since *P. sparsa* was barely quantifiable. In grapevine, downy mildew resistance is expressed as the production of necrotic lesions (Pezet et al., 2004), which could also explain the low level of infection in cv. Mesma. Whether the variation in *P. sparsa* strains influences symptom expression is an interesting question that cannot be answered without more detailed studies.

Preliminary field experience suggests that cv. Muuruska is at least somewhat tolerant to downy mildew, which is in line with the results obtained here from the inoculation experiment performed on *in vitro* grown plantlets. The results from microscopy and real-time PCR were also similar, confirming the applicability of the PCR method for *P. sparsa* quantification. The correlation between the *in vitro* results and field observations suggests that breeding material and cultivars might be evaluated with the plate assay before performing larger scale field experiments. However, more studies are needed before the *in vitro* assay can be considered as a reliable tool to detect downy mildew resistant arctic brambles. One reason is that in *in vitro* plantlets, the composition of leaf surface and defence reactions are not similar to those of outdoor grown plants. Leaf disc screening of downy mildew resistance has previously been established for other plants (Brown et al., 1999; Agnola et al., 2003).

In conclusion, the real-time PCR method described here was shown to be quantitative, sensitive and reproducible. Low amounts of *Peronospora* DNA could be quantified in field-grown arctic bramble as well as in *in vitro* inoculated plantlets. The method has the potential to be utilized as a novel tool in the breeding for resistance of several *Rubus* and *Rosa* species and in the evaluation of disease control methods and pathogen virulence.

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