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Quantitative analysis of phytopathogenic ascomycota on leaves of pedunculate oaks (*Quercus robur* L.) by real-time PCR

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Abstract

Leaves of oak trees are often infected by various pathogenic fungi. As it is difficult to remove such organisms quantitatively from the leaf surface and as it is often impossible to grow these organisms independently from their host, there are almost no molecular data available from these oak leaf specific pathogens. For the quantitative removal of the microorganisms a procedure was developed combining a wax and microorganism freezing method with a DNA extraction technique. For the development of a species specific detection, DNA of pathogenic filamentous fungi was isolated from hyphae of the upper leaf surface of *Quercus robur*. Three different species could be identified as (i) *Cladosporium* sp., (ii) *Ramularia* sp. and (iii) *Microsphaera alphitoides* by amplifying and sequencing an 18S–28S segment of their rDNA. For the final quantification a real-time PCR protocol was established allowing the species specific quantification of the three pathogenic filamentous fungi. The whole procedure was successfully applied to quantify the amount of the three species on oak leaves collected in autumn.

Keywords: Powdery mildew; Real-time polymerase chain reaction; *Quercus robur*; *Cladosporium*; *Ramularia*; *Microsphaera alphitoides*

1. Introduction

Pedunculate oaks (*Quercus robur* L.) are widespread forest trees in middle Europe. Oaks are often infected by a series of parasites. Especially on the leaf one can find a community of epiphytic pathogenic fungi and bacteria which can cause serious damage to their host leaves often accompanied by a reduction of the net assimilation rate and the total leaf chlorophyll content [1]. One of the most popular pathogenic organisms of oak leaves causing a serious fungal disease is the powdery mildew (*Microsphaera alphitoides*) [2,3]. There are some works describing the influence of moisture and fluctuating temperature or previous insect attacks on the leaves on the invasion rate of oak leaves by phytopathogenic organisms like the oak specific powdery mildew [1,4–6]. These investigations often lack a quantification and a species identification. This is due to the fact that pathogenic organisms as the powdery mildew are anchored in the epidermis by haustoria. Therefore they cannot be quantitatively removed from the leaf just by leaf washing which is often used to isolate other epiphytic microorganisms [7]. As in addition pathogenic microorganisms often do not grow in culture separated from the host tissue [3], a realistic quantification is quite difficult. Therefore the aim of the present work was to develop a sensitive method to quantify the degree of infection of oak leaves by phytopathogenic fungus species excluding any growth steps for these organisms.

2. Materials and methods

2.1. Amplifying and cloning of fungal rDNA segments

In order to amplify segments of fungal rDNA regions between 18S and 28S rDNA that cover the internally transcribed spacer sequences ITS1 and ITS2 and the 5.8 sRNA, the oligonucleotide primers o-its1 (5′ TCC GTA GGT GAA CCT GCG G 3′), o-its4 (5′ TCC TCC GCT TAT TGA TAT GC 3′), o-asc-for (5′ ATT AC(AC) GAG (CT)G(CT) GAG GCT CAG TCG 3′) and o-asc-rev (5′ CTA CCT GA(AT) TCG AGG TCA ACC TGT G 3′) were used. DNA for the PCR reactions was obtained by

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scratching hyphae from oak leaves collected at the end of October 2000, transferring the hyphae to 100 μl of sterile water and boiling the sample for 10 min. 1 μl of this sample was used for the PCR which contained in a final volume 50 μl of one of the forward/reverse primer pairs, 3.5 mM $MgCl_2$, dNTPs each 0.2 mM, 5 μl 10×PCR buffer (Life Technologies, Karlsruhe, Germany) and 1 U *Taq* DNA polymerase (Life Technologies). PCR was performed in 36 cycles (30 s 94°C, 30 s 48°C and 120 s 72°C). After verification of the size of the amplification products by agarose gel electrophoresis, 1 μl of the assays containing segments of the expected size was ligated into pCRII-Topo (Invitrogen BV, Groningen, The Netherlands) and cloned into *Escherichia coli* 10F′ (Invitrogen BV).

2.2. DNA sequencing

After purification of the fungal rDNA harbouring plasmid DNA by the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany), cycle sequencing dideoxy chain-termination reactions with Big Dye Terminators (PE Applied Biosystems, Weiterstadt, Germany) were performed for both cDNA strands, using universal forward and backward primers (Life Technologies, Karlsruhe, Germany). The sequences were analysed using an ABI Prism 310 System (PE Applied Biosystems). Sequences were submitted to the EMBL data base (accession Nos. AJ417495, AJ417496 and AJ417497).

2.3. Isolation of DNA from oak leaves

To be able to quantify the loss of DNA during the procedure, 10^5 copies of the plasmid pCR*myrS* carrying the gene of a myrcene synthase of *Quercus ilex* [8] were added as an internal standard to each leaf directly before DNA isolation. The upper surface of the leaf was frozen on sterile water in a metal dish cooling it by liquid nitrogen. After removing the leaf, all microorganisms and the wax layers of the leaf remained on the ice surface. This wax layer isolation procedure was first described by Dr. C. Neinhuis [9], improved by Dr. R. Jetter [10,11] and modified by Dr. L. Schreiber (Bonn, Germany). To isolate the DNA, the part of the ice was scratched off where the leaf was initially frozen and was transferred to a centrifugation tube. To minimize the loss of DNA of the microorganisms at the walls of the vials 1 μg of herring sperm DNA was added. DNA was extracted from the microorganisms incubating the tube for 5 min at 60°C in the presence of 1% SDS. After two times extraction by phenol/chloroform/isoamyl alcohol (25:24:1) the upper DNA containing phase was transferred to a new vessel. After two successive DNA precipitations in 70% ethanol and 0.5 M sodium acetate (pH 5.5) the DNA was dried and resuspended in 0.1 ml sterile water for quantification by real-time PCR.

2.4. Real-time PCR

In a final volume of 50 μl the assays contained 25 μl 2×SYBR-Green® PCR Master Mix (PE Biosystems, Weiterstadt, Germany), 300 nM of each primer and 5 μl of the leaf DNA sample. After a hot start (10 min, 95°C) 40 cycles were performed with a 15 s melting step at 95°C and a 1 min annealing/extension step at 60°C on a GeneAmp® 5700 Sequence Detection System (PE Biosystems). For the species specific amplification the oligonucleotide pairs o-clado-for (5′ GCG CGC CTC AAA GTC TCC 3′) with o-clado-rev (5′ TGA AAG ATT TAA CGG CCT CGA 3′), o-ramu-for (5′ ATC ATT ACT GAG TTA GGG AGC AAT CC 3′) with o-ramu-rev (5′ GGG GAA TGC CGT CGA AAC 3′) and o-micro-for (5′ CGC TGC TCC GCA AGG AC 3′) with o-micro-rev (5′ GCG CTC CAG CCG AAA CC 3′) were designed.

3. Results and discussion

A series of epiphytic pathogenic fungi and bacteria living on the leaf surface infect the leaves of oaks and often cause serious damage to the leaves, which reduces the net assimilation rate of this important forest tree. For instance the filamentous fungus powdery mildew (*M. alphitoides*) is known to cause a serious disease on pedunculate oak leaves [2,3]. As there are almost no sequence data of epiphytic pathogenic filamentous fungi of oak leaves available up to now, the scope of the present work was to develop a highly sensitive method based on molecular techniques allowing to distinguish the pathogenic fungus species and to quantify the degree of infection of oak leaves by these fungi even in early stages of invasion.

3.1. Isolation and cloning of rDNA sequences of filamentous pathogenic fungi from oak leaves

In order to obtain species specific sequence information of pathogenic oak leaf specific filamentous fungi, two oligonucleotide primers (o-asc-for, o-asc-rev) were designed to amplify the rDNA region between 18S rDNA and 28S rDNA on the bases of the conserved regions at the end of the 18S rDNA and the beginning of the 28S rDNA of gene bank available ascomycota rDNA sequences (see Section 2). Two additional oligonucleotide primers for these regions (o-its1, o-its4) were kindly supplied by Dr. G. Bahnweg (GSF, Neuherberg, Germany). In case of the combination of the oligonucleotide pair o-its1/o-asc-rev amplification products of the expected size of up to 600 bp were obtained by the use of heat denatured hyphae from *Q. robur* leaves (Fig. 1). The segments of the size from 500 to 600 bp were ligated into pCRII-Topo, and transformed into *E. coli* 10F′.

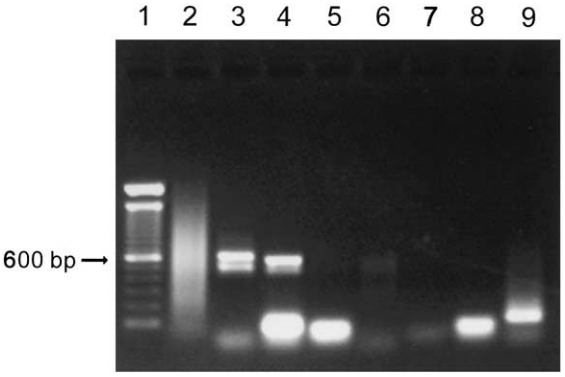

Fig. 1. PCR amplification of the rDNA region between 18S and 28S rDNA. Lanes: 1, 100-bp standard (Gibco BRL), 2–9, PCR assays with hyphae DNA from oak leaves and the following primer combinations: 2, o-its1/o-its2; 3, o-its1/o-asc-rev; 4, o-asc-for/o-asc-rev; 5, o-asc-for/o-its4; 6, only o-its1; 7, only o-its4; 8, only o-asc-for; 9, only o-asc-rev. 10 μl of the PCR assays were loaded on an 1.2% agarose gel.

3.2. Identification of rDNA sequences of pathogenic filamentous fungi

Sequencing of the plasmid inserts and a gene bank comparison led to the identification of three pathogenic filamentous ascomycota. One of the isolated rDNA sequences (Ascomyc1) with a size of 506 bp (EMBL accession No. AJ417495) was related to species of the genus *Cladosporium* (Table 1A), which belongs to the family of mitosporic Mycosphaerellaceae. These filamentous fungi are known to be epiphytic pathogens on leaves of various plants. However, as the distance in the matrix to the next related species available in the EMBL DNA library is more than 2 times higher (Table 1A, 47–58 exchanges) than the distances between the different *Cladosporium* species (Table 1A, 1–22 exchanges), it is probable that the species isolated from oak leaves belongs to another still unknown pathogenic genus of the Mycosphaerellaceae. The second isolated rDNA (Ascomyc2) of 495 bp (accession No. AJ417496) was most closely related to *Ramularia collo-cygni* (Table 1B, 19 exchanges), which also belongs to the mitosporic Mycosphaerellaceae and causes as epiphytic phytopathogenic fungus for instance the brown spots on barley leaves. The next related sequences derived from *Mycosphaerella* or other *Ramularia* species (Table 1B). The isolate of the oak leaf is probably a closely related variety of the barley specific *R. collo-cygni*. The third isolated rDNA segment (Ascomyc3) of 605 bp (accession No. AJ417496) was completely identical to *M. alphitoides* and *Microsphaera wallrothii* (Table 1C), which are powdery mildew of the Erysiphaceae described to be isolated from the leaves of *Q. robur* and of *Vaccinium hirtum*, respectively. This completely identical sequence of the isolated Ascomyc3 and the two sequences available in the gene bank (*M. alphitoides* and *M. wallrothii*) suggests that either *M. alphitoides* and *M. wallrothii* are one identical species which can infect two host plants, *Quercus* and *Vaccinium*, or that possible species specific differences in

Table 1
Distance matrices of the isolated rDNA sequences and related rDNA sequences[a]

(A) EMBL accession No./species	Ascomyc1	AJ300336	AF393703	AF393698	AF393711	L25432	AF393724
AJ417495 Ascomyc1	–	0.091	0.103	0.105	0.109	0.109	0.110
AJ300336 *Cladosporium* sp.	47	–	0.029	0.031	0.035	0.043	0.037
AF393703 *Cladosporium herbarum*	53	15	–	0.002	0.006	0.014	0.023
AF393698 *Cladosporium echinulatum*	54	16	1	–	0.004	0.016	0.025
AF393711 *Cladosporium macrocarpum*	56	18	3	2	–	0.019	0.029
L25432 *Cladosporium oxysporum*	56	22	7	8	10	–	0.037
(B) EMBL accession No./species	Ascomyc2	AF173310	AF297235	AF173312	AF222848	AF309601	AF222839
AJ417496 Ascomyc2	–	0.039	0.041	0.043	0.043	0.098	0.100
AF173310 *Ramularia collo-cygni*	19	–	0.027	0.029	0.031	0.096	0.086
AF297235 *Mycosphaerella fragariae*	20	13	–	0.025	0.039	0.091	0.089
AF173312 *M. fragariae*	21	14	12	–	0.055	0.094	0.087
AF222848 *Ramularia* sp. KC1	21	15	19	27	–	0.088	0.089
AF309601 *Mycosphaerella keniensis*	48	47	44	46	43	–	0.086
AF222839 *Mycosphaerella crystallina*	49	42	43	42	43	42	–
(C) EMBL accession No./species	Ascomyc3	AF298538	AB015930	AB015915	AF011302	AF073348	AB015920
AJ417497 Ascomyc3	–	0.000	0.000	0.002	0.023	0.023	0.026
AF298538 *Microsphaera alphitoides*	0	–	0.000	0.002	0.023	0.023	0.026
AB015930 *Microsphaera wallrothii*	0	0	–	0.002	0.023	0.023	0.026
AB015915 *Microsphaera pseudolonicerae*	1	1	1	–	0.024	0.024	0.028
AF011302 *Erysiphe liriodendri*	13	13	13	14	–	0.038	0.031
AF073348 *Erysiphe pisi*	13	13	13	14	22	–	0.035
AB015920 *Microsphaera syringae-japonicae*	15	15	15	16	18	20	–

[a]The distance matrices were calculated by the program PAUP 3.0 on the basis of a CLUSTAL W vers. 1.8 alignment of the most closely related sequences of the EMBL DNA library. Values below the diagonal are the absolute numbers of base exchanges, the values above the diagonal are relative distances.

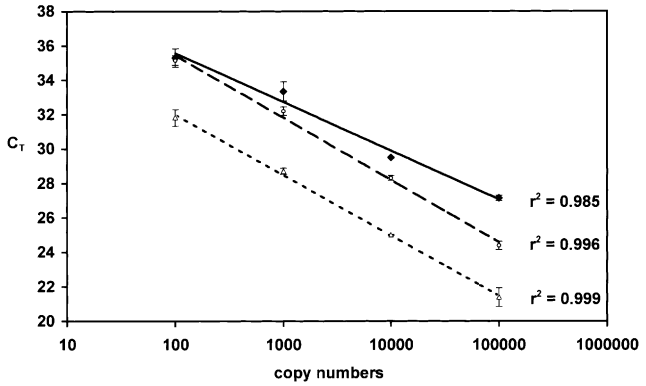

Fig. 2. Species specific standardization of the real-time PCR by assaying samples with known plasmid copy numbers. The real-time PCR was performed in the presence of SYBR Green (PE Biosystems) as fluorescence DNA staining dye using DNA from hyphae of oak leaves and the primer pair o-clado-for/o-clado-rev for the detection of the *Cladosporium* like oak leaf isolate (△), o-ramu-for/o-ramu-rev for the detection of the *R. collo-cygni* related isolate (○) and o-micro-for/o-micro-rev for the detection of the *M. alphitoides* isolate (◆). The C_T value corresponds to the PCR cycle at which the fluorescence signal rose above the background fluorescence threshold. Each value was determined in three independent assays.

the sequences of *M. wallrothii* and *M. alphitoides* are localized outside of the sequenced region. This assumption is the more probable one, as *M. alphitoides* is known to be species selective even in the genus *Quercus* where *Quercus petrea* and *Q. robur* can be infected but *Quercus rubra* and *Quercus cerris* are resistant [12]. As the isolate ascomyc3 was isolated from *Q. robur* and the sequence is identical to *M. alphitoides* it was deposited in the EMBL sequence library as a *M. alphitoides* sequence.

3.3. Establishment of a real-time PCR quantification for selected filamentous fungi

In order to identify the three pathogenic epiphytic fungi in samples of oak leaves, new species specific oligonucleotides o-clado-for/o-clado-rev, o-ramu-for/o-ramu-rev and o-micro-for/o-micro-rev were designed on the bases of the isolated *Cladosporium* (Ascomyc1), *Ramularia* (Ascomyc2) and *Microsphaera* (Ascomyc3) sequences, respectively (see Section 2). The oligonucleotides were chosen in a way to allow a species specific amplification of rDNA segments of about 100 bp for the real-time PCR at an annealing temperature of 60°C. To standardize the real-time PCR quantifications the plasmids containing the rDNA segments were diluted into the PCR assays to obtain final plasmid copy numbers from 1000 to 1 000 000 copies per assay. For each of the specific primer combinations this was done in three parallels with the DNA isolated from the hyphae from oak leaves. After the real-time PCR run, for each vial the cycle number (C_T value) was determined at which the fluorescence signal rose above a generally fixed noisy fluorescence background value and entered into the exponential phase (threshold value). Thus, plotting the C_T values against the copy numbers of the plasmids in the assays led to a standardization line, specifically for the primer pair used (Fig. 2). For each of the primer pairs a strong dependence of the C_T values on the logarithm of the copy number was obtained (Fig. 2) with high correlation values ($r^2 = 0.99$ for each primer pair). Therefore the copy numbers of the three species specific rDNA segments could now be quantified in each DNA sample using these standardization functions.

3.4. Establishment of a quantitative isolation method for epiphytic leaf microorganisms

In addition to a precise quantification of the DNA copies of species specific rDNA segments, a reproducible quantitative isolation method for the microorganisms on the leaf surface was necessary. With this purpose a freezing method was adopted to the leaves of *Q. robur* (see Section 2). The efficiency of the freezing isolation method was compared to a washing procedure with sodium chloride at a visibly infected *Q. robur* leaf (collected at 26 September 2001) by staining the remaining microorganisms with DAPI (4,6-diamidino-2-phenylindole) and detecting them by confocal laser scanning microscopy (Fig. 3). Whereas there were still remaining microorganisms visible after the leaf-washing procedure, there were almost no microorganisms left after the freezing procedure (Fig.

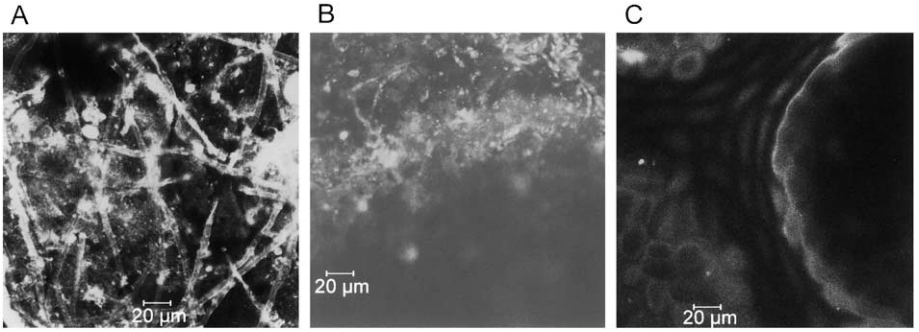

Fig. 3. Microscopic comparison of the efficiency of the freezing technique and the washing method in removing microorganisms from the leaf surface. Organisms on the pedunculate oak leaf surface were coloured by DAPI. The micrographs were taken after irrigation at 351 nm at an emission wavelength >470 nm. (A) Untreated leaf, (B) leaf after washing procedure, (C) leaf after applying the freezing technique.

3C). Therefore this procedure was chosen for further investigations.

3.5. Quantification of the amount of selected pathogenic fungi on Q. robur leaves

For the extraction of the DNA from the isolated microorganisms of the leaf surface a heat mediated (60°C) DNA isolation procedure was coupled to the freezing procedure using SDS as a detergent (see Section 2). To be able to calculate the DNA recovery during the whole procedure a defined amount of a standard DNA (100 000 copies of pCR*myrS* [8]) was added prior to freezing of the leaf surface, and the copy number of the standard DNA was quantified in an independent real-time PCR run after the complete DNA isolation. On the bases of this procedure the numbers of *Cladosporium* sp. (Ascomyc1 copies), *Ramularia* sp. (Ascomyc2 copies) and *M. alphitoides* (Ascomyc3 copies) genome copies were determined from 10 leaves collected at 26 September 2001 (Table 1). All the three pathogenic fungi were reproducibly detected on the leaves (Table 1). Whereas *Cladosporium* and *Ramularia* had low copy numbers ($(3.2 \pm 0.9) \times 10^6$ copies per m^2 and $(6.8 \pm 1.9) \times 10^6$ copies per m^2, respectively), the powdery oak mildew *M. alphitoides* had almost invaded the whole leaves with the high number of $(1.2 \pm 0.8) \times 10^{12}$ copies per m^2. The high copy number corroborated with the microscope view of the untreated leaf (Fig. 3A) where the surface was almost completely covered by hyphae. The result proved that the complete procedure can be used to quantify the degree of infection of oak leaves species specifically as well as quantitatively.

3.6. Concluding remarks

In the current work a powerful tool has been developed to isolate and quantify pathogenic fungi living on the surface of pedunculate oak leaves. The procedure enables a quantitative isolation and detection of the organisms and completely overcomes the problem that such organisms are often biotroph. It will allow to study the invasion of the leaf in dependence of environmental factors even in early stages of infection, which is a prerequisite to predict the development of a leaf concerning its health during the vegetation period in process based models used e.g. to calculate the emission of reactive volatile organic compounds from the leaves into the atmosphere [13,14].

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