Suitability of Quantitative Real-Time PCR To Estimate the Biomass of Fungal Root Endophytes^{∇†}

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A nested single-copy locus-based quantitative PCR (qPCR) assay and a multicopy locus-based qPCR assay were developed to estimate endophytic biomass of fungal root symbionts belonging to the *Phialocephala fortinii* sensu lato-*Acephala applanata* species complex (PAC). Both assays were suitable for estimation of endophytic biomass, but the nested assay was more sensitive and specific for PAC. For mycelia grown in liquid cultures, the correlation between dry weight and DNA amount was strong and statistically significant for all three examined strains, allowing accurate prediction of fungal biomass by qPCR. For mycelia colonizing cellophane or Norway spruce roots, correlation between biomass estimated by qPCR and microscopy was strain dependent and was affected by the abundance of microsclerotia. Fungal biomass estimated by qPCR and microscopy correlated well for one strain with poor microsclerotia formation but not for two strains with high microsclerotia formation. The accuracy of qPCR measurement is constrained by the variability of cell volumes, while the accuracy of microscopy can be hampered by overlapping fungal structures and lack of specificity for PAC. Nevertheless, qPCR is preferable because it is highly specific for PAC and less time-consuming than quantification by microscopy. There is currently no better method than qPCR-based quantification using calibration curves obtained from pure mycelia to predict PAC biomass in substrates. In this study, the DNA amount of *A. applanata* extracted from 15 mm of Norway spruce fine root segments (mean diameter, 610 μ m) varied between 0.3 and 45.5 ng, which corresponds to a PAC biomass of 5.1 ± 4.5 μ g (estimate $\pm$ 95% prediction interval) and 418 ± 264 μ g.

Interactions between fungi and plants are very common in nature and range from mutualistic to pathogenic (41). The outcome of a plant-fungus interaction largely depends on the extent of colonization by the fungus, independent of whether pathogenic or mutualistic fungal species are involved (6, 8). This is also true for endophytic species. In contrast to infections by pathogenic fungi, where disease symptoms are expressed after a comparatively short period of incubation, infection by endophytic fungi does not cause disease symptoms for prolonged periods, because once inside the tissue, endophytes assume a quiescent state either for the whole lifetime of the infected plant tissue or until the host is adversely affected by the arrival of biotic or abiotic stress (34, 38, 42, 45). Therefore, switching endophyte behavior from neutral to pathogenic or mutualistic can depend on the predisposition of the host tissue, environmental factors, and the extent of colonization. For instance, in conifer needles, the biomass of endophytic *Rhizodactylus parkeri* thalli increase over time (44). It has been postulated that the needles die as soon as the endophyte's biomass exceeds a certain threshold value (42). Therefore, attainment of the threshold usually coincides with natural senescence. However, the threshold can either be lowered or reached prematurely if host resistance is reduced by adverse factors. Thus, the health status of plants depends on the density of colonization by the endophyte, and vice versa.

Estimation of the extent of colonization is difficult, and there are certain conditions that must be met for techniques to determine fungal biomass. First, they should reproducibly combine target (i.e., species/genotype) specificity with accurate quantification of biomass. Traditionally, microscopy has been used to measure hyphal length or proportional colonization of host tissue (4, 28, 30). However, determination of fungal biomass by microscopy is very laborious, and results vary between investigators. Moreover, visual quantification is unspecific, as species designation is often difficult or impossible. Chemical methods measuring the amount of specific biomolecules stored inside fungal cells or released into the environment (e.g., the fatty acid ergosterol or the carbohydrate chitin) are also widely used (13, 49). Although these methods are much less laborious than microscopy, they are nonspecific and problems can arise if used for field samples (33), and the minimum sample size required is comparatively high (14, 31). Real-time quantitative PCR (qPCR) (23, 43) combines specificity at different taxonomic levels with accurate measurement of DNA copy number and allows quantification of DNA in very small samples. Different qPCR chemistries (TaqMan, SYBR green, or molecular beacons) and methods are available (22, 32). The choice of the locus used for qPCR assays largely depends on the aim of the study. While multicopy genes allow the detection of lower DNA amounts, single-copy genes give more precise measurements of DNA copy number, as the number of repeats of multicopy loci can differ between strains and even within a single individual strain (7, 24). In addition, sensitivity of qPCR can be increased by applying a nested approach, where the entire locus is initially amplified by conventional PCR, and the resulting product is then quantified with the specific primer-probe combination in a second step (11, 39). Diagnostic qPCR

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assays have been used for early and rapid detection of plant pathogens in the environment and in diseased tissues (9, 32, 50). However, little investigation has been done into the usefulness of qPCR to estimate fungal biomass, and considerable disagreement exists. For example, qPCR biomass estimates of multinucleate arbuscular mycorrhizal fungi (AMF) correlated only poorly with estimates obtained by visual assessment (12). This was proposed to be due to the multinucleate nature of *Glomeromycota*. On the other hand, a correlation between qPCR and mycelial dry weight was demonstrated for the Swiss needle cast-causing parasite *Phaeocryptopus gaeumannii* (52) and between qPCR and hyphal length for the ectomycorrhizal fungus (EMF) *Piloderma croceum* (36). Partial correspondence between ergosterol assays and qPCR has been demonstrated in needles infected with *P. gaeumannii* (51), and a high correlation between qPCR and ergosterol was found in tissues colonized by the conifer root pathogen *Heterobasidion annosum* (25).

In the present study we tested the suitability of qPCR to estimate the biomass of a common group of ascomycetous root endophytes. Members of the *Phialocephala fortinii* sensu lato-*Acephala applanata* species complex (PAC) (17, 46) are ubiquitous root symbionts in woody plants, especially in conifers and heathland shrubs, where they are the most prominent endophytes (1). PAC form loose networks of hyphae running mostly parallel to the root axis on the root surface but also grow inter- and intracellularly within the root cortex (47). Inside cortical cells and under certain culture conditions, PAC species can form microsclerotia, which are tight complexes of more or less isodiametric, to irregular, thick-walled cells that can endure harsh conditions and may therefore serve as resting spores and units of dispersal (2, 37, 53). The ecological role of PAC members is still controversial, despite several studies (21). The effects of PAC on their hosts were described as being pathogenic in some studies but beneficial in others. This variability in behavior was mainly due to the use of different, undefined isolates and a multitude of experimental designs which either favored PAC members or the host plants (21). Recently, isolates characterized by specific molecular markers have become available, making PAC-host interaction studies more meaningful.

In this study we aimed (i) to develop a specific qPCR method that allows detection of all PAC members, (ii) to test the method's suitability for biomass estimation in three different experimental systems *in vitro* (liquid fungal cultures, cellophane culture, and colonized roots of *Picea abies* seedlings) by using biomass estimates obtained by microscopy as reference, and (iii) to compare the reproducibility, sensitivity, and specificity of a nested single-copy qPCR assay and a multicopy qPCR assay.

MATERIALS AND METHODS

Mycelia were grown (i) in liquid cultures, (ii) in and on cellophane, and (iii) as endophytic thalli in roots of *in vitro*-grown Norway spruce (*Picea abies*) seedlings. One strain each of three PAC species was used: *A. applanata* (T1_58_1), *P. fortinii* sensu stricto (7_45_5), and *P. subalpina* (6_16_1) (17).

Mycelium preparation in liquid cultures. Strains were inoculated on terramycine-malt agar (TMA; 15 g liter⁻¹ malt extract [Hefe Schweiz AG, Stettfurt, Switzerland], 20 g liter⁻¹ agar, 50 mg liter⁻¹ terramycine) and incubated at 20°C in the dark. After 1 week, one colonized agar plug (diameter, 4 mm) from the margin of the growing colony was transferred to 50 ml 2% malt broth (20 g liter⁻¹ malt extract) in a 100-ml Erlenmeyer flask and incubated at 20°C on a

shaker at 100 rpm. After 3 weeks, the mycelium was harvested, washed twice with sterile water, and lyophilized. Twelve weight samples per strain were taken, ranging from 1 to 40 mg.

Cellophane system. Inocula were prepared on TMA as described above. After 10 days, five petri dishes per strain containing water agar overlaid with autoclaved cellophane were inoculated with a colonized agar plug from the growing margin of the colony and incubated at 20°C for 24 days in the dark. Nine 10-mm-long and 5-mm-wide pieces of cellophane were cut from the margin of the growing colony on each petri dish. Three pieces were pooled to represent one replicate for further analyses, resulting in a total of 15 replicates per strain. Each piece was cut in two squares of 5 by 5 mm, and one of each was randomly assigned to quantification by either microscopy or qPCR (see below).

In planta system. Mycelia were produced in malt extract broth as described above. Thalli were blotted dry on a sieve and weighed. The mycelium-to-water ratio was adjusted to 90 g liter⁻¹ (fresh weight) with sterile nanopure water. Thalli were homogenized with a blender for 30 s. Fifty-milliliter Falcon tubes containing a sterile 1:1 (vol/vol) vermiculite-peat mixture were inoculated with 2 ml of the homogenized PAC mycelia and incubated for 3 weeks at 20°C. Two-week-old, sterile spruce seedlings were planted in the tubes. Seedlings had been produced from surface-sterilized seeds. Surface sterilization occurred by immersion in 30% H₂O₂ for 30 min, followed by rinsing with sterile nanopure water. Germination occurred on water agar at 18°C in the dark. After planting into the Falcon tubes, plants were transferred to a phytotron (16-h day [120 to 140 μ E m⁻² s⁻¹]/8-h night; temperature, 22°C/10°C; relative humidity [rH], 45%/85%). Plants were watered every other day with 3 to 4 ml deionized water and fertilized every third to fourth week with 3 to 4 ml of a 0.2% (vol/vol) dilution of a complete fertilizer (Wuxal, Maag, Switzerland). Six plants per strain were harvested after 5 months. The shoot was cut off, and roots were washed under running tap water and dissected. Three 4-cm-long root pieces were excised from each root system, and lateral roots were removed. Each 4-cm-long root piece, constituting a replicate, was further subdivided into eight 0.5-cm-long segments. The outermost segments were surface sterilized (1 min in 30% H₂O₂, 10 s in 98% ethanol) and incubated on TMA to reisolate the inoculated fungal strain to accomplish Koch's postulates. From the six remaining segments, every other segment was assigned to qPCR and two of the three remaining ones to microscopic quantification. The three segments assigned to qPCR were pooled and considered one replicate. Correspondingly, the two segments assigned to microscopic quantification were considered one replicate.

Microscopic quantification of fungal biomass in cellophane and roots. Cellophane squares were stained with 0.5% trypan blue in lactophenol at 50°C overnight, mounted in 90% lactic acid on microscope slides, and analyzed with an Axiophot microscope (Zeiss, Switzerland). Pictures were taken with an AxioCam MRc5 camera at 200 $\times$ magnification, and images were acquired with the AxioVision (4.6.3.0) software. Five pictures were taken per cellophane square (one in each corner at a distance of 0.8 mm from both abutting sides and one in the center of the square, resulting in an analyzed area of 43,625 μ m², i.e., 0.17% of the total square area).

The extended focus option of the AxioVision software was used to visualize the mycelia at various depths in the cellophane in the same image: starting on the uppermost layer where hyphae grew, a picture was taken every 2 μ m, down to the hyphae that penetrated deepest into the cellophane, resulting in 3 to 15 layers. Light intensity, exposure time, and brightness were optimized to enhance contrast. Mean hyphal width of each strain was determined and used to categorize the width ranges for single hyphae ($n = 1$) and hyphal strands consisting of >1 hyphae. Lengths were measured for each category with the root analysis software WinRhizo (v2007d; Regent Instrument Inc., Canada) and multiplied by the corresponding n to obtain total hyphal length. Microsclerotia were characterized by parallel chains of short, wide cells. The width of these microsclerotial chains was several times that of single hyphae. Thus, the "length" of microsclerotia could be determined in the same way as that of hyphal strands.

For microscopy, root segments were destained in 10% KOH at 50°C overnight and at room temperature for one more day. Then, the segments were acidified in 2% HCl for 2 min, washed twice with nanopure water, and preserved in 90% lactic acid. The total length of the hyphae (l_{tot}) colonizing every individual root segment was estimated using a modified version of the line intersection method according to Newman (30) (see Method S1 in the supplemental material). Per root segment, the number of intersections ($n_{int,i}$) of the hairline of the ocular lens with hyphae was counted along $i = 6$ transects, and the length of each transect ($l_{trans,i}$) was measured. The silhouette area (A_{tot}) was estimated based on $l_{trans,i}$. Hyphal length was calculated according to the formula $l_{tot} = \pi/2 \times A_{tot} \times \sum n_{int,i} / \sum l_{trans,i}$. In the analyses l_{tot} of both root segments was summed up to represent one replicate. Moreover, the width of the microsclerotia ($l_{micro,i}$) along each transect line and the number of cells of the microsclerotia ($n_{micro,i}$) inter-

A) pPF-076:

DQ013117 CAACCGATTACAGACCACTGAA [331 bp] CGGATAGCTTCGCTGTGAATCG
P. subalpina [331 bp]
P. fortinii s.s. [331 bp]
A. applanata [331 bp]

DQ013117 CTTTGTTGACGTACAGGATGCTCCCTCTGGCATCTGAGACGGAGAAGCTCC
P. subalpina
P. fortinii s.s.
A. applanata

DQ013117 TTGAAAGATCTGCGT [102 bp] TTGTTCCATTGCTGCAGATTC
P. subalpina [101 bp]
P. fortinii s.s. [101 bp]
A. applanata [101 bp]

B) ITS:

AY033087 CGTGTTCATACATACTATTGTTGCTTTGGCGGGCCGTGGCCTCCACTGCGGG
P. subalpina
P. fortinii s.s.
A. applanata
 AY033087 CTCTGCTCGTGTGTCGCCGCCAGAGA
P. subalpina
P. fortinii s.s.
A. applanata

FIG. 1. Partial sequence alignments of the pPF-076 locus (A) and the ITS locus (B). Primers are underlined, the TaqMan probe is underlined and in bold, and deletions are marked as a dash.

secting with the transect line were measured per root segment. These measurements were used to estimate microsclerotial biomass.

Development of qPCR. A nested single-copy qPCR assay and a one-step multicopy qPCR assay were developed, with both based on TaqMan chemistry. The single-copy, noncoding PAC-specific locus pPF-076 was chosen for the nested assay (16, 18, 19). All available pPF-076 sequences of PAC strains were aligned, and the most conserved DNA region was selected to design specific primers and a probe using the Primer Express 3.0 software (Applied Biosystems, Switzerland). In the first step, a portion (561 bp) of the locus pPF-076 was amplified with the external primers pPF-076_F1 and pPF-076_R1 (15) in a conventional PCR. In the second step, the PCR product was subjected to the qPCR quantification step, using a specific pPF-076 qPCR primer-probe combination. Specificity of primers was tested by PCR amplification of DNA of PAC-free host plants and potential fungal contaminants in plant roots (see Table S2 in the supplemental material).

The multicopy gene assay was based on the internal transcribed spacer (ITS) sequence of the rRNA locus. Primers and probe for qPCR were developed as described above. However, DNA was quantified in a single step, without the preceding conventional PCR step.

DNA extraction and qPCR conditions. A few grains of DNA-free (dried 4 h at 180°C) silica sand were added to the cellophane and root samples to facilitate later disruption of cellulose and plant cell walls. Then, samples were frozen at -80°C and freeze-dried for 2 days. Lyophilized mycelia, colonized cellophane squares, and colonized fine root segments were homogenized using a bead mill. DNA was extracted with the DNeasy plant minikit (Qiagen, Basel, Switzerland) following the protocol of Grünig et al. (18). For the cellophane and root samples the protocol was adjusted for small sample sizes: only 250 µl lysis buffer and 3 µl RNase A were added to the ground tissue, and there was only one washing step with 500 µl chloroform-isoamyl alcohol (1:24). Extraction efficiency was estimated by extracting DNA from blank cellophane and PAC-free root samples spiked with known amounts of PAC DNA.

In step 1, PCRs were performed in a total volume of 25 µl containing 5 µl 1:25-diluted sample DNA, 2.5 µl 10× PCR buffer (GE, Switzerland), 200 µM deoxynucleoside triphosphates, 0.5 units of *Taq* polymerase (GE, Switzerland), and 500 nM each external primer. PCR amplification conditions were as follows: one cycle at 94°C for 2 min; 15 cycles at 94°C for 30 s, 60°C for 45 s, and 72°C for 30 s; and a final cycle at 72°C for 6 min. In step 2, reactions were performed in a total volume of 25 µl containing 5 µl of the PCR product of step 1, 12.5 µl of 2× reaction buffer (qPCR MasterMix Plus Low ROX; Eurogentec, Belgium), 600 nM each primer, and 150 nM probe. Optimum primer and probe concentrations had been determined previously by varying primer and probe concentrations. PCR amplification conditions consisted of a denaturation step at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. Fluorescence was monitored during the 60°C phase. As a standard, a serial dilution of genomic DNA with known concentrations was added to each sample plate. Each sample was run in triplicate. Reaction conditions and the setup of the qPCR step of the multicopy gene assay were exactly the same as for step 2 of the single-copy gene assay.

FIG. 2. Regression of C_T values against the logarithm of the DNA amount of serially diluted DNA. Triangles, *A. applanata*; circles, *P. fortinii* s.s.; squares, *P. subalpina*. Filled symbols represent the single-copy assay, and open symbols show the multicopy assay results.

Statistical analyses. All statistical analyses were performed with the R statistical package (35). A *t* test was used to test whether the slope of the regression line resulting from regression of the threshold cycle (C_T) values of the multicopy assay on the C_T values of the single-copy assay deviated significantly from 1. A full factorial analysis of variance (ANOVA) was applied to test relationships between the C_T value, strain, weight, or length of the mycelia and the area covered by microsclerotia. Only noncorrelated parameters were integrated in the models, to avoid inflation of the ANOVA. Weight, length, and microsclerotial parameters were log transformed to achieve homogeneity of variances. The residuals of each model were analyzed to detect violations of the normality assumption and points with high leverages.

RESULTS

Development of qPCR. Based on the sequence data of the pPF-076 locus, forward primer pPF-076_qPCR F (5'-CGGATAGCTTCGCTGTGAATC-3'), reverse primer pPF-076_qPCR R (5'-ACGAGATCTTTCAAGGAGCTT-3'), and fluorogenic probe pPF-076_qPCR P (5'-CTTTGTTGACGTACAGGATGCTCCCTCTG-3') were designed, yielding PCR products of 88 bp. The probe was labeled at the 5' end with the fluorescent reporter dye Yakima Yellow and at the 3' end with the quencher Black Hole Quencher 1 (BHQ1) (Fig. 1A). Correspondingly, forward and reverse primers of the ITS locus PF-ITS_qPCR F (5'-CGTGTTCATACATACTATTGTTGCTTGG-3') and PF-ITS_qPCR R (5'-TCTCTGGCGGGCACACA-3') and the probe PF-ITS_qPCR P (5'-CCGTGGCCTCCACTGCGGG-3') were used, yielding a product of 76 bp. The probe was labeled with the fluorescent reporter dye 6-carboxyfluorescein at the 5' end and with BHQ1 at the 3' end (Fig. 1B).

The efficiency ($E = 10^{(-1/\text{slope})} - 1$) determined with a serial dilution of genomic DNA was similar for each individual qPCR assay but differed significantly for the *A. applanata* strain in the pPF-076 assay (Fig. 2). The correlation between C_T values or DNA amounts of the single-copy and the multicopy assay was generally high but varied between different calibration methods, with DNA extracted from cellophane having the worst correlations (Table 1). The slope of the regression line of the regression of the C_T values of the multicopy assay on the C_T

TABLE 1. Correlations between qPCR measurements obtained using the single-copy (pPF-076) and the multicopy (ITS) gene markers

Strain	Pearson's correlation coefficient ^a			
	Genomic DNA	Liquid culture	Cellophane cuttings	Root segments
<i>A. applanata</i>	0.996***	0.993***	0.685**	0.955***
<i>P. fortinii</i> sensu stricto	0.995***	0.994***	0.484	0.955***
<i>P. subalpina</i>	0.998***	0.997***	0.726**	0.932***
Overall result	0.988***	0.963***	0.741***	0.889***

^a Correlations for genomic DNA were between C_T values from the two assays; otherwise, correlations were between DNA amounts determined with the two assays. Significance levels are indicated as follows: **, $P < 0.01$; ***, $P < 0.001$.

values of the single-copy assay did not deviate significantly from 1 ($t_{13} = 0.329$; $P = 0.374$), indicating that both assays were equally efficient. DNA extraction efficiency was about 70% for both cellophane and root samples. The single-copy assay was highly specific for PAC DNA. Even small amounts of

PAC DNA could be detected, and no product was obtained from non-PAC DNA (see Table S2 and Fig. S3 in the supplemental material). In contrast, the multicopy assay also amplified DNA of non-PAC strains, i.e., *Acephala* sp. strain 1 and *Phaeomollisia picea*.

Liquid cultures. The relationships between mycelium weight ($F_{1,26} = 560.20$; $P < 0.001$), strain ($F_{2,26} = 28.50$; $P < 0.001$), and the DNA amount as measured by single-copy qPCR were statistically significant in the global test with all three PAC strains. The DNA amount was regressed against mycelial dry weight for every strain individually, because strain had a significant but differential effect on the relationship between DNA amount and mycelial dry weight (Fig. 3A). The 95% prediction interval was narrow, indicating that DNA amount is a good predictor of fungal biomass. For a DNA amount of 1,000 ng, the width of the 95% prediction interval for mycelial dry weight was 7.8 mg for *A. applanata*, 13.0 mg for *P. fortinii* sensu stricto, and 37.5 mg for *P. subalpina*, indicating that the accuracy of the biomass prediction based on the DNA amount

FIG. 3. DNA amounts as measured by single-copy qPCR regressed on dry weight of mycelia grown in liquid cultures (A), hyphal length of mycelia grown on cellophane (B), and hyphal length measured in *P. abies* roots (C) (log scale axes). Dashed lines indicate the upper and lower 95% prediction intervals. In each graph the linear regression of y (the log[DNA amount]) against x (the log[weight or hyphal length]), r^2 , and P values are indicated.

FIG. 4. Fungal biomass estimates received from cellophane cultures. (A) DNA amounts as measured by single-copy qPCR; (B) hyphal length as measured with WinRhizo. Each box represents measurements from one petri dish, and the dotted lines indicate the means for each PAC strain.

was best in *A. applanata* and worst in *P. subalpina*. Correlation between DNA amounts estimated by the single-copy assay and the multicopy assay was high (Table 1), and the biomass estimates obtained with the multicopy assay were equivalent to those obtained with the single-copy assay. The width of the 95% prediction interval for mycelial weight was 8.8 mg for *A. applanata*, 6.7 mg for *P. fortinii* sensu stricto, and 18.6 mg for *P. subalpina*.

Cellophane system. In the cellophane system, the relationships between hyphal length ($F_{1,39} = 12.21$; $P = 0.001$), strain ($F_{2,39} = 14.77$; $P < 0.001$), and DNA amount were significant for the single-copy assay. Due to the significant strain effect, each strain was analyzed individually. The relationship between hyphal length and DNA amount was significant for *A. applanata*, and *P. fortinii* sensu stricto but not for *P. subalpina* (Fig. 3B). DNA amount and hyphal lengths varied considerably among cellophane squares within and among petri dishes (Fig. 4). The correlation between DNA amounts estimated with the single-copy and the multicopy assay was much lower than with the other test systems, and the correlation was not significant for *P. fortinii* sensu stricto (Table 1). The relationship between hyphal length and DNA amount was significant for *A. applanata* ($R^2 = 0.546$, $F_{1,13} = 15.65$; $P = 0.002$) but not for *P. fortinii* sensu stricto ($R^2 = 0.065$, $F_{1,13} = 0.91$; $P = 0.357$) and *P. subalpina* ($R^2 = 0.243$, $F_{1,12} = 3.85$; $P = 0.074$) in the multicopy assay.

In planta system. The PAC strains could be reisolated from the roots of every plant, confirming successful colonization. *A. applanata* was reisolated from 30 of 36 root segments, *P. fortinii* sensu stricto from all 36 root segments, and *P. subalpina* from 32 of 36 root segments. All microsclerotial parameters correlated highly with each other ($R^2 \geq 0.956$; $P < 0.001$). Therefore, only one parameter was selected and included in the model to prevent inflation. We selected the sum of the mean

silhouette area covered by microsclerotia of the two root segments originating from the same 4-cm-long root piece, because the fit of the ANOVAs was best when this parameter was used (data not shown). The area covered by microsclerotia per root segment has been defined as $s_a = 1/6 \times A_{tot} \times \sum(l_{micro,i}/l_{trans,i})$. In the global model, hyphal length ($F_{1,42} = 11.08$; $P = 0.002$) and strain ($F_{2,42} = 8.22$; $P < 0.001$) had significant influences on the DNA amount. In *A. applanata*, hyphal length ($F_{1,14} = 33.79$; $P < 0.001$), the area covered by microsclerotia ($F_{1,14} = 10.66$; $P = 0.006$), and the interaction between them ($F_{1,14} = 7.64$; $P = 0.015$) had significant influences on the DNA amount. For *P. fortinii* sensu stricto, only the interaction between hyphal length and area covered by microsclerotia was significant ($F_{1,13} = 7.83$; $P = 0.015$). As for the mycelia grown in and on cellophane, there was no fit of the model at all for the *P. subalpina* strain ($R^2 = 0.072$, $F_{3,14} = 0.36$; $P = 0.782$), and residual analysis revealed no possibilities to improve the model. When the DNA amount was regressed against hyphal length, ignoring microsclerotia, the model of *A. applanata* was significant, whereas the models of the other two strains were not (Fig. 3C). DNA amount, hyphal length, and area covered with microsclerotia varied strongly among individual plants (Fig. 5). The correlation between DNA amounts estimated with the single-copy assay and the multicopy assay was high (Table 1), and the biomass estimates obtained with the multicopy assay were equivalent to those obtained with the single-copy assay.

DISCUSSION

Real-time qPCR was introduced in fungal ecology and pathology to detect and quantify fungi in natural samples and to investigate host-fungus interactions. The method has been

FIG. 5. Fungal biomass estimates obtained from colonized roots. (A) DNA amounts as measured by single-copy qPCR; (B) hyphal length as measured by the Newman method; (C) area covered by microsclerotia. Each box represents measurements from one plant, and the dotted lines indicate the means for each PAC strain.

used to estimate the biomass of plant pathogenic fungi in colonized plant tissues (8, 9, 32), EMF growing in artificial medium and in seedlings (26, 36, 40), and AMF in plant roots (9, 12). However, the usefulness of qPCR and comparability with other methods to quantify fungal biomass in environmental samples has sometimes been controversial (12, 26). In the present study, the biomass of nonmycorrhizal fungal root symbionts was estimated by real-time qPCR and compared with estimates obtained by traditional visual inspection methods. In addition, two qPCR assays (single-copy locus versus multicopy locus) were compared.

Comparison of single-copy and multicopy qPCR assays and determination of specificities of both assays for PAC. The efficiencies of different qPCR assays must be equal to allow comparison. This was shown to be the case for the two assays tested (Fig. 2). qPCR measurements correlated well, except with DNA of *P. fortinii* sensu stricto extracted from cellophane (Table 1). Nonetheless, the C_T values of the single-copy qPCR were 6 to 10 times lower than those of the multicopy assay, indicating that the sensitivity of the single-copy qPCR was 100 to 1,000 times higher. Although nested qPCR assays are widely

used (10, 39), the additional PCR run increases the risk of cross-contamination and handling errors. However, the highly significant correlation between DNA amounts estimated with the single-copy and the multicopy assay showed that this risk was low for biomass estimation of mycelium grown in liquid cultures and endophytic mycelium in plant roots. There are several reasons for the low correlation between DNA amount estimates obtained with the two assays for DNA extracted from the cellophane samples: first, the density of mycelia was lower in cellophane than in roots or pure, freeze-dried mycelium, and therefore the DNA amount was probably at the detection limit in the multicopy assay. Second, cellophane is a glucose polymer and polysaccharides are known to hamper DNA extraction and PCR amplification (5, 48). Therefore, qPCR measurements of the single-copy assay are probably more accurate due to dilution of PCR inhibitors after the first amplification step.

Specificity for the target microorganism(s) is one of the strengths of the qPCR method, allowing detection and quantification of microorganisms in semisterile microcosms or in the field (32, 39, 43, 50). One aim was to develop a qPCR assay

specific on the PAC level to study the nature (mutualism/antagonism) of PAC-plant interactions *in vitro*. Species specificity was not essential, as the assay should allow quantification of strains of several PAC species. Specificity was tested by amplifying DNA from the host, frequent fungal contaminants, and close relatives of PAC (20, 29). While none of these control templates was amplified using the single-copy assay, the multicopy qPCR resulted in the amplification of DNA of close relatives of PAC (see Table S2 in the supplemental material). As some of these taxa are also known to colonize plant roots (20), it is possible that the multicopy assay will overestimate PAC DNA in semisterile or environmental samples. Therefore, due to the higher sensitivity and higher specificity, the nested qPCR assay is superior to the multicopy approach to quantify PAC DNA in natural samples.

Suitability of qPCR to measure biomass of pure fungal mycelium. The best measurement for fungal biomass is mycelial dry weight. The strong linear relationship between mycelial dry weight and qPCR estimates of biomass demonstrates that qPCR is a reliable method for PAC biomass estimation and that the regression lines are suitable as calibration curves (Fig. 3A). Similarly, a strong correlation between mycelial dry weight and qPCR was found for the ascomycete *Phaeocryptopus gaeumannii* (52). The PAC strain had a small but significant effect on measurements of DNA amount (Fig. 3A). A likely explanation for this is that the number of nuclei per unit of mycelium differs slightly among strains due to differences in cell size. Indeed, differences in growth response and cell lengths among PAC strains have been documented in previous studies (1, 2).

Correspondence of qPCR and microscopy in determining biomass of fungal mycelia growing in and on organic substrates. The weight of mycelia growing in and/or on organic substrates cannot be measured directly, as it is impossible to properly separate substrate and mycelia. Thus, indirect methods must be used to estimate fungal biomass. Biomass was measured in cellophane and in the *in planta* system by using microscopy and qPCR. Strength of correlation between microscopic measurements and qPCR differed strongly among the three PAC strains and between the two substrates, and correlations were lower than those obtained for mycelia grown in liquid cultures. The weak correlations between estimates obtained by qPCR and microscopy in the cellophane and the *in planta* system result from the weaknesses of either method. Nonetheless, a significant relationship between hyphal length and qPCR was found for *A. applanata* in both systems (Fig. 3B and C). Similarly, hyphal length correlated positively with the number of ITS loci of *Piloderma croceum* grown in agar on microscope slides (36).

The main reason for the weak correlation of the biomass estimates obtained by qPCR and microscopy for the other two PAC strains seems to be the presence of microsclerotia. It is technically demanding to extract DNA from microsclerotia, which form tight complexes of thick-walled cells (27). Therefore, proper homogenization of the sample is crucial. This was attempted by the addition of a few grains of silica sand. Ground root samples were shown to be properly homogenized, and incompletely crushed root fragments were rarely observed. Therefore, incomplete homogenization of samples was not the reason for the lack of congruence between estimates obtained

by qPCR and microscopy. In addition to variable cell dimensions, the number of nuclei per cell can be a source of variation of qPCR estimates. However, hyphae and microsclerotia of PAC are consistently monokaryotic according to our observations and information from R. L. Peterson (personal communication). Thus, differing numbers of nuclei per cell can be excluded as a source of variation.

On the other hand, fungal biomass estimates using light microscopy can be hindered by (i) increases in erroneous counts of hyphae and microsclerotial cells due to increasing colonization density, (ii) underestimation of microsclerotial biomass with increasing size of microsclerotia, and (iii) limited visibility of (hyaline) fungal structures. (i) Since the pictures taken at various depths in the cellophane were merged into a single picture, WinRhizo was not able to distinguish overlapping hyphae or microsclerotia. Similarly, some overlapping hyphae and microsclerotia in roots escaped counting, although fungal structures were examined on several focal planes separately. The denser the colonization, the more often fungal structures overlapped or were overlooked. (ii) Similarly, the difficulty in accurately measuring the vertical dimensions of microsclerotia (i.e., the extension of microsclerotia parallel to the optical path) increases with increasing size, density, and frequency of these structures. (iii) Hyphae of PAC are hyaline when young and thus may escape observation in both cellophane and roots, leading to an underestimation of fungal biomass. In addition, intercellular hyphae often grow parallel to host cell walls, rendering accurate counting difficult (53). Another source of error in the microscopic quantification of roots may be due to false positives (3). Since the seedlings were grown under semisterile conditions, colonization by non-PAC species occurred, albeit rarely (as confirmed by reisolation). Some of these species produced melanized mycelia (e.g., *Cladosporium* sp., *Virgariella* sp.) and could erroneously be considered PAC. However, the potential for false positives was minimized, as the PAC strains were inoculated in the substrate under axenic conditions before the seedlings were planted and grown in the phytotron. Furthermore, colonization of roots by non-PAC fungi is expected to be more important in plants only weakly colonized by PAC, due to less competition for infection sites. This was, however, not the case in our study, as the correlation between qPCR and microscopic biomass estimates was strongest for *A. applanata*, which formed less dense endophytic hyphal and microsclerotial networks than the other two strains (Fig. 5B). Finally, line intersect sampling was used to estimate fungal biomass in roots. This method is adequate for estimation of parameters of needle-shaped objects such as hyphae, but microsclerotia in roots can assume almost any shape and thus are less suitable for estimation by this method.

It is impossible to compare qPCR and visual inspection with regard to the accuracy of fungal biomass estimates, because there is no reference for the "true" amount of fungal biomass in cellophane and in roots. However, the specificity of qPCR is high and time consumption is low in contrast to visual methods. In addition, estimates of the biomass of pure mycelium obtained by qPCR were highly accurate, as indicated by narrow 95% prediction intervals (Fig. 3A). For example, the DNA amount from samples of three pooled 5-mm-long root segments (mean diameter, ~610 μ m) colonized by *A. applanata* varied between 0.3 and 45.5 ng (Fig. 3C). The upper and lower

limits of the 95% prediction interval of biomass for a DNA amount of 0.3 ng were 0.6 μg and 9.7 μg , and for a DNA amount of 45.5 ng they were 154 μg and 683 μg based on the calibration curve obtained from pure mycelium grown in liquid culture (Fig. 3A). Thus, there is currently no better method to predict PAC biomass in and on substrates than qPCR-based quantification with the calibration curves obtained from pure mycelium grown in liquid culture. The method can also be used to detect PAC and quantify fungal biomass as the DNA amount in field samples. Although significant strain differences were detected, estimated values were of the same order of magnitude, because differences in C_T values among strains were about 1 (Fig. 2). However, to gain insight into more complex interactions of PAC with their hosts in nature, primer-probe combinations would have to be developed to specifically detect single PAC strains or species.

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