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Real-time PCR mediated monitoring of *Fusarium foetens* in symptomatic and non-symptomatic hosts

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Abstract *Fusarium foetens* is a recently described aggressive vascular pathogen of *Begonia* x *hiemalis*. Since 2004, it has caused severe losses for *Begonia* growers in Northern Europe and North America. *F. foetens* is likely to be of exotic origin. Little is known about the accumulation of the fungus in *Begonia* plants before and during symptom expression and about its host range. We have optimised a molecular detection method for *F. foetens* by only using the plant part containing the largest amount of the pathogen and by optimising the tissue maceration and DNA extraction techniques. This allowed a reliable detection limit of 2310 spore equivalents per plant and a theoretical detection limit of as low as 84 to 167 spore equivalents per plant. Using this method, we demonstrated exponential accumulation of *F. foetens* DNA in *Begonia* roots, resulting in symptoms at a threshold of approximately 10^7 spore equivalents and levelling off at 10^9 spore equivalents per plant. The observed rate of accumulation and the amount of pathogen DNA in non-symptomatic plants can be combined to determine whether the cuttings were infected after delivery at the *Begonia* nursery and to calculate the estimated timing of symptom development. To test the host range, we applied the optimised molecular detection technique. During these tests, only *Begonia* x *hiemalis* plants became symptomatic, but many other plant species supported growth of the pathogen. This information can be used to aid pathogen control and has implications for pest risk assessment.

Keywords *Begonia* x *hiemalis* · Disease development · Host range · Latent infection · Wilt

Abbreviations

dpi	Days post inoculation
FSM	Fusarium selective medium
GE Kit	GenElute plant genomic DNA kit
ISP Kit	Invisorb spin plant mini kit
PDA/B	Potato dextrose agar/broth
QP (+ MC) Kit	Quick pick SML pant DNA kit (+ MagaCharc beads)
wpi	Weeks post inoculation

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Introduction

Fusarium foetens is a recently described pathogen of *Begonia* x *hiemalis* (*Begonia elatior* hybrids) that

causes an aggressive vascular disease (Schroers et al. 2004). The first symptoms are retarded growth and chlorosis. These symptoms are followed by wilting and dry rot of the stems. Ultimately, the stems collapse and a white mycelium, often with spore formation, appears on the leaves and stems (Schroers et al. 2004). Symptom development due to *F. foetens* has only been observed on *B.* x *hiemalis*, but it is possible that other plant species, such as *Cyclamen persicum* and *Saintpaulia ionantha* are latent carriers of the disease, based on recovery of the pathogen from symptomless plants (Schroers et al. 2004).

The origin of *F. foetens* is unknown, but given its growth optimum of 25–28°C (Schroers et al. 2004) and the tropical origin of wild *Begonia*, an origin in a high-temperature climate is suspected. It thus seems to be an introduced pathogen of exotic origin. Introduction via latently infected cuttings is suspected to be the main pathway of introduction (Baayen et al. 2002; Schroers et al. 2004). In greenhouses, secondary spread via the irrigation water, as in ebb and flow irrigation systems, has been demonstrated (Baayen et al. 2002; Elmer 2008), suggesting an infection pathway via the roots. By now, the pathogen has already been reported in a wide range of northern countries, such as the Netherlands (2000), the United Kingdom (2001), Germany (2001), the USA (2003), France (2005), Belgium (2006), Norway (2007) and Canada (2010) (Baayen et al. 2002; Elmer and Vossbrinck 2004; Anonymous 2007; Jones 2002; Tian et al. 2010). Although initially the disease caused significant losses in the main production areas, its impact is currently controlled via monitoring of plants for planting, strict sanitation and hygiene, and the use of less susceptible cultivars. Nevertheless, new findings still occur, often creating large problems for growers who are not familiar with the problem or who do not take the necessary sanitation measures.

F. foetens is difficult to distinguish morphologically from other fungi in the *Fusarium oxysporum* complex. Due to the time-consuming nature of identification with traditional plating methods, de Weerdt et al. (2006) designed PCR and real-time PCR detection methods based on the elongation factor 1α and the mitochondrial small subunit sequences, respectively. Despite having validated their methods using only isolates from the Netherlands and the UK, RAPD and sequence analyses indicate no genetic diversity within these or other, more recent *F. foetens* isolates from Canada, Germany and the USA (Tian et al. 2010; Tschöpe et al. 2007; Elmer and Vossbrinck 2004). These data indicate the specificity of the methods developed by de Weerdt et al. (2006); however, their methods were described for small plant samples (100 mg) only and the limit of detection was not defined. *B.* x *hiemalis* cultivars are usually grown from rooted or non-rooted cuttings provided by companies specialised in the production of propagation material. Given the danger for introduction of the pathogen via such cuttings or possibly via latently infected plant species other than *B.* x *hiemalis*, which may occur at very low concentrations (Baayen et al. 2002), it is essential to be able to detect very small amounts of the fungus in plant samples of a more practical size.

Real-time PCR, also known as qPCR, is a reliable, very sensitive and fast method to detect a fungal plant pathogen even at very low concentrations in plant samples. It can also be used to monitor fungal growth before (external) symptoms are present and to study the threshold levels for disease development (Demontis et al. 2008; Malvick and Impullitti 2007; Moradi et al. 2010; Rossi et al. 2007). The ability to detect very small amounts of a pathogen can also be useful when evaluating the resistance of cultivars against certain pathotypes (Markakis et al. 2009) or determining whether the pathogen can survive or even accumulate in non-symptomatic plants or non-hosts (Zellerhoff et al. 2006).

The specific objectives of this research were: 1) to develop a protocol that allows application of the existing molecular detection techniques to whole cuttings at a low detection limit; 2) to monitor the DNA accumulation rate of *F. foetens* DNA in *B.* x *hiemalis* and determine associated disease threshold levels; and 3) to use the optimised molecular detection protocol to determine the capacity of a range of plants to function as a (latent) host for *F. foetens*.

Materials and methods

F. foetens isolates and inoculum production

The different *F. foetens* isolates used and their origins are listed in Table 1. The isolates obtained during this study were isolated from symptomatic leaf samples using Fusarium Selective Medium (FSM) (Komada

Table 1 Origin of the *Fusarium foetens* isolates used

Isolate code	Cultivar	Host origin	Collector	Collection month and year
FF001	not known	Grower 1, France	DCP[a]	Dec, 2006
FF002	Bino	Grower 2, Belgium	this study	Mar, 2007
FF003	Bela	Grower 2, Belgium	this study	Mar, 2007
FF004	Baladin	Grower 2, Belgium	this study	Mar, 2007
FF006	Baladin	Grower 2, Belgium	this study	Apr, 2008

[a] *DCP* Diagnosecentrum voor Planten = ILVO's Diagnostic Centre for Plants

1975). Single spore isolates were prepared and maintained on Potato Dextrose Agar (PDA) (Formedium, England). Species identity was confirmed using the *F. foetens*-specific PCR from de Weerdt et al. (2006).

To produce inoculum, the *F. foetens* isolates were grown for 2 weeks in the dark at 26°C on PDA. A spore suspension was obtained by adding 10 ml of sterile water to each plate, scraping the colony with a Trihalski spatula, then filtering the remaining liquid through a 20 μm nylon mesh to remove the hyphae from the spore suspension. The spore concentration was determined using a haemocytometer and diluted to the desired concentration with water. The inoculum consisted of spores from one to four specific isolates, at an equal concentration, as specified below for each experiment. The viability of the inoculum was determined by plating serial decimal dilution series in triplicate onto PDA plates.

Plant material

Four *B.* x *hiemalis* cultivars were used: Berseba, Baladin, Dragone and Kristy. The breeders report the cultivar Kristy to be less sensitive to *F. foetens* than other cultivars. Rooted cuttings were taken randomly from a commercial shipment immediately after delivery, before entering the growing area. All cuttings were transferred to our research facility and planted in commercial potting soil (Aveve, Belgium), 3 days before inoculation.

For the host range experiment, 16 plant species other than *B.* x *hiemalis* were tested that could be divided in three groups based on the conditions under which they are normally produced and then kept by the end user. The first group contained plants often grown in the same greenhouses as *B.* x *hiemalis* cultivars: *Cyclamen persicum*, *Rosa* mini, *Exacum affine*, *Saintpaulia ionantha*, and *Euphorbia pulcherrima*. The second group consisted of house plants, which are also produced in heated greenhouses and later kept indoors: *Calathea roseo-picta*, *Calathea warsce-wiczii*, *Calathea makoyana*, *Cordyline* 'Red Star' and *Spathiphyllum* 'Alfa'. The third group included annual plants that are produced from seed in greenhouses, but that afterwards are planted outdoors: *Delphinium*, *Doronicum*, *Campanula isophylla*, *Rudbeckia*, *Valeriana officinalis* and *Aquilegia*. Plants were transferred into new commercial potting soil as described above for *Begonia*, except plants of the second group, which were transferred in a 4:6 mixture of coconut fibre and peat.

All plant experiments were conducted in a biosafety growth chamber at 25–28°C, 16 h light, and 65% relative humidity. The pots were placed in individual saucers and watered individually, carefully avoiding cross contamination via the water.

Sample selection and tissue maceration

The sample selection and preparation was optimised in two ways to produce a sample that was representative for the entire plant, while still allowing sensitive detection. The first optimisation was to only use the plant part that contained the largest proportion of *F. foetens* DNA. To determine the correct part, nine *B.* x *hiemalis* cv. Baladin plants were inoculated by pouring 10 ml of 5×10^5 spores ml^{-1} from isolates FF001, FF003, and FF004 (mixed at equal proportions; total inoculum viability of 76%) onto the potting soil close to the stem of each plant. Control plants were inoculated with water. After 3 weeks, leaves, stems and roots were separated with a flame-sterilized scalpel, washed in sterile water until debris or soil was removed, and weighed. All samples were macerated and processed as described below. In a separate experiment, the contribution of the original inoculum in the detection of *F. foetens* in and on the roots was evaluated. Two

plants were inoculated as described above with a spore suspension of 2×10^5 spores ml^{-1} FF001 (inoculum viability of 42%) and the amount of *F. foetens* DNA on the roots was determined 1 h after inoculation. Based on the amount of DNA detected in the different plant parts, only root samples were analysed in all further experiments.

The second optimisation was to develop a maceration technique that could easily process entire root systems of *Begonia* plants. Various maceration techniques were compared, including grinding in liquid nitrogen with a mortar and pestle, blending using a Grindomix GM200 (Retsch, Germany), and crushing using a Homex macerator (Bioreba, Switzerland). This optimisation needed to fulfil the following criteria: appropriate sample volume, quality of maceration, risk of inter-sample contamination, and ease of use. Ultimately, samples were macerated in plastic maceration bags (Bioreba, Switzerland) together with half their weight in 100 mM Tris HCl pH 8 buffer (e.g., 5 ml sterile buffer was added to a plant sample of 10 g) and crushed with a Homex macerator. These Bioreba bags contain a fine-meshed gauze that allows easy separation of the liquefied and the fibrous plant parts. The roots were macerated until the root structure was no longer visible. Maceration lasted approximately 30 to 180 s, depending on the size of the root system. The maceration liquid was collected, its volume measured, and a subsample (2 ml; see below) was centrifuged for 7 min at 14,000 rpm. The entire pellet was used immediately for DNA extraction and subsequent real-time PCR-based quantification of *F. foetens* DNA.

DNA extraction

Sufficiently sensitive detection of *F. foetens* DNA in a representative sample of an entire root system was accomplished through optimising DNA extraction. We compared DNA extraction efficiency of the following extraction methods: CTAB-based DNA extraction method by Gardes and Bruns (1993) and modified by Alaei et al. (2009), with and without an extra purification step using a PVPP column (Polyvinylpolypyrrolidone, Sigma, Belgium); GenElute Plant Genomic DNA Kit (GE Kit) (Sigma, Belgium); Quick Pick SML Plant DNA Kit (Bio Nobile, Belgium), with and without MagaCharc beads (QP or QP + MC Kit, respectively) (Bio Nobile, Belgium); and Invisorb Spin Plant Mini Kit (ISP Kit) (Invitek, Belgium). For each method, three 100-mg subsamples of the same liquid nitrogen-ground infected plant material were used. Sterile glass beads (100 mg) and the appropriate volume of extraction buffer for each DNA isolation method was added to each sample. The samples were vortexed for 1 min and shaken twice for 30 s at 30 Hz in a bead beater (Retsch, Germany). Then the samples were incubated for 1 min at 65°C and, if prescribed by the protocol, treated with Proteinase K for 30 s. After this step, each DNA extraction was continued according to each method's protocol. The resulting DNA concentrations were determined spectrophotometrically using a Nanodrop (Isogen Life Sciences, the Netherlands) and the samples were diluted to 1 ng DNA μl^{-1} before quantification of *F. foetens* DNA using the real-time PCR procedure described below. The total amounts of DNA extracted and the concentrations of *F. foetens* DNA were analysed statistically using ANOVA and Tukey's HSD test using Statistica 8.0 (Statsoft, Tulsa, USA).

Once the maceration technique had been optimised, the maximum volume of maceration liquid that could be handled by the best DNA extraction technique was determined. To do this, 250 *F. foetens* spores were added to 1, 2 or 4 ml of macerate (in triplicate), the DNA was extracted, and the detectable amount of *F. foetens* DNA was determined using real-time PCR.

To test for presence of PCR-inhibitory components, a dilution series (1/1, 1/2, 1/5, 1/10, 1/50 and 1/100) of the DNA extracted from 2-ml macerated infected plant samples was subjected to real-time PCR. The observed increase in Ct-value was compared to the theoretical increase. We also tested the presence of PCR-inhibitory compounds by adding the T4 gene 32 protein. This protein decreases the impact of such inhibitors and should therefore lower the Ct-values if inhibitors are present (Vandroemme et al. 2008).

Eventually, DNA was extracted in all further tests from 2-ml macerated root samples using the ISP Kit.

Reference DNA from *F. foetens* was obtained from 100 mg fresh mycelium of isolate FF001 which had been grown for 10 days while being shaken at 150 rpm at room temperature in Potato Dextrose Broth (PDB) (Formedium, England), then washed with sterile water, blotted dry, and extracted using the Puregene DNA purification Kit (Gentra - Qiagen, Belgium).

Real-time PCR and pathogen quantification

Real-time PCR was performed as described by de Weerdt et al. (2006) with the following modifications: the 30 µl PCR mix consisted of 15 µl TaqMan Universal Master Mix (Applied Biosystems, Belgium), 1.8 µl of 5 µM of both primers, 1.8 µl of 5 µM of the probe, labelled with 6-FAM at the 5' end (Applied Biosystems, Belgium), 4.6 µl water and 5 µl DNA extract. Five microlitres of a 10-fold dilution series of *F. foetens* genomic DNA (1 µg μl^{-1} to 0.1 fg μl^{-1}) was included in each run for quantification purposes. During real-time PCR optimisation, different DNA sample volumes were compared (5 versus 12.5 µl). Amplification and detection of fluorescence was performed using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Belgium).

To determine the relationship between Ct value and number of spores, a 10–fold dilution series of *F. foetens* conidia (5×10^2 to 5×10^5 spores ml^{-1}) in maceration liquid from pathogen-free *Begonia* roots was established in triplicate and the DNA was extracted from 2 ml samples. A spore-free sample was used as a control. Based on the relationship between the amount of *F. foetens* DNA detected and the number of added spores, quantification of the pathogen in all future experiments was expressed as spore equivalents. Given that the weight of each sample (= root system) was determined and fixed volumes were used for DNA extraction and real-time PCR, the number of spore equivalents per root system was determined.

Monitoring of *F. foetens* accumulation in *Begonia* roots

Begonia plants, cv. Baladin were inoculated with 5×10^4 spores per plant ("low" inoculation level), 5×10^6 spores per plant ("high" inoculation level), or sterile water (control). The inoculum was comprised of a mixture of isolates: FF001, FF002, FF003 and FF004 (inoculum viability 68%). The inoculum was suspended in 10 ml water then poured onto the potting soil around the base of each plant. Disease development was monitored and roots of three replicate plants per treatment were destructively sampled 0 h and 72 h after inoculation, and thereafter weekly over a period of 8 weeks. Roots were rinsed three times in sterile water, weighed and frozen at −20°C until processing. Buffer was added before thawing the roots. The samples were then macerated, DNA was extracted and real-time PCR quantification of *F. foetens* DNA was performed as described above. For the inoculated treatments, linear regression analysis of the $\log_{10}$-transformed DNA amounts (or $\log_{10}$-transformed numbers of spore equivalents), corresponding to exponential regression of the non-transformed data, was performed using Statistica 8.0 (Statsoft, Tulsa, USA). For the "low" inoculation level, no *F. foetens* DNA was detected for some of the samples up to 5 weeks post inoculation (wpi). The detection limit for these samples was calculated based on the proportion of macerate used and the very reliable DNA detection limit of 50 fg (see Results below). Regression analysis using the data of the "low" inoculation level was performed either using the upper bound limit (the $\log_{10}$-transformed value of the detection limit) or using the middle bound limit (the $\log_{10}$-transformed value of half the detection limit) for the values below the detection limit (Kroes et al. 2002). The lower bound limit (using a 0 for the values under the detection limit) was considered to be an underestimation, given that inoculum was added and that *F. foetens* was detected in each plant that was analysed at later time points. Similarly, omitting the data under the detection limit would result in an overestimation of the average amount of DNA. For the "high" inoculation level, for which a saturation level was observed and for which an S-shaped curve might fit the data better than the exponential regression, a logistic regression model $\left(y = L/\left(1 + b_0 \times e^{b1\times time}\right)\right)$ was also fit to the data. This was performed at a saturation level (L) of 1.5×10^9 spore equivalents per plant, just above the maximum observed number of spore equivalents.

Determination of the host range

In a first experiment, the host range of *F. foetens* was determined using the 16 plant species and cultivars listed under "plant material" and *B.* x *hiemalis* cv. Dragone, Baladin and Kristy as control plants. Five replicate plants per species or cultivar were inoculated with *F. foetens*, while two replicate plants per species or cultivar were inoculated with sterile water as controls. Inoculation with *F. foetens* was as described above, except that a mixture of the *F. foetens* isolates FF002, FF003, FF004 and FF006 was used with

inoculum doses adjusted to the estimated root volume of the different plant species. Specifically, 6×10^4 spores were added per cm³ of estimated volume taken up by the roots. This dose resulted in 5×10^6 spores (inoculum viability of 88%) per plant for the *Begonia* plants. Root mass volume was estimated during transplanting to fresh potting soil 3 days before inoculation. Spores were added in a minimum of 5 ml and a maximum of 15 ml water. Disease development was monitored weekly over a period of 12 weeks. Roots were sampled, weighed and examined for vascular discoloration. At the end of the experiment, qualitative assessment of *F. foetens* presence per plant was done by surface disinfecting washed roots in 70% ethanol for 10 s or in 1% NaOCl for 1 min, followed by two rinses with sterile water. Two disinfection methods were used to rule out recovery due to failed disinfection. Subsequently, root parts were placed onto FSM and incubated for 2 weeks at 26°C in the dark. The roots were removed from the medium and DNA was extracted from a small amount of aerial mycelium scraped from the plates using a rapid DNA extraction method described as follows. The mycelium was first placed into a 0.5 ml microtube containing 40 µl of 0.25 M NaOH. The tubes were placed in a boiling water bath for 30 s and the samples were then supplemented with 40 µl of 0.25 M HCl, 20 µl of 0.5 M Tris–HCl pH 8, and 20 µl of 0.25% (w/v) Nonidet P-40. The tubes were put back into a boiling water bath for 2 min (Klimyuk et al. 1993; Van Hemelrijck et al. 2010). DNA in the samples was quantified using a Nanodrop® spectrophotometer, diluted to 1 ng μl^{-1}, and stored at −20°C until needed. Presence of *F. foetens* DNA in these samples was verified using the real-time PCR method described above. *F. foetens* presence in washed roots was also quantified directly with the maceration, DNA extraction and real-time PCR methods described above. For samples in which no *F. foetens* was detected, the detection limit was again calculated based on the proportion of macerate used and the very reliable DNA detection limit of 50 fg (see Results, below).

In the second experiment, 10 replicate plants of *Aquilegia*, *Cordyline* 'Red Star' and *Delphinium* were inoculated with *F. foetens* using the same methods as in the first experiment. Four control plants were inoculated with water. Three days post inoculation (dpi) and 12 wpi, the roots of five *F. foetens*-inoculated plants and two control plants were sampled and analysed as described for the first experiment. For each plant species the statistical significance of the difference in pathogen load at 3 dpi and 12 wpi was tested using Student's *T*-test.

Results

Optimisation of the detection technique for practical samples

Sample selection and tissue maceration

Three weeks after inoculation, the highest proportion of *F. foetens* DNA was detected in the roots (25,840± 16,389 fg per 5 µl DNA extract), while only relatively small amounts were detected in the stems (100± 28 fg) and leaves (118±41 fg). The inoculated spores contained 3.9×10^6 fg DNA based on the standard curve (see "Monitoring of *F. foetens* accumulation in Begonia roots" above), but the amount of DNA in and on roots 1 h after inoculation was 2,443±452 fg, indicating that the amount of inoculum adhering to the roots does not contribute significantly to the amount of DNA detected in and on the roots at the 3-week time point. No *F. foetens* DNA was detected in the control plants.

The Homex system was chosen for tissue maceration, for the following reasons: the Bioreba bags could easily accommodate the total root volume (between 3 and 48 g), the system allowed a good detection limit, there was no inter-sample contamination, and the bags were easy to use. Grinding in liquid nitrogen or blending resulted in a similar detection limit, especially for small plants, but these methods were more labour intensive and risked inter-sample contamination (data not shown).

DNA extraction

We have compared different methods and kits for DNA extraction from the plant samples. The amount of total DNA and *F. foetens* DNA extracted from the plant samples with the different kits are summarised in Table 2. Relatively large amounts of *F. foetens* DNA were extracted with the QP Kit and the ISP Kit. The extra step with the MagaCharc beads did not increase the performance of the QP Kit protocol. We chose the

Table 2 Comparison of different methods to isolate *Fusarium foetens* DNA from the plant samples. The total DNA extracted from 100 mg plant material was measured spectrophotometrically. The amount of *F. foetens* DNA in the total sample was determined using real-time PCR. Three replicates per method were analysed and standard errors of the mean are given for all numbers. Within each column, treatments labelled with the same letter are not significantly different based on Tukey's HSD test ($p>0.05$)

Method[a]	Total DNA (ng per extraction)	*F. foetens* DNA	
		Concentration (fg per ng total DNA)	Total amount (ng per extraction)
CTAB	9293±781 c	169±41 a	1.5±0.3 ab
CTAB+	2582±62 b	474±153 a	1.1±0.2 ab
GE kit	450±733 a	21±6 a	0.01±0.004 a
ISP kit	1932±2567 ab	5107±1427 b	10.2±3.2 c
QP kit	3218±769 b	2287±183 a	7.6±2.2 bc
QP + MC kit	2613±63 b	1748±99 a	4.6±0.4 abc

[a] See Materials and Methods section for method abbreviations

ISP Kit due to its performance (significantly higher concentration and highest total amount of *F. foetens* DNA detected) and lower cost.

Using this method, increasing the sample volume up to 2 ml macerate resulted in a linear increase in detected *F. foetens* DNA, and this volume was chosen for all further analyses. No co-extracted PCR inhibitors were detected when using this volume, as the real-time PCR-based amount of *F. foetens* DNA decreased as theoretically expected in a dilution series of the DNA extract (data not shown). Addition of T4 gene 32 protein, which decreases the impact of PCR inhibitors, did not improve the detection sensitivity (data not shown).

Detection limit

When using genomic DNA from the *F. foetens* culture, the real-time PCR dose–response curve was $Ct = -3.4407 \times \log(\text{amount of DNA in fg}) + 42.2398$, with an R^2 of 0.9997. The theoretical detection limit (at a Ct of 40) was 4.5 fg of DNA. Fifty fg DNA corresponded to a Ct of 35.16±0.13 and was considered to be a very reliable detection limit, given the greater than 10-fold difference with the theoretical detection limit and the low standard deviation at this level. When using the dilution series of *F. foetens* spores in 2-ml plant macerate, the real-time PCR dose–response curve was $Ct = -3.1552 \times \log(\text{spore number}) + 40.6987$, with an R^2 of 0.9932 (Fig. 1). Given that these samples were analysed in the same PCR run as the genomic DNA, the number of spore equivalents of *F. foetens* can be calculated from the amount of DNA with the combined equation: number of spore equivalents $= 1E(1.0905 \times \log(\text{amount of DNA in fg}) - 0.4884)$. As long as the same maceration and DNA extraction protocol is followed, the number of spore equivalents can be calculated with this equation based on the amount of *F. foetens* DNA detected in any sample. At the very reliable detection limit of 50 fg DNA, this corresponds to 23.1 spore equivalents. At the theoretical detection limit of 4.5 fg, this corresponds to 1.67 spore equivalents per real-time PCR reaction.

To calculate the number of spore equivalents in the entire root system of a plant, the proportion of DNA extract used in the PCR reaction (5 out of 100 µl) and the proportion of macerate used in the DNA extraction (usually 20 to 40% of the total macerate) were taken into account. For a theoretical detection limit of 1.67 spores per PCR reaction, this amounted to a detection limit of

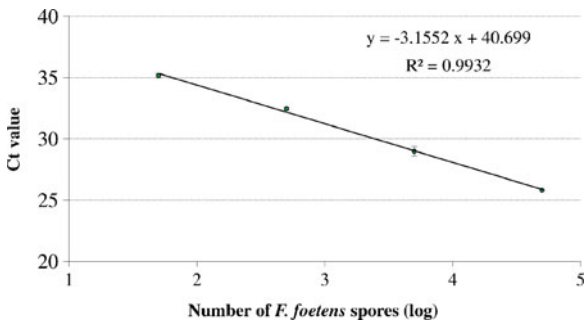

Fig. 1 Dose–response curve of real-time PCR quantification of *Fusarium foetens* spores. *Error bars* represent standard deviations from three replicate reactions

1.67×20×(2.5 to 5) = 84 to 167 spore equivalents per root system. For the very reliable detection limit of 23.1 spores per PCR reaction, this amounts to a detection limit of 23.1×20×(2.5 to 5) = 1155 to 2310 spore equivalents per root system. By using the exact percentage of the total macerate used, the exact detection limit can be calculated for each sample.

Monitoring of *F. foetens* accumulation in *Begonia* roots

We studied the accumulation of *F. foetens* DNA in *Begonia* cv. Baladin roots over time (Fig. 2). Given the significant linear regressions of the log amounts of *F. foetens* spore equivalents per plant over time (statistical data provided below), the amount of pathogen increased exponentially, up to a saturation level of approximately 10^9 spore equivalents per root system. For the "high" inoculation level, the regression curve on all data was as follows: $\log(\#\text{ of spore equivalents}) = 0.4565 \times \text{weeks} + 4.8781 (p = 3.2 \times 10^{-8}; R^2 = 0.67)$. Logistic regression resulted in the following equation: $\#\text{ of spore equivalents} = 1.5 \times 10^9/(1 + 19792.7 \times \exp(-1.09824 \times \text{weeks}))$, but did not result in a better fit ($R^2 = 0.62$). As the maximum average amount of DNA was reached after 6 weeks, the most appropriate estimation of the exponential accumulation is obtained after regression of the data up to 6 weeks. This resulted in the regression curve: $\log(\#\text{ of spore equivalents}) = 0.6323 \times \text{weeks} + 4.5548 (p = 1.1 \times 10^{-7}; R^2 = 0.73)$ (Fig. 2). For the "low" inoculation level, no saturation level was observed (Fig. 2) and only the exponential regression was applied. For the "low" inoculation level, no pathogen was detected in some of the plants up to 5 weeks after inoculation and either the upper bound or middle bound values were used for the estimation of these missing values. When using the upper bound values (= detection limit), the regression curve was as follows: $\log(\#\text{ of spore equivalents}) = 0.7045 \times \text{weeks} + 2.0437 (p = 3.1 \times 10^{-9}; R^2 = 0.72)$. If the more realistic middle bound values (= half the detection limit) were used for the missing values, the regression curve was: $\log(\#\text{ of spore equivalents}) = 0.7358 \times \text{weeks} + 1.8093 (p = 5.1 \times 10^{-9}; R^2 = 0.71)$, but neither the slopes ($p = 0.80$) nor the intercepts ($p = 0.67$) of these two variants were significantly different from each other (Fig. 2). More interestingly, the slope of the "high" inoculation level (based on the first 6 weeks' data) was not significantly different from those of the "low" inoculation level ($p = 0.57$ and $p = 0.45$ for the upper and middle bound version, respectively).

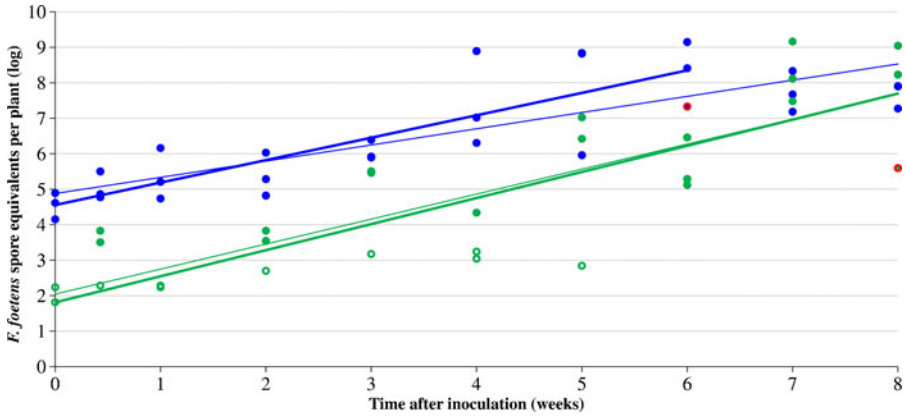

Fig. 2 Real-time PCR-based quantification of *Fusarium foetens* accumulation in *Begonia* x *hiemalis* cv. 'Baladin' after inoculation with either 5×10^4 (*green*) or 5×10^6 (*blue*) spores per plant. Each symbol represents a single replicate plant. *Empty symbols* represent plants in which no *F. foetens* DNA was detected, and correspond to the detection limit for the given plant. The upper two blue lines and the lower two green regression lines correspond to the "high" and the "low" inoculation level, respectively. Within the upper level, the thinnest regression line is based on all data, while the thickest line is based on the data up to 6 weeks only. Within the lower level, the thinnest regression line is based on the upper bound values (= detection limit) for the plants in which no *F. foetens* DNA was detected. The thickest regression line is based on the middle bound values (= half the detection limit) for the plants in which no *F. foetens* DNA was detected. Details on the regression lines are provided in the text. Except for the two plants represented by the data points marked with a red line, all plants containing more than 1.53×10^7 spore equivalents were symptomatic and all plants containing fewer than this amount were non-symptomatic

After 4 and 7 weeks, disease symptoms started to appear in plants inoculated with the high and low spore levels, respectively. Plants showing symptoms contained at least 1.53×10^7 spore equivalents per plant, with two exceptions (Fig. 2). No symptoms developed in the plants of the control treatment, but unexpectedly, *F. foetens* was detected in a total of 75% of these control plants starting 5 weeks after inoculation at a range of 4.0 to 5.3 log levels of spore equivalents.

Monitoring of *F. foetens* accumulation in non-symptomatic hosts

In the first host range experiment, typical symptoms developed on the *Begonia* plants, eventually resulting in plant mortality, while no symptoms were observed on the other test plants, nor on the water-treated *Begonia* plants. Differences between the *Begonia* cultivars were observed. Four weeks after inoculation, two of the inoculated *Begonia* cv. Baladin plants showed severe symptoms, and the other three plants started to show symptoms, while only one out of five plants of the two other cultivars (Dragone and Kristy) showed early wilting symptoms. Another 2 weeks later, all inoculated Dragone plants showed symptoms, while one inoculated Kristy plant remained symptomless for another 2 weeks.

F. foetens was isolated onto FSM from surface-disinfected root samples from 15 of the 16 pathogen-inoculated host plant species and cultivars (Table 3; Experiment 1). The average percentage of replicate plants with *F. foetens* detection via plating was 65%. No *F. foetens* was recovered from the root samples of the water-inoculated plants or from *C. warscewiczii*. Results of the real-time PCR analysis of a root subsample of each plant are shown in Fig. 3. Three groups of plant species could be distinguished based on the amount of *F. foetens* DNA detected in relation to the amount of spores added. The first group included *Aquilegia*, *Calathea roseo-picta*, *C. persicum* (small), *Doronicum*, *C. isophylla*, and *V. officinalis,* with an equal or a higher amount of *F. foetens* on the roots than the initial amount of spores added. The second group included plant species with at least three out of five plants in which no *F. foetens* was detected: *C. warscewiczii*, *C. makoyana*, *Cordyline* 'Red Star', *E. pulcherrima*, *Rudbeckia*, and *Spathiphyllum* 'Alfa'. The third group included the remaining plant species, with an average amount of *F. foetens* on the roots that was about 10 times smaller than the initial amount added (Fig. 3). Based on these results, one host plant species was selected from each group and analysed in more detail during a second experiment.

In the second host plant experiment, no symptoms of *F. foetens* infection were observed on the plants, except for the *Begonia* control plants. Qualitative detection of *F. foetens* in the roots, this time using various disinfection methods, again showed the presence of *F. foetens* in each of the host plants (Table 3). Direct real-time PCR quantification of *F. foetens* DNA in the roots now also included analysis at 3 dpi. The amounts of spore equivalents recovered at that time point are at least 3 log units smaller than those inoculated (Table 4). These increased with 1 to 1.5 log levels at 12 wpi and were significantly different from the 3 dpi-amounts for two of the three plant species.

Discussion

The first objective of this study was to make the molecular detection technique for *F. foetens* applicable to large plant samples such as an entire *Begonia* plant, while still providing a sufficiently low detection limit and limiting the risk of inter-sample contamination. Processing larger samples was necessary, as sampling issues impede the interpretation of results from 100 mg samples from possibly latently infected material. We attained our first objective by using Bioreba sample bags and the Homex macerator, using the most relevant plant part only, and optimising the DNA extraction and PCR conditions. Use of blended root material provided a comparable detection limit but this method requires rigorous inter-sample decontamination of the blending equipment. This is unpractical when processing large numbers of samples. Bead beating systems could not accommodate the required sample volume and were therefore not included in the tests. Analysis of the roots is less practical than that of the stem base, the plant part selected by de Weerdt et al. (2006). However, the relatively high Ct value observed by de Weerdt et al. (2006) when analysing the stem base of latently infected plants, as well as our data on the relative amounts of pathogen in the different plant parts, would indicate that roots should be used when small

Table 3 Qualitative detection of *Fusarium foetens* in a mixed root sample from the five replicate plants of the 16 plant species of the host range experiments (Exp. 1), and repeated for three plant species (Exp. 2). The roots were surface-disinfected with either EtOH or NaOCl to eliminate *F. foetens* attached to the outside of the roots. Roots of *Begonia* cultivars were not plated, as they were deteriorated due to severe necrosis at the time of analysis

Host species		% Plants with *F. foetens*[a]	
		EtOH-disinfected	NaOCl-disinfected[b]
Aquilegia	(Exp.1)	100	–
	(Exp.2)	40	80
Calathea roseo-picta		80	–
Calathea warscewiczii		0	–
Calathea makoyana		60	–
Campanulla isophylla		100	–
Cordyline 'Red Star'	(Exp.1)	40	–
	(Exp.2)	40	60
Cordyline persicum small		60	–
Cordyline persicum medium		80	–
Cordyline persicum large		60	–
Delphinium	(Exp.1)	80	–
	(Exp.2)	0	20
Doronicum		60	–
Euphorbia pulcherrima		50	–
Exacum affine		20	–
Rosa mini		100	–
Rudbeckia		50	–
Spathiphyllum 'Alfa'		80	–
Saintpaulia ionantha		50	–
Valeriana officinalis		100	–

[a] – =not tested

[b] In Exp.2 part of the roots was separately surface-disinfected with NaOCl to test that recovery of *F. foetens* was not due to a failing EtOH surface-disinfection

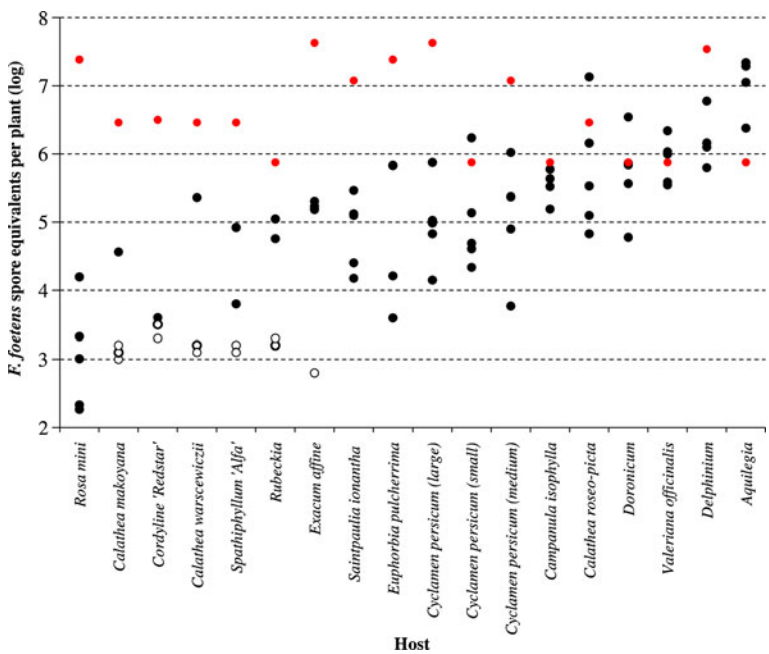

Fig. 3 Quantitative detection of *Fusarium foetens* (in spore equivalents) in root samples of 16 plant species, 12 weeks after inoculation (host inoculation experiment 1). The *red dots* represent initial amount of *F. foetens* spores added per plant. Each filled *black dot* represents the value of a replicate plant. *Empty black dots* represent the detection limit for plants in which no *F. foetens* DNA was detected. Plant species are sorted based on the average amount of *F. foetens* detected

Table 4 Quantitative monitoring of *Fusarium foetens* spore-equivalents in roots of three non-symptomatic plant species over time (host inoculation experiment 2). Data represent averages and corresponding standard error of the mean

Time (dpi)	*F. foetens* spore-equivalents per plant (log)		
	Aquilegia	*Cordyline* 'Red Star'	*Delphinium*
0[a]	5.8	6.5	7.5
3	2.7±0.3	3.0±0.8	3.4±0.1
84	4.2±1.0	4.0±1.6	4.5±0.7
p-value[b]	0.027[c]	0.25	0.00038[c]

[a] For time point 0 dpi, data represent initial amounts of *F. foetens* spores added per plant

[b] For each plant species, *p*-value of Students' *T*-test between data from 3 to 84 dpi. Values marked with [c] indicate a significant difference between the two time points ($p<0.05$)

amounts of the pathogen need to be detected, such as in latently infected rooted cuttings.

Using our optimised method, we obtained a sensitive theoretical detection limit of 4.5 fg DNA or 1.67 spore equivalents per PCR reaction, which translates to 84 to 167 spore equivalents per plant. This allowed us to study the presence and (latent) accumulation of the pathogen in symptomatic (second objective) as well as in non-symptomatic (third objective) plant species, at a scale which was not possible before and with a more limited risk of false negative detection results. These detection levels are at least as sensitive as in comparable studies: Atallah et al. (2007) detected 148 fg genomic DNA of *Verticillium dahliae*; Malvick and Impullitti (2007) detected 50 fg genomic DNA or 400 conidia of *Phialophora gregata* in *Glycine max*; and Debode et al. (2009) detected 50 fg genomic DNA of *Collectotrichum acutatum* in strawberry.

Although our optimisation was primarily geared to detect *F. foetens* in latently infected plants, it can also be applied to the sensitive detection of *F. foetens* in water from greenhouse irrigation systems via DNA extraction and real-time PCR of filtered propagules (Huvenne et al., unpublished data). Greenhouse irrigation systems can be an important pathway of spread according to Elmer (2008), as he found that as few as 100 conidia ml^{-1} irrigation water are sufficient to cause significant disease development.

The second objective of this study was to use the optimised protocol to monitor the fungus in the roots of *Begonia*. We accomplished this after artificially inoculating the substrate with spores at two inoculation levels to mimic a light or heavy natural infection of the root system. Pathogen accumulation was exponential for the majority of the time points and occurred at a similar rate for the two inoculation levels. Once the pathogen load reached 1.53×10^7 spore equivalents per plant, symptoms started to develop in all but one plant. The pathogen load continued to rise up to approximately 10^9 spore equivalents per plant, after which a decline was noticed. Roots and colonising *F. foetens* mycelium probably start to degrade during advanced stages of disease development. Our results are in accordance with those from Schroers et al. (2004), who found that sensitive *Begonia* plants inoculated with a high concentration spore solution will develop disease symptoms and die off within 10 weeks after inoculation. Logistic regression of the data could have been more appropriate for the "high" inoculation level due to the saturation part observed for that level. However, such a regression did not result in a better fit. This result and the need to directly compare the accumulation rates for the two inoculum levels led us to only use the exponential regression (linear regression after log-transformation of the data). For the "high" inoculation level, we used only the six-week data period that showed an accumulation of the DNA on average. As expected, these changes made the data of the "high" inoculation level fit better to the exponential regression curve.

Establishing the pathogens' growth rate for a specific *Begonia* cultivar under practical temperature and growing conditions and determining the amount of pathogen DNA at a given time point in a symptomless plant of that cultivar would make it possible to combine these data to calculate the pathogen's concentration at any previous point during the growing process. This has the practical implication that if this value is negative at the time point of delivery of the plants at the grower's site, it would imply that infection happened at the grower's site itself. When we applied this idea to the data of our own experiment, we observed a positive value for 78% of the control plants in which *F. foetens* was detected. Therefore, for most plants we cannot conclude that presence of the pathogen in our negative control plants is due to a contamination event in our growth chamber. It is therefore possible that these plants were already infected at low levels when we acquired them. This is consistent with the observation at the grower's site, where an estimated

25% of plants from the same batch eventually developed symptoms. However, to conclusively determine whether some of the plants were infected at the onset of the experiment, it would have been necessary to incubate them under quarantine conditions until symptoms could be evaluated. In commercial production, symptoms usually appear after eight to 12 weeks (growers' data). If we assume that the growth rate under practical conditions is similar to the one observed in our experiments, and with a start of symptoms corresponding to 1.53×10^7 spore equivalents per root system, we can calculate that cuttings may be infected before arrival at the nursery with less than 51 spore equivalents per root system. This is very low and below the detection limit of the test, questioning the value of direct molecular analyses to check the pathogen-free status of cuttings. Although post-delivery, on-site infection at a nursery is of course possible, sporadic re-entry with latently infected cuttings should also be considered a possibility. Elmer (2008) stipulated that fungus gnats (*Bradysia* spp.) might play an important role in the spread of the fungus. However, there were no fungus gnats or other pest insects observed during this experiment. Quantitative data on the amount of *F. foetens* in latently infected plants combined with growth rate data also allows calculation of the amount of pathogen DNA at a future time point. Given that symptoms start at approximately 1.53×10^7 spore equivalents per plant, one could also use the data to calculate when the symptoms will start to occur.

The growers' observation that some *Begonia* cultivars are more sensitive to *F. foetens* than others was confirmed in our inoculation experiment with multiple host plants. All inoculated Baladin plants showed symptoms after 4 weeks, while Kristy plants remained symptomless for up to 8 weeks. This is in contradiction with Elmer (2008), who tested seven cultivars that were all severely affected after 6 weeks. As Elmer (2008) did not include cultivars Dragone nor Kristy, the difference may be explained by the specific choice of cultivars. Future experiments will need to clarify whether either a smaller growth rate or a larger pathogen threshold level for disease are responsible for the delayed symptoms in the more resistant cultivars.

The third objective of this study was to verify the narrowness of the host range of *F. foetens*, and particularly, the possible survival of the pathogen in non-symptomatic hosts. We demonstrated that even though symptoms only developed on *Begonia*, *F. foetens* can survive and even accumulate on many plant species. This included plant species that are normally grown at cooler temperatures and also included two types of substrates. In some cases the pathogen is found at levels close to 1.53×10^7 spore equivalents per plant, the threshold at which symptoms occurred in *Begonia*. Using direct root analysis with real-time PCR, we could show more than a 10-fold increase of the pathogens' DNA amount between 3 days and 12 weeks after inoculation in non-symptomatic hosts. Analysis of surface-sterilised roots also demonstrated that the pathogen is present inside the roots, and not merely saprophytically on their surface. Schroers et al. (2004) tested the pathogenicity of *F. foetens* on *Impatiens walleriana, E. pulcherrima, S. ionantha* and *C. persicum.* They did not observe symptom development on these plants and stated that *F. foetens* may be relatively host specific. However, they could re-isolate the fungus from *S. ionantha* and *C. persicum,* consistent with our results. Based on our observations, we can state that many plant species can host *F. foetens* in a non-symptomatic manner, which can be detected easily if a sufficiently sensitive technique is used. These observations have important implications for pest risk assessment analysis: the fungus might survive and spread on plants other than *Begonia* to a level that has been previously underestimated. On a practical level, if a severe infection would take place in a glasshouse, decontamination measures might have to include host plants other than *Begonia*.

Using an optimised molecular detection method we have shown the latent accumulation of *F. foetens* in a symptomatic as well as in non-symptomatic host species. It suggests that host range experiments merely based on symptoms may be largely insufficient to determine the real risk for accumulation and spread of a pathogen. Accumulation on non-symptomatic hosts may be a more general phenomenon than previously thought, and much less species-specific than pathogenicity.

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