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A quantitative real-time PCR method for *in planta* monitoring of *Phytophthora infestans* growth

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Abstract

Aims: To establish a reliable and rapid protocol to simultaneously obtain high quality DNA from an infected host plant and the infecting pathogen. To develop an accurate and sensitive low-cost assay for the quantification and *in planta* monitoring of *Phytophthora infestans* growth.

Methods and Results: In this study, we describe a SYBR Green-based quantitative real-time PCR (qPCR) method for the quantification of *P. infestans*. The method is based on a simultaneous plant-pathogen DNA purification followed by a qPCR in which the relative quantification of pathogen and plant DNA is performed. Besides assuring an accurate quantification, the use of a plant gene provides a reliable indicator of sample quality, allowing the exclusion of inappropriate samples. By applying this methodology, we were able to detect *P. infestans* in potato leaf and tuber tissue before the first symptoms of the disease were observed and to monitor the *in planta* growth of the pathogen for 6 days.

Conclusions: This is a reliable low-cost assay that provides rapid, accurate and sensitive quantification of the late blight pathogen, allowing the *in planta* monitoring of *P. infestans* growth.

Significance and Impact of the Study: The quantitative nature of the assay described in this study may be useful in plant breeding programmes and basic research. The method is appropriate for the comparison of cultivars with different, and even subtle, degrees of pathogen resistance and in the screening of new anti-oomycete compounds. The method can be easily adapted to tomato and the model plant *Nicotiana benthamiana*.

Introduction

Late blight, caused by the oomycete *Phytophthora infestans*, is one of the world's most devastating plant diseases and the most destructive pathogen of potato (*Solanum tuberosum*) (Si-Ammour *et al.* 2003; Song *et al.* 2003; Haas *et al.* 2009), with severe outbreaks also affecting tomato (*Solanum lycopersicum*). Global potato crop losses caused by late blight are estimated at 6·7 thousand million dollars annually (Haverkort *et al.* 2008; Haas *et al.* 2009). Potatoes are currently the fourth most important food crop consumed worldwide and a critical alternative to the main cereal crops for feeding the world's population (Haas *et al.* 2009), besides being used as an animal feed (Dancs *et al.* 2008). Additionally, potato starch can be used to produce biodegradable plastics (Jobling 2004; Gonzalez-Gutierrez *et al.* 2010) as well as fuel-grade ethanol (Jobling 2004; Cardona and Sanchez 2007) and is widely used by the pharmaceutical, textile, wood and paper industries (Kraak 1992; Jobling 2004). Furthermore, the usefulness of potato plants as bioreactors for the production of therapeutic and other recombinant molecules

at high scales and low manufacturing costs has been demonstrated (Ma *et al.* 2003; Mullins *et al.* 2006).

Considering the economic importance of potato, early pathogen detection in the field and storage facilities is crucial because a few contaminated plants are enough to spread the infection and severely compromise production (Trout *et al.* 1997; Judelson and Tooley 2000; Jyan *et al.* 2002; Hussain *et al.* 2005). Besides being a threatening plant pathogen, *P. infestans* is also a model organism for oomycetes (Haas *et al.* 2009). Therefore, producers and plant breeders, as well as researchers, benefit from methodologies allowing the accurate measurement of *P. infestans* growth (Dorrance and Inglis 1997).

Traditionally, *P. infestans* detection is based either on visual and microscopic examination of infected plant tissue or on culturing the pathogen from plant tissue (Judelson and Tooley 2000). However, pathogen quantification by these methods is inconsistent (Nicolaisen *et al.* 2009), for example, mycelia in different focal planes might be imprecisely quantified during microscopic examination.

Moreover, contaminated plants can appear symptomless, or symptoms can be confused with those caused by other pathogens (Judelson and Tooley 2000). Additionally, *P. infestans* isolation can be difficult because of poor *in vitro* growth (Trout *et al.* 1997; Jyan *et al.* 2002). In recent years, several other techniques have been developed with the aim of improving the detection and quantification of *P. infestans*. Some of these are based on immunosensors (Skottrup *et al.* 2007), *P. infestans* transgenic strains (Kamoun *et al.* 1998; Si-Ammour *et al.* 2003), polymerase chain reaction (PCR) (Niepold and Schober-Butin 1995; Tooley *et al.* 1997; Trout *et al.* 1997; Judelson and Tooley 2000; Jyan *et al.* 2002; Wangsomboondee and Ristaino 2002; Hussain *et al.* 2005) and quantitative real-time PCR (Böhm *et al.* 1999; Eschen-Lippold *et al.* 2007).

Quantitative real-time PCR (qPCR) allows fast, reliable and accurate detection and quantification of plant pathogens (Martin *et al.* 2000). Additionally, microbiological detection methods based on qPCR provide greater sensitivity and less variability compared to culturing-dependent techniques (Li *et al.* 2008). By applying qPCR, detection of a single fungal spore has been reported (Alkan *et al.* 2004). Among all the detection technologies available, Taqman and SYBR Green chemistries are the most extensively used (Bustin 2005). The SYBR Green assay has a relatively low set-up and running cost (Pellissier *et al.* 2006; Arikawa *et al.* 2008), and it has proven to be as sensitive, specific and quantitative as the Taqman assay (Maeda *et al.* 2003; Arikawa *et al.* 2008). Considering that probe-based qPCR requires additional manipulation and extra cost for the probe, the SYBR Green qPCR may be more suitable for routine and large-scale examinations (Karsai *et al.* 2002; Maeda *et al.* 2003; Pellissier *et al.* 2006; Arikawa *et al.* 2008).

Here, we describe the development of a qPCR assay using SYBR Green technology for the *in planta* growth monitoring of the late blight pathogen *P. infestans*. The assay is based on a low-cost, quick and efficient simultaneous plant-pathogen DNA purification followed by a qPCR in which the relative quantification of pathogen and plant DNA is performed. The assay has potential applications in the development of late blight-resistant cultivars and the screening of new anti-oomycete compounds as well as in basic research by allowing the study of *P. infestans* growth.

Materials and methods

Plant and pathogen material

Wild-type potato plants (*S. tuberosum* var. Spunta) were grown in 4-litre pots in greenhouses (16-h light/8-h dark photoperiod and $25 \pm 3°C$), and tubers were harvested after the aerial parts of the plants had died. The isolate of *P. infestans* race $R_2R_3R_6R_7R_9$, mating type A2 (Andreu *et al.* 2006), was kindly provided by Dr Adriana Andreu (Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata, Argentina) and maintained on rye agar (Caten and Jinks 1968) at $19 \pm 1°C$ in the dark.

DNA extraction

To reduce the variability in DNA quality between samples, a single protocol was developed to isolate DNA from pure *P. infestans* cultures and from infected as well as noninfected potato leaves and tubers. Samples were mechanically homogenized at a ratio of 10 mg fresh weight to 1 ml lysis buffer (200 mmol l^{-1} Tris–HCl, pH 8, 100 mmol l^{-1} NaCl, 25 mmol l^{-1} Na_2 EDTA, 3% [w/v] SDS and 125 μg ml^{-1} proteinase K added just before use). The extracts were incubated at 37°C for 1 h and mixed with 1 volume of extraction buffer (100 mmol l^{-1} Tris–HCl, pH 8, 2·5 mol l^{-1} NaCl, 20 mmol l^{-1} Na_2 EDTA, 2% [w/v] CTAB and 2% [v/v] β-mercaptoethanol added just before use). The homogenates were incubated at 65°C for 15 min, mixed with 1 volume of phenol–chloroform–isoamylic alcohol [25 : 24 : 1] and centrifuged at 10 000 g for 10 min. The aqueous phases were transferred to new tubes and mixed with 100 μg ml^{-1} RNase A. Samples were left 30 min at 37°C followed by the addition of 0·25 volumes of 2 mol l^{-1} NaCl-4% [w/v] PEG_{6000} and 0·7 volumes of isopropyl alcohol. The mixtures were centrifuged at 15 000 g for 20 min, and the supernatants were discarded. After centrifugation, the pellets were washed with 1 ml cold 70% (v/v) ethanol, air-dried and

resuspended in 50 μl of TE pH 8. Purified DNAs were quantified by spectroscopy using a NanoDrop apparatus (Thermo Scientific, Waltham, MA, USA), and DNA integrity was evaluated by agarose gel electrophoresis. DNA samples were stored at −20°C until use.

Quantitative real-time PCRs

The EF-1αFwd (5′-TGAGGCAAACTGTTGCTGTC-3′) and EF-1αRev (5′-TGGAAACACCAGCATCACAC-3′) primers (Llorente *et al.* 2010) designed based on the sequence of the elongation factor 1-α gene of *S. tuberosum* (*Ef-1α*; GenBank accession number: AB061263.1) were used to quantify *S. tuberosum* DNA. The PiO8-3-3Fwd (5′-CAAT-TCGCCACCTTCTTCGA-3′) and PiO8-3-3Rev (5′-GCCT-TCCTGCCCTCAAGAAC-3′) primers (Eschen-Lippold *et al.* 2007; Halim *et al.* 2007) designed based on highly repetitive sequences from the *P. infestans* genome (Judelson and Tooley 2000) were used to quantify *P. infestans* DNA. Conditions for qPCRs were optimized with regard to Platinum *Taq* DNA polymerase (0·5 U per reaction for both amplicons; Invitrogen, Carlsbad, CA, USA), forward and reverse primers (1 μmol l^{-1} for *Ef-1α* and 0·75 μmol l^{-1} for *PiO8*; Alpha DNA, Montreal, Canada), MgCl$_2$ concentration (3 mmol l^{-1} for *Ef-1α* and 2 mmol l^{-1} for *PiO8*), dNTPs mix concentration (0·5 mmol l^{-1} per reaction for both amplicons; New England Biolabs, Ipswich, MA, USA) and signal acquisition temperatures. The qPCRs were performed in a total volume of 20 μl using SYBR Green technology (1 μl of a 1/1000 dilution in dimethyl sulfoxide of 10 000× SYBR Green I; Invitrogen) on a Rotor-Gene 6000 instrument (Corbett Life Science, Australia). Cycling conditions were 95° C for 10 min followed by 40 cycles of 10 s at 95°C, 15 s at 60°C (*Ef-1α*) or 58°C (*PiO8*), 20 s at 72°C and fluorescence signal acquisition temperatures at 79°C (*Ef-1α*) or 76°C (*PiO8*). Three technical replicas were performed, and the mean values were used. PCRs for each gene fragment were performed alongside standard dilution curves of DNA (Gomes *et al.* 2006). The qPCR efficiencies for *Ef-1α* and *PiO8* primers were obtained using standard dilution curves in triplicate of *S. tuberosum* and *P. infestans* DNA, respectively. The qPCR efficiencies were calculated according to the equation: $E = [(10^{(-1/\text{slope})}) - 1] \times 100$.

Pathogen inoculation and quantification

After growth for 10 days on rye agar medium in the dark (19 ± 1°C), *P. infestans* mycelia were harvested in 25 ml sterile water and stimulated to release zoospores by incubation at 4°C for 5 h. Following filtration through a 15-μm nylon filter cloth, sporangia suspension was observed under a light microscope for quantification and the concentration was adjusted to 25 sporangia μl^{-1} to be used as an inoculum. Leaves from 4-week-old greenhouse-grown potato plants (4th to 6th leaves counting from the top) were excised from the plants and transferred to a growth chamber kept at a 16-h light/8-h dark photoperiod and 19 ± 1°C. After 2 days, leaflets were inoculated on the abaxial side with equidistant droplets of 10 μl of water containing 250 sporangia. Leaf samples consisted of leaf discs (8 mm diameter) cut from the inoculation sites. Four-month-old tuber discs (8 mm diameter × 1 mm thick) were inoculated in the centre with 10 μl of water containing 250 sporangia and transferred to a plastic box at 19 ± 1°C in the dark for 6 days. The pathogen load was quantified from 20 infected leaf and tuber samples per time point by normalizing the *PiO8* values with the corresponding *Ef-1α* values for each individual sample. The mean values at 6-day postinoculation (dpi) were set at 100%, and all data were normalized to the values obtained for 6 dpi samples. Leaf and tuber pictures were acquired with a Canon Ixus 70 camera (Canon, Tokyo, Japan).

Statistical analysis

Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison test. Statistical analysis was performed using GRAPHPAD PRISM 5 (GraphPad Software, La Jolla, CA, USA).

Results

Quantitative real-time PCR efficiencies and linearity

Known concentrations of tenfold serially diluted DNA from *S. tuberosum* (200–0·02 ng) and *P. infestans* (20–0·002 ng) were used to construct standard curves (Fig. 1). These curves were evaluated with the *Ef-1α* primers (Llorente *et al.* 2010) and the *PiO8* primers (Eschen-Lippold *et al.* 2007) for potato and *P. infestans* DNA detection, respectively. The standard curves showed consistent amplification over the different amounts of DNA analysed for both target amplicons (Fig. 1a). The qPCR efficiencies (*E*) were calculated from the given slopes (*M*) in ROTOR-GENE 6000 software. The threshold was automatically set with the *Auto-Find Threshold* function of the ROTOR-GENE 6000 software, which delivers the best fit of the standard curve. The transcripts studied showed high qPCR efficiency rates (*E* > 98%) with high linearity (correlation coefficient $R^2 > 0.99$) (Fig. 1b).

Confirmation of primers' specificity

To confirm the specificity of the selected primers, both the *Ef-1α* and *PiO8* primers were tested with *S. tuberosum* and *P. infestans* DNA templates. Both *Ef-1α* and *PiO8*
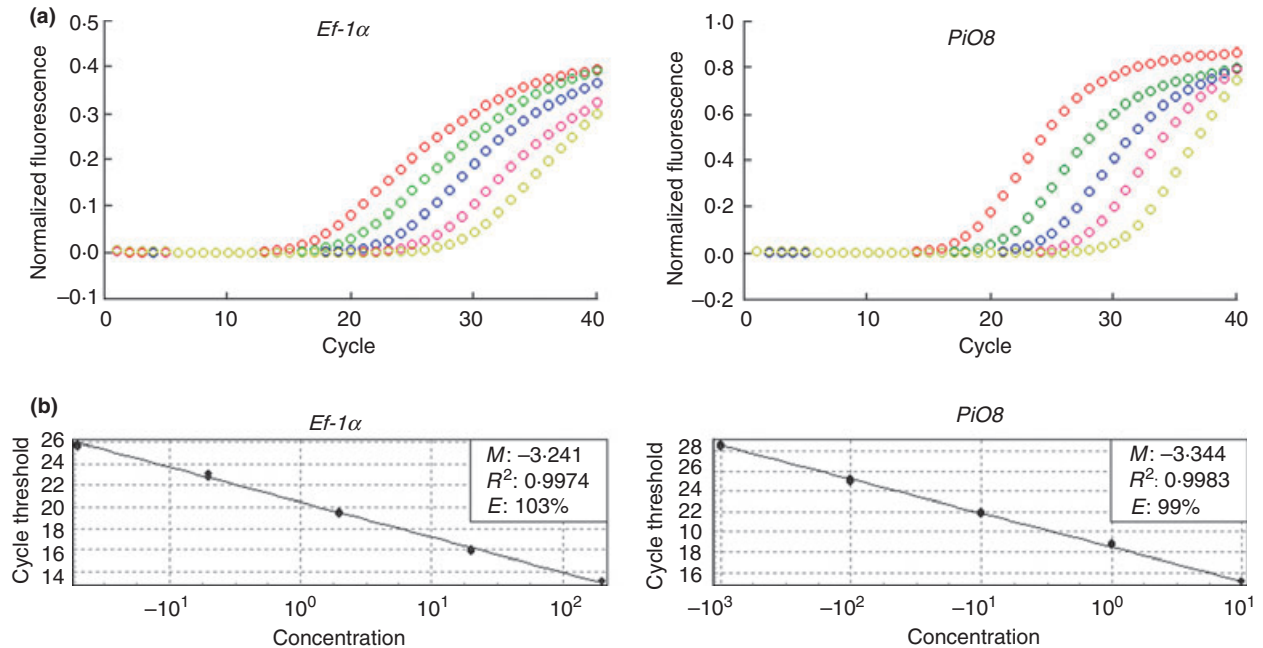
Figure 1 Quantification of serial diluted *Solanum tuberosum* and *Phytophthora infestans* DNA. (a) Quantitative real-time PCR (qPCR) amplification profiles of 10-fold serial dilutions of *S. tuberosum* (Red: 200 ng; Green: 20 ng; Blue: 2 ng; Pink: 0·2 ng; Yellow: 0·02 ng) and *P. infestans* (Red: 20 ng; Green: 2 ng; Blue: 0·2 ng; Pink: 0·02 ng; Yellow: 0·002 ng) DNA using *Ef-1α* and *PiO8* primers, respectively. Mean replicate values are depicted. (b) Standard curves for qPCR analyses of 10-fold serial dilutions of *S. tuberosum* and *P. infestans* DNA using *Ef-1α* and *PiO8* primers, respectively. *M*: slope; R^2: correlation coefficient; *E*: reaction efficiency. All standard deviations are below 5%.

primers showed amplification profiles when tested with *S. tuberosum* and *P. infestans* DNA templates, respectively (Fig. 2a). Additionally, melting curve analyses resulted in single dissociation curve peaks with specific melting temperatures for *Ef-1α* (83°C) and *PiO8* (81°C) (Fig. 2b). In contrast, no amplification was observed when *Ef-1α* primers were tested with *P. infestans* DNA or when *PiO8* primers were tested with *S. tuberosum* DNA, indicating no cross-reactivity (Fig. 2a,b). Furthermore, qPCR products were resolved in 4% agarose gel electrophoresis and resulted in single bands with the expected sizes for *Ef-1α* (126 bp) only when assayed with *S. tuberosum* DNA template and for *PiO8* (64 bp) only when assayed with *P. infestans* DNA template (Fig. 2c). The *Ef-1α* primers were also functional when tested in Bintje, Sarpo Mira and Danva cultivars (data not shown).

### *In planta* monitoring of *Phytophthora infestans* growth

The growth progress in *P. infestans* was studied for 6 days in inoculated *S. tuberosum* leaf and tuber tissues by qPCR using *Ef-1α* and *PiO8* primers. The assay allowed the detection of the pathogen DNA at 1-day postinoculation (dpi), while the first symptoms of the disease were observed at 2–3 dpi (Fig. 3). The quantified pathogen DNA increased progressively until the end of the bioassay

both in leaf and in tuber tissues, with slight variations until day 3 after inoculation and increasing sharply from that time point (Fig. 3).

To confirm that the monitored pathogen growth (Fig. 3) is not an artefact created by changes in plant DNA content, the variation in the cycle threshold values obtained for *Ef-1α* was compared during the course of the experiment. The cycle threshold values obtained for *Ef-1α* in samples 1–6 dpi were very close and not statistically different ($P \leq 0·05$) according to one-way ANOVA and subsequent *ad hoc* Tukey's multiple comparison test, suggesting that the amount of plant DNA is constant in samples from different stages of infection. Therefore, it can be expected that the assay provided an accurate quantification.

Discussion

Traditionally, potato late blight is detected by eye examination and culture isolation (Judelson and Tooley 2000). These methods are not quantitative and may lead to erroneous results because *P. infestans* may be confused with other pathogens (Judelson and Tooley 2000; Hussain *et al.* 2005). Numerous alternative methods to improve *P. infestans* detection and quantification have been reported, including qPCR (Böhm *et al.* 1999; Eschen-
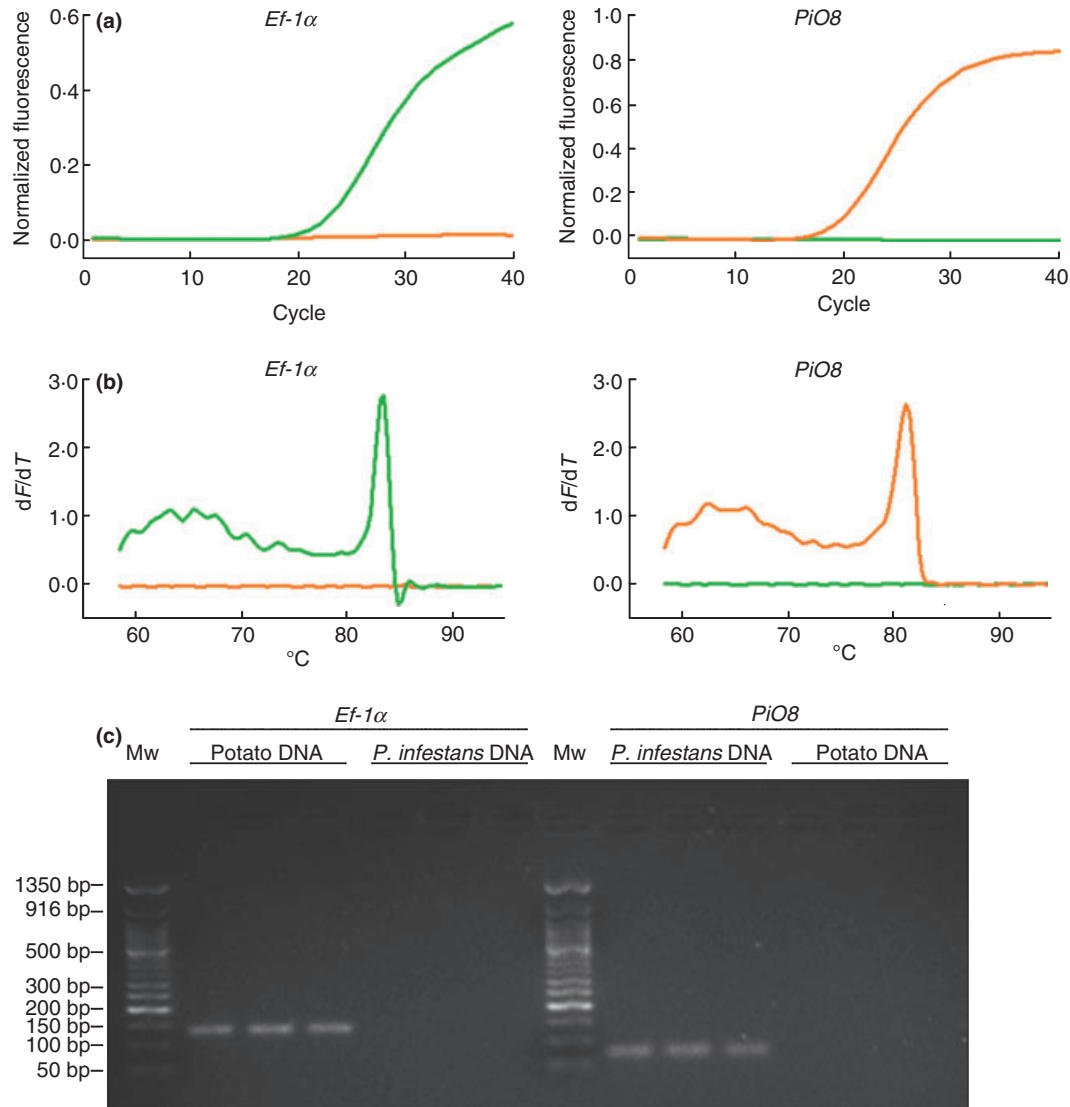
Figure 2 Specificity of *Ef-1α* and *PiO8* primers. (a) Quantitative real-time PCR amplification profiles of *Solanum tuberosum* and *Phytophthora infestans* DNA using *Ef-1α* and *PiO8* primers. (b) Melting curve profiles of qPCR amplifications of *S. tuberosum* and *P. infestans* DNA using *Ef-1α* and *PiO8* primers. *Solanum tuberosum* and *P. infestans* DNA are depicted in green and orange, respectively. (c) Electrophoretic analysis of *Ef-1α* and *PiO8* amplification products obtained with *S. tuberosum* and *P. infestans* DNA, respectively.

Lippold *et al.* 2007). Previous reports describing qPCR methods for the quantification of *P. infestans* (Böhm *et al.* 1999; Eschen-Lippold *et al.* 2007, 2009; Halim *et al.* 2007) have proposed the use of probe-based qPCRs, which are relatively expensive. Additionally, in those studies, DNA extractions were performed with commercial kits, which might be too expensive for large-scale applications, and data normalization was performed either by sample wet weight (Böhm *et al.* 1999), which may lead to high sample-to-sample variation, or by the addition of external standard DNA (Eschen-Lippold *et al.* 2007, 2009; Halim *et al.* 2007), which may underestimate the patho-

gen load and yield inaccurate results if samples are partially or totally degraded.

We developed a SYBR Green-based method to simplify and reduce assay costs (Pellissier *et al.* 2006). Additionally, as the pathogen DNA is directly normalized with the host plant DNA, an improvement in the accuracy of the quantification can be expected. Moreover, the use of a plant gene (*Ef-1α*) serves as an indicator of sample quality, allowing the exclusion of inappropriate samples. The methodology allowed the *in planta* monitoring of *P. infestans* growth in both leaf and tuber tissue, even before the first symptoms of the disease were observed.
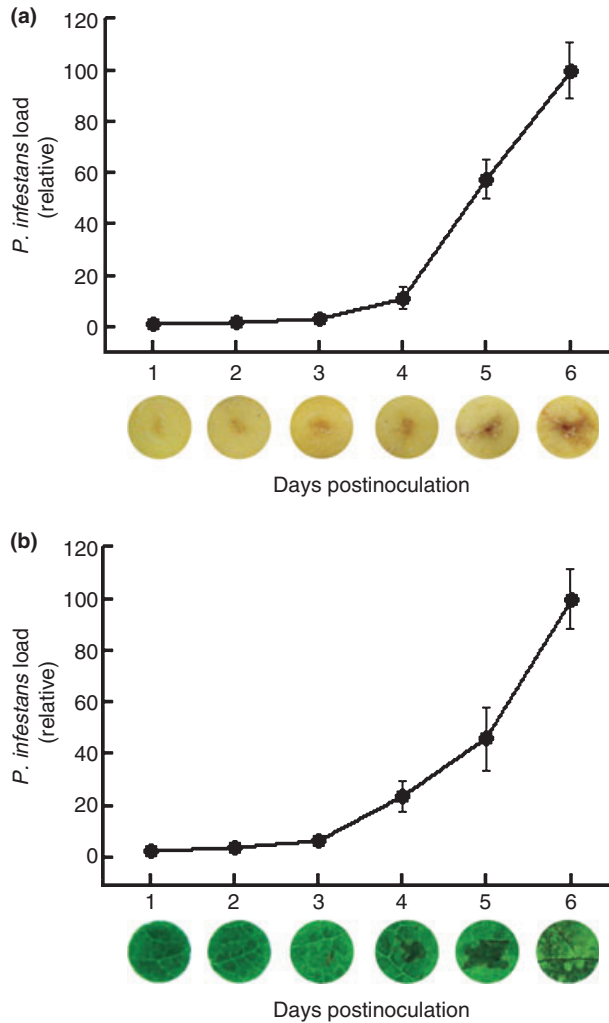
Figure 3 *Phytophthora infestans* growth in inoculated *Solanum tuberosum* leaf and tuber tissues. (a) Monitoring of *P. infestans* growth by qPCR in tuber tissue. Lower discs are representative tuber samples. (b) monitoring of *P. infestans* growth by qPCR in leaf tissue. Lower discs are representative leaf samples. The pathogen load was quantified from infected leaf and tuber samples by normalizing the *PiO8* values with the corresponding *Ef-1α* values for each individual sample. The mean values at 6 days post-inoculation were set at 100%. Error bars represent the ± SEM of 20 individual samples.

Our observations of the pathogen growth reflect the time course of infection (Judelson and Blanco 2005) and are in good accordance with preceding studies (Judelson and Tooley 2000; Halim *et al.* 2007; Eschen-Lippold *et al.* 2009). Given that the minimum amount of *P. infestans* DNA tested was 2 pg and considering that 10 pg is equivalent to 10–20 *P. infestans* nuclei (Tooley and Therrien 1987), it can be estimated that the assay could feasibly quantify as few as 2–4 pathogen cells. However, it is highly possible that the detection limit of the assay is way below this estimation because qPCR allows the detection

of genomic DNA in the order of fg (Qu *et al.* 2008) and the *PiO8* target is of high copy number (*c.* 14 000 copies per nucleus) (Judelson and Tooley 2000).

Early diagnosis of *P. infestans* by this method can contribute to the adoption of suitable measures for limiting the epidemics and the selection of appropriate control measures in the industry. The quantitative nature of the assay described in this study may be also useful in plant breeding programmes and should be valuable in basic research by allowing the study of *P. infestans* growth and the comparison of cultivars with different, and even subtle, degrees of pathogen resistance. Additionally, the simplicity and low cost of the assay holds applications for large-scale testing of new anti-oomycete compounds. Manual handling allows the processing of 50 samples per person in *c.* 6 h, and monitoring of *P. infestans* by this methodology should also be possible in tomato and the model plant *Nicotiana benthamiana* with the implementation of adequate plant primers.

Acknowledgements

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