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FULL RESEARCH PAPER

Assessment of real-time PCR as a method for determining the presence of *Verticillium dahliae* in different Solanaceae cultivars

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Abstract Real-time PCR was used to detect and quantify *Verticillium dahliae* and to assess the susceptibility of four *Capsicum annuum* cultivars (Luesia, Padrón, SCM331 and PI201234) and the *Capsicum chinense* cv. C118 to this pathogen. The symptoms which developed after infection included stunting and yellowing, and were more acute in the cv. SCM331, which also suffered defoliation in later stages of the disease and in C118, which suffered severe stunting. Quantification of the pathogen DNA in roots 23 and 34 days post-inoculation (dpi) revealed that there were significantly higher amounts of *Verticillium dahliae* DNA in C118 than in the other cultivars, followed by SCM331, Padrón and PI201234. The lowest amounts of fungal DNA in roots were found in Luesia. In hypocotyls, the highest amounts of fungal DNA were found in SCM331, while Luesia, Padrón and PI201234 had much lower amounts, and C118 had intermediate levels. When a compatible versus an incompatible system was studied, using the near-isogenic tomato lines LA3030 (susceptible) and LA3038 (resistant to *V. dahliae*), we were able to detect fungal DNA in both lines. As expected, the fungus/plant DNA ratio was lower in LA3038 than in LA3030 and it decreased with time in LA3038. The amount of *Verticillium dahliae* DNA in the roots of LA3030 remained constant between days 23 and 34 post-inoculation, but increased 10-fold in collars. Finally, when real-time PCR was applied as a diagnostic method to samples from pepper plants, soil and water collected from farms in northwest Spain, we were able to detect *V. dahliae* DNA in these samples even when symptoms of the disease were not evident.

Keywords *Capsicum annuum* · *Capsicum chinense* · *Lycopersicon esculentum* · Real-time quantitative PCR

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Introduction

Verticillium wilt, caused by the soil-borne fungus *Verticillium dahliae*, is a disease, which limits the production of a broad range of economically important crops (Pegg 1974). Among its hosts are pepper, aubergine, tomato, sunflower, potato and olive tree (Heale 1988). The first symptom of the disease is leaf chlorosis which can progress to necrosis. Other visible symptoms include stunting, epinasty, wilting, leaf abscission and, eventually, browning of the vascular system. In general, wilting is the result of a restricted water movement that is also mediated by

complex interactions between toxins, enzymes and hormones (Cooper 1984).

The genus *Capsicum* consists of approximately 22 wild species and five domesticated species, *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens* and *C. pubescens* (Bosland 1994). The cv. Padrón, one of the most widely cultivated breeds of pepper in Galicia (NW Spain), belongs to the species *C. annuum* var. *annuum*. Padrón, a locally grown breed in great demand and of high commercial value, is mainly cultivated in greenhouses. Verticillium wilt poses a serious threat to the production of pepper in this Spanish region (Pomar et al. 2001; Saavedra et al. 2003). Losses caused by this soil pathogen are significant, and no control procedure is currently available. Studies carried out to detect resistance to *Verticillium dahliae* within *C. annuum* have been unsuccessful (Barriuso et al. 1989; González-Salán and Bosland 1992; Palloix et al. 1990), making it necessary to broaden the search for sources of resistance in other pepper species. Although some authors attribute some degree of resistance against *V. dahliae* to some species such as *C. chinense*, *C. frutescens* and *C. baccatum*, according to virulence assays in which the symptoms of the disease were quantified (Gil Ortega et al. 1989), the only known resistance gene against *Verticillium* in Solanaceae is the tomato *Ve* gene. The *Ve* locus includes two tightly linked genes that code for cell-surface glycoproteins with receptor-mediated endocytosis-like signals. When used to transform potato, the individual genes were independently able to confer resistance against *Verticillium* (Kawchuk et al. 2001).

To evaluate the resistance or susceptibility of a plant to a pathogenic fungus it is necessary to quantify the degree to which it is colonised by that fungus. Of special interest among currently available methods for the direct detection and quantification of pathogens is PCR which has emerged as a major tool for the diagnosis and study of phytopathogenic fungi and has contributed to the alleviation of some of the problems derived from the detection and control of plant pathogens (Martin et al. 2000). PCR diagnostic assays use specific primers to amplify DNA from the species of interest. This approach does not require the fungus to be isolated from the infected tissue and is sensitive, rapid and less laborious than traditional methods. In the case of *Verticillium*, it has been used to quantify the rate of colonization of several crop species and has even made it possible to detect the fungus in soil (Hu et al. 1993; Nazar et al. 1991; Platt et al. 2000). In another report (Dan et al. 2001), the resistance or tolerance of different populations of potato to *V. dahliae* were evaluated according to the levels of fungal DNA. These authors used conventional PCR for the detection and quantification of the pathogen by amplification of the fungal internal transcribed sequences (ITS) (Nazar et al. 1991).

Real-time PCR technology provides new opportunities to detect and study phytopathogenicity (Silvar et al. 2005a). It facilitates the accurate, reliable and high throughput quantification of target fungal DNA in various environmental samples, including host tissues, soil, water and air, thus opening up new research opportunities for diagnosis, the study of inoculum threshold levels, epidemiology and host-pathogen interactions (Shena et al. 2004; Silvar et al. 2005a). Plants infected by *V. dahliae* only show symptoms in advanced stages of infection. Moreover, the general aspect of diseased plants is very similar to that of plants affected by *Phytophthora capsici*, or even by abiotic factors such as root hypoxia (Palazón et al. 1978). Because of its sensitivity, specificity and reproducibility, real-time PCR is suitable for identifying plant pathogens or for detecting minor changes in host resistance and susceptibility.

In the present study, real-time PCR-based diagnostics and measurements were used to quantify and compare the relative levels of *Verticillium dahliae* DNA in five cultivars of pepper and two inbred lines of tomato, one of them carrying the *Ve* resistance gene. We aimed to determine the extent of misdiagnosis based on symptom expression and whether tolerance to colonization can be satisfactorily distinguished using this quantitative approach.

Materials and methods

Plant material

Four different *C. annuum* cultivars (Luesia, Padrón, PI201234 and SCM331), one *C. chinense* cultivar (C118) and two lines of *Lycopersicon esculentum* were used. Tomato accessions LA3030 and LA3038 were requested from the CM Rick Tomato Genetics Resource Centre (UC Davis, CA, USA). LA3030 and LA3038 are near-isogenic lines that differ mainly in

the presence of the *Ve* and *I* (resistance to *Fusarium oxysporum* f. sp. *lycopersici*) genes in LA3038 plants. Plants were grown and kept in autoclave-sterilized vermiculite in a growth chamber at 25°C and a photoperiod of 16 h light and 8 h darkness. Sixteen-day-old plants were transplanted into individual pots with sterile soil-perlite (2:1) and inoculated 2 weeks later.

Fungal material and inoculation

The *Verticillium dahliae* isolate VD53 was collected in northwest Spain (La Coruña, Galicia) from infected pepper plants during a survey conducted in 1998 (Pomar et al. 2001). VD53 was found to be highly virulent in the different cultivars analysed (Novo et al. 2006). This isolate was maintained on water agar until inoculation. The inoculum was prepared by growing the isolate on PDA plates at room temperature for 20 days in the dark. Conidial suspensions were prepared by washing each plate with sterile water and scraping the cultures with a rubber spatula. Mycelial fragments were removed by filtration through a double layer of sterile cheese-cloth. The inoculum was adjusted to 10^7 conidia ml^{-1}. One ml of conidial suspension was directly pipetted over the soil surface for each pot. Control plants were mock-inoculated with sterile water. In each experiment, three plants were inoculated. The experiments were repeated three times.

Greenhouse samples from water, soil and plants

The sampling was carried out in two production areas of northwest Spain, Padrón and Betanzos, in July 2003. Three farms were sampled in Padrón (P1, P2 and P3) and five in Betanzos (B1, B2, B3, B4 and B5). The severity of the infection in different greenhouses was rated after visual inspection: non-affected, when no symptoms were observed; slightly affected, when the number of infected plants was <10%; relatively affected, when 10–25% of the plants showed some symptoms, and strongly affected, when >25% of the plants showed some degree of infection. Pepper plants cv. Padrón showing symptoms of Verticillium wilt and healthy plants were collected in the field from eight farms. Soil and water samples were also collected in the same farms where the plants had been harvested. From the plants, only the collar zone was collected, since this has been reported to be the penetration area. Water samples were directly taken from the irrigation system. Different numbers were assigned to the different zones of the greenhouses, and the plots were distributed using a table of random numbers. Water and soil samples were kept in sterile plastic containers until DNA extraction.

DNA extraction

Samples were taken at 23 and 34 days post-inoculation (dpi). In each of the three inoculation experiments roots and hypocotyls from several pepper plants, or roots and collars from tomato plants were analysed. The organs were detached and pooled into a sample that was frozen in liquid nitrogen. DNA extraction was performed by a CTAB method (Edwards et al. 1991). The same method was used for the extraction of DNA from *Verticillium dahliae* liquid cultures. Soil organisms were disrupted by grinding samples in liquid nitrogen with the natural abrasives present in soil. Losses due to degradation and absorption were largely eliminated by the addition of skim milk powder. DNA from disrupted cells was extracted with sodium dodecyl sulphate-phenol and collected by ethanol precipitation (Volossiouk et al. 1995). Five ml of irrigated water from each greenhouse was mixed with 25 ml of Czapec liquid medium and incubated at 20°C in the dark for 20 days. After centrifugation, DNA was extracted from the pellet as above.

Primer design

In real-time PCR, specific target sequences must be used for the detection and identification of species during analysis. We used a sequence characterized amplified region (SCAR) from *Verticillium dahliae* (Li et al. 1999). The *C. annuum*, *C. chinense* and *L. esculentum* primer sets were designed using the programme Primer-3 (Rozen and Skaletsky 2000), from sequences stored in Genbank. The primer sequences and the amplified genes are detailed in Table 1.

Verticillium DNA PCR

The concentration of DNA from infected plants was calculated spectrophotometrically (Spectrophotometer UV-Visible, Cary 3E, Varian). To check if an

Table 1 Sequences of primers used for quantification of *Verticillium dahliae* by real-time polymerase chain reaction

Primer name	Sequence (5′-3′)	Target	Accession number	Fragment length (bp)
VDS1	CACATTCAGTTCAGGAGACGGA	*V. dahliae*; SCAR	U23151	520
VDS2	CCGAAATACTCCAGTAGAAGG			
CAPR1F	GTTGTGCTAGGGTTCGGTGT	*C. annuum; PR1*	AF053343	301
CAPR1R	CAAGCAATTTAAACGATCCA			
CChPAL1F	AGCAATGTGCAATGGACAAG	*C. chinense; PAL*	AF081215	301
CChPAL1R	GCAGTATGCTAACTCCATGACAA			
LEPR1F	GCAACACTCTGGTGGACCTT	*L. esculentum;PR1*	X71592	272
LEPR1R	ATGGACGTTGTCCTCTCCAG			

excess of plant DNA could inhibit the amplification of fungal DNA, we prepared 10-fold serial dilutions of the samples. The dilutions were used as templates for conventional PCR with *Verticillium dahlia*e primers. The thermal cycling conditions were an initial denaturation at 95°C for 2 min followed by 50 cycles at 95°C for 30 s, 62°C for 30 s and 72°C for 1 min. A final extension step at 72°C for 5 min was added. Amplification reactions were run on a 1% agarose gel and visualized on a UV transilluminator (UV transilluminator 2000, Biorad). In most cases, no amplification product was visualized on an agarose gel. To check for fungal DNA amplification in real-time PCR experiments, we chose those dilutions where the fungal amplicon could be seen on an agarose gel.

Real-time PCR assays

Twenty-µl PCR samples were prepared by mixing 2.5 µl of DNA extract with 1X iQ SYBR Green Supermix (Biorad, Hercules CA, USA) and 0.3 µl of each primer at 10 µM. Negative control reactions contained 2.5 µl of sterile water instead of DNA. The thermal cycling conditions for the different plant primers were: an initial denaturation step at 95°C for 2 min followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. A final extension step at 72°C for 5 min was added. The thermal cycling conditions for the *Verticillium dahliae* primers were as described previously. Serial dilutions of pure genomic DNA were used to trace a calibration curve (Fig. 1). At least three independent calibrations were performed for each DNA sample. The standard curves obtained were highly reproducible, and all surpassed 100% efficiency. To determine the detection threshold for *Verticillium dahliae* DNA in plants, mixtures of pepper DNA at 100 ng μl^{-1} were prepared with fungal DNA at concentrations ranging from 0.01 to 10 ng μl^{-1}. These mixtures were used as templates for amplification using the *Verticillium dahliae* primers. Amplification of each sample was repeated twice. After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 60 to 95°C. Fluorescence of the target amplicon was detected at 84°C. All results are presented as pg *Verticillium dahliae* DNA/ng plant DNA. For conventional PCR, the same conditions of real-time PCR were employed, except for the use of Taq Polymerase (Roche Applied Science, Barcelona, Spain).

Statistical analysis

Data analyses were performed using a statistical analysis of variance (ANOVA) with the SPSS 11.5 software (Norusis and SPSS 1990). The model one-way ANOVA with non-randomized blocks best suited our results, considering the cultivars as the treatment and time after inoculation and organs as blocks, and taking into account that root and hypocotyl/collar samples came from the same plants. A *t*-student test was used to identify significant differences among groups, with $\alpha \leq 0.05$.

Results

Assay specificity

The usual real-time quantitative PCR controls were performed to check for the linearity of amplification over the dynamic range. Figure 1 shows the

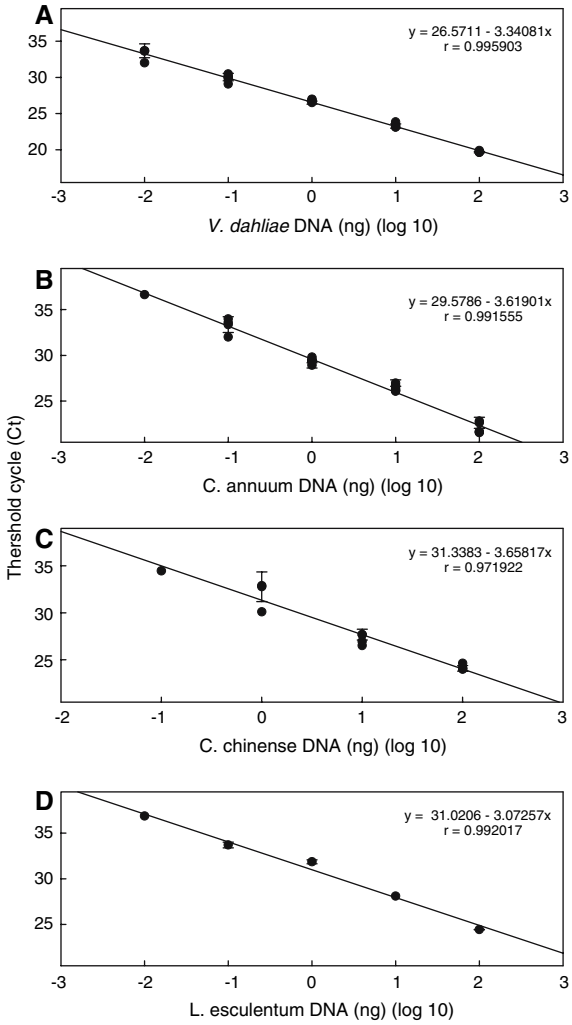

Fig. 1 Standard curves for real-time PCR of 10-fold serial dilutions of (**A**) *Verticillium dahliae*; (**B**) *L. esculentum*; (**C**) *C. annuum* and D, *C. chinense*. Cycle thresholds (Ct) were plotted against the log of known concentrations of genomic DNA standards and linear regression equations were calculated for the quantification of the unknown samples by interpolation

regression curves obtained after amplification of 1:10 serial dilutions of 100–0.01 ng of *Verticillium dahliae*, *C. annuum*, *C. chinense* and *L. esculentum* genomic DNA with the VDS1/VDS2, CAPR1F/CAPR1R, CChPAL1F/CChPAL1R and LEPR1F/LEPR1R primers, respectively. The standard curves obtained revealed high precision and reproducibility between replications as indicated by correlation coefficients ranging from $r = 0.972$ to $r = 0.996$. By means of conventional PCR, we checked that each set of primers was species-specific and did not amplify DNA from the remaining organisms used in this study or from other Solanaceae pathogens widely distributed in the Galician plantations of pepper (Pomar et al. 2001), such as *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *lycopersici*, *P. capsici*, *Rhizoctonia solani* and *V. albo-atrum* (Fig. 2). The use of mixtures of plant and fungal DNA as PCR templates allowed us to establish a detection threshold between 0.1 and 1 ng μl^{-1} for fungal DNA in infected plants. To check for primer specificity, the resulting amplification products were sequenced and the sequences were identical to those of the genes from which the primers were designed.

Detection and quantification of *Verticillium dahliae* in pepper plants

Four *C. annuum* cultivars (SCM331, Padrón, PI201234 and Luesia) and one *C. chinense* cultivar (C118) were inoculated with *Verticillium dahliae* and samples were taken 23 and 34 dpi. Fungal DNA was quantified in roots and hypocotyls by real-time PCR. The disease symptoms described in Table 2 were visible in plants 24 dpi. SCM331 defoliated, in contrast with the remaining cultivars, where stunting and the presence of a lesion in the collar were the most conspicuous symptoms. Stunting was particularly severe in C118. After DNA extraction and real-time PCR, root and hypocotyl samples from the

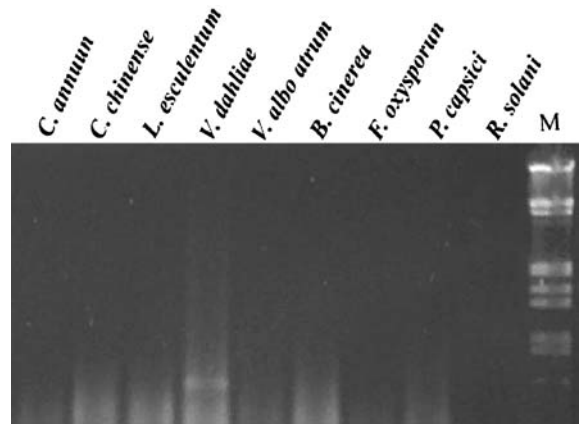

Fig. 2 Specificity of *Verticillium dahliae*-specific primers VDS1/VDS2. Lane 1, *C. annuum*; lane 2, *C. chinense*; lane 3, *L. esculentum*; lane 4, *Verticillium dahliae* VD53 (positive control); lane 5, *V. albo-atrum*; lane 6, *B. cinerea*; lane 7, *F. oxysporum*; lane 8, *P. capsici*; lane 9, *R. solani*; lane 10, M, molecular marker

different cultivars were analysed (Fig. 3). Significant differences in the *Verticillium dahliae* DNA/pepper DNA ratio at $\alpha = 0.05$ were found when the cultivars were compared. No significant differences in DNA ratio within roots of the cvs. SCM331, Padrón and PI201234 were found, nor between the ratios in roots of PI201234 and Luesia. In contrast, *C. chinense* C118 roots had a significantly higher ratio of fungus/plant DNA than all the *C. annuum* cultivars at both sampling times. As regards the DNA ratios in hypocotyls, no differences were found between Padrón, PI201234 and Luesia, although differences in ratios were observed between C118 and SCM331 hypocotyls, both of which differed from the ratios of the other cultivars. In hypocotyls, the highest ratio was observed 34 dpi in SCM331. A general increase was found in the *Verticillium dahliae* /plant DNA ratio, between 23 and 34 dpi both in roots and hypocotyls for all the cultivars tested. Fungal DNA could not be detected in PI201234 or Luesia roots 23 dpi, possibly because it remained below the detection threshold.

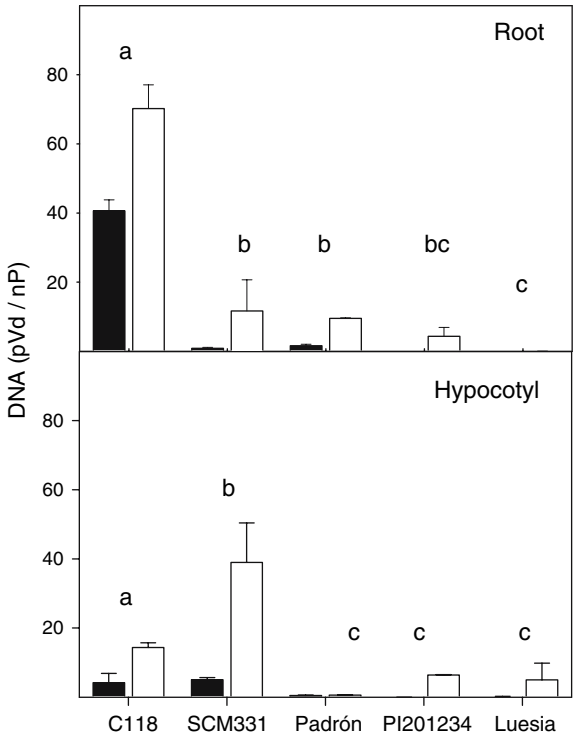

Fig. 3 Detection and quantification of *Verticillium dahliae* DNA in four cultivars of *C. annuum*, SCM331, Padrón, PI201234 and Luesia and one cultivar of *C. chinense*, C118, at 23 (■) and 34 (□) days after inoculation in roots and hypocotyls. The DNA content is expressed as pg fungal DNA ng plant DNA. The results are the mean of three independent real-time quantitative assays that included two replications for each DNA sample. Error bars represent the standard deviation. Cultivars labelled with the same letter are not significantly different at $\alpha = 0.05$

Detection and quantification of *Verticillium dahliae* in susceptible and resistant tomato plants

Two near-isogenic tomato lines, one susceptible (LA3030) and one resistant (LA3038), were inoculated with *Verticillium dahliae*, and fungal DNA was quantified in roots and collars by real-time PCR in samples taken 23 and 34 dpi. Significant differences in fungus/plant DNA ratios were found between the inoculated tomato cultivars when roots and collars were compared (Table 3). In LA3030, the fungal DNA/plant DNA ratio in roots remained constant during this time but the ratio in the collar increased 10-fold from 23 dpi to 34 dpi. In LA3038, the DNA ratios were significantly lower in both organs and decreased with time.

Detection and quantification of *Verticillium dahliae* in greenhouse samples

To verify the practical applicability of the method, a survey of Verticillium wilt of pepper was carried out in eight farms in northwest Spain, located in Betanzos

Table 2 Symptoms observed in the cultivars analysed 34 dpi

Species	Cultivar	Symptomatology
C. chinense	*C118*	Severe stunting
C. annuum	SCM331	Defoliation, stunting, mild lesion and hardening of the collar
	Padrón	Stunting, lesion and hardening of the collar
	PI201234	Stunting, mild lesion and hardening of the collar
	Luesia	Stunting, mild lesion and hardening of the collar
L. esculentum	LA3030	Stunting, mild lesion and hardening of the collar
	LA3038	Symptomless

Table 3 Detection and quantification of *Verticillium dahliae* DNA in two cultivars of *L. esculentum*, one susceptible (LA3030) and one carrying the resistance gene *Ve* (LA3038) ($\alpha = 0.05$)

	Symptom[a]	pg DNA fungus / ng DNA plant (Mean ± SD)[b]			
		23 days post inoculation		34 days post inoculation	
		Root	Collar	Root	Collar
LA3030	+	5.72 ± 1.24a	2.38 ± 0.67b	5.03 ± 3.13a	25.6 ± 0.81c
LA3038	–	0.55 ± 0.05d	0.006 ± 0.0007e	0.01 ± 0.006f	0.002 ± 0.002g

The results are the mean of three independent real-time quantitative assays that included two replications for each DNA sample

[a] (+) Plant with symptoms; (−) plant without symptoms

[b] Means followed by the same letter are not significantly different

(B) and Padrón (P) (Table 4). In greenhouses P3 and B5 no Verticillium wilt symptoms were observed; in greenhouses P2, B2 and B3 very few diseased plants were seen; in greenhouses P1 and B1 a few isolated diseased plants were found and, finally, greenhouse B4 presented extensive patches of plants showing symptoms of the disease, wilting and dwarfing.

Table 4 Detection and quantification of *Verticillium dahliae* in different farms of Betanzos (B) and Padrón (P) in northwest Spain

Farm[a]	Plant[b]	pg fungus DNA/pg plant DNA (Media ± SD)	Detection in soil[c]	Detection in irrigation water[c]
P1**	1^+	0.087 ± 0.002	+	+
	2^-	0.002 ± 0.0006		
P2*	1^-	–	+	+
	2^-	0.002 ± 0.0009		
P3–	1^-	0.002 ± 0.0009	+	–
	2^-	0.919 ± 0.199		
B1**	1^-	–	+	+
	2^+	0.009 ± 0.0003		
B2*	1^-	–	–	–
	2^-	–		
B3*	1^-	–	–	–
B4***	1^+	0.608 ± 1.03	+	–
	2^+	0.127 ± 0.0015		
B5–	1^-	–	–	–

[a] Ratio of infection of farm, (−) not affected, (*) < 10% infected plants, (**) >10 < 25% infected plants and (***) > 25% infected plants

[b] (+) Plant with symptoms; (−), plant without symptoms

[c] (+) Positive detection and amplification of fungus DNA and (−) negative detection. The results are the mean of two independent real-time quantitative assays that included two replications for each DNA sample

Collected samples were analysed and fungal DNA in plants quantified by real-time PCR. Amplification reactions were considered negative when no fluorescence signal could be detected after 40 cycles. *Verticillium dahliae* was detected in plants coming from the three greenhouses from Padrón, and also in soil and irrigation water. From the five greenhouses in Betanzos, *Verticillium dahliae* was only detected in plants and soil from greenhouse B4, where disease symptoms were evident, but fungal DNA was not detected in the irrigation water. In greenhouses P1 and B1, *Verticillium dahliae* was detected and quantified in most plants, and in the soil and irrigation water. In greenhouse P2, *Verticillium dahliae* was detected in one plant. In greenhouses B2 and B3 the pathogen was not detected. The pathogen was not detected in greenhouse B5, where disease symptoms had not been observed. Symptoms were not observed in greenhouse P3, but in this case the pathogen was detected in plant and soil. The results of the amplification of the different samples with the specific primers of *C. annuum* and *Verticillium dahliae* correlated well with the general aspect of the farms and the proportion of infected plants.

Discussion

Verticillium wilt has been reported as the main pathological problem in peppers growing in open fields of the Ebro Valley in Spain (Palazón and Palazón 1989) and in many Galician farms (Pomar et al. 2001; Saavedra et al. 2003). Since the disease produced by *Verticillium dahliae* cannot currently be controlled by chemicals, the use of resistant varieties

is considered to be the only efficient method of control. Initially, we looked for cultivars of *C. annuum* showing some degree of resistance against *Verticillium dahliae* and chose cv. Luesia based on previous reports (Barriuso et al. 1989; Pomar et al. 2004; Novo et al. 2006). The local *C. annuum* cv. Padrón was included because of its importance in Galicia, and also because its degree of resistance to *Verticillium dahliae* was unknown. We analysed the cvs SCM331 and PI201234 because of their known resistance against *P. capsici*, another pepper pathogen that displays symptoms resembling those produced by *Verticillium dahliae* (Bosland and Lindsey 1991; Gil Ortega et al. 1991). The *C. chinense* cv. C118 was included since some data in the literature suggested a certain degree of resistance to *Verticillium dahliae* in the field (Gil Ortega et al. 1989), although results obtained after inoculation in the laboratory were not conclusive. Finally, since no genes conferring complete resistance against *Verticillium dahliae* are known in pepper, we decided to include two near-isogenic lines of tomato, *L. esculentum*, one of them carrying the resistance gene *Ve*.

Bhat et al. (2003), studying Verticillium wilt in pepper plants, observed the appearance of symptoms approximately 24 dpi. In our experiments, plants did not show any visible symptoms until at least this time, and so we decided to collect samples 23 and then 34 dpi, when symptoms could be evident.

SCAR primers have been used for many fungi and have been shown to be particularly useful when closely-related species or specific strains need to be identified. For example, Vandermark and Baker (2003) used SCAR primers to monitor *Phytophthora medicans* in susceptible and resistant alfalfa. Li et al. (1999) described the development of a SCAR marker specific for *Verticillium dahliae*. This pair of primers did not amplify DNA from other fungi, including the closely related pathogen *V. albo-atrum*.

In the present study, *Verticillium dahliae* DNA was amplified with SCAR specific primers and pepper and tomato DNA with species-specific primers using defense genes as targets. Cycle threshold values depend on both the input of starting copies and the genomic organization of the target sequences (Böhm et al. 1999). For detection, we used SYBR Green I since this is a single and reliable low-cost method for monitoring PCR amplification and for quantifying template DNA. The amplification of single gene sequences from both the plant and the pathogen allowed us to obtain results correlated with the actual number of *Verticillium dahliae* genome copies invading the plant.

Several papers on the use of real-time PCR for the detection of pathogenic fungi in plants (Böhm et al. 1999; Gachon and Saindreman 2004; Hayden et al. 2004; Mercado-Blanco et al. 2003; Qi and Yang 2002; Vandermark and Baker 2003), irrigation water (Kong et al. 2003) and soil (Van de Graaf et al. 2003) have been published recently. For example, Van de Graaf et al. (2003) demonstrated that real-time PCR assays developed for the detection and quantification of *Spongospora subterranea* could detect the pathogen in water, soil and plant tissue. Real-time PCR technology was also applied to simultaneously quantify *Phaeocryptopus gaeumannii* and Douglas-fir DNA and to derive a relative measurement of pathogen colonization of host tissue (Winton et al. 2002).

Despite previous reports (Barriuso et al. 1992), cv. Luesia was found to be susceptible. It showed symptoms similar to those displayed by Padrón and PI201234, and the quantification of the fungus/plant DNA ratio in infected stems allowed us to group the three cultivars in the same susceptibility group. In roots, SCM331 behaved much like cvs Padrón and PI201234. As in SCM331, the fungal DNA content of Luesia hypocotyls was higher than the root content.

In summary, there was a correlation between the severity of the symptoms displayed by infected plants of the different genotypes and the relative amount of fungal DNA. Pepper plants of the cultivars showing milder symptoms (Padrón, PI201234 and Luesia) presented a lower fungus/plant DNA ratio. On the other hand, plants of the cvs C118 and SCM331, with more severe symptoms, had a higher pathogen/plant DNA ratio. We also found an uneven distribution of fungal DNA when comparing fungus/plant DNA ratios in roots and hypocotyls from different pepper cultivars (Fig. 3). Interestingly, the highest ratios in hypocotyls were found in cv. SCM331, where symptoms included defoliation (Table 2) and the highest ratios in roots appeared in C118, where stunting was most severe. Taken together, the results suggest that there is a correlation between the abundance of fungal DNA and the nature and severity of the symptoms.

As regards *L. esculentum*, the fungus/plant DNA ratio in the collar of the susceptible cv. LA3030 increased with time after inoculation, in contrast to that what was observed in roots and collars from the resistant cv. LA3038, where the fungus/plant DNA ratios decreased with time. The fungus penetrated the roots of both resistant and susceptible plants. The fungus/plant DNA ratio in LA3038 roots 23 dpi was 10-fold lower than in LA3030, but in the collar of the resistant cultivar fungal DNA was hardly detectable. From 23 to 34 dpi, the fungal/plant DNA ratio increased 10-fold in susceptible collars but remained constant in roots. The ratio of fungal to plant DNA in both roots and collars decreased in resistant tomatoes 34 dpi, suggesting some containment of the pathogen. The similar fungus/plant DNA ratios in LA3030 roots observed 23 and 34 dpi may reflect total colonization of the tomato roots by 23 dpi. At this stage of infection, susceptible root growth stops, and a very limited increase in root size, if any, is observed in roots between 23 dpi and 34 dpi. It is possible, then, that root infection cannot progress much beyond 23 dpi. Contrary to the common interpretation, the collar may not be the primary penetration area, at least in tomato or, alternatively, an active resistance response might be responsible for the hypertrophy of the resistant plant collar and, as a result, for the much lower fungus plant DNA ratio found in resistant roots compared with the roots of susceptible plants.

Finally, to verify the practical applicability of the PCR method, samples of pepper plants cv. Padrón, irrigation water and soil were collected from greenhouses. In farms where disease symptoms were observed, P1, B1 and B4, *Verticillium dahliae* was detected in individual plants, soil and, except for B4, in the irrigation water. In P2 and P3, where disease symptoms were not clear, the fungus was detected in plants, soil and, in the case of P2, in water. These samples were all positive for the detection of *P. capsici*. The symptoms presented by the plants affected by this pathogen are -similar to the symptoms of Verticillium wilt which can lead to confusion (Silvar et al. 2005b). We were unable to detect *Verticillium dahliae* in plots B2 and B3, where disease symptoms were slight, or in B5, where disease symptoms were not observed. Interestingly, *P. capsici* was detected in the two plants collected from greenhouse B2 (corresponding to Farm 4 in Silvar et al. 2005b).

Practical application of the described method seems clear, since it was possible to detect the pathogen in plants, soil and water before visible symptoms of the disease appeared. Due to the great difficulties in the eradication of *Verticillium dahliae*, all possible lines of action should be addressed to prevent contamination. In this sense, real-time PCR offers the possibility of the early detection of the fungus in plants, soil and water. Whenever suspicion of infection exists, screening for the presence of *Verticillium dahliae* should be carried out before the transport of plants or soil material is considered.

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References

Barriuso J, Gil Ortega, R., & Palazón C (1992) Influence of temperature on the expression of partial resistance in pepper after artificial inoculation with *Verticillium dahliae* Kleb. In: Proceedings of the VIII Meeting on Genetics and Breeding on Capsicum and Eggplant, Rome, Italy (pp. 201–204).

Barriuso, J., Palazón, C., Gil Ortega, R., & Delgado, I. (1989). Characterization of *Verticillium dahliae* Kleb. isolates on a set of *Capsicum* spp. *Capsicum Newsletter*, *11*, 37–38.

Bhat, R. G., Smith, R. F., Koike, S. T., Wu, B. M., & Subbarao, K. V. (2003). Characterization of *Verticillium dahliae* isolates and wilt epidemics of pepper. *Plant Disease*, *87*, 789–797.

Böhm, J, Hahn, A., Schubert, R., Bahnweg, G., Adler, N., Nechwatal, J., Oehlmann, R., & Obwald, W. (1999). Real-time quantitative PCR. Determination in isolate spores of the mycorrhizal fungus *Glomus mosseae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in the respective host plants. *Journal of Phytopathology*, *147*, 409–416.

Bosland, P. W. (1994). Chile: History, cultivation, and uses. In G Charalambous (Eds.) *Spices, herbs, and edible fungi* (pp. 347–366). New York, NY, USA: Elsevier Publisher.

Bosland, P. W., & Lindsey, D. L. (1991). A seedling screen for Phytophthora root rot of pepper, *Capsicum annuum*. *Plant Disease*, *75*, 1048–1050.

Cooper, R. M. (1984). The role of cell wall degrading enzymes in infection, damage. In R. K. S. Wood & G. J. Jellis (Eds.) Plant disease: Infection, damage and loss (pp. 13–27). Oxford, UK: Blackwell.

Dan, H., Ali-Khan, S. T., & Robb, J. (2001). Use of quantitative PCR diagnostics to identify tolerance and resistance to *Verticillium dahliae* in potato. *Plant Disease*, *85*, 700–705.

Edwards, K., Johnstone, C., & Thompson, A. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research*, *19*, 1349.

Gachon, C., & Saindreman, P. (2004). Real-time PCR monitoring of fungal development in *Arabidopsis thaliana* infected by *Alternaria brassicola* and *Botrytis cinerea*. *Plant Physiology and Biochemistry*, *42*, 367–371.

Gil Ortega, R., Palazón, C., & Barriuso, J. (1989). Búsqueda de resistencia a *Verticillium dahliae* en especies diferentes de *Capsicum annuum*. In Actas de las VII Jornadas de Selección y Mejora de Plantas Hortícolas, Badajoz, Spain (pp. 83–89).

Gil Ortega, R., Palazón, C., & Zueco, J. C. (1991). Genetics of resistance to *Phytophthora capsici* in the pepper line ''SCM-334''. *Plant Breeding*, *107*, 50–55.

González-Salán, M. M., & Bosland, P. W. (1992). Sources of resistance to Verticillium wilt in *Capsicum*. *Euphytica*, *59*, 49–53.

Hayden, K. J., Rizzo, D., Tse, J., & Garbeloto, M. (2004). Detection and quantification of *Phytophthora ramorum* from California Forest using real-time polymerase chain reaction assay. *Phytopathology*, *94*, 1075–1083.

Heale, J. B. (1988). *Verticillium* spp., the cause of vascular wilts in many species. In: J. H. Andrews & I. C. Tommerup (Eds.) Advances in Plant Pathology (pp. 291–312). London, UK: Academic Press Inc.

Hu, X., Nazar, R. N., & Robb, J. (1993). Quantification of *Verticillium* biomass in wilt disease development. *Physiological and Molecular Plant Patholology*, *42*, 23–36.

Kawchuk, L. M., Hachey, J., Lynch, D. R., Kulcsar, F., van Rooijen, G., Waterer, D. R., Robertson, A., Kokko, E., Byers, R., Howard, R. J., Fischer, R., & Prüfer, D (2001). Tomato *Ve* disease resistance genes encode cell surface-like receptors. *Proceedings of the National Academy of Sciences of the USA*, *98*, 6511–6515.

Kong, P., Hong, C., Jeffers, S. N., & Richardson, P. A. (2003). A specie-specific polymerase chain reaction assay for a rapid detection of *Phytophthora nicotiana* in irrigation water. *Phytopathology*, *93*, 822–831.

Li, K. N., Rouse, D. I., Eyestone, E. J., & German, T. L. (1999). The generation of specific DNA primers using random amplified polymorphic DNA and its application to *Verticillium dahliae*. *Mycological Research*, *103*, 1361–1368.

Martin, R. R., James, D., & Levesque, C. A. (2000). Impact of molecular diagnostic technologies on plant disease management. *Annual Review of Phytopathology*, *38*, 207–239.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., & Jiménez-Díaz (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. *Physiological and Molecular Plant Pathology*, *63*, 91–105.

Nazar, R. N., Hu, X., Schmidt, J., Culham, D., & Robb, J. (1991). Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of Verticillium wilt pathogens. *Physiological and Molecular Plant Pathology*, *39*, 1–11.

Norusis, M. J., & SPSS Inc. (1990). SPSS advanced statistics user's guide. SSPS Inc, Chicago, IL, USA.

Novo, M., Pomar, F., Gayoso, C., & Merino, F. (2006). Cellulase activity in isolates of *Verticillium dahliae* differing in aggressiveness. *Plant Disease*, *90*, 155–160.

Palazón, C., & Palazón, I. (1989). Estudios epidemiológicos sobre la ''tristeza'' del pimiento en la zona del valle medio del Ebro. *Boletín de Sanidad Vegetal Plagas*, *15*, 233–262.

Palazón, C., Gil, R., & Palazón, I. (1978). La ''tristeza o seca del pimiento. Estado actual del problema. *ITEA*, *32*, 56–62.

Palloix, A., Pochard, E., Phaly, P., & Daubèze, A. M. (1990). Recurrent selection for resistance to Verticillium dahliae in pepper. *Euphytica*, *47*, 79–89.

Pegg, G. F. (1974). *Verticillium* diseases. *Review of Plant Pathology*, *53*, 157–182.

Platt, H. W., Mahuku, G. S., Maxwell, P., & MacLean, V. (2000). Detection techniques for research on *Verticillium* species in potato soils. In E. C. Tjamos, R. C. Rowe, J. B. Heale & D. R. Fravel (Eds.) *Advances in Verticillium research and disease management* (pp. 140–143). Saint Paul, MN, USA: APS Press.

Pomar, F., Bernal, M. A., Collar, J., Díaz J, Caramelo, C., Gayoso, C., Novo, M., Prego, C., Saavedra, A., Silvar, C., & Merino, F. (2001). A survey of ''tristeza'' of pepper in Galicia and the fungal pathogens causing the disease. *Capsicum & Eggplant Newsletter*, *20*, 90–93.

Pomar, F., Novo, M., Bernal, M. A., Merino, F., & Ros Barceló, A. (2004). Changes in stem lignins (monomer composition and cross–linking) and peroxidase are related with the maintenance of leaf photosynthetic integrity during Verticillium wilt in Capsicum annuum. *New Phytolologist*, *163*, 111–123.

Qi, M., & Yang, Y. (2002). Quantification of *Magnaporthe grisea* during infection of rice plants using real-time polymerase chain reaction and Northern blot/phosphoimaging analyses. *Phytopathology*, *92*, 870–876.

Rozen, S., & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In S. Krawetz & S. Misener (Eds.) *Bioinformatics methods and protocols: Methods in molecular biology* (pp. 365–386). Totowa, NJ, USA: Humana Press.

Saavedra, A. M., Díaz, J., & Merino, F. (2003). Virulence of different isolates causing Verticillium wilt of pepper in the Padron region. *Spanish Journal of Agricultural Research*, *1*, 53–57.

Shena, L., Nigro, F., Ipolito, A., & Gallitelli, D. (2004). Real-time quantitative PCR: A new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology*, *110*, 893–908.

Silvar, C., Díaz, J., & Merino, F. (2005a). Real-time polymerase chain reaction quantification of *Phytophthora capsici* in different pepper genotypes. *Phytopathology*, *95*, 1423–1429.

Silvar, C., Duncan, J. M., Cooke, D. E. L., Williams, N. A., Díaz, J., & Merino, F. (2005b). Development of specific PCR primers for identification and detection of *Phytophthora capsici* Leon. *European Journal of Plant Pathology*, *112*, 43–52.

Van de Graaf, P., Lees, A. K., Cullen, D. W., & Duncan, J. M. (2003). Detection and quantification of *Spongospora subterranea* in soil, water and plant tissues samples using

real-time PCR. *European Journal of Plant Pathology*, *151*, 577–583.

Vandermark, G. J., & Baker, B. M. (2003). Quantifying *Phytophthora medicans* in susceptible and resistant alfalfa with a real-time fluorescent PCR. *Journal of Phytopathology*, *151*, 577–583.

Volossiouk, T., Robb, E. J., & Nazar, R. N. (1995). Direct DNA extraction for PCR mediated assays of soil organisms. *Applied and Environmental Microbiology*, *61*, 3972–3976.

Winton, L. M., Stone, J. K., Watrud, L., & Hansen, E. M. (2002). Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. *Phytopathology*, *92*, 112–116.