Real-Time Polymerase Chain Reaction Quantification of *Phytophthora capsici* in Different Pepper Genotypes

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ABSTRACT

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Reliable and sensitive quantification of *Phytophthora capsici* in pepper plants is of crucial importance in managing the multiple syndromes caused by this pathogen. A real-time polymerase chain reaction (PCR) assay was developed for the determination of *P. capsici* in pepper tissues. DNA levels of a highly virulent and a less virulent isolate were measured in different pepper genotypes with varying degrees of resistance. Using SYBR Green and specific primers for *P. capsici*, the minimal amount of pathogen DNA quantified was 10 pg. Pathogen DNA was recorded as early as 8 h

postinoculation. Thereafter, the increase was rapid in susceptible cultivars and slower in resistant ones. The amount of pathogen DNA quantified in each pepper genotype correlated with susceptibility to *Phytophthora* root rot. Likewise, there was a relationship between the virulence of the pathogen and the degree of colonization. Differences also were found in oomycete amount among pepper tissues, with maximal pathogen biomass occurring in stems. The real-time PCR technique developed in this study was sensitive and robust enough to assess both pathogen development and resistance to *Phytophthora* root rot in different pepper genotypes.

Additional keywords: *Capsicum annuum*, PI201234, Serrano Criollo de Morelos 331, Yolo Wonder.

In a plant–pathogen interaction where the result is disease, the detection and quantification of pathogen biomass in the plants is crucial. Traditionally, the common methods used to evaluate disease severity consisted of scoring visual symptoms displayed by the plant. For pathogens able to kill plants completely, survival rates are scored to evaluate disease resistance. Alternatively, for pathogens causing necrosis without killing the host, the degree of necrosis over time is used as a measure to monitor resistance (9). However, these techniques have several limitations. First, they can be used only when the pathogen–host interaction results in visible disease symptoms, which is a limiting factor in the earliest stages of infection. Second, there is not always a straightforward relationship between disease symptoms and pathogen development in plants (24,53). Indeed, a tolerant host can show no disease symptoms, without reduction in pathogen growth within the plant (12,42). Therefore, a method for direct assessment of pathogen development in infected plants was necessary.

Several techniques have been developed with the aim of quantifying pathogen biomass. Some of these are based on the measurement of compounds such as ergosterol or chitin (18,21,32,40,43) which are present in the pathogen but not in the host. The problem with these methods is the lack of specificity and the fact that they can be used only to assess colonization by pathogens that possess these constituents (3). Another group of techniques includes the use of immunological methods for pathogen biomass determination (13,22,29,38). However, among the drawbacks of these methods are the difficulty in obtaining antibodies with the required specificity for particular fungi (56), false-positive reactions (51,52), and interference of plant factors with the measured parameter (35,53).

In recent years, the polymerase chain reaction (PCR) method has been used successfully to detect and identify fungal pathogens

(48). Among the PCR-based methods, real-time PCR has proven to be a simple yet reliable technique to quantify viral, bacterial, fungal, and oomycete plant pathogens, and increasingly is being used in plant pathology (5,33,37,47,55). In this technique, the DNA of a specific target organism can be quantified by measurement of the intensity of fluorescence with time during the exponential phase of DNA amplification. Quantification of DNA in unknown samples will be possible by direct comparison with standards amplified in parallel reactions (36). The main advantage of this technique compared with the conventional one, end-point quantitative PCR, is that the amplification products can be monitored as they are accumulated in the log-linear phase of amplification. Therefore, it is more accurate, less time-consuming, and better suited, especially when the objective is to discriminate between slightly different levels of infection (58).

Phytophthora root rot, caused by *Phytophthora capsici* Leonian, is the most destructive disease of pepper crops in Spain (39,44). In susceptible plants, the pathogen penetrates through the collar, causing necrosis in that area. At the same time, the plant becomes withered while the pathogen advances systemically toward both the stem and the root, causing an obstruction in the vascular system. The infected tissues become dry, sunken, and parchment-like, and turn dark in color. Finally, the plant dies at the last stage of infection. Although the most effective method to combat the pathogen is based on the use of fungicides, some cultivars with different levels of resistance to *P. capsici* have been reported. Indeed, several different screening methods have been developed to distinguish *Phytophthora*-resistant individuals from susceptible ones (1,7,8,19). Different sources of resistance to *P. capsici* have been reported in *Capsicum annuum* L.; among the genotypes that exhibit resistance to *Phytophthora* root rot are United States Department of Agriculture (USDA) PI201234 and USDA PI201232, as well as Serrano Criollo de Morelos 331 (SCM331) and SCM334, the latter being more resistant to *P. capsici* than the former (4,20,41).

In a previous study (50), we developed a PCR procedure for the early detection of *P. capsici* in pepper plants. Specific primers for

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P. capsici, based on the internal transcribed spacer (ITS) region of ribosomal DNA, were designed and tested for their sensitivity and specificity as well as for their ability to detect the pathogen in artificially and naturally infected pepper plants (50). The development of a real-time approach enabled the quantification of pathogen biomass in plants over the course of infection. This method will facilitate the characterization of host–pathogen interactions. The aims of the present work were to (i) develop a real-time PCR assay to quantify *P. capsici* biomass in pepper plants and (ii) monitor pathogen growth in pepper plants in relation to different levels of pathogen virulence and host susceptibility.

MATERIALS AND METHODS

Pathogen material. In recent works carried out in our laboratory (C. Silvar, F. Merino, and J. Díaz, *unpublished data*), a population of Spanish *P. capsici* isolates was characterized according to their virulence. Two different isolates, one highly virulent (UDC196Pc) and one less virulent (UDC248Pc), were selected for this work. The isolates were maintained on potato dextrose agar (PDA) for further use. To obtain mycelium, the isolates were grown in still culture in pea broth (11). After 1 week of incubation at 24°C, mycelium was collected and lyophilized for extended storage.

Plant material. Three different cultivars of pepper plants were used: susceptible cv. Yolo Wonder, and SCM331 and PI201234, each with different levels of resistance to *P. capsici*, SCM331 being the most resistant (4). Moreover, a susceptible local cultivar (Padrón) was included because of its economic value in northwestern Spain. Plants were transferred into pots and grown separately in a chamber at 25°C and a photoperiod of 16 h of light and 8 h of darkness until they were 3 weeks old.

Pathogen inoculation, quantification bioassay, and disease assessment. A suspension of zoospores from UDC196Pc and UDC248Pc were obtained according to Larkin et al. (30). Pepper plants were inoculated with a concentration of 10^4 zoospores ml⁻¹ by adding 5 ml of the inoculum into the soil. For the quantification of pathogen DNA, samples of leaves, stems, and roots were collected at 8, 24, 48, and 72 h postinoculation in the different cultivars. Three independent experiments were carried out.

In parallel, assays were carried out with the aim of studying the disease progression in different cultivars during a time period. With this purpose, 10 plants of each cultivar were inoculated with the two different isolates (UDC196Pc and UDC248Pc). Disease severity was rated for 20 days after inoculation on a scale of 0 to 5, where 0 = no visible symptoms; 1 = ≤50% of stem with water-soaked lesions, ≤50% of leaves wilted, or both; 2 = >50% of stem with water-soaked lesions or >50% of leaves wilted; 3 = both ≤50% of stem with water-soaked lesions and >50% of leaves wilted or both >50% of stem with water-soaked lesions and ≤50% of leaves wilted; 4 = >50% of stem with water-soaked lesions and >50% of leaves wilted; and 5 = dead plant. Percentage of diseased plants (incidence) also was recorded during the time course of infection. In this case, two independent experiments were performed.

Isolation of *P. capsici* from artificially infected plants. Colonization of pepper plants by *P. capsici* was determined in each of the pepper cultivars studied by isolating the pathogen on PDA. During the host–plant infection assay, samples of plants were collected at 8, 24, 48, and 72 h postinoculation. Entire plants were surface disinfected with NaClO (10%) for 5 min, washed with distilled water, dried, and plated onto PDA medium. Plates were incubated at 24°C in the dark for 7 days.

DNA extraction. For DNA extraction, both pathogen and plant material (0.2 g fresh weight [FW]) were ground with liquid nitrogen to a fine powder using a mortar and pestle. All the powder was transferred to an Eppendorf tube containing extraction buffer (0.1 M Tris-HCl, pH 7, 1.4 M NaCl, 0.2% β-mercaptoethanol, 1% polyvinyl pyrrolidone, and 2% cetyltrimethylammonium bro-

mide). Subsequently, extraction with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol) was carried out. DNA was precipitated with isopropanol, washed with cold ethanol (70%), and resuspended in sterile distilled water.

Real-time PCR assay. Real-time PCR assays for quantification of both isolates in different cultivars were conducted using primer pair CAPFW/CAPRV1 (50), which defines an amplicon of 452 bp specific for *P. capsici*. Real-time PCR was performed in an iCycler (BioRad, Hercules, CA) apparatus and results were analyzed with the manufacturer's software (Optical System Software, v. 3.0; BioRad). Each reaction mixture (20 µl) contained 2.5 µl of undiluted DNA extract, 1× iQ SYBR Green Supermix (BioRad), and 0.3 µM of each primer. In negative controls, the DNA extracts were obtained from noninoculated plants of the same age and same culture conditions as inoculated ones. Additional negative control reactions contained the same mixtures with 2.5 µl of sterile water replacing the DNA. The thermal cycling conditions consisted of an initial denaturation at 95°C for 2 min followed by 40 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 1 min, and 84°C for 15 s. A final extension step at 72°C for 5 min was added. Fluorescence of the target amplicon (melting temperature (T_m) = 86.5°C) was detected at 84°C. After the final amplification cycle, a melting curve temperature profile was obtained by heating to 95°C, cooling to 65°C, and slowly heating to 95°C at 0.5°C every 10 s with continuous measurement of fluorescence at 520 nm. A standard curve was constructed by plotting the logarithm of 10-fold serial dilutions from 100 to 10⁻³ ng of *P. capsici* DNA against the cycle threshold (C_t) values. The C_t value is the cycle number at which the fluorescence emission of the PCR amplicon could be distinguished from the background.

In another assay, a 10-fold serial dilution of genomic DNA starting from 100 ng was added to samples containing 100 ng of pepper genomic DNA. This was done in order to test whether the presence of samples of plant DNA would interfere with the accurate quantification. When constructing a regression line with these data, a linear relationship was observed, as happened in the standard curve (data not shown). From these results, it was concluded that addition of host DNA has no effect on pathogen DNA quantification.

The amount of pathogen DNA in unknown samples was calculated by monitoring the C_t value at which the fluorescence emission could be differentiated from the background and comparing this C_t value with the point values of the linear regression line of the standard curve. Furthermore, to confirm that only one PCR product was amplified in the inoculated plants and no amplification product was obtained in the negative controls, all reactions were analyzed by electrophoresis in a 1% (wt/vol) agarose gel in 1× Tris-borate-EDTA buffer (31) and stained with ethidium bromide (5 µg ml⁻¹) for visualization.

Statistical analyses. Statistical analyses were performed using Statgraphics Plus for Windows, 5.1 Professional Version (StatPoint, Inc., Herndon, VA). Data of pathogen DNA quantification among cultivars were analyzed using the Kruskal-Wallis test ($P \leq 0.05$) followed by the multiple comparison procedure of Conover (10).

RESULTS

Disease assessment in different pepper cultivars. The capacity of the two *P. capsici* isolates to infect pepper plants was tested in a group of four pepper genotypes, differing in their degree of susceptibility to the pathogen. Data on disease severity were plotted over time to obtain disease progress curves (Fig. 1). In general, differences could be observed among cultivars, isolates, and cultivar–isolate interactions. Susceptible cv. Yolo Wonder showed high disease severity with both *P. capsici* isolates. Seedlings were attacked primarily in the collar region and the infection rapidly expanded up the hypocotyl, reaching the cotyledons within a few days. Host plants revealed the first visible symptoms

on the fourth day after inoculation, although the disease progress over time was higher in plants inoculated with UDC196Pc than in those infected with UDC248Pc. In the first case, a maximal infection (severity index = 4.8) was reached at 17 days after infection (Fig. 1A) and the percentage of infected plants at the end of the bioassay was 95% (data not shown). In the Yolo Wonder–UDC248Pc interaction, however, disease progression was slower and the severity rating achieved at the end of the ongoing period was only 1.5 (Fig. 1B), with only 30% diseased plants (data not shown). After inoculation with UDC196Pc, the infection pattern displayed by local cv. Padrón was similar to that in Yolo Wonder, but showed the highest degree of susceptibility (severity rate = 5 and percentage of infected plants = 100%) (Fig. 1A). However, after inoculation with UDC248Pc, the severity achieved by Yolo Wonder was higher than in Padrón (Fig. 1B).

In contrast, resistant genotypes showed the lowest disease severity. No disease symptoms were observed when the plants were infected with UDC248Pc, and slower progress curves were obtained in the PI201234– or SCM331–UDC196Pc system compared with those in susceptible cultivars. In the last case, more symptoms developed in PI201234 than in SCM331, with 40 and 20% disease percentage (data not shown) and 2 and 1 severity index, respectively, at the end of the trials. Noninoculated plants did not show any symptoms (data not shown).

P. capsici was isolated successfully from leaves, stems, and roots in all pepper genotypes inoculated with both isolates at 24, 48, and 72 h after inoculation. However, no pathogen isolation was possible at 8 h in any pepper–*P. capsici* system (data not shown).

Real-time PCR adjustment and DNA standard curves. Preliminary runs of real-time PCR without the step at 84°C for 15 s were carried out with the aim of detecting some possible non-

specific amplification products or primer-dimers. Melting curve profiles from those runs revealed the presence of such products, with a $T_m \leq 82^\circ\text{C}$. Therefore, to avoid any possible disturbance in the quantification of the target amplicon ($T_m = 86.5^\circ\text{C}$), an additional step (84°C for 15 s) was included in the real-time PCR amplification program and the reading of the fluorescence emission was set at this point. Additionally, to confirm the presence of the single expected amplicon of 452 bp, final products of real-time PCR from both standard curve and unknown samples were visualized in agarose gels (data not shown).

A standard curve was constructed by plotting known concentrations of *P. capsici* DNA against the C_t values obtained from real-time PCR (Fig. 2). In order to validate the reproducibility of the real-time PCR method, PCR amplifications were conducted with duplicate samples of pathogen DNA obtained from four different preparations. Highly reproducible C_t values with very small standard deviations were observed, the linear regression coefficient of the standard curve being $r = 0.968$. Within this standard curve, each 10-fold difference in initial DNA amounts was represented by approximately four-cycle differences in C_t . Under the PCR conditions tested, the minimum starting quantity of pathogen DNA that could be accurately quantified in our assays was 10^{-2} ng, which corresponded to a C_t value of 36.97 ± 1.06 . A C_t of 37 (6×10^{-3} ng of DNA according to the standard curve) was considered to be the threshold value suitable for quantification, because PCR efficiency at a higher number of cycles was far from the value 1 (100% efficiency).

Quantification of *P. capsici* DNA in pepper plants. Pathogen DNA was quantified in plants using real-time PCR with SYBR Green before first symptoms of the disease were observed in plants (Figs. 3, 4, and 5). Indeed, symptoms did not appear in infected plants until 4 days after inoculation; however, with the real-time PCR assay, pathogen DNA could be quantified as early as 8 h postinoculation in all the organs of the plant. From that time point, the pathogen DNA in pepper organs increased progressively until the end of the bioassay. Real-time PCR quantification showed important differences among the different organs. In general, stems were more heavily colonized than roots, and the latter more than leaves. In all cases, the maximal amount of pathogen DNA occurred at 72 h after inoculation. Although the pathogen was able to colonize all the susceptible and resistant pepper cultivars evaluated, the extent of colonization varied highly among cultivars with different levels of resistance. In most of the cases, higher amounts of pathogen DNA were present at 72 h after inoculation in susceptible cultivars (Padrón and Yolo Wonder) than in the more resistant ones (PI201234 and SCM331). In the susceptible cultivars, pathogen DNA was detected earlier

Fig. 1. Disease progress curves of pepper cultivars inoculated with A, UDC196Pc or B, UDC248Pc. Each plotted point is the mean value of 20 plants from two independent experiments. Padrón (●), Yolo Wonder (○), PI201234 (▼), and SCM331 (▽).

Fig. 2. Standard curve for real-time polymerase chain reaction analysis of a 10-fold serial dilution of *Phytophthora capsici* DNA.

and increased sharply over time whereas, in the resistant plants, the variations in pathogen development were slighter. Significant ($P \leq 0.05$) differences were found among resistant and susceptible cultivars as early as 24 h postinoculation in the stem for the UDC196Pc isolate (Fig. 4A) although, at 48 h, there was no significant differences. The differences were significant at 72 h postinoculation in all the organs and isolates, except for the combination of the roots and the isolate UDC196Pc (Fig. 5A). Comparing the cultivars separately, susceptible cvs. Padrón and Yolo Wonder displayed a similar pattern of pathogen invasion, but significant differences between them were found in the roots at 48 and 72 h postinoculation (Fig. 5A and B). For the resistant cultivars, significant differences were found in SCM331 compared with the partially resistant cv. PI201234 only in the case of roots at 48 h postinoculation (Fig. 5A and B). In any case, the accumulation of pathogen DNA in these resistant genotypes was slower than that in susceptible cultivars and the amount of pathogen DNA generally remained lower during the study.

With regard to the two *P. capsici* isolates, a different pattern of infection was observed. Up to 5.9 ng g⁻¹ FW of pathogen DNA was observed as soon as 8 h postinoculation in the Padrón- or Yolo Wonder-UDC196Pc interactions (Fig. 4A) whereas, in susceptible cultivar-UDC248Pc interactions, the maximal amount of DNA detectable was 0.2 ng g⁻¹ FW (Fig. 4B). In the same way, the average pathogen DNA in the resistant cultivar-UDC196Pc system was 1.4 ng g⁻¹ FW, whereas it could not be detected in PI201234 or in SCM331 until 24 h after infection, when the plants were inoculated with the least virulent pathogen (UDC248Pc). During the remaining period, the amount of UDC196Pc (the isolate previously characterized as more virulent) DNA was up to 10

times higher than UDC248Pc (less virulent) in all cultivars evaluated. These results correspond with those obtained in the severity assessment assay, where clear differences between isolates also were observed.

DISCUSSION

One of the objectives of this study was the development of an accurate quantification method of pathogen colonization in host tissue using real-time PCR technology and the use of this method for a better understanding of the interaction between different pepper genotypes and *P. capsici* isolates. With this purpose, four pepper cultivars were artificially infected with two *P. capsici* isolates, differing in their degree of virulence toward pepper plants. Pathogen DNA was monitored and quantified in a time course after inoculation using a quantitative SYBR Green PCR assay. In recent years, real-time PCR assays have been widely used for the detection and quantification of fungal plant pathogens (2,15,48). Similar studies have been carried out in *Phytophthora* spp. (5,6, 23,26,55). However, to the best of our knowledge, no attempts had been made to apply the real-time PCR technology for quantification of *P. capsici* in pepper plants.

In our study, the results showed that real-time PCR can be successfully implemented for the quantification of pepper colonization by *P. capsici*. The technique was highly reproducible, the correlation coefficient of the standard curve constructed with different replications being 0.968. At the same time, the method was quite sensitive, being able to readily detect, using the un-specific SYBR Green dye and specific primers to *P. capsici*, up to 10⁻² ng of pathogen DNA. Similar quantification limits have been

Fig. 3. *Phytophthora capsici* DNA quantification in leaves of different pepper cultivars inoculated with **A**, UDC196Pc or **B**, UDC248Pc. Vertical arrow (↓) indicates DNA samples (PI201234 and SCM 331) that could not be quantified. Data are the mean and standard error of three independent experiments. Within the same time postinoculation, different letters indicate significant differences between cultivars in a Kruskal-Wallis test ($P \leq 0.05$) followed by the multiple comparison procedure of Conover (10).

Fig. 4. *Phytophthora capsici* DNA quantification in stems of different pepper cultivars inoculated with **A**, UDC196Pc or **B**, UDC248Pc. Vertical arrow (↓) indicates DNA samples (PI201234 and SCM 331) that could not be quantified. Data are the mean and standard error of three independent experiments. Within the same time postinoculation, different letters indicate significant differences between cultivars in a Kruskal-Wallis test ($P \leq 0.05$) followed by the multiple comparison procedure of Conover (10).

found in other pathogen–plant interactions (15–17) as well as in other *Phytophthora* spp., although using different real-time chemistries (26). The Ct values depend on both the amount of starting DNA and the genomic organization of the target sequences recognized by the sequence-specific primer pair; therefore, the efficiency of the technique will improve if the target DNA is present in many copies in the genome, as is the case with rDNA. This sensitivity was similar to that achieved in previous works using conventional PCR but less than that obtained with a nested PCR assay (50). These results agree with those published recently by Bonants et al. (6) working on *P. fragariae*. They obtained equivalent detection limits by using either conventional PCR or a TaqMan assay but higher sensitivity with a nested PCR. In any case, with this sensitivity, pathogen abundance can be reliably quantified from the very beginning of the infection time course and even in tissues exhibiting a low degree of infection.

In a parallel assay, the influence of plant samples on quantification was tested (data not shown). Serial dilutions (10-fold) of pathogen DNA were mixed with an equal amount of plant DNA. This mixture possibly could reflect an infection from a range of 1:1 (a very heavy infection) to a range of 1:10⁶ (an early stage of infection). The presence of a host sample of DNA did not produce any interference in the quantification. Furthermore, with this technique, the sensitivity of the method was not affected by the presence of a plant DNA sample, as occurs with conventional and nested PCR (50).

In a real-time PCR assay, the accumulation of the amplicon can be detected using either specific detection methods such as TaqMan probes (25,54,57) or nonspecific methods like SYBR Green (36). The advantage of SYBR Green is that, in contrast to fluorogenic probes, it does not require the design of specific complementary fragments to the target DNA and, therefore, the reaction cost is limited compared with real-time PCRs that depend on hybridization of labeled sequence-specific probes. The main disadvantage of this method is that it binds double-stranded DNA and, therefore, it also can associate with primer-dimers and non-specific products, which can seriously disturb the interpretation of the results. However, this major drawback can be overcome with the use of melting curve temperature analysis at the end of the PCR assay (46). At the end of the cycling reactions, the products are melted by slowly increasing the temperature while continually monitoring the fluorescence. After melting curve analysis in our assay, the use of unspecific SYBR Green dye for quantifying the target amplicon (≈452 bp, T_m = 86.5°C) required a 84°C fluorescence emission reading step in the amplification program to eliminate any interference caused by the accumulation of unspecific PCR products. At that temperature, the nonspecific products will be denatured and only the fluorogenic emission from the target amplicon would result.

One of the results in this work is that the real-time PCR method developed here is able to quantify pathogen invasion in the absence of visible host plant symptoms. Although the first visible symptoms appeared in plants by day four after infection, quantification was possible at earlier times (as soon as 8 h postinoculation) in totally asymptomatic plants. Thereafter, the amount of pathogen DNA either increased sharply or varied more slightly during the study period, but in no case were the symptoms observed in inoculated plants. It must be pointed out that *P. capsici* is detected in leaves at 8 h postinoculation. It is surprising that the pathogen can invade and colonize the host so fast. This fact probably is due to the pathogen's ability to move through the vascular system and the small size of the plants at the time of the experiments. It was also an unexpected result that the pathogen was not isolated at 8 h after inoculation, taking into account that pathogen DNA was detected at that time. Maybe nonviable pathogen cells were present at that time. Another possibility is that the pathogen colonized only the outer layers of cells at that time, after which surface sterilization could have killed the pathogen.

The amount of pathogen DNA quantified 72 h after inoculation in each pepper genotype correlated with the level of susceptibility or resistance to disease caused by *P. capsici* (as indicated by severity of symptoms). Maximal absolute amounts of pathogen DNA were quantified in susceptible genotypes Yolo Wonder and Padrón (the most severely diseased genotypes), where measurements in stems ranged from 3.3×10^5 ng g⁻¹ FW in UDC196Pc–Yolo Wonder to 1.6×10^5 ng g⁻¹ FW in UDC196Pc–Padrón. Actual amounts of *P. capsici* DNA varied considerably for a given pepper genotype–*P. capsici* isolate interaction and, in some cases, between *P. capsici* isolates for a given pepper genotype. In the same way, smaller amounts of pathogen DNA were quantified in PI201234 and SCM331 plants, in that order. These two cultivars also produced fewer diseased plants or even none at all. Therefore, reduced disease severity in those cultivars considered resistant corresponded to the results of the quantitative analysis of pathogen DNA. In recent years, several different studies have demonstrated the utility of real-time PCR to discriminate between plant genotypes displaying different levels of resistance (9,17,34,45,59). Vandemark and Barker (55), studying the *P. medicaginis*–alfalfa interaction, were able to discriminate among three alfalfa populations with different levels of resistance. They also detected less pathogen DNA in highly resistant alfalfa populations than in more susceptible ones. Moreover, the results of our work corroborate others published previously which established clear differences in resistance among SCM331 and PI201234 (resistant cultivars) and Yolo Wonder (susceptible genotype) (4,19,20). The difference with the present work is that previous classifications of peppers susceptible or resistant to *Phytophthora* root rot were based on the evaluation of external symptoms and measurement of incidence and severity

Fig. 5. *Phytophthora capsici* DNA quantification in roots of different pepper cultivars inoculated with **A**, UDC196Pc or **B**, UDC248Pc. Vertical arrow (↓) indicates DNA samples (PI201234 and SCM 331) that could not be quantified. Data are the mean and standard error of three independent experiments. Within the same time postinoculation, different letters indicate significant differences between cultivars in a Kruskal–Wallis test ($P \leq 0.05$) followed by the multiple comparison procedure of Conover (10).

of plant symptoms, which is time consuming and sometimes inconclusive.

In terms of pathogen ability to infect the plant, it seemed clear that the more virulent isolate had a better capacity not only to infect the plant sooner but also to spread quicker during the time course of infection. In general, the average amount of pathogen DNA was higher for UDC196Pc-infected plants than for the UDC248Pc-infected ones. Moreover, the accumulation of pathogen DNA was rapid in susceptible genotype–most virulent isolate interactions whereas, in less compatible interactions, it was more progressive and notable increases in pathogen amount did not take place, the quantity of pathogen DNA being more constant over time. Considering all these results, the difference in virulence of the two *P. capsici* isolates could be attributed to differences in pathogen amount in infected plants, among other factors. However, a clear correlation between the virulence of a pathogen and the amount of pathogen present in the plant does not always exist. Sometimes, more virulent isolates do not involve an increase in pathogen colonization (34,53).

Another interesting aspect was the different amounts of pathogen DNA in the different tissues collected. Comparatively, the amount of pathogen DNA in leaves was much lower than that quantified in roots which, in turn, was less than that occurring in stems. This tissue difference suggests either a temporal sequence of systemic colonization by the pathogen or a differential response of the plant defense mechanisms. Differences in the level of colonization of different tissues by the pathogen have been reported in other plant–pathogen interactions (34,59). It has been reported that some plant defense-related genes, which encode pathogenesis-related proteins, display a differential expression in various pepper organs in response to pathogen infection (27,28,49). Such responses could be responsible for that differential distribution, preventing pathogen advance, especially in the cultivars that are more resistant.

A final conclusion of the present work was that the local pepper cv. Padrón displayed an infection and quantification pattern similar to that in Yolo Wonder, suggesting that it may have a similar degree of susceptibility. Padrón is a pepper cultivar of high economic importance in Galicia (northwestern Spain) (14). The results in this study have demonstrated its level of susceptibility to *Phytophthora* root rot, the most important disease of pepper crops in this region (44). The real-time PCR developed here, together with disease severity studies, will enable the selection of the most resistant plants within the Padrón cultivar for disease resistance breeding in the future.

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