Virulence Phenotypes on Chili Pepper for *Phytophthora capsici* Isolates from Michoacán, Mexico

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Abstract. *Phytophthora* blight of vegetables caused by *Phytophthora capsici* causes significant economic losses in production of Solanaceae and Cucurbitaceae crops in Mexico. The development of universal resistant chili pepper cultivars is challenging due to the diverse virulence phenotypes produced by *P. capsici*. The objective of the study was to characterize the diversity of phenotypic interactions for *P. capsici* isolates recovered from production fields in Michoacán, Mexico, to facilitate the development of resistant cultivars. Virulence phenotypes were characterized for 12 isolates of *P. capsici* using 26 *Capsicum annuum* New Mexico Recombinant Inbred Lines (NMRILs) in greenhouse conditions. Criollo de Morelos CM-334 and California Wonder were used as resistant and susceptible controls, respectively. Seedlings at the four to eight true leaf stage were inoculated with 10,000 zoospores per seedling and disease severity was evaluated at 20 days post-inoculation. Two of the *P. capsici* isolates did not infect any pepper host even though the isolate was less than a year old. The 10 virulent isolates were designated in 10 virulence phenotypes. The information generated by this study is of utmost importance for efforts of producing resistant cultivars specific for Michoacán producers.

The oomycete *Phytophthora capsici* is highly destructive to vegetable species in the Solanaceae, Cucurbitaceae, and Fabaceae families (Kamoun et al., 2015). Worldwide, it is the main pathogen limiting chili pepper (*Capsicum annuum*) production and can infect the roots, crown, stem, and fruits (Barchenger et al., 2018a; García-Rodríguez et al., 2010; Glosier et al., 2008; Lamour and Hausbeck, 2002). In Mexico, *P. capsici* primarily attacks the roots, and foliar blight is rare in production fields (Macías-Valdez et al., 2010). *P. capsici* has an asexual phase characterized by the rapid production of deciduous sporangia on infected tissues, which can quickly release swimming zoospores under wet conditions. The sexual phase requires the interaction of two mating types (A1 and A2), and outcrossing can greatly increase the genetic diversity of field populations and may increase the overall evolutionary potential and the ability to adapt to control measures (Lamour et al., 2012). Recently, variable ploidy has been reported in this pathogen and could affect its ability to adapt to different environments (Barchenger et al., 2017).

In Mexico, the presence of *P. capsici* has been reported in multiple vegetable production zones and is considered the main cause of chili pepper root rot (Silva-Rojas et al., 2009). The use of chemical fungicides has been a costly and ineffective strategy to control this pathogen and may be harmful to the environment (García-Rodríguez et al., 2010). Among the alternatives to control *P. capsici*, the development of resistant cultivars could be the best method to reduce the losses associated with this plant pathogen (Gómez-Rodríguez et al., 2017). However, the development of resistant cultivars and varieties to *P. capsici* has been difficult due to the presence of diverse virulence phenotypes of this pathogen (Barchenger et al., 2018b; Jiang et al., 2015; Oelke et al., 2003; Sy et al., 2008).

Virulence phenotype characterization requires a set of host plants carrying one or more resistant genes with recombinant inbred lines (RILs) often used for race-typing (Oelke et al., 2003). Regarding the *Capsicum annuum*–*P. capsici* pathosystem, Sy et al. (2008) reported the development of a series of differential host lines specifically for the characterization in *P. capsici* known as the New Mexico Recombinant Inbred Lines (NMRILs). The NMRILs were developed by crossing the landrace Criollo de Morelos CM-334 (considered a universally resistant host) with the highly susceptible variety Early Jalapeño. A subset of the resulting progeny was then selfed until the F₇ generation and 26 lines were further selected as useful to differentiate *P. capsici* isolates based on resistance/susceptibility reactions.

The NMRILs have been used to characterize *P. capsici* physiological races from isolates of different regions and are a valuable tool to characterize *P. capsici* isolates at a global scale. Recently, the term “race” has been replaced by the term “virulence

phenotype” to characterize the interaction of *P. capsici* isolates on RILs carrying various host resistance genes (Barchenger et al., 2018b). By using the NMRILs, 12 *P. capsici* root rot virulence phenotypes were characterized from isolates recovered from New Mexico, 11 from isolates recovered from Brazil, and 24 from isolates recovered from Taiwan (Barchenger et al., 2018b; da Costa Ribeiro and Bosland, 2012; Jiang et al., 2015). The NMRILs have also been used to characterize foliar blight virulence phenotypes (Monroy-Barbosa and Bosland, 2010; Monroy-Barbosa and Bosland, 2011).

Characterizing the virulence phenotypes present in production zones is useful in the development of resistant lines (Glosier et al., 2008). A promising approach is the introgression of specific resistance genes that could lead to multiresistant cultivars (da Costa Ribeiro and Bosland, 2012). In Mexico, there are no reports on the presence or characterization of virulence phenotypes of isolates of *P. capsici* recovered from production zones. Due to the high genetic diversity of this pathogen in Mexico (Castro-Rocha et al., 2016), it is likely that multiple virulence phenotypes exist in field populations. The objective of this study was to characterize the virulence phenotypes for *P. capsici* isolates recovered from Solanaceae and Cucurbitaceae production fields in Michoacán, Mexico using the NMRILs.

Materials and Methods

Differential hosts. For the present study, 26 NMRILs were used as differential hosts (Sy et al., 2008). The tested lines were: A, AA, AB, AC, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, V, X, and Z. The starting seeds of the NMRILs were provided by Dr. Paul Bosland of New Mexico State University. The NMRILs were grown in greenhouse conditions to obtain enough seed for the complete experiment. Criollo de Morelos CM-334 was used as resistant control and California Wonder (CW) as susceptible control (Seed brand “Los Molinos,” Morelos, Mexico). Seedlings were grown in germination trays divided in six sections of 100 cm³ using Sunshine Mix 3 (90% Canadian peatmoss, 10% plant starter mix) as substrate. Thirty days after germination, each plant was

fertilized once per week with 20 mL of Miracle-gro universal fertilizer (24N–8P–16K).

Collection of isolates of *Phytophthora capsici* and determination of mating type. Twelve *P. capsici* isolates were recovered from *Capsicum annuum*, *Solanum lycopersicum*, and *Cucurbita pepo* plants with root rot in eight production fields of the municipalities of Copándaro, Morelia, Queréndaro, Tacámbaro, and Tarímbaro in Michoacán state (Figs. 1 and 2, Table 1). The following isolates were collected from the same field: CPV-259 and CPV-260 from a field located in Copándaro; CPV-270, CPV-271, and CPV-272 from a field located in Tarímbaro; and CPV-277 and CPV-279 from a field located in Queréndaro. Plant tissues were rinsed with running tap water; small diseased tissue fragments were cut (1.0 × 0.5 cm), disinfested with 10% commercial chlorine solution (6% sodium hypochlorite) for 30 s, rinsed with sterile distilled water twice, blotted dry with sterile paper towels, transferred to NARPH-V8 plates [Delycid Instant (0.02 g·L⁻¹); (50% natamycin, 50% lactose)], ampicillin (0.27 g·L⁻¹), rifampicin (0.01 g·L⁻¹), pentachloronitrobenzene (0.10 g·L⁻¹), and hymexazol (0.075 g·L⁻¹) and incubated at 25 °C in the dark.

Mating type was determined by following the protocol described by Yin et al. (2012). All recovered isolates were placed in pairs with known A1 and A2 isolates on V8-agar medium in petri dishes at a distance of 3 cm from each other and incubated at 25 °C for five days. After the incubation period, isolates were examined for oospore formation.

Molecular characterization of *Phytophthora capsici* isolates. The isolates CPV-1, CPV-33 and CH11 were previously reported as *P. capsici* by SNP sequencing (Castro-Rocha et al., 2016). The isolates CPV-267, CPV-270, and CPV-277 were reported as *P. capsici* by whole genome sequencing (Reyes-Tena et al., 2019). Genomic DNA was extracted from the isolates CPV-259, CPV-260, CPV-271, CPV-272, CPV-273, and CPV-279, following the method described by Saghai-Marroof et al. (1984). Whole genomes were sequenced and assembled following the method reported by Reyes-Tena et al. (2019). A blast analysis was conducted to infer *cytochrome oxidase*

1 (cox1) and *cytochrome oxidase* 2 (cox2) genes and determinate the identity of the isolates.

Differential host's inoculation. Host inoculation was performed as described by Bosland and Lindsay (1991) with modifications. Each isolate was transferred to V8 agar petri dishes (150 × 15 mm). Once the growth media was completely colonized by the isolates, the agar was cut into 1 cm² fragments and transferred to new petri dishes afterward sterilized distilled water was added to each petri dish until the mycelium fragments were completely covered up to the margin. The cultured isolates were kept under white light at 25 °C and water was changed every 24 h over 3 consecutive days. Once the isolates produced sporangia, they were subjected to a temperature shock treatment of 30 min at 4 °C and 30 min at 24 °C to induce zoospore release. A sample of the zoospores containing liquid was used to quantify the zoospores in a Neubauer chamber using a drop of lactophenol blue to stain and immobilize them. A suspension containing 1 × 10⁴ zoospores/mL was prepared for each isolate used in the experiment. One milliliter of the suspension was used as the inoculum for the resistance/susceptibility tests. The experimental unit consisted of a single plant inoculated with zoospores of a single isolate of the pathogen. Each tray had six repetitions of the same experimental unit. After the inoculation with *P. capsici*, the trays were placed in closed trays that had 4 cm of water and were randomly distributed in greenhouse benches to diminish the effect of environmental factors on the development of disease symptoms. The complete experiment had 26 NMRILs, two controls (resistant and susceptible), 12 isolates, controls without inoculum, and 6 replications for a total of 28 × 13 × 6 = 2184 experimental units. Host plants were tested at the four to eight true leaf stage by inoculating them with 10,000 zoospores in the substrate next to the stem, with the aid of a dosing syringe (Ape; 50 mL). To allow a successful plant-pathogen interaction, the substrate of the host plants was saturated with water for 24 h after the inoculation. Noninoculated control plants were subjected to the same conditions but were not inoculated with zoospores. The pathogen was reisolated from plants with symptoms.

Fig. 1. Plants with symptoms caused by *Phytophthora capsici* in the field: (A) wilted pepper plants in a field in Queréndaro, (B) pepper plants with root rot and stem necrosis in Queréndaro, and (C) wilted zucchini plant in Morelia.

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Fig. 2. Map of the sites where the *Phytophthora capsici* isolates were collected.

Table 1. Municipality, host and collection date of isolates of *Phytophthora capsici* from Michoacán.

Isolate	Municipality ²	Host	Date of collection
CPV-1	Tacámbaro	<i>Capsicum annuum</i>	2002
CPV-33	Tacámbaro	<i>Solanum lycopersicum</i>	2002
CH11	Tarímbaro	<i>Capsicum annuum</i>	2011
CPV-259	Copándaro	<i>Capsicum annuum</i>	2016
CPV-260	Copándaro	<i>Capsicum annuum</i>	2016
CPV-267	Morelia	<i>Cucurbita pepo</i>	2016
CPV-270	Tarímbaro	<i>Capsicum annuum</i>	2016
CPV-271	Tarímbaro	<i>Capsicum annuum</i>	2016
CPV-272	Tarímbaro	<i>Capsicum annuum</i>	2016
CPV-273	Tarímbaro	<i>Solanum lycopersicum</i>	2016
CPV-277	Queréndaro	<i>Capsicum annuum</i>	2017
CPV-279	Queréndaro	<i>Capsicum annuum</i>	2017

²Municipality of Michoacán, Mexico.

Disease scoring. The severity of the disease was scored based on the following scale: 0 = no symptoms/healthy plant; 1 = yellow leaves without stem necrosis; 2 = minor stem necrosis; 3 = moderate stem necrosis and early signs of wilting; 4 = plant with severe stem necrosis and wilting; 5 = dead plant (Glosier et al., 2008).

The evaluation was performed at 20 d postinoculation (dpi). At this time, susceptible control plants exhibited severe stem necrosis and wilting. The average disease severity for each host*isolate interaction was calculated.

Virulence phenotype determination and data analysis. The *P. capsici* virulence phenotypes were determined based on the unique resistant/susceptible interaction pattern on the differential lines. A unique resistance/

susceptibility pattern on the differential hosts set was considered a unique virulence phenotype. The following criteria were used to determine if the plant hosts were resistant or susceptible: hosts with an average disease severity of 0 were considered resistant; hosts with an average disease severity greater than 0 were considered susceptible. In addition, the data obtained from the severity scale on differential hosts were compared with the results obtained from the resistant and susceptible controls by using a chi-square test of homogeneity at a confidence level of 99%. Differential hosts lines that differed statistically from the resistant controls were considered susceptible (Ho). Differential hosts lines that did not differ statistically from the susceptible controls were considered susceptible (Ha).

Results and Discussion

The *cox1* and *cox2* genes from the assembled genomes had at least 98% similarity to isolates of *P. capsici* in GenBank, and the isolates CPV-259, CPV-260, CPV-271, CPV-272, CPV-273, and CPV-279 were identified as *P. capsici*. Ten of the isolates were able to cause infection on at least one of the differential lines and each of these pathogenic isolates produced a unique virulence phenotype using the NMRILs as differential hosts (Table 2). Ten virulence phenotypes differ from all previously reported and the prefix “MX” has been appended to their designations to indicate their unique geographical occurrence. Not all NMRILs are needed for the identification of new virulence phenotypes, and the 10 new ones reported here are determined based on their differential reactions with six NMRILs (G, I, M, N, O, and AC; Table 3). In previous reports, 7–10 NMRILs were used to determine the virulence phenotypes of *P. capsici* isolates (Barchenger et al., 2018b; da Costa Ribeiro and Bosland, 2012; Jiang et al., 2015; Sy et al., 2008). Ideally, molecular markers could be used to determine virulence phenotypes and the use of a standardized set of differentials (e.g., NMRILs) is crucial to gain a reasonable estimate to support marker development (Barchenger et al., 2018b). Currently, *in planta* greenhouse experiments with differential hosts are the most effective

Table 2. Phenotypic response of the 26 New Mexico Recombinant Inbred Lines when tested for resistance against the isolates of *Phytophthora capsici*.

Host	Isolate ^z									
	CPV-279	CPV-277	CH11	CPV-260	CPV-1	CPV-272	CPV-271	CPV-259	CPV-33	CPV-267
CM-334 ^y	R ^v	R	R	R	R	R	R	R	R	R
NMRIL-T ^x	R	R	R	R	R	R	R	R	R	R
NMRIL-Z	R	R	R	R	R	R	R	R	R	R
NMRIL-P	S ^v	R	R	R	R	R	R	R	R	R
NMRIL-F	R	R	R	S	R	R	R	R	R	R
NMRIL-X	R	R	S	R	R	R	R	R	R	R
NMRIL-A	S	S	R	R	R	R	R	R	R	R
NMRIL-C	S	S	R	R	R	R	R	R	R	R
NMRIL-D	S	S	R	R	R	R	R	R	R	R
NMRIL-E	S	S	R	R	R	R	R	R	R	R
NMRIL-J	R	S	R	S	R	R	R	R	R	R
NMRIL-K	S	S	R	R	S	R	R	R	R	R
NMRIL-O	R	R	R	S	R	R	S	R	R	R
NMRIL-Q	S	R	R	R	R	S	R	R	R	R
NMRIL-R	S	R	R	S	R	R	R	R	R	R
NMRIL-S	R	S	R	R	R	R	R	S	R	R
NMRIL-AA	S	S	R	R	R	R	R	R	R	R
NMRIL-B	S	R	S	R	R	R	S	R	R	R
NMRIL-L	S	S	S	R	R	R	R	R	R	R
NMRIL-M	S	R	R	R	R	S	S	R	R	R
NMRIL-V	S	S	R	R	R	R	R	S	S	R
NMRIL-AB	S	S	R	R	S	R	R	R	R	R
NMRIL-G	S	S	R	R	S	R	R	R	R	S
NMRIL-H	R	R	S	R	S	S	R	S	R	R
NMRIL-AC	S	S	R	S	S	R	R	R	R	R
NMRIL-I	S	S	S	S	R	R	R	R	R	S
NMRIL-N	S	S	S	R	R	S	S	R	S	R
CW ^w	S	S	S	S	S	S	S	S	S	S

Chi-square test of homogeneity showed significant differences between all the infected hosts and the resistant controls.

^zIsolates from the Plant Pathology Laboratory culture collection (Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Mexico), ordered from most virulent to least virulent isolate.

^yResistant control: Criollo de Morelos CM-334.

^xNew Mexico Recombinant Inbred Lines from most resistant to least resistant.

^wSusceptible control: 'California Wonder'.

^vR = resistant host; S = susceptible host.

Table 3. Virulence phenotype designation based on virulence phenotype of 10 isolates of *Phytophthora capsici* on New Mexico Recombinant Inbred Lines differential hosts.

Isolate	CPV-279	CPV-277	CH11	CPV-260	CPV-1	CPV-272	CPV-271	CPV-259	CPV-33	CPV-267
Race	MX-1 ^z	MX-2	MX-3	MX-4	MX-5	MX-6	MX-7	MX-8	MX-9	MX-10
Mating type	A2	A1	A1	A2	A1	A2	A1	A1	A1	A2
NMRIL	Host response									
C ^y	S ^x	S	R	S	S	R	R	R	R	R
G	S	S	R	R	R	R	R	S	R	S
I	S	S	S	S	R	R	R	R	R	S
M	S	R	R	R	R	S	S	R	R	R
N	S	S	S	R	R	S	S	R	S	R
O	R ^x	R	R	S	R	R	S	R	R	R

^zVirulence phenotype designation for isolates of *Phytophthora capsici* from Mexico.

^yNew Mexico Recombinant Inbred Lines arranged in alphabetic order.

^xR = resistant host; S = susceptible host.

method due to the limited information concerning genes directly associated with each virulence phenotype.

NMRIL-N was the most susceptible differential host with 6 of the 10 isolates able to infect. Lines T and Z were resistant to all isolates. These results differ from Jiang et al. (2015), where line S was the most susceptible while lines X and AE were resistant to infection by

isolates from New Mexico. Barchenger et al. (2018b) reported lines A, S, and R as the most susceptible in their experiments and lines P and O as the most resistant against isolates from Taiwan. NMRILs resistant to many *P. capsici* isolates could be used as sources of resistance genes for local or regional programs (Barchenger et al., 2018a).

The disease symptoms produced by the virulent isolates ranged in severity (Fig. 3). CPV-279 was the most virulent isolate causing disease symptoms in 18 NMRILs. CPV-277 was the second most virulent isolate causing symptoms in 15 of the differential hosts. These isolates were recovered from the same cultivation field and had different mating types (Table 3). Isolates CPV-259

Fig. 3. Disease symptoms exhibited in New Mexico Recombinant Inbred Lines (NMRIL) inoculated with *Phytophthora capsici*. Controls: (A) = CPV-279: CW (first three pots) and CM334 (second three pots), (B) = CPV-277: CW (first three pots) and CM334 (second three pots), (C) = CPV-259: CW (first three pots) and CM334 (second three pots). Phenotypic response in NMRIL-AA: (D) = CPV-279: CW (first three pots) and the susceptible NMRIL-AA (second three pots), (E) = CPV-277: CW (first three pots) and the susceptible NMRIL-AA, (F) CPV-259: CW (first three pots) and the resistant NMRIL-AA. Phenotypic response in NMRIL-AC: (G) = CPV-279: CW (first three pots) and the susceptible NMRIL-AC (second three pots), (H) = CPV-277: CW (first three pots) and the susceptible NMRIL-AC, (I) CPV-272: CW (first three pots) and the resistant NMRIL-AC.

and CPV-260; CPV-270, CPV-271, and CPV-272 were recovered from the same field and had different mating types. Given that *P. capsici* is a heterothallic species, this finding suggests that sexual recombination could be occurring and may be important for the generation of new, better adapted, virulence phenotypes (Erwin and Ribeiro, 1996; Polach and Webster, 1972). This represents a challenge for producers because genetic recombination can lead to the emergence of strains capable of breaking the resistance barrier of the hosts (Jiang et al., 2015). The high genetic diversity of *P. capsici* in the center of Mexico (Castro-Rocha et al., 2016) and the high diversity of the *Capsicum* genus in this country (Aguilar-Meléndez et al., 2009) may drive the evolution of diverse virulence phenotypes in Michoacán. It is necessary to know the virulence phenotype makeup of the *P. capsici* populations in the production fields to be successful in the development of resistant cultivars (Jiang et al., 2015).

The isolates CPV-270 and CPV-273 were not virulent to any host. Nonvirulent *P. capsici* isolates have been reported previously when using the NMRILs as differential hosts (da Costa Ribeiro and Bosland, 2012). The isolate CPV-273 was isolated from *Solanum lycopersicum*, and it is possible that they were not pathogenic to *C. annuum* due to host specificity (Cohen 2002; Michalska

et al., 2016). The remaining isolates were virulent to CW and at least to one NMRIL. For the isolates that were able to infect at least one NMRIL, CW seedlings had obvious signs of infection thus supporting the use of CW as a suitable susceptible control (Candole et al., 2012). None of the resistant CM-334 control plants showed any symptoms. The chi-square test of homogeneity showed significant differences between the infected hosts and the resistant controls (Table 2).

This study provides information of phenotypic diversity on pepper for Mexican isolates of *P. capsici* recovered from three hosts. One limitation of this study was the low number of isolates used. Further studies will require characterization of a higher number of isolates from each host, with the goal of obtaining a more robust virulence phenotype structure. These three hosts are cultivated in the same fields in most of the sampled sites, an activity that is harmful to producers because it allows the survival of the virulence phenotypes of *P. capsici* throughout the seasons. A better crop rotation program is required to decrease the inoculum concentration. Crop rotation for 3 years with nonsusceptible hosts is recommended because the oospores can survive and remain virulent in soils (Babadoost and Pavon, 2013; Ristaino and Johnston, 1999).

Overall, our results concur with other studies where NMRILs were successfully used as differential hosts to identify *P. capsici* virulence phenotypes from different parts of the world including the United States, Brazil, and Taiwan (Barchenger et al., 2018b; da Costa Ribeiro and Bosland, 2012; Jiang et al., 2015; Sy et al., 2008). Also, the NMRILs are useful to designate virulence phenotypes in *P. capsici* isolates from non-*capsicum* hosts. This was previously demonstrated by da Costa Ribeiro and Bosland (2012) and might provide a basis for determining the existence of special forms. One of the main limiting factors for the development of universally resistant cultivars is the lack of a standardized system for *P. capsici* virulence phenotypes determination (Barchenger et al., 2018b). Some studies have used commercial cultivars as differential hosts (Glosier et al., 2008; Oelke et al., 2003) which makes it difficult to compare results. Commercial pepper cultivars are not available worldwide and have the risk of gene segregation (Monroy-Barbosa and Bosland, 2011), therefore the use of the NMRILs could be a viable alternative for a standardized test (Barchenger et al., 2018b).

The use of tolerant and resistant varieties is a low cost and environmentally friendly alternative that is easy to implement by producers (Hausbeck and Lamour, 2004). Creole

cultivars of “Huacle” and “Serrano” cultivars found in Mexico are resistant to some isolates of *P. capsici* (Gómez-Rodríguez et al., 2017; Palma-Martínez et al., 2017). Preliminary results show that native cultivars of “Pasilla” type chili pepper plants from the municipality of Queréndaro, Michoacán, are tolerant to some *P. capsici* isolates when inoculated with 1×10^5 zoospores (data not shown). Now that 10 *P. capsici* virulence phenotypes have been identified from the same geographic area, the next step would be to test the resistance of this tolerant plant material.

Our research provides important first results about the presence and composition of virulence phenotypes in the populations of *P. capsici* in production fields in Michoacán and is useful to producers of the state. The NMRILs were shown to be a valuable tool to designate virulence phenotype in *P. capsici* isolates of Mexico. The presence of multiple virulence phenotypes and of both mating types in the same production field suggests that outcrossing and sexual recombination may play a role in the emergence of new strains that could overcome host resistance. Knowledge of the virulence phenotypes composition within the populations of these production fields is useful in the decision-making process when developing resistant cultivars and disease management strategies. Further studies about the virulence phenotype structure in more regions from Mexico using the NMRILs, coupled with increased genetic diversity data, will help researchers and producers better understand the epidemiology of the disease in the country.

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