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Identification of sources of resistance in tomato to *Phytophthora infestans* at Mexico

Identificación de fuentes de resistencia a *Phytophthora infestans* en jitomate en México

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Abstract

Late blight caused by *Phytophthora infestans*, devastating disease in tomato (*Solanum lycopersicum* L.) worldwide. The present study was carried out to identify sources of resistance in wild genotypes of *Solanum lycopersicum* var. *cerasiforme* collected in Mexico, and experimental varieties. San Marzano and Rio Grande were susceptible controls, and the resistant control, accession LA2533 *pimpinellifollium*. Field and greenhouse trials were established for exposure to natural infections. Incidence and severity of the disease and the area under the curve of the disease (AUDPC) were estimated. In laboratory, leaf samples were inoculated with six pathogen strains isolated from potato plants, from Valle de México. It was calculated necrotic area percentage (NAP), sporulation degree (SD) and index disease (ID). Wild populations V115, 319 and 327 had similar behavior to resistant control, followed by experimental genotypes 1-1, 3-1, 1-12, 2-29, 3-31 and 2-14. In separated leaflets inoculated test with Toluca´s strains , V115 highlighted, followed by 3-3, 1-12, 2-29, 3-6, 1-1, 2-14 and 319 and moderated resistance were showed by 3-1, 3-31, LA2533, 3-33 and 327. Susceptible control San Marzano obtained higher values for AUDPC, NA, SD and ID.

***Late blight, Solanum lycopersicum* var. *cerasiforme*, genotypes, disease resistance, AUDPC**

Resumen

El tizón tardío causado por *Phytophthora infestans*, enfermedad devastadora en tomate (*Solanum lycopersicum* L.) a nivel mundial. El presente estudio se realizó para identificar fuentes de resistencia en genotipos silvestres de Solanum *lycopersicum* var. *cerasiforme* colectados en México, y variedades experimentales. San Marzano y Río Grande fueron los controles susceptibles, y el control resistente, la accesión LA2533 *pimpinellifollium*. Se establecieron ensayos de campo y de invernadero para la exposición a infecciones naturales. Se estimaron la incidencia y la gravedad de la enfermedad y el área bajo la curva de la enfermedad (AUDPC). En laboratorio, se inocularon muestras foliares con seis cepas del patógeno aisladas de plantas de papa, provenientes del Valle de México. Se calculó el porcentaje de área necrótica (NAP), el grado de esporulación (SD) y el índice de enfermedad (ID). Las poblaciones silvestres V115, 319 y 327 tuvieron un comportamiento similar al testigo resistente, seguidas por los genotipos experimentales 1-1, 3-1, 1-12, 2-29, 3-31 y 2-14. En el ensayo de inoculación de foliolos separados con cepas de Toluca, destacó V115, seguido de 3-3, 1-12, 2-29, 3-6, 1-1, 2-14 y 319 y mostraron resistencia moderada 3-1, 3-31, LA2533, 3-33 y 327. El control susceptible San Marzano obtuvo valores más altos para AUDPC, NA, SD e ID.

Tizón tardío, Solanum *lycopersicum* var. *cerasiforme*, genotipos, resistencia a la enfermedad, AUDPC

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Introduction

Late blight, caused by *Phytophthora infestans* (Mont.) de Bary, stands out for its destructive capacity on tomato (red tomato) and potato crops, which causes that worldwide about one billion dollars are spent annually in the application of products for its control (Abreu *et al*., 2008). In Sinaloa, Mexico, losses of up to 100% were reported in the 1967-68, 1978-79, 1980-81 (Retes, 1982) and 1991-92 cycles (Félix *et al.*, 2004). Following the onset of infection, entire tomato crops can be destroyed in 7 to 10 days (Merk *et al*., 2012).

Infection and development of this pathogen are optimal under conditions of high relative humidity (91 to 100 %) and temperatures between 15 and 20 °C (Agrios, 2005; Leyva *et al*., 2013).

P. *infestans*, belonging to the Oomycetes group, reproduces both sexually and asexually, behaving as a heterothallic organism that requires two types of mating, called A1 and A2 (Fry and Goodwin, 1997, Alarcón *et al*., 2013). However, homothallic types (A1/A2) with different allo-enzymatic genotypes also occur in Chapingo, Mexico (Alarcón *et al*., 2013).

The Toluca Valley, Mexico, has been proposed as the site of origin of this pathogen (Grünwald and Flier, 2005; Romero *et al.*, 2011) considering that the sexual form of the pathogen, reported by Graham, *et al*. (1959), had not been observed elsewhere. The pathogen population exhibits diverse virulence characteristics; all enzyme alleles known to date have been detected and populations from this locality contain all DNA fingerprinting bands (Fry and Goodwin, 1995; Andrivon, 1996). Thus, the world's greatest genotypic diversity of P. *infestans* is found in the Mexican central highlands and the Chapingo area, east of Lake Texcoco, is the second center of diversity of the oomycete (Alarcón *et al*., 2013).

A practical and economical method regarding disease control is based on the use of resistant genotypes, which has led to an intensified search for genetic resistance, using wild species for this purpose (Frías *et al*., 2001; Barquero *et al*., 2005a; Barquero *et al*., 2005b; Scott and Gardner, 2007).

Chunwongse *et al*. (1998), Gardner and Shoemaker (2004), Robertson and Labate (2007), mention the existence of three genes that condition resistance to specific races against *P. infestans* in tomato: Ph1, Ph2 and Ph3, from different populations and accessions of *Solanum* (=*Lycopersicon*) *pimpinellifolium*, located on chromosomes 7, 10 and 9, respectively. Foolad *et al.* (2006) found in this species a new gene (Ph5) located on chromosome 1 in accession PSLP153 of S. pimpinellifolium which confers resistance to no less than five different races of P. *infestans* (US8, US13, US14, US15 of A2 mating type), and numerous highly resistant selections of *S. habrochaites* (=*L. hirsutum*) have been made (Scott and Gardner, 2007).

In Mexico, the search for resistance to late blight is focused on potato accessions, so in tomato there is little scientific information available regarding the generation or discovery of resistant varieties. However, there is potential in native varieties and in wild populations of *S. lycopersicum* var. *cerasiforme*, widely distributed in Mexico from Sinaloa to the Yucatan Peninsula (Chavez *et al*., 2011), present in abandoned and cultivated fields, in tropical dry forests, coastal sites on the Pacific slope (300-1,100 m) of the Sierra Madre Occidental; with the largest populations collected at altitudes between 0 and 1,200 m (Sánchez *et al*., 2006; Álvarez *et al*., 2009).

Based on the above, the objective of this study was to identify sources of resistance to P. *infestans* among three wild genotypes of tomato *Solanum lycopersicum* var. cerasiforme (Dunal) Spooner, Anderson and R.K. Jansen collected in Mexico, and nine advanced lines, through exposure to natural and artificial infections using strains from the Valley of Mexico.

Materials and methods

Plant material

Since 2002, wild populations of tomato *S. lycopersicum* var. *cerasiforme* (Dunal) Spooner, G. J. Anderson et R. K. Jansen were collected in different regions of the country with the support of the National System of Phytogenetic Resources (SINAREFI-SAGARPA).

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As a result of the characterization and evaluation of the wild populations during the years 2004 to 2008 in field and greenhouse conditions, in the experimental area of the Centro Universitario de Ciencias Biológicas y Agropecuarias (CUCBA) of the University of Guadalajara, located in Las Agujas, municipality of Zapopan, Jalisco, Mexico at 20° 44' 44" N latitude, 103° 54' 62" W longitude and an altitude of 1,567 m asl, resistance and/or tolerance responses against late blight attack were observed, where populations V115, 319 and 327 stood out (Arellano *et al.* , 2013).

To confirm the above results, these 3 wild populations were used: V115 (originating from Veracruz, Mexico), 319 and 327 (originating from Nayarit, Mexico), in addition, nine experimental tomato materials developed through crosses, backcrosses and selection from the open-pollinated variety "Rio Grande" and a wild plant originating from Nayarit, Mexico were included; the denominations for the selected lines were:1-1, 1-12, 2-14, 2-29, 3-1, 3-3, 3-6, 3-31 and 3-33.

The Rio Grande and San Marzano varieties were used as controls, and the accession LA2533 belonging to the *Solanum pimpinellifolium* species, provided by the Tomato Genetics Resource Center (TGRC) of the University of California-Davis in Davis, California, reported to have genetic resistance to races 0 and 1 of *P. infestans* (Chetelat and Rick, 1998).

Exposure to natural infections. During the Spring-Summer 2012 cycle, three trials were established in the CUCBA experimental area: Trial I in a field area with a history of tomato planting; Trial II in an open field where corn had been grown; and Trial III in a greenhouse. Seedling production was carried out under greenhouse conditions, placing the seeds in 200-cavity polystyrene trays filled with a mixture of peatmoss (Sphagnum) and coconut fiber 50-50% vol/vol. At the beginning, they were irrigated with simple water (one week) and after the seedlings emerged they were irrigated with Steiner's Universal nutrient solution (Rodriguez, 2004) at 0.3 atm of concentration until transplanting, after 35 to 40 days.

The trials were established in a randomized complete block design with 2, 6 and 5 replications respectively for trial I, II and III. The experimental unit consisted of 5 plants per plot, with a plant spacing of 0.40 m and a row spacing of 1.20 m. In these trials, no fungicides were applied; cultivation tasks such as manual weeding, foliar fertilization and chemical pest control (whitefly and mites) were carried out.

Late blight occurred during the months of September and October, with daily minimum temperatures of 12 and 14 ºC and relative humidity of 80 to 90%, especially during the night and early morning hours (6 am to 10 pm). Taking into consideration the total number of plants in each experimental unit, and in order to determine the average severity of each experimental unit, visual readings of the percentage of disease severity were taken every seven days, according to the International Potato Center scale (Henfling, 1987): 1=0%, 2=3%, 3=10%, 4=25%, 5=50%, 6=75%, 7=90%, 8=97%, and 9=100%. According to Forbes *et al*. (2014), when the number of plants per plot is low, some researchers take severity data on a per plant basis. However, there is little evidence that this process confers any advantage, with the aggravating factor that it requires a significant amount of additional time. For this reason, CIP recommends taking data simply at the plot level.

Finalizing canopy damage ratings when the varieties used as susceptible controls reached 95 to 100% of leaf area damaged by *P. infestans* (Frías *et al*., 2001). In order to compare the behavior of each material, the area under the disease progress curve (ABCPE) was calculated with the data obtained each week in the experimental plots, to obtain a single data for statistical comparisons (Shaner and Finney, 1977; Campbell and Madden, 1990):

$$AUDPC = \sum_{i=1}^{n} \left[\frac{Y_{i+1}+Y_i}{2}\right] [X_{i+1} - X_i] \qquad (1)$$

Where:

Yi= Percentage foliage of affected tissue at each reading,
Xi= Time in days from transplanting to the time of evaluation, and
n = Total number of observations

Separated leaflet test. This test included six strains of *P. infestans* isolated from potato crops and characterized by Dr. Héctor Lozoya Saldaña and Dr. Norma M. Alarcón Rodríguez (Alarcón *et al*., 2014) from the Universidad Autónoma Chapingo, Mexico (Table 1). In experimental fields of this University, during the 2008 and 2009 rainfed crop cycles, isolates of *P. infestans* were randomly collected from simple, young, leaf and stem lesions, taking the tissue with the lesion once it appeared due to natural infec¬tion of the pathogen.

The diseased tissues were placed on healthy potato slices (var. Alpha) disinfested with 2% sodium hypochlorite in water (v/v) in petri dishes (10 cm diameter) to allow mycelial growth through the tissue at room temperature. On the third day, mycelial growth was observed microscopically, and the presence of the pathogen was confirmed by morphological observations on fixed preparations. The purified mycelium was transferred to solid agar-centene medium and incubated at 21 °C for characterization (Grünwald *et al*., 2001).

Compatibility was determined by crossing the *P. infestans* isolates with a known compatible type strain (A1 or A2) provided by the Programa Internacional Cooperativo del Tizón Tardío Tardío de la Papa (PICTIPAPA A. C.), Metepec, Mexico. On agar-centene medium, in a Petri dish (10 cm in diameter), the unknown strain and the known type were planted on opposite sides so that they would grow towards the center of the dish. After two to three weeks at 21 °C, the presence of oospores where the mycelia crossed with the A1 type identified the unknown isolate as A2, and crossing with the A2 type indicated that the unknown isolate was A1. If the same isolate formed oospores when crossed with the two known type strains, it was considered homothallic (Gilchrist *et al*., 2009).

Genotype identification by allozyme was performed using the method described by Goodwin *et al*. (1995). Mycelium of two to three weeks of active growth was transferred to a microcentrifuge tube with 30 µl of sterile distilled water and macerated with a plastic drill bit. After resting for 5 min, 10 µl was taken and placed on cellulose acetate plates (Titan III, Helena Laboratories). Electrophoresis was performed in Tris-Glycine buffer (pH 8.5).

Glucose-6-phosphate isomerase (Gpi) and Peptidase (Pep) enzymes were used, and each enzyme was revealed according to the methodology reported by Hebert and Beaton (1993). Parallel to field sowings of tomato materials established in greenhouses (experiment III), leaflets from the 5th and 6th subterminal leaves, fully developed, were taken from plants 6 to 8 weeks after transplanting. Subsequently, they were placed in transparent plastic boxes of 20×20×6.5 cm, in abaxial position, on a sterile paper towel placed on a metal sieve. To each box, 100 ml of sterile water was previously added. Each leaflet was inoculated with 25 µl of sporangium suspension, at a concentration of 40,000 sporangia ml-1. The boxes with inoculated leaflets were placed in the laboratory at 18±1°C and a photoperiod of 16 h light, as proposed by Barquero *et al*. (2005b) and Xuan and Byung (2007).

The test was done in duplicate and for each material 10 leaflets were used. The degree of resistance of the genotypes was determined by calculating the disease index described by Jeger *et al*. (2001), Pérez *et al*. (2001), and Barquero *et al*. (2005b) ,where three variables are considered in each leaflet with the following scales: leaflet necrotic area (NA): 1=no symptoms, 2=necrotic spots at the drop site, 3=necrotic spots of 2 mm, 4=1 cm, 5=50%, 6=51-65%, 7=66-75%, 8=76-85%, and 9= >85%; degree of sporulation (GE): 0=no sporulation, 1=Some sporangia in necrotic area, 2=50% of necrotic area with sporangia and 3= 100% of necrotic area with sporangia. Each leaflet received a disease index (DI) value corresponding to the product of the necrotic area and the degree of sporulation, considering that values from 1 to 9 correspond to highly resistant genotypes, 10 to 18 moderately resistant and 19 to 27, highly susceptible genotypes (Barquero *et al*., 2005b).

Statistical analysis

The data of the variable AUDPC, obtained from the three trials of exposure to natural infections in the field and greenhouse, were tested for normality using the Univariate procedure of SAS 8.1 and the NORMAL option based on the Shapiro-Wilks test.

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When the data did not meet the assumption of normality according to the test, transformations were performed on the ABCPE data, where the square root of ABCPE+1 allowed the values to adjust to a normal distribution. For the variables studied in the field, greenhouse and leaflet test, analysis of variance (ANAVA) was carried out individually and in combination. To separate genotypes into groups based on resistance, tolerance or susceptibility to late blight, a Tukey multiple means test was carried out for each observed variable. The statistical package SAS (Statistical Analysis System 8.1) was used.

Results and discussion

Exposure to natural infections

Analyses of variance for the variable Area Under the Disease Progress Curve in the three trials indicated highly significant differences ($P \leq 0.01$) among genotypes. The three wild populations, in the three trials were grouped together with the resistant control LA2533 (Table 2), followed by the 9 improved experimental materials and only in trial I genotype 3-31 reached similar levels to the susceptible genotypes. The V115 and 319 populations had lower ABCPE values than LA2533 in all three trials, 19 to 76 % lower and 82 to 94 % lower than the susceptible varieties.

When comparing the trials among themselves, the highest ABCPE values were found in the greenhouse (trial III), followed by trial I, where tomato had been grown in previous cycles, and thirdly in trial II, where corn had been grown previously.

In the combined analysis (Table 2), a clear separation between genotypes was observed. Group "d" corresponds to population V115 with the highest degree of resistance shown through experiments, followed by populations 319 and 327 (significance group cd) and accession LA2533 (group c). In group b, with intermediate resistance, were the experimental materials; and finally in group a, the susceptible controls, with the highest ABCDE values. The higher this value, the more susceptible the variety (Forbes *et al*., 2014). The data indicated that significant interaction between genotypes and trials varied in the response of genotypes in relation to the evaluation site.

Graph 1 shows that populations V115 and 319, showed a similar response in the three conditions, while population 327 showed greater susceptibility to the pathogen under greenhouse conditions, as did eight of the experimental varieties. On the other hand, variety 3-31 and accession LA2533 showed an increase of the disease in the sites with a history of tomato. In contrast, Rio Grande and San Marzano showed a lower level of disease in field conditions with a history of tomato cultivation and a very high susceptibility under greenhouse conditions, which could be due to very favorable conditions for pathogen expression, including greater leaflet development, influencing pathogen variability (Stewart *et al*., 1983), in addition to favoring pathogen expression due to the predominant humidity and temperature inside the greenhouse.

In field trials infected by late blight races, their aggressiveness, virulence and amount of inoculum are usually unknown and many climatic changes occur during the test season that can influence the behavior of pathogen-resistant genotypes by strongly depending on the environment (Michalska *et al*., 2011).

In the present study, the greenhouse did not have equipment for internal environmental control, so that during the months of September and October, during the night and early morning hours, temperatures tended to drop (below 15oC) and the predominant relative humidity was above 90%, favoring the presence of dew on the upper part of the greenhouse (roof and plastic walls), allowing the wetting of the leaves for a period of more than eight hours. According to Henfling (1987), late blight sporangia only form when relative humidity is above 95%; furthermore, if water (dew, dew, rain) is present on the leaf surface for a minimum of two hours, zoospores germinate and penetrate.

The response of the genotypes to the natural infection of *P. infestans* in the field and greenhouse conditions of Zapopan, Jalisco were consistent, in terms of the relative behavior among genotypes, which led to consider the wild populations of var. *cerasiforme* as the most resistant to the pathogen, together with accession LA2533.

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Separated leaflet test

Significant differences (P0.01) were obtained between genotypes in the ANAVA, while between strains and genotypes there was no interaction in the 3 variables evaluated (AN, GE, and IE), indicating that the genotypes presented a similar response when inoculated with the different strains.

The lowest values of necrotic area were found in the V115 population, and the experimental materials 1-12, 2-29, 3-3 and 3-6, while in degree of sporulation (DS) the lowest values were obtained in V115 and 3-3; with the exception of 327 and San Marzano, the other 10 materials had intermediate values (Graph 2). Genotypes V115, 3-3, 3-6, 1-12, 2-29, 1-1 and 2-14 were in the highest resistance group because they had the lowest disease index. Materials 319, 3-1, 3-31, LA2533, 3-33 and 327 were placed with an intermediate disease index (Graph 2).

According to Barquero *et al*. (2005b), disease index (DI) values from 1 to 9 correspond to highly resistant genotypes such as V115, 3-3, 3-6, 1-12, 2-29, 1-1, 2-14; from 10 to 18, medium resistance: 319, 3-1, 3-31, LA2533, 3-33 and 327; and from 19 to 27, highly susceptible genotypes such as the San Marzano (SM) control.

When analyzing the aggressiveness of the strains, expressed in the three variables evaluated, genotypes V115, 3-3, 1-12, 2-29, presented the lowest values, indicating greater resistance to the six strains with which they were inoculated. For genotype 2-14 the highest value of necrotic area and degree of sporulation was with strain 24. In general, strain 13 was the most aggressive for V115, 3-3, 319, 327 and strain 10 was the least aggressive. On average, strains 23, 24 and 31 were the most aggressive for the rest of the materials.

When subjected to the action of different strains in the leaflet test, the wild population V115 showed lower levels of necrotic area and sporulation than the resistant control, being characterized by maintaining its high resistance even when subjected to the action of the strains from Chapingo.

This response is of interest if it is considered that in the central part of Mexico is where the greatest diversity of this pathogen is found worldwide, while in other areas of Mexico and the world there are populations of late blight with low or moderate genotypic diversity (Grünwald *et al.,* 2001 and Flier *et al*., 2003).

These low values could indicate that the plant suppresses the action of the fungus on its foliage and at the same time reduces its sporulation capacity, while in the resistant control LA2533 the necrotic area has a higher value but a degree of sporulation similar to V115, which could indicate that the pathogen manages to affect the foliage of the plant and in response the plant tries to suppress its sporulation by developing a hypersensitivity reaction around the infected tissue. This hypersensitivity reaction is intended to protect or immunize the rest of the plant against a potential second infection (Lemus, 2009).

The two wild populations of var. *cerasiforme* from Nayarit showed intermediate levels in the disease index, close to the value of the resistant control and well below the value presented by the susceptible control. Population 319 showed less necrotic area and less sporulation than LA2533 and a lower disease index than LA2533, but higher than V115, while population 327 showed a lower necrotic area value but a higher sporulation index value, which places it with a higher disease index. In ABCPE the positioning of these two populations is reversed, 327 with higher resistance than 319.

When comparing the values obtained in natural infections (field) and those obtained with artificial inoculation of strains from the Chapingo area, the experimental materials showed greater resistance to artificial infections, and some of them (3-3, 3-6,1-12, 2-29,1-1 and 2-14) were superior to the resistant control LA2533 and genotypes 319 and 327.

These differences may be due to the fact that possibly the experimental materials present specific resistance to certain strains of the pathogen and when subjected to field evaluations with high pathogen pressures and climatic changes during the development of the same, made it possible to break the resistance.

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Since specific resistance in plants allows a clear differentiation between races of a pathogen, since it is effective against certain specific races of the same and ineffective against others (Lemus, 2009). Lesion size (% necrotic leaf area) and spore density (degree of sporulation) values measured in separate leaflet tests apparently correlate well with ABCPE values obtained in field experiments for late blight (Singh and Birhman, 1994). However, Vleeshouwers *et al*. (1999), using these two tests on several *Solanum* species, found no significant differences between them.

A disadvantage of selecting resistant genotypes under field evaluations versus selection under laboratory conditions is that the latter is less related to the environmental conditions prevailing in agricultural areas; since field resistance is directly evaluated including interactions of climatic conditions, cultural practices, pesticides, and other diseases (Horneburg and Becker, 2011). In many reported experiments with selection under artificial conditions, only one or a few strains of *Phytophthora infestans* have been applied to tomato (Michalska and Pazio, 2005).

To date, there is very little work reported on this crop using these methodologies (Foolad *et al*., 2008). However, laboratory trials can help breeders to discard part of the germplasm to minimize field treatments, which is necessary for the correct selection of resistant lines (Stewart *et al*., 1983; Dorrance and Inglish, 1997). Laboratory conditions are defined and stable conditions, a single strain is applied, they usually have complex virulence and high aggressiveness and a high concentration of inoculum is used (Michalska *et al.*, 2011).

Therefore, if we want to have reliable results on the genetic resistance of some genotypes to late blight, it is recommended to evaluate this resistance with strains from the Valley of Mexico, since in numerous studies the identification of resistance in wild species has been based on inoculations in artificial environments, or with exposure to limited genotypes of the pathogen (Lozoya *et al*., 2006).

Conclusions

It was confirmed that the three wild populations of *Solanum lycopersicum* var *cerasiforme* have high resistance to *P. infestans*, both in field and greenhouse tests and with exposure to strains from the Valley of Mexico, which can be incorporated into breeding programs as sources of resistance to late blight for the generation and development of commercial varieties.

The experimental materials showed a consistent response, intermediate in resistance, both in field and greenhouse trials, as well as in the leaflet test. Among them, it is possible to consider genotype 1-12 as a viable germplasm source, with comparable levels of ABCPE, necrotic area and degree of sporulation to LA2533.

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