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Inoculation methods and conidial densities of *Fusarium oxysporum* f. sp. *radicis lycoperici* in tomato

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Abstract

Fusarium oxysporum f.sp. *radicis-lycopersici* (Forl) is one of the most destructive necrotrophic pathogens affecting tomato crops, causing considerable field and greenhouse yield losses. The use of resistant cultivars is a safe, non-contaminating and reliable control method to eradicate or mitigate this disease. However, it is still necessary to determine the best inoculum density, sufficient to induce levels of resistance or susceptibility. In this study, inoculum concentrations of 3, 5 and 8 x 10^6 microconidia per mL of Forl were evaluated by root immersion and seed spraying in twenty tomato genotypes (*Solanum spp.)* in a completely randomized design. The germplasm was mainly introduced tomato species from the Genetic Resources Program of the University of California at Davis, USA. The inoculation was done by immersion of roots and by sprinkling of seeds at the time of sowing. For the inoculation by immersion of roots, tomato seedlings of 15 days emergence were used. The wounds were created in root tips then they submerged into the each conidial suspension. Finally, they transplanted into the plastic pots in a greenhouse. Regarding the inoculation by sprinkling of the seed, 20 seeds of each genotype were placed in plastic pots then sprayed with the corresponding conidial suspension. In both cases, the disease index was calculated. This allowed classifying the genotypes as resistant and susceptible. With the root immersion method all the genotypes evaluated showed susceptible. Significant differences were observed ($p \leq 0.01$) between genetic materials at different inoculum doses with the inoculation of seeds. This allowed classification of genotypes *Solanum parviflorum* LA 1326, *Solanum chesmanii* f. minor LA1401, *Solanum chmielewskii* LA1306, *Solanum pimpinellifolium* LA722, *Solanum pimpinellifolium* LA2184 as resistant to *Forl* with disease index of 3, 4, 5, 8 and 12% respectively, at a inoculum concentration of 3x10^6 microconidia per mL. These genotypes could be included in a genetic breeding program for resistance to crown and root rot caused by *Forl.*

Keywords: Crown rot, *Forl*, Immersion of roots, Inoculum density, Resistance.
Abbreviations: FORL_fusarium oxysporum f. sp. radicis lycopersici, PDA_potato dextrose agar, DI_Disease index, ANOVA_analysis of variance, SS_ sprinkling the seeds, RI_root immersion.

Introduction

Fusarium oxysporum f. sp. *radicis-lycopersici (Forl*) is a necrotrophic pathogen. It is a causal agent of crown and root rot tomato, a disease of global economic importance in the cultivation of tomato (*Solanum lycopersicum* L.) with significant losses in production in greenhouse and in the field (McGovern, 2015). The first reports on *Forl* came from Japan (1969) and California (1971) (Fazio et al., 1999).

Substantial crop losses in infected fields have given the international attention to disease. The host range of this pathogen comprises at least 36 other species (Menzies et al., 1990). Katan and Katan (1999) identified nine vegetative compatibility groups, indicating high genetic variation within *F. oxysporum* f. sp. *radicis-lycopersici.* The disease is characterized by a long period of incubation. The external symptoms appeared immediately, when the infection occurred immediately after sowing. However, if infection occurs during seedling production, the disease may manifest at the time of flowering (Ślusarski, 2000). The pathogen causes root rot and extensive necrotic lesions in the neck and stem base, wilt and plant death. The first symptoms of disease include a yellow discoloration of the older leaves that gradually progresses towards the younger leaves. As the disease progresses, the entire root system becomes brown and the primary root rots (Apodaca et al., 2004). *Radicis-lycopersici* constitutes of two special forms. These forms of *Fusarium oxysporum* are mostly important in tomato cultivation and are likely to coexist simultaneously in the same crop and even in the same plant. However, they present important genetic differences in epidemiology and symptomatology (Çolak and Biçici, 2013). During the evaluation of the evolutionary relationships between special forms associated with tomato, Lievens et al. (2009) found multiple evolutionary lineages for both *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici.* The phylogenetic analysis was based on the extension factor of 1α translation and the exopolygalacturonase genes. Although several methods have been used to control this pathogen, the use of resistant cultivars is the most

acceptable and economical control system (Szczechura et al., 2013). Identification of resistance sources by pathogenicity tests is an essential starting point for obtaining resistant varieties. Some field, greenhouse and laboratory techniques have been generated, including molecular techniques to evaluate tomato germplasm (Morid et al, 2012).

The penetration of the vascular elements of the tomato seedlings roots by *Forl* depends on the presence of damaged, senescent, necrotic cells or those provide the entrance through wounds. The efficiency of inoculation will increase if roots of seedlings are wounded (Beckman, 1987).

Jacobs & Van Heerden (2012) identified *Fusarium oxysporum* f. sp. *radicis-lycopersici* as the causal agent of crown and root rot of tomato in South Africa. They studied morphological and genetic characterization and pathogenicity assays by immersion of root (inoculation) at a concentration of 2 x 10^7 microconidia per mL for 3 minutes. Baysal et al. (2009) also inoculated tomato plants for 10 min in a conidial suspension of *Forl* of 7x10^7 conidia/mL. They were able to isolate and identify fourteen strains with characteristics of Forl, corroborating their identification through the use of microbiological and molecular markers.

Fusarium oxysporum f.sp. *radicis-lycopersici* (Forl) is one of the most destructive necrotrophic pathogens affecting tomato crops, causing considerable field and greenhouse. The use of resistant cultivars is a safe, non-contaminating and reliable control method to eradicate or mitigate this disease. Prior to this, it is necessary to determine the inoculum density sufficient to induce levels of resistance or susceptibility. In this study, inoculum concentrations 3, 5 and 8 x 10^6 microconidia per mL of *Forl* were evaluated by root immersion and seed spraying in twenty tomato genotypes (*Solanum spp.)* in a completely randomized design.

Results and discussion

Root immersion inoculation

All evaluated genetic materials were susceptible to fungi with disease rates higher than 50% (Table 2). However, some significant differences were observed among the different *Solanum* materials and among the inoculum doses (Table 3). In the diseased plants, the symptoms began with the appearance of a brown coloration in the root, based on the stem and vascular system that extended from three to six centimeters above the knot of the plant. In some genetic materials, we also observed weak and stunted plants with slight to moderate wilting.

The Walter variety which is a susceptible control showed an average disease rate of 79%. In tomato culture, root cell death occurred rapidly upon protoplast contact with fungal hyphae. This pathogen penetrates the roots mainly through wounds and proceeds into and throughout the vascular system, leading to functional collapse, systemic wilting and often the death of the infected plant (Steinkellner et al., 2005). This suggests that two centimeters damage to the roots by cutting prior to inoculation can facilitate the entry of the pathogen, subsequent invasion and death of the root cells. This usually causes a high severity of the disease.

In the wall of the cells of the cortex of tomato roots, aromatic compounds and suberine are formed as a normal response of resistant plants to the penetration of root pathogens and after facilitating the entry of the fungus into the tissues of roots, the pathogen enters easily (Nawrath et al., 2013). In this study, the method of inoculation of this pathogen with recommended doses of inoculum could not distinguish resistance responses between the studied genotypes.

Three days after inoculation, most of the tomato seedlings had several brown spots along the main root, including the crown. The network of hyphae invaded most of the root surface and spread to the tips, while the cells of the epidermal tissue are completely colonized by hyphae (Lagopodi et al., 2002). This colonization, although discontinuous because the plant's defense reactions limited the spread of the fungus, indicates the possibility that a resistant cultivar could be invaded and attacked when the disease is induced artificially so drastically. Therefore, defense mechanisms such as wall opposition, production of secondary metabolites, lignification and occlusion of vascular tissue and the formation of tylose (Alabouvette et al., 2009) may have lost efficacy to retain away the fungus.

Koyyappurath et al. (2015) employed histopathological preparations. They observed the preparations through wide field and multiphoton microscopy and showed that *F. oxysporum* penetrated the root hair region of roots, and then invaded the cortical cells, where it induced necrosis. The hyphae never invaded the root vascular system up to 9 days post-inoculation.

The response of the genetic materials to root immersion was “high susceptibility” in the three inoculum densities. To prevent evasions of genotypes susceptible to infection, it is common for researchers to use high inoculum densities. In these cases, it is easy to detect a high level of resistance but small differences in susceptibility tend to disappear.

The optimum inoculum density is the one that greatly allows plants to avoid evasion of pathogen. The identification of resistant materials and only the most susceptible genotypes are strongly affected (Elmer, 2002). The inoculum concentration of 3 x 10^6 conidia per mL was adequate to induce the disease. This method is efficient to evaluate germplasm of *Solanum* species for resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici.*

Spray inoculation on seeds

Spray inoculation on seeds at the time of planting is a method that has been little used in tomato, but is very effective in inducing *Fusarium oxysporum* diseases (Fazio et al. 1999). In this experiment, the rate of disease was lower than those obtained by inoculation of root immersion. The Walter variety was a susceptible control showed disease rates of 75, 75 and 87.5% for the concentrations of 3×10^6, 5×10^6, and 8×10^6 conidia per mL of suspension, respectively, with a mean disease index of 79%.

The analysis of variance for disease index indicated significant differences for inoculum doses and between genetic materials (Table 3). The differences enabled distinguishing of genetic materials from completely healthy (with 0%) to fully susceptible disease rates to disease rates higher than 50%. For discrimination of susceptible and/to resistant materials, those with an average disease index of 0 to 25% were considered to be resistant. The moderately susceptible to intermediate plants showed indexes of 26 to 50%. The susceptible plants were those that presented disease rates higher than 50%.

Table 1. Tomato genotypes (*Solanum spp*.) evaluated for their reaction to *Fusarium oxysporum* f. sp. *radicis lycopersici* by two inoculation methods and three inoculum densities.

Cultivars and species	Key PCRG-UC[1]	FC	Origin
S. lycopersicum cv. Creole	LA404 (90L335)	Red	Perú
S. lycopersicum cv. Creole	LA1251 (90L3575)	Red	Ecuador
S. lycopersicum cv. Creole	LA1021 (84L6594-1,2)	Red	Ecuador
S. lycopersicum cv. Creole	LA468 (83L4649)	Red	Chile
S. lycopersicum cv. Creole	LA172 (84L6491-4)	Yellow	Bolivia
S. lycopersicum cv. Creole	LA147 (90L3518)	Red	Honduras
S. lycopersicum cv. Saladette	LA2662 (88L1368)	Red	EUA
S. lycopersicum cv. Edkawi	LA2711 (86L9489)	Red	Egipto
S. lycopersicum cv. Walter		Red	EUA
S. lycopersicum cv. I_3R_3		Red	EUA
S. lycopersicum cv. Bonnie Best		Red	EUA
S. lycopersicum cv. Manapal		Red	EUA
S. pimpinellifolium	LA722 (86L29486)	Red	Perú
S. pimpinellifolium	LA2184 (87L0413)	Red	Perú
S. chmielewskii	LA1306 (87L0617)	Green	Perú
S. chesmanii f. *minor*	LA1401 (85L8098)	Red	Ecuador
S. parviflorum	LA1326 (81L572)	Green	Perú
S .lycopersicon var. *cerasiforme*	LA1673 (83L4805)	Red	Perú
S. hirsutum f. *glabratum*	LA1223 (86L9840)	Green	Ecuador
S. chilense	LA1963 (85L1851)	Green	Perú

[1]Program of conservation of genetic resources, University of California, Davis, Ca. FC= Fruit color.

Table 2. Disease index in 20 tomato genotypes (*Solanum spp*.) inoculated with three inoculum densities of *Fusarium oxysporum* f. sp. *radicis-lycopersici* and two methods of inoculation.

		RI				SS			
Solanum spp and key PCGR- UC[1]/ inoculum density		3*	5*	8*	Mean value	3*	5*	8*	Mean value
S. lycopersicum cv. Creole LA404	1	80	82.5	82.5	82ab	52.5	70	75	66ab
S. lycopersicum cv. Creole LA1251	2	80	80	90	83ab	60	65	65	63ab
S. lycopersicum cv. Creole LA1021	3	75	87.5	90	84a	57.5	65	65	63ab
S. lycopersicum cv. Creole LA468	4	67.5	82.5	90	80abc	57.5	57.5	70	62ab
S. lycopersicum cv. Creole LA172	5	67.5	80	75	74abc	47.5	47.5	60	52bcd
S. lycopersicum cv. Creole LA147	6	67.5	82.5	90	80abc	62.5	67.5	75	68ab
S. lycopersicum cv. Saladette LA2662	7	67.5	85	85	79abc	20	25	25	23e
S. lycopersicum cv. Edkawi.LA2711	8	72.5	87.5	87.5	83ab	25	62.5	77.5	55abc
S. lycopersicum cv. Walter	9	75	75	87.5	79abc	75	75	87.5	79a
S. lycopersicum cv. I_3R_3	10	80	70	87.5	79abc	70	65	75	70ab
S. l*ycopersicum* cv. Bonnie Best	11	50	62.5	62.5	58bc	62.5	72.5	87.5	74ab
S. lycopersicum cv. Manapal	12	60	67.5	67.5	65abc	57.5	57.5	85	67ab
S. pimpinellifolium. LA722	13	50	57.5	57.5	55c	7.5	7.5	10	8e
S. pimpinellifolium LA2184	14	50	55	87.5	64abc	5	5	25	12e
S. chmielewskii LA1306	15	50	55	62.5	56c	0	0	15	5e
S. chesmanii f. *minor* LA1401	16	50	57.5	82.5	63abc	0	0	12.5	4e
S. parviflorum LA 1326	17	60	67.5	60	63abc	5	0	5	3e
S. esculentum cv. Cerasiforme LA1673	18	80	67.5	82.5	77abc	57.5	60	67.5	62ab
S. hirsutum f. *glabratum* LA 1223	19	50	65	87.5	68abc	5	27.5	45	26de
S. chilense LA 1963	20	82.5	82.5	87.5	84a	20	27.5	40	29cde

[1]Program of conservation of genetic resources, University of California, Davis, Ca., * = Millions conidia per mL suspensión, RI = Root Immersion, SS = Spray to seed. Level of significance = 0.01, Figures followed by different letters are statistically different.

Table 3. Mean squares of the analysis of variance for disease index (DI) and percentage of germination. Inoculation by root immersion (IR) and by sprinkling the seeds at the time of planting (SS).

		RI	SS	
Source of variation	G de L	DI	DI	% Germination
Species of *Solanum*	19	309.05 **	2206.68 **	10.42 **
Density of inoculum	2	1034.48 **	1321.67 **	59.35 **
Error	38	50.27	53.57	0.66
C. V.		9.47	16.43	23.25

DI = Disease index, ** Highly significant differences, ns =non-significant differences.

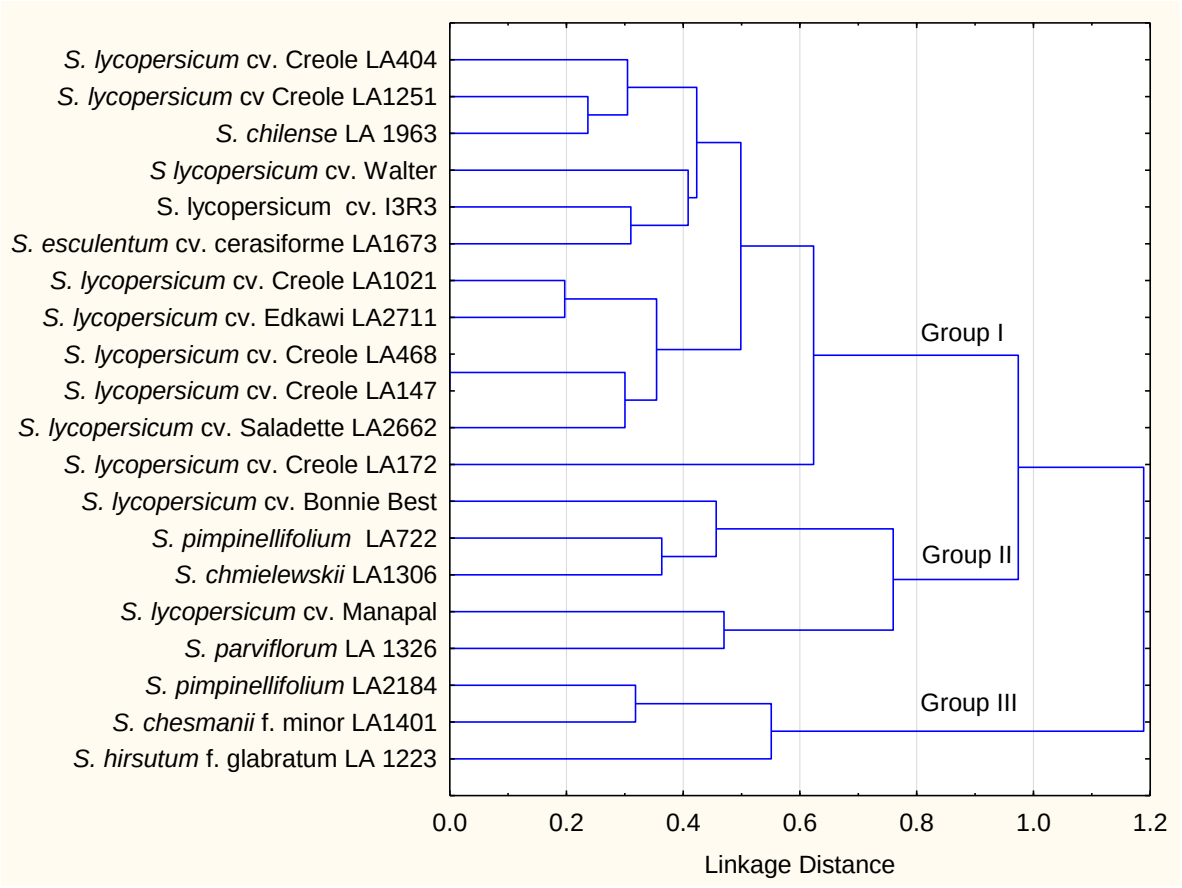

Fig 1. Dendogram of 20 tomato genotypes based on the disease index of *Fusarium oxysporum* f. sp. *radicis lycopersici.*

Based on these criteria, *Solanum parviflorum* LA 1326[2], *Solanum chesmanii* f. *minor* LA1401, *Solanum chmielewskii* LA1306, *Solanum pimpinellifolium* LA722, *Solanum pimpinellifolium* LA2184[2] and *Solanum lycopersicum* cv. Saladette LA2662 showed resistance to the fungus *Fusarium oxysporum* f. sp. *radici-lycopersici.* For its part, *Solanum hirsutum* f. *glabratum* LA 1223 and *Solanum chilense* LA 19632 showed moderate susceptibility. The other materials were susceptible with disease rates higher to 50%.

The average germination percentage of the seeds in this experiment was low. The analysis of variance indicated highly significant differences ($p \leq 0.01$) for inoculum doses with germination rates of 44.3%, 48.4% and 48.7% with inoculum doses of 3 x 10^6, 5 x 10^6 and 8 x 10^6 conidia / mL, respectively. It is possible that the fungus affected seed germination by reducing the percentage of germination regardless of inoculum doses applied. Apodaca et al. (2001) have successfully evaluated the response of tomato cultivars to the inoculation of *Fusarium oxysporum* f. sp. *lycopersici* in vitro, pre-inoculating the seeds.

For some soil borne pathogens, it has been observed that an increase in the inoculum population in the soil causes the disease to start earlier and the severity and incidence of the disease to be higher. In this experiment the highest values of disease index were observed, when the highest concentration (8 x 10^6) was used. However, it was also possible to observe that the 3x10^6 concentration was sufficient to induce the disease in tomato genotypes in greenhouse conditions.

Berbegal et al. (2007) observed that both the incidence and severity of wilt caused by *Verticillium dahliae* Kleb. in artichoke (*Cynara cardunculus* L. var. *Scolymus* (L.) Fiori) increased with increasing inoculum density up to 34 microsclerotia per gram of soil, with an average percentage of infected plants of more than 50%.

Khan et al. (2000) obtained significant correlations between inoculum density and disease severity and incidence in radish (*Raphanus sativus* L.) plants infected with *Verticillium dahliae* Kleb. However, they indicated that a prediction, only based on inoculum densities, may not be reliable under field condition when other factors are not under control and affect the inoculum-disease density ratio.

Cluster analysis

A cluster analysis was carried out considering the disease index value of the twenty genotypes, when inoculated with a density of 3 x 106 microconidia per mL of Forl. The twenty genotypes were classified into three large groups (I, II and III) (Figure 1). In group I, totally susceptible tomato genotypes were located. In group II and III, the tomato genotypes with resistance to Forl, mostly wild tomato species, were located. These genotypes could be included in genetic breeding program for resistance to crown and root rot caused by Forl. The wild species related to the tomato crop possess individual attributes of potential importance for the plant breeding of cultivated varieties by the introduction of important agronomic traits, such as resistance to diseases (Koenig et al., 2013).

Materials and methods

Plant materials

The study germplasm were 20 introductions of tomato species (*Solanum* spp.) from the genetic resources program of the University of California, Davis, USA (Table 1). Of these, six were cultivar creole from diverse origins, six obsolete commercial cultivars and eight correspond to different species originating in Peru and Ecuador. The Walter cultivar was included as a susceptible control.

Fungal strain used in the experiment

The strain of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, used in this study was isolated from the base of the stem of tomato plants that presented characteristic symptoms of this disease as yellowing of the leaves and sudden wilting during the ripening of the first fruits. For this, stem sections of approximately 3 mm were disinfected with 1% sodium hypochlorite solution for 3 minutes, seeded in a potato-dextrose-agar (PDA) culture medium in Petri dishes and incubated at 23 ± 2 ° C for six days. After this time, a tip transfer of hypha was carried out to obtain monosporic cultures. Increases of the pathogen were made and concentrations of 3, 5 and 8 x 10^6 microconidia per mL were adjusted to perform the corresponding inoculations.

Plant inoculation

The inoculation was done in two ways, by immersing the root tips in the spore suspensions and by sprinkling the seeds with the suspension of conidia at the time of planting.

Immersion of roots in suspensions of spores

Seeds of each of the genetic material to be evaluated were seeded in 200 cavities polystyrene trays with sterile soil. Fifteen days after germination twenty seedlings of each genetic material were inoculated with each of the inoculum concentrations used. For this, 2 cm of the root tips of the seedlings were cut and immersed in each of the suspensions of spores for 2 minutes, after which they were transplanted into 30 cm diameter plastic pots with sterile forest soil and kept in greenhouse for eight weeks until evaluation (Manzo et al., 2016).

Spray of seeds with the suspension of conidia at the time of planting

For this purpose, groups of four 30 cm diameter plastic pots were formed for each of the genetic materials. The pots also contained sterile forest floor. In each pot of the same group, 20 seeds of the same genetic material previously disinfected with 1% sodium hypochlorite and were placed in spaced form. Each pot was inoculated with three concentrations of inoculum, sprinkling the corresponding microspore suspension on the seeds and then covered with 0.5 cm layer of the same soil (Shin et al., 2014). The volume of the inoculum suspension was sufficient to cover the seeds completely.

Disease scoring

The evaluation was done eight weeks after inoculation according to the values of a severity scale where; 0 = healthy plants; 1 = slight root rot (less than 10% of the total area); 2 = dark lesions in 25% of the root; 3 = infection in the middle of the total area of the root, severe rot in the main root; 4 = infection in 75% of the total root area, crown lesions, wilting of older leaves, and 5 = severe infection in the total root area, wilt and death of young leaves (Çakır et al., 2014). Disease index (DI) was calculated using the following formula:

$$\text{Disease index} = \frac{\sum (\text{rating number x number of plants in the rating})}{\text{total number of plants x highest rating}} x\ 100$$

Experimental design and data analysis

In both experiments, a completely randomized design with factorial arrangement of two factors with two replications was used, where factor A corresponds to *Solanum* materials and factor B to inoculum concentrations (3, 5 and 8 x 10^6 microconidia per mL). First, an analysis of variance was performed with the values obtained on disease index and a comparison of means by the Tukey test, with a significance level of p≤0.01.

Cluster analysis

A cluster analysis was carried out considering the disease index values of the twenty genotypes when inoculated with a density of 3 x 106 microconidia per mL of Forl. The cluster analysis allowed classification of genotypes into resistant and susceptible

Conclusion

The methods of inoculation by root immersion and seed spray were very efficient to induce the crown and root rot disease caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in greenhouse. The highest values of disease index were observed when the concentration of 8 x 10^6 conidia per mL was used. However, the 3x10^6 concentration was sufficient to induce the disease with indices greater than 50%. It was possible to identify genotypes resistant to *Fusarium oxysporum* f. sp. *radici-lycopersici*, which can be included in subsequent genetic breeding works for resistance to this pathogen.

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