



Article

Tolerance Screening for *Phytophthora capsici* and *Fusarium solani* f. sp. *cucurbitae* in *Cucurbita* spp. and Gene Expression Responses in Mutant Families

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Abstract: *Cucurbita* species can be affected by soil-borne pathogens, such as *Phytophthora capsici* and *Fusarium solani* f. sp. *cucurbitae* (*Fsc*). Diverse commercial and conserved lines of *Cucurbita* spp. were tested. *C. pepo* subsp. *pepo* genotypes showed the highest susceptibility to both pathogens. The tolerance to *P. capsici* and *Fsc* was then screened in a zucchini mutant population. Two M3 mutant lines (Cp107 and Cp116) with a high occurrence of tolerant individuals to *Phytophthora capsici* were obtained from a screening of 160 M2 mutant lines. The M3 lines presented higher tolerance than the background MUCU-16. Furthermore, in the inoculated samples, both mutants overexpressed *CpDEF* and expressed more *CpPAL* and *CpChiIV* than the susceptible control. It has been previously shown that this expression pattern could be associated with tolerance in the *P. capsici* - *Cucurbita* spp. pathosystem. The M3 lines obtained could be applied in breeding programs, as they are likely to be compatible with the highly susceptible *C. pepo* subsp. *pepo* genotype.

Keywords: zucchini; squash; crown rot; soil; fungi; mutant



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1. Introduction

The *Cucurbitaceae* family includes a group of cultivable species within the *Cucurbita* genus, such as *C. moschata*, *C. maxima*, and *C. pepo*. There are several morphotypes for each species, with the zucchini morphotype (*C. pepo* subsp. *pepo*) being one of the most economically relevant. *Cucurbita* production can be affected by soil-borne pathogens, which can lead to plant death. Two important pathogens are the oomycete *Phytophthora capsici* [1] and the fungus *Fusarium solani* f. sp. *cucurbitae* (*Fsc*) [2]. Both *Fsc* and *P. capsici* are able to affect a wide range of cucurbit species and have analogous symptomatology, initially causing damage localized to the crown that leads to constriction and plant wilting due to the inability to transport water and nutrients [3–5]. These pathogens can remain in soil for long periods of time [1,5], and their management is currently through chemical treatment, crop rotation, or bio/solarization [5–7].

Screening of resistance to soil-borne pathogenic fungi and oomycetes (SBPFO) in cucurbits, mainly through existing germplasms and wild species, has been successfully used to inform breeding via rootstock candidates or introgression programs [3]. Grafting has proven to be very valuable in cucurbits, but plant resistance through resistance genes will always be the preferred objective, because it is an easy-handling solution, despite its complications [8]. Most studies on SBPFO in cucurbits have been focused on the *Cucumis* genus and the different formae speciales of *Fusarium oxysporum*, although there have been some focused on *Cucurbita* spp. with *P. capsici* [9–17] and *Fsc* [2,18]. *Cucurbita* spp. accessions have been identified with high frequency of individuals with resistance to *Fsc* [18]. Also, some *C. lundelliana* and *C. okeechobeensis* wild species [15], *C. pepo* [9], and *C. moschata* [11] genotypes showed reduced crown rot damage caused by *P. capsici*.

Currently, for cucurbits, artificially induced mutants are usually oriented towards viral diseases and the use of targeted strategies, such as CRISPR [19–21]. However, for pathogens with unknown resistance genes, such strategies are difficult to implement. In these circumstances, the use of mutagenic agents that produce high variability in numerous genes in a large population can be worthy. For this purpose, the ethyl methanesulfonate EMS mutagenic agent is especially useful in plant breeding [22]. New trait selection derived from mutations can be performed directly through screening, or indirectly by localizing the DNA changes in the genes of interest [23]. Previously, a method for generating zucchini EMS-mutants that exhibit high genetic variability and plant viability was developed [23,24]. Within *Cucurbita* spp. EMS-mutant collections, individuals with higher tolerances to abiotic [25,26] and biotic stresses [27] have been identified via screening, and the function of the altered genes with phenotypes has been obtained [28]. However, this has never been undertaken for soil-borne pathogen tolerance.

The tolerance to SBPFO phenotyping could be complemented by gene expression studies. Gene expression changes in *CpChiIV* (EP3 endochitinase) [29], *CpLPOX* (lignin-forming peroxidase) [30,31], *CpDEF* (*J1-2-like* defensin) [31,32], *CpPAL* (phenylalanine ammonia-lyase) [31,33], and *CpACO* (1-aminocyclopropane-1-carboxylate oxidase) [31] were previously tested in the *Cucurbita* spp. and *P. capsici* pathosystem [34]. Differential responses in *CpDEF* (higher upregulation with tolerance) and *CpPAL* (down-regulation with susceptibility) were found between tolerant and susceptible *Cucurbita* phenotypes inoculated with *P. capsici* [34].

The aim of this study was to evaluate different mutant cucurbit materials as a source of tolerance to *P. capsici* and *Fsc*, which could be suitable rootstocks or used for introgression programs in susceptible zucchini genotypes. For this, we carried out tolerance screenings with diverse commercial and conserved *Cucurbita* spp. genotypes, including a second-generation mutant (M2) population derived from the MUCU-16 (*C. pepo* subsp. *pepo*) genotype.

2. Materials and Methods

2.1. Plant Materials and Pathogen Isolates

A total of 18 *Cucurbita* genotypes were evaluated initially. These included *C. pepo* subsp. *ovifera* SCA (Scallop, Germplasm Bank Comunidad Valenciana BGV-5382); *C. pepo* subsp. *pepo* morphotype zucchini MUC (MUCU-16, IFAPA-La Mojonera collection); AMA (Amalthée, Gautier seeds), JED (Jedida, HM Clause), MIL (Milenio, Fitó seeds), NAT (Natura, Enza Zaden), VIC (Victoria, HM Clause), 2 marrow PIC (Piccolo, Tozer seeds), and ZEB (Zebra Cross, Tozer seeds); *C. moschata* M64 (64-064, Rijk Zwaan), M63 (64-063, Rijk Zwaan), TZ3 (TZ-3380, Tozer seeds), and TER (Butternut Sprinter, Tozer seeds); and hybrids of *C. maxima* × *C. moschata*, ROU (Routpower, Sakata seeds), SHI (Shintoza, Fitó seeds), CAM (Shintosa Camelforce, Bayer CropScience), AZM (Azman, Rick Zwaan), and HER (Hércules, Ramiro Arnedo).

Mutant germplasms were obtained from a second-generation mutant (M2) population of MUCU-16 *C. pepo* subsp. *pepo* [23]. The mutations were randomly distributed [23] in the 160 mutant families tested. Currently, the M2 and M3 families are conserved in the IFAPA-La Mojonera germplasm collection.

Phytophthora capsici (MI0211, GenBank MG012233) [6] and *Fsc* race 1 (Fs511, GenBank AM940070) isolates [35], obtained from infected plants in greenhouses in Almería province (Spain) were morphologically and molecularly described in previous studies [6,35]. Both pathogens were preserved in the IFAPA-La Mojonera laboratories and subsequently inoculated on *C. pepo*, and re-isolated and identified again from dead plants prior to inoculation.

2.2. Inoculation

P. capsici and *Fsc* inocula followed the same inoculation procedures. Two inoculation methods were used: root dipping and substrate infestation (Table 1). The first screening with different *Cucurbita* spp. genotypes and the initial M2 mutant germplasm were inocu-

lated by root dipping (Table 1). In this method, the radicles were immersed for 30 s in a suspension containing the pathogen propagules. This suspension was obtained by grinding several colonies fully covering the petri dish surface with each isolate, which had been previously grown for 10 days in PDA (1 petri dish per 400 mL of standard nutrient solution). The inocula concentrations were calculated by means of the dilution plate technique on PDA, obtaining ca. 10^6 UFC mL⁻¹ for *Fsc* and ca. 10^4 UFC mL⁻¹ for *P. capsici*.

Table 1. Summary of the inoculation tests in chronological order.

Test	Description	Inoculation Method	Inoculation Plant Stage	Pathogen	<i>Cucurbita</i> spp. Material	Duration (Days) ¹	N ² (rep. ³)
1 ⁴	<i>Cucurbita</i> spp. screening	Root dipping	Seedlings	<i>Pc</i> ⁵ <i>Fsc</i> ⁶	Commercial and Conserved genotypes	37	18 (16) 18 (16)
2	Mutant first screening	Root dipping	Seedlings	<i>Pc</i> <i>Fsc</i>	M2 mutant populations	27	160 (3) 160 (3)
3	Mutant second screening	Substrate infestation	2–3 true leaves	<i>Pc</i> <i>Fsc</i>	M2 selected mutants	27	50 (3) 26 (3)
4	Mutant third screening. Final selection	Substrate infestation	2–3 true leaves	<i>Pc</i>	M2 selected mutants AMA M64	34 (97 ⁸)	6 (10) 1 (4)
				<i>Fsc</i>	M2 selected mutants AMA ⁷ M64		1 (4) 1 (4)
5 ⁴	M3 tolerance and RNA expression	Substrate infestation	2–3 true leaves	<i>Pc</i>	Cp106 (M3) Cp117 (M3) MUCU-16 M63	14	1 (32) 1 (32) 1 (32) 1 (32)

¹ Test duration after inoculation. ² Number of different genotypes tested. ³ Number of total inoculated plant replicates for each genotype. ⁴ Test conducted twice, and results pooled. ⁵ *Phytophthora capsici*. ⁶ *Fusarium solani* f. sp. *cucurbitae*. ⁷ AMA susceptible control, M63 tolerant control. ⁸ Until day 34 in the chamber and 97 in the greenhouse for seed production.

The same procedure was used to obtain the inoculum for the subsequent mutant screenings and gene expression quantification analysis (test 3–5, Table 1). However, instead of radicle immersion, the inoculum was added by irrigation to the substrate at 2 cm depth near the plant with 50 mL of the inoculum suspension (substrate infestation method) once the plants reached the development stage of 2–3 true leaves (Table 1). The presence of *Fsc* macroconidia and *Phytophthora* sporangia on the dead plants' roots and crown was checked under a microscope, and they were reisolated for the following inoculations after each test. Control plants were watered with standard nutrient solution.

2.3. Test Conditions

All experiments were performed in a growth chamber with a 14 h photoperiod (>12,000 lux), 23–33 °C, and 40–75% relative humidity. The temperature and humidity were measured using a HOBO data logger. Before planting, the seeds were disinfected by immersing them in a sodium hypochlorite (35 g L⁻¹ active chloride) solution 1:1 for 20 min, and subsequently they were rinsed with autoclaved (30 min, 121 °C) sterile water and incubated at 28 °C in the dark until the roots reached 3–5 cm in length. Only germinated seeds were used.

In order to inoculate all plants at the same age, different sowing dates were applied depending on previous knowledge about the early development of each genotype. This step is important, because different plant development stages could affect their resistance responses [36]. The germination rate after one week for commercial and non-mutated germplasms was higher than 90%, while the M2 mutant germplasms had a germination rate between 25–95%, depending on the mutant family. Once the seeds had germinated, the seedlings reached 2–3 true leaves after 10–15 days. The substrate used for all of the experiments was vermiculite at field capacity, with a standard nutrient solution (1.5 dS m⁻¹). For all tests, all samples were randomly distributed in separate trays in the chamber beside non-inoculated controls. Non-inoculated controls were included in all of the tests. For

test 1, 8 control replicates per genotype were used. At least one plant per mutant family served as the control for tests 2, 3, and 4. Test 5 was completed with the same number (32) of control repetitions.

For the different *Cucurbita* spp. genotype tolerance screenings (test 1), two plants per 1 L pot (eight plants per pathogen, two trials) were used, and these plants were inoculated by the root-dipping method (Table 1). Susceptible and tolerant reference controls for tests 4 and 5 were chosen based on the responses of these plants (test 1).

The mutant screening and the selection of tolerant families consisted of three steps (Table 1): initial screening with seedlings (test 2), second screening with developed plants (test 3), and final selection (test 4). Initial mutant screening (test 2) was performed with 160 mutant families (three replicates) in 60 mL 96-well trays (Table 1). The mutant families that showed less symptomatology or that had tolerant individuals by day 14 post-inoculation were selected for the next test. Subsequent screenings were undertaken in 1 L pots (two plants per pot) with a decreasing number of selected families but increasing numbers of replicates per family (Table 1). For the next test, 50 selected families for *P. capsici* and 26 for *Fsc* were inoculated when the plants reached 2–3 true leaves (test 3). For the final mutant selection test (test 4), six families were selected for each pathogen, and genotypes M64 and AMA acted as the tolerant and susceptible controls, respectively. The mutant individuals that survived the *P. capsici* substrate infestation were reinoculated, transplanted into a 25 L pot, and developed in a greenhouse. This allowed the plants to reach a large enough size to produce, after self-pollination, a mature zucchini fruit with grown M3 seeds. The two M3 families with fertile seeds obtained in the study were used in the expression analysis test. For the gene expression analysis (test 5), the M3 mutant lines Cp107 and Cp116, and genotypes M63 (tolerant control) and MUCU-16 (susceptible control) were used (Table 1).

2.4. Tolerance Evaluation

The tolerance evaluation was based on three parameters: the disease severity index (DSI), percentage of plants with symptomatology, and relativized area under the disease progress curve (RAUDPC).

The DSI is based on a visual non-parametric scale ranging from 0 to 4: 0 = no damage, 1 = leaf epinasty/soaked crown, 2 = constricted/girdling crown, 3 = wilt/chlorosis, and 4 = death; this is analogous to other authors [6,9,18]. The percentage of plants with symptomatology plotted against time produced an area under the disease progress curve (AUDPC) that represented the disease evolution. The AUDPC was relativized (RAUDPC) using the sample with the highest AUDPC value in each trial [2,6,14]. In test 1, plants that had an RAUDPC between 0–10% were considered tolerant, 11–30% low susceptibility, 31–70% intermediate susceptibility, and 71–100% high susceptibility. The RAUDPC was submitted to an analysis of variance (ANOVA); when significant differences ($p < 0.05$) were found, Tukey's HSD was carried out. For two-level comparison, Student's *t*-test was used. The data were tested for a normal distribution and homoscedasticity by the Shapiro–Wilk and Levene tests. Statistix 9.0 software (Tallahassee, FL, USA) was used for all statistical analyses. Tests 1 and 5 were duplicated (Table 1), and the data were pooled. Additionally, the damage level caused by both pathogens was analyzed once the plant died (Figure A1).

2.5. qPCR Sample Collection and Conditions

The test conditions are shown in Table 1 (test 5). Root and crown samples were collected on 0, 3, 10, and 14 days post inoculation (dpi), as the disease evolution was observed. Each sample was extracted from eight different plants, which decreased the number of plants in the test by eight after each sample collection day. Sample collection, RNA extraction, and cDNA obtention were performed as described in previous work [34]. The primers (Sigma–Aldrich, St. Louis, MO, USA), except for the UFP and EFP-1 α reference genes [37], were obtained from previous work [34]. The qPCR cycling conditions were: 50 °C 2'; 95 °C 10'; 40 \times (95 °C 15'', 55 °C 15', and 60 °C 1''); and melting (95 °C 15'',

60 °C 1', and 95 °C 1"). Gene expression was relativized with UFP (ubiquitin fusion protein) and EFP-1 α (elongation factor-1 α) reference genes [37] following data processing with efficiency correction, multiple reference gene normalization, and the use of error propagation rules [38,39]. The normalized relative quantities (NRQ) for each sample were obtained and transformed with $\text{LOG}_{10}(\text{NRQ}_{\text{inoculated}}/\text{NRQ}_{\text{non-inoculated}})$. Sample data were pooled (3, 10, and 14 dpi and crown and root tissues) by genotype and compared against the susceptible control by applying Student's *t*-test ($p < 0.005$). Homoscedasticity and normal distribution were checked with Statistix 9.0 software.

3. Results

3.1. Cucurbita Genotype Tolerance Screening to *P. capsici* and *F. solani* f. sp. *cucurbitae*

The RAUDPC caused by *P. capsici* and *Fsc* for different *Cucurbita* spp. genotypes after 37 dpi is shown in Table 2. Both pathogens, inoculated by root-dipping, showed damage to all genotypes tested. However, the *C. moschata* species showed less damage for both *Fsc* and *P. capsici* (Table 2). According to the phenotype classification, M64 and M63 were considered tolerant to *P. capsici*, while TZ3 was tolerant to *Fsc* (Table 2). In contrast, many *C. pepo* plants showed high susceptibility to both pathogens, although the two marrow morphotypes analyzed (PIC and ZEB) presented intermediate susceptibility to *Fsc* (Table 2). Just one hybrid of *C. maxima* \times *C. moschata* showed high susceptibility to both pathogens, HER, while SHI showed intermediate susceptibility (Table 2). Significant differences in the RAUDPC were observed between the TZ3 tolerant and AZM, CAM, HER, MUC, MIL and NAT susceptible genotypes after *Fsc* inoculation (Table 2). For *P. capsici*, significant differences also appeared between M63, M64, TZ3, TER, and ZEB with the highly susceptible MIL, MUC, NAT, SCA, and VIC genotypes (Table 2).

Table 2. *Cucurbita* spp. genotype responses to *Phytophthora capsici* and *Fusarium solani* f. sp. *cucurbitae*.

Genotype	cv.	<i>Fusarium solani</i> f. sp. <i>cucurbitae</i>			<i>Phytophthora capsici</i>		
		RAUDPC ¹		Phenotype ²	RAUDPC		Phenotype
<i>Cucurbita moschata</i>	M63	0.29	ab	LS	0.09	c	T
	M64	0.18	ab	LS	0.08	c	T
	TZ3	0.04	b	T	0.27	bc	LS
	TER	0.23	ab	LS	0.25	bc	LS
<i>C. maxima</i> \times	SHI	0.67	ab	IS	0.62	abc	IS
	ROU	0.61	ab	IS	0.81	ab	HS
	AZM	0.79	a	HS	0.50	abc	IS
<i>C. moschata</i>	CAM	0.74	a	HS	0.64	abc	IS
	HER	0.84	a	HS	0.88	ab	HS
<i>C. pepo</i> subsp. <i>ovifera</i>	SCA	0.86	ab	HS	1.00	a	HS
<i>C. pepo</i> subsp. <i>pepo</i> (marrow)	PIC	0.63	ab	IS	0.74	abc	HS
	ZEB	0.67	ab	IS	0.29	bc	LS
<i>C. pepo</i> subsp. <i>pepo</i> (zucchini)	AMA	0.72	ab	HS	0.72	abc	HS
	JED	0.46	ab	IS	0.71	abc	HS
	NAT	1.00	a	HS	1.00	a	HS
	MUC	0.91	a	HS	0.97	a	HS
	MIL	0.97	a	HS	0.99	a	HS
	VIC	0.72	ab	HS	0.97	a	HS

¹ Relative area under the disease progress curve for 37 days (mean of two trials). Significant differences among genotypes for *Fsc* (ANOVA, $p = 0.0008$, replicates = 4) and *P. capsici* (ANOVA, $p < 0.0001$, replicates = 4) are grouped by letter (Tukey HSD). ² Plants with RAUDPC between 0.71–1 were considered highly susceptible (HS); 0.31–0.7 intermediately susceptible (IS); 0.11–0.30 low susceptibility (LS); and 0.0–0.10 tolerant (T).

3.2. Mutant Tolerance Screening to *P. capsici* and *Fusarium solani* f. sp. *cucurbitae*

The first mutant screening included 160 families (test 2, Figure 1). Both *P. capsici* and *Fsc* produced symptomatology (Figure 2) in the entire inoculated population after 27 dpi. In some cases, plants were unable to develop true leaves due to the disease. Families with less damage or with individuals without damage on day 14 post-inoculation were selected for the next test (Figure 1). For *P. capsici*, 50 families were selected, while for *Fsc*, 26 families were selected. Fewer families were selected for *Fsc* because this fungus produced more damage by day 14 than *P. capsici*. The selected families were inoculated in a new test (test 3). In this test, the six families with the lowest RAUDPC by day 27 post-inoculation were selected for the subsequent screening (test 4, Figure 1).

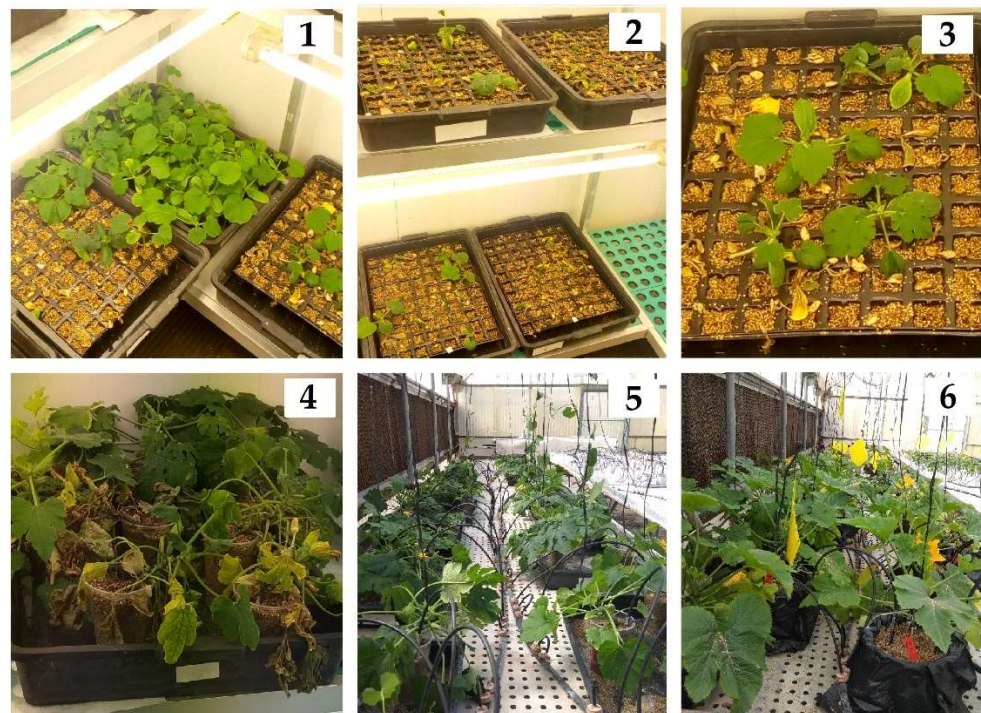


Figure 1. Tolerance screenings in the mutant population of MUCU-16. Screening with seedlings 14 days post-inoculation (dpi) (1–3): control tray (center) without inoculation (1); *Fusarium solani* f. sp. *cucurbitae* (left) and *Phytophthora capsici* (right) inoculated trays (2); individuals showing tolerance (3). Screening with selected plants (4–6): plant symptomatology 21 dpi—surviving individuals were moved to a greenhouse after 34 dpi and reinoculated (5); surviving plant growth until 97 dpi for fruit production (6).

For *P. capsici*, all the previously selected families from test 3 had individuals that survived to the inoculation of test 4 (Table 3); these plants were re-inoculated at 34 dpi and transferred to a greenhouse (Figure 1). In total, 15 plants from families Cp45 (1), Cp107 (2), Cp116 (3), Cp136 (3), Cp139 (2), and Cp144 (4) remained at 97 dpi. Mature fruits were obtained from families Cp107, Cp116, Cp139, and Cp144, but only Cp107 and Cp116 fruits had M3 fertile seeds. On the other hand, none of the six families selected for *Fsc* showed tolerance to the pathogen (Table 3). The tolerant (M64) and susceptible (AMA) controls responded (Table 3) as in the previous test for both pathogens (Table 2).

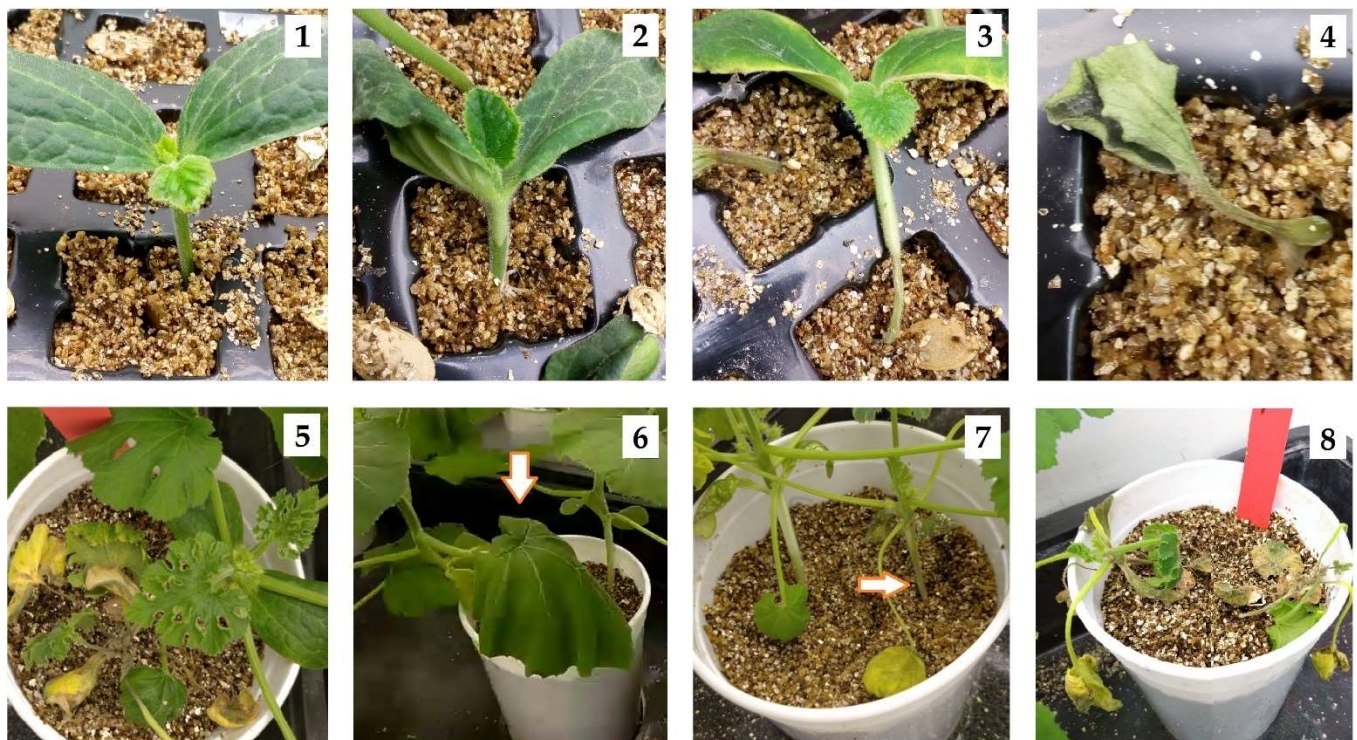


Figure 2. Disease symptoms caused by *Phytophthora capsici* and *Fusarium solani* f. sp. *cucurbitae* in *Cucurbita pepo*. Shoot symptomatology in seedlings 14 dpi (1–4): DSI 0 = no symptoms (1); DSI 1 = soaked crown (2); DSI 2 = constricted crown (3); DSI 4 = death (4). Shoot symptomatology 14 dpi in plants inoculated when they reached 2–3 true leaves (1–4). In some cases, pathogen damage was different between plants in the same pot (5): dead plant (left) and plant show no symptoms (right). Leaf epinasty (6), soaked and constricted crown (7), and wilted and dead (8) plants.

Table 3. *Phytophthora capsici* and *Fusarium solani* f. sp. *cucurbitae* disease evolution in selected mutant families.

Pathogen	Genotype	Days Post-Inoculation								RAUDPC ⁴
		7		15		27		34		
		DSI ¹	%s.p. ²	DSI	%s.p.	DSI	%s.p.	DSI	%s.p.	
<i>Fusarium solani</i> f. sp. <i>cucurbitae</i>	Cp005 ³	0.80	0.80	3.70	1	3.90	1	4.00	1	0.99
	Cp059	1.40	1	3.70	1	4.00	1	4.00	1	1.00
	Cp060	1.11	0.89	3.67	1	4.00	1	4.00	1	0.99
	Cp109	0.75	0.75	2.50	0.88	3.00	1	4.00	1	0.91
	Cp138	2.00	1	3.80	1	4.00	1	4.00	1	1.00
	Cp157	1.13	0.75	3.13	1	4.00	1	4.00	1	0.99
	AMA	1	0.75	3.25	1	4.00	1	4.00	1	0.99
	M64	0	0	0	0	0.30	0.25	0.50	0.25	0.24
<i>Phytophthora capsici</i>	Cp045	0.22	0.22	0.44	0.33	1.44	0.56	1.89	0.78	0.95
	Cp107	0	0	0	0	2.00	0.50	2.00	0.50	0.63
	Cp116	0	0	0	0	0.25	0.13	0.50	0.13	0.10
	Cp136	0.17	0.17	1.17	0.33	1.67	0.67	1.67	0.67	0.90
	Cp139	0	0	0	0	0.17	0.17	0.83	0.33	0.18
	Cp144	0	0	0.14	0.14	0.57	0.57	1	0.57	0.55
	AMA	0	0	0.50	0.25	2.50	0.75	4.00	1	1.00
	M64	0	0	0	0	0.25	0.25	1	0.50	0.26

¹ Disease severity index mean: 0 = no symptoms, 1 = leaf epinasty/soaked crown, 2 = constricted/girdling crown, 3 = wilt/chlorosis, 4 = death [40]. ² Percentage of the inoculated population with symptoms. ³ Mutant family (M2) code (CpXXX), susceptible control (AMA), and tolerant control (M64). ⁴ Relative area under the disease progress curve for 34 days.

The undergrown plant lesions evaluated after plant death indicated that both pathogens produced damage to the crown and root tissues. All dead plants analyzed for *P. capsici* and for *Fsc* presented more accentuated symptomatology in the crown, with at least soaking damage, while the secondary roots were the least affected tissue (Figure A1).

3.3. M3 Mutant Tolerance Analysis

The M3 Cp107 and Cp116 mutant families (derived from self-pollinated M2 plants that showed tolerance) presented less damage caused by *P. capsici* than the non-mutated MUCU-16 (M0) (Figure 3). The mutants showed significant differences in the RAUDPC against the susceptible control 14 dpi (Table 4). No damage was observed until day 10 after inoculation for Cp107, Cp116, and M63 while, for MUCU-16, the symptoms started on day 3 (Table 4).

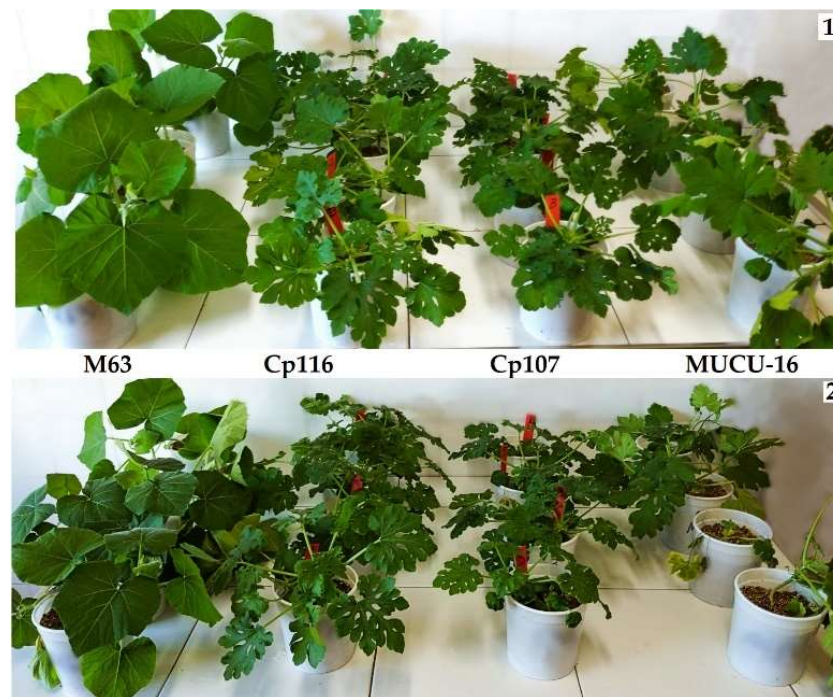


Figure 3. Randomly selected plants for the sample collection of the gene expression analysis 14 days post-inoculation. A total of eight roots and eight crowns per genotype for each non-inoculated (1) and inoculated (2) sample were collected from four pots (two plants/pot). Plants were inoculated with *P. capsici*. The symptomatology severity was higher in MUCU-16 (2, right) than that for the rest of the genotypes, although the damage was not homogeneous for all individuals.

Table 4. *Phytophthora capsici* disease evolution in M3 mutant families.

Genotypes	Cucurbita Species	Dpi 3 ($n^2 \geq 32$)		dpi 10 ($n \geq 24$)		dpi 14 ($n \geq 16$)		RAUDPC ⁵
		DSI ³	%s.p. ⁴	DSI	%s.p.	DSI	%s.p.	
M63	<i>Cucurbita moschata</i>	0	0	0.08	0.08	0.50	0.21	0.34b
Cp116	<i>Cucurbita pepo</i>	0	0	0.36	0.18	0.80	0.20	0.56b
Cp107	<i>Cucurbita pepo</i>	0	0	0.21	0.13	1.12	0.29	0.48b
MUCU-16	<i>Cucurbita pepo</i>	0.06	0.03	0.35	0.19	1.28	0.44	1.00a

¹ Days post-inoculation. ² Number of plants analyzed. ³ Disease severity index mean: 0 = no symptoms, 1 = leaf epinasty/crown scar, 2 = soaked/girdling crown, 3 = wilt/chlorosis, 4 = death [40]. ⁴ Percentage of affected plants in the inoculated population. ⁵ Relative area under the disease progress curve for 19 days, with significant differences (ANOVA $p = 0.0004$, $n = 3$) indicated with letters (Tukey's HSD).

3.4. M3 Gene Expression

The differential expression between inoculated and non-inoculated samples for all pooled samples (dpi: 3, 10, and 14) showed significant differences in MUCU-16 for the *CpDEF*, *CpChiIV*, and *CpPAL* genes. On the other hand, the *CpACO* and *CpLPOX* genes remained similar to the control (Figure 4). Mainly due to down-regulation of *CpPAL* in the susceptible after inoculation, the genotypes M63 ($p = 0.021$), Cp107 ($p = 0.042$) and Cp116 ($p = 0.043$) were expressed *CpPAL* more than MUCU-16 (Figure 4). No significant differences were found between the *C. pepo* genotypes (MUCU-16, Cp107, and Cp116) in *CpDEF* expression in the inoculated samples, while the tolerant control showed higher up-regulation ($p = 0.0117$, Figure 4) with respect to the susceptible control. However, only the Cp107 ($p = 0.046$) and Cp116 ($p = 0.035$) mutant families expressed the *CpChiIV* gene more in the inoculated samples versus the control (Figure 4).

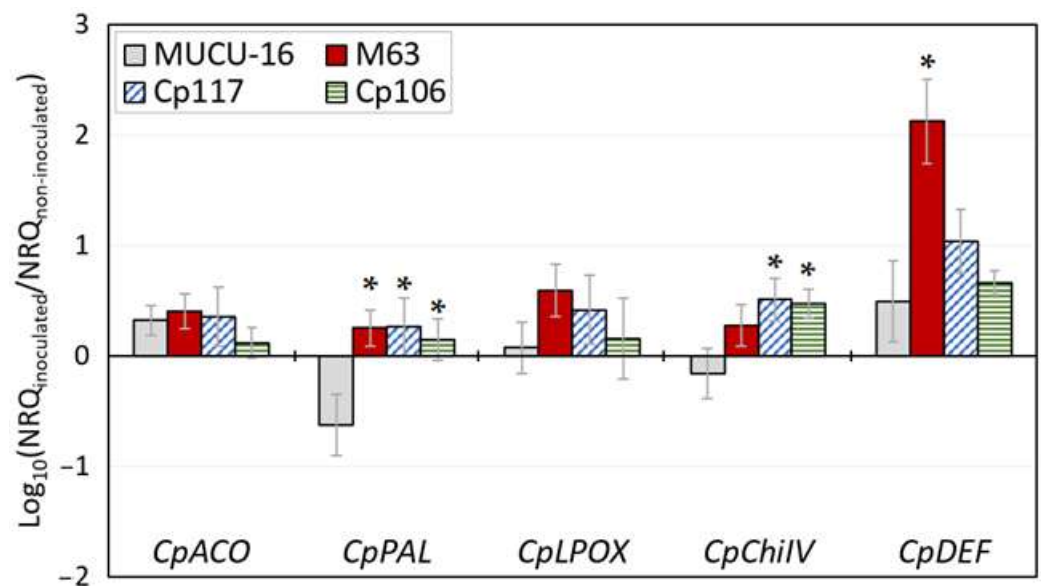


Figure 4. Differential gene expression between genotypes in inoculated and non-inoculated samples *Phytophthora capsici* for each genotype. Each bar represents the genotype (MUCU-16, M63, Cp117, or Cp106) mean of the normalized relative quantity transformations between the inoculated and non-inoculated samples ($\text{LOG}_{10}[\text{NRQ}_{\text{inoculated}}/\text{NRQ}_{\text{non-inoculated}}]$) from pooled samples of root and crown tissues and the days 3, 10 and 14 post-inoculation. Positive values are up-regulation and negative are down-regulation for the inoculated samples. Significant differences (*) from the MUCU-16 control were determined with Student's *t*-test ($p < 0.05$, number of samples compared = 12). Error bars represent the standard errors ($n = 6$).

4. Discussion

4.1. Tolerant Sources for *Fusarium solani* f. sp. *cucurbitae* and *Phytophthora capsici* in the *Cucurbita* Genus

Enzenbacher and Hausbeck [14] tested different isolates of *P. capsici* on *C. pepo* (zucchini and straightneck) and *C. moschata* (butternut) genotypes. Their results pointed out that the *C. pepo* straightneck morphotypes showed higher susceptibility and the *C. moschata* genotype showed higher tolerance [14]. Similarly, differences in the disease severity index were observed between the morphotypes of *C. pepo* and between the subspecies *C. pepo* subsp. *pepo* and *C. pepo* subsp. *ovifera* [4]. In this study, *C. pepo* subsp. *pepo* marrow morphotypes showed more tolerance to *P. capsici*, and the *C. pepo* subsp. *ovifera* were highly susceptible [4]. Our results indicated that the plant responses to these pathogens were also dependent on the species and morphotype. In our study, *C. moschata* was the most tolerant, while *C. pepo* subsp. *ovifera* was highly susceptible to *P. capsici*. The *C. pepo* zucchini morphotypes were the most susceptible, while the marrows were the least susceptible among the *C. pepo* subsp. *pepo* morphotypes. Furthermore, Perez-Hernandez et al. [2] tested

some pathogenic isolates of *Fsc* that were shown to be highly aggressive to zucchini plants, while other cucurbit hosts showed differential susceptibility to the pathogen. These results support ours, since, in this study, zucchini genotypes were found to be highly susceptible to *Fsc*, while some genotypes of *C. moschata* showed tolerance to the fungus.

It has been observed in different screenings for resistance to *P. capsici* that some genotypes of *C. lundelliana* and *C. okeechobeensis* wild species [15], *C. pepo* [9], and *C. moschata* [11] show reduced crown damage. Padley et al. [9] obtained, from a resistance screening in *C. pepo*, 16 accessions that were less damaged by *P. capsici*, and selected two of them because they were more resistant [9]. All these accessions showed high genetic proximity, but one line was also close to the susceptible *C. pepo* subsp. *pepo* and *C. pepo* subsp. *texana* genotypes [16]. The backcrossing and its phenotypic segregation analysis indicated that the resistance to *P. capsici* was controlled by three dominant genes (*R4*, *R5*, and *R6*) [17]. Furthermore, resistance to *P. capsici* has been introduced into *C. moschata* from wild *C. lundelliana* and *C. okeechobeensis*, and its segregation also suggests that this trait is controlled by three dominant genes (*Crr-1*, *Crr-2*, *Crr-3*) [10].

In addition, the genotypes that present more tolerance could be used for grafting other compatible cucurbits. *Cucurbita* spp. germplasms that present high frequency of individuals with tolerance to *Fsc* were identified as rootstock candidates [18]. Additionally, grafted *Citrullus lanatus* on Ferro, GV 100 (*C. maxima* × *C. moschata*), and Just (*Cucurbita* spp.) rootstocks showed tolerance to *Fsc*, although this response depended on the plant development at the inoculation moment [41]. For watermelon, the *Lagenaria siceraria* (bottle gourd) rootstocks FR-Strong, Emphasis, Macis, and WMXP-3938 were resistant to crown and root rot caused by *P. capsici* [42]. Our results suggest that there are rootstock candidates for tolerance to *P. capsici* (M63 and M64) and *Fsc* (TZ3), but compatibility studies with zucchini are needed.

4.2. Mutant Selection

To our knowledge, none of the previous SBPFO resistance screening studies in cucurbits were performed with artificially induced mutants. All were based on germplasm bank collections of wild, conserved, commercial, or hybrid genotypes [2,9,14,15,18]. This study presents the first results for mutant *C. pepo* subsp. *pepo* lines with tolerance to *P. capsici*.

Few studies have been conducted in the *Cucurbitaceae* family on biotic stress with EMS-mutated populations, despite this being an excellent tool for breeding [3,27]. For example, one bottle gourd (*Lagenaria siceraria*) M4 EMS-induced inbred line (117-8) resistant to zucchini yellow mosaic virus (ZYMV) was obtained and showed stable resistance and no virus presence among inoculated plants [27]. Our selected families (Cp107 and Cp116) presented more tolerance to *P. capsici* than the highly susceptible *C. pepo* subsp. *pepo* from which they originated (Table 4). However, some characteristics, such as plant architecture, seemed to be affected with respect to the MUCU-16 background M0 (Figure 3), although specific phenotyping analyses still have to be performed.

Genes for resistance to SBPFO have been reported in cucurbits [3,43]. However, the mutant tolerance to *P. capsici* could be due to diverse causes, given the nature (random and numerous) of mutations caused by EMS. Plant development, which affects plant tolerance [36], plant resistance genes that respond to pathogen virulence factors [3,30,31], or plant susceptibility genes, which are targeted by the pathogen [33,44–47], may have been affected by the mutations.

During soilborne disease-resistance screening with a large population, it is common to find individuals that show more tolerance, although the family as a whole may seem susceptible [18]. We also observed this situation, especially in the mutant population. In an M1 population, most of the randomly mutated genes would be in heterozygosis, and its segregation (M2) could show different phenotypes [23]. The individuals tolerant to *P. capsici* were self-fertilized and M3 seeds were obtained. This reduced the family phenotypic heterogeneity, although segregation studies should be performed to confirm this fact. It should be taken into account that, despite obtaining a mutant of interest, sometimes it will not be pos-

sible to produce seeds due to the difficulty some mutants have in generating fertile seeds, as random mutations can alter their formation [23]. Finding a mutant material with tolerance to a disease in the species to be cultivated would reduce the steps in introgression programs or grafting. Nevertheless, the method for producing a mutant population for each crop must be previously known to avoid the loss of desirable characteristics of the background M0, while still allowing it to acquire new ones. Vicente-Dólera et al. [23] observed that, with the mutagenic agent EMS at a concentration of 0.4%, plants had more viability than at higher concentrations. Also, with this concentration, the plant population incorporated a mutation frequency of 1/135 kb [23]. For this reason, we chose this concentration so that the plants would not lose the useful characteristics acquired during domestication.

4.3. qPCR

The *CpDEF* gene was highly overexpressed in the inoculated plants, and *CpPAL* down-regulation occurred only in the susceptible genotypes, which is in agreement with previous studies [34]. Moreover, The mutants Cp107 and Cp116 expressed more *CpChiIV* than MUCU-16 after inoculation. The mutant population may not have directly affected these genes, since this differential expression between the susceptible and tolerant phenotypes observed may be the consequence, rather than the cause, of tolerance.

Gene expression analysis has been demonstrated to be a useful approach to complement studies where the plant responds to biotic stress. Genes such as *CaPBR1*, *CaPO1*, *CaDEF1* [48], chitinase [29], lignin-forming peroxidase, ethylene, and jasmonic related genes [30], were found to be differentially expressed in the *P. capsici* and *Capsicum annuum* pathosystem. Additionally, defensins (*CIPDF2.1* and *CIPDF2.4*), PAL, chitinase, and ascorbate peroxidase were significantly induced in roots in watermelon during *Fusarium oxysporum* f. sp. *niveum* infection [32]. Also, a resistant genotype of *Cucumis* spp. showed up-regulation of ACO, PAL, defensins, and lignin-forming peroxidase genes in *P. capsici* inoculated roots [31].

5. Conclusions

The screening of an EMS-mutated population was proven to be a reliable method for obtaining new traits in complex phenomena, such as *C. pepo* subsp. *pepo* tolerance to *P. capsici*. The tolerance trait was found in the phenotype and gene expression analyses of the two M3 families obtained. These families presented significant differences in *P. capsici* tolerance, and in the expression of *CpPAL* and *CpChiIV* genes compared with the susceptible background.

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Appendix A



Figure A1. Underground symptomatology of *P. capsici* and *Fsc*. *P. capsici* (left) and *Fsc* (right) 14 dpi in seedlings (1). *P. capsici* (left) and *Fsc* (right) 21 dpi in plants inoculated when they reached 2–3 true leaves (2,3). Soaked and constricted crown and main root disaggregation caused by *Fsc* (4), main root browning caused by *P. capsici* (5) 14 dpi. Note that these cucurbits had a fibrous root system (generate more than one main root), but, in the young plants, a main root is clearly observable.

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