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Research article

Self-pollination, intra- and interspecific crosses in tomatoes

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ABSTRACT: Tomato genotypes (*Solanum* spp.) have genetic variability of most desirable features, such as resistance to biotic and abiotic stresses. However, incompatibility of crosses of wild genotypes with domesticated tomatoes, or even between wild genotypes, hinders the breeding process. Thus, knowledge of the reproductive biology of genotypes and conditions is necessary to maximize the success of artificial crossings. This study evaluated the compatibility of self-pollination, intra- and interspecific controlled crosses, stigma receptivity, and pollen viability in tomato genotypes. We used two commercial genotypes *S. lycopersicum* ('RVTM08' and 'Redenção') and seven accessions of wild tomato genotypes ('AF 26970', 'LA-1401', 'AF 19684', 'LA-1967', 'PI-127826', 'PI-134417', and 'LA-716'). We evaluated all crosses and their reciprocals, besides the self-pollinations. The variables evaluated were fruit index (FI), number of seeds per fruit (SN), and seed germination percentage (GP). Stigma receptivity and grains' pollen viability index (PVI) were also assessed. The results showed that 'LA-1967' was self-incompatible, had a low PVI, and generated fruit without seeds in most crosses. As female parents, 'RVTM08', 'Redenção', 'AF 26970', 'LA-1401', and 'AF 19684' showed higher FI and SN. There was a wide diversity of reproductive characteristics between the genotypes and crosses that did not influence GP. Compatibility of crosses in tomatoes is determined by the female parent choice and can be affected by stigma receptivity and the PVI.

Keywords: *Solanum lycopersicum*, artificial pollination, reproductive barriers, seed production, wild tomatoes

Introduction

The reproductive system plays a vital role in species diversity, as it is the primary source of genetic variability in tomatoes (*Solanum* spp.) (Peralta et al., 2008; Bergougnoux, 2014). Some genotypes of wild tomato species show self-incompatibility or can be compatible only with a specific group of genotypes of the same species or other species (Broz et al., 2017). This behavior decreases the efficacy of breeding programs to introduce genes from the wild into cultivated tomato species of *Solanum lycopersicon* L. (Jewell et al., 2020). Reproductive barriers occur due to the evolutionary process of plants, mainly genetic mechanisms developed to avoid hybridization between similar species (Li et al., 2010). These barriers can be expressed before or after fertilization, characterized in tomato plants as unilateral incompatibility or incongruity between species (Muñoz-Sanz et al., 2022).

Genotypes 'AF-26970' from *Solanum pimpinellifolium* L., 'LA-1401' from *Solanum galapagense* S.C. Darwin & Peralta, 'AF 19684' from *Solanum peruvianum* L., 'LA-1967' from *Solanum chilense* (Dunal) Reiche, 'PI-127826' from *Solanum habrochaites* S. Knapp & D. M Spooner var. *hirsutum*, 'PI-134417' from *Solanum habrochaites* S. Knapp & D. M Spooner var. *glabratum,* and 'LA-716' from *Solanum pennellii* Correll are of relevance for introgression of resistance genes in *S. lycopersicum* (Nyaku and Danquah, 2018).

The interspecific cross of some of these genotypes also allows the development of vigorous rootstocks resistant to biotic and abiotic stresses. However, there are variations in the mating systems of different tomato genotypes and many factors can influence the success of self-pollination and intra- and interspecific crosses (Bedinger et al., 2011; Muñoz-Sanz et al., 2022). Many reproductive barriers are genetic mechanisms that remain unclear despite previous studies (Chalivendra et al., 2013; Gao et al., 2015; Muñoz-Sanz et al., 2022). In addition, pollen viability and stigma receptivity also require attention in controlled self-pollinations and crosses, since these features may affect the fruit formation rate (Xu et al., 2012; Silva Junior et al., 2022). This study aimed to evaluate the compatibility of self-pollination, intra- and interspecific controlled crosses, stigma receptivity, and pollen viability in tomatoes to provide the basis for breeding programs.

Materials and Methods

Plant material, growth conditions, and crossing design

In this study, we used nine tomato genotypes, two from cultivated tomatoes ('RVTM08' and 'Redenção') and seven from wild species ('AF 26970', 'LA-1401', 'AF 19684', 'LA-1967', 'PI-127826', 'PI-134417', and 'LA-

716'). The species and reproductive system of these genotypes are shown in Table 1. Genotype 'RVTM08' displayed indeterminate growth habits and "Italian" type fruits. The commercial cultivar 'Redenção' with determinate growth habits has characteristics for industrial processing. These genotypes were used because of their potential for tomato breeding in tropical and subtropical regions (Zeist et al., 2018 a, b). Each genotype was sown in expanded polystyrene trays of 200 cells. Each cell was filled with a commercial growth medium made from bio-stabilized *Pinus* bark and the tray was kept in a floating hydroponic system for 24 days. The seedlings were kept under these conditions until reaching the 5th or 6th stage of six fully expanded leaves and were then transferred to polyethylene pots with capacities of 10 dm^{-3} and filled with sieved soil and cattle manure at a 3:1 ratio.

The plants were kept in a greenhouse and the phytosanitary control was performed with preventive sprayings according to the technical recommendations for the tomato crop. Foliar fertilization was performed weekly with commercial fertilizers based on calcium and boron (0.5 %). Irrigation was performed manually whenever necessary.

In the self-pollination and interspecific crosses, the nine tomato genotypes were combined in a diallelic design, resulting in 72 hybrids. Ten plants from each genotype were used to obtain each hybrid and in self-pollinations. For each genotype combination, 50 artificial crosses were made (and 50 manual self-pollinations). Pollen was collected from opened flowers in the morning to perform manual pollinations of the emasculated flowers of female parents to make the crosses. The same procedure was performed for self-pollination. After pollination, the flowers were properly identified.

Stigma receptivity and pollen viability

Plants were cultivated in a completely randomized design with five replications to evaluate stigma receptivity and pollen viability of the nine tomato genotypes used in this study. The growth conditions of plants were as described above. Pre-anthesis flower buds and opened flowers were collected in the morning from the 2nd to 7th

Table 1 – Parents used in the self-pollinations and intra- and interspecific controlled crosses of tomato genotypes (*Solanum* spp.).

Genotypes	Species	Reproductive system
'RVTM08'	Solanum lycopersicum	Autogamous
'Redenção'	Solanum lycopersicum	Autogamous
'AF 26970'	Solanum pimpinellifolium	Autogamous
'LA-1401'	Solanum galapagense	Autogamous
'AF 19684'	Solanum peruvianum	Allogamous
'LA-1967'	Solanum chilense	Allogamous
'PI-127826'	Solanum habrochaites var. hirsutum	Allogamous
'PI-134417'	Solanum habrochaites var. glabratum	Allogamous
'LA-716'	Solanum pennellii	Allogamous

inflorescences to test stigma receptivity,. Receptivity of ten stigmas of each genotype was performed using the protocol based on the peroxide enzyme test as proposed by Kearns and Inouye (1993).

Initially, anthers and calyx sepals were removed with sharp tweezers and the stigma was deposited on a glass slide. Immediately, three drops of the reagent were dropped directly over the stigma, which was then observed for one hour under a light microscope (Olympus SZ51). The evaluation was based on the presence or absence of bubbles in the stigmatic region and the speed of bubble formation. When the emission of bubbles was faster (due to peroxide breakdown by the action of the peroxidase enzyme), stigma receptivity was higher (Kearns and Inouye, 1993). The stigma was considered very receptive (VRS) when the release of bubbles was abundant and at high speed, allowing for visual counting. A receptive stigma (RS) was determined when the release of bubbles occurred at a moderate speed, thus allowing to perform visual counting (Figure 1A). A less receptive stigma (LRS) released fewer bubbles at a low speed, while a non-receptive stigma (NRS) occurred when no bubbles were released.

Furthermore, the stigmatic region where the bubble emission occurred was also observed and noted in the stigmatic slit, when the bubbles were released in the stigma slit, and the entire stigmatic region, when the bubbles were released throughout the stigmatic region.

The pollen viability test was performed according to Douglas and Freyre (2010). Pollen grains were collected from open flowers with tweezers and manual vibration and immediately disposed of on glass slides and a histochemical test was performed (Silva Junior et al., 2022). Three drops of dye solution 0.5 % chloride of 2,3,5-triphenyl tetrazolium (TTC) without sucrose were used in each glass slide. The TTC solution involves an enzymatic reaction, where the salts of the 2,3,5-triphenyltetrazolium chloride confirm pollen viability by the active enzyme, which is responsible for color changing of pollen grains to red in the presence of oxidative activity (Shekari et al., 2016), indicating a living cell (Figure 1B). After staining, pollen grains were evaluated under a light microscope (Olympus SZ51) with 10× magnification.
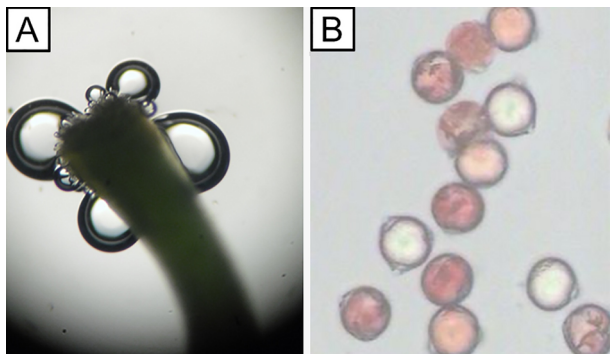
Five thousand pollen grains were evaluated from each genotype (1000 from each repetition). The pollen viability index (PVI) was determined by the percentage of viable pollen grains (in red color) in relation to the non-viable pollen grains (no color) (Abdelgadir et al., 2012).

Hybrid evaluation

To evaluate the potential of the pairwise combination of genotypes used in this study, three indices were determined from each crossing: fruiting index (FI), seed number (SN), and seed germination percentage (GP). The FI of artificial crosses was determined by counting the fruits developed from artificial pollination, expressed

Figure 1 – Glass slides under a microscope displaying (A) the emission of air bubbles during the stigma receptivity diagnosis using the hydrogen peroxide technique and (B) evaluation of the pollen viability by the histochemical test, with a solution of 0.5 % chloride of 2,3,5-triphenyl tetrazolium (TTC) without sucrose: grains in red are viable and colorless grains are unviable.

Table 2 – Aspects related to stigma receptivity and the pollen viability index (PVI) in tomato genotypes (*Solanum* spp.).

Genotypes	Stigma		Pollen grain
	Receptivity	Local	PVI (%)
'RVTM08'	VRS	SS	89 a*
'Redenção'	VRS	SS	90 a
'AF 26970'	VRS	SS	70 b
'LA-1401'	RS	SS	53 c
'AF 19684'	VRS	SS	82 a
'LA-1967'	VRS	ESR	2 d
'PI-127826'	LRS	SS	45 c
'PI-134417'	RS	SS	67 b
'LA-716'	VRS	ESR	61 b

VRS = very receptive stigma; RS = receptive stigma; and LRS = less receptive stigma. SS = stigmatic slit; and ESR = entire stigmatic region; *Means followed by the same lowercase letter in the column do not differ from each other according to the Scott-Knott test ($p < 0.05$).

as a percentage. Depending on the genotypes the ripe fruits were collected 50-70 days after pollination. After determining FI, the SN per fruit was counted.

For the germination test, seeds were treated with 0.7 % HCl, washed in tap water, and transferred to a forced air circulation oven at 32 °C for 48 h. The seeds germinated in plastic boxes filled with sterilized sand and kept in a germination chamber at a constant temperature of 25 °C and a photoperiod of 8-16 h light-dark cycle. The sand was moistened with distilled water until equivalent to 2.5 times its dry weight. From each cross, six repetitions of 25 seeds each were used, and the seedlings were counted from the 4th to the 25th day after planting. The germination percentage (GP) was calculated based on the number of germinated and planted seeds.

Statistical analysis

The PVI and GP were transformed into an arcsine of $(x/100)^{1/2}$ and tested for normality and homogeneity and were subsequently subjected to variance analyses by the F-test. The means of statistically significant results were grouped using the Scott-Knott test with a 5 % of probability using the software ASSISTAT version 7.7.

Results and Discussion

Stigma receptivity and pollen viability

The stigmas of 'Redenção', 'RVTM08', 'AF 26970', 'AF 19684', 'LA-1967', and 'LA-716' were classified as VRS, while those from genotypes 'LA-1401' and 'PI-134417' were classified as RS. However, the stigma of 'PI-127826' was observed to be LRS, with the emission of a few bubbles at low speed. In all the genotypes, air bubbles were released in the stigma slit or in the entire stigmatic region (Table 2).

The 'LA-1967' and 'LA-716' genotypes displayed stigma with good receptivity (Table 2), as female parents were incompatible with several crosses (Table 3). These findings corroborate with reports by Bedinger et al. (2011), who stated scientific evidence that genotypes of *S. chilense* and *S. pennellii* display reproductive barriers when used as female parents. The factors that influence the intra- and interspecific compatibility of these tomato species surpass the physiological barriers, which are primarily genetic mechanisms related to the evolution of the species (Chalivendra et al., 2013; Qin et al., 2018).

'AF 19684', 'RVTM08', and 'Redenção' presented the highest PVI at 82 %, 89 %, and 90 %, respectively (Table 2). On the other hand, 'LA-1967' displayed the lowest PVI (2 %), which diverged from the results of Gao et al. (2015), where the genotype 'LA 2-405', also from *S. chilense*, presented pollen grains with 90 % of viability. This issue of pollen viability may be specific to the genotype studied here.

Hybrid evaluation

The evaluation of FI showed no compatibility of genotype 'LA-1967' with the pollen from 'RVTM08', 'Redenção', and 'PI-134417'; genotype 'PI-127826' with the pollen from 'RVTM08', 'Redenção', 'AF 26970', 'LA-1401', 'PI-134417', and 'LA-716'; genotype 'PI-134417' with the pollen from 'RVTM08'; and genotype 'LA-716' with the pollen from 'AF 26970', 'LA-1401', 'LA-1967', and 'PI-134417'. However, this incompatibility does not occur in a reciprocal cross. In the crosses that resulted in fruit setting, all fruits experienced suitable development until maturation, where there was no occurrence of abortion during the developmental process (Table 3).

There is evidence in the literature showing that genotypes of *S. chilense*, *S. habrochaites*, and *S. pennellii* are incompatible with pollen from some other species (Moyle and Nakazato, 2008; Li and Chetelat, 2015).

Table 3 – Fruit index (FI) and number of seeds per fruit (NS) of self and intrapollination and interspecific artificial crosses in tomato genotypes (*Solanum* spp.).

Male parent	FI (%)								
	Female parent								
	M08	Red.	26970	1401	19684	1967	127826	134417	716
M08	86	92	72	60	36	0	0	0	38
Red.	84	100	88	78	48	0	0	42	46
26970	52	100	100	60	52	50	0	52	0
1401	78	92	28	98	26	20	0	54	0
19684	58	68	56	58	100	64	30	62	28
1967	64	72	64	52	50	0	34	68	0
127826	58	74	88	92	36	0	26	92	12
134417	42	68	40	46	60	52	0	60	0
716	38	68	32	28	36	38	0	26	48
Male parent	NS (fruit)								
	Female parent								
	M08	Red.	26970	1401	19684	1967	127826	134417	716
M08	33.4	22.9	56.2	22.4	14.5	-[1]	-	-	11.4
Red.	23.0	24.0	59.5	24.7	19.0	-	-	19.4	16.2
26970	24.0	23.5	83.4	27.0	27.0	0.0	-	23.7	-
1401	22.2	23.0	57.4	44.0	6.1	0.0	-	19.8	-
19684	28.9	21.0	48.3	16.5	58.2	0.0	11.3	21.3	17.2
1967	0.0	0.0	48.4	12.0	0.0	-	0.0	0.0	-
127826	26.5	22.4	53.4	34.5	7.9	-	32.0	24.3	14.1
134417	15.0	22.7	44.9	6.2	22.4	4.0	-	37.3	-
716	17.6	23.8	38.2	27.0	24.8	0.0	-	29.0	49.3

M8 = lineage 'RVTM08' from *S. lycopersicum*; Red = lineage Redenção from *S. lycopersicum*; 26970 = genotype 'AF 26970' from *Solanum pimpinellifolium*; 1401 = genotype 'LA-1401' from *S. galapagense*; 19684 = genotype 'AF 19684' from *Solanum peruvianum*; 1967 = genotype 'LA-1967' from *S. chilense*; 127826 = genotypes 'PI-127826' from *S. habrochaites* var. *hirsutum*; 134417 = genotype 'PI-134417' from *S. habrochaites* var. *glabratum*; and 716 = genotype 'LA-716' from *S. pennellii*; [1]There was not fruit development.

Although genotypes 'LA-1967' (*S. chilense*), 'PI-127826', 'PI-134417' (*S. habrochaites*), and 'LA-716' (*S. pennellii*) are allogamous, they show the capacity to reject the pollen from genotypes of related species. This reproductive barrier is part of the natural capacity of plants to avoid hybridization with related species (Li et al., 2010).

Our study shows that in crosses that use genotypes of *S. chilense*, *S. habrochaites*, and *S. pennellii* as female parents, pistils display reproductive barriers to pollen from other tomato species. In contrast, in the reciprocal crosses, the fruit set occurs successfully (Bedinger et al., 2011). Interactions between pollen and stigma play an essential role in restricting gene flow, especially between self-incompatible and self-compatible species, which often demonstrate unilateral interspecific incompatibility (Baek et al., 2015). In pollinations that resulted in fruit setting, all fruits had suitable development until maturation and no abortion occurred during the development process.

The FI showed a considerable variation among interspecific genotypes, demonstrating the importance of the knowledge of genotype compatibility, which has potential use in breeding programs. Plants that displayed better performance as female parents were the line 'RVTM08' and cultivar 'Redenção' (*S. lycopersicum*) and the genotypes of the wild species 'AF 26970', 'LA-1401', and 'AF 19684', resulting in a FI $\geq$ 26 % with pollen from all male parents (Table 3). These results follow Baek et al. (2015), where genotypes of cultivated tomato *S. lycopersicum,* wild species *S. pimpinellifolium* and *S. galapagense,* and other genotypes that are part of the species or section *Lycopersicon* have good compatibility with all tomato species when used as female parents.

The FI obtained in this study for 'AF 19684' (as both male and female parents; Table 3) disagrees with results for FI found in other studies since difficulties in fruit set in interspecific crosses are commonly reported for genotypes of *S. peruvianum* (Dall'Agnol and Schifino-Wittmann, 2002; Bedinger et al., 2011). However, genetic variability can be significant in the same tomato species, especially in *S. peruvianum* and *S. chilense*, , resulting in genotypes with different reproductive characteristics (Villand et al., 1998; Egashira et al., 2000; Bai and Lindhout, 2007). This shows that the genotype used in our study does not have genetic characteristics different from those evaluated in other experiments.

The intraspecific crosses performed between 'RVTM08' and 'Redenção' (*S. lycopersicum*) resulted in fruit setting as female parents, with FI $\geq$ 84 % for both genotypes. However, when crossed with genotypes 'PI-127826' and 'PI-134417', which belong to species *S. habrochaites*, there was only fruit setting when genotype 'PI-134417' was used as female parent, displaying an FI of 92 % (Table 3).

Regarding self-pollination, only genotype 'LA-1967' (*S. chilense*) displayed an FI of 0 %. Genotypes *S. chilense* are allogamous since they present genetic mechanisms of self-incompatibility (Rodriguez *et al.*, 2009). The same authors reported that some genotypes of *S. peruvianum*, S. *habrochaites,* and *S. pennellii* may also have total self-incompatibility. In our study, genotypes 'AF 19684', 'PI-127826', 'PI-134417', and 'LA-716' displayed FI of 100 %, 26 %, 60 %, and 48 %, respectively (Table 3).

The self-incompatibility is a mechanism of physiologic characteristics with a genetic basis, stimulating allogamy due to the failure of pollen grains from the same plant to penetrate or germinate through the stigma (Dall'Agnol and Schifino-Wittmann, 2002). Self-incompatibility in *Solanum* is S-RNase-based, gametophytic type, in which S-RNases determine S-specificity in the pistil and S-locus F-box proteins in pollen (Li and Chetelat, 2015). Although many studies have investigated self-incompatibility mechanisms,, little is known about how these mechanisms decompose in natural populations (Markova et al., 2017). In breeding programs, emasculations are not required when genotypes are self-incompatible and intended to be used as pollen recipients from other plants.

Genotype 'LA-1967' from *S. chilense* is self-incompatible and it also promotes the formation of seedless fruits in most crosses when used as both a female or male parent. The exceptions occurred only when the female parent was used in crosses with 'PI-134417' and as a male parent with 'AF 26970' and 'LA-1401', with an average SN of 4.0, 48.4, and 12.0 per fruit, respectively (Table 3). In the conventional hybridization process, besides incompatibility barriers where the stigma may not be compatible with pollen grains from other plants, postzygotic obstacles may occur, which are responsible for endosperm degeneration and, consequently, the death of the hybrid embryo (Li et al., 2010; Baek et al., 2015). This behavior has already been observed in crosses involving *S. chilense* and *S. peruvianum* (Chen and Imanishi, 1991; Table 3). In this sense, postzygotic mechanisms may act on crosses performed in our study, which involved *S. chilense* thus producing fruits without seeds.

Intrinsic postzygotic barriers manifest after successful fertilization and do not depend on environmental conditions or other extrinsic factors. Unlike prezygotic barriers that operate before fertilization, these barriers can hardly be overcome, making the speciation process irreversible (Roth et al., 2018). The broad definition of intrinsic postzygotic barriers encompasses a variety of developmental failures, which may occur at the beginning of seed development or, in some situations, even due to sterility or unviability of subsequent hybrid generations (Silva Junior et al., 2022).

The best result for SN was in the self-pollinated 'AF 26970' (Table 3). This genotype also produced many seeds when used as a female parent in crosses (Table 3). On the other hand, the other intra- and inter combinations of genotypes produced a maximum of 34.5 seeds per fruit (Table 3). Although 'AF 26970' can potentially to be a female parent in artificial crosses, this genotype displayed smaller flowers, requiring more attention and time for the emasculation technique.

The lowest values for SN occurred in 'RVTM08' and 'Redenção' compared to only self-pollinations (Table 3). The small number for self-pollination of these two *S. lycopersicum* lines was because they are breeding genotypes. The advance of the tomato breeding process has aimed to select plants with a small number of seeds per fruit, among other characteristics (Bai and Lindhout, 2007).

In all genotypes, self-pollination produced a higher SN than crosses (Table 3). In intra- and interspecific crosses, characteristics that may cause changes in seed production are somewaht variable, ranging from genetic mechanisms (Bedinger et al., 2011) to physiological aspects related to stigma receptivity and pollen quality (Barbara and Kubicki, 1985; Abdul-Baki and Stommel, 1995).

In this study, the GP of seeds obtained from artificial crosses and self-pollination showed no difference between all progenies, resulting in a GP above 84 %. Thus, the embryo developed normally for all crosses, despite the great diversity of the results for characteristics of the fruit index and seed numbers. However, interspecific crosses between genotypes present cases where reproductive barriers are related to endosperm degeneration and to a small number of germinated hybrid seeds, even *in vitro* cultivation (Aragão et al., 2002).

Breeding programs for cultivated tomatoes are becoming increasingly dependent on the genetic variability of genotypes of wild species, as the genetic bases of *S. lycopersicum* is very narrow (Peralta et al., 2008). Even basic questions about the mechanisms of intra- and interspecific reproductive barriers of tomato genotypes remain unanswered. In this sense, our study provides a detailed understanding of the capacity to combine tomato species, allowing for better use of the variability of wild tomato species in breeding programs.

Conclusions

In interspecific crosses, *S. lycopersicum* lines and genotypes of wild *S. pimpinellifolium*, *S. galapagense*, *S. peruvianum* show good compatibility with pollen of other species.

Solanum chilense genotype 'LA-1967' showed self-incompatibility and the formation of seedless fruits in interspecific crosses.

The stigma of *S. habrochaites* genotype 'PI-127826' was not very receptive and pollen grains of *S. chilense* genotype 'LA-1967' showed low viability.

Authors' Contributions

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