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Cucurbit Genetics Cooperative Reports

Watermelon and Related Species (*Citrullus* spp.)

Melon (*Cucumis melo*)

Cucumber (*Cucumis sativus*)

Squash, Pumpkins and Gourds (*Cucurbita* spp.)

Other Cucurbit Species and Reports on Multiple Species

Citrulline and Arginine in Fruit Flesh of Citron (*Citrullus amarus)* Accessions.

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Introduction

Citron watermelon *(Citrullus amarus*) is a wild or semi-wild relative of the cultivated watermelon (*Citrullus lanatus var. lanatus*). Citron offers a reservoir of genetic diversity and is used for watermelon breeding programs for traits such as fruit firmness, disease resistance and abiotic stress tolerance (Levi et al., 2001; Levi et al., 2007; Guo et al., 2019; Ngwepe et al, 2019; Mandizvo et al., 2021). In particular, the citron PI 296341 has been used as a grafting rootstock for cultivated watermelon and has resistance to *Fusarium oxysporum* f. sp. N (dwt)iveum, race 2 and tolerance to *Macrophomina phaseolina* (Edelstein et al., 2014).

Citrulline, an amino acid first identified in watermelon, has been of human health interest for some years. Citrulline is the precursor of arginine, helps remove ammonia in the urea cycle, and boosts the nitric oxide system and vasodilation (Allerton et al. 2018). Among cucurbits, watermelon generally has the highest content of citrulline (Fish and Bruton, 2010; Hartman et al., 2019). Production environment and cultivar can also affect citrulline and arginine content in watermelon (Rimando and Perkins-Veazie, 2005; Davis et al., 2011). Citrulline values of 6.9 to 44.3 and arginine values of 1.8 to 20.7 mg/g dry weight (dwt), with ratios of citrulline to arginine ranging from 2:1 to 6:1, were found across 107 watermelon accessions (Assefa et al., 2020).

The citrulline content in citron fruit may be as high or higher than that of watermelon. Fish and Bruton (2010) reported that Botswana watermelon (*C. amarus* PI 542113) contained 45.8 and 7.2 mg/g dwt citrulline and arginine, respectively, compared to 22.1 and 5.8 mg/g dwt for 'Dixilee' watermelon. Johnson et al. (2022) reported citrulline and arginine concentrations of 39.2 and 2.2 mg/g dwt in wild citron from Queensland Australia compared to 22.9 and 8.1 mg/g dwt of in red-fleshed watermelon. Citrulline and arginine accumulate in drought stressed watermelon as a means of preserving nitrogen, acts as an osmolyte (Song et al. 2020), and helps protect tissues from oxidative stress by scavenging free radicals (Akashi et al., 2001).

In this study, fruit from citron watermelon (*Citrullus amarus* or *Citrullus lanatus* var. *citroides*) were utilized to determine variation in soluble solids content (SSC), pH, citrulline and arginine. Most of the accessions used originated from Africa (Table 1). These data may help in identifying accessions higher in citrulline that have potential value for breeding programs.

Materials and Methods

Ripe fruit of citron accessions were grown at the U.S Vegetable Lab in Charleston, SC and transported to Kannapolis, NC. Watermelons were cut in half lengthwise (Figure 1A, B) and samples of 50 to 100 g of flesh per fruit were stored in plastic bags at -80°C until used. Remaining flesh was used to determine SSC and pH. Soluble solids content (SSC) from juice was determined by digital refractometer and pH determined by inserting a stainless steel pH probe into the melon flesh as described in Hartman et al. (2019).

Citrulline and arginine content were analyzed using 0.2 g of puree from thawed citron cubes mixed with a 1.2 ml aliquot of phosphoric acid (0.03M) following the method of Hartman et al. (2019). Filtered samples (5 ul) were injected onto a high-performance liquid chromatograph (Hitachi Elite LaChrom) equipped with a photodiode array detector and autosampler. A Gemini 3u C18, 110A, 250x4.6 nm column and guard column (C18 4x2.0 mm) (Phenomenex, CA, USA) were held at 25 °C and a mobile phase of 0.015 M phosphoric acid, 0.5 ml/min was used for peak separation at 195 nm. External standards of arginine and citrulline (Millipore Sigma, USA) were used to verify and quantify the amino acid peaks.

Results and Discussion

A total of 45 citron accessions were sampled in this study. The %SSC was low (1.1 to 4.6), with an average value of 2.5 and median value of 2.4 (Table 1). The fruit pH values were 4.8 to 5.6, indicating that most samples were near ripe to ripe when compared to the pH range of 5.0–5.6 of ripe watermelon (*Citrullus lanatus*) (Perkins-Veazie et al, 2006). No correlation

was found between citrulline and soluble solids content in this group of citron accessions although Assefa et al. (2024) reported a negative correlation between citrulline content and SSC in watermelon.

Average concentrations of the citron accessions were 54.6 and 11.6 mg/100g fresh weight (fwt) for citrulline and arginine, respectively (Table 1). Substantial variation in both citrulline and arginine contents was found among accessions. Citrulline content ranged from 2.1 to 294.6 mg/100 g fwt, while arginine content ranged from 0.5 to 64.3 mg/100 g fwt, with median values of 34.8 and 7.4, indicating a broad diversity among accessions. The citrulline content of citron in this study was often lower than that reported for watermelon but was higher than that reported for many other cucurbits, such as melon, cucumber, and squash (Hartman et al, 2019).

Five of the citron accessions in this study (PIs 482283, 482308, 482311, 482312, 50035) were reported to be drought resistant (Zhang et al. 2011). These accessions were medium to low in citrulline (21.5-50.1mg/100g fwt) and arginine (4.8-25.9 mg/100g fwt).

We were unable to follow the potential effects of ripeness among the citron accessions in this study; Johnson et al. (2022) indicated that citron could increase 3-fold between green and ripe stages. Assuming that citron has a dry weight near the 9.1% reported for watermelon, (https://fdc.nal.usda.gov/food-details/2747675/nutrients), PI 596696 had a citrulline content of 32.4 mg/g dwt, and was most comparable to that of Johnson et al. (2022).

Our study shows that citron can vary widely in citrulline and arginine content, with some accessions similar to that reported in cultivated watermelon. This information may be helpful in crosses of citron and watermelon if higher amounts of citrulline are desired along with disease and insect resistance.

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Figure 1. Variation in citron watermelon exterior appearance (left), and interior appearance (right).

Table 1. Composition of fruit flesh from citron watermelon (*C. lanatus* v. *citroides*) accessions

Accession	Source	%SSC	pH	Arginine	Citrulline
				mg/100g·fwt	
GRIF15896	Russian federation	2.4	5.1	11.9	61.3
GRIF16135	South Africa	4.2	5.2	64.3	153.2
GRIF17032	USA	1.6	4.9	6.8	104.9
PI270564	South Africa	2.7	5.2	17.6	34.5
PI271775	Transvaal, South Africa	2.1	5.0	1.4	37.7
PI295850	South Africa	1.9	5.1	1.9	16.2
PI296334	Limpopo, South Africa	1.2	5.0	2.2	34.4
PI296335	KwaZulu-Natal, South Africa	3.9	5.4	4.7	50.1
PI296337	South Africa	1.4	5.1	1.0	29.1
PI296339	Cape Province, South Africa	3.1	5.0	1.3	15.2
PI296341	Cape Province, South Africa	2.1	5.0	3.6	62.5
PI296343	Cape Province, South Africa	1.7	5.0	0.8	9.6
PI299378	Cape Province, South Africa	2.2	4.8	2.1	30.0
PI299379	Cape Province, South Africa	1.1	4.9	7.4	59.5
PI379243	North Macedonia	1.3	4.8	3.1	27.4

Table 1. (Continued)

Accession	Source	%SSC	pH	Arginine	Citrulline
				mg/100g·fwt	
PI482257	Zimbabwe	2.2	5.2	7.7	87.7
PI482259	Zimbabwe	4.6	5.2	16.6	96.9
PI482267	Zimbabwe	3.2	5.4	12.7	25.5
PI482276	Zimbabwe	2.1	5.2	1.4	5.1
PI482283	Zimbabwe	2.9	4.9	7.2	21.5
PI482293	Zimbabwe	2.6	5.3	13.2	91.3
PI482298	Zimbabwe	2.5	5.2	5.5	34.4
PI482299	Zimbabwe	2.9	5.2	10.3	50.1
PI482308	Zimbabwe	3.3	5.4	15.9	39.1
PI482311	Zimbabwe	2.4	5.3	4.8	32.0
PI482312	Zimbabwe	2.4	5.3	5.9	45.9
PI482326	Zimbabwe	2.9	5.3	3.8	9.0
PI482335	Zimbabwe	2.4	5.1	8.7	34.0
PI482336	Zimbabwe	2.5	5.2	20.6	30.7
PI482342	Zimbabwe	2.9	5.6	5.6	25.8
PI494817	Zambia	2.5	5.0	15.0	34.8
PI500303	Zambia	3.3	5.1	18.9	29.8
PI500308	Zambia	2.2	5.5	25.9	36.3
PI500335	Zambia	2.0	5.1	0.5	2.1
PI532624	Zimbabwe	1.7	5.1	17.9	50.9
PI532659	South Africa	2.4	5.3	1.8	6.7
PI532664	Eswatini	1.5	4.9	9.6	34.9
PI542119	Botswana	2.8	5.3	31.7	97.1
PI596656	South Africa	2.7	5.1	18.5	120.6
PI596659	South Africa	1.9	5.1	1.2	17.9
PI596665	Transvaal, South Africa	2.3	5.1	18.1	62.8
PI596666	Transvaal, South Africa	2.9	5.1	6.3	10.5
PI596668	Transvaal, South Africa	2.7	5.4	32.2	212.6
PI596669	Cape Province, South Africa	2.6	5.3	11.3	89.3
PI596696	Transvaal, South Africa	2.5	5.1	41.1	294.6
	Average	2.5	5.2	11.6	54.6
	Median	2.4	5.1	7.4	34.8
	Standard deviation	0.73	0.18	12.34	55.27
	Minimum	1.1	4.8	0.5	2.1
	Maximum	4.6	5.6	64.3	294.6

Assessment of Drought-Tolerant *Citrullus* Rootstocks for Watermelon Cultivation Under Full and Deficit Irrigation

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Introduction

The context of climate change that we are living in is driving the water scarcity in Mediterranean horticulture, and all over the world. Therefore, management that increases water use efficiency of crops while preserving yield and fruit quality is needed. Deficit irrigation is a key tool to reduce water usage (Fereres and Soriano, 2007). Watermelon (*Citrullus lanatus* L.) is a widely cultivated cucurbit crop, very sensitive to water limitations, especially in the early growth and fruit set stages (Morales et al., 2023).

Grafting is a commonly used strategy in watermelon production across Europe (including Spain) for improving tolerance to abiotic and biotic stresses and stabilizing productivity. The main rootstocks used are the interspecific *Cucurbita* hybrids contributing to plant vigor and a most efficient resource uptake, as well as giving protection against soilborne diseases (Liu et al., 2025, Louws et al., 2010). Negative effects on fruit quality of watermelons grafted onto *Cucurbita* hybrids have been previously reported (Alexopoulos et al., 2007, Soteriou et al., 2015).

Lately, *Citrullus* spp. germplasm is being tested as a promising drought tolerance source in cultivated watermelon, supported by recent analysis that have revealed substantial genetic variation and stress-related gene families associated with drought resilience. (Gkanogiannis et al., 2024). Also, physiological studies showed that the rootstocks derived from *Citrullus colocynthis* maintain the yield of the scion under water stress (Bikdeloo et al., 2021; Sehularo et al., 2025). Previous studies of the group found that *Citrullus*-based grafting generally maintains fruit quality much closer to that of non-grafted plants, compared to grafting onto *Cucurbita*, preserving sugar-acid balance and typical watermelon flavor profiles (Fredes et al., 2016). Others support that grafting can enhance yield production without compromising the soluble solids content (SSC), yet fruit quality outcomes are environmental-dependent, resulting in the need of evaluations under different commercial conditions (Devi et al., 2020; Jordana et al., 2023). Even so, SSC may increase under moderate water reduction stress due to concentration effects without necessarily impacting in the overall quality (Proietti et al., 2008).

In the current work, two watermelon cultivars were evaluated, grafted onto commercial *Cucurbita* and *Citrullus* rootstocks under 100% vs 50% irrigation in two commercial fields near Valencia, focusing on yield and SSC to identify combinations suitable for water-limited scenarios.

Materials and Methods

Experiments were conducted in summer 2024 in two open-field commercial sites in the Valencia horticultural belt (Spain): Museros (39°34'34.8"N 0°21'46.0"W) and Alcàsser (39°23'12.3"N 0°27'35.9"W). Each site included full irrigation according to the usual practices (100%) and deficit (50%) irrigation regimes; the 50% level represented a moderate reduction, commonly used to assess drought responses in cucurbits (Morales et al., 2023; Rouphael et al., 2008). Irrigation scheduling and application followed local commercial management at each site.

Two watermelon scions were used: commercial 'Style' and traditional 'Negra de Altura' (NA, BGV016458). Five rootstocks were evaluated: the commercial *Cucurbita* control 'Shintoza'; two *Citrullus* accessions (*C. colocynthis* VIR-1996 ('13') and *C. lanatus* var. *citroides* BGV0005167 ('14')), plus the hybrids BGV0005167 x VIR-1996 ('14x13') and BGV0005167 x *C. mucosospermus* PI 494527 ('14x18'). For each scion, self-grafted and non-grafted controls were included. At each location, and within each irrigation treatment, the 13 treatments were arranged in a randomized complete block design with two replications. Plots consisted of three plants. Grafting followed the standard cucurbit splice/one-cotyledon protocols to ensure high survival, uniformity and reproducibility of the commercial practices.

Four traits were recorded in each plot: fruit number, mean fruit weight (kg), yield (kg/plant) and soluble solids content (SSC), which was determined in pulp juice using a digital refractometer and expressed as ºBrix.

All statistical analyses were performed using StatGraphics Centurion (StatPoint Technologies Inc., Warrenton, VA, USA). Multi-factor ANOVAs (Type III sums of squares) were

conducted for each of the four traits evaluated (fruit number, mean fruit weight, yield and SSC), including site, irrigation, scion and rootstock as fixed factors, and followed by 95% LSD multiple-range tests and pairwise contrasts to identify significant differences among factor levels and combinations.

Results and Discussion

For all traits evaluated, there was a significant effect of the field site: every variable showed higher values in Museros compared with Alcàsser. Irrigation regime also had a significant effect on all traits: yield, number of fruits per plant and mean fruit weight were consistently higher under full irrigation (100%), whereas SSC increased under deficit irrigation (50%). Differences in plant vigor were also observed across field sites and irrigation regimes, with more vigorous growth under full irrigation in both sites (Figure 1). Regarding the scion, 'Style' produced a higher fruit number and registered higher SSC, while 'Negra de Altura' (NA) resulted in greater yield and mean fruit weight. In contrast, rootstocks did not show a significant effect in the global analysis. These results indicate a clear interaction between rootstock performance and environmental context, which has already been found in previous studies (Jordana et al., 2023, Rouphael et al., 2008,). The site-scion-irrigation interactions were significant for all variables; therefore, rootstock effects were examined separately for each specific combination.

In Museros with NA, all rootstocks performed similarly for yield (Figure 2A1) under each irrigation regime. For the remaining traits, differences were only significant under deficit irrigation. *Citrullus* rootstocks outperformed the commercial control 'Shintoza' for specific factors: rootstocks '13', besides self-grafted NA, increased SSC (Figure 2A2), and '14x13' enhanced fruit number (Figure 2A3). Conversely, 'Shintoza' and '14' produced larger fruits than the hybrids '14x13' and '14x28' (Figure 2A4). Although differences between irrigation levels were present for some rootstocks, these were less frequent than expected, likely because the irrigation supplied at Museros was high enough that 50% treatment did not impose strong water stress.

With 'Style' as the scion in Museros, in both irrigation regimes, 'Shintoza' rootstock consistently achieved high yield and fruit number (Figure 2A1, 2A3), not significantly different from '14x13' and '14x28' at full irrigation, and from '13' at 50% irrigation. All rootstocks produced fruits with similar weights (Figure 2A4). However, under full irrigation, for the self-grafted and when the hybrids '14x13' and '14x28' were used as the rootstock, the SSC was higher than with 'Shintoza' (Figure 2A2). For this Museros-'Style' combination, the non-grafted treatment was excluded because plants failed to survive transplanting in both blocks.

In Alcàsser, with NA under deficit irrigation, rootstocks '14' and '14x28' produced yields similar to 'Shintoza', higher than the self-grafted and non-grafted plants (Figure 2B1). For mean fruit weight (Figure 2B4), 'Shintoza' and '14' produced larger fruits than the self-grafted, while all other rootstocks performed similarly. Rootstock '14x28' exceeded the fruit number of '14x13', '13', and the non-grafted. Under full irrigation, '14' and '14x28' produced more fruits than both 'Shintoza' and '14x13' (Figure 2B3). No significant differences were found for SSC at Alcàsser with this scion (Figure 2B2).

For 'Style' in Alcàsser, no rootstock effects were detected for SSC (Figure 2B2) or mean fruit weight (Figure 2B4). Under deficit irrigation, '14x28' outperformed the self-grafted and non-grafted treatments for yield (Figure 2B1), and 'Shintoza' and '14x28' produced more fruits than the self-grafted and non-grafted treatments (Figure 2B3). Under full irrigation, rootstocks '14' and '14x28' produced higher yields than '13' and the non-grafted treatment (Figure 2B1), and 'Shintoza', '14', and '14x28' produced more fruits than the non-grafted plants (Figure 2B3). Similarly to what has been described for NA in Alcàsser, SSC remained stable with all rootstocks and for both irrigation levels (Figure 2B2). These results support the strong environmental influence on this quality trait, previously reported (Devi et al., 2020).

Moreover, water reduction had a consistent negative impact on watermelon performance in most site-scion combinations. In general, reducing irrigation from 100% to 50% significantly decreased yield and fruit number, except in Museros when cultivating the commercial scion 'Style', whose production remained stable under deficit irrigation. This reduction is consistent with previous studies reporting that watermelon production is highly sensitive to water limitation (Morales et al., 2023). Mean fruit weight was significantly lower in the 50% water deficit only in NA fruits grown in Museros; in all other combinations fruit weight was unaffected by irrigation level. SSC also remained stable, except for the SSC of the 'Style' fruits cultivated in Alcàsser, which increased under 50% irrigation. Similarly to our results, other studies reported that grafting doesn't consistently modify SSC under water stress, maintaining stable soluble solids accumulation across irrigation regimes (Rouphael et al., 2008).

Conclusions

This study demonstrates that watermelon responses to grafting and irrigation depend strongly on the interaction between cultivation conditions, scion and rootstock. Although no overall rootstock effect was detected across traits, notable context-specific differences were found. Differences observed between sites highlighted the importance of local irrigation practices, with higher water availability in Museros mitigating

yield losses under 50% irrigation. Effective rootstock selection requires considering specific production environments, as grafting effects are not universal but highly context dependent.

In the work here presented several experimental *Citrullus* rootstocks were identified that in some cases showed a performance comparable to the commercial *Cucurbita* rootstock 'Shintoza', either in full irrigation regime or with water deficit. Future analysis on fruit quality attributes of samples collected during this study will be carried out to determine the effect of grafting onto these experimental *Citrullus* rootstocks on quality parameters and confirm their usefulness as alternative to the classic *Cucurbita* hybrid rootstocks.

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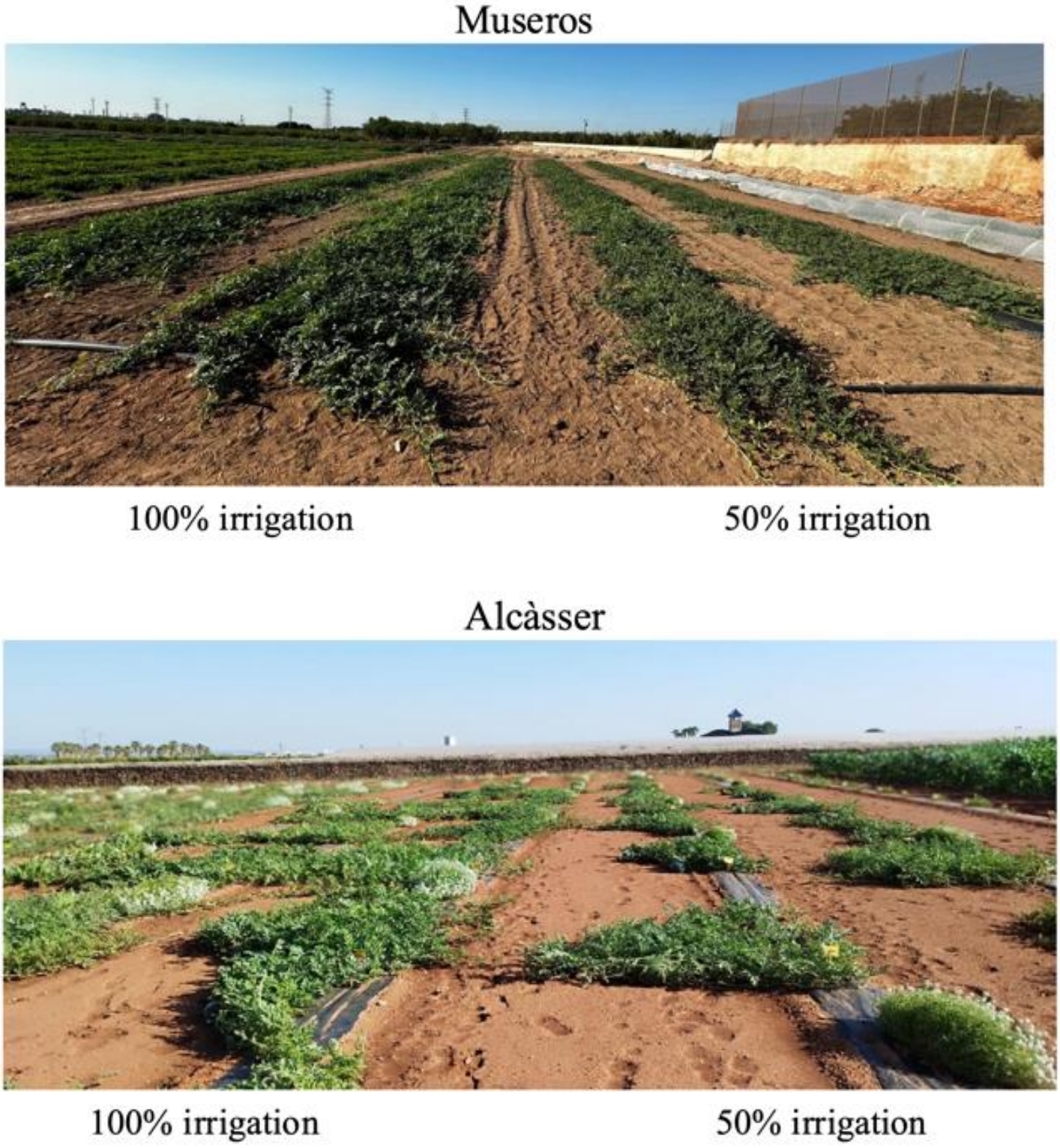

Figure 1. Differences in plant vigor in the two experimental fields, Museros (left) and Alcàsser (right), under full (100%) and deficit (50%) irrigation regimes.

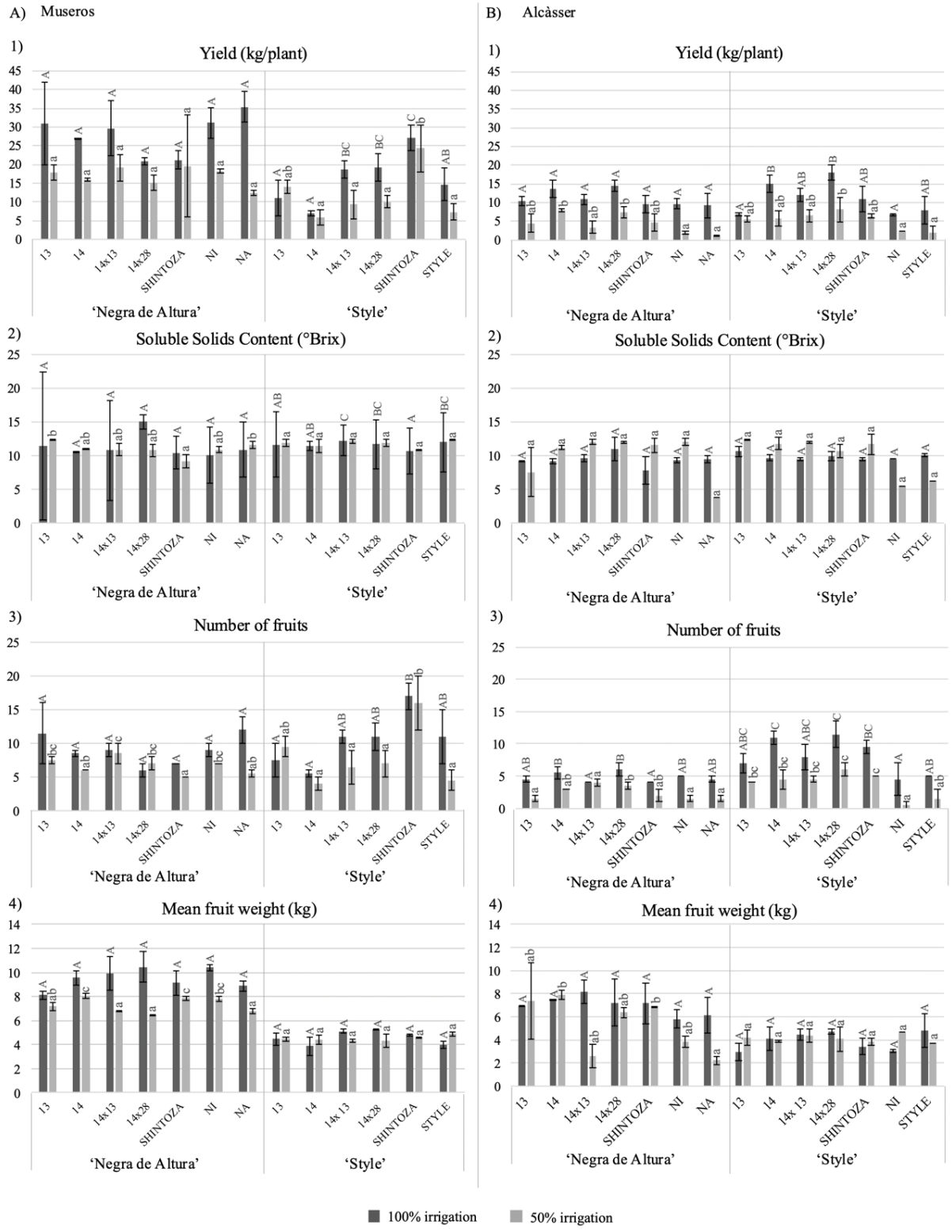

Figure 2. Mean yield (1), SSC (2), number of fruits (3) and mean fruit weight (4) for 'Negra de Altura' and 'Style' grafted onto different rootstocks under full (100%, dark bars) and deficit (50%, light bars) irrigation in A) Museros and B) Alcàsser. Rootstock included '13' (*Citrullus. colocynthis* VIR-1996), '14' (*C. lanatus* var. *citroides* BGV0005167), '14x13' (BGV0005167 x VIR-1996), '14x28' (BGV0005167 x *C. mucosospermus* PI 494527), the commercial control 'Shintoza', the non-grafted control ('NI') and the self-grafted controls ('NA' for 'Negra de Altura' and 'STYLE' for 'Style'). Bars represent means ± SE. Different letters indicate significant differences among rootstocks within each site-scion-irrigation combination (LSD, $p < 0.05$).

Evaluation of Resistance to Moroccan Watermelon Mosaic Virus (MWMV) in a Melon Recombinant Inbred line (RIL) Population Derived from TGR-1551

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Introduction

Cucurbit crops constitute an economically important horticultural group around the world but are severely affected by various viral diseases, particularly those caused by potyviruses (Martín-Hernández & Picó, 2020). Among them, the Moroccan watermelon mosaic virus (MWMV), a member of the genus *Potyvirus* (family *Potyviridae*), has emerged as a significant threat in the Mediterranean Basin, Africa, and several European countries, where its incidence and geographic range have steadily expanded (Miras et al., 2019).

MWMV was initially discovered in Morocco in 1972, but at that time, it was considered a watermelon mosaic virus (WMV) strain (Fischer & Lockhart, 1974); it was subsequently recognized as a distinct member (Lecoq et al., 2001). Symptoms associated with MWMV are characterized by mosaic, severe leaf and fruit deformations (Fischer & Lockhart, 1974), as well as severe vein clearing (De Moya-Ruiz et al., 2021). However, these symptoms can be exacerbated under natural conditions by mixed infection with other potyviruses (Jagunić et al., 2025).

Resistance to MWMV has been identified in previous works carried out by our research group in melon (*Cucumis melo* L.) accessions TGR-1551, PI 414723, and IC 274006 (personal communication). In melon, the source of virus resistance is usually found in accessions of the Asian Conomon, Momordica, and Acidulus Groups (Martín-Hernández & Picó, 2020). When these accessions present a multi-resistant profile, breeding programs are simplified, as a single donor parent can be used to introgress multiple resistance traits into a single genetic background (López Martín, 2023). The African accession TGR-1551 (acidulus group) has been reported as resistant to WMV, cucurbit yellow stunting disorder virus (CYSDV), cucurbit aphid-borne yellows virus (CABYV), MWMV, and powdery mildew (*Podosphaera xanthii*) races 1, 2, and 5 (Díaz-Pendón et al., 2005; Lecoq & Desbiez, 2012; López-Sesé & Gómez-Guillamón, 2000; Kassem et al., 2015, Palomares-Ríus et al., 2011, 2016; Yuste-Lisbona et al., 2010, 2011a, 2011b).

TGR-1551-derived resistance to the *potyvirus* WMV has been reported to provoke a drastic and significant reduction in virus titer, with infected plants being asymptomatic or showing only mild symptoms of the disease (Díaz-Pendón et al., 2005), with a strong early transcriptional defense response, despite the resistance mechanism being recessive (López Martín et al., 2024). A population of recombinant inbred lines (RILs) derived from the cross between the resistant genotype TGR-1551, and the susceptible Spanish cultivar 'Bola de Oro' (BO) has enabled significant advances in the detection of QTLs linked to WMV resistance. A major QTL on chromosome 11 and another minor QTL on chromosome 5 have been reported, revealing a complex genetic architecture influenced by environmental conditions (Palomares-Rius et al., 2011; Pérez-de-Castro et al., 2019).

A close genomic and biological relationship between WMV and MWMV has been previously reported (Jagunić et al., 2025). In this context, the objective of this study was to evaluate a population of RILs (TGR-1551 x BO) mechanically inoculated with MWMV to identify resistant lines and generate phenotypic data that will subsequently allow mapping of resistance and identification of candidate genes.

Materials and Methods

A recombinant inbred lines (RILs) population (F_7/F_8; 61 lines) derived from the cross TGR-1551 × BO was evaluated for resistance to MWMV. This set of 61 lines, previously genotyped by sequencing (GBS) covered 99.6% of TGR-1551 genome (Pérez-de-Castro et al., 2019). Plants (1–5 per line) were mechanically inoculated with a Mediterranean MWMV isolate, using infected tissue homogenized in inoculation buffer (1% PVP-10, 1% PEG-6000, 10% KH_2PO_4, pH 8). The inoculation was performed mechanically by rubbing one cotyledon and the first genuine leaf, previously sprinkled with carborundum. The plants were reinoculated 7 days later.

Symptoms were scored at 15, 21, and 28 days post-inoculation (dpi) on a 0–4 severity scale, where 0 indicates the plant does not show any disease symptoms, and 4 indicates the plant is highly affected (Figure 1, adapted from the symptoms described for MWMV infections (Miras et al., 2019)). Based on this scale, each line was classified as

resistant (R) or susceptible (S) according to the maximum severity value recorded during the experiment. Lines were considered resistant if symptom score was lower than 2, whereas those reaching scores of 2 or greater than 2 at any evaluation date were classified as susceptible.

The lines identified as resistant in this first assay, based on symptom scoring, were subsequently re-evaluated, together with both the resistant and the susceptible parents, TGR-1551 and BO. Leaf tissue was collected at 21 and 28 dpi, and stored at −80 °C until used to quantify MWMV accumulation.

Total RNA was extracted from leaf tissue using EXTRAzol® (Blirt). Samples were homogenized in 700 µL of EXTRAzol®, phase-separated with chloroform, and the aqueous phase was recovered. RNA was precipitated with isopropanol, washed twice with 70% ethanol, air-dried, and resuspended in 30 µL of nuclease-free water. Then, the RNA was quantified using a NanoDrop™ 1000 spectrophotometer (Waltham, Massachusetts, United States).

Once RNA quality and concentration were confirmed, samples were diluted to 100 ng/µL. RNA was then reverse-transcribed using the RevertAid™ Reverse Transcription Kit (Thermo Fisher Scientific) with random hexamer primers, following the manufacturer's protocol. The resulting cDNA was stored at −20 °C until RT-qPCR analysis.

Relative quantification of MWMV accumulation was performed by RT-qPCR using the FastStart Essential DNA Green Master (Roche). Each 15 µL reaction contained 7.5 µL of 2× Green Master Mix, 1.5 µL of each primer (10 µM), 3 µL of nuclease-free water, and 1.5 µL of cDNA. Two primer sets were used: one targeting the MWMV coat protein (CP). (F:5′CAACACCAGGGCAACTCAGA3′ and R:5′TGCACCACACCATGAAACCA3′, MWMV-CP primers designed in this study), and one targeting the endogenous reference gene Cyclophilin CYP7 (F:5′CGATGTGGAAATTGACGGAA3′ and R:5′CGGTGCATAATGCTCGGAA3′) (Sáez et al. 2022).

Ct values were used to calculate ΔCt, ΔΔCt, $2^{-\Delta\Delta Ct}$, and its logarithmic transformation log ($2^{-\Delta\Delta Ct}$) to estimate viral load. Statistical analyses (ANOVA and LSD 95%) were performed using GraphPad Prism.

Results and Discussion

A total of 61 recombinant inbred lines (RILs) were mechanically inoculated with MWMV. Symptom severity was evaluated at 15, 21, and 28 dpi using a 0–4 scale. According to the symptom score, each line was classified as susceptible (S) or resistant (R) (Table 1). Fifty-seven out of the 61 lines showed symptoms typically associated with MWMV (Miras et al., 2019), including leaf deformation, blistering, and severe mosaic, with severity scores of 3 or 4 during the evaluation period. These lines were therefore classified as susceptible. Four of the lines (195, 55, 60, 68) showed very low symptoms (range 0-1) during the evaluation period and were therefore classified as resistant (R). This finding is consistent with the previous works of the group reporting resistance to MWMV in accession TGR-1551.

A new trial was conducted with the previously classified resistant lines. Consistently, these lines either remained asymptomatic or showed only slight symptoms (range 0–1) similarly to the first evaluation. The accession TGR-1551, on the other hand, showed variability in its response: some plants remained practically asymptomatic (0–1), while others developed slightly more noticeable symptoms. Notably, some of the RILs included in this study were resistant, indicating that the RILs population was derived from a resistant TGR-1551 plant. Viral load was assessed at 21 dpi and 28 dpi. MWMV accumulation increased from 21 to 28 dpi, indicating a progressive rise in viral replication over time. This temporal trend is consistent with previous findings showing that viral load after inoculation with an infectious MWMV clone in melon plants steadily increased throughout the infection period, specifically at 6, 12, 18, and 24 dpi (De Moya-Ruiz et al., 2021).

In our study, differences in viral accumulation at 21 dpi were highly significant ($p = 0.021$). The four resistant lines (RILs) as well as the resistant control TGR-1551 showed virus accumulation lower than the susceptible parent BO (Figure 2A). At 28 dpi, viral accumulation in RILs 68, 60, and 195 was significantly lower than in BO, while viral accumulation in RIL 55 did not significantly differ from either that of TGR-1551 or BO (Figure 2B). The results indicated that the resistant lines exhibit reduced MWMV accumulation, consistent with their lack of symptoms. Previous studies on TGR-1551-derived resistance to the closely related potyvirus WMV, reported that this resistance consisted of a substantial decrease in viral titer and milder symptom expression compared with susceptible genotypes (Díaz-Pendón et al. 2005), thus, similar to resistance to MWMV characterized in the present study.

As previously stated, a GBS was available for the RILs population (Pérez de Castro et al., 2019). A preliminary haplotype analysis of the 61 evaluated lines, comparing resistant and susceptible lines, identified a TGR-1551 introgression on chromosome 11 shared by the four resistant lines. In contrast, susceptible lines predominantly carry the BO-derived allele for this region. The identified region, although longer, included the candidate region for the major QTL conferring resistance to WMV (López-Martín et al., 2024; Pérez-de-Castro et al., 2019). However, preliminary results by the research group with lines segregating for candidate region for TGR-derived WMV resistance suggest that both resistances

have different genetic control. The QTL responsible to TGR-derived resistance to MWMV could be in the candidate region on chromosome 11 identified here, but is different from the QTL for WMV resistance. In this sense, the study by Rodríguez-Hernández et al. (2012) showed that silencing the translation initiation factor eIF4E in melon conferred resistance to MWMV but not to WMV, suggesting the resistance to each of these two potyviruses is the result of two separate mechanisms.

TGR-1551 is a widely recognized multi-resistant accession, against various viruses and fungi affecting melon. Molecular markers are available for marker assisted selection for some of these resistances, including CYSDV (Pérez de Castro et al., 2020), WMV (Pérez de Castro et al., 2019) or powdery mildew (López-Martín et al., 2022). Thus, its use in breeding programs facilitate the introgression of multiple resistances in a common genetic background. In this report, we present evidence that TGR-1551 also confers resistance to MWMV. As a next step, segregating generation studies derived from the resistant RILs 195, 55, 60, and 68, will be conducted to confirm the candidate region conferring resistance to MWMV.

Acknowledgements

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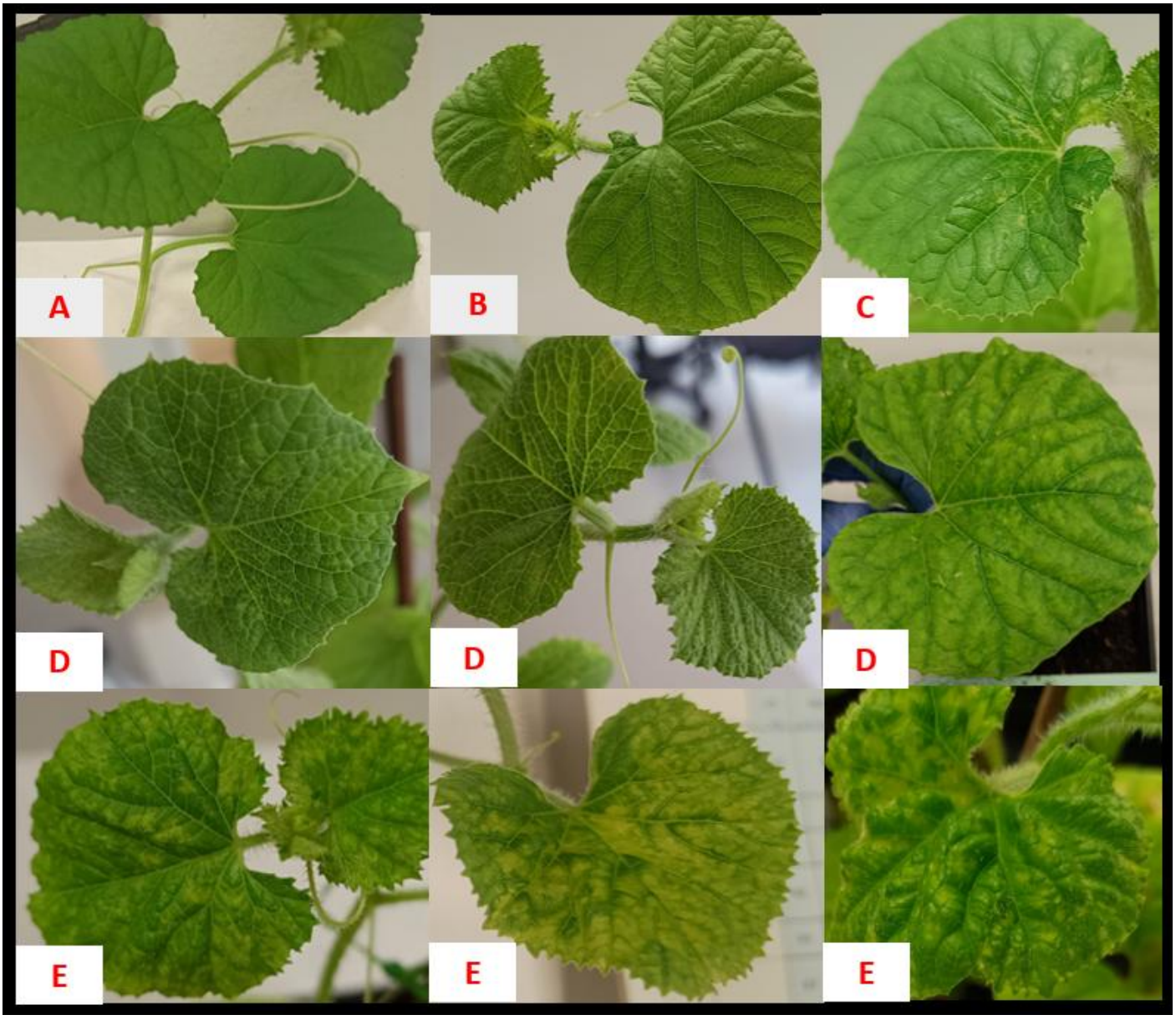

Figure 1 Symptoms after inoculation with Moroccan watermelon mosaic virus (MWMV) on a scale from 0 to 4. (A) No symptoms (0). (B) Mild symptoms (1). (C)Moderate symptoms (2). (D) Severe symptoms (3). (E) Very severe symptoms (4).

Table 1. Symptom severity ranges at 15, 21 and 28 days post inoculation (dpi) after MWMV inoculation in lines of the TGR-1551-derived recombinant inbred lines (RILs) population. Classification of the lines as resistant (R) or susceptible (S) (see text for description). N indicates the number of plants evaluated per line.

Line	N	Symptoms 15-dpi	Symptoms 21-dpi	Symptoms 28-dpi	Maximum severity	Classification
BO	5	(2 - 3)	(3 - 4)	(3 - 4)	4	S
TGR	5	(0 - 1)	(0 - 1)	(0 - 1)	1	R
7	3	(2 - 3)	(3 - 4)	(1 - 3)	4	S
9	3	(3 - 3)	(1 - 3)	(1 - 3)	3	S
17	3	(1 - 2)	(0 - 4)	(1 - 3)	4	S
21	2	(0 - 3)	(2 - 3)	(2 - 4)	4	S
28	3	(1 - 2)	(2 - 3)	(2 - 4)	4	S
35	3	(3 - 4)	(3 - 4)	(1 - 4)	4	S
49	1	3	3	3	3	S
50	3	(0 - 4)	(2 - 4)	(3 - 4)	4	S

Table 1 (continued)

Line	N	Symptoms 15-dpi	Symptoms 21-dpi	Symptoms 28-dpi	Maximum severity	Classification
55	5	(0 - 1)	(1 - 1)	(0 - 1)	1	R
60	5	(0 - 0)	(0 - 0)	(0 - 0)	1	R
67	3	(0 - 0)	(1 - 1)	(1 - 4)	4	S
68	5	(1 - 1)	(0 - 1)	(0 - 1)	1	R
69	4	(1 - 4)	(2 - 4)	(2 - 4)	4	S
73	4	(1 - 4)	(1 - 4)	(1 - 4)	4	S
85	2	(1 - 3)	(3 - 3)	(4 - 4)	4	S
87	5	(0 - 4)	(1 - 3)	(1 - 3)	4	S
101	1	3	4	4	4	S
104	2	(4 - 4)	(2 - 3)	(3 - 4)	4	S
109	5	(0 - 2)	(2 - 3)	(4 - 4)	4	S
110	2	(0 - 1)	(0 - 2)	(1 - 3)	3	S
114	1	2	4	2	4	S
131	4	(1 - 1)	(0 - 4)	(0 - 4)	4	S
143	1	1	1	4	4	S
147	3	(1 - 4)	(1 - 4)	(2 - 4)	4	S
151	5	(3 - 4)	(3 - 4)	(4 - 4)	4	S
156	3	(1 - 3)	(2 - 4)	(0 - 4)	4	S
157	2	(0 - 2)	(1 - 4)	(1 - 4)	4	S
158	5	(0 - 4)	(0 - 3)	(0 - 2)	4	S
159	2	(3 - 3)	(3 - 4)	(2 - 3)	4	S
161	3	(0 - 4)	(2 - 4)	(1 - 3)	4	S
164	2	(4 - 4)	(3 - 4)	(3 - 4)	4	S
165	4	(1 - 3)	(2 - 3)	(1 - 4)	4	S
167	4	(0 - 4)	(2 - 4)	(0 - 4)	4	S
172	2	(0 - 1)	(1 - 2)	(2 - 3)	3	S
172	2	(0 - 1)	(1 - 2)	(2 - 3)	3	S
176	3	(4 - 4)	(0 - 4)	(0 - 1)	4	S
182	3	(1 - 3)	(1 - 2)	(2 - 3)	3	S
183	4	(4 - 4)	(3 - 4)	(2 - 4)	4	S
186	3	(2 - 4)	(2 - 2)	(0 - 2)	4	S
192	3	(3 - 4)	(1 - 4)	(3 - 4)	4	S
193	3	(1 - 3)	(0 - 3)	(1 - 4)	4	S
195	5	(0 - 0)	(0 - 1)	(1 - 1)	1	R
215	5	(1 - 1)	(2 - 4)	(1 - 4)	4	S
246	5	(0 - 3)	(0 - 3)	(0 - 4)	4	S
253	4	(0 - 4)	(0 - 2)	(2 - 4)	4	S
258	1	4	4	2	4	S
263	5	(0 - 3)	(1 - 4)	(1 - 4)	4	S
271	1	3	4	4	4	S
272	3	(1 - 3)	(2 - 3)	(2 - 3)	3	S
275	3	(2 - 2)	(1 - 1)	(1 - 3)	3	S
278	2	(0 - 3)	(1 - 2)	(2 - 3)	3	S
313	1	4	4	4	4	S
398	1	0	3	3	3	S
401	3	(1 - 2)	(2 - 3)	(2 - 3)	3	S
435	2	(0 - 3)	(1 - 4)	(3 - 4)	3	S
478	5	(1 - 4)	(2 - 4)	(2 - 4)	4	S
513	3	(4 - 4)	(4 - 4)	(2 - 4)	4	S
520	3	(0 - 2)	(0 - 4)	(1 - 4)	4	S
556	3	(2 - 3)	(1 - 2)	(2 - 3)	3	S
562	5	(2 - 4)	(2 - 4)	(2 - 4)	4	S
579	5	(0 - 3)	(3 - 4)	(0 - 1)	4	S

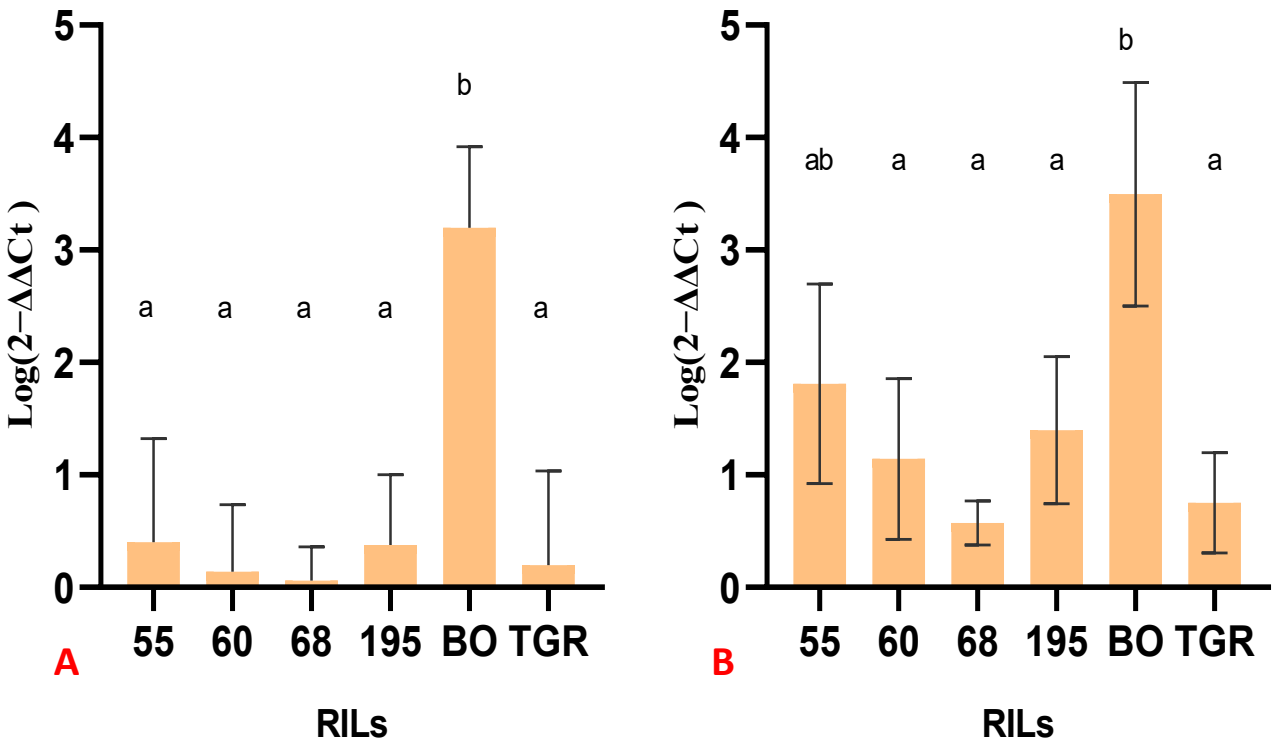

Figure 2. Mean viral accumulation log ($2^{-\Delta\Delta Ct}$) in melon recombinant inbred lines (RILs) derived from TGR-1551 (TGR) in 'Bola de Oro' (BO) genetic background, inoculated with Moroccan watermelon mosaic virus (MWMV) at 21 days post inoculation (dpi) (A) and 28 dpi (B). Vertical bars represent ± the standard error. Different letters indicate significant differences ($p < 0.05$, LSD test).

Breeding Program for the Introgression of Resistance to Viral and Fungal Diseases in Melon Landraces: Evaluation of Advanced Generations

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Introduction

Melon (*Cucumis melo* L.) production is near 30 million metric tons harvested worldwide. Production in Spain is characterized by high yields (35 t/ha in 2024), reaching approximately 594,000 tons annually, of which more than 397,000 tons are exported. This places Spain as the leading producer and exporter within the European Union. The melon types most widely grown in Spain belong to the *ibericus* group, which includes Piel de Sapo, Amarillo, Tendral, Rochet, and Blanco types. Efforts are currently underway to revive the cultivation of other melon landraces, such as snake melons. These non-sweet melons belong to the *flexuosus* group, and are given different names such as *alficoz*, *alficòs* or *cogombre*.

Biotic stresses currently represent the main limiting factor in melon (*Cucumis melo* L.) production. Among them, viral and fungal diseases are particularly important. In this context, watermelon mosaic virus (WMV) is among the most significant threats in Spain and in major melon-producing regions worldwide (Desbiez et al., 2020; López-Martín et al., 2024a; Pérez-de-Castro et al., 2019). WMV is a potyvirus transmitted by several aphid species and it induces symptoms such as mosaic, leaf deformation, and chlorosis, often accompanied by growth arrest, yield reduction and negative effect on fruit quality. Powdery mildew, mainly caused by the fungus *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff, is also widely distributed and causes significant economic losses worldwide. This fungus affects aerial plant tissues and is characterized by powdery spore masses on leaves, petioles, and stems. Infected leaves frequently wilt and plants undergo premature senescence. The globalization of agriculture and changes in cropping systems facilitate the spread of these pathogens, while current control strategies often provide limited effectiveness. Consequently, the development of resistant cultivars remains a key objective. However, despite the global importance of these pathogens, no cultivars providing a definitive solution are currently available.

In melon breeding, the genetic variability exploited is mainly intraspecific. Strong crossability barriers between wild species of the genus *Cucumis* and cultivated melon limit the use of interspecific variability. Fortunately, substantial genetic diversity exists within *C. melo* for many agronomic traits. For disease resistance, the main sources are exotic or wild accessions belonging to the *agrestis*, *momordica*, or *acidulus* groups, originating from regions such as India, the Far East, and Africa. Many of these accessions exhibit multiple disease resistances, as is the case for the accessions TGR-1551 (PI 482420) and PI 414723.

The Zimbabwean accession TGR-1551 belongs to the *acidulus* group. TGR-1551 exhibits resistance to WMV, cucurbit yellow stunting disorder virus (CYSDV), and powdery mildew. In addition, it is resistant to cucurbit aphid-borne yellow virus (CABYV) and to aphids of the species *Aphis gossypii* Glover and shows tolerance to the whitefly *Bemisia tabaci* Gennadius. Although partial resistance to WMV has been identified in other accessions, TGR-1551 displays the highest level of resistance reported so far, which is effective against several pathogen isolates. This resistance involves both reduced vector-mediated transmission and resistance to the virus itself. It is mainly controlled by a recessive gene, although additional modifying genes have also been described (Díaz-Pendón et al., 2005; Pérez-de-Castro et al., 2019). With respect to powdery mildew, TGR-1551 is resistant to races 1, 2, and 5, with resistance controlled by two genes showing dominant–recessive epistatic interaction (Yuste-Lisbona et al., 2011; López-Martín et al., 2022).

PI 414723 is an Indian accession, of the *momordica* group, showing resistance to WMV, zucchini yellow mosaic virus (ZYMV), tomato leaf curl New Delhi virus (ToLCNDV) and different races of powdery mildew (Anagnostou et al., 2000; Fazza et al., 2013; Sáez et al., 2017). Resistance to WMV is conferred by a dominant gene, *Wmv* (Gilbert et al., 1994). Resistance to ZYMV is also monogenic dominant, and both genes are linked, facilitating their introgression (Anagnostou et al., 2000). Regarding powdery mildew, *Pm-7* gene confers

resistance to race 1, and *Pm-x* confers resistance to race 2; this accession is also resistant to races 3 and 5, although the genetic control is unknown (Fazza et al., 2013).

A breeding program for the introgression of resistance into the main Spanish melon landraces was initiated using TGR-1551 and PI 414723, among other resistance sources (López-Martín, 2023). In this context, the objective of this work was the evaluation of the resistance to powdery mildew and to viral diseases in advanced materials derived from these two sources.

Materials and Methods

Advanced generations of the breeding program derived from initial crosses between different melon landraces and two resistance sources were tested. The two multi-resistant exotic melons used were the PI 414723 and the TGR-1551. The recurrent parents were accessions of the main Spanish melon types: 'Amarillo' (22AM-GO-BGV016451), 'Blanco' (29BL-BGV015753 and 26BL-BGV000444), 'Piel de Sapo' (11PS-BGV013188) and a snake melon, 'Alficoz' (*flexuosus* group, 05AL-BGV004853). The populations used corresponded to selfed generations from backcross populations ranging from BC3 to BC5, depending on the family. The families evaluated for resistance to powdery mildew included fixed lines and segregating generations. All the families tested for resistance to viruses were segregating generations. For fixed lines, 4 to 8 plants were tested, while around 30 plants were evaluated for the segregating generations. Plants were genotyped using the markers previously described as linked to the candidate regions for the resistance derived from the different sources (Pérez-de-Castro et al., 2019; López-Martín et al., 2022; López-Martín, 2023).

The assay for resistance to powdery mildew was carried out in greenhouse conditions in early spring season in Valencia. Plants were inoculated artificially by depositing a small amount of conidia at three spots on the second true leaf of each plant. The inoculum came from infected leaves collected in greenhouses in the same cultivation area, thus representing the races present in natural conditions. Symptoms were visually evaluated at 15, 30 and 45 days post-inoculation (dpi), according to the level of sporulation of the fungus, using a scale from 0 (no conidia germination) to 7 (profuse sporulation).

Evaluation for resistance to viral diseases was carried out in open field conditions, in Museros and Alcàsser (Valencia, Spain) in the spring-summer season, under natural infection conditions. Symptoms were evaluated at 30 and 45 days post-transplant (dpt), using a scale from 0 (asymptomatic plant) to 3 (plant showing severe symptoms). Samples were collected at these same dates, and viral accumulation was evaluated using qPCR or RT-qPCR, as previously described (López-Martín et al., 2024a).

Statistical analyses were performed using the Statgraphics Centurion XVII software (Statpoint Technolgies, Inc).

Results and Discussion

Resistance to powdery mildew was confirmed in the families fixed for the candidate regions from each of the sources, TGR-1551 and PI 414723. Symptoms were slight in most of the families throughout the assay. Even in the cases of families which showed higher mean symptoms in early evaluation dates, means were lower at 45 dpi, which indicated the control of the infection in the resistant genotypes (Figure 1).

In segregating generations, there was a positive significant low-to-moderate correlation (range 0.24-0.55) between the genotype for the candidate region (marker alleles) and the phenotype (symptom score) for the resistance to powdery mildew at the different evaluation dates. Correlation was higher at 45 dpi for both resistance sources. In families derived from each resistant parent, symptom scores were significantly lower in plants homozygous for the allele of the resistant parent when compared with those homozygous for the allele of the susceptible parent (Figure 2). The behavior of the heterozygotes differed depending on the resistance source. In generations derived from PI 414723, symptom scores in heterozygotes were not significantly different from homozygotes at 15 and 30 dpi, while at 45 dpi symptom scores were intermediate between the susceptible and resistant homozygotes. Resistance to different races derived from PI 414723 has been previously reported as dominant (Fazza et al., 2013). Results obtained here suggest incomplete dominance. In the case of generations derived from TGR-1551, symptom scores at 15 and 30 dpi in heterozygotes were comparable to those in homozygotes for the allele of the susceptible parent; at 45 dpi, symptom scores in heterozygotes did not significantly differ from those of the homozygotes for the allele of the resistant parent, as previously reported (López-Martín et al., 2022).

WMV was the only virus detected in the open field virus assays. ZYMV, CYSDV and ToLCNDV, were not detected in any of the samples. In previous studies by this group, WMV was the virus most prevalent (López-Martín et al., 2024a). Again, the genotypes were confirmed using the markers previously described as associated to the different candidate regions. Symptoms were evaluated at 30 and 45 dpi. Viral accumulation measured by qPCR was also determined at the same evaluation dates.

In the case of segregating generations with resistance derived from PI 414723 most of the homozygote resistant plants remained asymptomatic at 30 dpt, and those with

symptoms showed only slight mosaic (Figure 3). Similarly, symptoms in heterozygotes were mild. Mosaic and leaf deformation was more severe from 30 dpt in homozygotes susceptible. In any case, differences were not significant at this date (Figure 4). At 46 dpt mean symptom scores remained low in homozygote resistant and heterozygote plants, while in homozygotes susceptible symptoms increased, being significantly higher (Figures 3 and 4). The ability to recover from the infection in plant materials with resistance derived from PI 414723 has previously been reported, supporting these results (Anagnostou et al., 2000; Gilbert et al., 1994). Positive moderate significant correlation between the genotype and viral accumulation was identified at 30 dpt (0.52) and 45 dpt (0.56), with a gradient in the viral accumulation detected in homozygotes resistant, heterozygotes and homozygotes susceptible. These results are consistent with those previously reported for WMV PI 414723-derived resistance (Anagnostou et al., 2000); lines derived from this source exhibited dominant monogenic resistance to WMV, resulting in a reduction in symptom expression in plants carrying the allele. Subsequent studies confirmed that this accession acts by restricting the systemic spread of the virus and reducing viral accumulation (Adler-Berke et al., 2021; Anagnostou et al., 2000). Results presented here seem to indicate that viral accumulation in heterozygotes is intermediate between the parents, but sufficient to reduce the symptom severity ratings to the level of "resistant

Resistance to WMV in generations derived from TGR-1551 was also evaluated. Heterozygotes for the candidate region were grouped with the homozygotes susceptible, given the recessive nature of the resistance to WMV derived from this source. Plants homozygote for the allele of the resistant parent either remained asymptomatic or showed only slight symptoms at 30 dpt, while mean symptoms in plants classified as susceptible were significantly higher (Figures 3 and 4). At 46 dpt, symptoms increased in plants of both groups, but in the resistant plants were still significantly lower (Figure 4). This delay in symptom manifestation has relevant agronomic implications, as it may contribute to preserving fruit quality during critical stages of crop development (Díaz-Pendón et al., 2005; Pérez-de-Castro et al., 2019; López-Martín et al., 2024). There was a positive moderate significant correlation between the genotype and viral accumulation at 30 dpt (0.48), with lower viral accumulation in the homozygotes for the allele of the resistant parent. At 46 dpt, the correlation was not significant, probably due to higher variability in viral accumulation at later dates. Virus accumulation in asymptomatic plants has been described in lines with TGR-1551-derived resistance (López-Martín et al., 2024b). Thus, although the defensive response effectively limits replication of the virus in early stages, the virus accumulates at higher rates at later dates, explaining the lack of significant correlation between genotype and viral accumulation.

In conclusion, the effectiveness of the markers previously described as associated with resistances derived from TGR-1551 and PI 414723 for assisted selection in breeding programs for the introgression of these resistances has been confirmed. Additionally, the evaluated materials constitute advanced materials for the development of resistant varieties in different landrace backgrounds belonging to the most appreciated Spanish melon types. Recovery of the fruit quality after the backcrossing program has been confirmed in similar families (López-Martín, 2023). Future works will deal with the pyramidalization of resistance and evaluation of the resistance in lines combining resistance from both sources.

Acknowledgements

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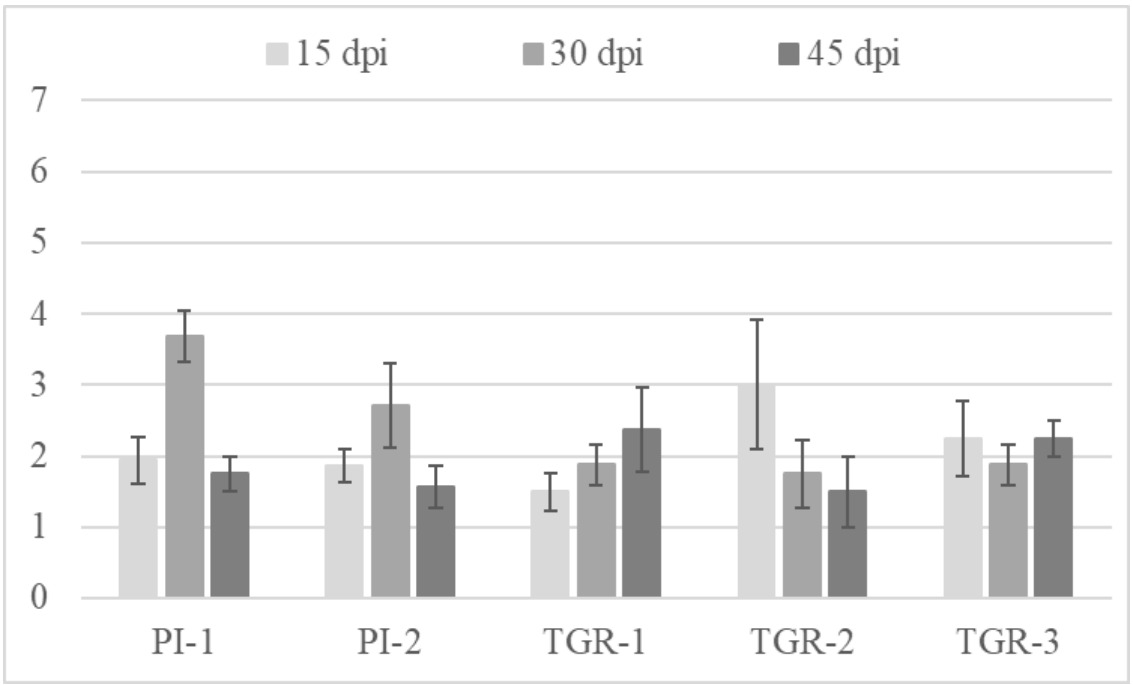

Figure 1. Mean powdery mildew (*Podosphaera xanthii*) symptom scores (scale from 1 to 7 - see text for description) after inoculation in families fixed for resistances derived from PI 414723 (PI-1 and PI-2) and TGR- 1551 (TGR-1, TGR-2, and TGR-3), at different days post-inoculation (dpi). Four to 8 plants per family were evaluated. Vertical bars represent ± the standard errors of the means.

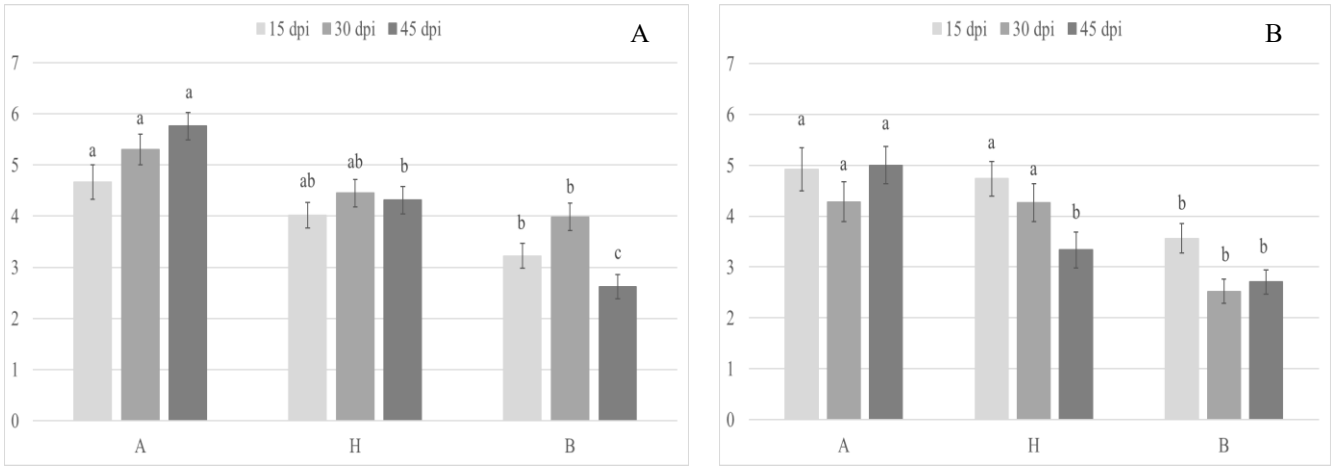

Figure 2. Mean powdery mildew (*Podosphaera xanthii*) symptom scores (scale from 1 to 7 - see text for description) after inoculation in families segregating for resistances derived from PI 414723 (A) and TGR-1551 (B) at different days post-inoculation (dpi). The genotype for the region associated with reaction to powdery mildew is indicated (A: homozygous for the susceptible parent allele; H: heterozygous; B: homozygous for the resistant parent allele). Approximately 30 plants per genotype were evaluated. Vertical bars represent ± the standard errors of the means. Different letters for the same evaluation date indicate statistically significant differences (LSD, $p<0.05$).

Figure 3. Resistant (R) and susceptible (S) plants in the field, 30 days post-transplant (dpt) (upper row) and 45 dpt (lower row), derived from PI 414723 (left panel) and TGR-1551 (right panel).

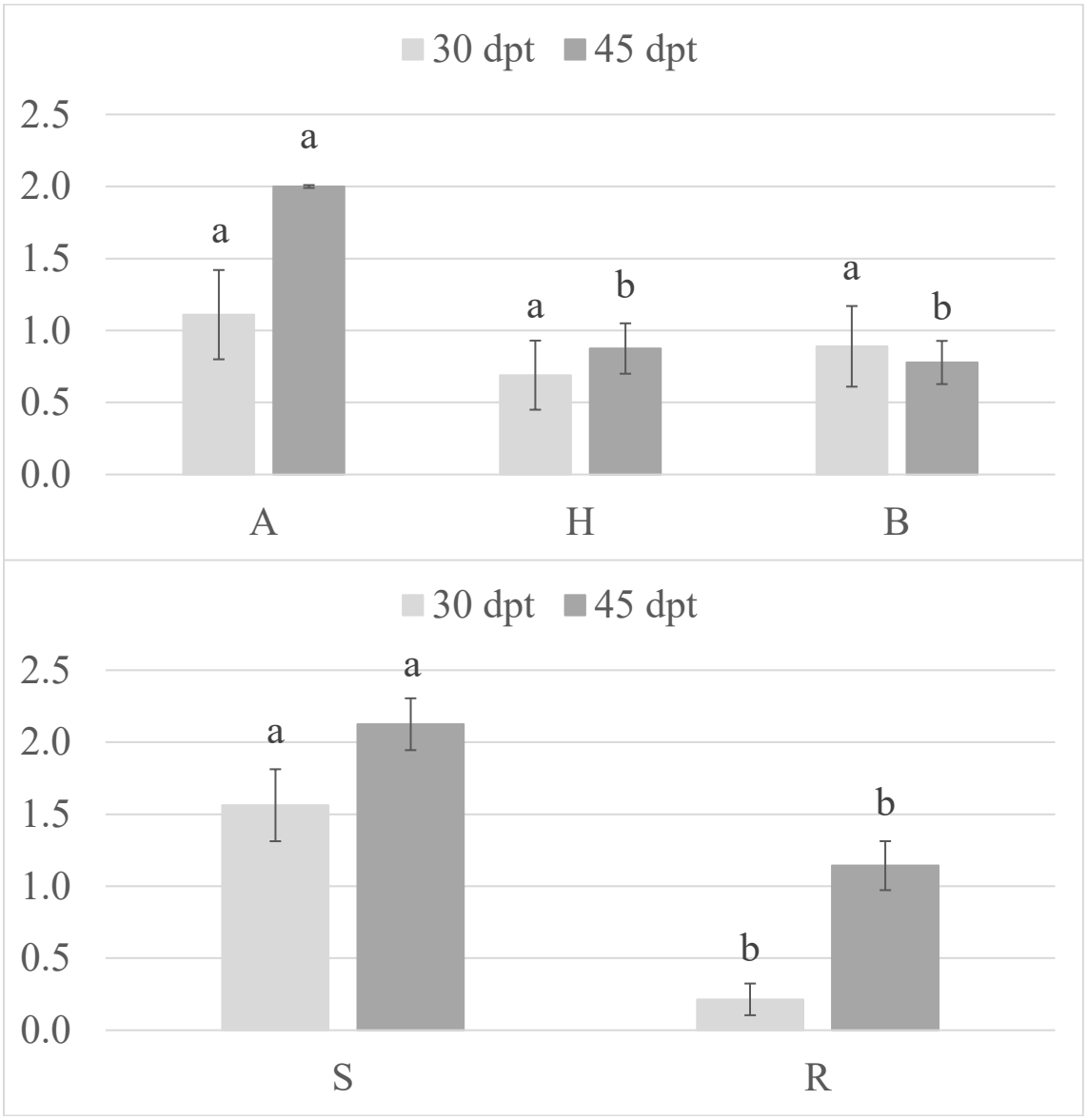

Figure 4. Average score of virus-like symptoms (scale from 0 to 3 - see text for description) after natural infection in field conditions in families segregating for resistance to viral diseases derived from PI 414723 (upper graph) and TGR-1551 (lower graph) at different days post-transplant dates (dpt). The genotype for the region associated with resistance to watermelon mosaic virus (WMV) is indicated (A: homozygous for the susceptible parent allele; H: heterozygous; B: homozygous for the resistant parent allele; S: homozygous for the susceptible parent allele or heterozygous; R: homozygous for the resistant parent allele). Approximately 30 plants per genotype were evaluated. Vertical bars represent ± the standard errors of the means. Different letters for the same evaluation date indicate statistically significant differences (LSD, $p<0.05$).

Emended Diagnosis, Description, and Type Citation for *Cucumis melo* subsp. *meloides*, the Progenitor of the African Domestication of *C. melo*

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This is a continuation of our efforts to clarify the taxonomy of *Cucumis*, which despite receiving a lot of attention from plant breeders and molecular-developmental biologists, is lacking modern taxonomic treatments. The only complete treatment of the genus, Kirkbride's (1993) monograph, is greatly outdated, covering only 32 of the 66 species currently recognized in *Cucumis* (Sebastian et al., 2010; Renner, 2024; and https://cucurbit.de/calycophysum/cucumis/). Two more species new to science have recently been discovered in Madagascar and await formal description (Andriamiharisoa et al., 2025).

Kirkbride synonymized no fewer than 522 names published under *Cucumis melo* and then subdivided the thousands of collections into two clusters, subspecies *melo* and subspecies *agrestis,* based on ovary pubescence. As shown with molecular characters, these two clusters are unnatural, with accessions instead clustering by geographic origin and not ovary pubescence or wild/cultivated origin (Endl et al., 2018). The study by Endl et al. (2018) showed that modern melon cultivars go back to two lineages that diverged ca. 2 million years ago. One is restricted to Asia (*Cucumis melo* subsp. *melo*), and the second, first described in their study, *C. melo* subsp. *meloides*, to Africa. The Asian lineage has given rise to the widely commercialized cultivar groups and their market types, while the African lineage gave rise to cultivars like the Tibish melons still grown in the Sudan region. The African sister clade to all other *C. melo* accessions has also been confirmed with more intense sequencing effort by Zhao et al. (2019), who found African wild melon accessions grouping with the tibish melons. Endl et al. (2018) also showed that *C. trigonus* Roxb., an overlooked perennial and drought-tolerant species from India, is among the closest living relatives of *C. melo*.

By oversight, Endl et al. (2018) failed to fulfill certain formal requirements when publishing the newly understood subspecies, creating a nomenclatural problem that we here remedy. This is important for two reasons. Firstly, the name *Cucumis melo* subsp. *meloides* is now being used in other studies (e.g., Imoh et al., 2025) and secondly, the repeated independent domestication of honey melons is the subject of ongoing research efforts that will benefit from an up-to-date system of taxon names, reflecting phylogenetic and geographic insights about relationships.

The name *Cucumis melo* L. subsp. *meloides* was proposed in Endl et al. (2018, Appendix S4), but that Appendix was a word document, while the Code of Botanical Nomenclature requires names to be published in printed form or in pdf file, with an ISSN.

We now provide a formal diagnosis and description of *Cucumis melo* L. subsp. *meloides* Endl & H.Schaef., subspecies nova, followed by a brief discussion of this subspecies.

Holotype: *K.G.T. Kotschy 107* (M), "Ad montem Cordofanum Arasch-Cool [Sudan] in planatie serpens; d. 2 Oct. 1839.", **Figure 1**. Isotypes: BR, K, M, MPU, TUB. Paratype: *K.G.T. Kotschy 352* (CAL, HAL, HBG, MPU, P, TUB).

Cucumis melo "melon sauvage d'Afrique" sensu Naudin (1859); *Cucumis ambigua* Fenzl ex Hook.f., Fl. Trop. Afr. [Oliver et al.] 2: 543. 1871, nom. inval.; *Cucumis cognata* Fenzl ex Hook.f., Fl. Trop. Afr. [Oliver et al.] 2: 543. 1871, nom. inval.; *Cucumis melo* L. var. *agrestis* auct., non Naudin (1859).

Diagnosis: Compared to C. *melo* subsp. *melo*, the middle lobe of the leaf is much longer, two to three times as long as the lateral lobes, while it is usually less than twice as long as the lateral ones in C. *melo* subsp. *melo*. In the nuclear ribosomal ITS region, the new subspecies differs from C. *melo* subsp. *melo* by 10 substitutions in ITS1 and ITS2 and an insertion-deletion of 4 nucleotides in ITS1.

Description (Figure 2): Monoecious annual or short-lived perennial creeper or climber with hispid shoots reaching a length of 1–3 m, becoming woody at base. Leaves ovate with cordate base, usually palmately 3(-5)-lobed with middle lobe 2–3× as long as lateral lobes, scabrid-hairy; petiole up to 10 cm long, scabrid-hairy. Male flowers solitary or in fascicles of up to 4 flowers, pedicels up to 2.5 cm long; receptacle-tube 3–6

mm long, its lobes linear-lanceolate, up to 6 mm long; petals yellow 5–10 × 3–8 mm. Female flowers solitary, pedicels up to 5 cm long, perianth similar to male flowers; ovary elliptic, densely pubescent. Fruits egg-shaped, small (3–5 × 2–3 cm, 20-40 g), smooth, light green with dark green dots or stripes, fruiting pedicel up to 5–6 cm long. The flesh is light green, very thin, moderately to strongly bitter; three placentas, no cavity between them. Seeds pale, elliptic, small, compressed, 5–8 × 2.5–4 x 1mm, in a gelatinous pulp.

Distribution: Africa, from the Cape Verde archipelago in the West to Sudan and Egypt in the East. The sub-Saharan distribution is still unclear due to confusion with escaped (feral) cultivated *C. melo* subsp. *melo* cultivars.

Habitat: Dry savanna and semidesert areas, in Cape Verde also found on disturbed ground close to settlements.

Pollination biology: In Cape Verde, flowers are visited by different fly species (Diptera) and small halictid bees (*Ceylalictus* sp., *Halictus* sp.).

Seed dispersal: In Cape Verde and probably elsewhere, the fruits are eaten by (feral) donkeys who disperse the seeds over long distances (Figure 3).

Unfortunately, there are several Kotschy collections with the same number but from different countries and representing different plant families. Thus, *Kotschy* 352 from Iran, with duplicates in at least 11 herbaria, is a lectotype of *Convolvulus acanthocladus* Boiss. & Kotschy, and another Kotschy 352 from Turkey and preserved in the Harvard herbaria is an isotype of the Caprifoliaceae *Pterocephalus pyrethrifolius* Boissier & Hohenacker.

Archaeobotanical and molecular evidence suggests that melon domestication occurred independently not only in Northeast Africa and in India (Endl et al., 2018), but perhaps a third time in the lower Yangtze region of China (Schaefer and Renner, 2021). The archaeobotanical evidence consists of the seeds, which prove the presences of melons at a particular site and time, and the increase of seed size during the domestication process. Other evidence comes from genetic diversity in particular regions, such as Sudan. Thus, a recent study of African melon germplasm (Imoh et al., 2025) sampled several accessions from Sudan, including two (CUM 287 and PI 185111) identified as belonging to *C. melo* subsp. *meloides* by sequence polymorphism at seven chloroplast regions and one nuclear region that matched the *meloides* sequences generated by Endl *et al.* (2018). Of these, CUM 287 belonged to a group with Tibish accessions. Among the 13 accessions in this group, four were small-fruited (cf. our Figure 1 of *C. melo* subsp. *meloides* fruits and seeds).

In conclusion, *Cucumis melo* subsp. *meloides,* described here, represents a taxon known at least since the times of Naudin and using its formal name helps communicate modern insights on trait evolution and domestication of *C. melo.*

Acknowledgements

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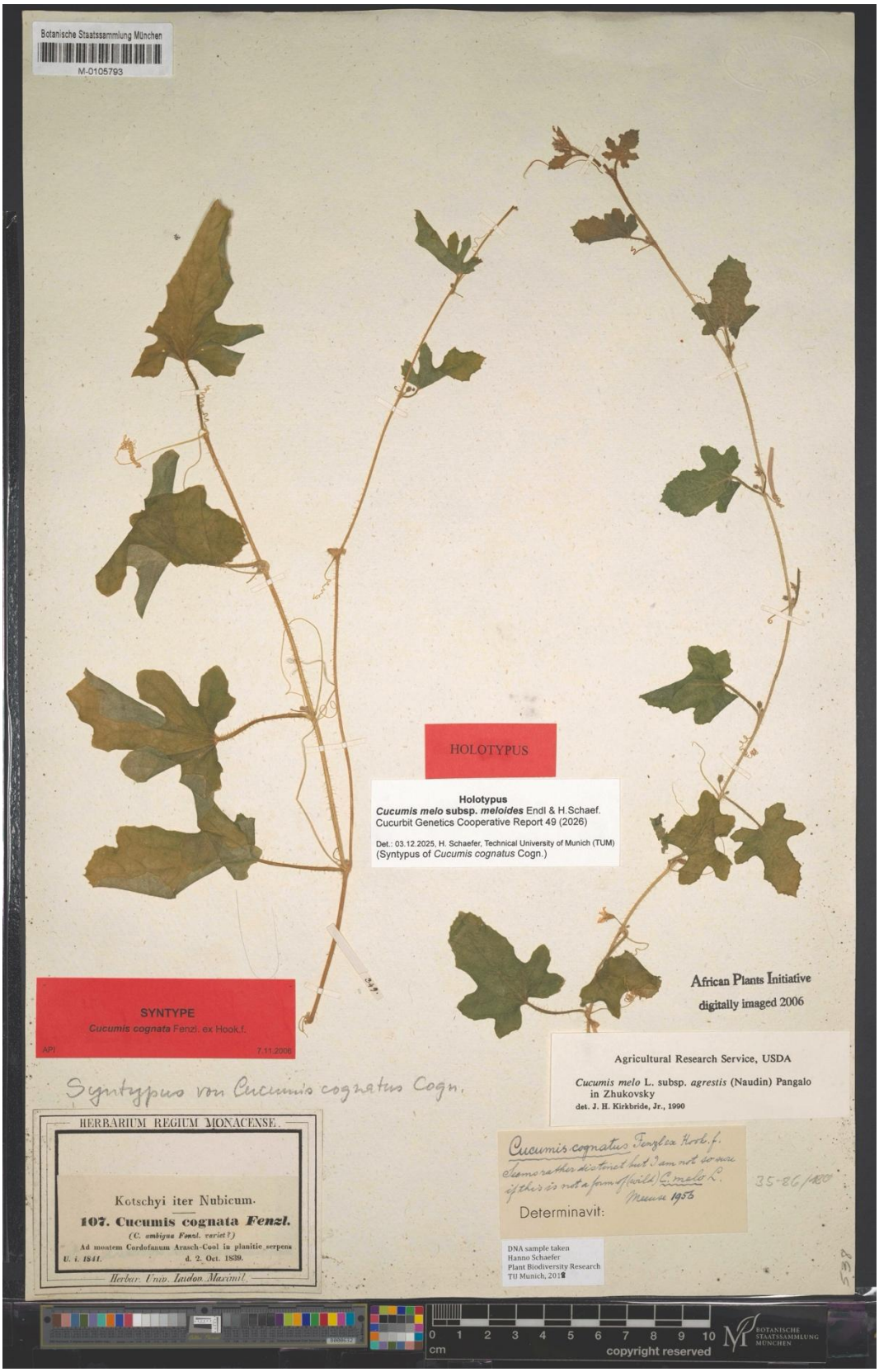

Figure 1. The type specimen of *Cucumis melo subsp. meloides* collected by G.C.T. Kotschy in Sudan, Province Sennar, and deposited in the Munich herbarium (acronym M).

Figure 2. *Cucumis melo* subsp. *meloides* shoot with male flower and fruit (left) and longitudinally cut fruit (3.5 cm long) showing the large number of small cream-white seeds (right); Sal, Cape Verde, Oct. 2015.

Figure 3. The natural habitat of *Cucumis melo* subsp. *meloides* in Boavista, Cape Verde, with feral donkeys (*Equus asinus*) that eat the fruits and disperse the seeds.

Optimizing Pickling Cucumber Multi-environment Trials for Current and Future Challenging Growing Conditions

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Introduction

Multi-environment trials (METs) are essential in a breeding program to identify high-performing and stable genotypes across different environments (Costa-Neto et al., 2021). However, trial sites are often chosen based on convenience rather than representativeness, leading to reduced relevance to actual grower environments and inefficient resource use (Prado et al., 2024). In this context, this study proposes a data-driven framework to optimize MET site selection for U.S. pickling cucumber breeding under both current and future climate scenarios.

Material and Methods

For this analysis, we integrated multi-location and multi-year historical yield data with 235 environmental covariates (ECs) derived from 16 years (2010–2025) of NASA POWER weather data (Costa-Neto et al., 2021). Then, as in Prado et al. (2024), we used stepwise regression to identify 14 key ECs, accounting for approximately 88% of the yield variation across environments and years (four were soil variables, and the rest were mainly temperature and solar radiation during early crop stages). These ECs were then used to cluster (K-means) the 11 major cucumber-producing counties (accounting for 95% of the USA pickling cucumber production) into three distinct mega-environments (clusters) depicted on the left side of Figure 1. Finally, to evaluate the resilience of these sites under future growing conditions , we followed Fradgley et al. (2024) by comparing present-day environmental clusters with projections for two future planting windows: March-June 2050-2065 and April-July 2050-2065.

Results and Discussion

The comparative analysis revealed that nine of the 11 counties are likely to remain environmentally similar in the future while San Joaquin County (CA) and Arenac County (MI) may experience unique climatic shifts (Figure 1). Despite these shifts, our analysis confirmed that the same optimized set of counties would remain representative across both present and future production scenarios. Trial optimization (one trial per cluster, the one with the highest heritability) reduced the number of sites by 57% (from 7 to 3) while maintaining 100% representativeness of production zones and maintaining high Cullis heritability of 0.98 for yield. These results should improve the efficiency of cucumber breeding programs, reduce costs, increase accuracy, and align field evaluations with current and future farmer needs.

Acknowledgments

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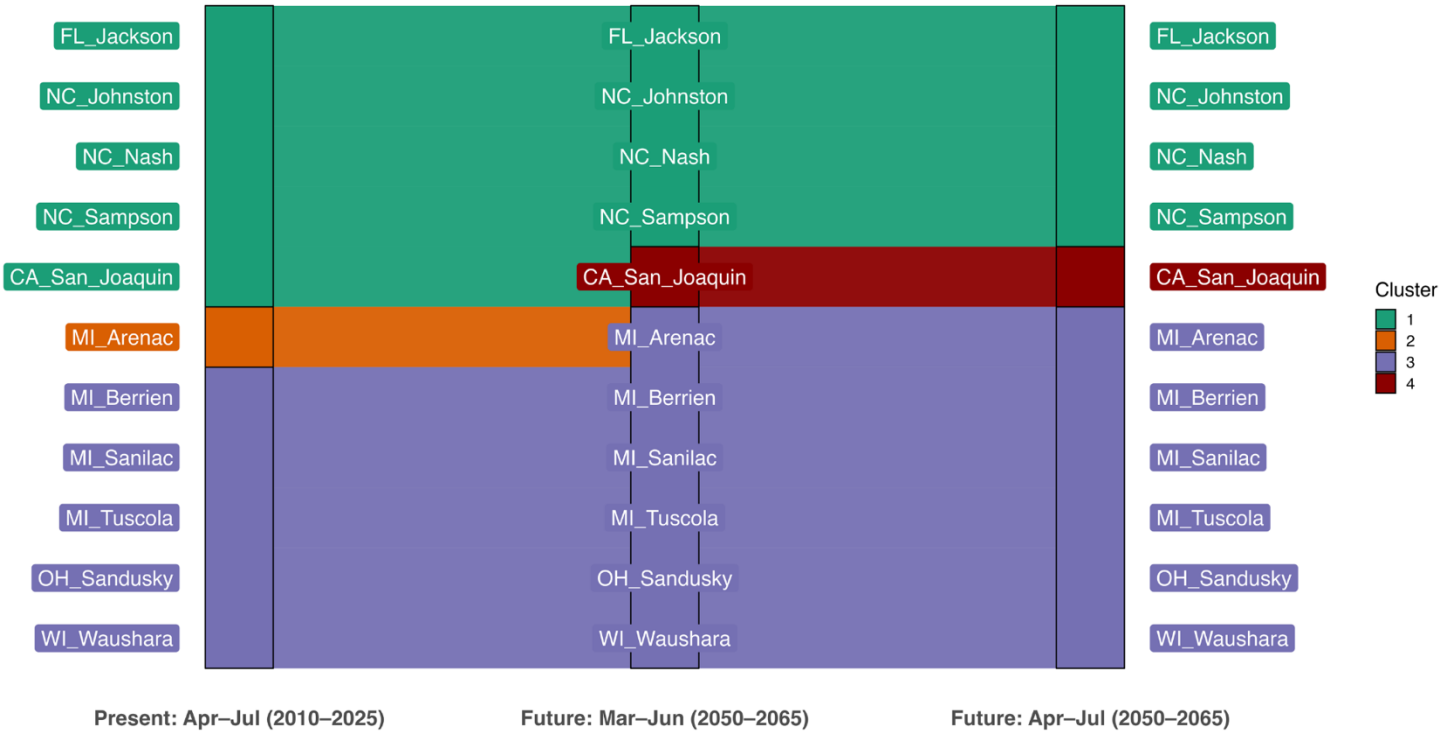

Figure 1. Predicted temporal progression of the mega-environments regarding the USA cucumber production and breeding. On the left, we have the present scenario based on average environmental data from 2010 to 2025. In the middle and on the right, respectively, we see an early planting scenario (March to June) and a normal planting scenario (April-July) predicted for 2050-2065. The flow paths show how counties shift between clusters. Most stay stable, but some move to different clusters as the environment changes. FL, Florida; NC, North Carolina; CA, California; MI, Michigan; OH, Ohio; WI, Wisconsin.

Free-living *Cucurbita pepo* in Maryland

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Cucurbita pepo L. is native to North America. It is widely cultivated on that continent for production of its relatively large, edible fruits, referred to as pumpkins if they are round and squash if they are not round. Some *C. pepo* bear small, inedible, often bitter fruits, and these are referred to as gourds. Pumpkins and gourds are also grown and marketed for decorative purposes. Most squash are used for culinary purposes when the fruits are young and tender, several days past anthesis.

Two subspecies of *Cucurbita pepo* are widely cultivated. One of them, subsp. *pepo*, is thought to have originated in central or southern Mexico and was domesticated approximately 10,000 years ago. However, no wild-growing plants of this subspecies have been documented. The other, subsp. *ovifera* (L.) D.S. Decker, is native to the woodlands of the eastern half of the United States and was domesticated approximately 5,000 years ago. Indeed, there have been numerous sightings of plants of subsp. *ovifera* growing wild in the southern and central states (Smith, 2006; Kistler et al., 2015; Khoury et al., 2020).

We document and describe herein the occurrence of a small but phenotypically highly diverse free-living population of *Cucurbita pepo* in Howard County, Maryland, that we sighted in the early autumn of 2024. This population was on an islet in the Middle Patuxent River. We did not wish to access this population physically so as not to trample or disturb it in any way, so we observed it from the opposite riverbank and shallow water. Moreover, this population was surrounded by a deep accumulation of detritus, flotsam, and heavy logs piled in water of uncertain depth, which would have made closer physical access hazardous (Figure 1). The Middle Patuxent River (https://mapcarta.com/W964155444/Map) is situated in the Maryland Fall line, where the Piedmont Plateau flattens into the Atlantic Coastal Plain, and is a tributary of the Patuxent River (Maryland Department of the Environment, 2010). The river traverses forested, agricultural, urban and suburban landscapes. Runoff has eroded the riverbanks, increased sediment levels, and introduced nutrients and pollutants to the river water and nearby soils, which are sandy clay loam or fine sandy loam. Intense, short-duration rainfall events can lead to dramatic increases in the river water levels, depositing plant debris along the river (Maryland Department of the Environment, 2010; Paul and Meyer, 2008).

We observed this free-living population of *Cucurbita pepo* plants on the north-facing, upstream end of a small islet (~0.25 ha) in the river (Figure 1). Numerous dead tree trunks and thick branches accumulated at this location following an intense rain event in December 2023. The islet and adjacent riparian habitat featured a community of eastern deciduous forest hardwood species as well as numerous invasive plant species. While some individual plants were rooted in alluvial soils of the islet, others appeared to have rooted in the fine detritus found between the logs that constituted the north end of the islet. The plants were partially intertwined with those of a native cucurbit, oneseed bur cucumber (*Sicyos angulatus* L.), and the invasive orange jewelweed (*Impatiens capensis* Meerb., Balsaminaceae), and experienced light conditions of partial shade.

The number of plants comprising the population of *Cucurbita pepo* is uncertain, but the minimum would be four, based on the observed fruit phenotypes. Fruits were observed over much of the site, with some hanging off the deadwood above the water (Figure 2). Perhaps the most prominent of the fruits in this population was a pumpkin (subsp. *pepo*) about the size of the pie pumpkins that are widely sold in autumn in the United States (Figure 3). This pumpkin, though, differed from those typically found at marketplaces by having a light orange exterior instead of the typical dark, intense orange. The light color suggests that it may have been derived from crossing. Its large stylar scar confirmed its taxonomic identity as subsp. *pepo*. Several bicolor and striped, more-or-less pyriform gourds at different stages of development were also observed (Figure 4). These may have derived from the same plant. The shape, striping, and bicolor pattern suggest that the ornamental gourd 'Bicolor Pear' (subsp. *ovifera*) was in the parentage. Another bicolor fruit appeared to have been rounder and had less amount of yellow-orange on the bicolor fruit surface, perhaps it was from a different plant (Figure 4). Two young, round, light green fruits, one with a short, dark

green peduncle and the other with a long, light green peduncle were observed (Figure 5). The starkly differing peduncles indicate that these two fruits must have been borne by different plants but perhaps one of them was derived from the same plant as the large, nearly mature light orange pumpkin (Figure 3). Another fruit was larger, warted, and dark green, but only partially visible to us through the foliage and fallen leaves, and it was in the close vicinity of the maturing light orange pumpkin (Figure 5).

The minimum number of plants comprising this population would have been four, two pumpkins and at least two gourds. None of the fruits closely resembled those of a distinct cultivar. It is difficult to account for the strange, mixed assemblage constituting this small, free-living population. Both pie pumpkins and ornamental gourds are frequently sold at markets during the autumn. Growers and consumers can simply discard these fruits when they have outlived their usefulness or save seeds from them and plant them the following spring in their gardens. Clearly, not all of the variants in this spontaneous population could have been derived from a single pumpkin plant and a single plant of 'Bicolor Pear'. The pumpkin, with its plain dull coloration and large stylar scar, could not have been derived from a first-generation cross between a typical pie pumpkin and a 'Bicolor Pear' gourd. If the variants were derived from the same field or garden or household, then a minimum of one other parental plant, besides a pumpkin and 'Bicolor Pear', would have been necessary to account for the fruit variation seen in this population. In this regard, it should be mentioned that the nearest houses are approximately 250 m away and the riverbank is part of the Middle Patuxent River Trail. So, it is possible that this population is derived from the nearby residences or the users of the trail. Or that this population is downstream from other possible seed sources, leading to two other scenarios that could perhaps better explain its constitution. One would be that the population did not have a single origin, but rather had two or more derivations and indeed, rotting logs, flotsam, and detritus were observed to have accumulated at this site. Another distinct possibility would be that this was at least a second-generation population, implying that it had been derived from at least one previous spontaneous generation reproducing itself in an uncultivated habitat. Or, more specifically, that this population has reproduced itself at this same or nearby site at least once.

We did not find *Cucurbita pepo* at this location or in the immediate vicinity in 2025. Only time will tell if the population we have described here was merely ephemeral. However, the natural distribution of *C. pepo* subsp. *ovifera* may have been considerably wider in the past, extending beyond the central and southern states into the northeastern states, but was contracted by human activities in the distant and recent past (Kistler et al., 2015; Khoury et al., 2020; Reamer, 2024). On the other hand, if, ironically, *C. pepo* does reappear here, it would be a propagule derived from modern human activities leading to the restoration of this species in what may have been a part of its former natural range.

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Figure 1. View looking southwest to the free-living population of *Cucurbita pepo* on an islet in the Middle Patuxent River in Howard County, Maryland. Plants were growing on top of a large mass of dead plant matter accumulated at the location during an intense rain event in late 2023. Photographed 14 September 2024.

Figure 2. Fruits of *Cucurbita pepo* hanging off deadwood above the water, on an islet in the Middle Patuxent River, Howard County, Maryland. Notice the young, light green pumpkin and fully grown, quadricolor (striped and bicolor) gourd. Photographed 28 September 2024.

Figure 3. Light orange pumpkin growing in the spontaneous population of *Cucurbita pepo*. Photographed 28 September 2024.

Figure 4. Bicolor striped, more-or-less pyriform gourds. Top left: large, maturing fruit. Top right: fruit approximately two weeks past anthesis. Bottom left: young fruit day after flowering. These three may have been borne by the same plant. Bottom right: bicolor, shape of fruit not clear, may or may not have been borne by the same plant as the other three fruits. Top photographs from 28 September 2024. Bottom photographs from 14 September 2024.

Figure 5. Top: young pumpkin fruits. Left, with short, dark green peduncle and right, with long, light green peduncle. The differing peduncles indicate that these two fruits could not have been derived from the same plant. Bottom: Partial view of warted, dark green gourd (left foreground) and a maturing light orange pumpkin (right background). Photographs from 28 September 2024.

Some Observations on Methods of Scoring Symptom Severity to Papaya Ringspot Virus in Tropical Pumpkin

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Introduction:

A reliable and efficient method of distinguishing between resistant vs. susceptible plants (or sometimes additional categories such as intermediate resistance) is essential when using phenotypic selection for resistance and for inheritance and other genetic studies relating to disease resistance. The development of molecular markers also depends on associating a genotype (the marker) with the correct phenotype. Developing a reliable method of phenotyping for resistance/susceptibility is often a challenging endeavor. It is therefore not surprising how often phenotyping has been a topic of discussion in meetings of breeders and pathologists. Plant breeders and pathologists have developed many ways of phenotyping disease response in plants. An ideal phenotyping method should allow the breeder to study or select for resistance as early as possible in the life cycle of the plant.

Papaya ringspot virus watermelon strain (PRSV-W) and *Zucchini yellow mosaic virus* (ZYMV) are potyviruses that affect cucurbit crops worldwide. Because PRSV and ZYMV produce similar symptoms on squash and pumpkins (*Cucurbita* spp.), researchers often use similar severity scales or scoring systems when carrying out inheritance studies or selecting for resistance to these two viruses. While the details of these phenotyping methods often vary, in my review of the literature I have noted that researchers always seem to consider the entire plant (seedling) when scoring for symptom severity.

Researchers in Brazil (Maluf et al., 1997; Menezes et al., 2015; Oliveira et al., 2003) studied resistance to PRSV in various *Cucurbita* spp. These researchers gave a single severity score to whole plants at anywhere from 10 to 42 days after inoculation (DAI) using a 0-5 scale developed by Maluf et al. (1997) where 0=no symptoms; 1=most leaves with no symptoms, few leaves with mild symptoms usually vein clearing, 2=mild symptoms (mostly vein clearing) on majority of leaves, 3=majority of leaves with mosaic, varied symptoms (vein clearing, sparse chlorotic spots, chlorosis up to 50% of leaf area), 4=almost all leaves with severe mosaic, coalescence of chlorotic spots up to 50% of leaf area, and 5=almost all leaves with severe mosaic, at least one leaf with more than 50% of area affected or severely distorted. These researchers treated symptom severity as a quantitative variable (albeit with only 6 possible values) and used a biometrical approach (generation mean analysis) to study inheritance.

Other researchers have taken a more qualitative approach to scoring resistance/susceptibility in inheritance studies. Paris et al. (1988) evaluated *C. moschata* inoculated with ZYMV. They scored plants at the 3rd to 4th true leaf stage at 19 to 20 days after planting and classified plants as resistant (could have chlorotic spots up to several mm in diameter, usually 1-2 mm) or susceptible (strongly blistered or mosaic).

Paris and Cohen (2000) investigated inheritance of ZYMV resistance in *C. pepo.* They noted that symptoms changed as new leaves developed. Some plants initially showed symptoms but then recovered. When only mild symptoms were observed after the 5th leaf plants were classified as resistant. Plants classified as susceptible sometimes had no symptoms up to the 3rd leaf, but later developed symptoms such as mosaic, blistering and stunting. They concluded that scoring should be done 20-22 days after inoculation (DAI) when *C. pepo* plants had 8 leaves.

Brown et al. (2003) studied resistance to four cucurbit viruses in *C. moschata*, including PRSV and ZYMV. For each of the four viruses, plants were classified into one of two categories: resistant or susceptible. However, they did not describe the criteria used for classification.

Pachner and Lelley (2004) used a 1 to 5 scale to evaluate symptom severity in *C. moschata* populations inoculated with ZYMV. To study inheritance of resistance they then classified plants scored with a 1 or 2 (chlorotic dots or spots; light marbling) as "resistant" and plants rated 3, 4, or 5 (blistered, distorted and/or stunted) as "susceptible."

Pachner et al. (2011) scored plants inoculated with ZYMV as susceptible when symptoms included mosaic, overall yellowing, stunting, blistering, distortions or leaf strapping, and as resistant if plants had only chlorotic dots, spots or streaks. However, they then went on to classify *C. moschata* lines into three categories: highly resistant, resistant and susceptible. In photographs in the paper, two lines classified as highly resistant showed only small spots or streaks while the two lines classified as resistant had much larger chlorotic spots or mosaic. The leaf from the line classified as susceptible ('Waltham Butternut') was distorted and blistered.

Pachner et al. (2015) studied resistance to ZYMV in *C. pepo* using a scale of 0 (symptomless) to 9 (very severely distorted, growth discontinued, plus a score of 10 for stunted plants that later recovered. Plants were evaluated at 10, 17 and 24 DAI (likely up to the 7rd or 8th leaf, although not specified in the paper). Two categories, resistant and susceptible, were formed by grouping plants that scored ≤4 (plus certain plants with a score of 10 that later recovered) as resistant and the remaining plants as susceptible. They then used a classical qualitative approach to determine the genetic basis for resistance to ZYMV

Shrestha et al. (2022) identified two KASP markers associated with resistance to PRSV-W in *Cucurbita moschata.* Plants in the F_2 population were scored for symptom severity at 7, 14, 21, and 28 DAI on a 0-3 scale (0=asymptomatic, 1=light yellowing on the leaves, 2=moderate yellowing and mottling and 3=severe leaf yellowing, mottling, stunted plant growth). Four phenotypic classes were evident at 28 DAI. Plants scored 0 or 1 were categorized as resistant while plants scored 2 or 3 were categorized as susceptible.

The studies described above all used a "whole plant" approach to phenotyping resistance, that is, scoring was based on the appearance of the entire seedling. Early on in our tropical pumpkin (*Cucurbita moschata* Duchesne) breeding program at the Agricultural Experiment Station, University of Puerto Rico, we also used that approach. For example, McPhail et al. (2012) and Sierra-Rivera (2012) scored severity of PRSV and ZYMV, respectively, on a scale of 0 to 3 on a whole plant basis, evaluating seedlings with four to five leaves 16 to 20 DAI. However, when seedlings were transplanted to the field or allowed to continue to grow in the greenhouse, plants with few or no symptoms on the 4th leaf would sometimes exhibit symptoms of susceptibility on later leaves. Inheritance studies also proved challenging. When converting a multiple point scale (0 to 3 or 0 to 4, for example) into two classes for an inheritance study, the number of plants in each class can vary widely depending on which severity scores are classified as "susceptible" vs. "resistant".

Here I describe a severity scoring method for PRSV where each seedling leaf is scored independently from leaf 1 (1st leaf above the mechanically inoculated cotyledons) to leaf 7. Data from leaves 1 to 3 is not presented since I have found that that data is not informative. Data beyond the 7th leaf is difficult to gather in the greenhouse since plants grow too large. My objective is to attempt to answer the following questions: (1) How early can symptom severity for PRSV-W be evaluated on tropical pumpkin seedlings to reliably classify a plant as resistant or susceptible? (2) How should one convert a 0 to 4 severity scale into a two-class system that would be useful for an inheritance study on resistance to PRSV?

Materials and Methods:

The PRSV-W susceptible tropical pumpkin lines 'Taína Dorada' and 'Verde Luz' were crossed to resistant 'Nigerian Local' to produce two F_2 populations segregating for resistance to PRSV-W. Taína Dorada x Nigerian Local (TDxNL) (n=112) and Verde Luz x Nigerian Local (VLxNL), (n=199) were grown in the greenhouse and cotyledons were mechanically inoculated with PRSV-W five days after seeding following the protocol described by Seda-Martínez et al. (2021). A solution with a concentration of 4 g of 20N-8.74P-16.6K (800 ppm N) in 1 L of water was used to fertilize seedlings every 5 to 6 days. Daytime greenhouse temperatures were generally around 35 °C. The study was conducted in the months of January to April in Mayagüez, Puerto Rico, when daylength varies from about 11 hours to 12.5 hours with clear sunny days about 80% of the time.

For each seedling, symptom severity was scored separately on the 4th to the 7th true leaf above the cotyledons. The 4th leaf was scored on 21-day-old seedlings (16 DAI). The 5th and 6th leaves were scored on 26- to 28-day-old seedlings. The final reading on the 7th leaf was taken on 30- to 35-day-old leaves. Symptom severity was scored on a 0 to 4 scale (Figure 1) where 0=absence of virus symptoms, 1=a few small flecks or chlorotic spots, or faint mosaic, 2=numerous small flecks or chlorotic spots or moderate mosaic, 3= a few large chlorotic lesions causing puckering especially in leaf veins or severe mosaic covering most of the leaf area, 4= vein chlorosis, blistering, puckering, and/or leaf distortion.

The analysis involved showing how severity scores on the 4th, 5th and 6th leaf compared to the severity score on the 7th leaf on the same plant. In my experience scoring PRSV, severity scores generally change very little after the 7th leaf. In the analysis some severity scores were combined. A score of 0 or 1 was combined and categorized as "resistant", a score of 2 was categorized as "intermediate" and a score of 3 or 4 was combined and categorized as "susceptible." For each resistance category (resistant, intermediate or susceptible) at each of the lower leaf positions (4th, 5th or 6th leaf), the number of plants later categorized as resistant, intermediate or susceptible based on the 7th leaf was counted, then converted to a percentage (Table 1).

Results and Discussion:

PRSV severity score in leaf 4 vs. leaf 7: Plants with a severity score of 0 or 1 (resistant) in the 4th leaf maintained that score in the 7th leaf in only 57% of the cases in the TDxNL F_2 and in an even lower percentage of cases (13%) of the cases in the VLxNL F_2 (Table 1). Likewise, a severity score of 2 (intermediate) in the 4th leaf was followed by that same score in the 7th leaf in only 39% (TDxNL) and 16% (VLxNL) of those plants. By contrast, almost all plants in both F_2 populations

(96% and 99%) with a severity score of 3 or 4 (susceptible) in the 4th leaf also had a severity score of 3 or 4 on the 7th leaf of those same plants. This demonstrates that a very young seedling with only its first four leaves can be reliably classified as susceptible when strong early symptoms (a score of 3 or 4) appear on the 4th leaf. However, the scoring of the 4th leaf cannot reliably be used to classify a plant as resistant or intermediate at this early stage. Scoring PRSV symptoms at the 4th leaf stage will be useful for the breeder who wants to eliminate susceptible plants as early as possible but would not give reliable counts of resistant vs susceptible plants for an inheritance study. Under our tropical conditions in Puerto Rico, the 4th true leaf expands about 21 days after planting, but expansion of the 4th leaf likely occurs later in more temperate climates.

PRSV severity score in leaves 5 and 6 vs. leaf 7: As with the 4th leaf, severity scores of 3 or 4 (susceptible) in the 5th and 6th leaves allowed a plant to be reliably classified as susceptible (that is, with a score of 3 or 4) in the 7th leaf in 90% to 100% of the cases (Table 1). For plants of TDxNL scoring resistant (0 or 1) in the 5th or 6th leaf, the percentage of those plants correctly predicting the score in the 7th leaf was much better than the predictive ability of the 4th leaf. Compared to the 4th leaf, the 5th and 6th leaves of VLxNL also had an improved ability to predict the severity score in the 7th leaf, but in a much lower percentage of the cases compared to the TDxNL population.

Combining severity scores for testing gene models: The testing of gene models often requires categorizing plants into two classes: susceptible and resistant (or sometimes more). Scaling systems with intermediate scores present a challenge in inheritance studies. How symptom severity scores are grouped can dramatically change the ratios of resistant:susceptible (R:S). In a subsample (n=101, Table 2) of plants from the VL x NL population the ratio of R:S was 32:69 when plants with a score of 2 were combined with those scoring 0 or 1. A very different ratio of 7:94 (R:S) resulted when plants with a score of 2 were combined with those scoring 3 or 4. Neither ratio is an obvious fit to simple gene models. Other combinations of the five phenotypic classes for symptom severity are also possible. Considering the data in Table 1, when the 4th, 5th and 6th leaves had an intermediate severity score (=2), those plants were much more likely to maintain that score or receive a higher score in the 7th leaf than they were to have a lower score suggesting that plants with an intermediate score might best be combined with those in the susceptible category.

Conclusions:

Under conditions like those in the greenhouse in Puerto Rico, the presence of strong symptoms (score of 3 or 4) on the 4th leaf of tropical pumpkin seedlings can be used to eliminate plants susceptible to PRSV. Under our conditions this occurs about 16 DAI. Plants exhibiting strong symptoms of PRSV early on very rarely produced later leaves with few or no symptoms. However, few symptoms on the 4th leaf cannot be used to select in favor of resistance at this early stage. The evaluation of the 5th or 6th leaf will give a clearer sense of a plant's resistance although even at this stage a low score will not guarantee that the plant will not show future symptoms. Additional tests such as ELISA or RT-PCR can be helpful beyond visual observation. These techniques would help to minimize the number of lines to be tested in the field. For an inheritance study of PRSV resistance, severity scores of the 5th or 6th leaf should be used. How to simplify a severity scale with more than two categories is still not completely clear, but plants scoring 2, 3 or 4 on a 0-4 scale might best be combined into the susceptible class.

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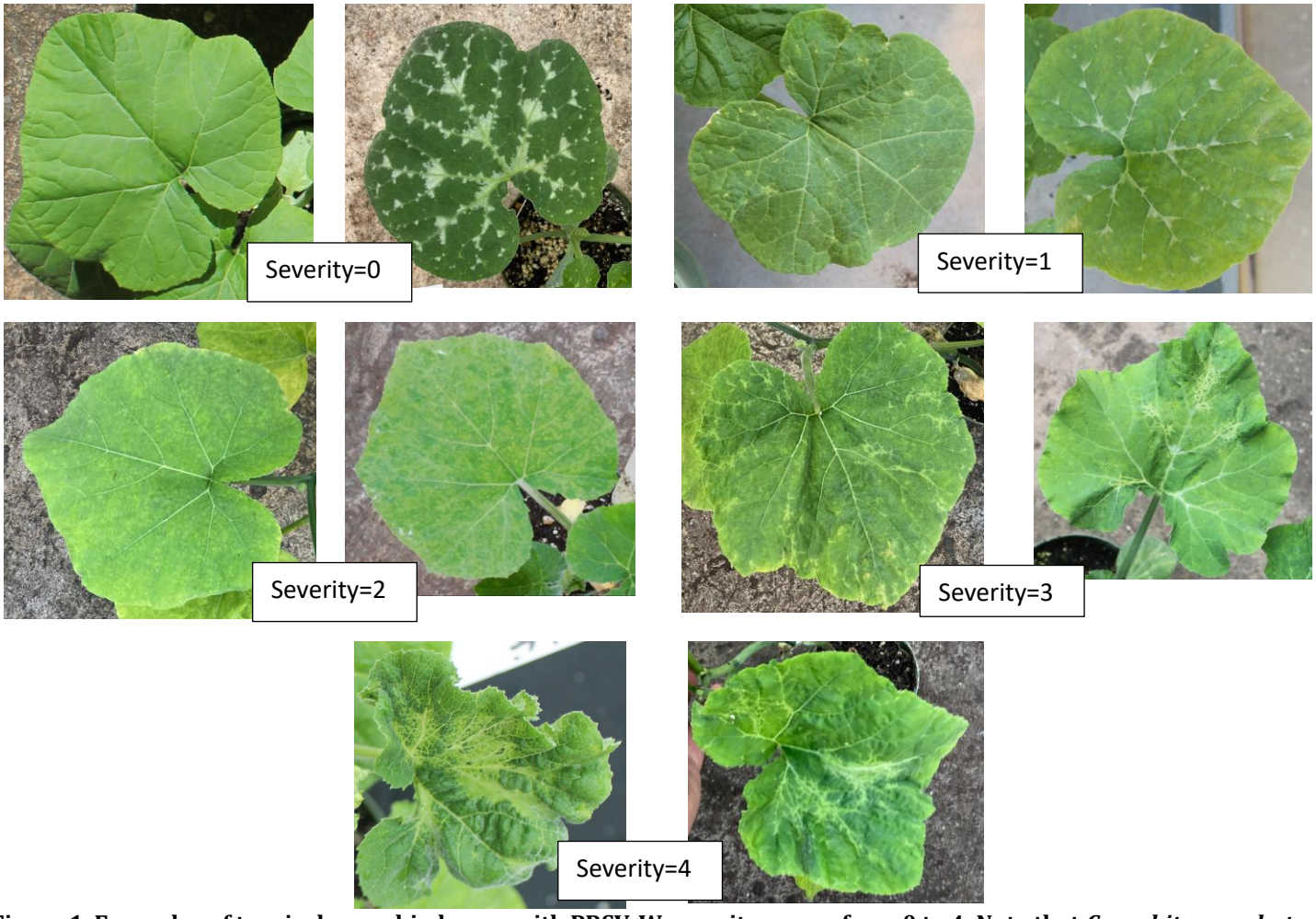

Figure 1. Examples of tropical pumpkin leaves with PRSV-W severity scores from 0 to 4. Note that *Cucurbita moschata*, including tropical pumpkin, can have completely green leaves or varying degrees of silver-grey patches in the axials of leaf veins (the *Mottle-leaf* trait). This type of leaf mottle is not related to PRSV-W vir

Table 1. The table presents the percentage of times a symptom severity score in the 4th, 5th or 6th leaf correctly predicts the severity score in the 7th leaf. Data from two F_2 populations of tropical pumpkin (*Cucurbita moschata*) seedlings inoculated with PRSV-W are presented. For each F_2 population, table values within a row correspond to the percentage of plants with a common severity score in early leaves of tropical pumpkin (the 4th, 5th or 6th true leaf above the cotyledons) and the 7th leaf. The diagonals (shaded values) correspond to the percentage of plants were the severity score in the 4th, 5th or 6th leaf matched the severity score observed in the 7th leaf.

Symptom severity score (and resistance category) of 4th, 5th and 6th leaf	Symptom severity score (and resistance category) of 7th true leaf					
	Taína Dorada x Nigerian Local F_2 (n=112)			Verde Luz x Nigerian Local F_2 (n=199)		
	Leaf 7 = 0 or 1 (Resistant)	Leaf 7 = 2 (Intermediate)	Leaf 7 = 3 or 4 (Susceptible)	Leaf 7 = 0 or 1 (Resistant)	Leaf 7 = 2 (Intermediate)	Leaf 7 = 3 or 4 (Susceptible)
Leaf 4 = 0 or 1 (Resistant)	57%	26%	17%	13%	33%	54%
Leaf 4 = 2 (Intermediate)	20%	39%	41%	1%	16%	83%
Leaf 4 = 3 or 4 (Susceptible)	0%	4%	96%	0%	1%	99%
Leaf 5 = 0 or 1 (Resistant)	85%	15%	0%	30.5%	30.5%	39%
Leaf 5 = 2 (Intermediate)	12%	64%	24%	0%	39%	61%
Leaf 5 =3 or 4 (Susceptible)	2%	7%	91%	0%	1%	99%
Leaf 6 = 0 or 1 (Resistant)	96%	4%	0%	60%	40%	0%
Leaf 6 = 2 (Intermediate)	18%	70%	12%	2%	69%	29%
Leaf 6 = 3 or 5 (Susceptible)	0%	10%	90%	0%	0%	100%

Table 2. The distribution of symptom severity in a sample (n=101) of plants from a tropical pumpkin F_2 population (Verde Luz x Nigerian Local) inoculated with PRSV.

Symptom severity score of leaf 5	Number of plants
0 or 1	7
2	25
3 or 4	69

Sponge Gourd (Loofah, *Luffa cylindrica*) in Tunisia: An Underutilized Cucurbit Crop

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The genus *Luffa* (Cucurbitaceae) includes several wild and cultivated species that are widely recognized for their agricultural, nutritional, medicinal, and industrial value. Six wild species have been reported within the genus, namely *Luffa graveolens* Roxb., *L. echinata* Roxb., *L. tuberosa* Roxb., *L. umbellata* Roem., *L. quinquefida* Seem., and *L. astorii* Svans. (Seshadri and More, 2009). Subsequently, *L. saccata* was discovered in the Northern Territory of Australia, further expanding the known diversity of the genus (Telford et al., 2011).

Only two species, sponge gourd or loofah (*Luffa cylindrica* Roem., syn. *L. aegyptiaca* Mill.) and ridge gourd (*Luffa acutangula* (L.) Roxb.), have been domesticated and are the most common and economically important representatives of the genus (Silva et al., 2012). Both species are cultivated mainly in tropical and subtropical regions and play a significant role in local food systems and rural economies (Dhillon et al., 2020). The edible pulp of sponge gourd, characterized by its white color, is consumed as a vegetable after peeling and is valued for its culinary versatility (Swetha and Muthukumar, 2016).

Sponge gourd is recognized as a nutritionally rich vegetable, containing carbohydrates, vitamin C, riboflavin, and niacin (Swetha and Muthukumar, 2016; Manikandaselvi et al., 2016). It also provides essential amino acids, minerals such as Mg, Ca, Na, K, Fe, Cu, Zn, and Mn, and several bioactive compounds including tannins, oxalates, phytin phosphorus, and phytic acid, supporting its potential contribution to human nutrition and vegetable protein intake (Dhillon et al., 2020). In addition, compounds such as charantin and specific peptides present in sponge gourd have been reported to exhibit insulin-regulatory properties, contributing to the reduction of blood and urine glucose levels, while the high dietary fiber content promotes digestive health (Swetha and Muthukumar, 2016).

Beyond its nutritional importance, *Luffa cylindrica* has long been used as a medicinal and multipurpose plant. Different plant parts, including leaves, seeds, and fruits, are traditionally used in the treatment of inflammatory disorders, diarrhea, viral infections, and for their immunomodulatory effects (Wu et al., 2020). Moreover, sponge gourd fibers have numerous non-food applications. Oboh and Aluyor (2009) documented the worldwide utilization of *Luffa* in agriculture, medicine, environmental engineering, and biotechnology, with applications ranging from personal hygiene products and household cleaning sponges to shock absorbers, soundproof materials, packaging, handicrafts, industrial filters, biodiesel production, and chemical extraction processes. The use of loofah sponges is widespread in many countries (Silva et al., 2012), including Tunisia.

In Tunisia, *Luffa cylindrica* is considered a minor and underutilized cucurbit species. It is commonly grown in home gardens as a climbing plant, primarily for the production of natural sponges that are sold in local markets, particularly in the Centre-East regions of the country. Despite its cultural, economic, and ecological importance, the genetic diversity present in Tunisian *Luffa* landraces remains largely unexplored. To date, no comprehensive studies have focused on the characterization of these local populations, although several reports have highlighted the overall genetic diversity of Tunisian cucurbit species (Chikh-Rouhou and Garcés-Claver, 2021, 2023, 2024; Chikh-Rouhou et al., 2021, 2023).

A small collection of Tunisian *Luffa* landraces is maintained at the Regional Research Centre on Horticulture and Organic Agriculture – CRRHAB (Figure 1), and efforts toward their valorization are ongoing. Documenting existing knowledge and assessing genetic diversity therefore represent essential steps toward the conservation, valorization, and sustainable utilization of Tunisian *Luffa cylindrica* germplasm at both regional and national levels.

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Figure 1. Fruits and seeds of a representative Tunisian *Luffa cylindrica* accession maintained at CRRHAB.

Rhizosphere Microbiome Diversity and Breeding Relevance in Cucurbit Crops from Tunisia

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The Cucurbitaceae family, including melons, watermelons, and pumpkins, represents a major group of dietary staples worldwide. Understanding their microbiomes has far-reaching implications for crop sustainability and quality. Root-associated microbiomes play a fundamental role in plant growth, nutrient acquisition, disease resistance, and overall crop performance (Sandrini et al. 2022). However, cucurbit breeding programs have traditionally focused on plant genetics alone, largely overlooking the contribution of rhizosphere microbiota to agronomic performance. Recent studies demonstrate that plant genotypes actively shape their associated microbiome communities and that these communities, in turn, influence yield and fruit quality (Sandrini et al. 2022; Aydi-Ben-Abdallah et al. 2021). This growing understanding opens new perspectives for holobiont-based breeding strategies that integrate both plant and microbiome traits to enhance productivity while supporting sustainable agriculture (Kusstatscher et al. 2021).

To explore the potential of microbiome-assisted selection in cucurbit breeding, three complementary studies conducted at CRRHAB-Tunisia characterized rhizosphere microbiome diversity in melon and snake melon (*Cucumis melo* L. and *C. melo* var. *flexuosus*), watermelon (*Citrullus lanatus*), and pumpkins (*Cucurbita* spp.). In total, 64 genotypes were assessed for key rhizosphere microbiome components, including total bacteria, actinomycetes, total fungi, *Pseudomonas* spp., *Trichoderma* spp., and *Aspergillus* spp., and their relationships with yield and fruit quality traits (Aydi-Ben-Abdallah et al. 2021; 2023; 2024).

In melon and snake melon, Aydi-Ben-Abdallah et al. (2021) showed that genotypes selectively recruit distinct rhizosphere microbiome communities. The microbiomes of most genotypes exhibited significantly higher bacterial and actinomycete populations compared with the control (a bulk soil sample taken prior to planting and not directly attached to plant roots) while total fungal populations remained similar. Twelve genotypes were characterized by enrichment of *Pseudomonas* spp., a group known for plant growth promotion and disease suppression. Superior agronomic performance, including increased plant height and fruit weight, was observed in specific melon genotypes (H0, H7, H11, H17, H52), while snake melon genotypes (H47 to H50) displayed the highest yields. Overall, top-performing genotypes tended to harbor higher bacterial populations (especially *Pseudomonas* spp.), abundant actinomycetes**,** and elevated populations of *Trichoderma* spp., suggesting a strong association between microbiome composition and crop productivity.

Watermelon accessions also displayed genotype-dependent rhizosphere microbiome profiles (Aydi-Ben-Abdallah et al. 2023). Significant variation was detected in actinomycetes and *Trichoderma* spp. populations, and hierarchical analyses grouped accessions according to their microbiome composition. Notably, rhizosphere soil properties, particularly pH and electrical conductivity, varied among accessions, suggesting that plant genotype influences both soil characteristics and microbiome structure. The top-performing accessions (P1 and P8), which produced the highest fruit weight and sweetest taste, were characterized by the highest actinomycetes and *Aspergillus* spp. populations in their rhizosphere. These associations suggest a potential role of actinomycetes and *Aspergillus* spp. in enhancing watermelon yield and fruit quality. These findings support the use of associated microbiota as complementary selection criteria in watermelon breeding programs.

Similarly, pumpkin accessions across three species (*C. pepo*, *C. maxima*, and *C. moschata*) revealed strong genotype- and species-level effects on rhizosphere microbiome composition (Aydi-Ben-Abdallah et al. 2024). The top-performing *C. maxima* accessions (C5, C23, C14.2, C6.2) combined superior fruit weight and yield with the highest populations of bacteria, actinomycetes, *Trichoderma* spp., and *Aspergillus* spp., alongside the lowest total fungal populations in their rhizosphere. Positive correlations were observed between bacterial populations and fruit weight, and between *Trichoderma* spp. and yield, while total fungal populations were negatively associated with both traits. These patterns indicate that productive pumpkin genotypes maintain distinctive microbiome communities characterized by the highest number of beneficial microorganisms.

Overall, these studies demonstrate that cucurbit genotypes consistently shape their rhizosphere microbiome communities and that microbiome composition is closely associated with yield and fruit quality (Table 1). In particular, the enrichment of beneficial microbial groups—especially

actinomycetes, *Pseudomonas* spp., and *Trichoderma* spp.—in top-performing genotypes (Table 2) suggests a potential functional role of microbiome recruitment in enhancing nutrient availability, growth promotion, and disease suppression.

From a plant breeding perspective, the results highlight the potential value of integrating rhizosphere microbiome profiling into breeding pipelines as an early-screening tool to identify high-performing genotypes alongside traditional phenotypic assessments. Genotypes associated with the recruiting of beneficial microbial communities could be prioritized as parents in crossing schemes, supporting the development of holobiont-based selection strategies that consider the plant and its associated microbiome as an integrated biological system.

Beyond breeding applications, such microbiome-associated traits may also contribute to more sustainable production systems by reducing dependence on external inputs. Genotypes enriched in beneficial microbiota could potentially require lower applications of fertilizers and fungicides, an advantage that is particularly relevant for cucurbit cultivation in semi-arid environments such as Tunisia, where soil fertility constraints and input-use efficiency are critical challenges.

However, the microbiome–performance relationships reported across these studies remain correlative. Although consistent across species and traits, the underlying causal mechanisms have yet to be confirmed through controlled inoculation experiments and mechanistic studies. Future research should therefore validate these associations under diverse environmental conditions and further elucidate the functional roles of the key microbial taxa underlying the observed plant benefits.

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Table 1. Summary of rhizosphere microbiome composition in cucurbit crops (melon, watermelon, and pumpkin) grown at the Sahline Experimental Station of CRRHAB, Tunisia. (from Aydi-Ben-Abdallah et al. 2021; 2023; 2024)

Crop	BACTERIAL POPULATIONS			FUNGAL POPULATIONS				
	Total Bacteria	**Actinomycetes**	***Pseudomonas* spp.**	**Total Fungi**	***Trichoderma* spp.**	***Aspergillus* spp.**	***Penicillium* spp.**	***Fusarium* spp.**
Melon/Snake melon (*C. melo*)	**High** in most; **Highest** in top-yielding accessions	**High** in most; **Abundant** in superior accessions	**Elevated** in top accessions	Comparable to control	**Elevated** in top snake-melon accessions	**Elevated** in top-yielding snake melon accessions	**Present** in some top accessions	**Absent** in most; **Absent/low** in best accessions
Watermelon (*C. lanatus*)	Variable among accessions	**Highest** in top-yielding accessions	-	**Elevated** in top accessions	**Elevated** in some accessions	**Highest** in top accessions	-	Variable, no significant difference
Pumpkin (*Cucurbita* spp.)	**Highest** in top accessions; Positive correlation with fruit weight	**Highest** in top-accessions	-	**Lowest** in top accessions; negative correlation with fruit weight and yield	**Highest** in top accessions; positive correlation with yield	**Highest** in top accessions	-	-

Table 2. The rhizosphere microbial community structure in cucurbit accessions grown at Sahline Experimental Station of CRRHAB, Tunisia. The table presents culturable populations present in the rhizosphere of cucurbit accessions (mean across samples) compared to populations present in bulk (control) soil samples taken prior to planting. Accessions were classified as "top-performing" based on fruit weight, yield, and microbiome profile. Watermelon values are mean log CFU g^{-1} fresh soil. Pumpkins values are mean CFU g^{-1} fresh soil. *Pseudomonas* spp. and *Penicillium* spp. were assessed in melon only.

Accession	Bacterial Populations			Fungal Populations					Yield / Fruit quality
	Total Bacteria	Actinomycetes	*Pseudomonas* spp.	Total Fungi	*Trichoderma* spp.	*Aspergillus* spp.	*Penicillium* spp.	*Fusarium* spp.	
Melon & Snake Melon (*Cucumis melo*) - Aydi-Ben-Abdallah et al. 2021 [n=45 melon + 7 snake melon genotypes]									
Bulk soil (control)	1.70×10^8 CFU g^{-1}	0.66×10^4 CFU g^{-1}	0	21×10^4 CFU g^{-1}	0	11×10^4 CFU g^{-1}	0	2.3×10^4 CFU g^{-1}	—
Top melon genotypes: H0, H7, H11, H17, H52 Top snake melon genotypes: H47–H50	59–68% higher than bulk soil	28.5–92.6% higher than bulk soil	~3× bulk soil (enriched in 12 genotypes)	Similar to bulk soil	3–23× higher than bulk soil	36.8–53.8% higher than bulk soil (H49, H50)	Present in selected genotypes	Absent in most	Highest fruit weight, plant height & yield per plant positive correlation fruit weight with *Pseudomonas* & actinomycetes
Other genotypes (lower performers)	Similar to bulk soil	Moderate increase vs. bulk soil	Low or absent	Similar to bulk soil	Low or absent	Low	Variable	Variable	Lower fruit weight & yield
Watermelon (*Citrullus lanatus*) - Aydi-Ben-Abdallah et al. 2023 [n=7 accessions evaluated]									
Bulk soil (control)	6.15 (log CFU g^{-1})	3.26 (log CFU g^{-1})	—	3.53 (log CFU g^{-1})	0.76 (log CFU g^{-1})	1.60 (log CFU g^{-1})	—	0.89 (log CFU g^{-1})	—
Top watermelon accessions: P1 & P8	5.49–5.81 (log CFU g^{-1}) Variable vs. bulk soil	3.66–3.71 (log CFU g^{-1}) Highest among top accessions	—	3.52–3.99 (log CFU g^{-1}) Low to intermediate	0.40–1.53 (log CFU g^{-1})	2.05–3.09 (log CFU g^{-1}) Highest among top accessions	—	0.60–0.65 (log CFU g^{-1})	9.5–10 kg fruit weight / 9.46–9.8 °Brix (sweetest) positive correlation with actinomycetes & *Aspergillus* spp.
Remaining accessions (P2, P6, P14, P15, P16)	5.47–5.89 (log CFU g^{-1}) Variable	3.37–3.60 (log CFU g^{-1}) Lower than top accessions	—	3.50–4.02 (log CFU g^{-1}) Variable	0.67–2.94 (log CFU g^{-1}) Variable	1.78–2.35 (log CFU g^{-1}) Lower than top accessions	—	0.23–1.04 (log CFU g^{-1}) Variable	2.35–5.17 kg fruit weight; 2.56–8.5 °Brix

Accession	Bacterial Populations			Fungal Populations					Yield / Fruit quality
	Total Bacteria	Actinomycetes	*Pseudomonas* spp.	Total Fungi	*Trichoderma* spp.	*Aspergillus* spp.	*Penicillium* spp.	*Fusarium* spp.	
Pumpkin (*Cucurbita* spp.) - Aydi-Ben-Abdallah et al. 2024 [n=12 accessions, 3 species]									
Bulk soil (control)	2.99 ×10^7 CFU g^{-1}	0.95 ×10^4 CFU g^{-1}	—	1.62 ×10^4 CFU g^{-1}	1.12 ×10^3 CFU g^{-1}	0.12 ×10^3 CFU g^{-1}	—	0.18 ×10^3 CFU g^{-1}	—
Top *C. maxima* accessions: C5, C23, C14.2, C6.2	1.62–3.35 ×10^8 CFU g^{-1} Highest among top accessions; positive correlation with fruit weight	1.13–1.68 ×10^5 CFU g^{-1} Highest among top accessions	—	0.93–1.32 ×10^5 CFU g^{-1} Lowest total fungi	1.75–2.08 ×10^4 CFU g^{-1} Highest among top accessions positive correlation with yield	2.75–3.75 ×10^4 CFU g^{-1} Highest among top accessions	—	0.07–1.50 ×10^4 CFU g^{-1} No significant difference	4.18–8.48 kg fruit weight and 5.31–6.21 kg $plant^{-1}$ yield negative correlation total fungi with fruit weight & yield
Remaining accessions (*C. pepo* & *C. moschata* + lower *C. maxima*)	1.48–2.66 ×10^8 CFU g^{-1} Lower or comparable	0.74–1.08 ×10^5 CFU g^{-1} Lower than top accessions	—	1.20–1.69 ×10^5 CFU g^{-1} Higher total fungi	0.33–1.58 ×10^4 CFU g^{-1} Lower Trichoderma spp.	1.92–3.33 ×10^4 CFU g^{-1} Variable	—	0.07–1.66 ×10^4 CFU g^{-1} Variable	2.53–3.9 kg fruit weight and 0.91–3.96 kg $plant^{-1}$ yield

Fruits that Develop Underground in the Genus *Cucumis* and other Cucurbitaceae

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In most Cucurbitaceae, the pedicels of pollinated female flowers elongate and the fruits then mature dangling in the air or laying on the ground. In at least four cucurbit species, however –the Mexican *Echinopepon arachoideus* (Dieterle, 1974), the Australian *Cucumis umbellatus* (Telford et al., 2011), the South African *Cucumis humifructus* (Meeuse, 1962; Hollmann et al., 1995; Johnson et al., 2025), and *Kedrostis psammophila* (Bruyns, 1993)– the pedicels of pollinated flowers grow into the ground and the fruits then develop entirely below ground. Such underground fruit development, geocarpy, evolved in an estimated 30 species of flowering plants, with new cases still being discovered (Kuhnhäuser et **al. 2023).** Here we report the first successful *ex situ* cultivation of any geocarpic Cucurbitaceae.

The first author has twice grown *Cucumis humifructus* outdoors in his garden in western France (1994 – 1995 one generation only; 2025 – 2026: successful seed production), using seeds received from Pretoria and Namibia. Seeds were planted in mid-April in 50-cm-wide mounds of soil as commonly used for squash or melon plantings. The soil used was a sandy clay with a little compost added. A drip irrigation system was installed but used for only 5 min/week. Later in the summer, plants were watered during exceptionally hot days. The mounds were covered with black plastic mulch to reduce temperature fluctuations. Seeds germinated in about a week, at which point the mulch was removed. The plants appeared resistant to spider mite attacks but were susceptible to white powdery mildew in early autumn.

Cucumis humifructus is a monoecious perennial with hispid shoots that reach 2.5 m in length when flowering and over 4 m when fruiting (Fig. 1). Male flowers are borne solitary or in fascicles, female flowers are borne solitary. Cross pollination in France occurred in June and July and involved native species of halictid bees as well as bumble bees. The pedicels of fertilized flowers undergo negatively phototropic and positively gravitropic growth to push the fertilized ovary into the loose ground. Further pedicel elongation by at least 30 cm (Fig. 1), and fruit maturation seems to be triggered by the darkness and humidity conditions underground and perhaps mechanical stimuli. Fruits do not develop above ground.

Fully mature fruits were soft and contained ca. 30 seeds, each surrounded by a gelatinous bag. They were dug up in late January 2026.

Cucumis humifructus is native in semi-deserts of southern sub-Saharan Africa, where it has been documented in Angola, the Central African Republic, Ethiopia, Kenya, Senegal, Namibia, South Africa, and Zimbabwe (www.gbif.org). Very few of those populations have been confirmed more recently (https://www.inaturalist.org/observations?taxon_id=15256 71) but the species is easily overlooked and probably still widespread throughout its former range, which likely overlaps that of its specialized seed disperser, the aardvark (*Orycteropus afer*). The aardvark is a nocturnal ant- and termite-specialist that digs up the fruits of *C. humifructus*, thereby swallowing and spreading the plant's seeds through its feces (Hollmann et al., 1995; Johnson et al., 2025: video!).

The adaptive value of geocarpy is thought to lie in the protection of the developing seeds from herbivores, combined with the benefit of the seeds being dispersed inside piles of dung of relatively large mammals.

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Figure 1. Left: Mature fruit of *Cucumis humifructus* in the hand of Jean-Luc Gatard. Western France, January 2026. Fruit diameter: around 7 cm. Right: **Fertile seeds of *Cucumis humifructus.* Seed size: 2 x 0.8 cm.**