Neus Ortega Albero

Development of experimental populations in Solanaceae crops for interesting traits, response to climate change, and associated genetic factors

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PhD Thesis

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València, September 2025

Advisors:

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UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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Institute de Conservación y Mejora de la Agrodiversidad Valenciana

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A dissertation for the degree of Doctor of Philosophy within the biotechnology program of the Universitat Politècnica de València

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A tots aquells que han format part d'aquest viatge.

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"Qui perd els orígens, perd la identitat". *Raimon*

Mama, Papa, gràcies per tot. Sense vosaltres, res seria possible.

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Resum

La família de les solanàcies inclou espècies hortícoles de gran importància econòmica a nivell mundial, entre ells l'albergínia (*Solanum melongena*) i el pimentó (*Capsicum* spp.). El cultiu d'aquestes espècies s'enfronta a nombrosos estressos abiòtics accentuats pel canvi climàtic, la qual cosa exigeix el desenvolupament de varietats més tolerants per a garantir una producció sostenible i estable en el temps. L'èxit dels programes de millora depèn de la disponibilitat de germoplasma adequat, el coneixement de mecanismes de tolerància i la identificació de gens que controlen els caràcters agronòmics d'interès. No obstant això, la complexitat d'aquests caràcters, generalment controlats per múltiples *loci*, requereix poblacions de mapatge adequades i l'aplicació d'eines biotecnològiques avançades.

En aquest context, l'albergínia té un gran potencial de millora degut a la gran variabilitat genètica que presenten les seves espècies silvestres relacionades (CWRs). L'estudi d'aquestes espècies amb aproximacions multiòmiques (fenotípics, fisiològics i transcriptòmics) permetria ampliar el coneixement base sobre caràcters com la tolerància a estressos abiòtics. Per això, en el primer capítol de la present tesi doctoral, es va dur a terme un estudi fisiològic de la resposta a la salinitat de l'espècie conreada *S. melongena*, les espècies silvestres relacionades *S. insanum* i *S. dasyphyllum*, així com de l'híbrid interespecífic *S. Insanum* × *S. melongena*, identificant fonts de variació genètica útils per a la seva incorporació en programes de millora. Addicionalment, es va realitzar un estudi transcriptòmic comparatiu en *S. melongena, S. insanum*, i el seu híbrid interespecífic, confirmant els resultats fisiològics obtinguts i diferenciant els mecanismes de resposta específics de cada genotip. El segon capítol es va centrar en la qualitat de fruit de varietats conreades d'albergínies sota condicions de salinitat moderada però prolongada. Els resultats van mostrar que la qualitat interna del fruit no es veu compromesa significativament, confirmant la seva capacitat productiva en ambients salins.

Quant al pimentó, encara que és un dels cultius més importants dins de la família de les solanàcies, existeix una menor disponibilitat d'estudis sobre el seu germoplasma i de poblacions experimentals per a la millora. Per això, el tercer capítol d'aquesta tesi doctoral es va enfocar en la caracterització fenotípica i genotípica d'una col·lecció de varietats tradicionals de pimentó (*C. annuum*) de les Illes Balears, amb l'objectiu d'avaluar el seu potencial com a materials d'inici per a programes de millora. Així mateix, en aquest capítol es descriu la construcció de la primera població MAGIC (*Multi-parent Advanced Generation InterCross*) en pimentó que combina vuit varietats de dues espècies (*C. annuum* i *C. chinense*) amb l'objectiu d'augmentar la base genètica del cultiu i facilitar la identificació de *loci* associats a caràcters agronòmics. Després dels creuaments inicials i quatre cicles d'autofecundació, es van avaluar la tercera i quarta generació d'autofecundació mitjançant fenotipat i genotipat. A més, es va avaluar l'estructura poblacional i es van relacionar caràcters senzills amb gens candidats mitjançant una anàlisi d'associació gènic (*Genome-wide association analysis*).

En conjunt, els resultats obtinguts en aquesta tesi posen de manifest la importància d'ampliar i caracteritzar el germoplasma disponible per a la millora de cultius de solanàcies. Tant les espècies silvestres com les varietats tradicionals constitueixen recursos genètics valuosos per al desenvolupament de cultivars més resilients i sostenibles. A més, la població MAGIC desenvolupada representa una nova eina prometedora per a la comunitat científica, amb un enorme potencial intrínsec per la seva variabilitat genètica com per la seva utilitat en la identificació de gens candidats associats a caràcters agronòmics.

Resumen

La familia de las solanáceas incluye especies hortícolas de gran importancia económica a nivel mundial, entre ellos la berenjena (*Solanum melongena*) y el pimiento (*Capsicum* spp.). El cultivo de estas especies se enfrenta a numerosos estreses abióticos acentuados por el cambio climático, lo que exige el desarrollo de variedades más tolerantes para garantizar una producción sostenible y estable en el tiempo. El éxito de los programas de mejora depende de la disponibilidad de germoplasma adecuado, el conocimiento de los mecanismos de tolerancia y la identificación de genes que controlan los caracteres agronómicos de interés. Sin embargo, la complejidad de estos caracteres, generalmente controlados por múltiples *loci*, requiere poblaciones de mapeo adecuadas y la aplicación de herramientas biotecnológicas avanzadas.

En este contexto, la berenjena tiene un gran potencial de mejora dada la gran variabilidad genética que presentan sus especies silvestres relacionadas (CWRs). El estudio de estas especies con aproximaciones multiómicas (fenotípicos, fisiológicos y transcriptómicos) permitiría ampliar el conocimiento base sobre caracteres como la tolerancia a estreses abióticos. Por ello, en el primer capítulo de la presente tesis doctoral, se llevó a cabo un estudio fisiológico de la respuesta a la salinidad de la especie cultivada *S. melongena,* las especies silvestres relacionadas *S. insanum* y *S. dasyphyllum*, así como del híbrido interespecífico *S. insanum* × *S. melongena,* identificando fuentes de variación genética útiles para su incorporación en programas de mejora. Adicionalmente, se realizó un estudio transcriptómico comparativo en *S. melongena*, *S. insanum*, y su híbrido interespecífico, confirmando los resultados fisiológicos obtenidos y diferenciando los mecanismos de respuesta específicos de cada genotipo. El segundo capítulo se centró en la calidad de fruto de variedades cultivadas de berenjenas bajo condiciones de salinidad moderada pero prolongada. Los resultados mostraron que la calidad interna del fruto no se ve comprometida significativamente, confirmando su capacidad productiva en ambientes salinos.

En cuanto al pimiento, aunque es uno de los cultivos más importantes dentro de la familia de las solanáceas, existe una menos disponibilidad de estudios sobre su germoplasma y de poblaciones experimentales para la mejora. Por ello, el tercer capítulo de esta tesis doctoral se enfocó en la caracterización fenotípica y genotípica de una colección de variedades tradicionales de pimientos (*C. annuum*) de la Islas Baleares, con el objetivo de evaluar su potencial como materiales de inicio para programas de mejora. Asimismo, en este capítulo se describe la construcción de la primera población MAGIC (*Multi-parent Advanced Generation InterCross*) en pimiento que combina ocho variedades de dos especies (*C. annuum* y *C. chinense*) con el objetivo de aumentar la base genética del cultivo y facilitar la identificación de loci asociados a caracteres agronómicos. Tras los cruces iniciales y cuatro ciclos de autofecundación, se evaluaron la tercera y cuarta generación de autofecundación mediante fenotipado y genotipado. Además, se evaluó la estructura poblacional y se relacionaron caracteres sencillos con genes candidatos mediante un análisis de asociación génico (*Genome-wide association analysis*).

En conjunto, los resultados obtenidos en esta tesis ponen de manifiesto la importancia de ampliar y caracterizar el germoplasma disponible para la mejora de cultivos de solanáceas. Tanto las especies silvestres como las variedades tradicionales constituyen recursos genéticos valiosos para el desarrollo de cultivares más resilientes y sostenibles. Además, la población MAGIC desarrollada representa una nueva herramienta prometedora para la comunidad científica, con un enorme potencial intrínseco por su variabilidad genética como por su utilidad en la identificación de genes candidatos asociados a caracteres agronómicos.

Abstract

The Solanaceae family includes horticultural species of great economic importance worldwide, including eggplant (*Solanum melongena*) and peppers (*Capsicum* spp.). The culture of these species is facing numerous abiotic stresses accentuated by the climate change, requiring the development of more tolerant varieties to ensure sustainable and stable production over time. The success of breeding programmes depends on the availability of available germplasm, the knowledge of tolerance mechanisms and the identification of genes controlling the agronomic traits of interest. However, the complexity of these traits, usually controlled by multiple loci, requires appropriate mapping populations and the application of advanced biotechnological tools.

In this context, eggplant has a great potential for improvement given the high genetic variability of its wild related species (CWRs). The study of these species with multiomics approaches (phenotypic, physiological and transcriptomic) would allow to expand the available knowledge about traits like tolerance to abiotic stresses. Therefore, in the first chapter of this doctoral thesis, a physiological study of the response to salinity of the cultivated species *S. melongena*, the related wild species *S. insanum* and *S. dasyphyllum*, as well as the interspecific hybrid *S. insanum* × *S. melongena* was carried out, identifying sources of genetic variation useful for their incorporation in breeding programmes. Additionally, a comparative transcriptomic study was performed on *S. melongena, S. insanum*, and their interspecific hybrid, confirming the physiological results obtained and differentiating the specific response mechanisms of each genotype. The second chapter focused on the fruit quality of eggplant varieties grown under moderate but prolonged salinity conditions. The results showed that the internal quality of the fruit is not significantly compromised, confirming eggplant's productive ability in saline environments.

As for peppers, although it is one of the most important crops within the Solanaceae family, there is less availability of studies on its germplasm and experimental populations for breeding. Therefore, the third chapter of this thesis focused on the phenotypic and genetic characterisation of a collection of traditional pepper (*C. annuum*) varieties from the Balearic Islands, with the aim of evaluating their potential as starting materials for breeding programmes. This chapter also describes the construction of the first MAGIC (Multi-parent Advanced Generation InterCross) population in pepper that combines eight varieties of two species (*C. annuum* and *C. chinense*) with the objective of increasing the genetic base of the crop and facilitating the identification of loci associated with agronomic traits. After the initial crosses and four autopollination cycles, the third and fourth autopollination generations were evaluated by phenotyping and genotyping. In addition, population structure was assessed, and highly inheritable traits were related to candidate genes by Genome-Wide Association Analysis (GWAS).

Overall, the results obtained in this thesis highlight the importance of expanding and characterising the available germplasm for the improvement of solanaceous crops.

Both wild species and traditional varieties constitute valuable genetic resources for the development of more resilient and sustainable cultivars. In addition, the MAGIC population developed represents a promising new tool for the scientific community, with enormous intrinsic potential due to its genetic variability and its usefulness in the identification of candidate genes associated to agronomic traits.

General Introduction

1. Relevance of Solanaceous crops

1.1 Economic importance

Solanaceae is a worldwide known family distributed on all continents except Antarctic, specially diversified in Central and South America, where its origin is placed. Species within the family can be grown on a wide range of habitats, from deserts to tropical rainforests (Motti, 2021). Among angiosperm families, Solanaceae is one of the most important for human nutrition, as it comprises well-known crops as tomato, potato, tobacco, pepper, or eggplant.

The production of Solanaceae species consumed for their fruits reached 650×10^6 t in 2022 according to Food and Agriculture Organization for the United Nations, cultivated in almost 27×10^6 ha (FAO, 2023). The most economically important crop, tomato (*Solanum lycopersicum* L.) produced more than 185×10^6 t in 2022 in almost 5×10^6 ha. The following most produced solanaceous crops are eggplant (*Solanum melongena* L.), with an annual production of almost 60×10^6 t in 2×10^6 ha and peppers (*Capsicum* spp.) with 35×10^6 t in 2×10^6 ha (FAO, 2023). All solanaceous crops have significantly increased their production in the last century (Figure 1). For example, in the last 30 years, tomato doubled its production, and eggplant and pepper almost triplicated it. The main premise for this production increase, even when the productive land has remained almost the same, is the improvement of cultivation techniques as irrigation, fertilisation, and pest management, as well as the introduction of commercial breeding varieties with enhanced yield (Daunay, Crosby, Diez, & Nuez, 2008).

The production of eggplant and peppers is remarkable in Spain. Regarding eggplant, Spain is the first exporter in the market worldwide, with total production of 275×10^3 t, representing one third of the global exports (Gobierno de España, 2024). In the case of peppers, Spain had a production of almost 2×10^6 t in 2023, and almost the half for exports, it has become the main producer of peppers in the European Union.

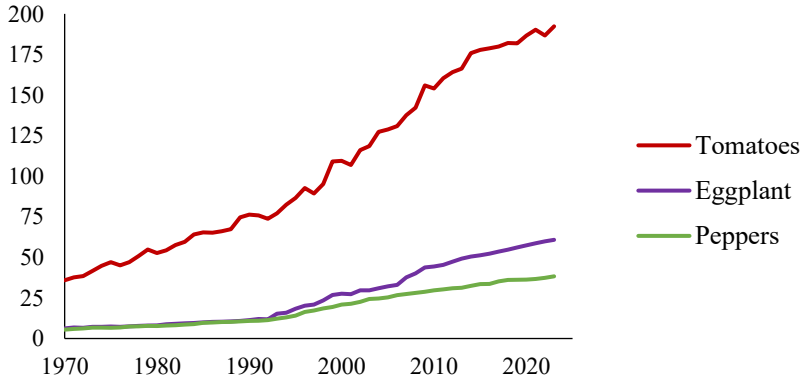

Figure 1. Total production (million tonnes, Mt) worldwide of tomato, eggplant and pepper from 1970 to 2023. Data from FAO (2023).

1.2 Uses

Solanaceae species have been used in many fields comprising from medicinal compounds and culinary purposes to ornamental plants, or, more recently, model species for genetic studies (Gebhardt, 2016; Olmstead et al., 2008). Most of the species are widely known for their culinary use, including fresh consumption, cooked preparations, or as a spice in the case of pepper. However, tobacco remains one of the most economically important plants globally due to its psychoactive alkaloid content, consumed via cigarettes, cigars, and pipes.

Solanaceae species are remarkable for presenting a wide range of biologically active compounds used for human health as terpenes (carotenoids, sterols, or glycosides), phenolics (flavonoids, tannins, or lignin) and nitrogen-containing compounds (alkaloids and glucosinolates). These secondary metabolites have been studied for their anti-inflammatory, and antimicrobial properties (Moghe & Last, Robert, 2015; Zaynab et al., 2018). It is also important to note the Solanaceae wild species' relevance, used in traditional cuisine, consuming their leaves or fruits because of the presence of these compounds in the edible part in the correct dose. *Jaltomata repanddentata* (Dunal) Hunz., and *Cestrum nocturnum* L. leaves are consumed cooked, while fruits of *Acnistus arborescens* L. Schltdlt., *Solanum stramonifolium* Jacq., *Lycianthes asarifolia* (Kunth and Bouché) Bitter, and *L. lycioides* (L.) Hassl are consumed fresh (Lim, 2012; Motti, 2021; Samuels, 2015).

However, less is commonly known about the medicinal uses of these species, which content in alkaloids ranges from innocuous, to irritating and, in great quantities, fatal for humans. Historically, many species have been used therapeutically for their content in this compound, including *Datura.* spp., *Mandragora autumnalis* Bertol., *Withania somnifera* (L.) Dunal, or *Atropa belladonna* L. which contain tropane alkaloids with analgesic, antispasmodic or sedative properties (Jaiswal, Liang, & Zhao, 2016). While most of these species have been abandoned for modern medicine due to toxicity, eggplant relatives like *Solanum insanum* L. are used in Asian tribes for almost any imaginable application like diuretic, lowering blood sugar, relieving cough, activate immune system, among many others (Meyer, Bamshad, Fuller, & Litt, 2014).

Solanaceae species are also displayed for their ornamental characteristics including genera like *Petunia, Browallia, Lycianthes* or *Nicotiana,* and species like *Solanum jasminoides* Spreng (Motti, 2021). In particular, petunia highlights as one of the most popular annual bedding plants worldwide. Introduced in Europe from South America and, after two hundred years of breeding and selection, a great morphological diversity is observable nowadays within this genus (Gerats & Vandenbussche, 2005). Moreover, over the last century, tomato, tobacco and petunia species have been widely used as model plants in classic, cellular and, lastly, molecular genetics (Lin et al., 2014). On the other hand, potato, eggplant and pepper have been predominant for solving agricultural problems like finding resistant and tolerant cultivars. Other genera like *Datura, Hyoscyamus* and *Physalis* have been less used in research, however, they

contributed to the first development of double haploid genotypes and biochemical mutants (Gebhardt, 2016).

2. Taxonomy, origin, and diffusion of Solanaceae

Solanaceae family is assigned to the order Solanales in the APG IV classification. It is the largest plant family after Poaceae and Fabaceae, comprising four main clades at the subfamily level – even there are unresolved subfamily levels –, 14 clades at the tribe level, nearly 100 genera, and more than 2,500 species (Motti, 2021; Olmstead et al., 2008). The Solanaceae family seemed to have diversified between 50 and 60 million years ago in South America and spread in short and long-distance events to other continents during millennia (Dupin et al., 2017). Many of the previously cited species belong to the genus *Solanum*, one of the most diverse within the family, comprising nearly 1,400 species around the globe, growing in different environments and agronomic conditions (Knapp, Vorontsova, & Prohens, 2013; Motti, 2021). The origin of the family is settled in South America, with many hints suggesting the Andean and Chilean region. Several important species such as tomatoes and chili peppers were introduced to Europe from the New World by maritime routes through Spain in the fifteenth and sixteenth centuries. Other solanaceous crops, like eggplant, were already introduced during the Middle Ages via Arab trade routes (Dupin et al., 2017).

2.1 Eggplant

Solanum melongena L. is classified within the *Leptostemonim* clade, one of the major *Solanum* clades with, approximately, 500 species. Near 6,500 accessions of eggplant are conserved in leading genebanks according to AVGRIS and Genesys databases; nevertheless, wild species are still under-represented in these collections (Syfert et al., 2016). Eggplant is a common name for three domesticated species: brinjal eggplant *S. melongena*, with origin in southeast Asia, scarlet eggplant *S. aethiopicum* L. and gnoba eggplant *S. macrocarpon* L., from the Sub-Saharan Africa (Meyer, Karol, Little, Nee, & Litt, 2012). The oldest evidence of the cultivation of eggplant is placed in Sanskrit documents from 300 years B.C.E.; however, its origin seems to be placed in the Holocene (8,000 – 7,000 B.C.E.). After domestication from wild ancestor *S. insanum* L., in the Indian subcontinent and Southeast Asia (Page, Gibson, Meyer, & Chapman, 2019), eggplant spread to the Middle East as early as in the IXth century. It was introduced into the Mediterranean region and North Africa through Arab trade and cultural exchange during the early Middle Ages, with cultivation in Islamic Spain documented by the Xth century. Eggplant reached more distant parts of Eucope by the XIVth century. It was later introduced in America following European colonization, likely during the XVIth or XVIIth centuries (Barchi et al., 2019; Page et al., 2019). However, more recent studies propose the existence of two to four domestication centres in India and South-East Asia, as well as four major historical migrations. Moreover, a possible reversion of *S. melongena* to weedy form resembling *S. insanum* has also been proposed (Barchi et al., 2023).

Domestication led to increased fruit size and morphological diversity, as the main domestication syndrome. Moreover, eggplant exhibits a large variability, including fruit characters as colour, size, shape, and, also, agronomic traits like adaptation to a plethora of ecosystems (Kouassi et al., 2021; Toppino, Barchi, & Rotino, 2022). Nevertheless, eggplant domestication was accompanied by a genetic bottleneck of more than 45% of allelic loss (Page et al., 2019). This loss can be considered moderate compared to other species as rice (62%) or bean (72%) (Bitocchi et al., 2013; Caicedo et al., 2007). However, a close genetic relationship and signs of admixture has been observed between *S. melongena* and *S. insanum* indicating the occurrence of gene flow between both species during first selection processes. This type of intercrosses has been also reported for other crops and described as adaptative introgression (Barchi et al., 2023).

2.2 *Capsicum* spp.

The genus *Capsicum* includes more than thirty species – even this is still subject of controversy – with different chromosome number and ploidy level, from which only five are considered as cultivated: *C. annuum* L., *C. baccatum* L., *C. frutescens* L., *C. chinense* Jacq. and *C. pubescens* Ruiz & Pav. (Pickersgill, 1991; Tripodi et al., 2021). Approximately 15,000 wild and cultivated genotypes are maintained in the largest genebanks, USDA (United States of America) and AVRDC (Taiwan). However, other outstanding genebanks over the world hold thousands of accessions which have been analysed with different phenotypic, molecular, and genetic methods for their potential (Lee et al., 2016). Within Solanaceae family, genus *Capsicum* is believed to be one of the first domesticated in South America, with the oldest evidence dated between 5,000 – 6,500 B.C.E. in Mesoamerican and Andean regions (DeWitt & Bosland, 1996).

Brought to Europe through Spain, it was rapidly incorporated into the Spanish culture and domesticated, becoming a secondary centre of diversity (González-Pérez et al., 2014). Different centres of diversification have been proposed for pepper, with a clear differentiation between European and Asian accessions. The pepper expansion seemed to be a complex net of trading routes including the Silk Road in Europe and Asia and the Ottoman trade routes for Middle East to Central and Eastern Europe. However, the genetic similarities between Europe and Mesoamerican peppers indicate interchanges during the first transatlantic Spanish trades in the XVIth century (Tripodi et al., 2021).

The first genetic bottleneck due to domestication is placed in Mesoamerica, followed by accumulation of new genetic diversity and interbreeding processes of cultivated species (Tripodi et al., 2021). Curiously, African peppers show high coincidence with those in South America and in East and Central Asia as well, indicating that slave trades with Portuguese maritime routes have also been key for peppers expansion (DeWitt & Bosland, 1996). The domestication syndrome in pepper included transition to non-deciduous pendant fruit, reducing fruit pungency, increase fruit weight and morphology diversity, as it has been also reported for other *Solanum* species (Kumar et al., 2018). Historically, pepper selection and differentiation have been primarily driven

by cultural factors, consumption preferences, and region economies. Consequently, pepper producers preserve a broad genetic and morphological diversity, which has been further expanded in recent decades through breeding programmes for developing new cultivars.

3. Eggplant breeding

3.1 Germplasm available

Crop improvement in traditional breeding requires the existence of naturally available resources with desirable features and their selection. Thus, genetic diversity is crucial for agricultural improvement. Cultivated eggplant shows a limited genetic variance, as other domesticated crops. However, eggplant has many wild and cultivated relatives or Crop Wild Relatives (CWRs) that might become a source of genetic diversity. They present a reservoir of genes and alleles which might be missing in cultivated species and can be moved into cultivated gene pools through breeding techniques (Gramazio et al., 2023; Plazas et al., 2016; Prohens et al., 2017; Rakha et al., 2020). In fact, many close species have been used as rootstock for grafting and improve eggplant performance (Daunay & Hazra, 2012; Gisbert et al., 2011; Rakha et al., 2020).

Eggplant related species have been classified according to their proximity and crossability with *S. melongena* into three gene pools (Harlan & de Wet, 1971) (Figure 2). The primary gene pool is composed by *S. melongena* and *S. insanum*, the ancestor of *S. melongena* which can produce fertile hybrids in their interspecific cross (Davidar et al., 2015; Knapp et al., 2013; Syfert et al., 2016). The secondary gene pool is constituted by African and Southeast Asian species, characterised by showing prickles in their leaves (Vorontsova & Knapp, 2016; Vorontsova, Stern, Bohs, & Knapp, 2013). The species of this pool show different crossability degrees with *S. melongena* and can be divided into three clades: I) "eggplant clade", with species genetically similar to *S. melongena,* includes: *S. campylacantum* Hochst. Ex. A. Rich., *S. incanum* L., *S. linnaeanum* Hepper & P.-M-L- Jaeger, and *S. lichtensteinii* Willd.; II) "anguivi clade", with species which can be fully crossed with *S. melongena* and obtain fertile hybrids, includes: *S. anguivi* Lam., *S. dasyphyllum* Schumach. and Thonn., *S. aethiopicum* L., *S. macrocarpon* L., and *S. tomentosum* L.; and III) "Madagascar clade", with more distant species like *S. pyracantos* Lam. (Daunay & Hazra, 2012; Plazas et al., 2016; Rotino, Sala, & Toppino, 2014). Hybrids of species like *S. incanum* have been widely used for their vigour as rootstock for cultivated eggplant (Gisbert et al., 2011).

The species with great difficulties to intercross with cultivated eggplant are catalogued in the tertiary gene pool, producing sterile or low-fertility hybrids after using biotechnological tools like embryo rescue or somatic hybridisation (Daunay & Hazra, 2012; Kouassi et al., 2016; Plazas et al., 2016; Rotino et al., 2014). They are the most phylogenetically distant of the *Leptostemonum* subgenus, and includes species like *S. torvum* Sw., *S. sisymbriifolium* Lam. and *S. eleagnifolium* Cav. One relatable species is

S. torvum, endemic of Centre and South America, it has become an invasive weed worldwide for its capacity to live in any shrub environment (Musarella, 2020).

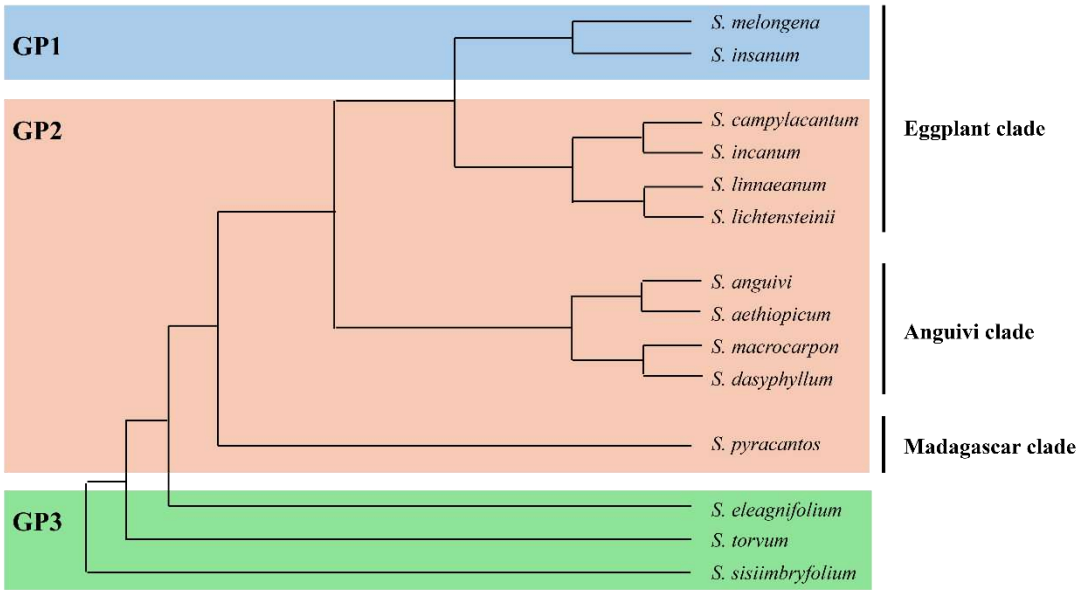

Figure 2. Phylogenetic tree of the most relevant species of primary (GP1), secondary (GP2), and tertiary (GP3) genepools of eggplant. Based on Daunay & Hazra, (2012); Vorontsova & Knapp (2016); Gramazio et al. (2023).

3.2 Breeding populations and tools

From related species and intrapecific germplasm, germplasm core collections, recombinant inbred lines (RILs), advanced backcross lines (ABLs), and ILs populations have been generated (Gramazio et al., 2017; Mangino et al., 2022; Prohens et al., 2017; Toppino et al., 2020). These collections increase the genetic information available for breeders, becoming essential tools to identify and exploit genes related with traits of interest, especially, quantitative characters as those related to environmental changes (Prohens et al., 2017). Different *S. melongena* populations have been obtained with relatives as *S. aethiopicum* with resistance to *Fusarium oxysporum* (Toppino, Valè, & Rotino, 2008), *S. linnaeanum* with resistance to *Verticillium* (Liu et al., 2015), and *S. incanum* with improved tolerance to abiotic stresses (Prohens et al., 2017). Also, new genomic tools as high-throughput genotyping, reference genomes, pangenomes, etc., contribute to enlarge the knowledge of genetic resources, thereby enhancing their utility in breeding projects. They have allowed to perform rapid and easy genotyping of experimental population with thousands of markers and evaluate quantitative trait loci (QTLs) related to interesting traits (Mangino et al., 2022; Toppino et al., 2020). Moreover, the publication of first eggplant genome in 2014 (Hirakawa et al., 2014), and the late assemblies (Barchi et al., 2019; Li et al., 2021; Wei et al., 2020) are expected to allow the identification of potential genes and the acceleration of breeding programmes.

The first reference genome draft of eggplant Asian cultivar "Nakate-Shinkuro" was released in 2014 (Hirakawa et al., 2014) and covered 833.1 Mb assembled in 33,873 scaffolds, completing 74% of eggplant genome. More recent assembled genomes are the "67/3" (Barchi et al., 2019) covering 1.06 Gb in 12 pseudomolecules which included 34,916 proteins, the "HQ-1315" (Wei et al., 2020), with 1.17 Gb and 36,582 predicted coding genes, and the "GUIQIE-1" (Li et al., 2021) with 1.15 Gb and 35,018 annotated genes. At this moment, different groups are working on releasing reference genome drafts on CWRs like *S. aethiopicum* (Song et al., 2019), which are expected to increase genetic and breeding resources for eggplant.

Despite their importance to widening eggplant genetic diversity, genomic investigation in CWRs is still limited. Few RNA-based studied have been performed in eggplant relatives, as the whole transcriptome assembly of *S. torvum* (Syfert et al., 2016) with 38,185 unigenes, very interesting because of its resistance to many pathogens and tolerance to other abiotic stresses; and the novo transcriptome of *S. aculeatissimum* Jacq., from the tertiary genepool, with 69,824 unigenes annotated, interesting for its resistance to root-knot nematode and *Verticillium dahlia* and tolerance to low temperatures (Yang et al., 2017). Also, transcriptome assemblies of *S. incanum* and *S. aethiopicum* were released in 2016 with 83,905 and 97,085 unigenes respectively, because of their resistance to several biotic streses like fungi, bacteria, insects, and root-know nematodes (Collonnier et al., 2001; Gramazio et al., 2016). Latest transcriptome released is of *S. sisymbriifolium*, from the tertiary genepool, with 41,189 unigenes annotated, considered resistant to many Solanaceae species' pathogens (Wixom et al., 2018).

3.3 Breeding advances in eggplant

Genetic improvements performed in eggplant have been based on yield and fruit quality traits. For instance, absence of prickles on the plant, earliness, and fruit visual traits like black colour, epidermal shininess, and lower flesh browning (Daunay & Hazra, 2012; Kumar, Sharma, Jain, & Kaushik, 2020; Prohens, Rodríguez-Burruezo, Raigón, & Nuez, 2007). Breeding in this crop has relied historically on landrace and variety selection and F1 hybrid development as these hybrids show traits of heterosis and heterobeltosis (Kumar et al., 2020). Thus, parental selection is key for the development of efficient F1 hybrids. Major challenges in eggplant breeding are obtaining resistant varieties for abiotic stresses and main diseases of the crop (Toppino et al., 2022).

Despite great phenotypic, physiological, and biochemical variability is still found in eggplant cultivars, only intraspecific variability and cultivated relatives have been exploited for breeding (Muñoz-Falcón, Prohens, Vilanova, & Nuez, 2009). Therefore, wild eggplant related species are an important source of allelic variation for many agronomic and fruit quality traits as well as for adaptation to concerning climate change scenarios (Daunay & Hazra, 2012; Rotino et al., 2014). The main problem in the use of wild relatives in eggplant breeding are intercross incompatibilities due to incorrect pairing of chromosomes during meiosis. Only few species have been used efficiently for obtaining F1 progenies and successfully backcrossed to obtain *S. melongena* cultivars

(Daunay & Hazra, 2012; Plazas et al., 2016). Novel biotechnological tools have been tested for eggplant breeding as somatic hybridization, embryo rescue, or genetic transformation followed by *in vitro* culture with great prospections to transfer interesting genes overcoming sexual barriers (Daunay & Hazra, 2012; Rotino et al., 2014).

In regard to biotic stresses, CWRs have presented resistance to most important fungal, bacterial diseases and nematodes. For instance, *S. aethiopicum* has shown resistance to *Phomopsis, Fusarium,* and *Verticillium* (Collonnier et al., 2001); *S. macrocarpon* has shown resistance to fungi and some insects (Sossou et al., 2024); *S. incanum* has shown resistance to nematode and *Verticillium* wilt (Gisbert et al., 2011); *S. linnaeanum* is considered a source of resistance for *Verticillium, Phytophthora capsici* and *Ralstonia* (Liu et al., 2015); and *S. torvum* and *S. sisymbriifolium* showed resistance to root nematodes (Daunay & Hazra, 2012).

In regard to abiotic stresses, eggplant has presented a mild tolerance to drought and salinity and other stresses, partially reducing yield and quality (Brenes et al., 2020a; Brenes et al., 2020b; Kirnak, Tas, Kaya, & Higgs, 2002; Kouassi et al., 2021; Ortega-Albero et al., 2023; Shahbaz, Mushtaq, Andaz, & Masood, 2013). However, to avoid yield and quality loss, CWRs have been proposed as the main option for eggplant breeding as donors of tolerance genes due to the adaption to different ecological conditions (Gramazio et al., 2017; Kouassi et al., 2021; Plazas et al., 2016). *S. insanum* is cultivated in Southeast Asia, Madagascar, and Mauritius Islands, reported to grow well in dry areas (Knapp et al., 2013) and thus, many tests have been carried out for tolerance to abiotic stresses as salinity and drought with promising results (Kouassi et al., 2016; Brenes et al., 2020a; Ortega-Albero et al., 2023). Species from the secondary eggplant gene pool are cultivated in very diverse habitats, including proximity to sea and desertic areas, and have been described as tolerant to various stresses (Gramazio et al., 2017; Knapp et al., 2013; Vorontsova & Knapp, 2016). In this group, we find cultivated eggplants *S. aethiopicum, S. macrocarpon,* and *S. linnaeanum,* which display traits associated with domestication syndrome, thus, their use in breeding becomes easier (Plazas et al., 2014). Although their challenges for crossability with *S. melongena*, species of the tertiary eggplant gene pool are interesting genetic resources with great prospect for eggplant breeding with new breeding strategies (Gousset et al., 2005; Öçal, Özalp & Devran, 2018; Brenes et al., 2020a). For instance, *S. torvum* have been proved to be tolerant to salinity (Brenes et al., 2020a) and *S. eleagnifolium* showed great tolerance to drought (Fita et al., 2015). These sources for tolerance genes could play an essential role in eggplant adaptation to climate change conditions, a growing concern in many countries. Nevertheless, it is still necessary to check the profit of these lines when breeding for commercial cultivars.

4. *Capsicum* breeding

4.1 *Capsicum* species and diversity

Gene banks and *in situ* cultivated materials are valuable repositories of seeds, which genetic variation can be used for genome-wide improvements. Cultivated peppers have shown a narrow genetic variance, hence, genetic diversity is essential in breeding programs to introduce new beneficial alleles. *Capsicum* wild species are grown in a very diverse range of environments, from rain forests to drier areas; even some of them are established in a very narrow area, considering them vulnerable species like *C. tovarrii* Eshbaugh, Smith & Nickrent or *C. pereirae* Barboza & Bianch. Therefore, there is a need to analyse the genetics of these materials as it has been done for populations of interest in Spain, Mexico, or collective efforts from Asia, Africa or America (Pereira-Dias et al., 2019; Taitano et al., 2019; Taranto et al., 2016).

The *Capsicum* species can be organised in three main genetic complexes or genepools: the *annuum, baccatum,* and *pubescens* pools, determined by the genetic proximity and intercross compatibility (Figure 3). The first complex includes the species which can be directly hybridised with *C. annuum* and main pepper producer species for fresh consumption producing fertile hybrids like *C. annuum, C. frutescens, C. chinense,* or *C. galapagoense* Hunz. The secondary genepool includes related species which can hybridise with *C. annuum,* but the progeny is partially sterile or presents difficulties during their development like *C. baccatum,* or *C. tovarii*. The tertiary complex holds species which can be crossed with *C. annuum,* but the hybrids need to be recovered through embryo rescue like *C. pubescens, C. cardenasii* Heiser & P.G.Sm., or *C. eximium* Hunz. (Carrizo García et al., 2016; Manzur, Fita, Prohens, & Rodríguez-Burruezo, 2015; Srivastava & Mangal, 2019). Conventional pepper breeding has been done by hybridisation with the primary *Capsicum* complex and selection. However, other strategies have been developed like introgression of genes of interest by backcrossing with species from the first and the secondary complex, induced mutagenesis to obtain new genetic variants, and somatic hybridisation for new genomic combinations. Moreover, modern biotechnological tools focused on gene and allele selection have been used on peppers with promising results including marker assisted selection (MAS), polyploid and haploid breeding and transgenesis (Srivastava & Mangal, 2019). Also, a wide variability can be found within *C. annuum* species, with many traditional varieties cultivated worldwide. These constantly evolving genotypes encompass profitable genetic diversity that can be used for breeding efforts.

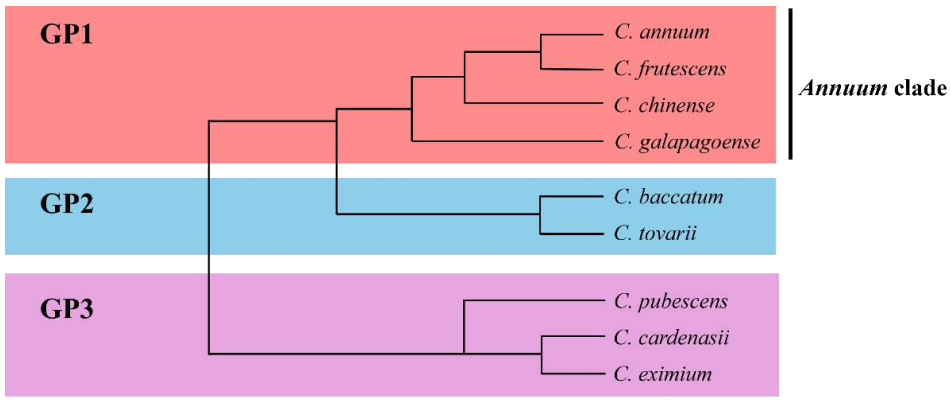

Figure 3. Phylogenetic tree of the most relevant species of *Capsicum* clades *annuum, baccatum, pubescens.* Adapted from Carrizo García et al. (2016)

4.2 Current breeding efforts in *Capsicum*

Currently, the main objetive of *Capsicum* breeders is developing new genotypes with higher genetic potential including improved productivity thought heterosis exploitation, introgressing disease and pathogen resistance through hybridization and increasing content of bioactive compounds in fruit (Srivastava & Mangal, 2019). These tasks have typically been performed through intraspecific crosses and subsequent backcross to introgress qualitative interesting traits into commercial varieties (Rao, Ben Chaim, Borovsky, & Paran, 2003). Crosses between species from the same clade are usually possible with fertile hybrids' production, but the genotype is determinant for the success (Manzur et al., 2015). However, interspecific crosses are problematic due to chromosomic imbalances. Embryo rescue and other biotechnological techniques have been of great importance to overcome with embryo abortion; however, pepper is recalcitrant to *in vitro* culture limiting the use of this technique.

In regard of genomic tools, pepper genome (2n=2x=24) is a complex genome with 3.5 Gb and a large number of repetitive elements compared to other solanaceous species like tomato or potato with genome sizes of 900 Mb and 844 Mb, respectively (Sato et al., 2012; Xu et al., 2011). This is the main reason why the sequence-based resources in this species have been delayed in comparison to other relatives in the family. First transcriptomes reported in pepper were developed with 454 sequencing technology in 2012 (Ashrafi et al., 2012; Lu, Cho, & Park, 2012; Lu et al., 2011). However, first draft genomes of *Capsicum* were not released until 2014. In fact, several genomes were released in the same year. Kim et al. (2014) released the whole-genome sequencing and assembly of *C. annuum* hot pepper 'Serrano Criollo de Morelos' (hereafter CM334) and also *C. chinense* accession PI159236 with an interspecies comparative phylogenetic analysis. These two accessions were selected for their resistance to nematodes, fungi like some species of *Phytophthora* and virus like Tobacco mosaic virus (TMV), Potato Y Virus (PYV), and Pepper Mottle Virus (PepMoV). The authors reported a total of 34,903

protein-coding genes in a 3.45 Gb genome length. In the same year, Qin et al. (2014) published the genomic sequence of *C. annuum* cv. Zunla-1 and its wild parent *C. annuum* var. *glabriusculum* Chiltepin with an assembly of 3.35 Gb for Zunla-1 and 3.48 Gb for Chiltepin with 35,336 and 34,476 protein-coding genes, respectively. In both Kim and Qin works, more than 80% of pepper genome seemed to be transposable elements (TEs), most of which are LTR retrotransposons. From this day on, new sequencing and resequencing projects are coming out like the sequencing and assembly of *C. baccatum* PBC81 and a new version of *C. chinense* PI159236 (Kim et al., 2017) achieving total genomes of 3.2 and 3.0 Gb length. Also, approximately 35,000 were annotated in both genomes. However, it was in 2018 when Hulse-Kemp et al. applied the linked-read sequencing to *C. annuum* to generate a highly ordered and more contiguous reference genome of 3.21 Gb (Hulse-Kemp et al., 2018).

These efforts have been the base for developing multiple high-throughput technologies as genomic chips and arrays with SNP data (Ashrafi et al., 2012; Hulse-Kemp et al., 2016). The availability of these resources has facilitated molecular breeding, marker development, and MAS for improvement in pepper. In this regard, quantitative or complex traits can be examined with genetic mapping strategies like linkage analysis and genome wide association studies (GWAS) by identifying regions affecting the phenotype. GWAS has proved to be an effective tool for discovering multiple alleles, as reported by several authors for complex traits as capsaicinoid content, fruit shape or fruit weight (Tripodi & Kumar, 2019).

Global warming, water resources limitations, and increasing salinity are presented as future limiting factors for pepper cultivation. As a tropical crop, peppers optimal cultivation temperature range is 20-30 ºC. This crop is frost-sensitive, and low night temperatures during plant growth can affect pollen viability and quantity, and development of female organs in the flower (Cruz-Huerta, Williamson, & Darnell, 2011). Nevertheless, heat stress also has a negative impact on pollination and fertilisation and overall productivity (Guo et al., 2014). Sources of tolerance to heat stress and low temperature have been detected in pepper cultivar as Habanero pepper (*C. chinense)*, capable of modify leaf metabolism when the temperature increases, and several QTLs have been identified for tolerance against thermic stress including heat shock proteins, or hormone and calcium signalling (Li et al., 2015; Wang, Niu, Zhai, & Lu, 2017).

Regarding drought, *C. chinense* was reported to accumulate phenolics and other secondary metabolites with antioxidant activities to cope with water and turgency loss, and alterations in the photosynthetic machinery, showing some tolerance. Also, high pungent cultivars seemed to have a lower reduction in fruit yield due to higher water retention capacity (Phimchan, Techawongstien, & Bosland, 2012). Salt stress is a major concern for pepper breeding, affecting dramatically water and nitrogen use efficiency and limiting plant growth and productivity (Gupta & Huang, 2014). Genotypic variation has been studied in *Capsicum* accessions for improved response to salinity. Some *C. annuum* cultivars have been reported to be tolerant to salinity as ‘Early Jalapeño’ and ‘Cayenne’, by accumulating less sodium ions in the shoot and increasing enzymatic

antioxidants (Aktas, Abak, & Eker, 2012). Further, Al Hattab et al. (2015) developed a salt-tolerant chili with high capsaicin content. However, further research is necessary to unravel the genetic control of this tolerance.

5. Drought and salinity as major challenges for horticultural crops

Plants endure adverse edaphoclimatic conditions during their life cycles. These environmental conditions comprise biotic stresses such as herbivores attack and infection by several pathogens (fungi, nematodes, bacteria or viruses); and abiotic stresses which include heat, cold, drought, salinity, or heavy metals in the soil. Climate change is a well-known effect originated due to fossil fuel burning and other anthropogenic activities and it is increasing with concentration of greenhouse gases (GHGs) in the atmosphere (Dutta, Chakraborti, Chaudhuri, & Mondal, 2020). This global effect is expected to progress drastically over the next decades and cause extreme climatic conditions as record-breaking temperatures, CO_2 increase, decline of rainfall reducing hydric resources and water quality for consumption and irrigation and, therefore, increase of drought and salinity in worldwide soils, including those for cultivation. These climatic conditions endanger all agroecosystems and future food security (Bailey-Serres et al., 2019) (Figure 4).

Regarding abiotic stresses, drought and salinity are the most concerning for crop cultivation, by causing a severe reduction in crop yield (Fahad et al., 2017; Isayenkov, 2019). On one hand, the main effects of water deficit on plants are: poor germination, reduced seedling growth, reduced nutrient availability and uptake, reduced activity of photosynthetic machinery and evapotranspiration, oxidative stress, and, thus, reduced plant growth, yield, and survivance (Farooq et al., 2009). On the other hand, salinity is currently affecting more than 109×10^6 ha of cropland worldwide, reducing potential yields up to a 50% in productive areas (Daliakopoulos et al., 2016; Shahid, Zaman, & Heng, 2018). Although salt effects on plants are similar to droughts', including nutritional imbalance, osmotic alteration, oxidative stress, photosynthesis and transpiration limitation, and growth and yield reduction; it also includes a toxicity effect, due to the accumulation of Na^+ and Cl^- ions (Figure 4). The response of the plant is dependent on different aspects, such as duration and severity of the stress, but, mainly, the genotype (Sumalan et al., 2020). In this regard, responses to abiotic stresses in plants show very complex regulatory paths, affecting numerous metabolic processes and altering the expression of many genes. The responses can be divided into non-adaptative, reflecting damaged inflicted by the stressor, and adaptative responses, to increase resistance and repair the induced damage (Zhang et al., 2020). Genetic, biochemical and molecular studies have been developed for identifying responses in many species to different stresses. It seems that, in most cases a multilevel response is activated, including stress sensing, signal transduction, transcription regulation, transcript processing, translation and post-translational protein modifications (Zhang, Zhu, Gong, & Zhu, 2022).

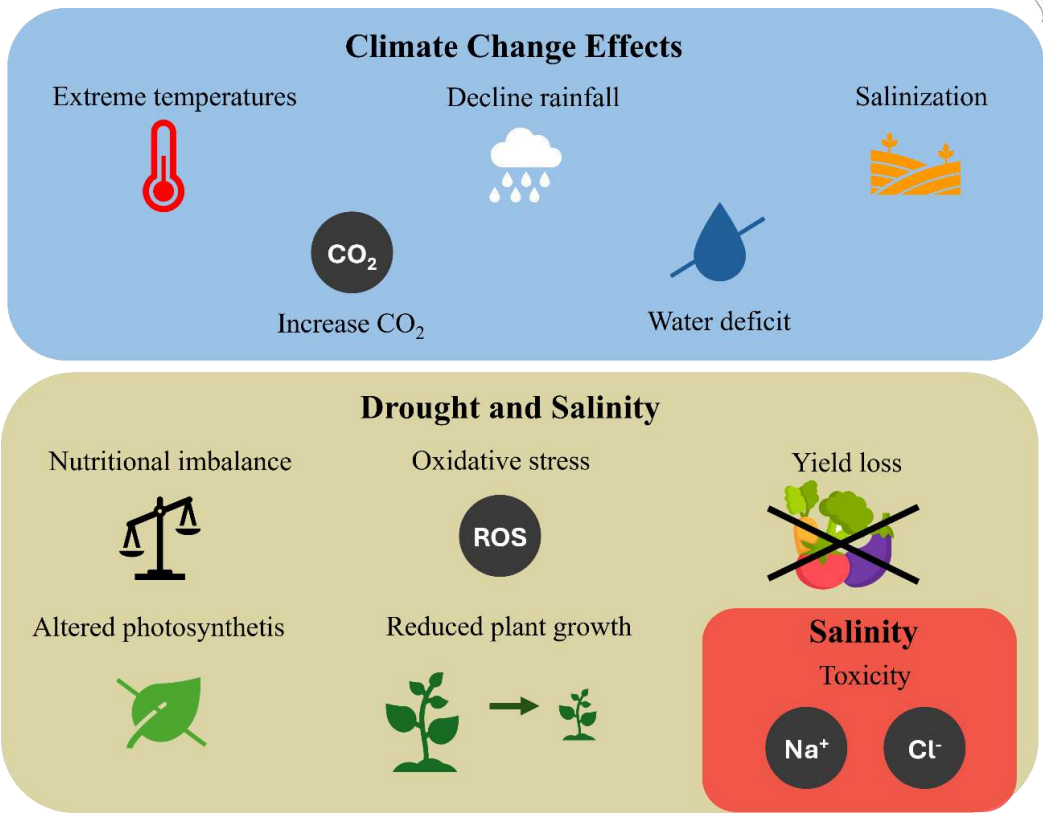

Figure 4. Representation of rising stresses derived from climate change and their effect on plant species.

Solanaceae family includes, as mentioned before, a large number of economically important vegetables such as tomato, potato, eggplant or pepper, essential for human nutrition worldwide (FAO, 2023). The impact of climate change and derived abiotic stresses threaten these vegetables' production and, thus, future nutrient availability and food security. Breeding for Solanaceae main crops has been focused on obtaining resistance to pests and other biotic stresses so far. However, tolerance to abiotic stresses is recently emerging as a major objective for breeding these species. For instance, the success obtained in tomato for enhanced production as well as other resistances to diseases and suited to different environments, sets the basis for breeding in other economically important crops of the family as eggplant and peppers (Daunay et al., 2008).

6. Breeding for complex traits, the role of diversity and experimental populations

Traditional methods in plant breeding have been used for decades to obtain a plethora of commercial varieties for many horticultural crops. These methods include mass, pure-line, and pedigree selection, as well as backcrossing or hybrid breeding for introgression. They have been usually used through hybridisation of selected genotypes

within the primary pool of a genus and identification of an interesting commercial phenotype in the segregating progenies (Srivastava & Mangal, 2019). In this context, although variation exists at the DNA level, selection is primarily based on phenotypic expression, which is influenced by environmental conditions. As a result, cultivars developed through traditional methods may exhibit undesirable traits inadvertently inherited from parental lines due to indirect selection. These methods, in the current knowledge of plant genetics, are not precise, fast, and cost effective enough to explode the potential of a genotype. In fact, more than 10 years are necessary in some projects to release a new variety into the market, and narrowed genetic diversity has proved to be one of the consequences of decades of this type of breeding methods (Srivastava & Mangal, 2019).

To avoid dragging undesirable traits during a species' breeding, other strategies have been proposed. Classic selection has proved to be effective for qualitative traits, while selection for quantitative or complex traits is often worthless due to the environmental influence. Molecular markers were developed to cope with this problematic initiating the era of direct, DNA-based selection. Although is not a gene selection, marker-assisted selection (MAS) has been highly used for plant breeding as it can be easily used in early stages of a single plant accelerating the breeding process (Collard & Mackill, 2008; Jena & Mackill, 2008). DNA markers have been developed and used for increasing productivity, diversity analysis, evolution history assessment, and gene location (Costa et al., 2016; Hossain, Rahman, Alam, & Singh, 2015; Jeong et al., 2015; Tanaka et al., 2014). However, in the current multi-omics era, the usage of different and integrative methods is considered the main solution for plant breeding improvement. These methods include high throughput phenotyping (HTP), high throughput genotyping and transcriptome analysis with next generation sequencing (NGS) technologies, together with proteomics, metabolomics and even environmental essays known as enviromics (Crossa et al., 2021; Jamil et al., 2020).

Identifying QTLs associated with agronomically important traits in wild populations remains a complex task due to the genetic complexity, environmental instability, and limited control over crossing. Environmental variation introduces phenotypic noise, complicating reliable mapping of loci with small effects (Mackay & Powell, 2007). Furthermore, wild populations often exhibit extensive population structure, which difficulties QTL detection and reduce the power of association studies (Kremling et al., 2018). As a result, new types of populations have been developed in many crops to gather many genetic and phenotypic resources (Scott et al., 2020). Introgression lines (ILs) obtained by a series of backcrossing with MAS are one of the proposed populations. An ILs population is a group of lines with identical genetic background and different introgression from another genotype (ideally those introgressions cover the whole genome from the introgressed parental). This population was proposed to broaden the genetic base of existing cultivars while developing new commercial lines with one improved trait. They have a higher mapping resolution than classic bi-parent crosses for QTLs due to their low genetic background noise (Cavanagh, Morell, Mackay, & Powell, 2008). However, their main drawback for finding QTLs in

these ILs is the limited number of recombinations and the limited number of alleles (determined by the two parentals). To address this problem, multi-parent populations (MPPs) were proposed to capture additional recombination of many genotypes, increasing mapping resolution and QTL identification because of the lower linkage disequilibrium or population structure (Arrones et al., 2020). The two most popular MPP designs in plants are nested association mapping (NAM) and multi-parent advanced generation intercross (MAGIC). NAM populations are based on the crosses between a recurrent founder line and other founder lines finishing in a population of recombinant inbred lines (RILs) with different genomic combinations of the recurrent founder line (Figure 5). In contrast, MAGIC populations are based on the intercross of more than four founder lines (typically 4, 8 or 16) followed by a funnel breeding design to obtain a population of RILs with unique genomic combination of all the founder lines (Arrones et al., 2020; Scott et al., 2020) (Figure 5). The main disadvantage of this type of populations is that they are time- and space-consuming. Also, the key point for their success is the selection of the founder lines to maximize genetic diversity and minimize population structure while taking in account crossability barriers. These populations have been developed in economically important crops like tomato and eggplant (Mangino et al., 2022; Pascual et al., 2015), cereals like maize, wheat, sorghum, and rice (Bandillo et al., 2013; Dell'Acqua et al., 2015; Kumar et al., 2023; Mackay et al., 2014) and legumes like chickpea or cowpea (Huynh et al., 2018; Thudi et al., 2024) obtaining a large number of new QTLs related to interesting complex traits like yield and resistance to biotic and abiotic stresses.

Lastly, enhanced crop varieties have been historically developed through gene introgression, however, recent advances in genomics and genome editing increase the possibilities of plant breeding, in addition with new high-throughput sequencing techniques and bioinformatic tools release (Khan, 2019). In this field, genome editing tool CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) might revolution the gene editing techniques to generate fresh genetic diversity to speed up crop breeding. Unfortunately, plant transformation is not well developed in some species, particularly in peppers.

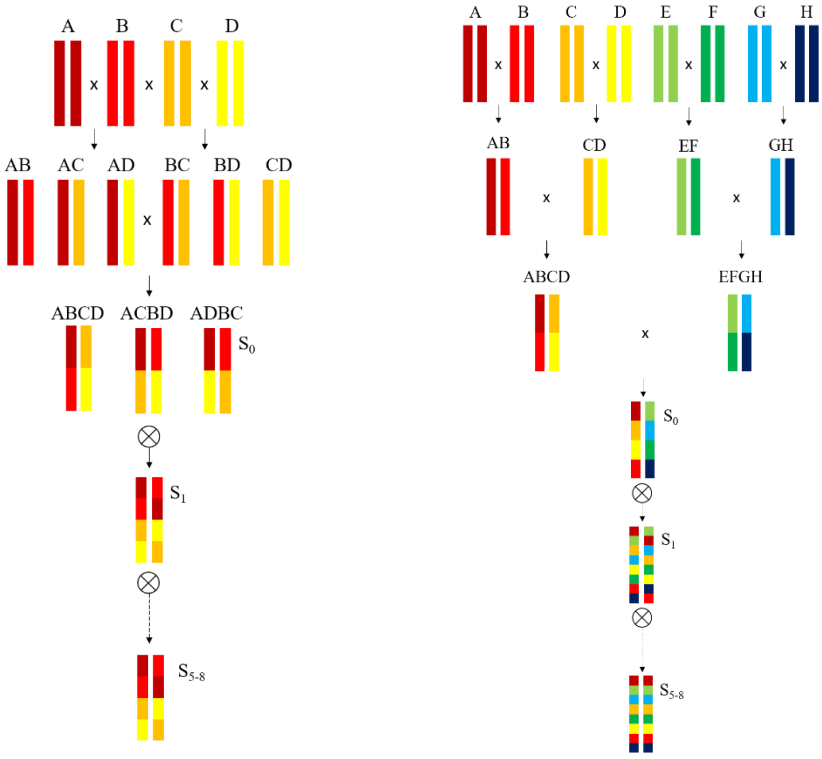

Figure 5. Diagram of a Nested Association Mapping (NAM) (left) and a Multi-parent Advanced Generation InterCross (MAGIC) populations (right).

Objectives

Although significant progress has been made in Solanaceae breeding, some gaps remain in the common knowledge of crops like eggplant and pepper. In eggplant, there is a growing need to explore new sources of tolerance to abiotic stresses, specially under the increasing pressure of climate change. Many underutilised and uncharacterised CWRs within the *Solanum* genus hold genetic variation that could be exploited to improve tolerance. In pepper, while there is a wider range of diversity, a more comprehensive understanding of its distribution, and potential for breeding is still required. Furthermore, the development of mapping populations is essential to accurately identify loci associated with traits of agronomic interest.

Forthcoming breeding programmes require expanding genomic knowledge and genetic resources for developing new varieties adapted to up-coming climate and soil changes. It is mandatory to create and analyse plant materials for traits of interest which can ensure crop productivity and sustainability for future vegetable consumption.

Therefore, we proposed the following specific main objectives:

Objective 1. Evaluation of eggplant CWRs for their prospect as parental species for new hybrids in breeding for abiotic tolerance.

Objective 1.1 Assessment of the physiological and biochemical responses of *Solanum melongena, S. insanum* and their interspecific hybrid *S. insanum* × *S. melongena* to salt stress.

Objective 1.2 Comparative analysis of the transcriptomic response of *S. melongena, S. insanum* and interspecific hybrid *S. insanum* × *S. melongena* to salt stress.

Objective 1.3 Assessment of the prospect of *S. dasyphyllum* as donor of tolerance genes for *S. melongena*.

Objective 2. Estimation of the effects of salinity on quality in different eggplant germplasm.

Objective 3. Analysis of the diversity within *Capsicum annuum* germplasm and creation of useful mapping populations.

Objective 3.1 Evaluation of the diversity of a collection of insular landraces.

Objective 3.2 Development of a *Multi-parent Advanced Generation InterCross* (MAGIC) population in *Capsicum* spp. and its morphological, molecular characterisation.

Results

This section presents the main results obtained during the development of this doctoral thesis, organised in three chapters. Each chapter is aligned with one of the presented objectives and provides a comprehensive overview of the research.

Chapter I focuses on the morphological, biochemical and transcriptomic responses to salt stress in cultivated eggplant and related species. The results are presented as three research articles:

Responses to salt stress of the interspecific hybrid Solanum insanum × Solanum melongena *and its parental species.*

First transcriptomic profile of the interspecific hybrid Solanum insanum × S. melongena *under salt stress.*

Biometric and biochemical responses to salt in Solanum dasyphyllum*, a potential donor of tolerance for eggplant.*

Chapter II evaluates the effect of prolonged moderate salt stress on the internal quality of eggplant fruits. The results are summarized in published paper:

Internal fruit quality is maintained in eggplant under mild long-term salt treatment.

Chapter III explores the phenotypic and genetic variability of a collection of *C. annuum* landraces and describes the development and analysis of the first MAGIC population in *Capsicum*. The results obtained in this chapter are displayed in research articles:

*Genetic diversity, population structure, and phylogeny of insular Spanish pepper landraces (*Capsicum annuum *L.) through phenotyping and genotyping-by-sequencing.*

First interspecific multi-parent advanced generation intercross (MAGIC) population in Capsicum *peppers: development, phenotypic evaluation, genomic analysis, and prospects.*

Chapter I.

Salinity response mechanisms in eggplant and wild relatives

Research article

Responses to salt stress of the interspecific hybrid *Solanum insanum* × *Solanum melongena* and its parental species

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PhD candidate contribution

Neus Ortega Albero has a main role in performing the experiments, sample and data collection, data analysis and visualization, drafting manuscript, revision and editing.

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Abstract

Soil salinity is becoming one of the most critical problems for agriculture in the current climate change scenario. Growth parameters, such as plant height, root length and fresh weight, and several biochemical stress markers (chlorophylls, total flavonoids and proline), have been determined in young plants of *Solanum melongena*, its wild relative *S. insanum*, and their interspecific hybrid, grown in the presence of 200 and 400 mM of NaCl, and in adult plants in the long-term presence of 80 mM of NaCl, in order to assess their responses to salt stress. Cultivated eggplant showed a relatively high salt tolerance, compared to most common crops, primarily based on the control of ion transport and osmolyte biosynthesis. *S. insanum* exhibited some specific responses, such as the salt-induced increase in leaf K^+ contents (653.8 μmol g^{-1} dry weight) compared to *S. melongena* (403 μmol g^{-1} dry weight) at 400 mM of NaCl. Although there were no substantial differences in growth in the presence of salt, biochemical evidence of a better response to salt stress of the wild relative was detected, such as a higher proline content. The hybrid showed higher tolerance than either of the parents with better growth parameters, such as plant height increment (7.3 cm) and fresh weight (240.4% root fresh weight and 113.3% shoot fresh weight) at intermediate levels of salt stress. For most biochemical variables, the hybrid showed an inter-mediate behaviour between the two parent species, but for proline it was closer to *S. insanum* (ca. 2200 μmol g^{-1} dry weight at 200 mM NaCl). These results show the possibility of developing new salt tolerance varieties in eggplant by introducing genes from *S. insanum*.

Keywords

Eggplant, heterosis, ion homeostasis, osmolyte, oxidative stress, proline, salinity, tolerance

1. Introduction

Global food production should increase by 50% by 2050 to feed a population that will rise to 10×10^9 people (FAO, 2022). However, soil salinity affects over $10^9 \times 10^6$ ha of cropland worldwide, reducing yields in more than 50% of the surface of the most productive agricultural areas–those cultivated under irrigation in arid and semi-arid regions (Daliakopoulos et al., 2016; Mahajan & Tuteja, 2005; Munns, 2005; Shahid, Zaman, & Heng, 2018; Wicke et al., 2011). The source of high salt concentration is primarily the presence of salt in irrigation water and the accumulation of Na^+ and Cl^- in soils (Shahid, Zaman, & Heng, 2018).

The effects of soil salinity on plants vary widely depending on multiple factors, but salt tolerance is mainly controlled by genotype (Hesami, Bazdar, & Shahriari, 2020; Sumalan et al., 2020). An operational threshold has been established to classify species into salt-sensitive (glycophytes) and salt-tolerant (halophytes), as well as those able to complete their life cycle under soil salinities equivalent to more than 200 mM NaCl (Flores, Troke, & Yei, 1977; Himabindu et al., 2016; Tang et al., 2015). Different species have developed protective mechanisms to cope with salt stress, from the physiological and biochemical to the molecular level, involving complex networks of genes, proteins and metabolites (Kumar et al., 2013).

Salinity induces detrimental effects in glycophytes, such as (i) reduced water availability, (ii) ion toxicity, (iii) oxidative stress and (iv) K^+ deficiency (Blumwald, 2000; Shabala & Cuin, 2008), which lead to a reduction in plant growth and biomass accumulation, and eventually, plant death (Gupta & Huang, 2014; Munns & Tester, 2008). Salt stress alters the normal metabolic processes of the plant: photosynthesis and energy production, lipid metabolism, nutrient acquisition, the integrity of cellular membranes and the activity of enzymes (Nishiyama & Murata, 2014). Plant growth under such stressful conditions depends on the efficiency of the mechanisms of stress responses in each species. In this regard, tolerance mechanisms can be divided into two major groups: defence against osmotic stress and ion toxicity, which includes the control of ion transport and osmolyte biosynthesis and defence against oxidative stress, which includes the activation of antioxidant mechanisms.

To maintain intracellular osmotic balance, some plants accumulate toxic ions such as Na^+ and Cl^- in the vacuoles (Flowers & Colmer, 2008; Kumari, Das, Parida, & Agarwal, 2015). An excessive accumulation of Na^+ is generally accompanied by K^+ deficiency by competition between the two cations because of their similar physicochemical properties. Thus, maintaining Na^+/K^+ homeostasis is crucial to developing normal metabolic processes in the cytoplasm, such as enzymatic reactions and protein synthesis (Shabala & Cuin, 2008). In addition, different metabolites are involved in responses to osmotic stress such as osmolytes and osmoprotectants, including sugars, polyalcohols, amino acids, ammonium compounds, betaines and sulphonium compounds. Sugars are direct products of

photosynthesis that play essential functions in the cell; their increase could be a response to stress or a signal for activating other cellular processes. During abiotic stress, their primary role is stress mitigation by osmoprotection, carbon storage and the scavenging of Reactive Oxygen Species (ROS). Amino acids also have some regulatory and signaling functions. In stress tolerance mechanisms, a significant role is played by the flagship compatible solute proline (Pro) (Gil et al., 2014; Hildebrant, 2018; Kumari, Das, Parida, & Agarwal, 2015; Szabados & Savouré, 2010).

Toxic ions absorbed by roots move into photosynthetic organs, causing harmful nutritional imbalances and the generation of oxidative stress by an increase in the production of ROS. Through the oxidation of fatty acids, amino acid residues in proteins and the DNA bases, ROS accumulation leads to membranes degradation, protein inactivation and DNA mutations, causing cellular damage, and eventually, cell death (Apel & Hirt, 2004; Demidchik & Maathuis, 2007; Shabala, 2009). To cope with oxidative stress, plant cells activate antioxidant systems, including the activation of redox regulatory enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) (and other peroxidases) or glutathione reductase (GR), and the synthesis of antioxidant metabolites, such as phenolic compounds (Ozgur, Uzilday, Sekmen, & Turkan, 2013).

Eggplant (*Solanum melongena* L.) is one of the most economically important crops worldwide, reaching 1.86×10^6 cultivated hectares and an annual production of over 54×10^6 tonnes (FAO, 2022). Eggplant is moderately sensitive to salinity (Ünlükara et al., 2010), which partially reduces growth and yield. *Solanum melongena* can be crossed with many wild relatives from the primary, secondary and tertiary gene pools, adapted to a wide range of environments (Plazas et al., 2016). Therefore, it should be possible to identify new sources of genetic variation in wild relatives adapted to saline areas. The responses of *Solanum insanum* L. to salt stress have recently been studied, and the wild genotype seems more tolerant than the cultivated eggplant, as it can stabilise its growth and photosynthetic rate under saline conditions (Brenes et al., 2020). In *S. insanum*, toxic Na^+ and Cl^- ions are transported to the leaves at high external salinity, where they most likely accumulate in vacuoles, according to the 'ion compartmentalisation hypothesis' (Maathius, 2014; Peng et al., 2016), whereas K^+ concentrations are maintained. In the presence of high salt concentrations, compared with cultivated eggplant, the wild species also shows a higher accumulation of proline, an excellent marker of a stress tolerance phenotype (Ünlükara et al., 2010).

Little is known about the inheritance of quantitative or complex traits such as stress tolerance. However, introgression can be used to introduce favorable characteristics in cultivated *S. melongena* (Kouassi et al., 2016; Mangino et al., 2020; Prohens et al., 2017) for future breeding prospects. This study compares the response

of cultivated eggplant (*S. melongena* L.), a wild relative (*S. insanum* L.) and their interspecific hybrid, under salt stress conditions in shortand long-term treatments by determining growth parameters and the levels of several biochemical stress markers –such as ions, osmolytes or antioxidant compounds –and the activity of antioxidant enzymes. This work represents the first step in assessing the possibilities of success of the introgression of salt tolerance traits from the wild relative into the cultivated species.

2. Results

2.1 Substrate electrical conductivity

The electrical conductivity ($EC_{1:5}$) of the substrate in the pots was measured at the end of the short-term salt treatments of young plants. The obtained values were in concordance with the treatments applied: 4.9 dS m^{-1} on average for the control, 23.5 dS m^{-1} on average for irrigation with 200 mM of NaCl and 31.5 dS m^{-1} for irrigation with 400 mM of NaCl. No significant differences were found in these levels among the three genotypes within each treatment (Table 1).

Table 1. Electrical conductivity values ($EC_{1:5}$ mean ± SD; n = 5) in the pot substrates of *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB), when applying the salt treatments. Different lowercase letters within the same row indicate statistically significant differences between treatments, and different uppercase letters within the same column indicate statistically significant differences between genotypes ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

		Treatment (NaCl Concentrations)		
		Control	**200 mM**	**400 mM**
$EC_{1:5}$	MEL	5.2 ± 1.6 ^{aA}	21.8 ± 3.2 ^{bA}	32.2 ± 4.1 ^{cA}
	HYB	5.2 ± 2.001 ^{aA}	26.4 ± 3.2 ^{bA}	31 ± 5.9 ^{bA}
	INS	4.4 ± 1.9 ^{aA}	22.1 ± 2.9 ^{bA}	31.8 ± 3.9 ^{cA}

2.2 Young plant growth parameters

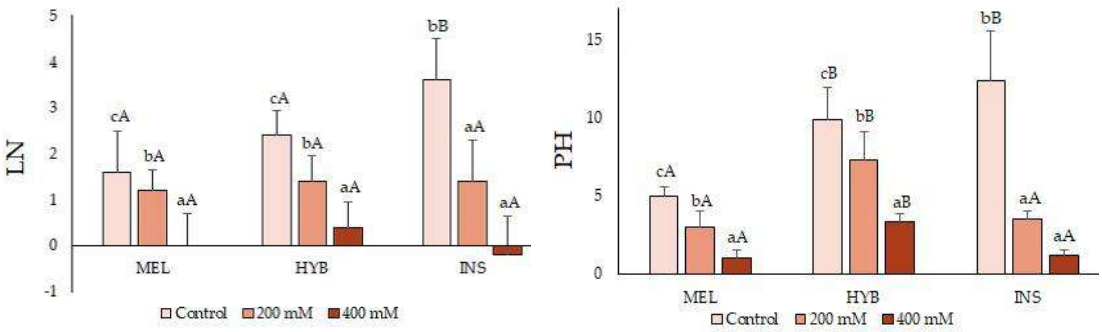
Salt stress inhibited growth in both parents and the hybrid (Figure 1). For a better comparison among genotypes, the leaf number (LN) and the plant height (PH) are shown in the figures as their increase compared with the corresponding values at the beginning of the treatment. Root length (RL), stem diameter (SD) and leaf surface (LS) are expressed in percentages of the corresponding mean values of the control, non-stressed plants, considered as 100%.

(a) (b)

(c)

Figure 1. *Solanum melongena* (a), hybrid (b) and *Solanum insanum* (c) plants at the end of the four-week growth period, in the control, and the 200 mM NaCl and 400 mM NaCl salt treatments (from left to right).

Under control, non-stress conditions, *S. insanum* (INS) plants apparently grew faster than the *S. melongena* (MEL) counterparts, as indicated by a larger increase in LN (Figure 2a) and PH (Figure 2b) during the four-week period, whereas values that were intermediate between those of the parents were obtained for the hybrid (HYB) plants. The three genotypes showed a significant concentration-dependent reduction in both parameters in the presence of salt (Figure 2a,b). However, the relative salt-induced growth inhibition suggested that the hybrid is somewhat more tolerant than either parent, especially at the highest salinity tested (Figure 2).



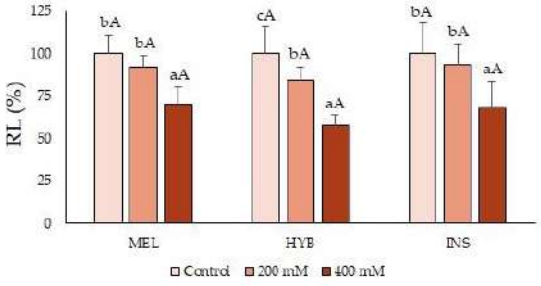
(a) (b)

Figure 2. Increase in leaf number (LN) (a) and plant height (PH) (b) (mean ± SD; n = 5) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in comparison with the beginning of the salt treatment (control, 200 and 400 mM of NaCl, as indicated). Different lowercase letters within the same genotype indicate statistically significant differences between treatments, and

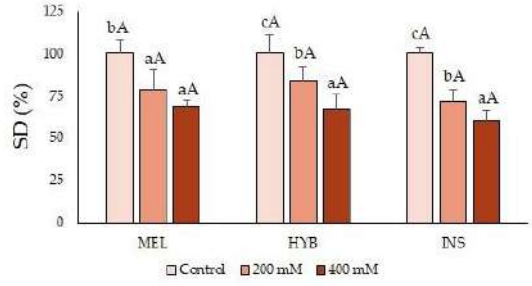
different uppercase letters within the same treatment indicate statistically significant differences between genotypes ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

Similarly, the salt treatments led to a concentration-dependent reduction in RL (Figure 3a), SD (Figure 3b) and LS (Figure 3c) in all plants, compared with the corresponding non-stressed controls. These differences were always statistically significant in the presence of 400 mM of NaCl but also, in most cases, in response to the lower salinity (200 mM NaCl) treatment. Regarding these growth parameters, however, no significant differences were detected between the three tested genotypes (Figure 3).

Water content (WC) was homogeneous in all plants and treatments, around 80%, indicating that the salt stress did not cause dehydration in the treated plants, except for slight water stem loss for INS (Table 2). In fact, MEL and the hybrid seemed to accumulate more water in the roots and showed better maintenance of humidity than INS in both roots and leaves. Nevertheless, fresh weight (FW) was considered the most relevant growth parameter. Root fresh weight (RFW, Figure 4a) was not affected by any of the treatments in the parent species; interestingly, however, the plants of the hybrid showed a significant increase in root growth in response to the 200 mM NaCl treatment (Figure 4a). On the other hand, stem fresh weight (SFW, Figure 4b) and leaf fresh weight (LFW, Figure 4c) were significantly reduced with increasing salt concentrations. Nevertheless, the hybrid was less affected at 200 mM than any of the parents, supporting the notion of its relatively higher salt tolerance.



(a)



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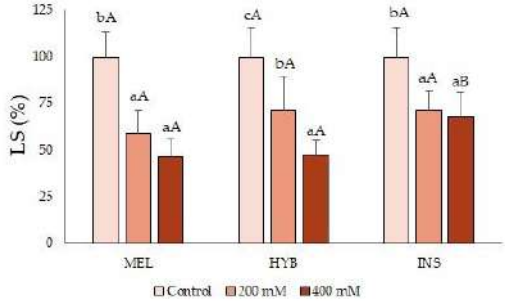
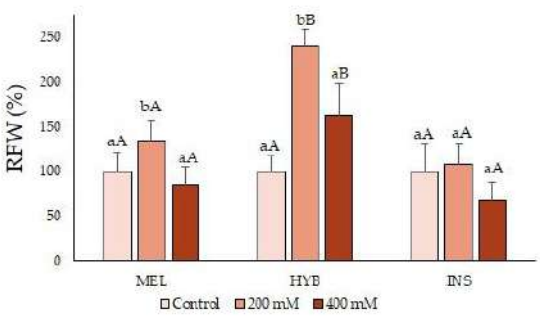


(c)

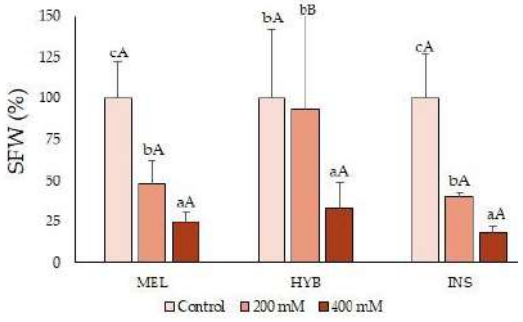
Figure 3. Root length (RL) (a), stem diameter (SD) (b) and leaf surface (LS) (c) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control, 200 mM and 400 mM of NaCl, as indicated). Percentages (mean ± SD; $n = 5$) refer to the corresponding control as 100%, with absolute values for RL (cm): [MEL = 30.4, HYB = 31.7, INS = 26.9], SD (mm): [MEL = 4.7, HYB = 5.2, INS = 5.3] and LS (cm^2): [MEL = 187.6, HYB = 207.9, INS = 121.3]. Different lowercase letters within the same genotype indicate statistically significant differences between treatments, and different uppercase letters within the same treatment indicate statistically significant differences between genotypes ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

Table 2. Root (RWC) (a), stem (SWC) (b) and leaf (LWC) (c) water content (%) (mean ± SD; $n = 5$) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control 200 mM and 400 mM of NaCl, as indicated). Different lowercase letters within the same genotype indicate statistically significant differences between treatments, and different uppercase letters within the same treatment indicate statistically significant differences between genotypes ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

		Treatment (NaCl Concentrations)		
		Control	**200 mM**	**400 mM**
RWC	MEL	77.6 ± 1.4 ^{aA}	84.2 ± 1.2 ^{bB}	83.5 ± 0.9 ^{bB}
	HYB	75.4 ± 1.6 ^{aA}	83.1 ± 0.5 ^{bB}	83.6 ± 0.4 ^{bB}
	INS	77.5 ± 1.8 ^{aA}	78.3 ± 0.4 ^{aA}	78.2 ± 0.3 ^{aA}
SWC	MEL	90.1 ± 4.5 ^{bA}	84.3 ± 1.7 ^{aA}	83.5 ± 0.9 ^{aA}
	HYB	79.9 ± 11.3 ^{aA}	77.3 ± 26.2 ^{aA}	75.1 ± 18.9 ^{aA}
	INS	78.7 ± 3.02 ^{bA}	76.2 ± 1.8 ^{bA}	71.1 ± 0.9 ^{aA}
LWC	MEL	81.8 ± 5.1 ^{aA}	86.1 ± 2.4 ^{aA}	85.6 ± 1.4 ^{aB}
	HYB	87.9 ± 6.3 ^{aA}	85.9 ± 0.9 ^{aA}	83.6 ± 0.4 ^{aB}
	INS	82.6 ± 3.2 ^{aA}	83.8 ± 1.3 ^{aA}	81.2 ± 1.5 ^{aA}



(**a**)



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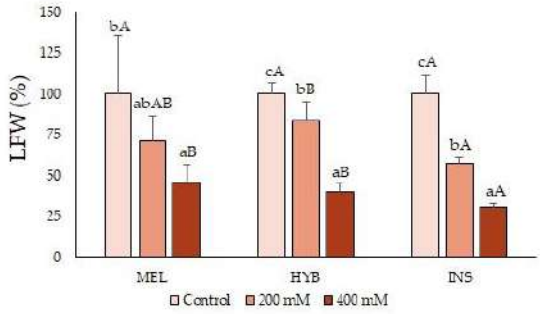


(**c**)

Figure 4. Root (RFW) (a), stem (SFW) (b) and leaf (LFW) (c) fresh weight in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control 200 mM and 400 mM of NaCl, as indicated). Percentages (mean ± SD; $n = 5$) are referred to the control as the 100% with absolute values for RFW (g): [MEL = 13.3, HYB = 12.2, INS = 12.2], SFW (g): [MEL = 6.8, HYB = 7.7, INS = 7.4] and LFW (g): [MEL = 13, HYB = 13.1, INS = 16.2]. Different lowercase letters within the same genotype indicate statistically significant differences between treatments, and different uppercase letters within the same treatment indicate statistically significant differences between genotypes ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

2.3 Photosynthetic pigments

In general, no significant differences were found between treatments or genotypes in the major pigments under most experimental conditions tested (Table 3). For photosynthetic pigments, only chlorophyll *a* (ChlA) showed significantly higher values in the hybrid than in the parents in the presence of 400 mM of NaCl. The carotenoid (Caro) contents did not vary between the three genotypes, although the values calculated for INS plants were significantly lower at 400 mM of NaCl than in the control or at 200 mM of NaCl.

Table 3. Chlorophyll *a* (ChlA) (mg g^{-1} dry weight), chlorophyll *b* (ChlB) (mg g^{-1} dry weight) and carotenoids (Caro) (mg g^{-1} dry weight) values (mean ± SD; $n = 5$) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control, 200 mM and 400 mM of NaCl). Different lowercase letters within the same row indicate statistically significant differences between treatments, and different uppercase letters within the same column indicate statistically significant differences between genotypes for each measured compound ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

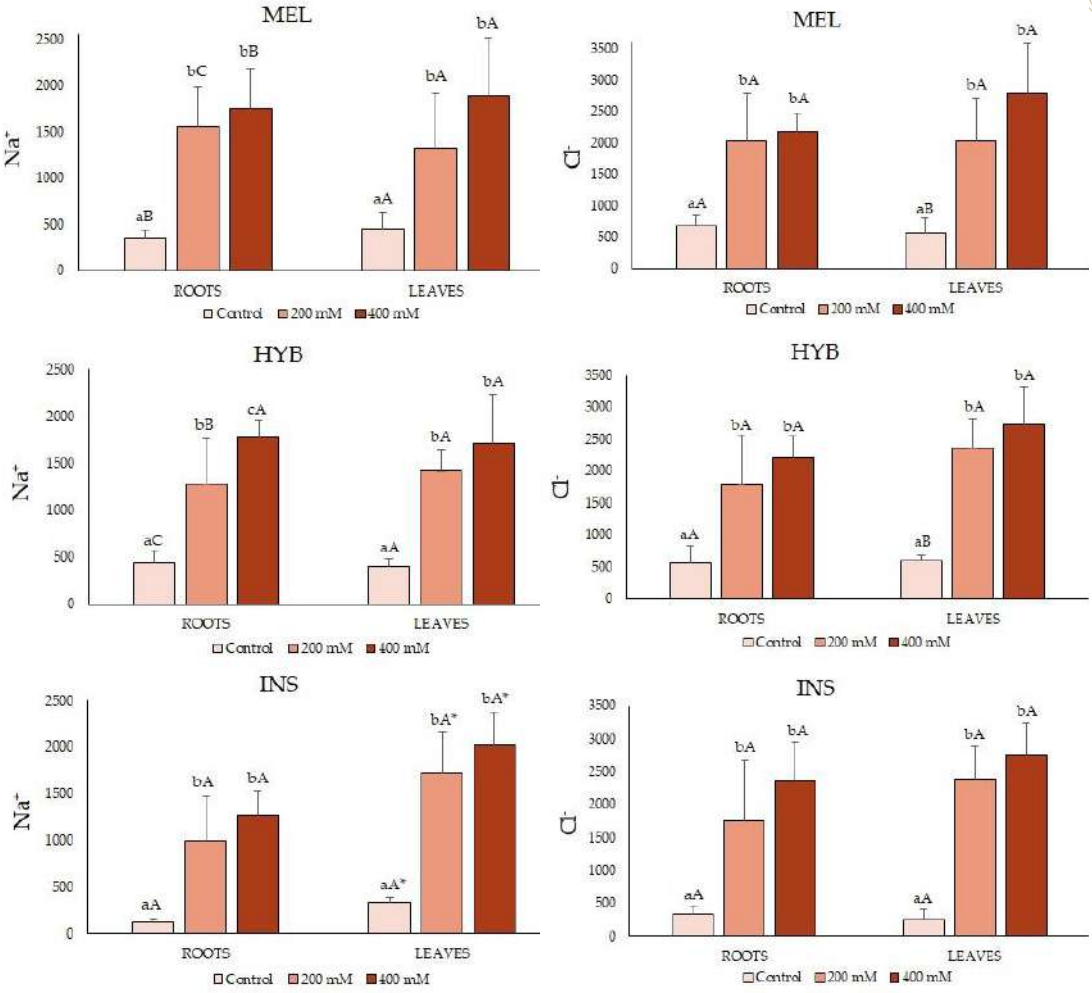
		Treatment (NaCl Concentrations)		
		Control	200 mM	400 mM
ChlA	MEL	2.8 ± 0.8 ^{aA}	3.1 ± 1.7 ^{aA}	2.2 ± 0.9 ^{aA}
	HYB	2.7 ± 1.3 ^{aA}	2.7 ± 1.4 ^{aA}	4.4 ± 1.9 ^{aB}
	INS	1.5 ± 0.9 ^{aA}	2.9 ± 1.9 ^{aA}	1.8 ± 1.1 ^{aA}
ChlB	MEL	0.9 ± 1.4 ^{aA}	1.6 ± 1.3 ^{aA}	0.8 ± 0.9 ^{aA}
	HYB	0.5 ± 0.4 ^{aA}	0.3 ± 0.1 ^{aA}	0.7 ± 0.5 ^{aA}
	INS	1.0 ± 1.1 ^{aA}	0.9 ± 0.3 ^{aA}	0.4 ± 0.2 ^{aA}
Caro	MEL	0.8 ± 0.4 ^{aA}	0.9 ± 0.4 ^{aA}	0.6 ± 0.4 ^{aA}
	HYB	1.0 ± 0.3 ^{aA}	0.6 ± 0.2 ^{aA}	0.8 ± 0.3 ^{aA}
	INS	0.9 ± 0.3 ^{bA}	1.0 ± 0.2 ^{bA}	0.5 ± 0.2 ^{aA}

2.4 Ion contents

The mean Na^+ contents significantly increased with increasing external salinity in a concentration-dependent manner; the patterns of Na^+ accumulation were roughly the same in roots and leaves and for the three genotypes (Figure 5a), and the absolute Na^+ levels were also similar for each treatment, with a few exceptions. For example, the differences between 200 and 400 mM of NaCl were always non-significant, except in the roots of the hybrid, with values of 1778 and 1284 µmol g^{-1} DW, at 400 and 200 mM of NaCl, respectively. Moreover, the Na^+ contents in INS were significantly higher in leaves than in roots for all treatments; this eggplant wild relative also showed somewhat lower Na^+ levels in the roots compared to MEL and the hybrid (Figure 5a). The observed Cl^- content patterns were similar to those of Na^+, although its absolute concentrations were somewhat higher for each treatment in both organs and the three genotypes (Figure 5b).

In the plants of all three tested genotypes, salt treatments did not cause any significant change in K^+ concentrations in roots compared to the corresponding non-stressed controls (Figure 5c). In leaves, however, different K^+ accumulation patterns were observed in the two parent species upon the salt treatments: a significant salt-induced decrease of about 50% in MEL, whereas K^+ levels significantly increased by more than 40%, in INS plants subjected to 400 mM of NaCl—although the lower salinity treatment (200 mM) had no effect. In the hybrid, leaf K^+ concentrations neither decreased nor increased in response to the salt treatments and were maintained around 500 µmol g^- DW in the control and the stressed plants (Figure 5c)

The Ca^{2+} contents followed the same pattern in both parents, in leaves and roots, significantly increasing in the presence of NaCl. In the hybrid, the measured values were intermediate between those of the parents; they showed a significant difference between 200 mM and 400 mM of NaCl. The average Ca^{2+} concentrations were higher in roots than in leaves for each salt concentration; in the case of INS, these differences (about two-fold) were statistically significant (Figure 5d).


MEL
Na+
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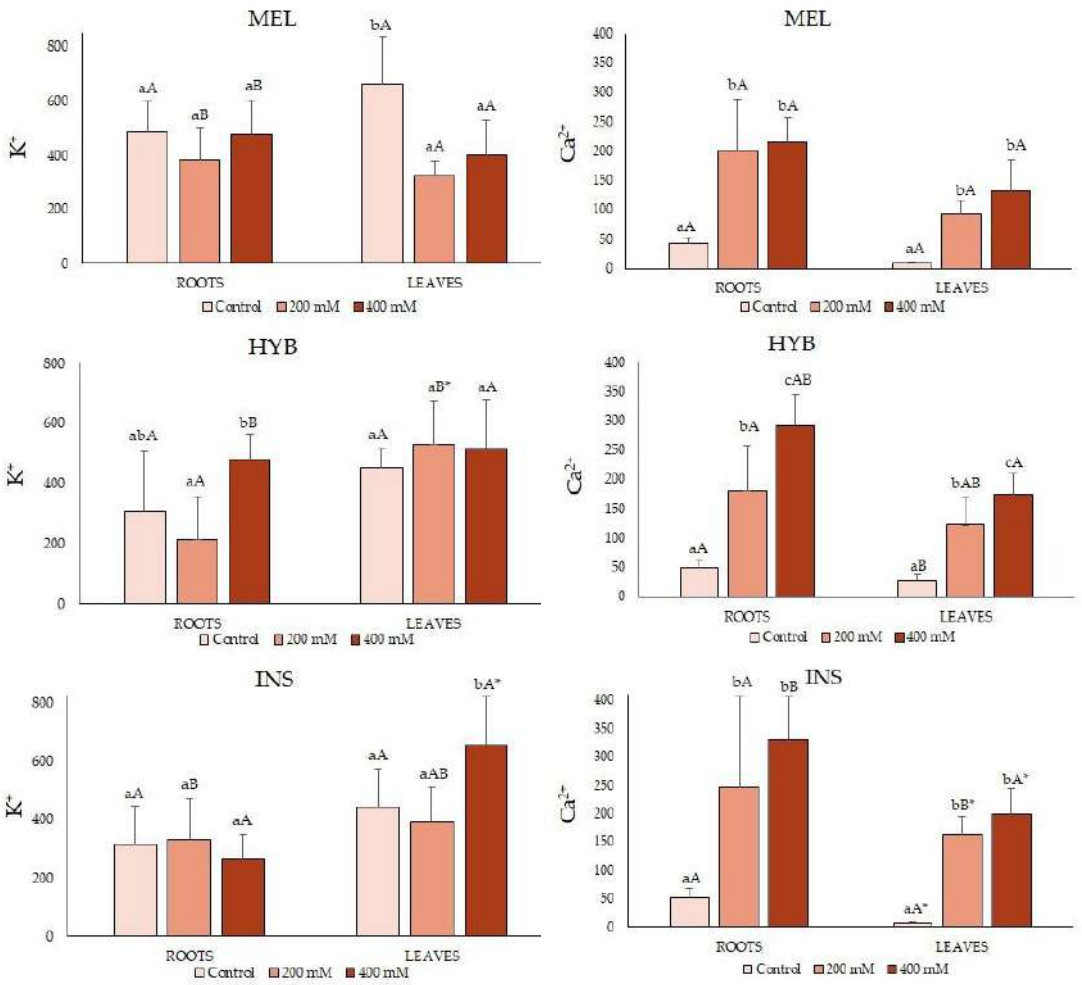
(a) (b)



(c) (d)

Figure 5. Na^+ (a), Cl^- (b), K^+ (c) and Ca^{2+} (d) content (μmol g^{-1} dry weight) in roots (left) and leaves (right) (mean ± SD; $n = 5$) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control, 200 mM and 400 mM of NaCl). Different lowercase letters within the same genotype indicate statistically significant differences between treatments, different uppercase letters within the same treatment indicate statistically significant differences between genotypes, and an asterisk indicates statistically significant differences between roots and leaves for the same treatment and genotype ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

The Na^+/K^+ ratio values were similar under the control conditions, near 1, in all samples except in hybrid roots, near 2. The ratios were higher in the salt-treated plants, with some differences between genotypes (Figure 6). In leaves, the hybrid and INS showed lower values than MEL. In roots, however, a higher average ratio was

calculated for the hybrid than for both parents, at 200 mM of NaCl, although the differences were not statistically significant (Figure 6).

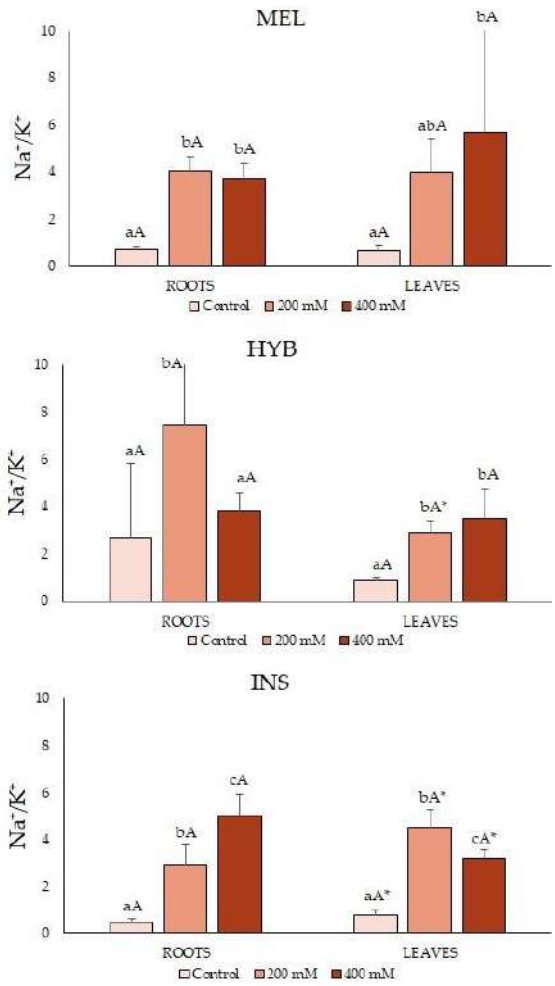


Figure 6. Na^+/K^+ relation in roots (left) and leaves (right) (mean ± SD; $n = 5$) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control, 200 mM and 400 mM of NaCl). Different lowercase letters within the same genotype indicate statistically significant differences between treatments, different uppercase letters within the same treatment indicate statistically significant differences between genotypes, and an asterisk indicates statistically significant differences between roots and leaves for the same treatment and genotype ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

2.5 Osmolyte quantification, MDA, H_2O_2 and antioxidant compounds

Proline (Pro) is a reliable stress marker in most species of the Solanaceae family. Leaf Pro contents increased in response to the salt treatments in the three genotypes but reached absolute values about 25-fold higher in INS and the hybrid than in MEL (Table 4). In the non-stressed controls, the hybrid tended to accumulate more Pro than both parents. Under salt stress conditions, both the hybrid and INS accumulated more Pro than MEL.

The total soluble sugars (TSS) contents did not vary significantly between treatments or genotypes, with values around 200 mg equiv. glu g^{-1} DW in all cases (Table 4). Similarly, the levels of oxidative stress biomarkers (MDA, H_2O_2) and total phenolic compounds (TPC, representative antioxidant metabolites), in general, did not significantly increase in response to the salt treatments, except for H_2O_2 in INS plants watered with 400 mM of NaCl (Table 4). Total flavonoids (TF) levels showed significant differences between genotypes in the control and 200 mM NaCl treatments, with the values of the hybrid closer to the wild parent species than the cultivated eggplant; however, no differences were detected between treatments for each genotype (Table 4).

Table 4. Proline (Pro) (µmol g^{-1} dry weight), total soluble sugars (TSS) (mg equiv. glucose g^{-1} dry weight), malondialdehyde (MDA) (nmol g^{-1} dry weight), hydrogen peroxide (H_2O_2) (µmol g^{-1} dry weight), total phenolic compounds (TPC) (mg equiv. gallic acid g^{-1} dry weight), and total flavonoids (TF) (mg equiv. catechin g^{-1} dry weight) values (mean ± SD; $n = 5$) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control, 200 mM and 400 mM of NaCl). Different lowercase letters within the same row indicate statistically significant differences between treatments, and different uppercase letters within the same column indicate statistically significant differences between genotypes for each measured compound ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

		Treatment (NaCl Concentrations)		
		Control	**200 mM**	**400 mM**
Pro	MEL	11.9 ± 1.4 ^{aA}	85.7 ± 13.9 ^{bA}	134.1 ± 22.6 ^{cA}
	HYB	107.8 ± 42.4 ^{aB}	2155.9 ± 258.9 ^{bB}	3217.5 ± 697.9 ^{bB}
	INS	27.3 ± 35.5 ^{aA}	2233.5 ± 265.5 ^{bB}	3717.1 ± 318.1 ^{cB}
TSS	MEL	160.2 ± 39.7 ^{aA}	133.0 ± 63.7 ^{aA}	224.1 ± 60.6 ^{aA}
	HYB	205.8 ± 69.9 ^{aA}	180.9 ± 50.1 ^{aA}	209.9 ± 32.8 ^{aA}
	INS	227.4 ± 120.0 ^{aA}	144.6 ± 78.9 ^{aA}	187.9 ± 31.4 ^{aA}
MDA	MEL	623.7 ± 277.5 ^{aA}	544.4 ± 119.8 ^{aA}	493.9 ± 77.3 ^{aA}
	HYB	658.3 ± 111.6 ^{aA}	437.2 ± 147.5 ^{aA}	443.3 ± 162.0 ^{aA}
	INS	569.1 ± 323.2 ^{aA}	597.3 ± 131.9 ^{aA}	477.9 ± 171.3 ^{aA}

H_2O_2	MEL	6.6 ± 0.9 ^{aA}	5.6 ± 1.8 ^{aA}	11.4 ± 7.33 ^{aAB}
	HYB	5.5 ± 1.6 ^{aA}	5.1 ± 2.8 ^{aA}	9.4 ± 4.7 ^{aA}
	INS	28.5 ± 6.7 ^{aB}	21.3 ± 5.5 ^{aB}	50.8 ± 11.8 ^{bB}
TPC	MEL	23.3 ± 8.6 ^{aA}	26.7 ± 9.6 ^{aA}	36.2 ± 11.4 ^{aA}
	HYB	37.8 ± 11.6 ^{aB}	25.2 ± 3.3 ^{aA}	32.5 ± 14.2 ^{aA}
	INS	31.2 ± 6.5 ^{aAB}	34.5 ±4.6 ^{aA}	41.3 ± 11.3 ^{aA}
TF	MEL	4.6 ± 3.5 ^{aA}	7.8 ± 4.4 ^{aA}	11.8 ± 6.6 ^{aA}
	HYB	11.9 ± 4.2 ^{aB}	11.3 ± 2.8 ^{aAB}	9.7 ± 5.5 ^{aA}
	INS	12.3 ± 1.5 ^{aB}	15.2 ± 2.7 ^{aB}	16.6 ± 5.1 ^{aA}

2.6 Antioxidant enzymes

The salt treatments did not increase the specific activity of relevant antioxidant enzymes, such as superoxide dismutase (SOD), aspartate peroxidase (APx) or glutathione reductase (GR) in any of the studied genotypes (Table 5), as should be expected, since no salt-induced oxidative stress was observed. Under the assay conditions, no catalase (CAT) activity could be detected (data not shown). The enzymes activities showed great variability within the same genotype and treatment, as shown by the relatively high SD values.

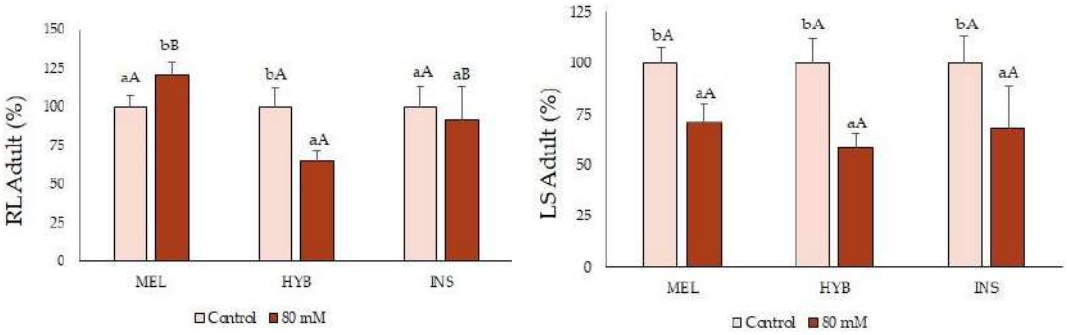
Table 5. Superoxide dismutase (SOD), ascorbate peroxidase (APx) and glutathione reductase (GR) specific activities (units mg^{-1} protein) (mean ± SD; $n = 5$) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control, 200 mM and 400 mM of NaCl). Different lowercase letters within the same row indicate statistically significant differences between treatments, and different uppercase letters within the same column indicate statistically significant differences between genotypes for each measured compound ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

		Treatment (NaCl Concentrations)		
		Control	**200 mM**	**400 mM**
APx	MEL	48.9 ± 29.2 ^{aA}	89.9 ± 58.2 ^{aB}	87.4 ± 28.2 ^{aA}
	HYB	98.5 ± 65.3 ^{aA}	124.5 ± 20.7 ^{aB}	267.2 ± 161.8 ^{aA}
	INS	62.7 ± 64.0 ^{aA}	16.7 ± 11.2 ^{aA}	83.0 ± 28.6 ^{aA}
SOD	MEL	90.2 ± 47.3 ^{aA}	84.3 ± 34.9 ^{aA}	89.2 ± 35.3 ^{aAB}
	HYB	144.2 ± 55.0 ^{aB}	92.3 ± 24.8 ^{aA}	128.3 ± 34.4 ^{aB}
	INS	61.9 ± 20.1 ^{aA}	56.3 ± 13.1 ^{aA}	55.2 ± 21.7 ^{aA}
GR	MEL	948.1 ± 203.5 ^{aA}	703.7 ± 196.8 ^{aAB}	638.4 ± 68.6 ^{aA}
	HYB	1018.9 ± 363.5 ^{aA}	863.9 ± 369.0 ^{aB}	1175.6 ± 438.7 ^{aA}
	INS	349.1 ± 172.7 ^{aA}	313.6 ± 157.0 ^{aA}	359.7 ± 232.6 ^{aA}

2.7 Adult plant performance

To complement the previous data, adult plants at the pre-flowering stage were subjected to longer (eight weeks) treatments with lower (80 mM of NaCl) salt concentrations. After the treatment, growth parameters, root and leaf ion contents, and leaf Pro levels were determined in the plants of MEL, INS and their hybrid.

Regarding root length (RL), the salt treatment caused a small but significant increase in MEL plants, whereas it did not affect INS; on the other hand, RL was significantly reduced in HYB plants in the presence of 80 mM of NaCl (Figure 7a). The mean leaf surface (LS) values saw a similar decrease in the three genotypes, although to a somewhat lesser extent in MEL plants (Figure 7b).



(a) (b)

Figure 7. Root length (RL) (a) and leaf surface (LS) (b) (mean ± SD; $n = 5$) in adult plants of *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in control conditions and under long-term (eight weeks) 80 mM NaCl irrigation. Values are shown as percentages of the corresponding controls of each genotype, taken as 100%, with absolute values for RL (cm): [MEL = 65.2, HYB = 68.6, INS = 71.6] and LS (cm^2): [MEL = 141, HYB = 172.4, INS = 140.8]. Different lowercase letters within the same genotype indicate statistically significant differences between treatments, and different uppercase letters within the same treatment indicate statistically significant differences between genotypes ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

No effect of the salt treatment on biomass accumulation was observed at the root level (Figure 8a). However, salt stress significantly reduced the growth of the aerial part of the plants to a similar degree for MEL, INS and their hybrid, as shown by the decrease in SFW and LFW (Figure 8b,c).

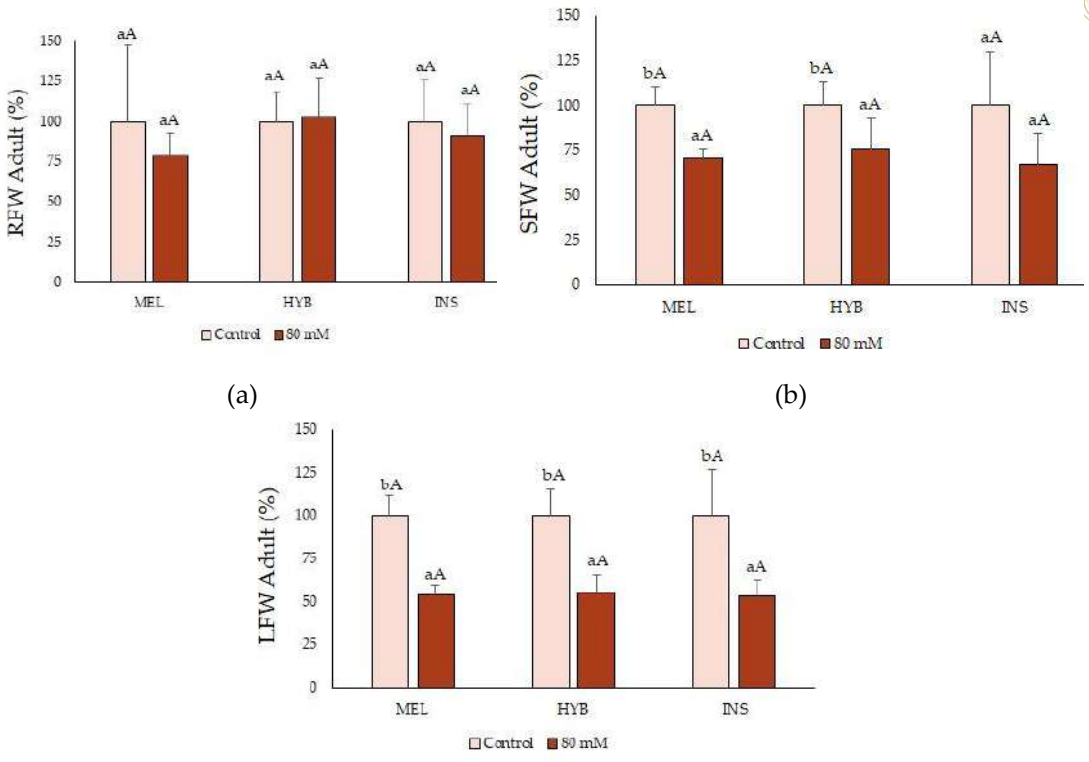


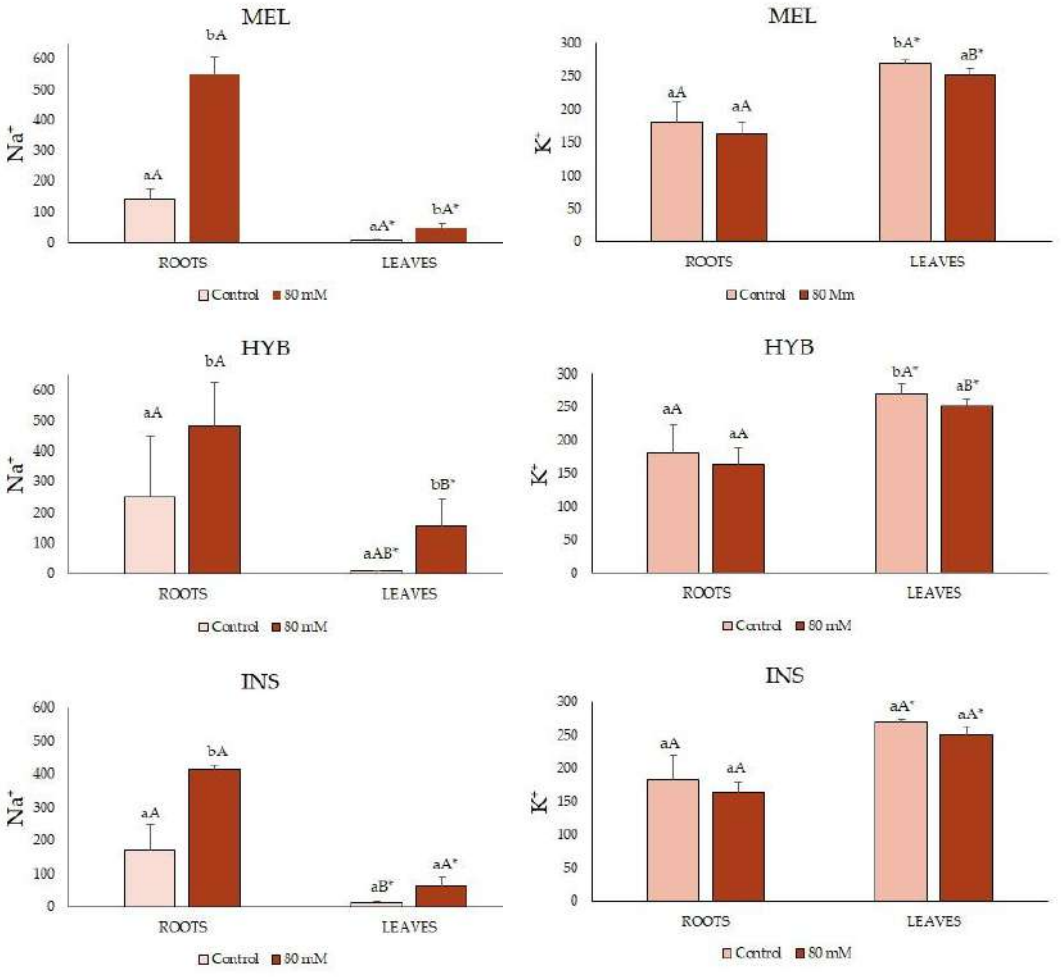
Figure 8. Root (RFW) (a), stem (SFW) (b) and leaf (LFW) (c) fresh weight (mean ± SD; n = 5) in adult plants of *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in control conditions and under long term (eight weeks) 80 mM NaCl irrigation. Values are shown as percentages of the corresponding controls of each genotype, taken as 100%, with absolute values for RFW (g): [MEL = 129.9, HYB = 177.4, INS = 111.2], SFW (g): [MEL = 120.1, HYB = 185.3, INS = 131.2] and LFW (g): [MEL = 130.2, HYB = 195.1, INS = 174]. Different lowercase letters within the same genotype indicate statistically significant differences between treatments, and different uppercase letters within the same treatment indicate statistically significant differences between genotypes ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

The adult plants showed significant differences between leaf and root ion content for all analysed ions and the three tested genotypes. Thus, Na^+ concentrations were much higher in roots than in leaves; K^+ and Ca^{2+}, on the contrary, accumulated to significantly higher levels in leaves than in roots, although the differences between organs were relatively small compared to those observed for Na^+ (Figure 9).

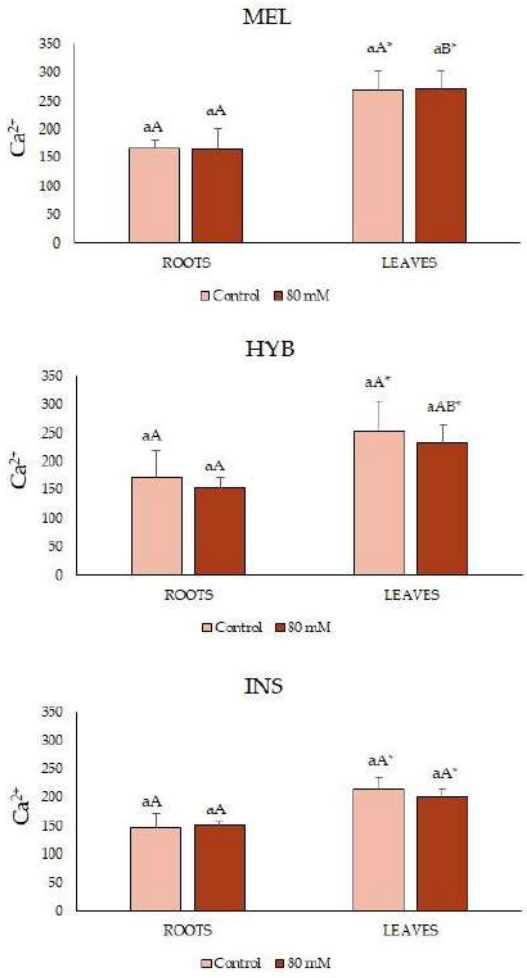
Regarding the effect of the salt treatment on ion levels, Na^+ concentrations increased after eight weeks in the presence of 80 mM of NaCl, as expected; the differences with the controls were statistically significant in all samples, except in the

leaves of INS plants (Figure 9a). Interestingly, the leaf Na^+ contents were significantly higher in the hybrid than in its two parents (Figure 9a). Contrary to Na^+, mean K^+ (Figure 9b) and Ca^{2+} (Figure 9c) concentrations varied little in response to the salt treatment, neither in the roots nor in the leaves of the plants of the three genotypes. Indeed, a slight NaCl-induced reduction in leaf K^+ contents in MEL and HYB plants was the only significant difference detected (Figure 9b). It should also be pointed out that the mean K^+ and Ca^{2+} leaf contents were higher than those of Na^+ for the three analysed genotypes (Figure 9).

The Na^+/K^+ ratios remained low, below 1.0, under the control conditions for the three genotypes tested, especially in leaves, which showed much lower values than roots. These ratios significantly increased, in all cases, in response to the NaCl treatment (Figure 10).



(a) (b)



(c)

Figure 9. Na^+ (a), K^+ (b), and Ca^{2+} (c) content (µmol g^{-1} dry weight) in roots (left) and leaves (right) (mean ± SD; $n = 5$) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control, 80 mM of NaCl). Different lowercase letters within the same genotype indicate statistically significant differences between treatments, different uppercase letters within the same treatment indicate statistically significant differences between genotypes, and an asterisk indicates statistically significant differences between roots and leaves for the same treatment and genotype ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

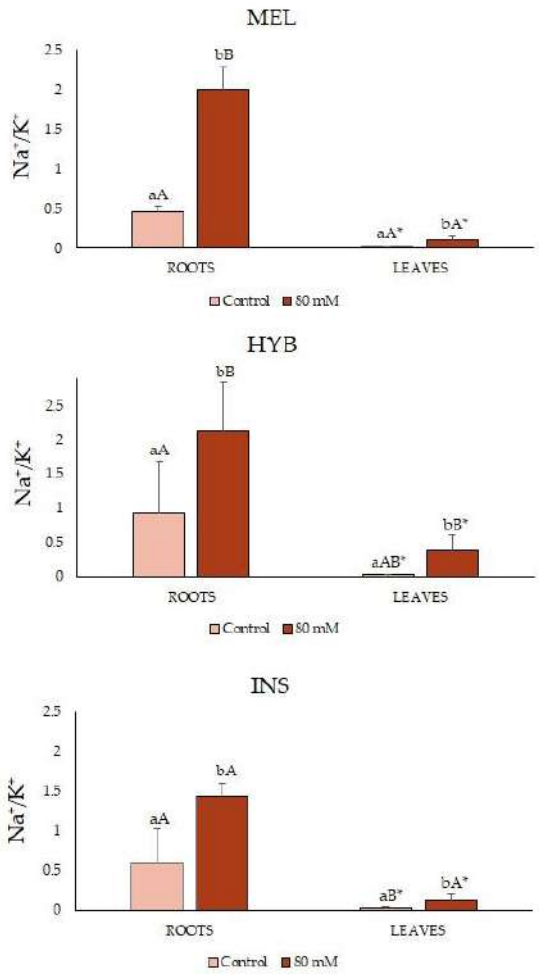


Figure 10. Na^+/K^+ ratio in roots (left) and leaves (right) (mean ± SD; $n = 5$) in *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in the conditions tested (control, 80 mM of NaCl). Different lowercase letters within the same genotype indicate statistically significant differences between treatments, different uppercase letters within the same treatment indicate statistically significant differences between genotypes, and an asterisk indicates statistically significant differences between roots and leaves for the same treatment and genotype ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

The leaf Pro contents increased significantly in response to the salt treatment in MEL and HYB plants, about ten- and five-fold, respectively, with respect to the corresponding controls. The mean Pro content also increased in salt-treated INS plants, but the difference with the control was not statistically significant (Table 6). Therefore, in the hybrid, absolute Pro levels under control and salt stress conditions, and their relative increase, were intermediate between those of the parent species.

Table 6. Proline (Pro) values (μmol g^{-1} dry weight) (mean ± SD; $n = 5$) in adult plants of *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid (HYB) in control conditions and under long-term (eight weeks) 80 mM NaCl irrigation. Different lowercase letters within the same row indicate statistically significant differences between treatments, and different uppercase letters within the same column indicate statistically significant differences between genotypes ($p < 0.05$), according to the Student–Newman–Keuls multiple range test.

		Treatment (NaCl Concentrations)	
		Control	**80 mM**
Pro	MEL	13.6 ± 3.7 ^{aA}	125.1 ± 54.1 ^{bB}
	HYB	9.8 ± 6.1 ^{aA}	46.5 ± 22.8 ^{bA}
	INS	6.7 ± 4.8 ^{aA}	18.6 ± 18.8 ^{aA}

3. Discussion

Recent studies have revealed that, even though *Solanum melongena* has been described as moderately susceptible –or tolerant– to salt stress, the response largely depends on the genotype (Hannachi & Van Kabeje, 2018; Mustafa, Ayyub, Amjad, & Ahmad, 2017; Plazas et al., 2019; Zayova, Philipov, Nedev, & Stoeva, 2017). The effects of salt stress on plant development are well established (Gupta & Huang, 2014; Munns, 2005), although specific response mechanisms depend on the genotype and the experimental conditions tested (Brenes et al., 2020). The present work compared the behaviour of an eggplant cultivar (MEL), the wild relative *Solanum insanum* (INS) and their hybrid (HYB), with the aim of establishing possible differences in salt tolerance between the tested genotypes and the inheritance of those tolerance mechanisms. Our general assumption was that both parents, closely related genetically, would not differ in the type of responses to salt stress but could differ in the magnitude or efficiency of those responses.

High salt concentrations cause growth inhibition in plants (Hannachi & Van Labeke, 2018; Ünlükara et al., 2008), which was also observed in the present work for the three studied genotypes, especially at 400 mM NaCl. Some quantitative differences between both parents were detected in our experiments; for example, the wild relative seemed to grow more rapidly than the cultivated eggplant in the absence of salt, as shown by the larger increase in leaf number and plant height. However, considering most measured growth variables, MEL and INS plants did not differ substantially in terms of their relative growth inhibition under salt stress, indicating that the degree of salt tolerance was similar for the tested *S. melongena* cultivar and *S. insanum.*

However, in a previous study, another *S. melongena* cultivar was shown to be less salt-tolerant than the wild relative at high salinities (Brenes et al., 2020),

confirming the genotype dependence of this trait in eggplant. On the other hand, our results pointed to a higher tolerance of the hybrid than either parent: a smaller relative reduction in the leaf surface and plant height and, most importantly, a relatively higher biomass accumulation (FW of stems and leaves) in the presence of 200 mM of NaCl. Moreover, while root FW did not change in response to the salt treatments in MEL and INS plants, it significantly increased in hybrid plants treated with 200 mM of NaCl. Since the primary root length actually decreased, the intermediate salt treatment most likely induced the growth of secondary roots to enhance water uptake from the soil, as has been observed in some other species (Franco et al., 2011; Julkowska et al., 2017; Wang, Li, & Li, 2009; Zolla, Heimer, & Barak, 2010), suggesting a specific adaptation to salt stress in the hybrid. The higher efficiency of the defence mechanisms against salt stress observed in the hybrid may relate to the well-known phenomenon of heterosis that hybrids show in comparison to their parents, leading to improving important agronomical quantitative traits, such as growth vigour, seed production, yield or even stress survival (Bernardes, Stelkens, & Greig, 2017; Botet & Keurenties, 2020). Heterosis has been observed and studied in many different plant species, including, for example, maize, rice or tomato (Cheng et al., 2007; Krieger, Lippman, & Zamir, 2010; Kutka, 2011), as well as in eggplant (Kumar, Sharma, Jaim, & Kaushik, 2020). This hybrid vigour would be of great interest in future eggplant breeding prospects, but the genetic basis of heterosis is still under study.

Salinity is generally associated with the degradation of photosynthetic pigments, which are more pronounced in less tolerant species, reducing photosynthesis activity in the plant. Consequently, chlorophylls *a* and *b* (ChlA and ChlB), and carotenoid (Caro) contents, might be used as stress biomarkers (Hamani et al., 2020; Sudhir & Murthy, 2004). In the present work, however, we did not observe a significant salt-induced reduction in ChlA, ChlB or Caro in any of the genotypes, thus supporting the moderate salt tolerance of eggplant and its wild relative. Interestingly, the ChlA contents were higher in the hybrid than in both parents in the presence of 400 mM of NaCl, which was probably also due to a heterotic effect.

Na^+ and Cl^- contents accumulated in leaves and roots when salinity increased, with similar patterns for *S. melongena, S. insanum* and the hybrid, as previously reported in different eggplant cultivars (Shahbaz, Mushtaq, Andaz, & Masood, 2013; Shaheen, Naseer, Ashraf, & Akram, 2013). However, we did not observe significant differences between roots and leaves, except for *S. insanum*, with a more significant Na^+ accumulation in leaves. This result supports the idea that in *S. insanum*, Na^+ is transported from roots to leaves and is sequestered in leaf vacuoles with a somewhat higher efficiency than in the cultivated eggplant.

An increase in Na^+ contents in response to increasing salinity would generally promote a decrease in K^+ concentration, as both cations compete for the same

transport proteins of the membrane (Hannachi & Van Lebeke, 2018; Shahbaz, Mushtaq, Andaz, & Masood, 2013; Shaheen, Naseer, Ashraf, & Akram, 2013). For this reason, the Na^+/K^+ ratio has been reported as a possible indicator of salt tolerance in plants (Shabala & Cuin, 2008; Zhu, 2003). Some studies revealed a limited Na^+ accumulation in eggplant leaves to maintain lower Na^+/K^+ ratios, reducing Na^+ uptake or compartmentalising it into root vacuoles (Ashraf & Akram, 2009; Li et al., 2019). In this study, we observed that the plants of the three genotypes maintained root K^+ levels under salt stress, which likely contributes to salt tolerance in eggplant, its wild relative and their hybrid. Changes in leaf K^+ concentrations were also not detected in salt-treated HYB plants, which in this respect showed an intermediate behaviour between its parent species. The Na^+ contents measurements indicated that ion transport in response to salt differs in both parental species. In INS, Na^+ levels were higher in leaves, whereas Na^+ predominantly accumulated in roots in MEL and the HYB, which in this respect was more similar to the cultivated eggplant than the wild relative.

The Ca^{2+} contents increased in the presence of salt, with higher accumulation in roots than in leaves, supporting the notion that Ca^{2+} is beneficial for maintaining Na^+ and K^+ homeostasis, at least partly via the SOS pathway (Mahajan, Pandey, & Tuteja, 2008). Moreover, due to its critical regulatory and signaling roles in plant growth and development (Hepler, 2005), increasing Ca^{2+} in the leaves of salt-stressed plants is important to maintain essential metabolic and cellular processes.

Proline (Pro) is a reliable marker of abiotic stress in many plant species, increasing its concentration in stressed plants in relation to its background levels in non-stressed controls (Munns, 2008). In addition to its role in osmotic adjustment, Pro is an osmoprotectant because of its function as a low-molecular-weight chaperon, maintaining protein structure and membrane integrity, as well as ROS detoxification under stress conditions (Alvarez, Savouré, & Szabados, 2022). Several reports have shown a positive correlation between Pro accumulation and stress tolerance when comparing related taxa, suggesting the direct participation of Pro in the mechanisms of tolerance (Al Hassan et al., 2016; Dar et al., 2016; Szabados & Savouré, 2010). In other cases, however, Pro contents simply indicate the relative degree of stress affecting the plants, accumulating at higher levels in the more-stressed, less-tolerant genotypes, indicating that Pro is not directly involved in relevant tolerance mechanisms (Kaur & Asthir, 2015; Mustafa, Ayyub, Amjad, & Ahmad, 2017; Plazas et al., 2019). Our results showed higher Pro contents in the control plants of the hybrid than in *S. melongena* or *S. insanum*, suggesting a constitutive mechanism of stress response. The salt treatments led to significant, concentration-dependent increases in Pro accumulation in all three genotypes; however, the absolute concentrations reached were much higher, around 25-fold, in INS and HYB than in MEL. Therefore, regarding Pro biosynthesis and accumulation in response to salt stress, the hybrid was much closer to the wild relative than the cultivated eggplant.

Soluble sugars (TSS) play a role in osmoregulation under stress environments in some plant species, amongst other multiple functions unrelated to specific responses to stress (Ozturk et al., 2021; Pipatsitee et al., 2022). This report does not show any significant variation in TSS contents in response to salt stress, indicating that sugar accumulation is not a relevant mechanism of salt tolerance in eggplant.

Malondialdehyde (MDA), a product of lipid peroxidation, is an excellent marker of oxidative stress generated as a secondary effect of high salinity and other abiotic stresses due to the increase in cellular ROS levels (Gouiaa et al, 2012). Therefore, in general, it should be expected that MDA contents increase in plants in the presence of salt, as should the levels of antioxidant compounds and enzyme activities, in order to counteract oxidative stress (Aleem et al., 2022; Birhanie et al., 2022; De Rossi et al., 2021). In this study, however, MDA contents did not vary significantly in response to the salt treatments. Similarly, no salt-induced increase in H_2O_2 contents was detected, in agreement with the maintenance of low ROS levels, but for a slight increase in INS at 400 mM of NaCl. These data suggest that salt stress responses based on the control of ion transport and Pro accumulation are efficient enough to avoid the generation of oxidative stress in salt-treated MEL, INS or HYB plants under our experimental conditions. In the absence of oxidative stress, the activation of antioxidant enzymes or the synthesis of antioxidant metabolites, such as phenolic compounds, was not detected, as should be expected.

The responses of adult plants to lower salt concentrations during longer treatment were qualitatively similar, in most cases, to those described above for younger plants, albeit generally weaker. This behaviour could be explained by the increase in stress tolerance with plant age, which has been observed in many plant species (Johnson, Smith, & Dobrenz, 1992; Ragab & Taba, 2016; Vicente et al., 2004). The salt treatment also caused an inhibition of growth in adult plants, mainly reflected in a reduction in the FW of the aerial part, stem and leaves, but without significant differences between the three genotypes.

The Na^+ contents significantly increased in salt-treated plants with respect to the non-treated controls, in roots and leaves. However, contrary to what was observed in younger plants, Na^+ concentrations were much lower in leaves than in roots. It seems that adult plants can efficiently block the transport of the toxic ions to the aerial part of the plants. On the other hand, K^+ and Ca^{2+} concentrations were slightly (but significantly) higher in leaves than in roots and did not vary in response to the salt treatment or between genotypes. Compared to young plants, where Ca^{2+} is accumulated primarily in roots, Ca^{2+} concentrations were slightly but significantly higher in leaves in adult plants. Moreover, Na^+/K^+ ratios were significantly lower in adult hybrid roots compared to young hybrid roots. The relatively low accumulation in the leaves of Na^+ ions, and the higher contents of K^+ and Ca^{2+}, partly counteracting the deleterious effects of salt stress, most likely contribute to the higher tolerance of adult plants.

Finally, Pro contents increased in the plants treated with 80 mM of NaCl, although the relative increase over control levels and the maximum values reached were lower than in young plants. In this case, however, the lowest Pro concentrations were measured in INS plants and the highest in MEL, with the hybrid showing intermediate values. Since toxic Na^+ accumulated in leaves at much lower levels than in young plants, it seems logical to assume that lower Pro concentrations are also required for osmotic adjustment.

4. Materials and methods

4.1 Plant material, seed germination and plant growth

Seeds of *Solanum melongena* (MEL), *Solanum insanum* (INS) and their hybrid *S. insanum* × *S. melongena* (HYB), originally obtained from Sri Lanka and maintained in the COMAV eggplant collection (Universitat Politècnica de València, Spain) (Plazas et al., 2016)), were sown following the protocol of Ranil et al. (2015). The seeds were immersed for 24 h in distilled water, followed by 24 h in a solution of 1.4 mM of gibberellic acid (GA). After the treatment, the seeds were placed in standard Petri dishes (90 mm in diameter) with a sterile cotton layer covered with filter paper and wet with 10 mM of potassium nitrate (KNO_3). The seeds were incubated at 4 ºC covered with aluminum foil for seven days, followed by 24 h at 37 ºC, before placing them in the germination chamber (25 ºC, 78% humidity and a photoperiod of 16/8 h with a mean light intensity of 1000 lux).

Plants with four leaves were transplanted in the greenhouse into pots of 14.5 cm in diameter, 12.5 cm in height (1.3 L Series CD Alto Thermoformed Pots) with commercial soil (Neuhaus Huminsubstrat S), and a 1 cm layer of vermiculite; the pots were placed in plastic trays distributed in groups of ten. One week later, treatments were started.

4.2 Salt treatments

Five plants of each genotype were watered with solutions containing either 200 or 400 mM of NaCl, or with water for the controls, every three days for four weeks ($n = 5$). At the beginning of the treatments, the number of leaves (NL) and plant height (PH) (cm) were measured in all plants. At the end of the experiment, the same parameters were measured, plus the following: stem diameter (SD) (mm), length of the primary root (LR) (cm), leaf surface (LS) (cm^2), and the electrical conductivity of the substrate (EC) (dS m^{-1}). Once these parameters were determined, the plants were taken into the laboratory to measure the leaf fresh weight (g) (LFW), shoot fresh weight (g) (SFW) and root fresh weight (g) (RFW). Part of the fresh leaf material was deep-frozen in liquid N_2 to perform some of the biochemical assays. Part of the fresh material was weighed and dried for three days at 65 ºC and then

weighed again. The water content (WC) percentage was calculated using Equation (1) (Gil et al., 2014).

$$WC\ (\%) = [(FW - DW)/FW] \times 100 \qquad (1)$$

4.3 Substrate electrical conductivity

The EC of the substrate was measured at the end of the treatments using a 1:5 soil:water suspension prepared in deionised water and stirred for two h at 600 rpm and 4 ºC. $EC_{1:5}$ (dS m^{-1}) was measured with a Crison Conductivity-meter 522 (Crison Instruments SA, Barcelona, Spain).

4.4 Photosynthetic pigments

Total carotenoids (Caro), chlorophyll *a* (ChlA) and chlorophyll *b* (ChlB) were measured following the procedure of Lichtenthaler and Wellburn (1983), as modified by Yangen et al. (2019). Pigments were extracted from 0.05 g of fresh leaf material in 10 mL of 80% ice-cold acetone. After mixing overnight and centrifuging for 10 min at 13,300 × *g*, the supernatant was collected, and its absorbance was measured at 663, 646 and 470 nm. Pigment concentrations were calculated following the equations given by Lihtenthaler and Wellburn (1983), and pigment contents were expressed in mg g^{-1} DW.

4.5 Ion content measurement

The ion contents were determined in samples of 0.05 g of dry leaf, stem and root material after extraction in 15 mL of H_2O, according to the original protocol of Weimberg (1987), with minor modifications (González-Orenga et al., 2019). The samples were heated at 95 ºC for one hour in a water bath, mixed overnight in a rocker shaker, and centrifuged for 10 min at 13,300 × *g*. Cations (Na^+, K^+ and bioavailable Ca^{2+}) were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), and Cl^- was measured using a chloride analyser Corning 926 (Sherwood Scientific Ltd., Cambridge, UK).

4.6 Osmolyte quantification

Proline (Pro) was extracted from 0.05 g of fresh leaf material with two mL of 3% (*w*/*v*) sulphosalicylic acid and quantified according to the ninhydrin method (Bates, Waldren, & Teare, 1973). The extract mixed with acid ninhydrin was heated at 95 ºC in a water bath for one hour, cooled on ice and extracted with toluene; the absorbance of the organic phase was measured at 520 nm. Samples with known Pro amounts were assayed in parallel to obtain a standard curve. Pro concentrations were expressed as µmol g^{-1} DW.

The total soluble sugars (TSS) were quantified using 0.05 g of fresh leaf material. The samples were extracted with 3 mL of 80% (*v*/*v*) methanol and mixed overnight. The extracts were centrifuged for 10 min at 13,300 × *g*, concentrated

sulphuric acid and 5% phenol were added to the supernatant, and the absorbance was measured at 490 nm. The TSS contents were expressed as equivalents of glucose, used as the standard (mg eq. gluc g^{-1} DW) (Dubois et al., 1956; Gill, Sharma, Singh, & Bhullar, 2002).

4.7 MDA, H_2O_2 and antioxidant compounds

The malondialdehyde (MDA) contents were determined following the method described by Hodges et al. (1999), with some modifications (González-Orenga et al., 2019). Leaf methanol extracts (80% *v*/*v*) were supplemented with 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA), or with 20% TCA without TBA for the controls, and incubated for 15 min at 95 ºC in a water bath. The reaction was stopped on ice, and the absorbance was measured at 440, 600 and 532 nm. The MDA concentrations were calculated using the equations described by Taulavuori et al. (2001).

The hydrogen peroxide (H_2O_2) contents were quantified using the method described by Loreto and Velikova (2001). An amount of 0.05 g of fresh leaf material was extracted with a 0.1% (*w*/*v*) TCA solution and centrifuged for 10 min at 13,300 × *g*. The supernatant was mixed with one volume of potassium phosphate buffer (10 mM, pH 7.0) and two volumes of 1 M potassium iodide. The absorbance was measured at 390 nm, and H_2O_2 concentrations were calculated against a standard calibration curve and expressed as µmol g^{-1} DW.

Total phenolic compounds (TPC) and total flavonoids (TF) were determined in the same methanol extracts used for the TSS measurement. TPC were quantified by reaction with Folin–Ciocalteu reagent (Blainski, Lopez, & De Mello, 2013). The absorbance was measured at 765 nm, and the results were expressed as equivalents of gallic acid, used as the standard (mg equiv. acid gallic g^{-1} DW). TF were measured following the method described by Zhishen et al. (1999), based on the nitration of phenolic rings containing a catechol group, followed by reaction with $AlCl_3$ at basic pH. The absorbance was measured at 510 nm, and TF contents were expressed in equivalents of the standard catechin (mg equiv. cathechin g^{-1} DW).

4.8 Antioxidant enzymes activities

Crude protein extracts were prepared from leaf material, as previously described by Szabados and Savouré (2010), and stored at −75 ºC. The protein concentration was determined according to Bradford (1976), using the Bio-Rad reagent and bovine serum albumin (BSA) as the standard. The specific activities in the protein extracts of the four selected antioxidant enzymes were determined by spectrophotometric assays.

Superoxide dismutase (SOD) activity was determined by monitoring, at 560 nm, the inhibition of nitroblue tetrazolium (NBT) photoreduction in reaction mixtures containing riboflavin as the source of superoxide radicals (Beyer &

Fridovich 1987; Lee, Kim, & Lee, 2001). One SOD unit is defined as the amount of enzyme causing 50% inhibition of the NBT photoreduction.

Catalase (CAT) activity was measured following the consumption of H_2O_2 added to the extracts by the decrease in absorbance at 240 nm (Aebi, 1984; Lee, Kim, & Lee, 2001). One CAT unit is defined as the amount of enzyme necessary to decompose one mmol of H_2O_2 per min at room temperature. Ascorbate peroxidase (APx) activity was determined following the decrease in absorbance at 290 nm, caused by oxidation of the ascorbate present in the plant extract (Nakano & Asada, 1981; Neto et al., 2005). One APx unit is defined as the amount of enzyme catalysing the consumption of one mmol of ascorbate per min at room temperature.

Glutathione reductase (GR) activity was quantified by the decrease in absorbance at 340 nm, the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH), and the cofactor in the GR-catalysed reduction in oxidised glutathione (GSSG) (Connel & Mullet, 1986; Madhu et al., 2022). One GR unit is defined as the amount of enzyme required to oxidise one mmol of NADPH per min at room temperature.

4.9 Adult plant evaluation

Before the salt treatments, ten plants per genotype were transplanted into pots (33 cm diameter, 28.5 cm height) with coconut fibre. A controlled-release fertiliser, CoteN Mix, with NPK = 20−5−10, was added to each pot. Fertilisation was repeated after four weeks. Five plants were maintained until the pre-flowering stage when a long-term salt treatment started, by watering with a solution containing 80 mM of NaCl (n = 5). Automatised irrigation was set to water each plant with 1.65 litres daily. Once a week, the plants were irrigated with a control solution without salt to avoid salt accumulation in the coconut fibre and the pot.

After eight weeks, growth parameters (RL, LS, LFW, SFW and RFW) were determined, as well as the DW and WC of leaves, stem and roots, as described above for young plants.

Considering the results obtained for young plants, only ions and Pro were quantified in adult plants. Na^+, K^+ and Ca^{2+} were measured using 2 g of dry leaf or root material in an Agilent ICP-EOS 710 photometer (Agilent Technologies, Santa Clara, CA, USA). Pro contents were determined following the protocol described in 4.7.

4.10 Statistical analysis

Data was analysed using the software Statgraphics Centurion v.XVII (Statpoint Technologies, Warrenton, VA, USA). A factorial analysis of variance was performed for all parameters analysed in the plants, considering two factors of variability—treatments and genotypes—and their interaction. An analysis of variance was carried

out separately for each species. Differences between the treatments were evaluated by the Student–Newman–Keuls test.

5. Conclusions

Our results confirm the relatively high tolerance of eggplant to moderate salinity compared to most common crops, primarily based on the control of ion transport and Pro accumulation. These mechanisms seem efficient enough to avoid the generation of oxidative stress and, therefore, the activation of antioxidant systems as an additional defence mechanism.

The hybrid *S. melongena x S. insanum* showed a heterotic effect on plant growth, being more salt-tolerant than either parent. It appeared closer to the wild species in some crucial responses, such as the NaCl-dependent Pro accumulation in young plants. Wild relatives have been exploited to introgress novel genes that increase the fitness of the cultivated species. The results presented here suggest that *S. insanum* and the hybrid can be used as new sources of variation for breeding programmes, in order to obtain eggplant varieties that are better adapted to salt stress and, consequently, more sustainable.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Author contributions

Conceptualization, AF and OV; Methodology, SG-O and NO-A; Software, SG-O and AF; Validation, OV, AR-B and AF; Formal analysis, SG-O and NO-A; Investigation, NO-A and SG-O; Resources, AR-B and OV; Data curation, AF and NO-A; Writing—original draft preparation, NO-A; Writing—review and editing, AF and OV; Visualization, SG-O, OV and AF; Supervision, OV and AR-B; Project administration, AF; Funding acquisition, AF.

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Research article [Under Review]

First transcriptomic profile of the interspecific hybrid *Solanum insanum* × *S. melongena* under salt stress

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PhD candidate contribution

Neus Ortega Albero has a main role in performing the experiments, sample and data collection, data analysis and visualization, drafting manuscript, revision and editing.

Abstract

Salt accumulation in the soil is one of the main threats in current and future agriculture that leads to production loss. Many works describe the morphological and physiological response to salinity stress in eggplant. However, our work aims to study the changes in gene expression of cultivated eggplant (*Solanum melongena*), and its wild relative *S. insanum* under salt stress; and explore the inheritance of the tolerance by studying the interspecific hybrid *S. insanum* × *S. melongena*. We performed an RNA sequencing study (RNA-seq) of the transcriptomes of the three genotypes after 12 hours of salt stress (150 mM) in comparison with the non-treated plants. The number of differential expressed genes (DEGs) among the three genotypes was dissimilar, being the wild relative who showed a higher amount of DEGs. In this regard, high similarities were found between *S. melongena* and the hybrid, indicating the absence of maternal effect. The transcriptome analysis revealed that each genotype, even showing some common functions, activated their own metabolic routes to cope with the salt stress. Moreover, the interspecific hybrid inherited some key metabolic routes of its parental lines but triggered new mechanisms to respond to the abiotic conditions, including the activation of *MYB*-transcription factors, very relevant under osmotic stresses. This work presents inheritance patterns of eggplant species regarding tolerance to abiotic stress.

Keywords

Comparative transcriptome, DEGs, eggplant, ion metabolism, RNA-Seq, salinity

1. Introduction

Soil salinity has increased in the last decades affecting more than 100 x 10^6 ha of cropland and limiting crops' yield around the globe. Moreover, soil salinization is expected to further increase in the immediate future (FAO, 2023). The presence of salt in soils affects the metabolism of most plant species by two main types of stress: osmotic stress, causing incapacity of water uptake and loss of turgence, and ion toxicity, causing nutritional imbalance. These stresses trigger morphological, physiological, and molecular response mechanisms in plants, which usually result in cellular damage caused by reactive oxygen species (ROS), and reduction in plant growth, yield and fruit quality. Furthermore, high long-term salt stress may lead to plant death (Gupta & Huang, 2014; Isayenkov, 2019; Munns & Tester, 2008). Plants have evolved adapting certain mechanisms to respond to abiotic stresses, including modification of ion transport, ion compartmentalization, production of osmoprotectants, and activation of antioxidant enzymes, among others (Kumar et al., 2013; Peng et al., 2016).

Eggplant (*Solanum melongena* L.) is the eighth most important crop worldwide and the third most important Solanaceae species in the market after tomato and potato (FAO, 2023). It has been described as moderately tolerant to salinity, partially reducing growth and fruit production under this stress (Ünlükara et al., 2010). Crop wild relatives (CWRs) have been revealed as fundamental sources of genetic variation for plant breeding to improve tolerance to abiotic stresses in commercial plant species (Prohens et al., 2017). Moreover, eggplant CWRs are distributed throughout the globe, adapted to a wide range of environmental conditions, showing interesting genetic features for eggplant breeding (Vorontsova & Knapp, 2016). In this regard, *S. insanum* L., considered the direct ancestor of *S. melongena,* is distributed in south and southeast Asia, and some parts of Africa, and is adapted to a great diversity of geographic, climatic and soil conditions (Knapp, Vorontsova, & Prohens, 2013). Hereof, *S. insanum* has shown better performance under drought and salinity in recent reports compared to *S. melongena*, maintaining growth rate and photosynthetic machinery by altering ion transport and protectant osmolyte accumulation (Brenes et al., 2020; Ortega-Albero et al., 2023).

Previous studies of our group revealed some new physiological mechanisms in the interspecific hybrid *S. insanum* × *S. melongena* when applying high levels of salt stress (Ortega-Albero et al., 2023). Specifically, the hybrid showed a larger main root under salinity compared to the parents to ensure water and nutrient uptake. It also maintained growth under moderate salt stress, similarly to *S. insanum*. Differences in the biochemical assays showed that the hybrid rather than having intermediate values was similar to the cultivated parent for some parameters and similar to the wild parent in others. This may reflect genome rearrangements resulting in gene expression patterns.

The response to abiotic stresses involves hundreds of metabolic pathways in plants, together with gene regulation machinery. High-throughput sequencing together with transcript identification and quantification, commonly known as RNA-seq analysis, have arose as a main activity in molecular studies, providing a significant characterisation of mRNA transcripts of a specific tissue and time, and allowing to obtain

information about regulation at the genomic level (Conesa et al., 2016). Some transcriptomic studies have been published in diverse species in response to a plethora of abiotic stresses like drought (Bai et al., 2022; Kaur et al., 2023; Villanueva et al., 2023), salinity (Acharya et al., 2022; Dugasa et al., 2021; Kumar et al., 2021), or heavy metals (Fang et al., 2023; Tang et al., 2024). But the transcriptomic response of interspecific hybrids remains unexplored, yet understanding their gene expression patterns is essential for uncovering the genetic mechanisms behind stress tolerance. Since future eggplant breeding projects will rely on interspecific crosses with CRWs, this knowledge is fundamental for developing stress-resilient cultivars (Li et al., 2019; Villanueva et al., 2023).

In this work, we aimed to understand the expression differences underlying the salt responses of *S. melongena*, *S. insanum,* and their interspecific hybrid. For this purpose, we analysed the transcriptomes of cultivated *S. melongena,* its direct ancestor *S. insanum,* and the interspecific hybrid *S. insanum* × *S. melongena* after a 12–hour salt stress. Our aim was to identify the genes and the metabolic pathways involved in the response to salinity in eggplant and wild relatives and how they are inherited in interspecific hybrids after sexual crossing.

2. Materials and methods

2.1 Plant material and growing

Seeds from *S. melongena* accession MEL5 (MEL), *S. insanum* accession INS1 (INS), and interspecific hybrid *S. insanum* × *S. melongena* (HYB) were germinated according to Ranil et al. (2015) protocol. Plant with the two cotyledons were transplanted into 1.5 mL Eppendorf tubes without the cap and with the tub end cut and filled with 0.2% agar in tap water acting like a matrix system for the developing plants (Renau-Morata et al., 2014). The tubes were placed in a pierced plastic cover over opaque 10 L containers filled with a Hoagland nutritive solution (Hoagland & Arnon, 1938). Plant were growth in a chamber with 16/8 h light/dark photoperiod, 25ºC temperature and 70–75% of humidity.

2.2 Salt treatment and sampling

The salt treatment was performed on fully developed 4 leaf-stage plants by adding new Hoagland solution including diluted 150 mM NaCl. 8 plants per genotyped were treated with nutritive solution supplemented with NaCl and 8 plants were resupplied with clean nutritive solution as a control. Leaf samples were taken after 12 hours of treatment for each plant, including 8 control and 8 treated plants per genotype. Immediately, leaf samples were deep-frozen in liquid nitrogen and stored at −80ºC for RNA extraction.

2.3 RNA extraction, sequencing and data processing

Leaf samples were grinded separately with liquid nitrogen in clean mortar and pestle. Bulks were prepared to avoid bias in the RNA-seq assay due to developmental or culture bias. Thus, 1.5 mL Eppendorf tubes were filled with 100 mg from 2 random plants per treatment and genotype which constituted 4 final samples per treatment and genotype. Total RNA was extracted from each bulk using TRIzol^{TM} Reagent (Invitrogen, Carlsbad, CA, USA) and genomic DNA was removed with DNase I, RNase-free (Thermo Fisher Scientific Inc, Waltham, MA, USA). RNA library was performed by Novogene Co., LTD (Beijing, China) and sequenced on an Illumina NovaSeq 6000 (paired–end 150 bp). A quality analysis was realized in the raw reads, and overall quality, total bases, total reads, error rate (%), GC content (%) and Q20 (%) and Q30 (%) were calculated. Afterwards, the raw reads were trimmed to remove adaptors and low-quality reads, PCR duplicated, and contaminant DNA were removed. Trimmed reads were mapped to the eggplant reference genome "67/3" V3 (Barchi et al., 2019) with HISAT2 and Stringtie was used for transcript assembly (Kim et al., 2019; Pertea et al., 2015). Abundance of transcripts was calculated in the read count and the gene expression profiles were normalized as FPKM (Fragment per Kilobase of transcript per Million mapped reads) and TPM (Transcripts Per Kilobase Million). All raw data were deposited in the Short Read Archive (SRA) of the National Library of Medicine repository.

2.4 Transcriptomic analysis

Normalized data was pre-filtered previously to transcriptomic analysis to remove genes with low read count. Differentially expressed genes (DEGs) analysis was performed using the DEseq2 R package (Love, Huber, & Anders, 2014), and the resulting *p*-values were adjusted for controlling the false discovery rate. Genes with adjusted *p*-value < 0.05 and $|\log_2 (\text{fold change})| > 2$ were considered as differentially expressed. DEGs were annotated based on the function annotation of reference genome "67/3" V3 (Barchi et al., 2019).

Differences among the genotypes in each treatment were presented through a principal component analysis (PCA) performed with R packages ggplot2 and dplyr (Wickham, 2016; 2019). DEGs main results were presented with MA-plots, Venn diagrams, volcano plots, and plot counts of selected genes using R packaged DEseq2 R package, devtools, and ggplot2 (Love et al., 2014; Wickham, 2016). Finally, Gene ontology (GO, https://www.geneontology.org/) and Kyoto Encyclopaedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/) enrichment analysis were performed with R packages topGO (Alexa, Rahnenführer, & Lengauer, 2006) and clusterProfiler (Yu, Wang, Han, & He, 2012) using GO terms annotated in the eggplant reference genome and KEGG ortholog codes obtained from the KOfam database (https://www.genome.jp/tools/kofamkoala/).

3. Results

3.1 Physiological response to salinity

Under control hydroponic culture, the three genotypes showed turgor and a good development (Figure 1). INS plants were smaller than the others due to its slowed growth rate (Brenes et al., 2020; Ortega-Albero et al., 2023). In regard to vegetative growth, INS developed smaller leaves and stems and a high density of spikes, which are also present in HYB but with a lower density and absent in the cultivated eggplant. Nevertheless, MEL showed the higher root density in control conditions, and HYB showed an intermedium root density between both parents. After applying a 150 mM NaCl treatment for 12 hours on the same hydroponic nutrition media, visual differences were observed among the studied genotypes. Both parental genotypes and the interspecific hybrid showed loss of firmness in leaves and stems. However, MEL and HYB showed other stress symptoms as changes in the leaf colour, which were not observed in INS.

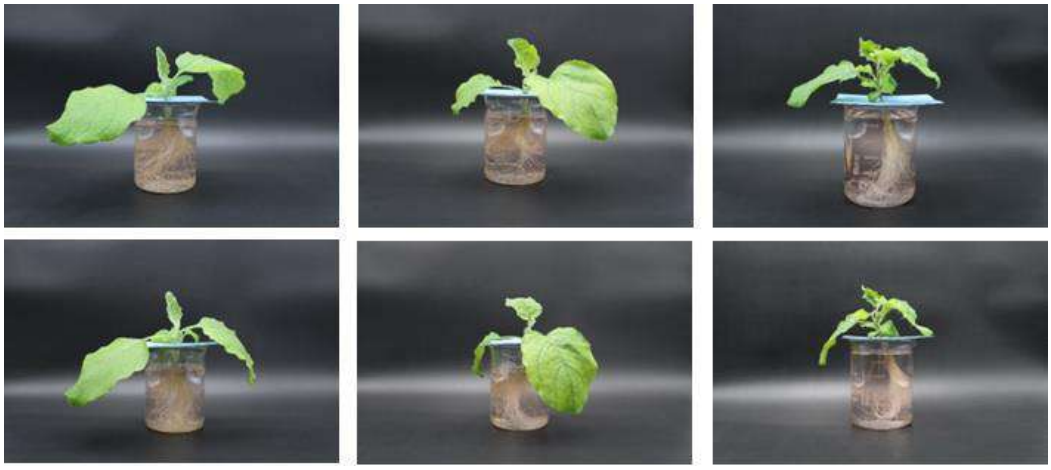

Figure 1. From left to right, representative visual aspect of *Solanum melongena,* interspecific hybrid *S. insanum* × *S. melongena*, and *S. insanum* under control (up) and 12 hours of 150 mM NaCl culture (down).

3.2 Expression patterns by genotype

A total of 7.2 Gpb were sequenced in the 24 samples with more than 45 M reads per sample. After trimming raw data, filtered sequencing data showed an average Q30 value of 94.63% and an average GC content of 44.07%. More than 98.5% of reads were mapped to the reference "67/3" genome in each sample.

The observed expression pattern was different among genotypes and changed clearly when applying the NaCl treatment. This result is represented with the principal component analysis, which explained the 83% of the samples' total variation in the two first principal components indicating a minor effect of other factors not included in the experiment (Figure 2). The first component (PC1) explained 54% of the samples' variability, segregating the samples grown in control and salt conditions. This indicates

that the treatment was the main reason of the samples' variability. The second component (PC2) explained 29% of the samples' variability, differentiating between genotypes, indicating that the genotype was the second main reason of the samples' variability. Samples of each genotype and treatment clustered together and were clearly different from the other clusters. HYB samples were closer to the cultivated parent MEL, indicating closer transcriptomic response to salt stress to this parental.

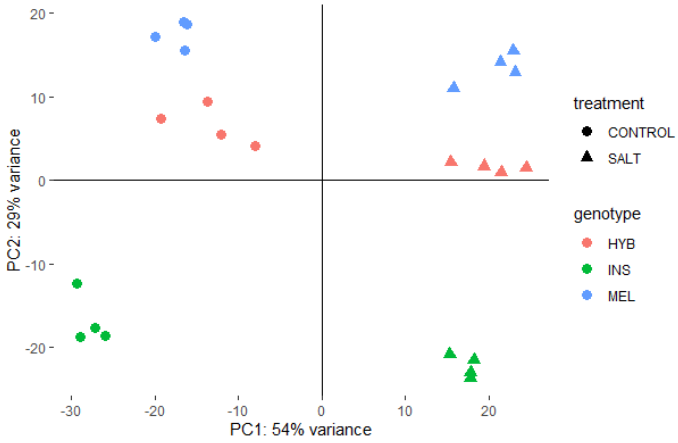

Figure 2. Principal component analysis (PCA) of *Solanum melongena* (MEL), *S. insanum* × *S. melongena* (HYB), and *S. insanum* (INS) samples in control and 150 mM NaCl stress with transcript abundance normalized data.

All genotypes modified their expression after 12 hours of salt treatment, as shown in the MA-plots (Figure 3). Hundreds of transcripts, and, thus, genes, were up– and downregulated in response to the stress.

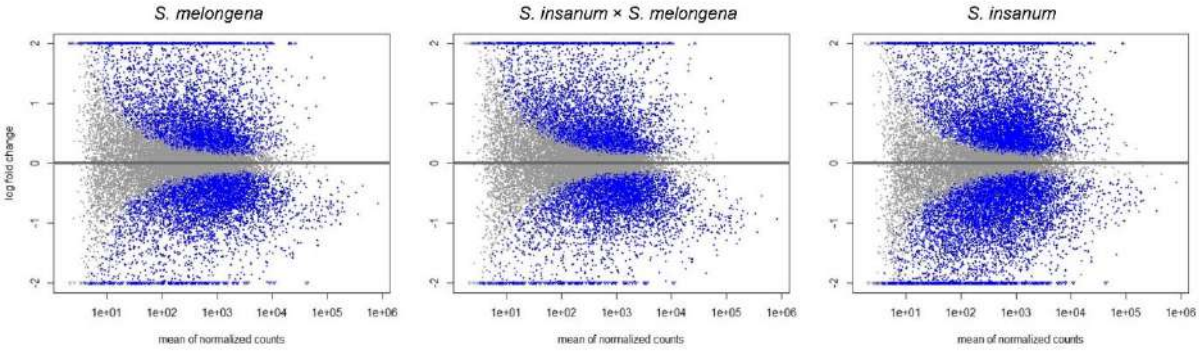

Figure 3. MA-plots of *Solanum melongena*, *S. insanum* × *S. melongena,* and *S. insanum* normalized transcripts abundance in 150 mM NaCl referred to control conditions.

3.3 Differential gene expression analysis in response to salt stress

A differential expressed genes (DEGs) analysis was performed with the filtered and normalized data (Figure 4). DEGs with an adjusted *p*-value < 0.05 and $|\log_2$ (fold change)| > 2 were selected by performing pairwise comparisons of each genotype under salinity stress at 12 hours versus the control/non-stressed samples. The number of DEGs up– and downregulated in MEL, HYB, and INS compared to their respective control samples are summarized in Table 1. INS showed the maximum number of total DEGs of the three genotypes, as well as the highest number of upregulated genes, while HYB showed the lowest values of total and upregulated genes. Although total DEGs number of HYB was more similar to MEL, HYB also showed the lowest value of upregulated genes, indicating that its response to salinity is based on deactivating some genes, instead of activating other metabolisms, like both parental lines seem to do (Figure 4).

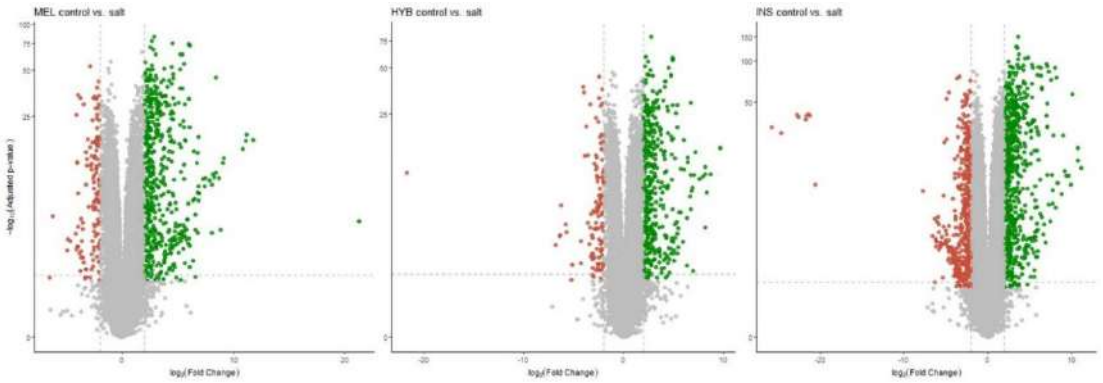

Figure 4. Volcano plots of *Solanum melongena*, *S. insanum* × *S. melongena,* and *S. insanum* DEGs in 150 mM NaCl referred to control conditions.

From these DEGs, 312 were shared by the three genotypes as shown in the Venn plot (Figure 5). INS showed the highest number of unique DEGs, with more than 650, followed by MEL and HYB. HYB shared few DEGs with both parental lines individually, 60 approximately, while both parental lines shared 124 DEGs in response to salinity.

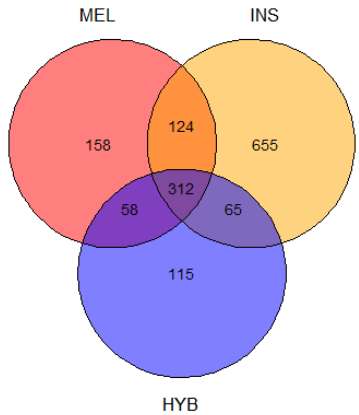

Figure 5. Venn plot of *Solanum melongena* (MEL), *S. insanum* × *S. melongena* (HYB), and *S. insanum* (INS) DEGs in 150 mM NaCl conditions referred to control conditions.

Most of DEGs with exacerbated expression (over |log2 (fold change)| > 6 in salt-treated compared to control conditions) were shared among the three genotypes (Table 2) and were predominantly upregulated. Among them can be mentioned genes such of TAS14 a stress-responsive dehydrin which improve the osmotic stress in solanaceous crops (Kawamoto et al., 2024), oleosins which play a crucial role in stabilizing oil bodies and modulating lipid metabolism in response to salt stress, contributing to the plant's tolerance mechanisms (Tailor & Bhatla, 2023), EXLB1, expansin like B1, or proteins abundant in late embryogenesis (LEAs). Some DEGs were common but only overpassing the threshold of |log2 (fold change)| > 6 in some genotypes like ATHB-7 a leucine zipper transcription factor, in INS, AVT6C an amino acid transporter protein in MEL. From this list (Table 2), the only common downregulated genes RADIALIS-like 6 (RL6) and OCT-3 which are transcription factors. In addition, some DEGs showed extreme differential expression with more than $|\log_2$ (fold change)| > 20. For instance, gene SAC3B, was expressed in the three genotypes, but only overexpressed in MEL. SAC3B is a component of the TREX-2 complex which interact with the nuclear pore complex and is involved in plant stress response through the control of mRNA/protein nucleo-cytopastmic trafficking (Seidler & Sträßer, 2024; Yang et al., 2017). Another gene involved in modulating the gene expression through posttranscriptional modulation, LSM2, was downregulated in HYB under salt stress. LSM2 has been identified in salt tolerance mechanisms (Catalá et al., 2019). Among the DEGs with $|\log_2$ (fold change)| > 20 and known function, nine were found downregulated for INS under salt conditions. Those were: UBP12, with hydrolase functions, RNALX, with ribonuclease function, COG3, related with Golgi intermembrane transport, PME51, related to pectin degradation in cell wall, and TPS8, related to sucrose metabolism.

Table 1. Number of total DEGs, up– and downregulated in *Solanum melongena* (MEL), *S. insanum* × *S. melongena* (HYB), and *S. insanum* (INS) under 12h of 150 mM NaCl stress.

	Total DEGs	Upregulated	Downregulated
MEL	652	520	132
HYB	550	119	431
INS	1156	667	489

3.4 GO and KEGG enrichment

Significant gene ontology (GO) terms in DEGs of both parental lines, MEL and INS, were similar, sharing important processes as response to water and auxins, lipid transport, or glutamine amino acid metabolic process (Figure 6). However, some

processes were only present in one of the parental lines like glycerol ether metabolic process and cell redox homeostasis in MEL and inorganic ion homeostasis, response to abscisic acid, and carbohydrate metabolic process in INS.

On the first hand, MEL and HYB mostly showed common functions except for lipid metabolic process, response to auxin and glycerol ether metabolic process, only present in MEL; and systemic acquired resistance, metal ion transport, response to external stimulation, inorganic ion homeostasis, hydrotropism, and chitin catabolic process only present in HYB (Figure 6). On the other hand, INS and HYB also shared many processes, like response to auxin and abscisic acid, proteolysis, and carbohydrate metabolism, only present in INS, and systemic acquired resistance, hydrotropism, metal ion transport, and response to external stimulus. Moreover, these functions of the HYB seem to be absent the parents, thus, it demonstrates that there are new mechanisms in the interspecific hybrid in response to salinity.

DEGs seemed to be working mainly in the monolayer-surrounded lipid storage body for the three genotypes (Figure 6). However, regarding cellular components, MEL also show DEGs in the lipid droplet, while HYB showed mainly DEGs in the extracellular space and membrane, the same results obtained for INS.

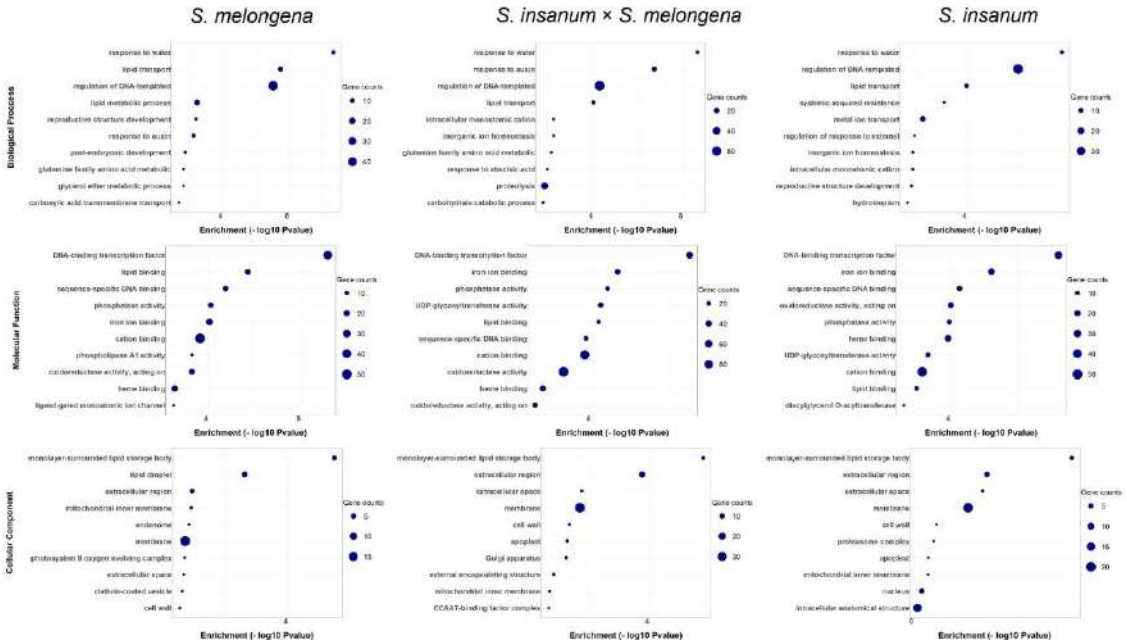

Figure 6. Gene ontology (GO) terms enrichment in DEGs of *Solanum melongena* (A), *S. insanum* × *S. melongena* (B), and *S. insanum* (C) DEGs in 150 mM NaCl referred to control conditions.

In regard to KEGG enrichment, some terms were common to cultivated and wild parent lines like protein phosphatase, kinase protein and other protein modification functions (Figure 7). However, they differed in many functions like activation of heat shock proteins, and glutamate receptor only on MEL, and abscisic acid receptor or

benzyl alcohol transferase only in INS. Most KEGG terms in the HYB were found in one or both the parental species, like heat shock proteins, abscisic acid receptor, peroxygenase, or leucine zipper protein (Figure 7). However, other functions were found only in the HYB, like diacylglycerol O-acyltransferase wax synthase, transcription factor MYB, pathogen-inducible salicylic acid glucosyltransferase, cytochrome P450, and basic endochitinase.

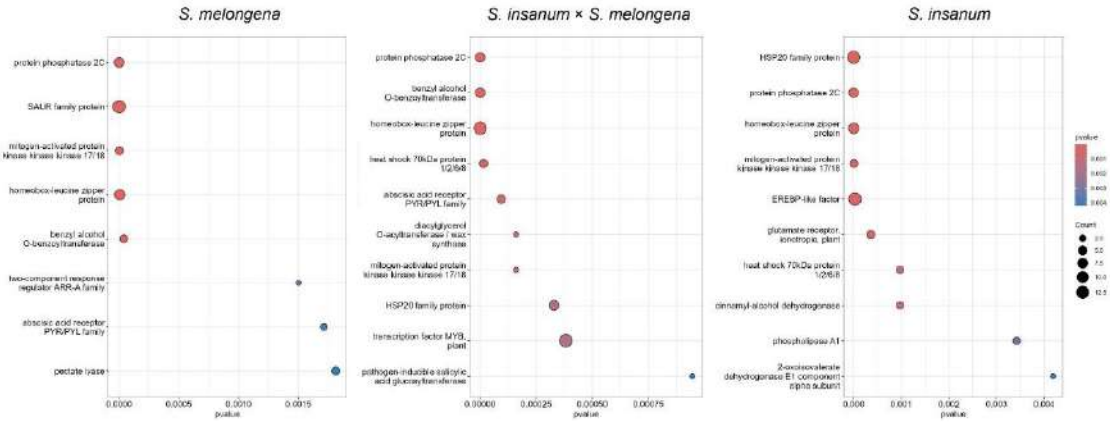

Figure 7. Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment in DEGs of *Solanum melongena* (A), *S. insanum* × *S. melongena* (B), and *S. insanum* (C) DEGs in 150 mM NaCl referred to control conditions.

Table 2. Differentially expressed genes ($|\log_2$ (fold change)$| > 6$) under salt stress in *Solanum melongena* (MEL), *S. insanum* × *S. melongena* (HYB), and *S. insanum* (INS). The table includes Gene ID, regulation status (upregulated or downregulated), annotated gene function, and fold change values for each genotype.

Gene ID	Status	Gene function	Fold change		
DEGs shared among genotypes			**MEL**	**HYB**	**INS**
SMEL4_05g003140.1	down	Organic cation/carnitine transporter 3 [OCT3]	< 6	< 6	–7.73
SMEL4_05g018920.1	down	Protein RADIALIS-like 6 [RL6]	< 6	< 6	–6.37
SMEL4_07g000540.1	up	60S ribosomal protein L10 [RPL10]	< 6	< 6	7.07
SMEL4_02g009210.1	up	Abscisic acid and environmental stress [TAS14]	11.05	6.75	10.71
SMEL4_06g009120.1	up	Amino acid transporter [AVT6C]	6.37	< 6	< 6
SMEL4_02g017700.1	up	Berberine bridge enzyme-like 28	6.34	< 6	< 6
SMEL4_08g005770.1	up	Cytochrome C oxidase subunit 6b-like	6.75	< 6	< 6
SMEL4_02g025520.1	up	Dehydrin [XERO 1]	< 6	< 6	7.54
SMEL4_08g010900.1	up	E3 ubiquitin-protein ligase [RZFP34]	< 6	< 6	6.31
SMEL4_09g011280.1	up	EID1-like F-box protein 3 [EDL3]	7.78	< 6	8.13
SMEL4_08g005400.1	up	Expansin-like B1 [EXLB1]	9.04	8.21	9.56

SMEL4_11g004930.1	up	Glucan endo-1,3-beta-glucosidase, acidic isoform PR-Q	6.65	6.47	< 6
SMEL4_11g008500.1	up	Heavy metal-associated isoprenylated plant protein 20 [HIPP20]	6.84	< 6	7.69
SMEL4_08g001510.1	up	Homeobox-leucine zipper protein [ATHB-7]	< 6	< 6	7.99
SMEL4_01g035110.1	up	Late embryogenesis abundant protein	7.49	7.06	9.85
SMEL4_03g008010.1	up	Late embryogenesis abundant protein [DC3]	11.73	7.24	11.16
SMEL4_10g001090.1	up	Late embryogenesis abundant protein [LEA46]	6.00	6.02	6.19
SMEL4_02g015280.1	up	Late embryogenesis abundant protein 76	6.79	8.35	6.67
SMEL4_03g031380.1	up	Linamarin synthase 1 [UGT85K4]	10.79	< 6	7.61
SMEL4_11g016330.1	up	Low temperature-induced protein [LT101.2]	< 6	< 6	7.27
SMEL4_03g018390.1	up	Low-temperature-induced protrin [LTI65]	8.36	6.74	10.11
SMEL4_03g029210.1	up	Lysine-specific demethylase [JMJ706]	6.05	< 6	< 6
SMEL4_09g003530.1	up	Membrane protein [PM19L]	< 6	< 6	7.71
SMEL4_07g016580.1	up	Mitogen-activated protein kinase kinase [MAPKKK18]	< 6	< 6	6.38
SMEL4_12g000730.1	up	NADP-dependent malic enzyme	6.53	< 6	< 6
SMEL4_05g001340.1	up	Non-specific lipid-transfer protein 2	< 6	6.85	< 6
SMEL4_09g000470.1	up	Oil body-associated protein 1A [OBAP1A]	< 6	< 6	7.05
SMEL4_03g011110.1	up	Oleosin H2	9.11	8.23	9.31
SMEL4_06g016080.1	up	Oleosin L	8.66	8.75	8.75
SMEL4_01g017960.1	up	Oleosin L	< 6	< 6	7.04
SMEL4_00g007360.1	up	Oleosin L	8.12	7.92	< 6
SMEL4_05g020370.1	up	Peroxygenase [SOP1]	6.45	< 6	< 6
SMEL4_09g003210.1	up	Peroxygenase [SOP1]	< 6	< 6	7.39
SMEL4_11g023860.1	up	Protein [LE25]	< 6	< 6	6.02
SMEL4_05g015620.1	up	Protein BIG GRAIN 1-like A	7.37	6.69	6.37
SMEL4_05g019500.1	up	Protein NRT1/ PTR FAMILY 1.2 [NPF1.2]	< 6	< 6	8.24
SMEL4_03g013880.1	up	Protein REDOX 2	< 6	6.36	< 6
SMEL4_09g007280.1	up	Translocator protein homolog [TSPO]	6.04	< 6	7.05
SMEL4_09g007290.1	up	Translocator protein homolog [TSPO]	< 6	< 6	6.99
SMEL4_08g022580.1	up	UDP-glucosyltransferase 29 [UGT29]	8.01	6.89	< 6
SMEL4_02g018460.1	up	Unknown	6.74	6.46	9.14
SMEL4_02g000500.1	up	Unknown	< 6	< 6	7.79
SMEL4_02g002670.1	up	Unknown	< 6	< 6	7.22
SMEL4_03g012040.1	up	Unknown	< 6	< 6	7.03

SMEL4_04g019520.1	up	Unknown	6.84	< 6	8.91
SMEL4_06g027400.1	up	Unknown	11.18	9.72	10.78
SMEL4_06g019010.1	up	Unknown	< 6	< 6	6.96
SMEL4_08g014870.1	up	Unknown	6.87	7.66	< 6
SMEL4_08g009380.1	up	Unknown	< 6	< 6	6.61
SMEL4_11g004950.1	up	Unknown	< 6	< 6	6.28
SMEL4_12g017230.1	up	Unknown	7.23	6.02	8.31
SMEL4_03g004890.1	up	Unknown	8.38	< 6	< 6

DEGs found in one genotype			**MEL**	**HYB**	**INS**
SMEL4_11g010280.1	down	Alpha-trehalose-phosphate synthase 8 [TPS8]	–	–	–21.29
SMEL4_12g009580.1	down	Carboxylesterase 15 [CXE15]	–	–	–6.29
SMEL4_07g007870.1	down	Conserved oligomeric Golgi complex subunit 3 [COG3]	–	–	–6.26
SMEL4_07g007880.1	down	Conserved oligomeric Golgi complex subunit 3 [COG3]	–	–	–21.47
SMEL4_11g025750.1	down	Cytochrome P450 71D7 [CYP71D7]	–	–6.83	–
SMEL4_11g025760.1	down	Cytochrome P450 71D7 [CYP71D7]	–	–6.42	–
SMEL4_00g008490.1	down	Feruloyl CoA ortho-hydroxylase [F6H1-3]	-	–	–6.26
SMEL4_08g012740.1	down	Histidine-containing phosphotransfer protein [AHP4]	–	–6.31	–
SMEL4_07g000810.1	down	Intracellular ribonuclease LX	–	–	–21.80
SMEL4_01g031560.1	down	Pathogenesis-related protein 1A1	–6.25	< 6	–
SMEL4_10g016470.1	down	Pectinesterase/pectinesterase [PME51]	–	–	–22.78
SMEL4_01g034070.1	down	Polyneuridine-aldehyde esterase [PNAE]	–	–	-6.38
SMEL4_11g019490.1	down	Pyruvate kinase 2C	–	–	–6.61
SMEL4_01g002690.1	down	Secoisolariciresinol dehydrogenase	–	–6.25	–
SMEL4_09g008450.1	down	Sm-like protein [LSM2]	–	–21.72	–
SMEL4_05g004440.1	down	Ubiquitin carboxyl-terminal hydrolase 12 [UBP12]	–	–	–21.64
SMEL4_05g004450.1	down	Ubiquitin carboxyl-terminal hydrolase 12 [UBP12]	–	–	–6.15
SMEL4_01g026190.1	down	Unknown	–	–	-6.10
SMEL4_03g015430.1	down	Unknown	–	–	–24.70
SMEL4_03g016720.1	down	Unknown	–	–	–25.79
SMEL4_03g033760.1	down	Unknown	–	–	–22.60
SMEL4_06g015900.1	down	Unknown	–6.53	–	–20.59
SMEL4_03g018050.1	up	CEN-like protein 1 [CET1]	6.35	–	< 6
SMEL4_09g001060.1	up	Geraniol 8-hydroxylase [CYP76B6]	< 6	–	6.84
SMEL4_06g027850.1	up	Heat shock protein 17.7 kDa class I	8.01	–	–

SMEL4_01g004950.1	up	Protein ADP-ribosyltransferase [PARP3]	–	6.35	–
SMEL4_03g015850.1	up	Protein phosphatase 2C 24	< 6	< 6	6.40
SMEL4_07g000180.1	up	SAC3 family protein B [SAC3B]	21.28	–	–
SMEL4_02g020790.1	up	Senescence-specific cysteine protease [SAG39]	6.75	8.22	–
SMEL4_08g025770.1	up	Serine racemase [SERR]	6.57	–	–
SMEL4_05g000230.1	up	Transcription factor [MYB21]	6.52	7.01	–
SMEL4_09g008300.1	up	Unknown	–	6.78	–
SMEL4_12g005570.1	up	Unknown	< 6	–	6.22
SMEL4_06g003830.1	up	Unknown	6.06	–	–
SMEL4_03g010900.1	up	Unknown	8.82	-	< 6

4. Discussion

S. melongena has been described as moderately tolerant to salinity (Ünlükara et al., 2010). However, previous works from our group indicated that there were existing differences between *S. melongena* and its direct ancestor *S. insanum* in response to salt stress, regarding physiological parameters (Ortega-Albero et al., 2023). Hence, in this work we analysed the differences in gene expression after a salt stress shock between both genotypes and their interspecific hybrid.

This study revealed that the transcriptomic profiles of the three studied genotypes changed in response to salt irrigation. Also, there were unequivocal differences in number of differentially expressed genes (DEGs) among both parent lines and the hybrid. Hereof, INS showed almost the double of DEGs than MEL after salinity shock, and more than the doubled than HYB. Our results suggested that HYB was more similar to MEL in number and function of DEGs, despite MEL being the pollen donor in the intercross. This could indicate the absence of a maternal effect, which has been described as a transgenerational form of phenotypic plasticity, transmitted without modifications in the DNA sequence, determined by the maternal genotype (Herman & Sultan, 2011; Li & Li, 2015).

Our transcriptomic comparison of the three eggplant genotypes after salt treatment revealed both conserved and divergent gene expression patterns. More than 300 of these DEGs were shared among the three genotypes, indicating that many underlying mechanisms to salinity stress might be common in related *Solanum* species. All genotypes activated key stress-responsive genes such as EXLB1, related to cell wall modifications, Oleosin family, related to lipid stability, TAS14, related to signalling after response to environmental stress, like LTI65, related to low temperature response, and late embryogenesis abundant proteins (LEAs), which have a role in protecting cells from dehydration processes (Jia et al., 2022; Kawamoto et al., 2024; Krishnamurthy et al., 2019; Liu et al., 2023; Muthusamy et al., 2020). Moreover, both parental lines shared more than 100 DEGs in response to salt shock. However, INS showed the highest number of unique DEGs, which are supposed to be determinant in the salinity tolerance

of this genotype. INS was distinguished for upregulating transcription factors like ATHB-7, and NPF1.2. MEL, in contrast, exhibited some heat shock proteins, enzymes like SERR, and amino acid transporters like AVT6C. HYB showed the lowest number of unique DEGs, since the vast majority were shared with one or both of its parental lines. However, these 115 unique DEGs like rybosiltransferase PARP3, indicate that the interspecific hybrid had some new recombinant mechanisms against salinity.

GO enrichment analysis revealed that gene functions like phosphatases, kinases and transcription factors were common and very important among MEL and INS, indicating modifications in the cell activity. Moreover, these activities are potentially related to the amplification of cell signalling for gene regulation and promotion of protective mechanisms in the plant (Li et al., 2019). Our findings agree with the latest studies in eggplant which emphasise the importance of the transcription factors in eggplant roots under salt stress (Shen, Zhao, Liu, & Yang, 2022; Sun et al., 2025). However, INS showed an overrepresentation of genes involved in peroxygenase activity, consistent with previous results reporting non oxidative stress response under salinity (Ortega-Albero et al., 2023). In contrast, MEL showed enrichment in abscisic acid receptor activation, suggesting a differential mechanism of plant signalling. Some of these functions were also inherited by HYB, like phosphatase activity and activation of transcription factors for cell activity remodelling and signalling activation. However, HYB also showed unique functions absent in its parental lines like MYB transcription factors and glycosyltransferase activity. These results are in agreement with previous publications showing that HYB tends to accumulate more sugars in response to a moderate salt treatment (Brenes et al., 2020; Ortega-Albero et al., 2023).

In regard to biological processes, all the genotypes showed gene functions related to water responses, confirming that salt stress triggers similar response processes to water stress. These results are in agreement with the common effects that present water and salinity stress like reducing soil water potential, ability of the plant to take water, limiting photosynthetic rate or cease cell expansion (Munns, 2011). Furthermore, lipid transport was a common gene function found in the three genotypes, indicating that avoiding lipid peroxidation of the cellular membranes is important to maintain cellular integrity and plant metabolism. Hereof, Yu et al. (2020) have reported that modifications in the lipid cellular content can be an adaptative mechanism for tolerance to salinity. Inorganic ion homeostasis was particularly important for HYB and INS, confirming that ion regulation has great importance in salinity tolerance (Ortega-Albero et al., 2023). For instance, recent transcriptomic studies showed that eggplants increase the absorption of K^+ rather than excluding Na^+ since K^+ transporters were upregulated under salt stress (Li et al., 2019).

In summary, the distinct transcriptional responses observed among the three eggplant genotypes highlight the genotype specificity of salinity response mechanisms. MEL appears to activate stress-responsive pathways primarily through kinase cellular signalling, whereas HYB relies more on post-transcriptional regulation via RNA degradation processes. In contrast, INS modulates its response through the accumulation of metabolites and alterations in sugar metabolism.

5. Conclusions

This is the first time that *S. insanum*, a tolerance donor for eggplant transcriptome has been analysed under salt stress. We identified significant differences among the three studied genotypes in the DEGs and the metabolic pathways activated after the salt treatment. *S. melongena* and *S. insanum* showed some unique responses to salinity in some fundamental metabolic pathways even being closely related phylogenetically. Despite sharing some metabolic processes, important functions determine the effectiveness of the response of each genotype. Moreover, even most metabolic activities are inherited by their interspecific hybrid, *S. insanum* × *S. melongena* also showed particular important functions in response to salinity compared to its parental lines, determining non-inherited mechanisms of response to salt. Moreover, with the expression results, we can dismiss a maternal effect in this intercross. In short, these divergent strategies underscore the complexity of plant responses to salt stress and provide valuable insights on the development of salt-tolerance eggplant cultivars.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data availability

The raw data analysed in this study are available in the Short Read Archive (SRA) of the National Library of Medicine repository under the accession number PRJNA1300365.

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Author contributions

Conceptualization, NO-A and AF; Methodology, NO-A; Software, NO-A; Validation, AR-B and AF; Formal analysis, NO-A; Investigation, NO-A; Resources, AF; Data curation, NO-A and AF; Writing—original draft preparation, NO-A; Writing—review and editing, AF; Visualization, AR-B; Supervision, AR-B; Project administration, AF; Funding acquisition, AF.

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Research article

Biometric and biochemical responses to salt in *Solanum dasyphyllum*, a potential donor of tolerance for eggplant

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Neus Ortega Albero has a main role in performing the experiments, sample and data collection, data analysis and visualization, drafting manuscript, revision and editing.

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Abstract

Soil salinity is a major constraint on crop cultivation, affecting millions of hectares of land and increasing drastically worldwide. Identifying sources of tolerance within the crops and their wild relatives is imperative. Recently, *Solanum dasyphyllum* L. has been identified as source of tolerance to drought for eggplant (*S. melongena* L.). In this article, the potential use of *S. dasyphyllum* as a source of tolerance to salinity is investigated through the characterization of young plants' performance under three salt stress treatments, well water (control), as well as 200 mM and 400 mM NaCl. Biometric parameters such as leaf and radicular biomass, plant height, root length, and biochemical parameters—such as photosynthetic pigments, main ions accumulation, proline, total soluble sugars, malondialdehyde, total phenolics, flavonoids, and antioxidant enzymes' activity—were quantified. The results showed a certain reduction in leaf and stem plant growth up to 60% in response to extreme salinity, while root biomass was maintained under mid-salt stress. Salt stress caused toxic ions to accumulate in plant organs, up to 1600 mmol g^{-1}dry weight Na^+ and a 2250 mmol g^{-1} dry weight Cl^- in leaves under extreme salinity exposure. However, *S. dasyphyllum* maintained K^+ levels at around 450 mmol g^{-1} in leaves and roots and 750 mmol g^{-1} in stems, indicating a mechanism related to ion transport to cope with ion toxicity. The biochemical response indicated osmotic adjustments and antioxidant activity without the need of activating antioxidant enzymes. *S. dasyphyllum* has proved to be a valuable genetic tool for new eggplant breeding programs regarding salt stress, with somewhat improved performance regarding biometric parameters and ion transport.

Keywords

Abiotic stress, antioxidant enzymes, ion transport, photosynthetic pigments, proline, salinity, *Solanum dasyphyllum*, wild relative

1. Introduction

Eggplant (*Solanum melongena* L.) is the sixth most cultivated vegetable in the world, with a production of over 54 × 10^6 tons in more than 1.8 × 10^6 hectares (FAO, 2024). It has been reported as moderately tolerant to abiotic stresses such as drought and salinity (Kouassi et al., 2021). However, the new climatic conditions force us to seek further tolerance for extreme events. Hybridization with crop wild relatives (CWRs) has been the main source of variation for cultivated eggplant during decades (Gramazio et al., 2019; Plazas et al., 2016; Rakha et al., 2020). Tolerance to abiotic and biotic stresses has been reported among eggplant CWRs due to adaptation to differentiated climates in the region of origin (Gisbert et al., 2011; Rakha et al., 2020). Thus, hybridization with these CWRs is a valuable tool to increase eggplant variation and enhance its performance in response to a multitude of stresses.

Solanum is one of the most diverse genera within angiosperms, comprising over 1400 species adapted to a large variety of edaphoclimatic conditions. There are more than 450 species in the *Solanum* subgenus *Leptostemonium* Bitter, which comprises *Solanum melongena* (Knapp, Vorontsova, & Prohens 2013). Its CWRs are classified into primary, secondary, and tertiary genepools according to the phylogenetic distance and intercross ability with cultivated eggplant. The primary genepool comprises *S. insanum* L., the wild eggplant ancestor, which produces fully fertile inter-specific hybrids (Knapp, Vorontsova, & Prohens 2013). The secondary genepool comprises African and Southeast Asian species like *S. incanum* L., as well as *S. dasyphyllum* Schumach. and Thonn. (Plazas et al., 2014; Vorontsova & Knapp, 2012; Vorontsova et al., 2013), which can produce inter-specific hybrids with different degrees of crossability and fertility (Plazas et al., 2016). The third genepool includes species distributed around the globe which show very low crossability with cultivated eggplant like *S. torvum* Sw. and *S. sisymbriifolium* Lam. (Kouassi et al., 2016; Plazas et al., 2016). Species from the secondary pool have been evaluated for their survivability under a plethora of environmental stresses and are considered good candidates as gene donors in tolerance breeding projects in eggplant because of their tolerance and crossability (Brenes et al., 2020a; Knapp, Vorontsova, & Prohens 2013; Kouassi et al., 2016; Plazas et al., 2016).

Solanum dasyphyllum, from the secondary genepool, grows in a wide range of tropical areas of Central and South Africa and is considered the wild ancestor of *S. macrocarpon* L. and *S. aethiopicum* L. (Kouassi et al., 2021; Plazas et al., 2014; Plazas et al., 2022). *S. dasyphyllum* has been used as a donor to create an introgression lines (IL) population in a *S. melongena* background (Plazas e al., 2020). The knowledge of all the interesting features about this species is crucial to explore the potential of this ILs-resultant population. Few studies have been published about this species regarding abiotic stresses, and only its tolerance under drought conditions has been evaluated (Kouassi et al., 2021; Villanueva et al., 2023).

Generally, drought and salinity tend to generate the same responses in plants, although some differences exist depending on the genotype tolerance (Brenes et al., 2020b; Martínez-Cuenca et al., 2020; Plazas et al., 2022). Both stresses generate osmotic stress on the plant, limiting nutrient uptake and reducing photosynthetic activity and growth (Zayova, Phulipov, Nedev, & Stoeva, 2017). Nevertheless, salt stress is also associated with a toxic effect due to ion accumulations causing nutrient imbalance and metabolic damage (Shabala, 2009). *S. dasyphyllum* was previously studied under drought conditions, but there is no previous description of its response under salt stress conditions.

Salinity represents a major threat to agriculture in the following decades due to the increase of saline soils and salinized water for irrigation worldwide, which can reduce the yield and quality of the currently cultivated crops (Shahid et al., 2018). Salt stress induces negative morphological, physiological, and biochemical responses in plants, and may even result in plant death under an intense or prolongated application (Forni, Duca, & Glick, 2017). Nonetheless, the damage caused is highly dependent on the genotype and the response mechanism (Hesami, Bazdar & Shahriari, 2020; Ortega-Albero et al., 2023a; Sumalan et al., 2020). Salinity alters the normal function of metabolic processes in plants, including photosynthesis, nutrient acquisition, or enzyme activities (Nishiyama & Murata, 2014). In a sense, plants respond by producing defense metabolites against osmotic stress, activating antioxidant mechanisms and modifying ion transport to avoid ion toxicity (Kumari, Das, Parida, & Agarwal, 2015).

Improving the stress tolerance of cultivated eggplant by hybridization with CWRs is one of the new challenges in breeding programs (Gramazio et al., 2017; Kouassi et al., 2021). In this work, we have undertaken the first phenotypic and biochemical study in young *S. dasyphyllum* plants under different salt stress levels to assess this species' potential as a gene donor for new breeding programs for salt tolerance in eggplant due to salinity tolerance and high crossability.

2. Materials and methods

2.1 Plant material

Seeds of *S. dasyphyllum* were obtained from the germplasm bank of the Institute of Conservation and Improvement of Valencian Agrodiversity (COMAV, UPV, València, Spain). Following the germination method described by Ranil et al. (2015), seeds in Petri dishes were placed in a germination chamber (25ºC, 80% humidity and 16 h light/8 h dark photoperiod) after treatment with gibberellic acid (The Merck KGaA, Darmstadt, Germany) (500 ppm) and a thermic shock.

Plants with two cotyledons were transplanted into plastic trays and were grown in the germination chamber. Once the plants grew to the 4-leaf stage, 70 days

after sowing, selected plants with homogeneous size were transplanted under greenhouse conditions in 2 L-pots with one layer of vermiculite over commercial substrate (Neuhaus Huminsubstrat S, C. Deilmann GmbH and Co., Osterberg, Germany). The trial started after one week of acclimatation.

2.2 Experimental procedures

The treatments consisted of irrigation of five plants, three times per week, with constant watering of 25 mL per pot of solutions containing 200mM NaCl (The Merck KGaA, Darmstadt, Germany), 400 mM NaCl, or well water that is commonly used for crop irrigation as control, while also ensuring that any pot became dry in the late days of the experiments due to evaporation of bigger plants. Before the treatment started, leaf number (LN) and plant height (PH, cm) were measured. After 4 weeks of salt treatment and 15 weeks after sowing, the previous parameters were measured to calculate the growth in plant height (ΔPH) and the growth in number of leaves (ΔLN). In addition, the length of the primary root (RL, cm), stem diameter (SD, mm), and leaf surface (LS, cm^2) were measured. Fresh weight (FW) of roots (RFW, g), stems (SFW, g) and leaves (LFW, g) were also determined. After deep-freezing some leaf material to perform biochemical assays, the rest of the leaves, as well as the stem and root material, were dried for three days at 65ºC until constant weight was reached, thereby obtaining the dry weights (DW) of the roots (RDW), stems (SDW) and leaves (LDW), from which we can calculate the water content using the following equation:

$$WC\ (\%) = [(FW - DW)/FW] \times 100$$

2.3 Soil electrical conductivity

A Crison Conductivity-meter 522 (Crison Instruments SA, Barcelona, Spain) was used for measuring the electrical conductivity of the soil. For the measurements, a 1:5 soil:water dilution was performed using distilled water after the end of the salt treatment for each pot and $EC_{1:5}$ was expressed as dS m^{-1}.

2.4 Biochemical assays

2.4.1 Ion content

Ions were extracted in an aqueous solution from 0.05 g of dry leaf, stem, and root tissue following Weimberg's method (1987). After 1 h incubation in a water bath Ultraterm 200 (J.P.Selecta, Barcelona, Spain) at 95 ºC and centrifugation (centrifuge 5415R, Eppendorf Ibérica S.L.U., Madrid, Spain) for 10 min at 13,300× *g*, cations Na^+, K^+, and Ca^{2+} were quantified with a flame photometer

PFP7 (Jenway Inc., Burlington, VT, USA), and anion Cl^- was quantified using a chloride analyser Corning 926 (Sherwood Scientific Ltd., Cambridge, UK).

2.4.2 Photosynthetic pigments

Alternatively, photosynthetic pigments were extracted from 0.05 g fresh leaf tissue in 1 mL 80% acetone (Labbox Labware, S.L., Barcelona, Spain), mixed overnight, and centrifuged for 10 min at 13,300× *g*. The absorbance of the supernatant was measured at 663, 646, and 470 nm with a spectrophotometer UV-3100PC (VWR International, LLC., Radnor, PA, USA) to calculate chlorophyll a (ChlA), chlorophyll b (ChlB), and carotenoid (Caro) concentrations using Lichtenthaler and Wellburn's equations (1983). Concentrations were expressed in mg g^{-1} DW.

2.4.3 Osmolyte contents

Proline (Pro) and total soluble sugars (TSS) were extracted from 0.05 g fresh leaves. Proline was extracted with 3% sulfosalicylic acid (PanReac Quimica, S.L.U., Barcelona, Spain) and quantified using the acid ninhydrin (The Merck KGaA, Darmstadt, Germany) method and expressed as µmol g^{-1} DW (Bates, Waldren, & Teare, 1973). After incubation in a water bath at 95 ºC for 1 h and cooling, the organic phase was separated, and absorbance at 520 nm was measured. TSS were extracted with 80% methanol (PanReac Quimica, S.L.U., Barcelona, Spain), mixed overnight, and centrifuged. The supernatant was combined with sulfuric acid (Labbox Labware, S.L., Barcelona, Spain) and 5% phenol (Labbox Labware, S.L., Barcelona, Spain), and absorbance was measured at 490 nm, with concentrations expressed as glucose equivalents (Dubois et al., 1956).

2.4.4 Oxidative stress markers

Oxidative stress markers malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) were quantified in fresh leaf tissue. MDA was measured by extracting 0.05 g fresh leaf tissue in 80% methanol and adding thiobarbituric acid (TBA) (PanReac Quimica, S.L.U., Barcelona, Spain) in trichloroacetic acid (TCA) (Labbox Labware, S.L., Barcelona, Spain), followed by incubation at 95 ˚C for 15 min. The absorbance was measured at 440, 532, and 600 nm to quantify MDA, using equations from Taulavori et al. (2001), and the results were expressed in nmol g^{-1} DW. Hydrogen peroxide (H_2O_2) content was quantified by extracting fresh leaf material with 0.1% TCA, followed by centrifugation. The supernatant was mixed with phosphate buffer (The Merck KGaA, Darmstadt, Germany) and potassium iodide (Labbox Labware, S.L., Barcelona, Spain), and the absorbance at 390 nm was

used to calculate H_2O_2 concentrations based on a standard curve, expressed as µmol g^{-1} DW.

2.4.5 Antioxidant compounds

Cell protective antioxidant compounds' total phenolics (TPC) and flavonoids (TF) were extracted from 0.05 g fresh leaf tissue with 80% methanol. TPC were quantified using the Folin–Ciocalteu reagent (PanReac Quimica, S.L.U., Barcelona, Spain) (Blainski, Lopez, & De Mello, 2013), while absorbance was measured at 765 nm. TPC were expressed as gallic acid equivalents (mg eq. GA g^{-1} DW). TF were determined using a method based on the reaction of catechol groups with $AlCl_3$, with absorbance measured at 510 nm. Results were expressed as catechin equivalents (mg eq. C $^{-1}$ DW) following Jia et al.'s method (1999).

2.4.6 Antioxidant enzyme activities

Crude protein extracts were prepared from fresh leaf tissue, and protein concentrations were measured using Bradford's method (1976) (PanReac Quimica, S.L.U., Barcelona, Spain) with BSA (PanReac Quimica, S.L.U., Barcelona, Spain) as the standard. Enzymatic activities of superoxide dismutase (SOD), ascorbate peroxidase (APx), and glutathione reductase (GR) were measured spectrophotometrically. SOD activity was determined by inhibiting the photoreduction of nitroblue tetrazolium (NBT) (The Merck KGaA, Darmstadt, Germany) at 560 nm. APx activity was measured by monitoring ascorbate (The Merck KGaA, Darmstadt, Germany) oxidation at 290 nm. GR activity was determined by monitoring NADPH (The Merck KGaA, Darmstadt, Germany) oxidation at 340 nm. Activity units were defined as the amount of enzyme that catalyzes the conversion of 1 mmol of substrate per minute at room temperature (Aebi, 1984; Connel, Mullet, & Pea, 1986; Nakano & Asada, 1981).

2.5 Statistical analysis

A factorial analysis of variance in all the measured parameters was performed with the Statgraphics Centurion v.XVII software (Statpoint Technologies, Warrenton, VA, USA). Statistical differences were calculated with the Student–Newman–Keuls test with $p < 0.05$.

3. Results

3.1 Growth measurements

Values of soil electrical conductivity ($EC_{1:5}$) before starting the experiment had a mean of 4.1 dS m^{-1} among all the experimental pots. After the salt experiment, $EC_{1:5}$

values showed a large increase from control treatment with well water ($EC_{1:5}$ = 8.5 dS m^{-1}) compared to 200 mM and 400 mM salt treatments ($EC_{1:5}$ = 24.4 dS m^{-1} and $EC_{1:5}$ = 37.4 dS m^{-1}, respectively), showing significant differences between treatments.

S. dasyphyllum development was affected under salt stress (Figure 1). In the 200 mM NaCl treatment, the growth of the plant in height (ΔPH) was reduced to half in comparison with the control, whereas under 400 mM, growth was practically interrupted (Figures 1 and 2). The same results were obtained regarding the production of new leaves (ΔLN), even observing leaf loss with respect to the initial number in plants treated with 400 mM NaCl (Figure 2). Both salt treatments caused reduction in stem diameter (SD) and leaf surface (LS), although there were no significant differences between the salt concentrations (Figure 2).

Figure 1. *Solanum dasyphyllum* plants after four-week treatment with tap water, as well as 200 mM and 400 mM salt treatment (from top to bottom).

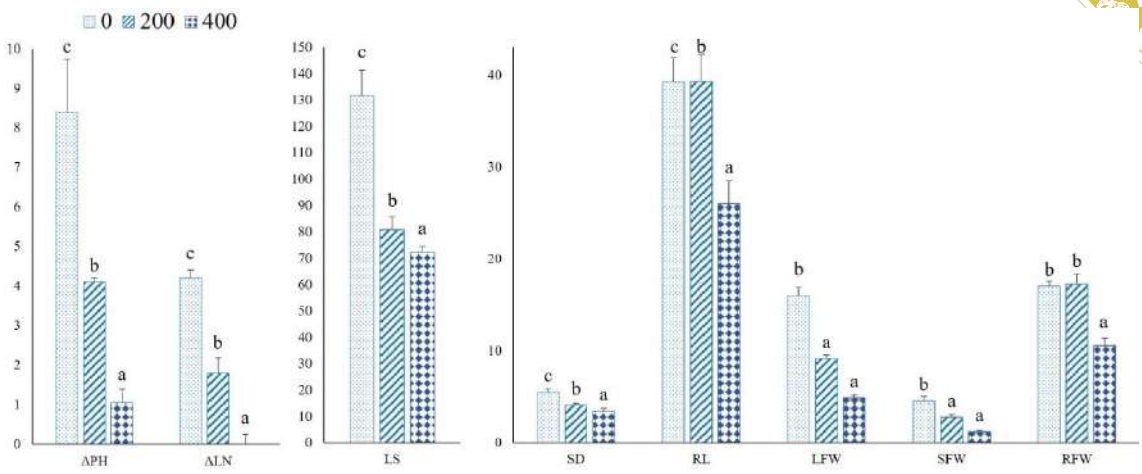

Figure 2. Increment of plant height (ΔPH), increment of leaf number (ΔLN), stem diameter (SD, mm), leaf surface (LS, cm^2), root length (RL, cm), and root (RFW), stem (SFW), and leaf (LFW) fresh weight (g) (mean ± SD; $n = 5$) of *Solanum dasyphyllum* plants treated for one month with the indicated NaCl concentrations. Significant differences between treatments are labelled with different letters ($p < 0.05$).

No differences were found in primary root length (RL) between control plants and plants treated with 200 mM NaCl, indicating that *S. dasyphyllum* maintained root development and osmotic equilibrium (Figure 2). However, 400 mM NaCl caused a RL reduction to 65% of the control values. Similarly, root fresh weight (RFW) was only reduced at the highest salt concentration tested (Figure 2). Leaf (LFW) and stem (SFW) fresh weights followed the same pattern as ΔLN, LS, and SD, decreasing by almost 50% of the control in the presence of 200 mM NaCl and by almost 75% with 400 mM NaCl (Figure 2).

Uniform water content (WC) was found within the treatments, averaging at 78% for roots and 72% for stems, whereas the leaf water content decreased, slightly but significantly, from the 400 mM NaCl treatment, with an average of 83% in control and 200 mM and 80% in 400 mM, indicating that fresh weights are a good indicator of the condition of the plants.

3.2 Ion contents

Ion content was one of the most affected parameters under salinity stress. Na^+ increased significantly under 200 and 400 mM NaCl irrigation, almost 7-fold in roots and 3.5-fold in leaves, when the salt concentration was the highest (Figure 3). Na^+ values in the stem were lower than in the root and leaf; however, this organ showed higher differences between the tested salt treatments, indicating ion movement through the plant vessels. Cl^- levels showed a 6-fold increase in leaves and a 15-fold increase

in stems for salt treatments. However, roots showed a 4.5-fold increase for 200 mM and a 3.2-fold increase for 400 mM NaCl treatment. No reduction was observed in root, stem, or leaf K^+ contents under the salt treatments (Figure 3), but Na^+/K^+ ratios increased in roots, stems, and leaves under salt treatment (Figure 3). The divalent cation Ca^{2+} showed an increase in roots, stems, and leaves under salt stress, although without significant differences between the two salt concentrations (Figure 3). Under control conditions, roots showed more than double Ca^{2+} concentration than stems and leaves.

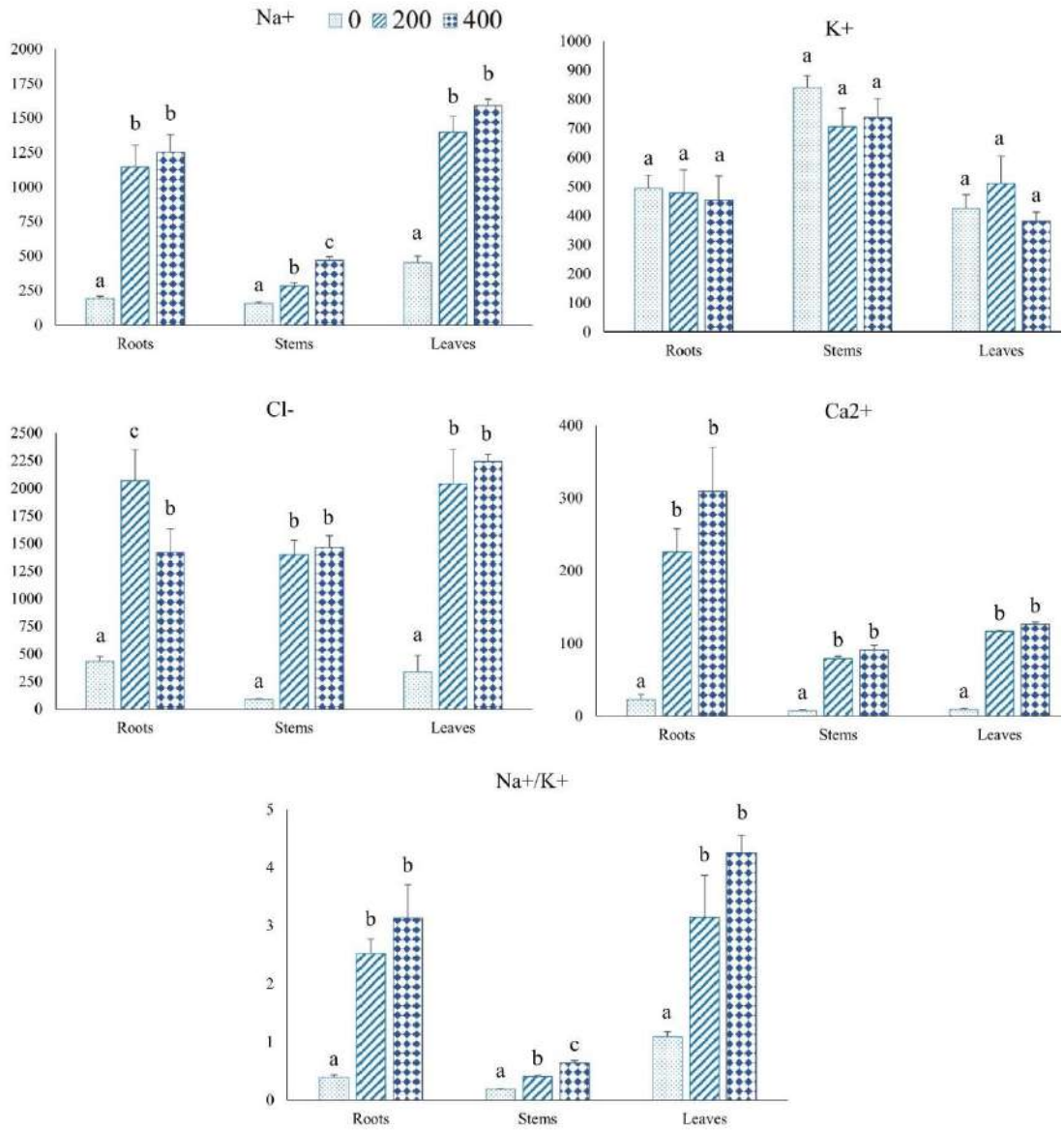

Figure 3. Na^+, K^+, Cl^-, Ca^{2+} contents (mmol g^{-1} DW) and Na^+/K^+ ratio (mean ± SD; n = 5) of *Solanum dasyphyllum*. Significant differences between

treatments are labelled with different lowercase letters and significant differences between plant organs are labelled with different capital letters ($p < 0.05$).

3.3 Biochemical measurements

Chlorophyll a (ChlA) and chlorophyll b (ChlB) average values decreased with increasing salinity, but the differences were not statistically significant. Regarding carotenoids (Caro), no differences were observed in response to salt irrigation (Figure 4). These results indicate the ability of *S. dasyphyllum* to maintain photosynthetic machinery under salt presence.

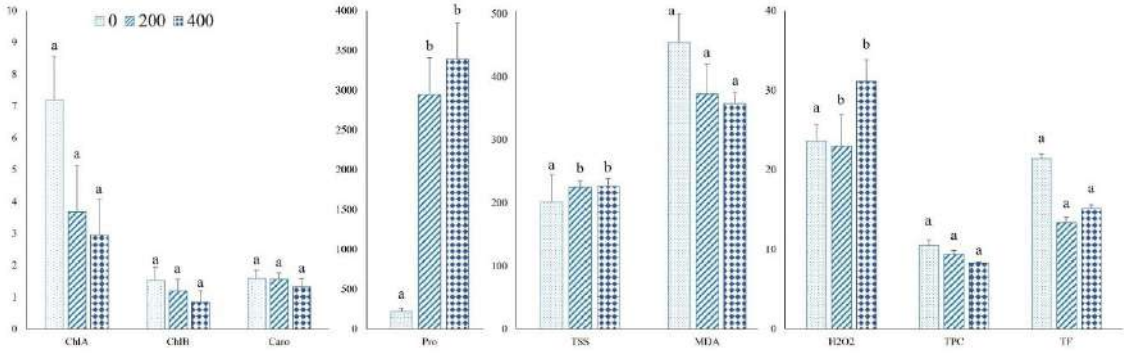

Figure 4. Chlorophyll a (ChlA, mg g^{-1} DW), chlorophyll b (ChlB, mg g^{-1} DW), carotenoids (Caro, mg g^{-1} DW), proline (Pro, µmol g^{-1} DW), total flavonoids (TF mg eq. C g^{-1} DW), total phenolic compounds (TPC, mg eq. GA g^{-1} DW), total soluble sugars (TSS, mg eq. Glu g^{-1} DW), malondialdehyde (MDA, nmol g^{-1} DW), hydrogen peroxide (H_2O_2, µmol g^{-1} DW) contents (mean ± SD; $n = 5$) of *Solanum dasyphyllum* plants treated for one month with the indicated NaCl concentrations. Significant differences between treatments are labelled with different letters ($p < 0.05$).

Osmolyte accumulation due to salt stress was mainly in the form of proline (Pro), which drastically increased under salt stress by more than 15-fold under 400 mM NaCl irrigation compared to control plants (Figure 4). On the contrary, total soluble sugars (TSS) did not show differences between the control and any salt condition (Figure 4). The stress provoked by the salt treatments was not enough to activate the oxidative stress markers malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), which showed no significant differences between treated and untreated plants (Figure 4). However, antioxidant molecules like total phenolic compounds (TPC) and total flavonoids (TF) were significantly reduced after NaCl application (Figure 4).

Regarding antioxidant enzymes, no significant differences were found in *S. dasyphyllum* plants between control and salt treatments for analysed antioxidant enzymes ascorbate peroxidase (APx) and glutathione reductase (GR). Only superoxide dismutase (SOD) showed an increase in its activity under 400 mM NaCl irrigation, but no statistical differences were observed between control treatment and the 200 mM NaCl irrigation (Figure 5). These results indicate that other mechanisms are enough to cope with salt stress in *S. dasyphyllum*, which did not activate antioxidant enzymes.

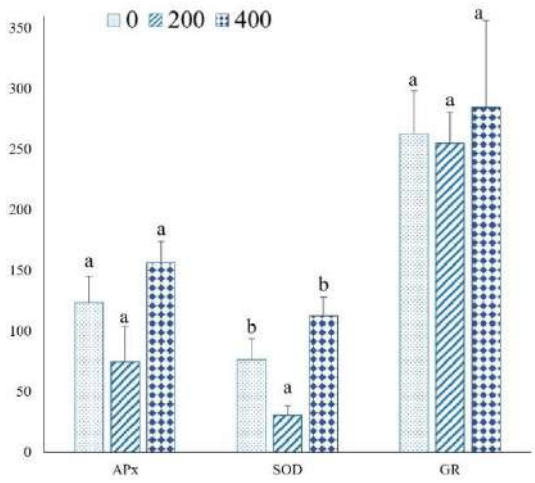

Figure 5. Enzymatic activities (units mg^{-1} protein) of ascorbate peroxidase (APx), superoxide dismutase (SOD), and glutathione reductase (GR) (mean ± SD; $n = 5$) of *Solanum dasyphyllum* plants treated for one month with the indicated NaCl concentrations. Significant differences between treatments are labelled with different letters ($p < 0.05$).

3.4 Meta-analysis of salinity response in *Solanum* species

Eggplant CWRs have been evaluated for different abiotic stresses. Table 1 shows a comparison between the responses to salinity of two *S. melongena* genotypes, and several *Solanum* species from the primary, secondary, and tertiary genepool, including our results in *S. dasyphyllum*. This comparison includes biometric and biochemical parameters and the increase or reduction in percentage with respect to the control as 100%. Most species tend to show a reduction in biomass; however, most *S. melongena, S. insanum*, and *S. dasyphyllum* cope with salinity stress by maintaining root biomass, and only *S. dasyphyllum* maintained its root length completely at 200 mM.

Regarding ion accumulation, *S. dasyphyllum* increased Na^+ in leaves similar to *S. melongena* at 200 mM and accumulated the same levels as *S. aethiopicum*. Species from the secondary genepool also accumulated more K^+ in leaves than

other species, trying to maintain K^+ homeostasis and were similar to *S. insanum* for K^+ accumulation in roots. Cl^- accumulation in *S. dasyphyllum* was lower than the accumulation of *S. insanum* but higher than *S. melongena*.

Photosotynthetic pigments seemed to be maintained in *S. melongena* and *S. aethiopicum*¸ while they seemed to be increased in *S. melongena* and reduced in *S. dasyphyllum* and *S. torvum*. Nevertheless, this was dependent on the pigment. *S. dasyphyllum* showed the highest increase in Pro after *S. torvum* at 200 mM NaCl but not at 400 mM NaCl. Any *Solanum* species seemed to accumulate TPC under salt stress.

Table 1. Comparison of biometric and biochemical values in different Solanum species under salinity treatment, presented as percentages relative to control (100%), and bibliographic references.

	S. melongena				**Primary Genepool**	
	MEL1		**MEL5**		***S. insanum***	
	200 mM	**400 mM**	**200 mM**	**400 mM**	**200 mM**	**400 mM**
LFW	60	30	60	30	58	30
RFW	130	79	119	86	108	68
RL	70	56	92	70	94	68
Na^+ leaves	312	299	293	418	524	617
Na^+ roots	215	297	443	505	745	954
K^+ leaves	116	326	49	61	88	147
K^+ roots	45	64	79	99	106	84
Cl^- leaves	331	324	356	488	981	1133
Cl^- roots	195	224	300	322	509	686
ChlA	117	133	109	77	205	123
ChlB	81	80	160	83	138	60
Caro	89	94	141	92	112	55
Pro	524	1061	578	1028	817	13,610
TPC	84	83	100	100	100	100
Refs.	Ortega-Albero et al. (2023b)		Ortega-Albero et al. (2023a)			

	Secondary Genepool		**Tertiary Genepool**			
	S. dasyphyllum		***S. aethiopicum***		***S. torvum***	
	200 mM	**400 mM**	**200 mM**	**400 mM**	**200 mM**	**300 mM**
LFW	57	30	60	33	40	30
RFW	102	62	74	49	45	40
RL	100	66	95	81	80	60
Na^+ leaves	308	351	468	517	1101	835
Na^+ roots	605	660	645	605	522	429

K^+ leaves	121	90	162	185	56	75
K^+ roots	97	92	63	105	62	59
Cl^- leaves	604	664	413	463	875	649
Cl^- roots	479	328	379	323	337	371
ChlA	51	41	109	120	64	74
ChlB	77	55	108	109	59	58
Caro	99	84	64	67	66	92
Pro	1362	1568	369	1082	3676	4282
TPC	89	79	100	100	82	132
Refs.			Ortega-Albero et al. (2023b)		Brenes et al. (2020a)	

4. Discussion

Eggplant CWRs have proven to be tolerant to different abiotic stresses (Brenes et al., 2020a; Knapp, Vorontsova, & Prohens, 2013; Kouassi et al., 2016; Plazas et al., 2016). Studying their performance under these stresses and understanding the molecular mechanisms underlying tolerance is essential for establishing new breeding programs for cultivated eggplant.

4.1 Previously available information about *S. dasyphyllum*

Drought stress responses have been previously studied in *Solanum dasyphyllum*, which showed a better performance than *S. melongena* under this stress condition, modifying expression in metabolic paths related to plant growth, plant signaling, and phenylpropanoid synthesis (Plazas et al., 2014; Prohens et al., 2013; Villanueva et al., 2023). Moreover, previous reports *in vitro* on *S. dasyphyllum* after salt application described a better callus development compared to *S. macrocarpon*, its direct cultivated descendant, showing good capacity to maintain turgor and cell expansion under these conditions (Hannachi et al., 2021). Nevertheless, to our knowledge, there is no previously available information on *S. dasyphyllum* plants responding to salt stress. Also, *S. dasyphyllum* is the donor parent in a ILs population, and this type of information is key to exploding this resource (Plazas et al., 2020). This work presents a three-level salinity (well water, 200 and 400 mM of NaCl) study on young plants to assess *S. dasyphyllum* tolerance under soil and greenhouse conditions. Salt concentrations used in this study represent a gradient that spans from no salt to moderate and high levels of salinity. Regarding available data, 200 mM NaCl was selected to be the concentration where most eggplant genotypes started to show stress symptoms but can still adapt and grow. 400 mM NaCl was selected to display stress effects in tolerant genotypes and to understand *S. dasyphyllum*'s capacity to adapt to extreme saline conditions.

4.2 Growth performance of *S. dasyphyllum* under salt stress

Generally, salinity triggers morphological, physiological, biochemical, cellular, and molecular mechanisms in plants, causing severe reduction in plant growth and crop production of glycophyte species (Brenes et al., 2020b; Ortega-Albero et al., 2023a; Plazas et al., 2020). In our experiment, well water used for irrigation resulted in a slightly salinized substrate in the control pots. This is a common side effect of using bad-quality water, as it is being studied in many areas near the Mediterranean Sea (de Paz et al., 2004; 2011). However, young *S. dasyphyllum* plants showed a good visual appearance under control conditions, despite receiving salinized water and having only started limiting their growth and development with salinity treatment as high as 200 mM NaCl. However, salt tolerance is a complex trait involving different components at physiological, biochemical, and genetic levels (Kuar et al., 2013). Interestingly, plants managed to maintain root biomass at 200 mM NaCl, as previously reported in other Solanum species. Moreover, *S. dasyphylum* seemed to maintain root biomass by maintaining root length (Julkowska et al., 2017; Ortega-Albero et al., 2023a).

4.3 Impact of salt stress on photosynthetic pigments

Salt stress typically modifies photosynthetic metabolism, resulting in the degradation of photosynthetic pigments; therefore, they are useful as stress biomarkers (Hamani et al., 2020). *S. dasyphyllum* showed a slight reduction in chlorophyll *a* and chlorophyll *b* contents, as with other eggplant wild relatives such as *S. torvum* (David-Rogeat, et al., 2024). However, the reduction was not significant, suggesting tolerance of the studied species to salinity, even at high salt concentrations, as it strives to maintain the carbon assimilation process as observed in *S. melongena*, *S. insanum*, and *S. aethiopicum*.

4.4 Ion accumulation and comparison with other CWRs

S. melongena and other related species such as *S. insanum*, *S. torvum*, and *S. aethiopicum* accumulate Na^+ and Cl^- in leaves, stems, and roots in the presence of salt (Brenes et al., 2020a; 2022b; David-Rogeat, et al., 2024; Ortega-Albero et al., 2023b; Shahbaz et al., 2013; Shaheen et al., 2013). Na^+ competes with K^+ for membrane transport proteins, and as salinity increases, K^+ levels decrease (Shahbaz et al., 2013; Shaheen et al., 2013). In eggplant and CWRs, under salt stress, different mechanisms have been observed to avoid toxicity and maintain K^+ homeostasis. *S. melongena* seems to accumulate Na^+ in leaves, increasing the Na^+ leaf/root ratio while attempting to maintain the K^+ leaf/root ratio (Ortega-Albero et al., 2023a; Li et al., 2019). Other species, such as *S. insanum*, showed an increase in the K^+ leaf/root ratio, suggesting a better osmotic adjustment due to their ability to activate K^+ transport to the photosynthetic tissues (Brenes et al., 2020b; Ortega-Albero et al., 2023a).

Also, higher K^+ accumulation in leaves and a lower Na^+/K^+ ratio in leaves have been reported in tolerant genotypes, indicating that Na^+ compartmentalization in vacuoles and K^+ accumulation in leaves might be relevant for tolerance (Assaha et al., 2015; Li et al., 2019). Our results showed that *S. dasyphyllum* maintained K^+ concentration in roots and leaves under salt treatment, as observed in other CWRs like *S. aethiopicum* (David-Rogeat et al., 2024). Moreover, *S. dasyphyllum* showed a stable K^+ leaf/root ratio in the three experimental conditions, whereas its Na^+ leaf/root ratio was close to 1 with salinities of 200 mM and 400 mM. Also, it seemed that the Na^+ and K^+ compensation triggered by salinity followed the same pattern at 200 mM and 400 mM treatments to cope with ion toxicity, indicating that the activated response is independent from the concentration of NaCl. In regard to Ca^{2+}, it helps to control Na^+/K^+ homeostasis by modulating the SOS metabolic pathway (Mahajan, Pandey, & Tuteja, 2008), so noticing Ca^{2+} increase under salt stress for all organs and salt concentrations tested might be a plant response to protect metabolic and cellular processes, which is the case for *S. dasyphyllum*.

4.5 Osmoprotectants and oxidative stress

The stress-related osmoprotectant proline (Pro) has been described as a marker of abiotic stress because of its capacity for maintaining membrane stability, protein architecture, and ROS detoxification (Alvazez, Savouré, & Szabados, 2022). Pro has been reported to be overproduced in more tolerant genotypes; however, this does not seem to be a universal phenomenon (Szabados & Savouré, 2010). Pro significantly increased in *S. dasyphyllum* under 200 mM and 400 mM salt concentrations, which could indicate a higher tolerance, compared with cultivated eggplant and other CWRs Hannachi et al., 2021). As was previously found in *Solanum* species (Ortega-Albero et al., 2023a), no changes were noticed in other stress-related metabolites such as MDA and TSS due to the salinity, suggesting that their function is not relevant for salt tolerance in this genus.

Furthermore, no response in antioxidant enzymes was found, indicating that *S. dasyphyllum* suffered from less oxidative stress than other CWRs (Hannachi et al., 2021). These results reinforce the idea that changes in ion transport and compartmentalization, as well as osmolyte accumulation are the main mechanisms in *S. dasyphyllum* to face salinity and allow plant development and survival.

5. Conclusions

The response to salt irrigation of *Solanum dasyphyllum*, a wild eggplant relative, is reported in this study. Growth parameters were affected at a high salinity of 200 mM and plant development seemed to stop at 400 mM. However, root length was maintained, and it is settled as a major mechanism in regard to plant growth to cope with the stress. Differentiated ion transport mechanisms and proline

production seem to be key to coping with oxidative stress and avoiding further cell damage. Here, we settled the importance of the underestimated *S. dasyphyllum* as a gene reservoir for salt tolerance for cultivated eggplant. These, alongside previous results, reinforce the idea of the importance of CWRs from the secondary and tertiary genepools for eggplant breeding. Future experiments including *S. dasyphyllum, S. melongena*, and other CWRs should be performed, including biometric, biochemical, and expression analysis to give a complete vision of the response of *S. dasyphyllum* to salt stress and the differences found with other eggplant species.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Author contributions

Conceptualization, AF; Methodology, SG-O and NO-A; Software, SG-O and NO-A; Validation, AF and OV; Formal analysis, SG-O and NO-A; Investigation, SG-O and NO-A; Resources, OV and AR-B; Data curation, AF and NO-A; Writing—original draft preparation, NO-A; Writing—review and editing, SG-O and OV; Visualization, SG-O and OV; Supervision, AR-B; Project administration, AF and AR-B; Funding acquisition, AF.

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Chapter II.

Impact of salinity on fruit quality of eggplant cultivars

Research article

Internal fruit quality is maintained in eggplant under mild long-term salt treatment

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Neus Ortega Albero has a main role in performing the experiments, sample and data collection, data analysis and visualization, drafting manuscript, revision and editing.

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Abstract

Modern *Solanum melongena* varieties have been developed to improve the content of phenolics, sugars, and nutritionally relevant minerals in fruit. However, fruit composition might be altered due to abiotic stresses like salinity. Physiological and fruit quality traits were evaluated in four eggplant landraces under usual irrigation and moderately salty irrigation conditions (80 mM NaCl). Growing parameters measured included root length, leaf surface, and fresh weight, while fruit composition traits included sugars, phenolics, and mineral content determinations. Few differences were observed for agronomic traits, probably due to the mild tolerance of eggplant to salinity. Some varieties showed signs of salt tolerance like an increase in primary root length to overcome salt stress. Glucose was the metabolite most affected by the salt treatment in the fruit, while phenolic compounds and other metabolites studied were not altered. Significant differences were observed in the main minerals Na, K, Ca, P, and Mg, both between genotypes and treatments. Although salinity produced changes in some physiological and developmental traits, the composition of the fruit was not significantly modified for the accessions tested. Mineral, sugar, and phenolic contents were not particularly altered in unripe fruits, indicating tolerance of eggplant varieties to salinity in terms of fruit quality.

Keywords

Chlorogenic acid, minerals, phenolic compounds, salt stress, *Solanum melongena*, sugars

1. Introduction

Eggplant, or brinjal (*Solanum melongena* L.), is one of the main vegetable crops worldwide, with a total production of over 50×10^6 t (FAO, 2023). Eggplant fruits are known for their high phenolic content, mostly anthocyanins, in the peel and chlorogenic acid in the flesh (Docimo et al., 2016). In addition, eggplant is a good source of diverse minerals, whereas it has a moderate sugar content (Raigon, Rodríguez-Burruezo, & Prohens, 2010). Eggplant has a wide natural variation between genotypes for its nutraceutical composition, which could be of interest to new quality breeding programs (Mennella et al., 2012; Plazas et al., 2013; Prohens, Rodríguez-Burruezo, Raigón, & Nuez, 2007).

In many agricultural areas of the world, deficient use of irrigation water, application of fertilisers, poor drainage systems, and accumulation of Na^+ and K^+ ions in soils may cause severe problems like salinity, which affects the production of many economically important crops (FAO, 2023; Zhao et al., 2021). Moreover, soil salinization is one of the consequences of climate change due to the alteration in patterns of precipitation and the dramatic reduction in water availability and water use efficiency because of CO_2 accumulation (Corwin, 2021).

Under saline conditions, plant growth is impaired, and many physiological parameters are affected, such as photosynthesis, nutrient acquisition, and transportation. Also, normal cellular and enzymatic activity can be altered due to the reduction in water availability, ion toxicity, and K^+ deficiency, which induces osmotic imbalances in the cells and oxidative stress in plants (Blumwald, 2000; Nishiyama & Murata, 2014). If the stress is prolonged during the season, even mild salinity can remarkably alter yield and fruit quality (Mady et al., 2023).

As salt affects the metabolic profile of the plant, it also may affect fruit composition. Interestingly, this effect is not always negative for fruit quality. In tomatoes, for instance, an improvement in the fruit quality when plants are subjected to moderately saline irrigation, particularly in sugar and acid contents, as a result of osmotic adjustments has been reported (Agius, von Tucher, & Rozhon, 2022; Meza et al., 2020). In response to salt stress, tomato plants decrease plant growth and yield and start accumulating specific metabolites, some of them in the fruit, through reducing water content, which is commonly known as the "concentration effect" (Galli et al., 2016; Massaretto et al., 2018; Meza et al., 2020).

Although being described as a glycophyte, eggplant has been demonstrated to have a higher tolerance to salt than other Solanaceae species (Brenes et al., 2020; Ortega-Albero et al., 2023; Ünlükara et al., 2010). Moreover, eggplant rootstocks are commonly used to alleviate salinity stress in tomatoes (Sanwal et al., 2022). In a saline environment, eggplant changes the absorption, transport, and accumulation of ions (Ortega-Albero et al., 2023). This competition causes a loss in yield and plant growth

and can cause a deviation compared to normal mineral accumulation in fruit. However, information on how salinity affects fruit quality in eggplant is scarce or almost nil.

The aim of this work is to identify the differences in fruit composition due to eight weeks of salt irrigation in four cultivated *Solanum melongena* L. genotypes by analysing plant growth parameters, total phenolic compounds, sugars, and mineral composition of the fruit; hence, understanding the importance of eggplant in future meal plans when cultivated in changing environments.

2. Materials and methods

2.1 Plant material and seed germination

Four *Solanum melongena* commercial cultivars were used for this experiment. MEL1 originated in the Ivory Coast, and MEL5 originated in Sri Lanka, as referred to in Plazas et al. (2016). ALM and LdG are two Spanish landraces referred to in Vilanova et al. (2011). To improve germination, seeds were sown following the protocol described by Ranil et al. (2015). Seeds were immersed in distilled water for 24 h and then in gibberellic acid 500 ppm for 24 h. Afterward, seeds were placed in Petri dishes (90 mm in diameter) over sterile cotton and filter paper watered with KNO_3 1000 ppm. A hot shock of 37 ºC was applied for 24 h after a cold treatment at 4 ºC for 7 days. Petri dishes were placed in a germination and growing chamber at 25 ºC, 78% humidity, and a 16/8 photoperiod.

2.2 Plant growth and salt treatment

Ten plants per genotype at the 4-leaf stage were transplanted into the greenhouse to 33 × 29 cm pots (15 L capacity) with coconut fiber. Fertiliser CoteN Mix (Haifa Negev Technologies Ltd., Haifa, Israel) with NPK = 20-5-10 and control release was added to each pot every 3 to 4 weeks. They were grown until the pre-flowering stage, when the salt treatment started. Plants were watered through automated irrigation of 1.65 L daily with 4 L^{h-1} emitters and 3 irrigation times of 5 min. Two treatments were tested: 5 plants were watered with a non-saline (control) solution, and the other 5 with a solution containing 80 mM of added NaCl. Once a week, plants were irrigated with the control solution to simulate eventual natural rain on the crop. In this way, we could achieve the electrical conductivity of high–moderate saline areas in eastern Spain, which ranges from 4 to 6 dS m^{-1} (De Paz et al., 2004; 2011).

After 8 weeks of salt treatment, the following parameters were measured: primary root length (RL, cm) determined with the clean root extended from the neck to the apex, leaf surface (LS, cm^2), determined with Digimizer image analysis software version 6.4.0 (MedCalc Software Ltd., Ostend, Belgium), root fresh weight (RFW, g), and leaf fresh weight (LFW, g). Part of the material was dried for three

days at 65 ºC and weighted to calculate water content (WC, %) using the following formula: WC (%) = [(FW − DW)/FW] × 100

2.3 Substrate electrical conductivity

The electrical conductivity ($EC_{1:5}$) of the substrate was determined at the end of the experiment with 5 g of the dried coconut fiber from each pot diluted with 30 mL of MilliQ water. The solution was shaken for 2 h at 600 rpm and filtered for big particles. $EC_{1:5}$ (dS m^{-1}) was measured with a Crison conductivity meter 522 (Crison Instruments SA, Barcelona, Spain).

2.4 Fruit weight

Measurements of fruit weight (FruW) (g) were taken from the fruit production for all genotypes. Fruit collection started after 1 week of salt treatment. The quantity of fruits per genotype was variable, with a minimum of five fruits under salinity treatment and a maximum of ten fruits. After weighting, fruit flesh was lyophilized (VirTis Genesis, SP Scientific, Warminster, PA, USA) for further measurements. Fruit yield was not recorded, as the pollination in the greenhouse was poor, and, therefore, the fruit setting was not representative of a commercial eggplant crop.

2.5 Mineral content quantification

Mineral content was extracted from 2 g samples of dry root, leaf material, and lyophilized fruit, as described by Raigon et al. (2010), and quantified with an inductively coupled plasma spectrometer ICP-EOS 710 (Agilent Technologies, Santa Clara, CA, USA). Ion concentrations were expressed in mg 100 g^{-1} DW.

2.6 Phenolic compounds quantification in fruit

Total phenolic compounds (TPCs) were extracted from 125 mg of lyophilized fruit weight, following the Folin–Ciocaltieu method, and quantified based on a colorimetric reaction measuring absorbance at 750 nm (Kumari et al. 2022), referring to chlorogenic acid as control. TPCs were expressed in mg TPC g^{-1} DW.

2.7 Chlorogenic acid and sugar contents in fruit

For the sugar determinations, 0.100 g of lyophilized material was diluted in 1.5 mL of ultrapure water. The samples were extracted with a vortex agitator set at maximum rpm rotational speed for 1 min and centrifugated for 5 min at 12,000 rpm. The supernatant was filtered through 0.22 µm PVDF syringe filters and analysed in an Agilent 1220-Infinity HPLC System (Agilent Technologies, Santa Clara, CA, USA). The compound separation was performed with a Luna® Omega SUGAR

LC column (150 mm × 4.6 mm i.d., 3 µm particle size) (Phenomenex, Torrance, CA, USA) and a guard column (SUGAR, 4 mm × 3.0 mm i.d.). Quantifications were based on calibration curves prepared for glucose (GLU), fructose (FRU), and sucrose (SUC) by the external standard method from 0.3 g L^{-1} to 20 g L^{-1}, and quantities were expressed in mg g^{-1} DW (Guijarro-Real et al., 2023).

Chlorogenic acids were extracted and quantified according to Plazas et al. (2014). First, 0.05 g of lyophilized samples were homogenized with 1.5 mL of methanol/water (80:20, *v*/*v*) plus 0.1% (*w*/*v*) of 2,3-tert-butyl-4-hydroxyanisole (BHT). The total extract was vortexed vigorously, sonicated for 1 h at room temperature, and then centrifuged at 10,000 rpm for 5 min. The supernatant was filtered through 0.22 µm PTFE syringe filters and analysed by HPLC-UV at 325 nm. The analysis was performed on a Brisa C18 column (3 µm; 150 × 4.6 mm) (Teknokroma Analítica S.A., Barcelona, Spain). Quantification was based on external calibration obtained by serial dilutions of commercial standard chlorogenic acid covering the concentration ranges from 20 to 500 mg L^{-1}, and quantities were expressed in mg g^{-1} DW.

2.8 Data analysis

Data statistical analysis was made with Statgraphics Centurion XVII.I (Statpoint Technologies, Warrenton, VA, USA). A factorial analysis was performed for all the parameters quantified considering as factors genotype, treatment, and their interaction. Statistical differences between treatments and genotypes were evaluated with a Student–Newman–Keuls test. Correlations between ion accumulation in different organs were also calculated.

3. Results

3.1 Substrate electrical conductivity

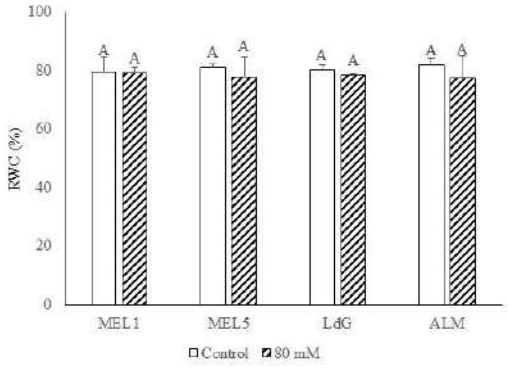
Electrical conductivity was measured after 8 weeks of salt treatment ($EC_{1:5}$). The values obtained in both treated and non-treated plant pots were similar for all genotypes. However, ALM showed a slightly higher salt presence in control plant pots compared to MEL1, probably due to the different water requirements between them (Table 1).

Table 1. Electrical conductivity ($EC_{1:5}$ mean ± SD; $n = 5$) in the pots after 8 weeks of treatment of *Solanum melongena* accessions under control and saline irrigation (80 mM) treatments. Different letters indicate statistically significant differences between genotypes ($p < 0.05$) within each treatment. The asterisk indicates statistically significant differences between treatments ($p < 0.05$) on the corresponding genotype, according to the Student–Newman–Keuls multiple range test.

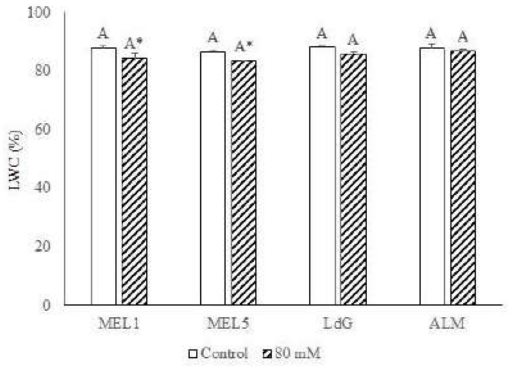
	Genotype	Control	Salt Treatment
$EC_{1:5}$	MEL1	1.86 ± 1.1^{A}	$4.36 \pm 0.4^{A,*}$
	MEL5	1.2 ± 0.1^{AB}	$5.61 \pm 0.8^{A,*}$
	LdG	1.78 ± 0.2^{AB}	$5.3 \pm 0.3^{A,*}$
	ALM	2.32 ± 0.2^{B}	4.02 ± 2.1^{A}

3.2 Plant growth parameters

The water content of the genotypes was not greatly affected by the salt treatment (Figure 1). Therefore, the water content of the roots remained around 75%, no matter the genotype or treatment (Figure 1a), whereas the leaf water content was around 85% except for MEL1 and MEL5, which showed a reduced leaf water content under stress (Figure 1b).



(a)



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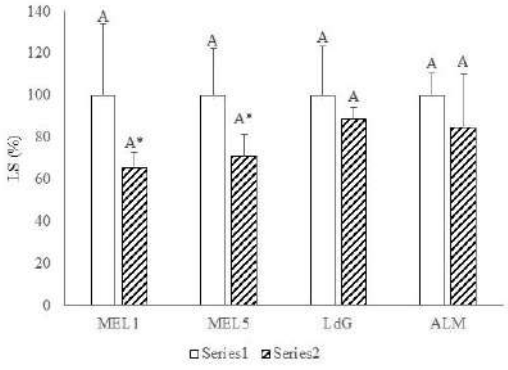
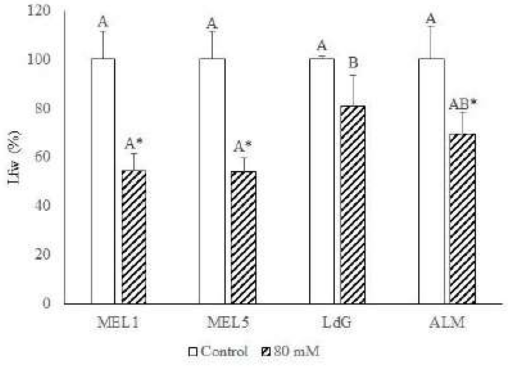
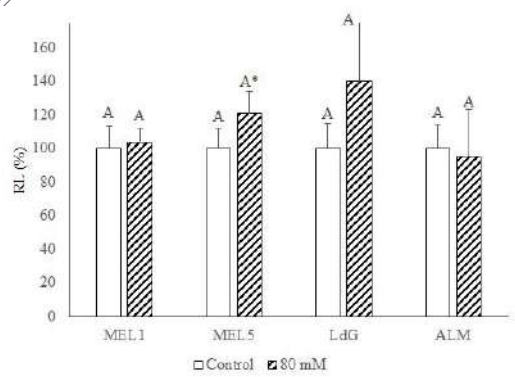


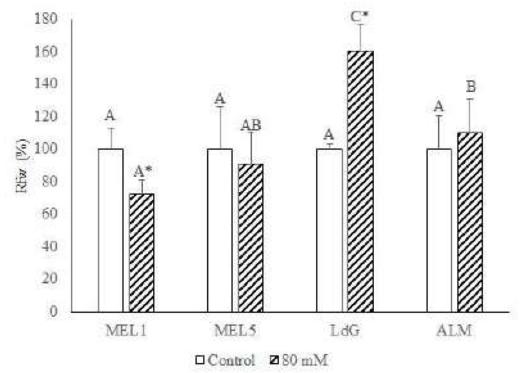
(c)



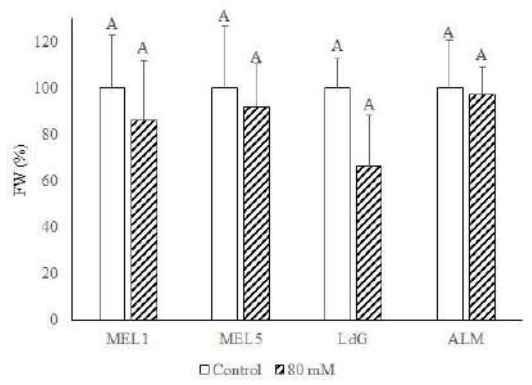
(d)



(e)



(f)



(g)

Figure 1. (a) Root water content (RWC), (b) leaf water content (LWC), (c) leaf surface (LS), (d) leaf fresh weight (LFW), (e) root length (RL), (f) root fresh weight (RFW), and (g) fruit weight (FruW) (mean± SD; n = 5) for *Solanum melongena* accessions under control and saline irrigation (80 mM) treatments. Different letters indicate statistically significant differences between genotypes ($p < 0.05$) within each treatment. The asterisk indicates statistically significant differences between treatments ($p < 0.05$) in the corresponding genotype, according to the Student–Newman–Keuls multiple range test.

As different varieties, the accessions showed differences in their aerial part size in control conditions and were affected by salt stress in different ways. Thus, MEL1 and MEL5 significantly reduced their leaf surface (LS) (Figure 1c) and their leaf fresh weight (LFW) (Figure 1d). This was not the case for LdG, which was not affected by the salt treatment in the aerial traits. Finally, ALM was affected in LFW but not in LS. However, LdG and ALM were less affected than MEL cultivars.

Both control and stress conditions did not affect the weight of roots. The only exception was MEL5, which increased the root length (RL) under salt treatment, and MEL1 and LdG, which showed opposite responses to salt (Figure 1e). The first one

decreased considerably RFW under saline conditions, while the second one increased RFW (Figure 1f).

As expected, fruit weight (FruW) differed greatly among accessions under control conditions, and the salt treatment only produced a slight decrease in LdG fruit weight (Figure 1g).

3.3 Mineral composition in leaves, roots and fruits

The tested genotypes showed significant differences in their root mineral composition under control conditions for P, Mg, B, and Mn and in their leaf mineral composition for Na, Ca, P, Mg, B, and Mn. Therefore, K, Fe, and Zn showed similar basal values among genotypes (Table 2). The treatment with saline irrigation modified those mineral contents in a genotype-dependent manner.

Na concentration increased considerably under salt treatment. Thus, all the genotypes doubled (MEL1 and ALM) or even tripled (MEL5 and LdG) their Na content in the roots under the salt treatment (Table 2). In comparison, the response to the salt treatment observed in the leaves was still more diverse. Thus, MEL1 and LdG accumulated 17-fold Na content in the leaves of salt-treated plants in comparison to the control, whereas MEL5 and ALM accumulated 8.6- and 4.1-fold Na, respectively. By contrast, the levels of K present in roots and leaves were barely affected by the salt treatment, except for 0.9-fold in the leaves of MEL1 and MEL5 under salt stress (Table 2).

Regarding the other minerals assessed, the effects of saline stress were scarce, apart from some exceptions (Table 2). For example, the most affected genotype by the salt treatment at the mineral level was MEL1, which showed lower levels of Ca, P, Mg, Fe, B, and Zn root concentrations and P in the leaves in comparison to control conditions. ALM, on the contrary, only decreased leaf mineral composition for Ca and B and showed an increase in Fe and Zn under this condition. ALM also increased its root concentration of Mn under salt treatment. MEL5 was affected at Mg root concentrations and Fe leaf concentrations. Finally, LdG was the less affected genotype, with changes only at leaf B concentration (Table 2).

Similar to the roots and leaves, the basal levels of Na and K concentrations in the fruits were similar among genotypes; however, Na levels were highly increased under salt treatment, especially for MEL1 and LdG (13.2and 5.3-fold, respectively) (Table 2). The K fruit concentration was significantly reduced only for MEL1 and ALM. Fruit mineral concentration for Ca, P, Fe, B, and Mn was different among genotypes at the control treatment and was not altered by the salt treatment except for the concentration of Mn, which was reduced in the case of MEL5 (Table 2). The concentrations of Mg and Zn were not significantly different among genotypes under control conditions, and although they became different under salt treatment, the

differences were not enough to consider them different from their control values (Table 2).

Table 2. Mineral composition (mg 100 g^{-1} DW) in root, leaf, and fruit (mean ± SD; $n = 5$) of *Solanum melongena* accessions under control and saline irrigation (80 mM) treatments. Different letters indicate statistically significant differences between genotypes ($p < 0.05$) within each treatment. The asterisk indicates statistically significant differences between treatments ($p < 0.05$) on the corresponding genotype, according to the Student–Newman–Keuls multiple range test.

		NaCl Treatment (mM)						Fold-Change Salt vs. Control		
		Root		Leaf		Fruit		Root	Leaf	Fruit
		0	80	0	80	0	80			
Na	MEL1	4.22 ± 1.6 ^{A}	10.29 ± 0.7 ^{A}	0.17 ± 0.1 ^{AB}	2.88 ± 0.7 ^{B}	0.34 ± 0.2 ^{A}	4.45 ± 0.6 ^{A}	2.44 *	17.44 *	13.19 *
	MEL5	3.25 ± 0.8 ^{A}	12.65 ± 1.4 ^{A}	0.13 ± 0.1 ^{AB}	1.09 ± 0.4 ^{A}	0.45 ± 0.2 ^{A}	2.36 ± 1.1 ^{A}	3.89 *	8.66 *	5.29 *
	LdG	4.12 ± 1.7 ^{A}	13.47 ± 0.5 ^{A}	0.09 ± 0 ^{A}	1.52 ± 0.1 ^{AB}	0.76 ± 0.4 ^{A}	4.02 ± 0.3 ^{A}	3.27 *	17.64 *	5.31 *
	ALM	5.7 ± 1.2 ^{A}	12.25 ± 2.2 ^{A}	0.22 ± 0.1 ^{AB}	0.9 ± 0.6 ^{A}	0.38 ± 0.2 ^{A}	2.54 ± 0.9 ^{A}	2.15 *	4.13 *	6.69 *
K	MEL1	7.79 ± 0.8 ^{A}	7.07 ± 2.1 ^{A}	11.12 ± 0.6 ^{A}	0.26 ± 0.5 ^{A}	9.66 ± 0.3 ^{A}	9.01 ± 0.4 ^{A}	0.91	0.92 *	0.93 *
	MEL5	7.09 ± 1.2 ^{A}	6.37 ± 0.7 ^{A}	10.54 ± 0.2 ^{A}	9.82 ± 0.4 ^{A}	9.52 ± 0.4 ^{A}	9.71 ± 0.5 ^{A}	0.9	0.93 *	1.02
	LdG	7.13 ± 0.5 ^{A}	5.24 ± 1.2 ^{A}	10.46 ± 0.3 ^{A}	10.75 ± 0.7 ^{B}	9.82 ± 0.1 ^{A}	9.13 ± 0.5 ^{A}	0.74	1.03	0.93
	ALM	7.69 ± 0.7 ^{A}	6.75 ± 1.9 ^{A}	10.37 ± 0.3 ^{A}	0.49 ± 0.5 ^{A}	9.41 ± 0.2 ^{A}	8.75 ± 0.4 ^{A}	0.88	1.01	0.93 *
Ca	MEL1	11.52 ± 1.1 ^{A}	8.8 ± 1.5 ^{A}	20.55 ± 2.2 ^{AB}	8.78 ± 2.6 ^{A}	3.25 ± 0.9 ^{B}	2.96 ± 0.8 ^{A}	0.76 *	0.91	0.91
	MEL5	13.27 ± 1.2 ^{A}	13.17 ± 2.9 ^{B}	21.58 ± 2.8 ^{AB}	21.7 ± 2.7 ^{BC}	2.63 ± 0.2 ^{AB}	2.4 ± 0.3 ^{A}	0.99	1.01	0.91
	LdG	13.3 ± 1.4 ^{A}	12.68 ± 3.6 ^{B}	25.76 ± 3.9 ^{BC}	22.86 ± 2.8 ^{Bt}	2.85 ± 0.6 ^{AB}	3.23 ± 0.4 ^{A}	0.95	0.89	1.13
	ALM	12.44 ± 0.9 ^{A}	14.05 ± 1.6 ^{B}	28.33 ± 2.5 ^{C}	25.25 ± 1.5 ^{C}	2.2 ± 0.4 ^{AB}	2.09 ± 0.2 ^{A}	1.13	0.89	0.95
P	MEL1	3.91 ± 1 ^{B}	2.42 ± 0.3 ^{A}	7.6 ± 0.3 ^{B}	5.87 ± 1.5 ^{A}	4.11 ± 0.4 ^{A}	4.49 ± 0.3 ^{A}	0.62 *	0.77 *	1.09
	MEL5	2.51 ± 0.4 ^{A}	2.39 ± 0.4 ^{A}	5.8 ± 0.2 ^{A}	5.72 ± 0.2 ^{A}	4.78 ± 0.3 ^{AB}	4.88 ± 0.4 ^{AB}	0.95	0.99	1.02
	LdG	2.8 ± 0.1 ^{A}	2.51 ± 0.2 ^{A}	6.76 ± 0 ^{B}	7.39 ± 0.8 ^{A}	4.84 ± 0.2 ^{AB}	5.12 ± 0.6 ^{AB}	0.89	1.09	1.06
	ALM	2.63 ± 0.4 ^{A}	2.56 ± 0.5 ^{A}	6.73 ± 0.5 ^{B}	6.69 ± 0.8 ^{A}	5.36 ± 0.4 ^{B}	5.5 ± 0.2 ^{B}	0.97	0.99	1.03
Mg	MEL1	4.25 ± 0.3 ^{AB}	3.41 ± 0.5 ^{A}	5.54 ± 0.3 ^{A}	5.17 ± 0.4 ^{A}	2.94 ± 0.2 ^{A}	2.79 ± 0.3 ^{AB}	0.80 *	0.93	0.95
	MEL5	4.09 ± 0.4 ^{AB}	3.54 ± 0.4 ^{A}	6.22 ± 0.5 ^{A}	6.43 ± 0.4 ^{BC}	2.75 ± 0.1 ^{A}	2.94 ± 0.2 ^{AB}	0.87 *	1.03	1.07
	LdG	4.63 ± 0.2 ^{B}	3.93 ± 0.4 ^{A}	6.21 ± 0.2 ^{A}	5.76 ± 0.4 ^{AB}	3.11 ± 0.2 ^{A}	3.22 ± 0.2 ^{B}	0.85	0.93	1.03
	ALM	4.11 ± 0.3 ^{AB}	3.79 ± 0.7 ^{A}	7.5 ± 0.4 ^{B}	7.01 ± 0.4 ^{C}	2.72 ± 0.2 ^{A}	2.57 ± 0.1 ^{AB}	0.92	0.93	0.95
Fe	MEL1	0.71 ± 0.1 ^{A}	0.67 ± 0.1 ^{A}	0.09 ± 0 ^{A}	0.08 ± 0 ^{A}	0.03 ± 0 ^{B}	0.04 ± 0 ^{A}	0.94 *	0.85	1.23
	MEL5	0.98 ± 0.3 ^{A}	0.91 ± 0.2 ^{A}	0.09 ± 0 ^{A}	0.08 ± 0 ^{A}	0.03 ± 0 ^{AB}	0.02 ± 0 ^{A}	0.93	0.82 *	0.96
	LdG	0.73 ± 0.1 ^{A}	0.74 ± 0.4 ^{A}	0.09 ± 0 ^{A}	0.09 ± 0 ^{B}	0.03 ± 0 ^{AB}	0.03 ± 0 ^{A}	1.01	0.94	1.12
	ALM	0.53 ± 0.3 ^{A}	0.72 ± 0.3 ^{A}	0.08 ± 0 ^{A}	0.1 ± 0 ^{C}	0.02 ± 0 ^{A}	0.02 ± 0 ^{A}	1.35	1.39 *	1
B	MEL1	0.01 ± 0 ^{A}	0.012 ± 0 ^{A}	0.05 ± 0 ^{AB}	0.05 ± 0 ^{B}	0.02 ± 0 ^{B}	0.02 ± 0 ^{A}	0.79 *	1	0.76
	MEL5	0.011 ± 0 ^{A}	0.011 ± 0 ^{A}	0.06 ± 0 ^{B}	0.06 ± 0 ^{C}	0.02 ± 0 ^{AB}	0.02 ± 0 ^{A}	0.92	1.03	1.06
	LdG	0.011 ± 0 ^{A}	0.01 ± 0 ^{A}	0.05 ± 0 ^{AB}	0.04 ± 0 ^{A}	0.02 ± 0 ^{AB}	0.02 ± 0 ^{A}	0.91	0.73 *	1
	ALM	0.014 ± 0 ^{B}	0.011 ± 0 ^{A}	0.06 ± 0 ^{B}	0.05 ± 0 ^{B}	0.02 ± 0 ^{AB}	0.01 ± 0 ^{A}	1.20 *	0.84 *	0.88
Mn	MEL1	0.06 ± 0 ^{AB}	0.05 ± 0 ^{A}	0.07 ± 0 ^{A}	0.06 ± 0 ^{A}	0.02 ± 0 ^{B}	0.02 ± 0 ^{A}	0.88	0.84	0.91
	MEL5	0.07 ± 0 ^{B}	0.07 ± 0 ^{A}	0.11 ± 0 ^{B}	0.07 ± 0 ^{A}	0.02 ± 0 ^{AB}	0.02 ± 0 ^{A}	1.01	0.65 *	0.89 *
	LdG	0.05 ± 0 ^{AB}	0.05 ± 0 ^{A}	0.12 ± 0 ^{BC}	0.08 ± 0 ^{A}	0.02 ± 0 ^{B}	0.02 ± 0 ^{A}	1.11	0.64 *	0.96
	ALM	0.03 ± 0 ^{A}	0.05 ± 0 ^{A}	0.14 ± 0 ^{C}	0.11 ± 0 ^{B}	0.02 ± 0 ^{AB}	0.02 ± 0 ^{A}	1.50 *	0.77 *	0.95
Zn	MEL1	0.02 ± 0 ^{A}	0.01 ± 0 ^{A}	0.09 ± 0.1 ^{A}	0.03 ± 0 ^{A}	0.04 ± 0 ^{A}	0.05 ± 0 ^{B}	0.64 *	0.35 *	1.18
	MEL5	0.02 ± 0 ^{A}	0.01 ± 0 ^{A}	0.08 ± 0 ^{A}	0.11 ± 0.1 ^{A}	0.04 ± 0 ^{A}	0.03 ± 0 ^{AB}	0.82	1.36	0.76
	LdG	0.01 ± 0 ^{A}	0.02 ± 0 ^{A}	0.12 ± 0.1 ^{A}	0.08 ± 0 ^{A}	0.03 ± 0 ^{A}	0.03 ± 0 ^{A}	1.42	0.64	0.76
	ALM	0.02 ± 0 ^{A}	0.04 ± 0 ^{A}	0.05 ± 0 ^{A}	0.11 ± 0 ^{A}	0.03 ± 0 ^{A}	0.03 ± 0 ^{AB}	1.86	2.15*	1.28

Statistically significant correlations between ion accumulation in different organs were calculated (Table S1). A total of 18 correlations were found within fruit minerals, 17 were found within leaf minerals, and 12 were found within root minerals. More correlations were found between fruit and leaf (16) than between fruit and root (7) or leaf and root (14). However, root showed a smaller number of significant correlations indicating a differential performance under salt stress than fruit and leaf. As expected, negative correlations were found with Na and other main ions for plant metabolism such as K, Mn, and Mg. When increasing Na in roots and leaves, K, Mn, and Mg levels decreased in both plant organs. On the contrary, when Na increased in the fruit, K also seemed to accumulate more in the fruit.

3.4 Fruit antioxidant accumulation

Total phenolic compounds (TPC) values did not show significant differences between genotypes within the same treatment or between control and treated plants, with values ranging from 7.42 mg g^{-1} DW for ALM in control conditions to 11.81 mg g^{-1} DW in ALM in treated plants. Control values ranged from 7.42 in ALM to around 8 in MEL5 and LdG and almost 10 mg g^{-1} DW for MEL1. Further, TPC obtained values under the salt treatment ranged from around 7.50 for MEL5 and LdG to around 11.5 mg g^{-1} DW for MEL1 and ALM.

Chlorogenic acid (CA) content was not significantly altered in fruit under salt stress for any of the genotypes tested. CA values ranged from 4.26 mg g^{-1} DW for LdS to 7.65 mg g^{-1} DW for MEL1, with no differences between genotypes. Under salinity conditions, CA values ranged from around 6 for MEL5, LdG, and Alm to 7.4 mg g^{-1} DW for MEL1.

3.5 Sugar quantification

Sugar accumulation in fruit showed different performances depending mainly on the genotype (Figure 2). MEL1 fruit sugar content was not affected by the salt treatment. In the case of MEL5, glucose (GLU) contents were reduced under salt treatment (Figure 2a). Contrarily, LdG showed the highest values of GLU both for control and stress conditions. Fructose (FRU) levels in fruits were similar within genotypes, and only MEL5 showed a significant reduction in FRU accumulation under salt stress (Figure 2b). MEL5 and LdG were the most affected by salt, drastically reducing GLU production. MEL1 accumulated lower sucrose (SUC) values in treated and non-treated plants (Figure 2c). Genotypes generally increased SUC levels in fruits under salt stress; however, LdG showed higher production values under control conditions and were reduced under salt stress. Curiously, ALM increased significantly SUC accumulation during salt treatment while it did not show modifications in FRU or GLU.

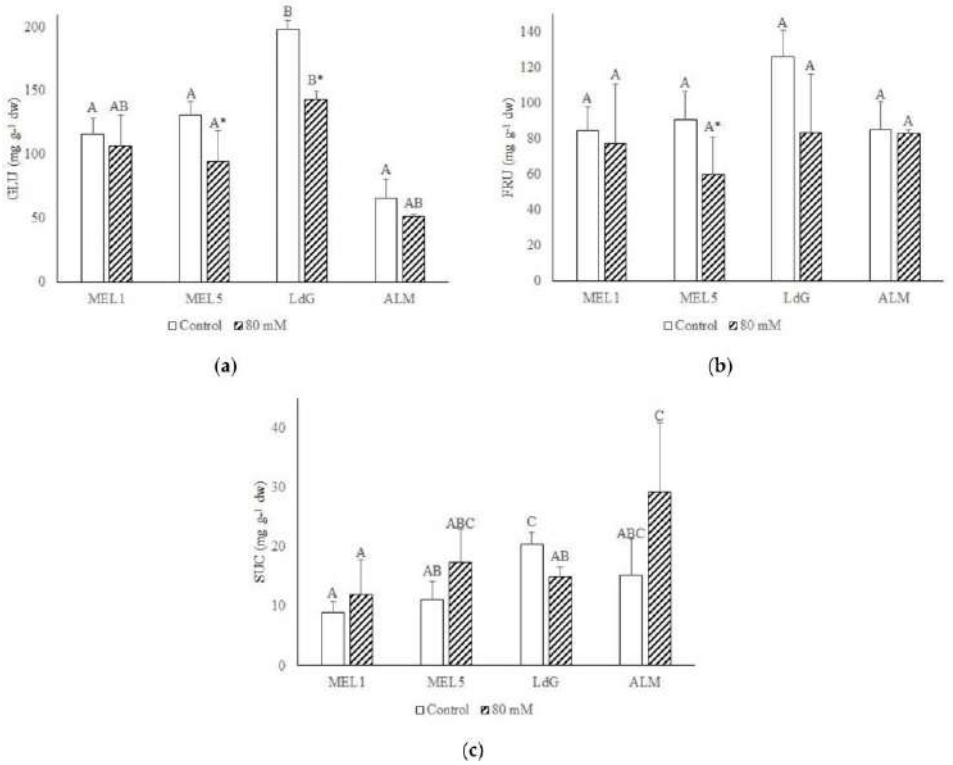


Figure 2. (a) Glucose (GLU), (b) fructose (FRU), and (c) sucrose (SUC) quantification (mean ± SD; $n = 5$) for *Solanum melongena* accessions under control and saline irrigation (80 mM) treatments. Different letters indicate statistically significant differences between genotypes ($p < 0.05$) within each treatment. The asterisk indicates statistically significant differences between treatments ($p < 0.05$) in the corresponding genotype, according to the Student–Newman–Keuls multiple range test.

4. Discussion

This work compares the fruit quality of four eggplant genotypes (*Solanum melongena* L.) under long-term salt stress. Salt has a great variety of effects on plant species, with a limitation in plant growth being the first indicator of stress (Brenes et al., 2020; Ortega-Albero et al., 2023). However, as has been previously reported, great stress is necessary for the eggplant cultivars to significantly reduce their growth parameters (Plazas et al., 2022). In fact, the capability of maintaining or even elongating primary and secondary roots (Mady et al., 2023) in response to drought has been noticed, as was observed in this work in some genotypes. In this regard, eggplants share some response mechanisms for drought and salinity, such as growth inhibition, ion movement alteration, and osmotic stress (Brenes et al., 2020; Ortega-Albero et al., 2023). However, compared to other salt-tolerant species, such as quinoa (*Chenopodium quinoa*), *S. melongena* reduces plant biomass and limits its metabolism under salt stress with lower NaCl concentrations. Moreover, ion accumulation in quinoa is barely altered

in salinity under moderate salt concentrations (Derbali et al., 2020; Jaramillo-Roman et al., 2021).

Fruit quality changes under abiotic stress are highly dependent on the genotype (Quinet et al., 2019). To determine whether fruit quality is affected in eggplant cultivars, a constant and long-term 80 mM salt treatment was used in four *S. melongena* varieties in which mineral composition, phenolic compounds, and sugars were measured. Concentrations over 100 mM are considered lethal for the plants, as they lead to metabolism inhibition (Singh, 2022; Tang et al., 2015). Thus, 80 mM was considered moderate in this study.

Eggplants show a huge diversity in fruit morphology, including shape and size (Mennella et al., 2012), which can explain the natural differences in fruit weight observed in this study. However, it is likely to find fruit weight differences due to salinity (Mady et al., 2023) that were not found in this study. Our hypothesis is that a longer or stronger salinity treatment and a large fruit set are necessary to induce significant changes in fruit weight.

Interestingly, there were no great changes in most mineral components analysed, while there was significant diversity among the cultivars, as reported by previous authors (Prohens, Rodríguez-Burruezo, Raigón, & Nuez, 2007). The main affected ions were related to salt response, such as Na and K (Galli et al., 2016; Ortega-Albero et al., 2023), and to cell signaling in root and leaf, such as P (Amtmann & Armengaud, 2009; Nilsson, Müller, & Nielsen, 2010). Ca deficiency can create some physiological disorders and depreciation of the fruits with the appearance of so-called blossom end rot (Flores, Borges, Almeida, & Prado, 2015). Fortunately, the presence of Na did not affect the Ca accumulation in the fruit. Na accumulation in roots and leaves causes toxicity in the plant and leads to an alteration of the ion movement, which has been reported to be dependent on the genotype (Blumwald, 2000; Plazas et al., 2016). Regarding correlation data, the expected alteration of the main ion of plant nutrition and metabolism has been found as a reduction in K, Mn, and Mg when Na presence increased in plant organs. Curiously, fruits proved to have a different metabolism regarding ion accumulation, with an increase in both Na and K. One hypothesis could be the protection of the fruit and, thus, the seeds through the translocation of Na to the roots instead of relocation to non-photosynthetic or "sink" plant tissues such as flowers and fruits with the activation of HKT transporters as in other species, such as pepper (Blom-Zandstra, Vogelganz, & Veer, 1998). Another alternative reported in tomatoes could be the overexpression of vacuolar Na^+/H^+ antiporters to sequester sodium into the vacuoles, avoiding the movement to other organs and tissues (Baghour et al., 2019). Ion transporter studies in fruit are rare or nonexistent and only reported for fruit development (Song et al., 2015). Nevertheless, it would be interesting to establish studies for Na and K transports in the fruit in response to salt stress and ion translocation.

Phenolic compounds are one of the main quality descriptors in eggplant fruit, specially chlorogenic acid, the main phenolic compound in unripe eggplant fruit. It has been reported by Martinez-Ispizua et al. (2021) that the accumulation of phenolic compounds in eggplant fruit is highly dependent on the genotype. In our study, the cultivars tested were similar for the phenolics and minerals contents in the fruit. It has been demonstrated that environmental conditions, including abiotic stresses, could influence eggplant growth and composition and affect fruit quality (Ozgur, Uzilday, Sekmen, & Turkan, 2013; Sumalan et al., 2020). However, the accessions tested did not show differences in their phenolic and mineral composition in this experiment, as reported previously (Raigón, Prohens, Muñoz-Falcon, & Nuez, 2008; Stommel, Whitaker, Haynes, & Prohens, 2015).

Sugars did not show huge changes under salt stress, differentiating eggplant from other Solanaceae species, such as tomato or pepper (Meza et al., 2020), and other species, like strawberry (Galli et al., 2016), especially on sucrose metabolism. A lack of response to the salt treatment was previously reported in eggplant (Baghour et al., 2019) and can be explained by the fact that eggplant fruit is harvested when immature when starch has not been hydrolyzed. An increase in soluble solids such as total sugars has been observed in tomatoes treated with moderate salt stress due to what is known as the "concentration effect" or a different movement in the plant of assimilates (Massaretto et al., 2018). In eggplant, differences between varieties on their sugar profile have been observed (Martínez-Ispizua et al, 2021), and a higher sugar content has been described as a result of a selection process (San José, Sánchez, Cámara, & Prohens, 2013). Sucrose synthase in coordination with sucrose phosphate synthase are described as important enzymes related to sugar accumulation in eggplant fruit, with high contents of fructose and glucose and low sucrose content indicating considerable invertase activity (Boo, Kim, & Lee, 2010). Also, the up-regulation of sucrose synthase under salt stress has been described in other species like tomatoes (Agius, von Tucher, & Rozhon, 2022; Mascellani et al., 2021; Tang et al., 2020). In our case, two genotypes reduced the quantity of monosaccharides, and one, ALM, increased the amount of the disaccharide sucrose, indicating activation of different metabolic pathways. The maintenance of the quality of the fruit has been associated with more tolerant genotypes (Darko, Yuan, Sekyere, & Liu, 2019; Suarez et al., 2021). In this work, all genotypes showed a similar tolerance to salt, probably due to the moderate stress applied. Nevertheless, each genotype showed unique performances for the fruit composition and quality of analysed traits. Special differentiated performance was observed in LdG, specifically in regard to sugars and chlorogenic acid. That means that there is genetic diversity to be explored in the quality response of eggplant to salt stress.

5. Conclusions

To summarize, eggplant genotypes show an intraspecific diversity in their response to salt irrigation. Nevertheless, little difference was found in fruit quality due to salt stress. These results were in consonance with small alterations in growth parameters, which were not remarkably affected in low salt concentrations and may be due to a partial tolerance to salinity of eggplant species. Moreover, as eggplant fruit is consumed unripe, inappreciable changes were seen in mineral and phenolic contents and very few in sugar content under mild salt stress. Altogether, these results indicate the resilience of eggplant in terms of fruit quality to the changing environment.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Author contributions

Conceptualization, AF; Methodology, AMA-M, MDR and VC-Z; Software, NO-A; Validation, AR-B; Formal analysis, NO-A and AMA-M; Investigation, NO-A, AMA-M and MDR; Resources, AR-B and MDR; Data curation, NO-A and AMA-M; Writing—original draft, NO-A and AF; Writing—review and editing, AMA-M, MDR and VC-Z; Visualization, AF and AR-B; Supervision, AR-B and VC-Z; Project administration, AF and AR-B; Funding acquisition, AF.

Supplementary materials

Supplementary materials are available at https://www.mdpi.com/article/10.3390/agriculture14060871/s1

Table S1. Mineral correlations between plant organs.

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Chapter III.

Capsicum annuum germplasm analysis and creation of the first MAGIC population in peppers

Research article

Genetic diversity, population structure, and phylogeny of insular Spanish pepper landraces (*Capsicum annuum* L.) through phenotyping and genotyping-by-sequencing

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Neus Ortega Albero has a main role in performing the experiments, sample and data collection, data analysis and visualization, drafting manuscript, revision and editing.

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Abstract

Pepper (*Capsicum* spp.) is one of the most important crops worldwide. Understanding the species' genetic background is key to preserving agrodiversity on-farm, to contribute to a more diverse and resilient agrifood sector, and to find new sources of variation that could be useful in future breeding programs. In this regard, varietal groups bred in insular environments have gained special interest as they have evolved quite isolated from continental forms, with a limited genetic exchange. The present work explores the diversity of a plethora of Balearic landraces, corresponding to different local varietal types, through phenotyping and genotyping-by-sequencing (GBS). Mallorca and Eivissa landraces were phenotyped according to a comprehensive list of descriptors for plant, leaf, flower, fruit, pollen, and seed and were genotyped with single nucleotide polymorphism (SNP) markers; population structure and their patterns of diversity were studied. The results showed a considerable morphological diversity for most traits analysed, within and between landraces. On the whole, in regard to genetic patterns, relatively low levels of heterozygosity and moderate genetic diversity for the studied landraces were found although some of them exhibited diverse patterns. The materials were not grouped in specific clusters associated with each island, but mainly according to varietal types. These findings can serve as the basis for studying divergent evolutionary patterns associated with the corresponding populations. Finally, the results can contribute to further elucidation of the genetic basis of Balearic landraces and serve as an inspiring case of study for other insular endemisms of cultivated species.

Keywords

Agrodiversity, cultivated endemism, genetic background, heterozygosity, phenomics, single nucleotide polymorphism, vegetable crops

1. Introduction

Peppers and chilies, among other terms, are recognizable names for *Capsicum* spp., one of the most important cultivated vegetables in the world for fresh consumption and as a spice with more than 40 Mt of fruit production and 3 Mha of harvested area (Jarret et al., 2019; FAO, 2023). More than 30 species are compressed in the *Capsicum* genus, from which only five are recognized as cultivated species, namely, *Capsicum annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L., and *C. pubescens* Ruiz & Pav. Among them, *C. annuum* is the most diverse and economically relevant including well-known varietal types like 'bell', jalapenos', 'numex', and 'ancho' (Pickersgill, 1991; Bartolomé Garcia et al., 2015; Pereira-Dias et al., 2019, 2020).

Capsicum cultivated species present a broad diversity in morphological and agronomic traits mostly in Central and South America, their geographical origin, as a result of evolution, domestication, and artificial and natural selection in primary and secondary centers of diversity (DeWitt & Bosland, 1996; Bartolomé Garcia et al., 2015; Tripodi et al., 2021). Indeed, more than five centuries of pepper selection in Europe have resulted in a great range of landraces adapted to a wide range of agroclimatic conditions (Bartolomé Garcia et al., 2015).

Spain is considered an important secondary center of diversity of peppers that were brought into the country from Mexico since the XV century as an alternative to the Asian black pepper (*Piper nigrum* L.). In this regard, Spain has the largest number of European Union Protected Designations of Origin (PDO) such as, among others, 'Pemento de Herbon and 'Pimiento del Piquillo de Lodosa' and Protected Geographical Indications (PGIs) such as 'Pemento do Couto' and 'Pimiento asado del Bierzo,' and many local varieties can be found on each Spanish region, including the Canary Islands and the Balearic Islands (Rodríguez-Burruezo et al., 2016).

In this context, the demands of the consumers for improved tastes, or the recovery of the "old ones," and the need to develop resilient vegetables adapted to diseases and changing environments are the main reasons why landraces are being reintroduced into the market. Moreover, these varieties have great importance because they are available, preserved *in-situ* materials for conservation and breeding programs that may help to revert agrodiversity loss due to genetic erosion. They have also proved to be key for farmers and communities' rights preservation and empowerment (Lanteri et al., 2003; Parisi et al., 2007; Brugarolas & Ruiz, 2009; Rodríguez-Burruezo et al., 2016). In this frame, farmers from the Balearic Islands Mallorca (Majorca) and Eivissa (Ibiza) have been very active in growing and preserving endemic landraces for centuries. As "living laboratories," these high added value landraces are still grown and preserved by these farmers in their socioeconomic environment, constantly adapting and evolving to insular environments, helping to the study of evolution processes (Gillespie & Baldwin, 2010).

Moreover, landraces have proved to be useful for understanding and predicting how species may respond to uncertain climate changes (Emerson, 2002; Farrell et al., 2015).

The knowledge on the available germplasm diversity, phylogenetic relations between accessions, and the genetic basis of the adaptation to specific conditions are major challenges for establishing pepper breeding programs (Prohens et al., 2017). However, despite its importance, genetic information for *Capsicum* has been obtained later than other Solanaceae species such as tomato and potato, mainly due to its large genome (3.5 Gb) and the high amount of repeated elements (70%–80%) (Ashrafi et al., 2012; Qin et al., 2014). Through the last decades, the development of high-throughput sequencing technologies allowed us to obtain fast and low-cost data (Metzker, 2010; Edwards et al., 2013). Among them, genotyping-by-sequencing (GBS) plays an important role in new scientific insights across species or populations, particularly for its relative simplicity (Elshire et al., 2011; Poland & Rife, 2012). Thus, GBS has been recently used in *Capsicum* peppers for studies focused on germplasm diversity, population structure, and genomic analysis as a result of domestication or local adaptation (Taranto et al., 2016; Pereira- Dias et al., 2019; Chae et al., 2022).

Here, we present a genomic characterization performed with GBS and a morphological characterization, using conventional descriptors of a collection of pepper landraces from Mallorca and Eivissa. Our goals were to analyse i) the morphological variation of insular landraces to contribute to their varietal typification, promotion, and preservation; ii) the genetic relations of a collection of Balearic landraces, historically conserved and multiplied *in situ* by farmers; and iii) the genetic patterns of Balearic landraces, specifically adapted to insular conditions.

2. Materials and methods

2.1 Plant material, DNA extraction, library preparation, and sequencing

Seeds from 73 accessions corresponding to 11 landraces from Mallorca and Eivissa, plus six reference varieties used as external controls and representing a wide variability of *Capsicum* morphologies, were sown in seedling trays in January 2023 (Figure 1; Table 1; Supplementary Table S1). Young tissue samples were taken from one plantlet per accession at the four- leaf stage, before transplanting. DNA was then extracted from 100 mg of deep-frozen leaf tissue with the SILEX method, developed by Vilanova et al. (2020).

DNA digestion was performed from 100 ng of genomic DNA with ApeKI enzyme and 3.6 mg of adapters. GBS library construction and sequencing were carried out by The Elshire Group Ltd. (Palmerston North, New Zealand) following the protocol by Elshire et al. (2011), including library amplification with 18 PCR cycles and sequencing with Illumina NovaSeq 6000 platform, using paired-end 150 pb reads.

Figure 1. Illustration of the varietal types evaluated. Pebrera Blanca (A), Citró de Matances (B), Banya de Cabra (C), Blau (D), Cirereta (E), D'Envinagrar (F), Fulla d'Olivera (G), Ros (H), Ros Gruixat (I), Ros Prim (J), Tap de Cortí (K), California Wonder (L), Chile Serrano (M), Bola (N), Pasilla Bajío (O), and Piquillo (P). White bar indicates 5 cm length.

Table 1. Studied varietal types, with the corresponding abbreviations, origins, and number of phenotyped and genotyped accessions.

Varietal type/Local name	Abbreviation	Number of phenotyped accessions	Number of genotyped accessions
Eivissa/Ibiza			
Pebrera Blanca	PB	6	26
Citró de Matances	CdM	9	21
Mallorca			
Banya de Cabra	BdC	3	2
Blau	B	3	2
Cirereta	C	1	1
D'Envinagrar	DE	2	1
Fulla d'Olivera	FO	3	2
Ros	R	3	2
Ros Gruixat	RG	4	3
Ros Prim	RP	2	2
Tap de Cortí	TdC	5	5

Reference controls			
California Wonder (Breeding line, Spain)	CW	1	1
Chile Serrano (Mexico)	CS	1	1
Bola (Murcia, Spain)	BOL	1	1
Pasilla Bajío (Mexico)	PAS	1	1
Serrano Criollo de Morelos (Mexico)	SCM	1	1
Piquillo (Navarra, Spain)	PIQ	1	1

2.2 Mapping and SNP calling

Raw reads were demultiplexed and barcodes were removed using Kevin-Murray's AX-demux algorithm (Murray & Borevitz, 2018). Demultiplexed reads were aligned to the reference *C. annuum* genome CM334 (Criollo de Morelos version 1.55) (Kim et al., 2014) using BWA-mem (Li, 2013) with default parameters except for avoiding multiple-mapping reads. GATK haplotype- caller 4.1.9 (Depristo et al., 2011) was used to perform the SNP calling on the BAM files and select SNPs with minimum mapping quality ($Q > 30$). VCFtools (Danecek et al., 2011) with missing data <15% and mean minimal read depth >7 was used for selecting good quality SNPs. Finally, heterozygosity was also calculated for each accession with VCFtools (Danecek et al., 2011). For further analysis, SNPs with missing data per site <25% and MAF >5% were retained with bcftools (Li, 2011) and plink2 (Chang et al., 2015).

2.3 Evaluation of phenotypic traits and statistical treatment

To assess the phenotypic variation in the Balearic collection, 41 accessions representing the group of target landraces and the six control varieties were transplanted in March 2023 to 30 cm diameter pots with coconut fiber, under experimental greenhouse conditions of the Universitat Politècnica de València at the Campus de Vera (València, Spain) (Table 1). Plants were trained with vertical strings, drip-irrigated, and fertilised by fertigation, following the standard practices for this crop.

Phenotyping was performed in one plant per accession in the Spring–Summer season 2023, using 29 descriptors for *Capsicum*, related to plant (6), inflorescence/flower (6), fruit (14), pollen (2), and seeds (1) as described on Table 2, including their abbreviations to facilitate their presentation in the rest of tables and figures. Most traits corresponded to *Capsicum* descriptors from Bioversity International (International Plant Genetic Resources Institute, IPGRI, 1995), and fruit wall consistency and pollen morphological and biological viability were included as commonly used traits for germplasm characterization in *Capsicum*. Fruit wall consistency was estimated by applying pressure to the pericarp with the hand. We considered consistent fruit walls when the fruit did not deform permanently after finger pressure, while non- consistent or soft fruit walls were those which lost their shape permanently after pressure and/or showed permanent bruising. Pollen morphological

and biological viabilities were measured using a light microscope SE (Nikon Instruments Inc., Melville, NY, USA), with pollen samples from four flowers per plant, dyed with tetrazolium (Norton, 1966; Manzur et al., 2015). Pollen samples were kept for 15 min in the darkness, after which four fields were observed per sample, with a total magnification of ×100. Morphological viability was estimated as the ratio between turgent grains against total grains (turgent + deformed grains). Biological viability was obtained in the same way but only considering turgent tetrazolium-dyed grains against total grains (turgent-dyed + turgent-non-dyed + deformed). Each pollen viability value corresponds to the average of four fields in each sample.

One plant per accession, representative of the accession and whose DNA was previously extracted, was characterized. For qualitative traits, one observation was made to establish a unique genotype–phenotype relationship. For fruit quantitative traits, five measurements per plant were made. Fruit color was measured using a Minolta CR-300 colorimeter (Minolta Corporation, Osaka, Japan) and expressed according to CIE L*a*b* 1976 space. For this fruit trait, 10 measures per accession were taken in homogeneous spots, i.e., two measures per fruit in five fruits. In a few accessions, no fruits with enough quality were obtained due to their lack of adaptation to the experimental conditions of this work. Therefore, in such cases, it was not possible to present the results of the corresponding traits.

Phenotypic data were normalized, and outliers were removed. For qualitative traits, the percentages of each trait level were calculated per accession and per landrace. Statgraphics Centurion XVII software (StatPoint Technologies, Warrenton, VA, USA) was used to estimate means and standard deviations for each quantitative trait, as well as to perform principal component analysis (PCA) with both qualitative and quantitative traits. Finally, intertrait correlations were calculated with all phenotypic data and Pearson coefficient <0.05.

Table 2. List of qualitative and quantitative traits measured, corresponding abbreviation, IPGRI descriptor number, and units or scale, as necessary.

Descriptor	Abbreviation	IPGRI Number	Units/Scale
Plant descriptors			
Stem colour	SC	7.1.2.2	1 = Green, 2 = Green with purple stripes, 3 = Purple
Nodal anthocyanin	NA	7.1.2.3	1 = Green, 3 = Light purple, 5 = Purple, 7 = Dark purple
Stem pubescence	SP	7.1.2.5	3 = Sparce, 5 = Intermediate, 7 = Dense
Stem lenght	SL	7.1.2.9	mm
Leaf colour	LC	7.1.2.14	1 = Yellow, 2 = Light green, 3 = Green, 4 = Dark green, 5 = Light purple, 6 = Purple, 7 = Variegated
Leaf shape	LS	7.1.2.15	1 = Deltoid, 2 = Ovate, 3 = Lanceolate

Inflorescence/Flower descriptors			
Number of flowers per axil	NFA	7.2.1.2	-
Flower position	FP	7.2.1.3	3 = Pendant, 5 = Intermediate, 7 = Erect
Anther colour	AC	7.2.1.8	1 = White, 2 = Yellow, 3 = Pale blue, 4 = Blue, 5 = Purple
Filament colour	FC	7.2.1.10	1 = White, 2 = Yellow, 3 = Green, 4 = Blue, 5 = Light Purple, 6 = Purple
Stigma exsertion	SE	7.2.1.12	3 = Inserted, 5 = Same level, 7 = Exserted
Calyx margin	CM	7.2.1.15	1 = Entire, 3 = Intermediate, 5 = Dentate
Fruit descriptors			
Anthocyanin spots or stripes	AS	7.2.2.2	0 = Absent, 1 = Present
Fruit color at intermediate stage	FCI	7.2.2.3	3 = Light green, 5 = Green, 7 = Dark green
Fruit color at mature stage	FCM	7.2.2.6	1 = White, 2 = Lemon-yellow, 3 = Pale orange-yellow, 4 = Orange-yellow, 5 = Pale orange, 6 = Orange, 7 = Light red, 8 = Red, 9 = Dark red, 10 = Purple, 11 = Brown, 12 = Black
Fruit color lightness (L)	FL	-	0 = Black to 100 = White
Fruit color green/red value (a)	FA	-	Negative = green to positive = red
Fruit color blue/yellow value (b)	FB	-	Negative = blue to positive = yellow
Fruit shape	FS	7.2.2.7	1 = Elongate, 2 = Almost round, 3 = Triangular, 4 = Campanulate, 5 = Blocky
Fruit length	FLE	7.2.2.8	mm
Fruit width	FWI	7.2.2.9	mm
Fruit weight	FW	7.2.2.10	g
Fruit shape at blossom end	FSBE	7.2.2.15	1 = Pointed, 2 = Blunt, 3 = Sunken, 4 = Sunken and pointed
Number of locules	NL	7.2.2.18	-
Pedicel with ripe fruit persistence	PP	7.2.2.20.2	3 = Slight, 5 = Intermediate, 7 = Persistent
Fruit wall consistency	FWC	-	0 = Non consistent, 1 = Consistent
Pollen descriptors			
Pollen morphological viability	PMV	-	-
Pollen biological viability	PBV	-	-
Seed descriptors			
100-seed weight	SW	7.3.5	g

2.4 SNP diversity analysis

To describe the structure of the population, a PCA was performed with Rpackage SNPrelate (Zheng et al., 2012). To elucidate the relationship between individuals, a phylogenetic tree was obtained with IQ-TREE (Minh et al., 2020), and a kinship matrix was obtained with SNPrelate (Zheng et al., 2012). An admixture analysis was built with AdminPipe v2.0 (Mussmann et al., 2020) to understand the genomic admixture of the studied population. For the analysis, 10 subpopulations (*K*) were used and each *K* run was statistically analysed with Pong statistical software (Behr et al., 2016) using 19 replicates to obtain the best statistical run.

3. Results and discussion

3.1 Study of phenotypic variation

3.1.1 Qualitative traits

On the whole, a considerable diversity was found in both the reference group of control varieties and the collection of Balearic accessions for most traits. By contrast, some traits were found monomorphic, in both the reference group and Balearic accessions, like stem color and fruit wall consistency (Tables 3, 4).

However, for most qualitative traits, variation was found within and between varietal types. Thus, there were traits where variation was found in both groups, the non-insular varieties and the Balearic peppers. In this regard, there were traits like nodal anthocyanin, leaf color, leaf shape, flower position, anther color, stigma exsertion, calyx margin, or fruit shape, where the ranges of variation observed in the Balearic landraces were similar to those found in the reference group (Tables 3, 4). Some qualitative variation was found in the Balearic peppers in traits like the number of flowers per axil, filament color, or anthocyanin spots, where the control varieties were found monomorphic. These findings indicate that variation can be found in many traits like leaf shape, flower position, or anther color, even in a genetic background of a few varietal types native to these islands, in the same level as that observed in a more variable group of non-insular varietal types.

Accessions from groups of Pebrera Blanca or Citró de Matances showed a considerable variation in traits like stigma exsertion or fruit shape (Figure 2*;* Tables 3, 4). Also, Citró de Matances was relatively variable for stigma exsertion and fruit shape, Banya de Cabra for flower position and filament color, Blau for stigma exsertion, or Tap de Corti for leaf color and stigma exsertion, taking into account the limitations in the number of accessions in the last three varietal groups (Tables 3, 4). On the contrary, there were also cases of monomorphism or high uniformity within varietal types in other traits like fruit color at the intermediate stage or filament color.

In terms of flower morphology, reference varieties differed in stigma exsertion among them. Also, variation was found in stigma exsertion among reference varieties, as much as both among and within varietal types (Tables 3, 4). Adaptation to the environment is a key factor for flower morphology (Yamada & Maki, 2014). Thus, flower trait variations among landraces could be due to adaptation to different ecological conditions. Finally, fruit color at the intermediate stage showed high variation between varietal types. For instance, unripped commercialized landraces such as Pebrera Blanca, Ros, Ros Prim, or Ros Gruixat showed the lightest color at the intermediate stage, indicating a historical farmer selection to improve the visual performance of these landraces.

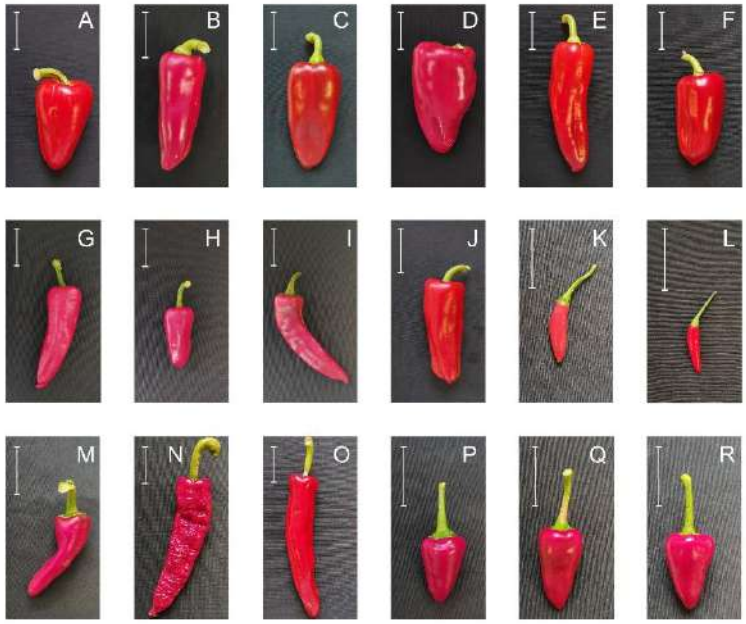

Figure 2. Examples of phenotypic variation in fruit morphology among accessions of Pebrera Blanca (A-F), Citró de Matances (G-L), Banya de Cabra (M-O) and Tap de Cortí (P-R). White bar indicates 5 cm length.

Table 3. Phenotypic values in plant and flower qualitative traits of the landraces and the reference varieties.

Varietal type/Local name	SC[1]	NA	SP	NFA	FP	AC	FC	SE	CM
Pebrera blanca	Green	Light purple (17%); Purple (83%)	Sparce	1	Intermediate (60%); Erect (40%)	Blue (50%); Purple (50%)	White	Inserted (50%); Same level (33%); Exserted (17%)	Intermediate
Citró de matances	Green (89%); Purple (11%)	Light purple (22%); Purple (78%)	Sparce (78%); Intermediate (11%); Dense (11%)	1 (89%); 2 (11%)	Intermediate (33%); Erect (67%)	Blue (67%); Purple (33%)	White	Inserted (11%); Same level (33%); Exserted (56%)	Enture (11%); Intermediate (78%); Dentate (11%)
Banya de cabra	Green	Purple	Sparce	1 (67%); 2 (33%)	Pendant (33%); Intermediate (33%); Erect (33%)	Blue	White (67%); Light purple (33%)	Same level (33%); Exserted (67%)	Intermediate (67%); Dentate (33%)
Blau	Green	Purple	Sparce	1 (67%); 2 (33%)	Intermediate (67%); Erect (33%)	Blue (33%); Purple (67%)	White (67%); Light purple (33%)	Inserted (33%); Same level (33%); Exserted (33%)	Intermediate
Cirereta	Green	Purple	Sparce	1	Erect	Purple	White	Same level	Intermediate
D'envinagrar	Green	Light purple	Sparce	1	Intermediate	Yellow (50%); Purple (50%)	White	Inserted	Dentate
Fulla d'olivera	Green	Light purple (33%); Purple (67%)	Sparce	1	Erect	Blue	White	Same level (33%); Exserted (67%)	Intermediate (67%); Dentate (33%)
Ros	Green	Light purple (33%); Purple (67%)	Sparce	1 (67%); 2 (33%)	Intermediate (67%); Erect (33%)	Blue	White	Same level (67%); Exserted (33%)	Intermediate
Ros gruixat	Green	Purple	Sparce	1	Intermediate (75%); Erect (25%)	Blue (75%); Purple (25%)	White	Inserted (50%); Same level (50%)	Intermediate (75%); Dentate (25%)
Ros prim	Green	Purple	Sparce	1	Intermediate	Blue (50%); Purple (50%)	White	Inserted (50%); Same level (50%)	Intermediate (50%); Dentate (50%)
Tap de cortí	Green	Green (20%); Purple (80%)	Sparce	1 (40%); 2 (60%)	Erect	Pale blue (20%); Blue (40%); Purple (40%)	White	Inserted (20%); Same level (60%); Exserted (20%)	Intermediate
California Wonder	Green	Light purple	Sparce	1	Pendant	Blue	White	Inserted	Intermediate
Chile Serrano	Green	Light purple	Intermediate	1	Pendant	Purple	White	Exserted	Dentate
Bola	Green	Purple	Sparce	1	Pendant	Blue	White	Inserted	Intermediate
Pasilla Bajío	Green	Light purple	Sparce	1	Pendant	Purple	White	Exserted	Dentate
Serrano Criollo de Morelos	Green	Light purple	Dense	1	Erect	Pale blue	White	Exserted	Dentate
Piquillo	Green	Purple	Sparce	1	Intermediate	Pale blue	White	Same level	Enture

[1] SC: stem colour, NA: nodal anthocyanin, SP: stem pubescens, NFA: number of flowers per axil, FP: flower position, AC: anther colour, FC: filament colour, SE: stigma exsertion, and CM: calyx margin.

Table 4. Phenotypic values in leaf and fruit qualitative traits of the landraces and the reference varieties.

Varietal type/Local name	LC[1]	LS	AS	FCI	FCM	FS	FSBE	PP	FWC
Pebrera blanca	Green	Deltoid (17%); Ovate (83%)	Absent (33%); Present (67%)	Light green	Red	Triangular (50%); Blocky (50%)	Sunken	Intermediate (17%) Persistent (83%)	Consistent
Citró de matances	Green (89%); Dark green (11%)	Deltoid (11%); Ovate (78%); Lanceolate (11%)	Absent (67%); Present (33%)	Green	Red (44%); Dark red (56%)	Elongate (33%); Triangular (44%); Blocky (22%)	Pointed (22%); Blunt (11%) Sunken (67%)	Intermediate (78%) Persistent (22%)	Consistent
Banya de cabra	Green	Ovate	Absent (67%); Present (33%)	Green	Red (33%); Dark red (67%)	Elongate (67%); Triangular (33%)	Pointed (25%); Sunken (75%)	Persistent	Consistent
Blau	Green (67%); Dark green (33%)	Ovate	Absent (67%); Present (33%)	Green	Dark red	Triangular		Intermediate	Consistent
Cirereta	Green	Ovate	Absent	Green	Dark red	Elongate	Pointed	Intermediate	Consistent
D'envinagrar	Light green (50%); Green (50%)	Ovate	Absent	Light green	Light red	Elongate	Pointed	Intermediate	Consistent
Fulla d'olivera	Dark green	Ovate (33%); Lanceolate (67%)	Absent	Light green (67%); Green (33%)	Red (67%); Dark red (33%)	Elongate	Pointed	Intermediate	Consistent
Ros	Light green (33%); Green (67%)	Ovate	Absent (33%); Present (67%)	Light green	Red	Triangular (33%); Blocky (67%)	Sunken	Intermediate (33%) Persistent (67%)	Consistent
Ros gruixat	Green	Ovate	Present	Light green	Red	Triangular	Pointed (20%); Sunken (80%)	Intermediate (25%) Persistent (75%)	Consistent
Ros prim	Light green (50%); Green (50%)	Ovate	Present	Light green	Red (50%); Dark red (50%)	Triangular	Sunken	Intermediate	Consistent
Tap de cortí	Light green (20%); Green (60%); Dark green (20%)	Ovate	Absent (60%); Present (40%)	Light green (25%); Green (75%)	Dark red	Triangular	Sunken	Intermediate	Consistent
California Wonder	Light green	Deltoid	Absent	Light green	Lemon-yellow	Blocky	Sunken	Persistent	Consistent
Chile Serrano	Dark green	Lanceolate	Absent	Dark green	Red	Elongate	Blunt	Slight	Consistent
Bola	Green	Ovate	Absent	Green	Red	Blocky	Blunt	Persistent	Consistent
Pasilla Bajío	Green	Lanceolate	Absent	Dark green	Brown	Elongate	Pointed	Intermediate	Consistent
Serrano Criollo de Morelos	Green	Lanceolate	Absent	Light green	Red	Triangular	Blunt	Intermediate	Consistent
Piquillo	Green	Ovate	Absent	Green	Dark red	Triangular	Sunken	Persistent	Consistent

[1] LC: leaf colour, LS: leaf surface, AS: anthocyaninic spots, FCI: fruit colour immature, FCM: fruit colour mature, FS: fruit shape, FSBE: fruit shape at blossom end, PP: pedicel persistence, FWC: fruit wall consistency.

3.1.2 Quantitative traits

The quantitative traits analysed showed a remarkable diversity. Moreover, diversity was found within and between all the studied varieties (Table 5; Supplementary Table S2). Monomorphic traits were few, and only the number of locules and fruit shape at blossom end showed very little variation among the studied varietal types. On the contrary, high variability was found within varietal types (Supplementary Table S2). As was observed for qualitative traits, some quantitative traits such as pollen morphological and biological viability and seed weight had similar ranges of variation when comparing Balearic landraces and the control group. Also, seed weight was found quite discriminating to discriminate among varietal types, in particular, the Balearic collection. Thus, most reference varieties ranged between 0.60 and 0.80 g/100 seeds, with the only exception of Pasilla Bajío, which achieved 1.29 g/100 seeds (Table 5). The means of the Balearic types ranged between 0.40 and 0.50 g/100 seeds for Blau, Cirereta, D'Envinagrar, and Fulla d'Olivera and 0.70 g/100 seeds of Pebrera Blanca and Banya de Cabra, and even a remarkable intravarietal variation was found in Citró de Matances (0.28–0.99 g/100 seeds) (Table 5; Supplementary Table S2).

As mentioned, a relevant variation between varietal groups was observed for fruit traits such as fruit length, width, fruit color at the mature stage, and fruit weight. Regarding fruit morphology, bigger fruits were observed in those landraces whose fruits are marketed at the unripe stage (Pebrera Blanca, Ros, Ros Prim, and Ros Gruixat), while the smallest fruits corresponded to those landraces whose fruits are used at the ripe stage (Cirereta, D'Envinagrar, or Fulla d'Olivera) (Table 5).

As observed for qualitative traits, Pebrera Blanca and Citró de Matances showed great variation for quantitative traits, while Mallorca landraces showed less variability within varietal types, taking into account variation limitations due to a small number of accessions of some Mallorca landraces (Figure 2; Supplementary Table S2). This wide variation between and within insular varieties has also been found in other collections such as the Ramellet tomato (*Solanum lycopersicum*) accessions in the Balearic Islands, with great diversity especially for leaf and fruit morphology (Bota et al., 2014). Bota et al. (2014) also observed significant differences between Ramellet accessions and a group of external accessions in tomato. Moreover, barley collections from the Canary Islands have also presented great variation among accessions within and among islands and a considerable diversification from continental accessions (Hagenblad et al., 2019).

Pollen viability was very variable among the reference varieties, ranging from very low values in the California Wonder breeding line (7% morphological, 5% biological) to very high in the Serrano types and Piquillo (≥80%) (Table 5; Supplementary Table S2), in correspondence to their varietal diversity and adaptation to different environments. A similar pattern was found among the Balearic accessions and varietal types, with means comprised between Ros Prim (approximately 50%)

and Ros (86%), appearing extreme values in accessions like Ros-X6 (94%) or Blau-P50 (16%) (Supplementary Table S2).

3.1.3 Principal component analysis

The first two PCA components contributed to explain 42.30% of the observed variation, 26.82% by PC1 and 15.48% by PC2 (Figure 3A). According to this distribution, traits like fruit width, fruit shape, number of locules, leaf color, and leaf shape, among others, appeared as the most discriminant in terms of PC1 (i.e., the ones which appear in the extremes of PC1), while fruit color, and nodal anthocyanin in the stem were the more discriminant for PC2 (Figure 3B). Therefore, these traits seem to be the determinants to differentiate between accessions. In comparison to other reports, like those from Bota et al. (2014) in tomatoes or Pereira-Dias et al. (2020) in peppers, our findings are quite similar, confirming that these kinds of traits are usually the most useful to characterize accessions and discrimination among Solanaceae genotypes.

The first and second principal components, obtained with all the phenotypic data, enabled us to differentiate among the reference varieties, which appeared clearly separated among them and widely distributed in the PCA, mainly based on fruit descriptors such as fruit length, fruit width, and fruit weight (Figure 3). Fruit color at the fully ripe stage was key to differentiating California Wonder, i.e., yellow color, against the rest of them (Figure 3B). Regarding the Balearic landraces, Citróde Matances showed two clusters of accessions in the PCA, suggesting intravarietal variability. Thus, in general, Pebrera Blanca and all the Mallorca varietal types also differed among them and distributed widely in the PCA with some overlapping, but their intravarietal variation was smaller than that of the Citró de Matances, appearing quite close in the PCA. In this regard, we found that Citró de Matances, D'Envinagrar, and Banya de Cabra, as well as Ros and Ros Gruixat varietal types, partly overlapped, despite appearing on specific areas of the PCA (Figure 3A). Moreover, Pebrera Blanca or Ros types, used for their unripe and bigger-sized peppers were located in the upper left part of the PCA, separated from the rest of the landraces grown for their smaller fruits, mainly used fully ripe (Figure 3A).

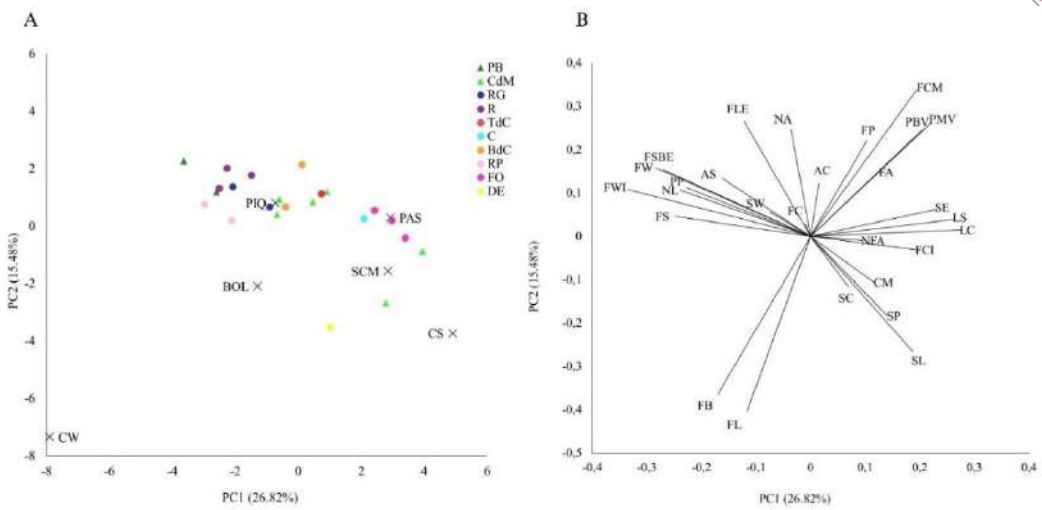

Figure 3. Principal component analysis using phenotypic data. (A) First (PC1) and second (PC2) principal components representing the selected accessions and the reference varieties and (B) loading plot of the PC1 and PC2.

Table 5. Mean values (mean ± standard deviation) in the studied quantitative traits of the landraces and the reference varieties. NA indicates missing data.

Varietal type/Local name	SL[1]	FL	FA	FB	FLE	FWI
Pebrera Blanca	166 ± 87	35.29 ± 1.9	36.42 ± 4.1	21.99 ± 4	131 ± 17	54 ± 12
Citró de Matances	325 ± 15	35.88 ± 3.5	37.66 ± 4.7	19.25 ± 4.6	87 ± 40	2.5 ± 11
Banya de Cabra	252 ± 164	33.58 ± 2.9	34.12 ± 4	16.3 ± 3.5	154 ± 46	35 ± 5
Blau	372 ± 24	NA	NA	NA	NA	NA
Cirereta	255	35.1 ± 1.7	40.16 ± 1.4	18.06 ± 1.5	102 ± 20	17 ± 4
D'Envinagrar	495 ± 17	42.73 ± 2.1	42.46 ± 3.3	31.79 ± 5.8	86 ± 9	22 ± 3
Fulla d'Olivera	212 ± 16	36.22 ± 3.9	39.62 ± 2.1	18.82 ± 3.6	102 ± 21	15 ± 3
Ros	160 ± 66	35.5 ± 1.8	38.22 ± 3.8	20.53 ± 3.7	126 ± 28	46 ± 6
Ros Gruixat	225 ± 52	36.5 ± 3	38.61 ± 4.2	22.7 ± 5.1	144 ± 18	44 ± 5
Ros Prim	218 ± 4	35.75 ± 2.6	39.43 ± 2.7	22.54 ± 3.5	142 ± 21	46 ± 4
Tap de Cortí	184 ± 20	33.3 ± 1.8	32.48 ± 2.3	14 ± 1.9	61 ±6	28 ± 2
California Wonder	245	59.12 ± 3.3	-0.08 ± 2.5	56.24 ± 3	72 ± 4	62 ± 4
Chile Serrano	85	41.93 ± 2.4	40.5 ± 1.1	26.95 ± 4	36 ± 4	13 ±1
Bola	435	36.5 ± 1.3	35.59 ± 2.7	18.86 ± 1.5	33 ± 2	27 ± 3
Pasilla Bajío	485	28.03 ± 0.8	3.12 ± 1.1	3.73 ± 0.8	155 ± 12	29 ± 2
Serrano Criollo de Morelos	23	39.9 ± 2.6	39.83 ± 1.7	23.65 ± 2.4	101 ± 9	2 ± 1
Piquillo	25	36.13 ± 1.9	34.63 ± 3.5	17.2 ± 2.9	71 ± 9	41 ± 3

Varietal type/Local name	FW	NL	PMV	PBV	SW
Pebrera Blanca	151.67 ± 40.8	3.2 ± 0.6	0.74 ± 0.1	0.74 ± 0.1	0.71 ± 0.1
Citró de Matances	24.92 ± 19.7	2.42 ± 0.6	0.74 ± 0.1	0.68 ± 0.2	0.61 ± 0.2
Banya de Cabra	53.02 ± 23.6	3.27 ± 0.5	0.82 ± 0.1	0.82 ± 0.1	0.72 ± 0.1
Blau	NA	NA	0.61 ± 0.4	0.61 ± 0.4	0.52
Cirereta	12.79 ± 5.6	2.2 ± 0.4	0.72	0.72	0.42
D'Envinagrar	13.79 ± 2.2	2.7 ± 0.5	0.78	0.78	0.5 ± 0.1
Fulla d'Olivera	10.68 ± 5.8	2.4 ± 0.5	0.81 ± 0.1	0.8 ± 0.1	0.48 ± 0.1
Ros	104.28 ± 31.6	3.27 ± 0.7	0.86 ± 0.1	0.86 ± 0.1	0.63
Ros Gruixat	114.65 ± 25.1	2.5 ± 0.5	0.68 ± 0.1	0.68 ± 0.1	0.63 ± 0.1
Ros Prim	118.07 ± 21.9	2.9 ± 0.3	0.5 ± 0.1	0.49 ± 0.1	0.65 ± 0.1
Tap de Cortí	21.81 ± 3.7	2.55 ± 0.5	0.81 ± 0.1	0.81 ± 0.1	0.55 ± 0.1
California Wonder	93.15 ± 8.1	3	0.07	0.05	0.78
Chile Serrano	3.24 ± 1	2.4 ± 0.5	0.79	0.78	0.6
Bola	14.61 ± 5.4	2.6 ± 0.5	0.39	0.26	0.7
Pasilla Bajío	27.06 ± 7.7	2.2 ± 0.4	0.68	0.66	1.29
Serrano Criollo de Morelos	16.52 ± 4.3	2.2 ± 0.4	0.88	0.83	0.74
Piquillo	41.8 ± 9.3	2.6 ± 0.9	0.81	0.76	0.77

[1] SL: stem length (mm), FL: fruit colour lightness, FA: fruit colour green/red, FB: fruit colour blue/yellow, FLE: fruit length (mm), FWI: fruit width (mm), FW: fruit weight (g), NL: number of locules, PMV: pollen morphological viability (%), PBV: pollen biological viability (%), SW: seed weight (g).

3.1.4 Intertrait correlations

Many correlations were found between traits with the phenotypic data of the studied populations (Figure 4). Positive correlations were found for plant descriptors such as stem color and stem pubescence, indicating that purple stems tend to show more density of the hairs. One negative correlation was found for flower descriptors, between flower position and calyx margin, indicating that a pendant flower position is related to a dentate calyx margin in the developing fruit. Morphological and biological pollen viabilities were highly positively correlated indicating that both parameters were estimating pollen grain viability in an equivalent way. A large number of correlations were found between fruit traits. For instance, darker fruits in the immature stage seemed to be related to the absence of anthocyanin spot and to darker fruits in the mature stage. Also, numerous positive correlations were observed for fruit shape; for example, larger fruits were related to wider fruits, indicating that, in the studied populations, landraces with bigger fruits tend to be more blocky-shaped fruits. Some correlations were found between different organ traits, e.g., longer stems and round leaves were correlated with smaller (lower length, width, and weight) and elongated fruits. Moreover, lower

pollen viabilities were correlated with the presence of anthocyanin spots in the fruit and an inserted stigma in the flower. Finally, a higher seed weight seemed to be related to a pendant flower, an elongated, blocky-shaped fruit with numerous locules.

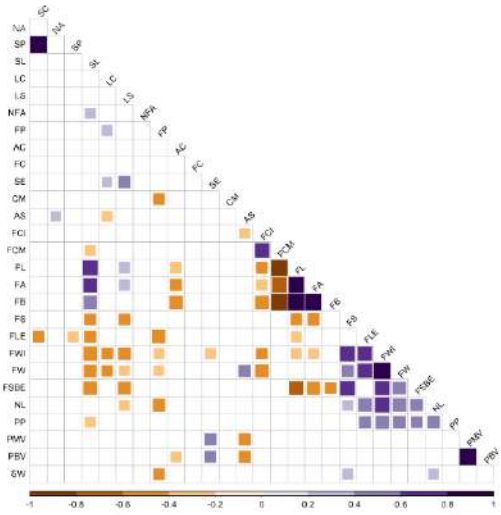

Figure 4. Inter-trait correlations (Pearson coefficient $p < 0.05$) among plant (6), flower (6), fruit (14), pollen (2) and seed (1) traits phenotyped from 47 peppers accessions. Positive values are indicated in blue (increasing in value with darkness, i.e. from pale to dark blue). Negative values are indicated in orange (decreasing in value with darkness, i.e. from pale orange to dark orange/brown).

SC: stem colour, NA: nodal anthocyanin, SP: stem pubescence, SL: stem length, LC: leaf colour, LS: leaf shape, NFA: number of flowers per axil, FP: flower position, AC: anther colour, FC: filament colour, SE: stigma exsertion, CM: calyx margin, AS: anthocyaninic spot, FCI: fruit colour immature stage, FCM: fruit colour mature stage, FL: fruit brightness, FA: fruit colour green/red, FB: fruit colour blue/yellow, FS: fruit shape, FLE: fruit length, FWI: fruit width, FW: fruit weight, FSBE: fruit shape at blossom end, NL: number of locules, PP: pedicel persistence, PMV: pollen morphological viability, PBV: pollen biological viability.

3.2 Genotyping-by-sequencing characterization

3.2.1 SNP calling and sequencing

The sequencing of the Balearic and reference accessions produced over 90 million raw paired-end reads and approximately 10 Gb of data. After demultiplexing, cleaning, and trimming, selected reads were aligned to the pepper genome CM334 (Serrano Criollo de Morelos version 1.55) (Kim et al., 2014). In otal, 1,197,532 polymorphic sites, distributed over the 12 chromosomes, were retained after SNP calling and basic filtering, and 9,508 high-quality SNPs were selected for diversity analysis as described in Section 2.4.

Heterozygosity was calculated using the 1,197,532 SNPs retained after basic filters. Heterozygosity was comprised between 0.38% and 1.95% in the Balearic collection (Table 6, Supplementary Table S3). Low heterozygosity values are usually expected for autogamous species, despite being exposed to open pollination like landraces, as has been observed for other autogamous species such as tomato (Bota *et al.*, 2014). In pepper, also an autogamous species, low values for heterozygosity have previously been reported. For instance, Taranto et al. (2016) described mean heterozygosity values of 2.4% in a collection of *C. annuum* with different origins. Similarly, Pereira-Dias et al. (2019) found heterozygosity mean values of 2.97% for a collection of *C. annum* varieties. Even more, higher mean heterozygosity values for other pepper populations have been obtained when including wild-related species (Ibiza et al., 2012; Cheng et al., 2016; Lee et al., 2016), which are exposed to conditions where allogamy is favored.

Regarding Eivissa accessions, Pebrera Blanca showed slightly higher heterozygosity values than Citró de Matances, with a mean value of 0.80% in the former and a mean value of 0.65% in the latter (Table 6). For Mallorca varietal types and accessions, heterozygosity values varied a little bit more in a general view, maybe due to differences in the number of accessions genotyped for the Mallorca landraces (Table 6, Supplementary Table S3).

Table 6. Heterozygosity mean values (Het, %) per varietal types and heterozygosity range of values among the accessions (minimum-maximum) within each varietal type.

Accession	Het	Range
Pebrera Blanca	0.80	[0.55-1.14]
Citró de Matances	0.65	[0.38-1.11]
Banya de Cabra	0.88	[0.72-1.03]
Blau	0.80	[0.77-0.84]
Cirereta	0.82	-
D'Envinagrar	1.95	-
Fulla d'Olivera	0.77	[0.69-0.86]
Ros	0.74	[0.74-0.75]
Ros Gruixat	0.81	[0.75-0.90]
Ros Prim	0.74	[0.72-0.76]
Tap de Cortí	0.71	[0.56-0.92]

3.2.2 Principal component analysis (PCA)

The reference varieties, as expected for their genetic divergence, appeared widely distributed throughout the PCA, with California Wonder, Bola, and Piquillo in the central area (i.e., PC1 = 0, PC2 = 0) and Chile Serrano, Pasilla Bajıó, and,

particularly, Serrano Criollo de Morelos, in the most extreme positions, and clearly separated from the main cloud of accessions (Figure 5). Regarding the Balearic materials, the Citró de Matances accessions covered a wide distribution in the PCA, with several accessions located in the main cloud but others appearing in far positions close to Chile Serrano, Bola, and Pasilla Bajío (Figure 5). These findings are in agreement with the phenotyping intravarietal variation found within this type (Supplementary Table S2) and confirm that, on occasions, one local name may encompass a range of phenotypes as well as different genetic strains, as reported by Pereira-Dias et al. (2020) in peppers, Nuijten and Almekinders (2008) in Baras rice in Gambia, or Mar and Holly (2000) in beans in Hungary, whose common names refer to just one main morphological or agronomical trait but actually encompass a plethora of genotypes and morphologies for other traits.

Finally, in the case of Pebrera Blanca accessions, they appeared in the corner of positive values of PC1 and PC2 although they seem to be divided into two groups slightly separated, suggesting two differentiated genetic subpopulations with the available accessions. These results agree with the diversity found for phenotypic data and fruit morphology for this varietal type (Tables 3, 4).

3.2.3 Phylogenetic tree and kinship matrix

The dendrogram showed accessions clustered by varietal group, but not clearly clustered by origin, indicating the existence of interisland crosses due to the interchange of seeds between Mallorca and Eivissa farmers (Figure 6). However, some landraces were clustered together and clearly differentiated like Tap de Cortí or Blau, indicating a unique genetic pattern that will be further discussed in Section 3.2.4. On the other hand, some varietal types like Fulla d'Olivera and Cirereta appeared in the same dendrogram branch, which could be explained because both landraces show similar fruit shapes and are usually used as a spice. Interestingly, cultivars usually harvested and sold at the unripe stage like Ros, Ros Gruixat, Ros Prim, and Pebrera Blanca, clustered and were not differentiated clearly by varietal group, suggesting selection and genetic exchange in these materials. Moreover, as it has been seen in the PCA and the phenotyping data, differentiated genetic lineages may be observed in Pebrera Blanca and Citró de Matances, with various genetic clusters of accessions each, one cluster per varietal type very close and some accessions mixed with other landraces (Figures 5, 6). The group of reference varieties also appeared close in the dendrogram, with the only exception of Bola. However, some accessions of Banya de Cabra, Fulla d'Olivera, and Citró de Matances appeared clustered together with the reference varieties. This might suggest that, despite most Mallorca and Eivissa landraces had a different breeding process, some materials could conserve genetic similarities with the controls or even had experienced genetic exchange.

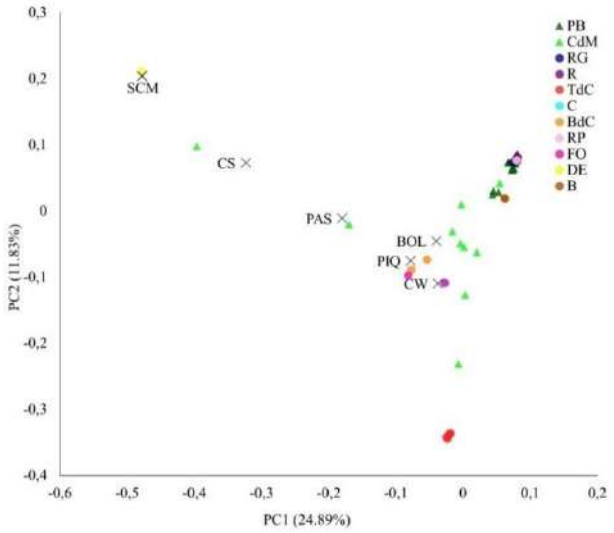

Figure 5. Principal component analysis. First (PC1) and second (PC2) principal components representing the Balearic accessions and the reference varieties, based on the 9,508 filtered SNPs.

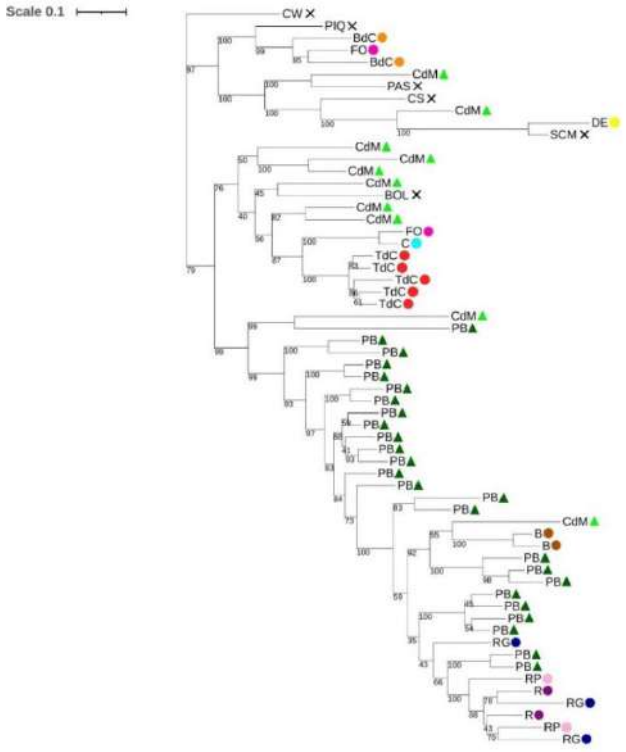

Figure 6. Dendrogram created from 1000 bootstrap replicates for the studied Balearic accessions and the reference varieties, based on the 9,508 filtered SNPs.

The kinship matrix confirmed the data obtained in the dendrogram and the PCA. Thus, the reference controls were clustered and differentiated from Balearic landraces, with the only exception of some Citró de Matances accessions, whose high genetic intravarietal diversity, as observed in the PCA, explains such overlapping with the reference varieties (Figures 5, 7). All Tap de Cortí accessions were clustered unequivocally, suggesting breeding and selection processes very close to this varietal type. Also, Banya de Cabra and Fulla d'Olivera were clustered together, suggesting little genetic differences between both landraces.

Fruit traits such as fruit morphology and color were key to differentiate cultivars, as it was found in both phenotypic and genotypic PCAs (Figures 3A, 5). In this regard, landraces used for the unripe commercialized peppers Ros, Ros Gruixat, and Ros Prim were clustered together, indicating great genetic similarities. These results could be associated with quality traits in these unripe-used peppers due to historical farmer selection preferences and/or genetic exchange (Figure 7). As it has been previously mentioned, no clear separation was observed between Eivissa and Mallorca blocks. Thus, despite considering that some genotypes include few accessions, we cannot contemplate the occurrence of different genetic backgrounds among both islands with the available information.

3.2.4 Admixture analysis

For further elucidation of the genetic structure of the collection of landraces, an admixture analysis was performed (Figure 8). The estimated cross-validation (CV) error (Figure 8A) indicated that K= 5 was the best model for the analysis of the studied collection. This means that a model based on five genetic clusters provides the most accurate explanation for the genetic variation observed in our study. In this regard and considering the K = 5 model, the admixture analysis confirmed the findings of the PCA (Figure 5) and provided some more details of interest (Figure 8B). Thus, some landraces such as Tap de Cortí, Banya de Cabra, and D'Envinagrar showed genetic patterns without admixture. Moreover, the group of Ros accessions, i.e., Ros, Ros Gruixat, and Ros Prim, also showed a unique genetic background (green) with the only exception of a very little occurrence (<10%) of an additional background (orange), and the same was found in Banya de Cabra, mainly blue background with very few of other backgrounds (Figure 8B). Such findings suggest that Ros, Ros Gruixat, and Ros Prim might share a common origin despite their seeds being obtained from different sources or locations. In short, they may have some morphological differences, but this is barely reflected in their genome.

On the contrary, other varietal types like Blau, Cirereta, or Fulla d'Olivera showed more complex patterns, with a mixture of genetic backgrounds (Figure 8B). This fact was even more remarkable in the case of the Pebrera Blanca and, particularly, Citró de

Matances varietal groups. Nevertheless, it is necessary to take into account the possible existence of differences due to the different number of accessions available on-farm to represent each varietal group.

In the case of the Pebrera Blanca population, the admixture analyses showed two clearly different lines, one which could be considered almost a unique background (purple) with a limited presence of other minor backgrounds (<10%) and another which appears as a mixture of two main backgrounds (green and orange) (Figure 8B). These results are in agreement with the PCA based on the SNP polymorphisms (Figure 5), confirming the presence of two subpopulations of this varietal type. Finally, also in agreement with the PCA, the Citró de Matances accessions encompassed a plethora of genetic patterns, which confirms the intravarietal heterogeneity of this varietal group observed at both the phenotypic and genetic levels (Tables 3–5; Figures 3A, 5). This high diversity found in landraces like Citró de Matances and Pebrera Blanca could determine the necessary efforts for typification and to protect these landraces as relevant representatives of agrodiversity in the islands. In this regard, the possibility of new allele combinations during *in-situ* conservation needs to be taken into account, mainly due to gene exchange if grown open field together with other varieties, as often occurs in these agrifood systems. On the contrary, varieties with low diversity (and therefore clearly defined in phenomic terms) like Tap de Cortí could be conserved easier by farmers and directly used for breeding purposes and gene introgressions.

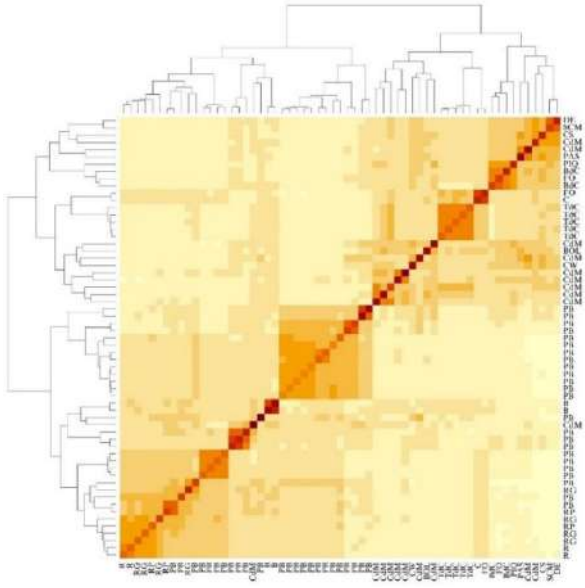

Figure 7. Heatmap of kinship matrix with the tree shown on the top and left of the studied Balearic accessions and the reference varieties, based on the 9,508 filtered SNPs.

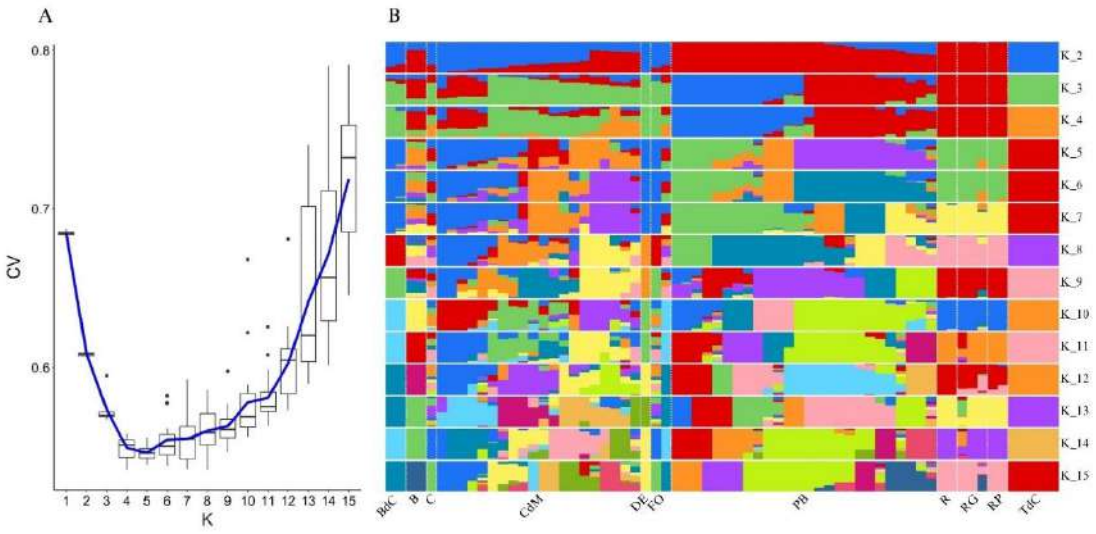

Figure 8. Admixture population structure analysis using the filtered SNPs of Mallorca and Eivissa landraces. (A) Cross-validation (CV) error plot to estimate the best fitting *K* value, and (B) structuration of the population according to the number of clusters *K* (2- 15). Colors represent different assigned clusters.

4. Conclusions

Our study on endemic peppers representative of the Balearic Islands has revealed a very diverse picture in terms of morphological variation and genetic structure. The on-farm cultivation and multiplication of these landraces has driven different genetic lineages, not always necessarily toward heterogeneous varieties as pepper is considered an autogamous crop. Thus, varietal types like Citró de Matances from Eivissa showed a plethora of morphologies and genetic backgrounds among their accessions, as well as a range of heterozygosity values (0.38%–1.11%) in comparison to other varietal types, revealing that under a common term, many lineages can be encompassed in local materials. In other cases, varietal types like Tap de Cortí or the Ros group were quite uniform in morphologies and presented a unique genetic background, with low levels of heterozygosity (<0.90%). Finally, other types like Pebrera Blanca represent intermediate cases, where two genetic subpopulations or lineages appeared, in agreement with slight morphological differences as can be observed in their rectangular elongated fruits (e.g., some accessions with fruits a bit more elongated vs. some others a bit shorter). These results suggest, on the whole, a diverse genetic structure, probably due to differences in farmers' practices during multiplication and breeding, or cross-pollination levels. This genetic and phenotypic diversity could be of great interest for future breeding projects to improve resilience in commercial peppers by broadening the genetic background of the breeding materials. Finally, farmers could also benefit

from the information obtained in this work, which can be used for developing genetic profiles for each landrace, determining a more efficient on-farm and *ex situ* conservation and speeding up the selection process. Moreover, this work could be used for future typification and legal protection of these local materials for scaling up toward more commercial exploitation.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data availability

The raw data analysed in this study are available in the Short Read Archive (SRA) of the National Library of Medicine repository under the accession number PRJNA1173395.

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Author contributions

Conceptualization, AR-B; Methodology, NO-A, MD and FM; Software, LB and MD; Validation, AF and AR-B; Formal analysis, NO-A and LB; Investigation, NO-A and MD; Resources, LB and J-ML-P; Data curation, NO-A and LB; Writing—original draft, NO-A, AF, MD and AR-B; Writing—review and editing, LB, FM and JML-P; Visualization, J-ML-P; Supervision, AR-B; Project administration, AR-B; Funding acquisition, AF and AR-B.

Supplementary materials

Supplementary materials are available at https://www.frontiersin.org/articles/10.3389/fpls.2024.1435427/full#supplementary-material

Supplementary table 1. List of studied accessions with corresponding code, genebank code, varietal type, and origin.

Supplementary table 2. Phenotyping values (mean ± standard deviation, in traits where several measurements were possible) for the quantitative traits analysed in all the selected accessions and reference varieties. NA indicates missing data.

Supplementary table 3. Observed (O) and expected (E) number of heterozygous sites, fixation index (F), and estimated heterozygosity values (Het, %) for all the studied accessions.

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Resesarch article

First interspecific multi-parent advanced generation intercross (MAGIC) population in *Capsicum* peppers: development, phenotypic evaluation, genomic analysis, and prospects

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Neus Ortega Albero has a main role in performing the experiments, sample and data collection, data analysis and visualization, drafting manuscript, revision and editing.

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Abstract

This work presents the first eight-way multi-parental advanced generation intercross (MAGIC) population in pepper. This interspecific MAGIC population was built with six *Capsicum annuum* accessions and two *C. chinense* accessions, selected for encompassing a representative and wide genetic diversity, and being complementary for morphological, agronomic, and fruit quality traits. The population in its third selfing generation has been phenotyped with reliable descriptors and genotyped using genotyping-by-sequencing (GBS) to assess its overall diversity, homozygosity, parental contributions, and genetic structure. A great variability was found in the phenotyping study, showing many forms of recombination of all the founder lines. Even more, new phenotypic combinations were found, as well as transgressive inheritance in quantitative traits. The S3-generation contained a balanced distribution of the parental genomes and each S3-individual seemed to contain a unique genomic combination of the founder lines reaching a high homozygosity. In this regard, a preliminary genome-wide association study (GWAS) was performed for highly heritable traits to evaluate the potential of this population for future breeding prospects. Strong associations were found for most traits analysed, like stem pubescence and fruit colour at maturity stage, with associated genes related to response to stress and defence functions; or fruit wall consistency, with associated genes related to lipid metabolism. Our results show that this first *Capsicum* MAGIC population is a valuable genetic resource for research and breeding purposes in peppers, by identifying genomic regions associated to traits of interest and its potential for future GWAS in more complex agronomical and fruit quality traits.

Keywords

Capsicum annuum, Capsicum chinense, genome-wide association study (GWAS), genotyping-by-sequencing, homozygosity, phenotyping

1. Introduction

Capsicum peppers (*Capsicum* spp.) are one of the most profusely grown vegetables worldwide for fresh fruit consumption and as a spice (Jarret et al., 2019) and they encompass a huge variation in morphological and agronomic traits. Among the cultivated species, *C. annuum* L. is the most economically important and diverse. Also, other cultivated species like *C. chinense* Jacq. displays a volatile profile which enhances fruit flavour and overall quality. *C. chinense* is phylogenetically close to *C. annuum* and sexual crosses are possible among them (Manzur, Fita, Prohens, & Rodríguez-Burruezo, 2015). In fact, *C. chinense* has been extensively used as donor for *C. annuum* breeding for fruit quality traits (Jeong et al., 2015; Wei et al., 2019).

Present variation in the *Capsicum* genus offers an outstanding genetic background for breeding commercial *C. annuum*. In this frame, a better understanding of the genetic basis of these traits has a paramount importance to predict and establish breeding programs. *C. annuum* has one of the largest genomes from the Solanaceae family, with around 3.5 Gb and between 75 and 80% of repetitive elements (2n=2x=24) (Hulse-Kemp et al., 2018). Considerably larger than other Solanaceae relatives like tomatoes or eggplants (Barchi et al., 2019; Hosmani et al., 2019). This is a major reason why elucidating peppers coding regions and genes has been a challenge for decades and molecular tools have been developed later than other Solanaceae crops (Kim et al., 2014; Qin et al., 2014). First draft genomes of *Capsicum* were released in the last decade. Kim et al. (2014) released the whole-genome sequencing and assembly of *C. annuum* hot pepper 'Serrano Criollo de Morelos' (hereafter CM334) reporting a draft genome of 3.45 Gb and *C. chinense* accession PI159236, with an estimated genome of 3.2 Gb. In the same year, Qin et al. (2014) published the genomic sequence of *C. annuum* cv. Zunla-1 and its wild parent *C. annuum* var. *glabriusculum* Chiltepin with assemblies of 3.35 and 3.48 Gb, respectively. However, it was in 2018 when Hulse-kemp et al. (2018) applied the linked-read sequencing to *C. annuum* to generate a highly ordered and more contiguous reference genome of 3.21 Gb.

Usually, genetic mapping has been performed in segregating progenies from bi-parental crosses, with a short number of haplotypes and recombination events. Although these experimental populations allowed to identify many major genes, they are quite limited in terms of recombination events and, hence, the discovery of quantitative traits loci (QTLs) (Mohammadi et al., 2020). In this regard, *Multi-parent Advanced Generation InterCross* (MAGIC) populations proposed by Mackay and Powell (2007), have been developed to gather and explode the phenotypic and genetic variability from a group of more than two founder lines to increase mapping resolution and QTL discovery because of the lower linkage disequilibrium or population structure (Arrones et al., 2020). MAGIC populations have been even used successfully in early generations to assess a preliminary vision of the population structure and its perspectives, as well as to understand the levels of intercross of founder lines' genomes and to identify potential genomic regions related to traits (Bandillo et al., 2013; Mangino et al., 2022; Pascual et al., 2015). And has been evaluated in later generations for identifying genes and

mutations responsible for the expression of more complex traits like production or resistance to stresses (Arrones et al., 2022).

MAGIC populations have been developed in some relevant cereals such as maize, wheat, and rice (Bandillo et al., 2013; Dell'Acqua et al., 2015; Mackay et al., 2014), legumes like chickpea or cowpea (Huynh et al., 2018; Thudi et al., 2024), and some Solanaceae species like tomato and eggplant (Mangino et al., 2022; Pascual et al., 2015). However, no MAGIC population has been reported in *Capsicum* peppers to date, and most genome-wide association studies (GWAS) in this genus have been based on collections of accessions or experimental populations limited to two founder parents (Leprévost et al., 2023; Navara & Smith, 2014). In the present work, we report the first interspecific MAGIC population in peppers, derived from interspecific crosses of six *C. annuum* genotypes and two *C. chinense* genotypes to integrate a plethora of genetic backgrounds and morphological, agronomic and fruit quality traits. This population has been phenotyped for qualitative and quantitative traits and genotyped to evaluate the parental markers distribution along the population and to preliminarily explore the occurrence of QTLs related to some highly inheritable traits evaluated.

2. Results and discussion

2.1 Multi-parent advanced generation population development

Six genotypes of *C. annuum* and two genotypes of *C. chinense* were selected for their interesting phenotypic traits as founder lines for developing the first interspecific MAGIC pepper population. Following the scheme of Figure 1, a total of 420 eight-way hybrid individuals, from which 284 derived from ABCD as a mother and 136 derived from EFGH as a mother. The S3-MAGIC population was comprised by 350 individuals obtained following a single seed descent (SSD) program. The introduction of multiple founders from two different species with high genetic and phenotypic diversity enabled the accumulation of a plethora of recombinant events, increasing mapping accuracy of interesting genes (Arrones et al., 2020). The loss of 70 progenies occurred during the first and even second selfing generations, which might be due to the inclusion of two *C. chinense* founder genotypes, despite being used as male parents (B and D). In this regard, previous MAGIC populations have been reported to lose progenies during first developmental generations as a result of genome rearrangements causing infertility or non-viable hybrids as occurred in the eggplant population, which included a wild related species (Mangino et al., 2022). In fact, after three selfing generations, the current S3-population has reached high percentages of pollen fertility, as will be explained in the next section.

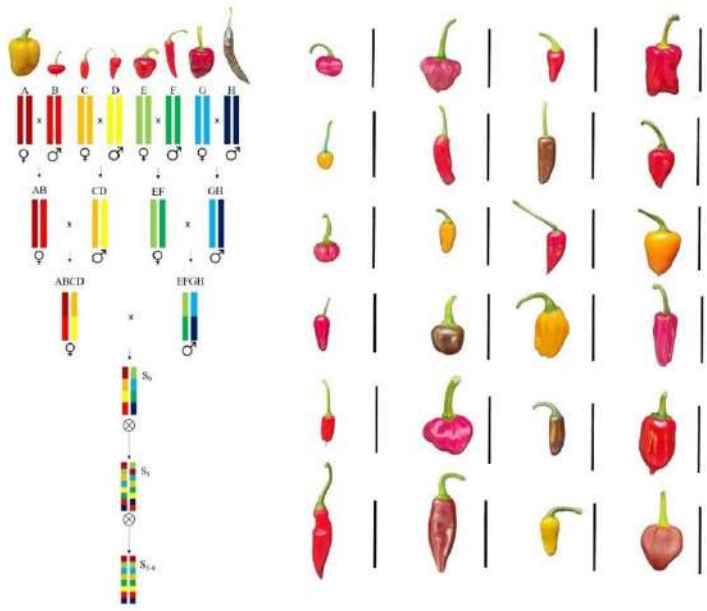


Figure 1. (A) MAGIC population construction process, founder lines, crosses, and development. A: California wonder (*Capsicum annuum*), B: Ají dulce (*C. chinense*), C: Chile Serrano (*C. annuum*), D: Ecu-994 (*C. chinense*), E: Bola (*C. annuum*), F: Serrano Criollo de Morelos (*C. annuum*), G: Piquillo (*C. annuum*), H: Pasilla Bajío (*C. annuum*). (B) Illustrative representation of the variation among the S3 progeny. Black bar indicates 5 cm length.

2.2 Phenotypic variation and inter-trait correlations

2.2.1 Qualitative traits

Most evaluated traits showed variation among the founder lines like, nodal anthocyanin, stem pubescence, leaf shape, flower position, fruit colour, fruit shape parameters, and pedicel persistence. Even, a new phenotype was described for fruit shape in Ají Dulce (phenotype 'Disc'). Differences were between *C. annuum* and *C. chinense* included absence of nodal anthocyanins, two flowers per axil and purple filament colours, only found in *C. chinense* parents. Very few traits were found monomorphic (or almost) in the parent lines including stem colour, neck at the base of the fruit, fruit cross-section corrugation, and anthocyaninic spot in the intermediate-stage of the fruit (Supplementary table 1).

F_1 hybrids showed intermediate phenotypes to those of the corresponding parents, the phenotype of one of the parents and, even, some new phenotypes (Supplementary table 1). The findings relative to stigma exsertion, fruit colour in mature fruits, and fruit shape, indicated the dominance of triangular shapes, red colour at the fully ripe stage, due to described recessive alleles for yellow and brown colours, and the exsertion of stigma, as a wild trait which favours crossed pollination in nature against inserted

stigmas (Jeong et al., 2019; Zhou et al., 2017). A few cases of new phenotypes were detected in some F_1 crossings. For example, intermediate leaf density and dark green leave in F_1 Piquillo x Pasilla, which came from sparce-density and green leaves parents, or the lanceolate leaves and erect flowers of F_1 California x Ají Dulce, whose parents were deltoid and ovoid-shaped leave and pendant and intermediate flowers (Supplementary table 1). The occurrence of new phenotypes has been pointed out by evolutionary biologists during interspecific hybridization, due to genome recombination of varieties and species together with genetic and epigenetic changes (Rodionov et al., 2019).

As expected, the S3-progeny showed a huge variation for all the studied traits and, even, the occurrence of new phenotypes was recorded in some traits. Some qualitative traits showed a balanced segregation of the different phenotypes, in proportion to their occurrence in the founder lines, like nodal anthocyanin, stem pubescence, leaf density, leaf colour, leaf shape, number of flowers per axil, or flower position (Supplementary table 1). In other cases, the segregation of the different phenotypes was different to that observed in the founder parents, like anther colour or stigma exsertion, with a 30% of each phenotype in the S3-population while one phenotype appeared just in one parent line (Supplementary table 1), confirming the dominance phenomena observed in the F_1 hybrids for, example, for stigma exsertion. Finally, some cases of new phenotypes, not present in the founder lines, were recorded in traits like stem colour, filament colour, anthocyaninic spots in the intermediate-stage fruit, fruit colour at the mature stage, or fruit shape at pedicel attachment, which suggest the occurrence of genetic complementation or specific recombinations that produced these phenotypes hidden under recessive and complementation effects (Kumar et al., 2021; Mackay et al., 2014; Thudi et al., 2024). Qualitative traits were also evaluated in the S4-generation, confirming, in most traits, the distribution observed in the earlier S3 generation (Supplementary table 1). This can be observed in traits like stem pubescence, leaf colour, or fruit colour at intermediate stage. Only very few more complex traits like fruit shape, or fruit section corrugation showed slight differences in the phenotype distribution over the individuals, which could be due to the different number of evaluated lines in the S4 against the S3. In fact, the expression observed for qualitative traits in individual lines of the S4 generation coincided to the previously observed in the corresponding S3 lines, suggesting that these traits, at least phenotypically, were already fixed in S3.

2.2.2 Quantitative traits

All the quantitative traits showed a great variation among the founder lines, confirming that they encompass a great phenotypic diversity. Most variable traits among founder lines seemed to be stem length, fruit length, width and weight, and seed weight (Supplementary table 2). *C. chinense* showed considerable differences compared to *C. annuum* like a lower fruit weight, pedicel length, fruit shape triangle and seed weight values. Few traits like proximal and distal fruit blockiness, fruit shape triangle and number of locules showed little variation (Supplementary table 2). Generally, morphological pollen viability showed the highest values compared to biological viability values, being the germinative viability the one with the lowest values. These

results agree with other results previously reported in *Capsicum* (Manzur et al., 2015). Founder lines' viabilities were found to be considerably high, with the only exception of California Wonder (Supplementary table 2).

As observed for qualitative traits, F_1 hybrids showed a different performance depending on the trait. In some cases, an intermediate value between the corresponding founder lines was observed, e.g. leaf length, leaf width, and fruit length (Supplementary table 2). For other traits, the phenotype in the F_1 was closer to one of the corresponding parents, e.g. for fruit width, pedicel length, or number of locules of the fruit (Supplementary table 2). Other traits showed transgressive values, like stem length, internode length, fruit weight, the pollen viabilities (Supplementary table 2). Pollen viability was particularly low in interspecific F_1 hybrids between *C. annuum* and *C. chinense,* which is probably due to deleterious phenomena during genome rearrangements (Sui & Hu, 2015). These events are key to understand the difficulties that interspecific experimental populations face during the first generations, even losing some non-viable lineages.

Generally, the distribution of the traits in the S3-population was within the ranges of variation in the founder lines, as it was found for internodal length, leaf length and width, fruit length and width, or number of locules (Supplementary table 2). Nevertheless, a great diversity among the S3-population was found, resulting in the appearance of transgressive individuals for most traits. This event was observed in internode length with a coefficient of variation (CV) of 58%, internode number (CV=30%), leaf length (CV=17%), leaf width (CV=19%), pedicel length (CV=36%), locule number (CV=18%), and, especially, in fruit weight with CV=102% (Supplementary table 2). These recombination effects have also been reported by other authors in multiparent populations (MPPs) of several species, including studies about transgressive events (Bandillo et al., 2013; Kumar et al., 2021; Thudi et al., 2024). In this regard, new trait combinations and heterosis in fruit traits like fruit weight have been previously observed in interspecific *C. chinense* × *C. annuum* descendant populations (Naves et al., 2022a; 2022b). Pollen morphological viability showed relatively high values in the S3-progeny, with a mean value of 41% and a maximum value of 97%. Viability was lower in biochemical and germinative measurements, as expected, with means of 33% and 11%, respectively, although very high values were observed in some individuals, with a 96% and 69%, respectively.

According to our findings, the S3-MAGIC progeny looks very useful to study the recombination of the traits. Values of the S4 population have been studied in most traits, confirming the distribution observed in the S3 generation (Supplementary table 2). Most quantitative traits maintained the mean values displayed by the S3 individuals. Thus, the evaluation of the S4 progeny was a check point to confirm that the phenotypic distribution was maintained for most traits among both generations. However, the overall variation observed in some quantitative traits made us decide to evaluate only qualitative traits in the preliminary GWAS analysis.

2.2.3 Inter-trait correlations

Significant inter-trait correlations (Pearson $p < 0.05$) were detected considering all the studied populations (i.e. S3-generation, founder lines, and F_1 and $F_1 \times F_1$ hybrids) (Figure 2). Strong correlations were found within the same group of descriptors. CIELab colour coordinates were highly correlated with qualitative colour descriptors for both leaf and fruit, indicating that the values obtained in the CIELab space are accurate indicators of the visual classification. High correlations were found between plant descriptors, like the positive correlation between stem colour and nodal anthocyanin, or between plant height and internode length. Fruit traits showed the highest number of correlations among them. For instance, fruit length and fruit width showed high positive correlations with fruit weight, and peduncular persistence seemed to be related with consistent fruit walls, that means, that the softer the fruit, the lower the persistence, indicating common processes associated to fruit ripening and senescence (Figure 2). Also, biochemical and morphological pollen viabilities as well as germinative viability showed strong positive correlation values. Finally, correlations were also found between different group of descriptors. As an example, positive correlations were observed between leaf length and fruit weight and between leaf length and fruit length indicating that plants with larger leaves tend to have bigger fruits, as well as the contrary, i.e. plants with smaller leaves usually produce small or light fruits. This event has previously reported in *Capsicum* wild types like Chiltepín (*C. annuum* var. *glabriusculum*) or cultivated Cayenne types (*C. annuum* var. *annuum*) (Hayano-Kanashiro et al., 2016).

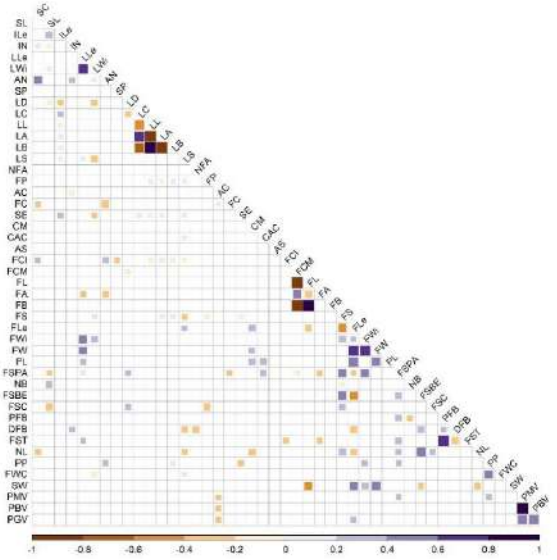


Figure 2. Statistically significant inter-trait correlations with correlation coefficients (Peason $p < 0.05$), with a size-scale of the magnitude of the correlation and a colour-scale of the significance.

2.2.4 Principal component analysis

The first and second principal components explained 10.5% and 9.1% of the observed variation (Figure 3). Founder lines appeared mainly located in the borders of the cloud of S3-individuals, indicating that the offspring integrated and displayed most phenotypic variation of the parents. F_1 hybrids were distributed close to their corresponding founder lines, while $F_1 \times F_1$ hybrids were located in the centre of the cloud of the S3-population and were separated by the PC1. Finally, some S3-lines appeared apart from the main cloud, even surpassing the founder lines, with extreme phenotypic values in some traits (Figure 3).

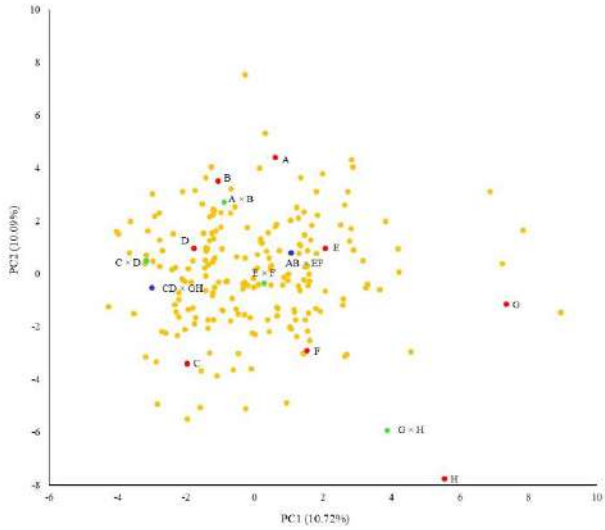


Figure 3. First (PC1) and second (PC2) principal components, representing founder lines (red points), F_1 (green) and $F_1 \times F_1$ hybrids (blue) and the S3-progeny (orange), analysed with phenotypic data.

2.3 Genotyping-by-sequencing results

2.3.1 Sequencing and SNP calling

The S3-MAGIC lines were sequenced obtaining 900 million raw paired-end reads and about to 110 Gb of data. After de-multiplexing, cleaning and trimming, clean reads were aligned to the reference pepper genome CM334 (Serrano Criollo de Morelos version 1.55) (Kim et al., 2014). A total of 1,197,532 polymorphic sites were identified after SNP calling and basic filtering distributed over the 12 chromosomes and 37,748 were selected for further analysis.

Then, the 1,197,532 polymorphic sites obtained after SNP calling and basic filtering were used for calculating the population homozygosity. Homozygosity values for the selected founder lines ranged from 99.1% in Serrano Criollo de Morelos and 99.68% in Chile Serrano, Bola, and Pasilla Bajío. All the founder lines of the population showed more than 99% of homozygosity, as they have been selected and bred through generations of auto-pollination. Regarding the F_1 hybrids, the homozygosity values drop to 96.87%, being the interspecific *C. annuum* × *C. chinense* hybrids, the ones showing the lowest values, indicating that interspecific crosses, might result in higher number of genomic recombination. This high homozygosity in the F_1 hybrids suggest that, even phenotypically the founder lines differ remarkably, there is a huge amount of shared genetic background.

Homozygosity values in the S3-progeny ranged from 97.7 and 99.8% and showing a mean value of 99.38% indicating that, after three selfing generations, the population has evolved fast in their fixation. Moreover, despite including *C. chinense* genetic background might have caused some genome deconstruction and reordering and generating miss-crossing during meiosis, our S3-population does not show apparently further difficulties to recover homozygous individuals (Sui & Hu, 2015).

2.3.2 Genetic relationship and structure of the S3 generation

2.3.2.1 Principal component analysis

37,748 SNPs were selected after quality filtering (missing data < 15% and mean minimal read depth > 7, missing data per site < 25%, minor allele frequency (MAF) > 5%) to assess the genetic relationship between the S3-lines and the founder lines. It should be noted that the two first components of the principal component analysis only explained a 10.87% of the variation (Figure 4), indicating the huge genetic variability encompassed by our S3-MAGIC population. *C. annuum* parent genotypes appeared clustered in the positive area of the PC1, while *C. chinense* genotypes appeared in the opposite location of PC1, confirming genetic differences between both genetic strains. In this sense, commercial peppers California Wonder and Bola were located together and slightly separated from the other genotypes, especially Serrano peppers, which are considered the closest cultivated types of wild *C. annuum* var. *glabrisculum* i.e. the least domesticated *C. annuum* var. *annuum* (Pereira-Dias et al., 2019).

In the case of F_1 hybrids, *C. annuum* intra-specific hybrids clustered with *C. annuum* genotypes due to the shared genomic background, while interspecific *C. annuum* × *C. chinense* hybrids appeared in the middle of both parents' taxons, indicating that these hybrids embedded, in a balanced way, part of their parent genomes (Figure 4). Also, the $F_1 \times F_1$ hybrids were located in the PC1 halfway between inter- and intra-specific F_1 hybrids, indicating, again, a balanced admixture of the founder genomes in the offspring, in agreement with our findings in the phenotypic characterization. Nevertheless, both selected $F_1 \times F_1$ separated considerably along the PC2, indicating also considerable differences at the genomic level between those hybrids. Finally, the S3-progeny appeared widely distributed in the PCA, although, in terms of PC1, the cloud of

individuals was located closer to the *C. annuum* group of parents, as expected in an offspring which should encompass 75% of these genomes vs. 25% of the *C. chinense* background. Furthermore, the S3-progeny distributed widely in PC2, much wider than observed in the founder lines, F_1 and $F_1 \times F_1$ hybrids (Figure 4). Such an outbreak of diversity, particularly in the PC2, indicates new recombination events in the MAGIC population.

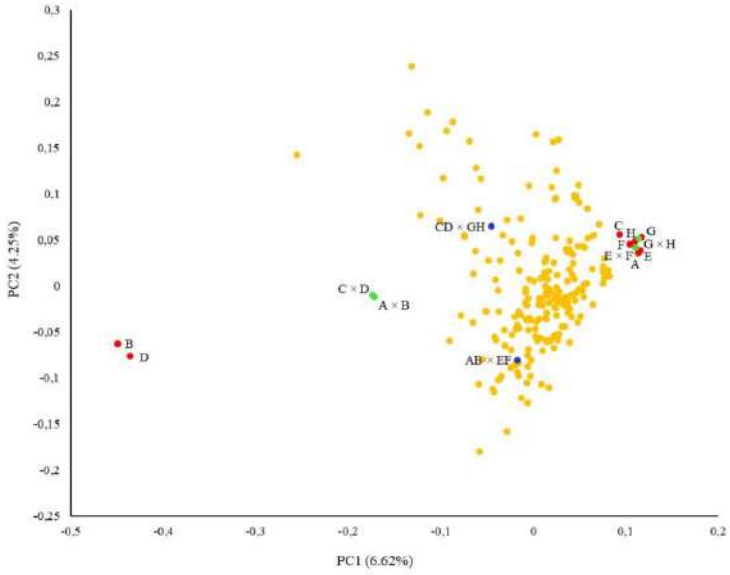


Figure 4 First (PC1) and second (PC2) principal components, representing founder lines (red points), F_1 (green) and $F_1 \times F_1$ hybrids (blue) and the S3-progeny (orange), analysed with GBS data.

2.3.2.2 Parental contributions

The parental genomic contributions were calculated for the S3-population with the good quality SNPs. Our results showed that all the parental genomes were represented in, at least, a 10% of the S3 lines, indicating a well-balanced reorganization of all the founder lines (Figure 5). The representation of the *C. annuum* species was, in average, a 78.5%, distributed as 14.2% of 'California Wonder', 12.4% of 'Chile Serrano', 12.7% of 'Bola', 10.1% of 'Serrano Criollo de Morelos', 14.7% of 'Piquillo', and 14.5% of 'Pasilla Bajío'. *C. chinense* genome was present in a 21.5% distributed as 10.8% 'Ají Dulce' and 10.6% 'Ecu-994'. This distribution agrees with the expected values of 12.5% of each founder line, and even *C. chinense* genomes were represented in almost 25%. These results agree with the results observed in Figure 4, the cloud of the S3 lines displays genetically in a region which is placed in the half side closer to the *C. annuum,* but not overlapping, suggesting a 25% *C. chinense* and a 75% of *C. annuum* mixture.

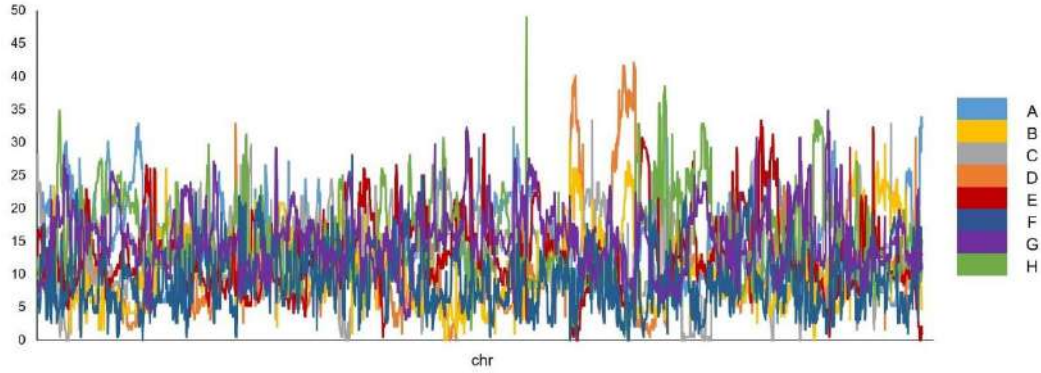


Figure 5. SNP position along the genome (x-axis) and the percentage of founder contribution (y-axis) in the S3 population. Each point represents founder contribution at a specific SNP, illustrating ancestral contributions across the genome. Legend: colour code associated with each founder.

2.3.2.3 Dendrogram and kinship matrix

No clear relationships or clusters were observed in the phylogenetic tree among the S3-lines as they appeared widely distributed in agreement with the findings of the PCA (Supplementary figure 1A). On the contrary, lines were equally distributed, and the founder lines were placed between the descendants, including *C. annuum* and *C. chinense,* although both species were genetically distant according to the SNP data (Supplementary figure 1A). Moreover, S3-lines showed more similarities among them and the founder lines than F_1 and $F_1 \times F_1$ hybrids, which showed differentiated genomes compared to the S3-population. Furthermore, the kinship matrix showed that S3-lines were unique recombination events, as, even genetic similarities were found between groups of individuals, no specific clusters were observed (Supplementary figure 1B). These results confirmed the results of the PCA, indicating that each individual has a unique genetic pattern resulting from the recombination of the founders' genomes. And, therefore, the whole S3-population appears to encompass a wide and well-balanced genetic diversity with a lack of genetic structure as reported in other MAGIC populations of Solanaceae species, like tomato and eggplant (Arrones et al., 2022; Mangino et al., 2022).

2.3.2.4 Admixture analysis

An admixture analysis was performed to elucidate the genetic patterns and structure of the S3-population. The estimated cross-validation error indicated that $K = 9$-11 were the best statistical models to fit the available SNP data (Figure 6A). Considering $K = 9$ model as the simplest one, each individual of the S3-population is made of an admixture of different genomic regions, confirming the results obtained in the kinship matrix (Figure 6B). Very few individuals showed no admixture or recombination or, even, similar genome patterns. Also, 9 genetic subpopulations fit quite accurately the eight founder lines and confirms a balanced genome and allelic admixture. High diversity has been found for admixture and substructure in MAGIC populations. For

instance, other MAGIC populations like chickpea that have reached up to 13 subpopulations in the admixture analysis (Thudi et al., 2024), indicating that this analysis is highly dependent on the underlying genome complexity, crossed species, etc. while MAGIC populations of sorghum have shown four subpopulations or groups (Kumar et al., 2021).

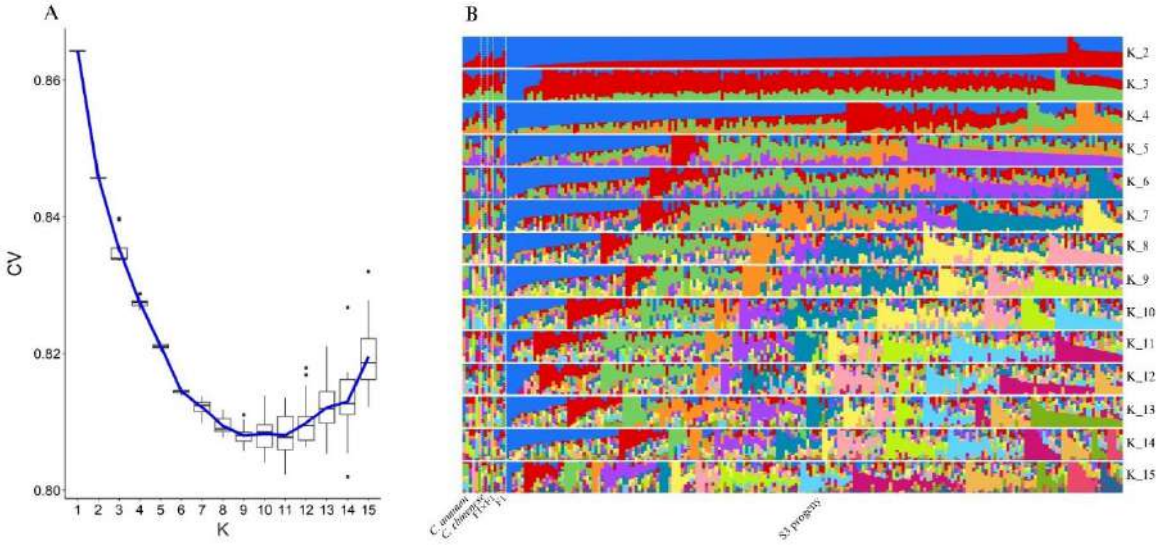


Figure 6. Admixture structure analysis for MAGIC pepper population including founder lines, F_1 hybrids, $F_1 \times F_1$ hybrids and 200 S3-individuals. A: Cross-validation (CV) plot to estimate the best fitting *K* value and B: structure of the population.

2.4 Genotype-wide association study

2.4.1 Linkage disequilibrium

A diagram of r^2 against physical distances showed a pattern of LD decay that allowed us to stablish the inter-marker separation in 500 kilobase pairs, when the r^2 magnitude was reduced to $r^2 > 0.2$ (Supplementary figure 2). LD generally has great variation depending on the chromosome as reported by Thudi et al. (2024) and it is also very variable at which point LD will decay to $r^2 < 0.2$, when two markers are considered independent.

Reported LDs were also variable depending on the species, for instance, chickpea showed a mean LD of 1.2 Mb, while barley had a LD decay value around 19 Mbp, and extremely low 40 kb for a 19-way sorghum MAGIC population (Kumar et al., 2021; Thudi et al., 2024). LD decay tends to decrease in each generation, next generations of this MAGIC population will be used to narrow the genetic basis of QTL associated to traits and, thus, stablishing candidate genes.

2.4.2 QTL mapping and analysis

Significant QTLs were found for nine of the qualitative traits analysed, using BLINK and MLMM statistical models (Supplementary table 3; Supplementary figure 3). Based on our data, a higher number of QTLs were found with the BLINK model, although some QTLs were also reported using the two statistical models. This result has been also reported in other GWAS analysis (Kumar et al., 2021), with higher number of QTLs obtained with BLINK than MLMM. As expected for genetically simple traits, all the QTLs related to the corresponding trait were located in the same chromosome, with the only exception of i) stem pubescence, where QTLs were located in chromosomes 2 and 10, ii) anthocyanin spot in the intermediate stage fruit, with QTLs in chromosomes 4 and 6, iii) fruit colour at maturity stage in chromosomes 1, 4, and 6, and iv) fruit wall consistency with QTLs in chromosomes 5, 10 and 12 (Figure 7).

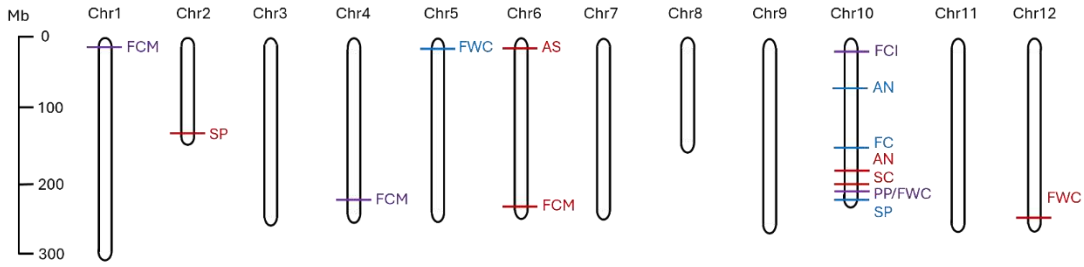


Figure 7. Distribution of the QTLs in a pepper physic map. Coloured bars show the location of the QTLs found in each trait and each statistical model (red: BLINK, blue: MLMM, purple: both models). SC: stem colour, AN: nodal anthocyanin, SP: stem pubescence, FC: filament colour, AS: anthocyanin spot, FCI: fruit colour at intermediate stage, FCM: fruit colour at mature stage, PP: pedicel persistence, FWC: fruit wall consistency.

Once the significant QTLs for plant related traits were identified, the associate genes in those regions were investigated (Figure 7). For stem colour several genes were identified in chromosome 10; however, the most remarkable finding corresponded to the esterase/lipase gene *CPRD49,* presumably related to lipid metabolism (Supplementary table 3). In this regard, tissue pigmentation has been previously associated with globular lipid inclusions and proteins associated with these lipid molecules, embedded with anthocyanins in chromoplasts (Middleton et al., 2020; Rey et al., 2000). In the case of nodal anthocyanin, several genes related to the response and adaptation to changes in temperature have been found in chromosome 10, like *BTB/POZ* domain-containing protein, *HSP17,* and *APRR7,* which are involved in protein modification (Supplementary table 3). In this regard, in agreement with our findings, anthocyanin accumulation has been recently associated with protein modification and gene regulation (Fu et al., 2022). We also identified a thylakoid membrane protein gene (*TERC*) associated with nodal anthocyanin. In these terms, anthocyanin transport into the vacuoles through activation of transport systems has been previously reported (Wang et al., 2022). Our results for colouration patterns in plant organs agrees with previous reports in *Capsicum*, indicating

the existence of a major gene in chromosome 10 determinant for pigmentation in plant, foliage, and flowers (Lopez-Moreno et al., 2023). Candidate genes in chromosome 10 needs an in-depth study for their function in anthocyanin accumulation in plant tissues. For stem pubescence, genes related to defence were detected in chromosomes 2 and 10, e.g. *R1B-23* and zinc finger CCCH domain-containing protein 14, (Supplementary table 3). These results confirm previous reports indicating QTLs related with trichome accumulation in the stem located in chromosomes 2 and 10 in peppers (Chunthawodtiporn, Hill, Stoffel, & Van Deynze, 2018). Publications about this trait are scarce or nil, although some authors proposed that hair density could be determinant for plant protection against different biotic and abiotic stresses like cold and drought (Moles et al., 2020). Moreover, regions in chromosome 2 of *Capsicum* have been related to resistance to pathogens as well as tolerance to abiotic stresses (TS et al., 2023).

Regarding flower traits, only filament colour showed significant QTLs in chromosome 10 (Supplementary table 3). These QTLs included genes related with phenol and photosynthetic metabolism and the production of isoflavone formonotenine, e.g. *D7OMT* isoflavone 7-O-methyltransferase and *HEMA1* glutamyl-tRNA reductase 1. These findings suggest that accumulation of phenolic compounds as precursors could be determinant for the final pigmentation of the filament with purple anthocyanins and reinforce the idea that chromosome 10 accumulates many genes related to pigmentation of plant and, also, flower tissues (Chunthawodtiporn et al., 2018; Lopez-Moreno et al., 2023).

Finally, significant QTLs were found for fruit descriptors like anthocyanin spot, fruit colour at the intermediate stage, fruit colour at the fully ripe stage, pedicel persistence, and fruit wall consistency (Figure 7). All QTLs reported for anthocyanin spot in immature fruits were located in chromosome 6 and most genes were associated to defence against pathogens, especially, genes for late blight resistance (*R1B-14, R1A-4* or *R1A-10*) (Supplementary table 3). For fruit colour at the intermediate stage, several QTLs related to cell metabolism like development or protein modification were found in chromosome 10, e.g. *SD25* G-type lectin S-receptor-like serine/threonine-protein kinase or *TBL39* Protein trichome birefringence-like 39 (Supplementary table 3). In this regard, anthocyanin accumulation has been reported to increase cold tolerance (Sudheeran et al., 2018), even gene family *CaMYB*, transcription factors regulating development and stress responses, have been related to accumulate anthocyanins and increase cold tolerance in purple peppers and located in chromosome 10 (Eggink et al., 2014; Wu et al., 2023; Zhang et al., 2020). Also, gene *Ca3GT* has been described in chromosome 10 for determining fruit colour at the unripe stage, indicating that fine mapping of chromosomic region reported in our work would lead to the same gene (Liu et al., 2020). On the whole, our study detected a strong association between QTLs related to response to biotic and abiotic stress (including response to temperature changes), with tissue colour traits, like nodal anthocyanin and unripe fruit colour, indicating that presumably simple traits might be modulated by environmental conditions. Furthermore, we have confirmed the existence of a hot spot of genes in chromosome 10 determining

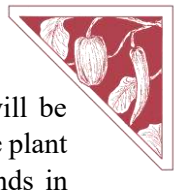
anthocyanin accumulation in plant organs, flowers, and fruits; and we have proposed new candidate genes for future validation.

A large number of significant QTLs were found for fruit colour at fully ripe stage, including regions in chromosomes 1, 4, and 6 (Figure 7). Although in the first studied on inheritance and gene control, fruit colour was described as qualitative or controlled by few major genes, several studies have later confirmed that variation in colour and intensity can be determined in a quantitative manner, involving many regulating genes (Brand et al., 2012). Some of the identified genes in this work were related with fruit development, ripening and pigment accumulation like ethylene-responsive proteinase inhibitor 1, alpha-trehalose-phosphate synthase 7, and capsanthin/capsorubin synthase, the last one previously reported in peppers by Jeong et al. (2020) (Supplementary table 3). Our results regarding chromosomes 1 and 4 coincide with phytoene synthase *CaPSY1* and *CaPSY3* genes described by Wei et al. (2021) in the same genomic regions for fruit colour in ripe fruits in *Capsicum*. Other QTLs found for fruit colour were highly related to plant development like cyclin-dependent kinase C-1, protein Fantastic Four 3 or ankyrin repeat-containing protein *BDA1*. Finally, QTLs related to lipid metabolism and hormone response and signalling were also associated to fruit colour at mature stage like phosphopantothenoylcysteine decarboxylase and lipid transfer protein GPI-anchored 2 associated to lipid metabolism, and protein *RALF*-like 5, protein ethylene insensitive 3 and vesicle-associated membrane protein 714, associated to hormone signalling.

For pedicel persistence and fruit wall consistency, polygalacturonase were the most remarkable associated genes, in agreement with the findings of Zhai et al. (2021) (Supplementary table 3). These genes have been preliminarily located in regions of chromosomes 10 of *Capsicum* (Chaim et al., 2001; Islam et al., 2023). Fruit wall consistency also showed several genes related to lipid metabolism distributed along all the genome. Moreover, some interesting functions were found related to glucose metabolism, response to biotic and abiotic stresses for fruit wall consistency (Supplementary table 3). Overall, these results indicate that chromosome 10 is a hot spot of genes determining fruit ripening, a key trait for post-harvesting quality improvement.

3. Conclusions

In conclusion, in this work we present the first MAGIC interspecific population achieved in *Capsicum* as a valuable tool for future breeding and genetic studies in peppers. A wide phenotypic diversity has been observed within the population, increasing the available *Capsicum* resources. Also, unique genetic patterns have been found in the population, indicating a plethora of recombinant processes, but representing at the same time a well-balanced distribution of the eight parental genomes in the developing MAGIC population. Moreover, with this MAGIC population, genomic regions and candidate genes have been preliminarily associated with highly inheritable traits stem colour, nodal anthocyanin, stem pubescence, filament colour, anthocyanin spots in the fruit, fruit colour at the intermediate and mature stage, pedicel persistence and fruit wall consistency to show the potential of this population. These findings will be exhaustively investigated, and candidate genes will be confirmed for these traits in



further experiments. Moreover, future generations of the MAGIC population will be used to also define candidate genes for more complex traits in pepper breeding like plant architecture, root traits, volatile profile, and accumulation of bioactive compounds in fruit as well as response to biotic and abiotic stresses.

4. Materials and methods

4.1 Development of the MAGIC population

Founder lines of the MAGIC population included six *Capsicum annuum* L. genotypes, corresponding to four cultivated genotypes ('Bola', 'California Wonder Yellow', 'Pasilla Bajío', 'Piquillo') and two semi-wild types ('Chile Serrano' and 'Serrano Criollo de Morelos'); and two *C. chinense* Jacq. genotypes ('Ají dulce' and 'Ecu-994') (Figure 1). These lines were chosen to maximize phenotypic, agronomic, and genetic diversity in the progenies. Among other traits, *C. annuum* lines like 'California Wonder' and 'Piquillo' were selected because of their high commercial value, with a thick flesh of the fruit. Also, 'Piquillo' has an improved root architecture, showing a good enzymatic activity in the soil and a great absorption, which are good indicatives for adaptation to organic and low input conditions (Pereira-Dias et al., 2020). 'Pasilla Bajío' is one of the peppers varieties with the highest accumulation in antioxidants in fruit like ascorbic acid (vitamin C), carotenoids and flavonoids (Ribes-Moya et al., 2018; Rodríguez-Burruezo, Prohens, Raigón, & Nuez, 2009). 'Bola', on the contrary, has been selected for its precocity in fructification, thin flesh, and high-water content, and improved production traits. 'Chile Serrano' and 'Serrano Criollo de Morelos' (cv. SCM334), have been selected for their plant architecture, showing a higher plant with open branches, enabling an easier harvest, as well as for being the wildest representation of *C. annuum* taxon, and well-known for their resilience to hard pedoclimatic conditions (Moreno-Peris et al., 2020; Rodríguez-Burruezo et al., 2010). *C. chinense* 'Ají Dulce' and 'Ecu-994' have been added as founder lines of this MAGIC population because they have shown a different volatile profile in fruits compared to *C. annuum* varieties, improving fruit quality and flavour, including pungency as a high-added value. Moreover, these accessions had shown a satisfactory crossability in previous works compared to other cultivated peppers and they have been proposed as gene donors for *C. annuum* breeding for many traits of interest like capsaicinoid content, resistances to multiple pests, and introgressing multiple-flower traits to improve yield (Devi et al., 2021; Jeong et al., 2015; Manzur et al., 2015; Pereira-Dias et al., 2019; Tanksley & Iglesias-Olivas, 1984; Uncu, 2019). These varieties have been used as male parents in their corresponding F_1 crossings to prevent cytoplasmic deleterious phenomena (dwarfism or virus-like syndrome) that may appear when *C. chinense* is used as female (Manzur et al. 2015). F_1 crosses were made with individual plants of each founder line, previously selected for their highest homozygosis after several selfing processes and GBS-genotyping analysis (Pereira-Dias et al., 2019). Only 'Serrano Criollo de Morelos' has been selected for resistance purposes, carrying the *Me1* genes responsible for resistance to *Meloidogyne arenaria* and other genetic factors for resistance to *Phytophthora capsici.*

Since 2016, four parental crosses were performed between the accessions as indicated in Figure 1. Then, F_1 hybrid 'California Wonder' (A) x 'Ají Dulce' (B) and F_1 'Chile Serrano' (C) x 'Ecu-994' (D) were crossed to obtain 175 4-way individuals (ABCD). On the other branch, F_1 hybrid 'Bola' (E) x 'Serrano Criollo de Morelos' (F) and F_1 'Piquillo' (G) x 'Pasilla Bajío' (H) were crossed to obtain 160 4-way hybrids (EFGH). Then, the two double hybrids were intercrossed in reciprocal crosses to obtain the 8-way or quadruple hybrid generation (Figure 1). As a result, 420 unique lines were obtained to constitute the S0 generation. These lines were then propagated following a single seed descent (SSD) procedure for three generations to obtain a S3-generation of 350 lines on the 2021/22 season.

Seeds of S3-lines, founder genotypes, the original F_1 hybrids and two representative $F_1 \times F_1$ hybrids as controls, were sown in seedling trays with commercial substrate after disinfection with 30% bleach for 30 minutes and irrigated with tap water. Trays were maintained at room temperature and watered every three days. Once plants reached the four-leaves stage, they were transplanted into 30 cm diameter pots, grown under glasshouse-controlled conditions, trained with vertical strings, drip irrigated and fertigated following agronomic recommendations for peppers.

4.2 Phenotyping traits evaluation and statistics

Phenotypic variation in unique S3-individuals of the MAGIC population was determined in Spring and Summer 2023 under greenhouse experimental conditions in the Universitat Politècnica de València (València, Spain) with 47 morphological descriptors of *Capsicum,* corresponding to plant (14), inflorescence/flower (7), fruit (22), pollen (3), and seed (1) traits (Table 1). Also, a collection of 200 S4-individuals was phenotyped in the Spring-Summer 2024 under the same conditions with a selection of descriptors for plant (7), inflorescence/flower (7), fruit (18), pollen (3), and seed (1) traits to check the reproducibility and consistency of the data among both generations. Most descriptors were obtained from Bioversity International (Institute International Plant Genetic Resources, 1995). Also, other common traits used in peppers breeding characterisation and/or registration, like internode number, internode mean length, fruit wall consistency, and pollen morphological, biological and germinative viability were added. Fruit shape was also estimated through three ratios: proximal fruit blockiness, distal fruit blockiness, and fruit shape triangle. Proximal fruit blockiness was calculated as the ratio between highest fruit part width (mm) and the centre of the fruit (mm). Distal fruit blockiness was calculated as the ratio between the lowest fruit part width (mm) and the centre of the fruit (mm). Finally, fruit shape triangle was measured as the ratio between highest fruit width (mm) and lowest fruit part width (mm). Fruit wall consistency was estimated by applying pressure to the fruit pericarp with the fingers. Those pericarps flexible which retained the original shape were considered consistent, while fruits with permanent deformation or bruising were considered non-consistent.

Pollen morphological and biological activity was measured in a light microscope, with pollen samples from four flowers per plant, dyed with 2,3,5-triphenyl tetrazolium chloride (TTC) and kept in darkness for 15 minutes (Rodriguez-Riaño & Dafni, 2000).

Turgent pollen grains in the case of morphological analysis and dyed turgent grains in the case of biological analysis were counted in four fields per sample, with a total magnification of 100× and referred to the total grains. Finally, for pollen germinative viability, pollen samples were stirred for 5 hours in a solution of H_3BO_3 100 ppm and 10% sucrose, dyed with acetocarmine with 1% glycerine jelly and incubated as described in Díez et al. (2004). Four fields per sample were also observed, with a total magnification of 100× and viability was estimated as the proportion of pollen grains germinated referred to the total grains.

Three measurements per each S3-individual were made for qualitative traits, including plant, flower, and fruit traits. Regarding quantitative traits, three measurements were taken per plant for plant, flower, and seed descriptors; five measurements were made for leaf descriptors, and ten measurements were made for fruit descriptors. Leaf and fruit colour were measured using a Minolta CR-300 colorimeter (Minolta Corporation, Osaka, Japan) and expressed according to CIE L*a*b* 1976 space. Finally, 100 seeds weight was measured with an electronic balance PLJ 600-2GM (Kern & Sohn GmbH, Balingen, Germany).

Statistical analyses were performed using R software (R Core Team, 2015). Phenotypic data was removed of outliers and correlations between traits were calculated using the Pearson coefficient with statistical packages (Wickham, 2016). Moreover, mean, standard deviations and variation coefficient were estimated for each trait in the studied genotypes with Statgraphics Centurion XVII software (StatPoint Technologies, Warrenton, VA, USA).

Table 1. List of the traits considered, corresponding abbreviation, IPGRI descriptor number, and units/scale (if applicable).

Descriptor	Abbreviation	IPGRI Number	Units/Scale
Plant descriptors			
Stem colour	SC	7.1.2.2	1 = Green, 2 = Green with purple stripes, 3 = Purple
Stem length	SL	7.1.2.9	mm
Internode length	ILe	-	mm
Internode number	IN	-	-
Mature leaf length	LLe	7.1.2.18	mm
Mature leaf width	LWi	7.1.2.19	mm
Nodal anthocyanin	AN	7.1.2.3	1 = Green, 3 = Light purple, 5 = Purple, 7 = Dark purple
Stem pubescence	SP	7.1.2.5	1 = Sparce, 3 = Intermediate, 5 = Dense
Leaf density	LD	7.1.2.13	3 = Sparce, 5 = Intermediate, 5 = Dense
Leaf colour	LC	7.1.2.14	1 = Yellow, 2 = Light green, 3 = Green, 4 = Dark green 5 = Light purple, 6 = Purple, 7 = Variegated
Leaf colour lightness	LL	-	0 = Black to 100 = White

Leaf colour green/red value	LA	-	Negative = green to positive = red
Leaf colour blue/yellow value	LB	-	Negative = blue to positive = yellow
Leaf shape	LS	7.1.2.15	1 = Deltoid, 2 = Ovate, 3 = Lanceolate
Inflorescence/Flower descriptors			
Number of flowers per axil	NFA	7.2.1.2	-
Flower position	FP	7.2.1.3	3 = Pendant, 5 = Intermediate, 7 = Erect
Anther colour	AC	7.2.1.8	1 = White, 2 = Yellow, 3 = Pale blue, 4 = Blue, 5 = Purple
Filament colour	FC	7.2.1.10	1 = White, 2 = Yellow, 3 = Green, 4 = Blue, 5 = Light Purple, 6 = Purple
Stigma exsertion	SE	7.2.1.12	3 = Inserted, 5 = Same level, 7 = Exserted
Calyx margin	CM	7.2.1.15	1 = Enture, 2 = Intermediate, 3 = Dentate
Calyx annular constriction	CAC	7.2.1.16	0 = Absent, 1 = Present
Fruit descriptors			
Anthocyanin spots at intermediate stage	AS	7.2.2.2	0 = Absent, 1 = Present
Fruit colour at intermediate stage	FCI	-	3 = Light green, 5 = Green, 7 = Dark green
Fruit colour at mature stage	FCM	7.2.2.6	1 = White, 2 =Lemon-yellow, 3 = Pale orange-yellow, 4 = Orange-yellow, 5 = Pale orange, 6 = Orange, 7 = Light red, 8 = Red, 9 = Dark red, 10 = Purple, 11 = Brown, 12 = Black
Mature fruit colour lightness	FL	-	0 = Black to 100 = White
Mature fruit colour green/red value	FA	-	Negative = green to positive = red
Mature fruit colour blue/yellow value	FB	-	Negative = blue to positive = yellow
Fruit shape	FS	7.2.2.7	1 = Elongate, 2 = Almost round, 3 = Triangular, 4 = Campanulate, 5 = Blocky
Fruit length	FLe	7.2.2.8	mm
Fruit width	FWi	7.2.2.9	mm
Fruit weight	FW	7.2.2.10	g
Pedicel length	PL	7.2.2.11	mm
Fruit shape at pedicel attachment	FSPA	7.2.2.13	1 = Acute, 2 = Obtuse, 3 = Truncate, 4 = Cordate, 5 = Lobate
Neck at base of fruit	NB	7.2.2.14	0 = Absent, 1 = Present
Fruit shape at blossom-end	FSBE	7.2.2.15	1 = Pointed, 2 = Blunt, 3 = Sunken, 4 = Sunken and pointed
Fruit cross-sectional corrugation	FSC	7.2.2.17	3 = Slightly corrugated, 5 = Intermediate, 7 = Corrugated
Proximal fruit blockiness	PFB	-	-
Distal fruit blockiness	DFB	-	-
Fruit shape triangular	FST	-	-

Number of locules	NL	7.2.2.18	-
Pedicel with ripe fruit persistence	PP	7.2.2.20.1	3 = Slight, 5 = Intermediate, 7 = Persistent
Fruit wall consistency	FWC	-	0 = Absent, 1 = Present
Pollen/seed descriptors			
Morphological viability	PMV	-	%
Biochemical viability	PBV	-	%
Germinative viability	PGV	-	%
Weight of 100 seeds	SW	7.3.5	g

4.3 DNA extraction, sequencing, mapping and SNP calling

Leaf tissue samples were obtained from individual plants of a random selection of 200 S3-lines among the previously phenotyped lines, representative of the S3-MAGIC population, together with the founder lines, and F_1 and $F_1 \times F_1$ hybrids individual plants at the four leaves stage. DNA was extracted using the SILEX method (Vilanova et al., 2020). GBS library construction and sequencing was performed by The Elshire Group Ltd. performed the GBS library construction (Elshire et al., 2011) including the following changes: 100 ng of genomic DNA were used, 3.6 mg of total adapters, digestion with ApeKI enzyme and amplification with 18 PCR cycles. DNA libraries were sequenced with Illumina Novaseq 6000 platform using paired-end 150 bp reads. Kevin-Murray's AX-demux was used for de-multiplexing raw reads and removing barcodes (Murray & Borevitz, 2018). BWA-mem with default parameters but avoiding multiple-mapping reads (Li, 2013) was used to align de-multiplexed reads to the reference *C. annuum* genome CM334 (version 1.55) (Kim et al., 2014). BAM files were used for Single Nucleotide Polymorphism (SNP) calling and select minimum mapping quality SNPs ($Q > 30$) with GATK haplotype-caller 4.1.9 (Depristo et al., 2011). Afterwards, good quality SNPs (missing data < 15% and mean minimal read depth > 7) were retained with VCFtools (Danecek et al., 2011) for further analyses. Homozygosity was calculated for all the genotypes with VCFtools (Danecek et al., 2011). The distribution of the parental markers in the population and the parental contribution was calculated with R-package HaploBlocker (Pook et al., 2019).

4.4 Genetic diversity and structure analyses

For elucidating phylogenetic relationships within the MAGIC population, low-quality SNPs (missing data per site > 25% and MAF < 5%) were removed with bcftools (Li, 2011) and plink2 (Chang et al., 2015). To have an illustrative description of the genetic variability of the S3-population and to assess the possible occurrence of a cluster effect and the founder lines, a PCA was first performed with Rpackage SNPrelate (Zheng et al., 2012). Further, a phylogenetic tree and a kinship matrix were performed with IQ-TREE2 (Minh et al., 2020) and SNPrelate (Zheng et al., 2012), consecutively, to visualize genetic relationships and the genetic distribution of the S3-population. An admixture analysis was run with AdminPipe v2.0 (Mussmann, Douglas, Chafin, &

Douglas, 2020) to elucidate the level of genomic mixing of the parents in the MAGIC population, with the following parameters: number of subpopulations (*K)* ranging from 1 to 10, and each *K* run with 16 replicates submitted to Pong software (Behr et al., 2016) to elucidate the best statistical run.

4.5 Genome-wide association study (GWAS)

Phenotypic and genotypic data were used to perform a Genome-Wide Association Study (GWAS) with GAPIT (version 3) (Wang & Zhang, 2021). In the view of the number of individuals which constitute the S3-MAGIC population, only traits expected to be coded by few genes and previously described as highly inheritable, were chosen for the GWAS analysis. Genetic control and regulation of supposed more complex traits will be further studied in the next S5-S6 generations, when the population is expected to be fully fixed throughout the genome. For the association study, multiple loci mixed model (MLMM) and Bayesian-information and linkage-disequilibrium iteratively nested keyway (BLINK) were conducted and corrected with the Bonferroni and the false discovery rate (FDR) methods (Benjamini & Hochberg, 1995) at 0.05 significance level. PopLDDecay (Zhang et al., 2019) was used to determinate the linkage disequilibrium (LD) and haplotype structure. LD correlation coefficient (r^2) was used to stablish the intervals between significant independent SNPs with a limit value of 0.2 and distance of 5,000 kbp. Candidate genes were retrieved from the CM334 (version 1.55) reference genome (Kim et al., 2014) with BEDtools (Quinlan & Hall, 2010).

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data availability

The raw data analysed in this study are available in the Short Read Archive (SRA) of the National Library of Medicine repository under the accession number PRJNA1279249.

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Author contributions

Conceptualization, AR-B; Methodology, NO-A and MD-R; Software, LB; Validation, NO-A, AF and AR-B; Formal analysis, LG and LB; Investigation, NO-A and MD-R; Resources, AR-B; Data curation, LB; Writing—original draft, NO-A and MD-R; Writing—review and editing, AR-B; Visualization, NO-A and AR-B; Supervision, AF and AR-B; Project administration, AF and AR-B; Funding acquisition, AF and AR-B.

Supplementary materials

Supplementary materials are available at https://academic.oup.com/hr/advance-article/doi/10.1093/hr/uhaf182/8203318.

Supplementary figure 1. Population structure analysis for MAGIC population, including founder lines in red colour A: California wonder (*Capsicum annuum*), B: Ecu-994 (*C. chinense*), C: Chile Serrano (*C. annuum*), D: Ají dulce (*C. chinense*), E: Bola (*C. annuum*), F: Pasilla Bajío (*C. annuum*), G: Serrano Criollo de Morelos (*C. annuum*), H: Piquillo (*C. annuum*) (red), F_1 hybrids (green), $F_1 \times F_1$ hybrids (blue) and the 200 S3-individuals (orange) in A: maximum likelihood phylogenetic tree and B: kinship matrix.

Supplementary figure 2. LD decay (r^2) in Kb off all SNPs pairs related to the physical distance of pairs of SNPs.

Supplementary figure 3. QQ plots and Manhattan plots built with the BLINK and MLMM statistical models for stem colour, nodal anthocyanin, stem pubescence, filament colour, anthocyanin stripes, fruit colour at intermediate stage, fruit colour at mature stage, pedicel persistence with the fruit, and fruit wall consistency. Upper and lower horizontal lines represent, respectively, FDR significance threshold at $p = 0.01$ and $p = 0.05$.

Supplementary table 1. Qualitative trait values for founder lines A: California wonder (*Capsicum annuum*), B: Ají Dulce (*C. chinense*), C: Chile Serrano (*C. annuum*), D: Ecu-994 (*C. chinense*), E: Bola (*C. annuum*), F: Serrano Criollo de Morelos (*C. annuum*), G: Piquillo (*C. annuum*), H: Pasilla Bajío (*C. annuum*), F_1 and $F_1 \times F_1$ hybrids; and phenotype distribution observed in the S3 and S4 progenies.

Supplementary table 2. Quantitative trait values (mean ± standard deviation) for founder lines A: California wonder (*Capsicum annuum*), B: Ají Dulce (*C. chinense*), C: Chile Serrano (*C. annuum*), D: Ecu-994 (*C. chinense*), E: Bola (*C.* annuum), F: Serrano Criollo de Morelos (*C. annuum*), G: Piquillo (*C. annuum*), H: Pasilla Bajío (*C. annuum*), F_1, $F_1 \times F_1$ hybrids, and the S3 and S4 progenies; and coefficient of variation (CV %) for the later generations.

Supplementary table 3. Significant QTLs, chromosome, QTL start, QTL end, FDR adjusted value, statistical model, gene function, and metabolic pathway for stem colour, nodal anthocyanin, stem pubescence, filament colour, anthocyanin spot in the immature fruit, fruit colour at the immature stage, fruit colour at the mature stage, pedicel persistence, and fruit wall consistency.

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General Discussion

Solanaceae family is known for its great importance worldwide. Within solanaceous crops, eggplant and peppers have been studied for its great phenotypical and genotypical diversity, stablishing many breeding projects for countless goals. Historically, the main objectives of these programmes have been increasing productivity, obtaining resistance to biotic stresses and, more recently, tolerance to abiotic stresses. However, climate change might increase the challenges of crop improvement, demanding the exploration of uncharted genetic resources and the development of novel technologies. In this regard, remarkable advances have been made in the application of molecular tools to enhance our understanding of genetic basis of tolerance. Moreover, the creation of experimental populations has proved to be a valuable tool for understanding the basis of complex characters. Nevertheless, genetic control of many interesting traits is still unknown, requiring further investigation to be able to improve these traits in actual breeding projects.

The main objectives of this study were: i) assessment of the morphological, biochemical, and gene expression response to salinity of the cultivated and wild related species of eggplant; ii) evaluation of the effects of salt stress on the fruit quality of eggplant cultivated varieties; and iii) analysis of the diversity within a collection of *Capsicum annuum* landraces, and creation, development, and analysis of the first MAGIC population in *Capsicum* spp. The first goal corresponds to Chapter I, comprising the information obtained with *S. melongena,* its direct ancestor *S. insanum*, and their interspecific hybrid (*S. insanum* × *S. melongena*) in response to different levels of salt stress, measuring morphological and biochemical parameters. Moreover, a gene expression analysis is included in this chapter to compare the genetic and metabolic response to salt of these species. This analysis was carried out with the RNA-seq technology, discovering differences among the genotypes and associating them with the previously obtained biochemical results. Chapter I also includes the physiologic evaluation of *S. dasyphyllum* and its comparison with other CWRs under salt treatment. Chapter II displays the fruit composition of different *S. melongena* cultivars under long-term mid-stress salt conditions emphasising the potential of this crop for future agricultural systems. Chapter III discusses the existent phenotypic and genetic variability in a collection of pepper landraces adapted to an insular microclimate and the prospects of these materials evolving partially isolated from commercial varieties and under changing climatic conditions. Finally, Chapter III also presents the creation of a MAGIC population, built to maximize genome reorganization and to ensure a superlative diversity, analysed with phenotypic and genetic markers in its third and fourth autopollination generation and, more interestingly, used to perform the association of some traits with genetic regions through genome-wide association analysis (GWAS).

This research presents valuable knowledge regarding eggplant response to salinity and the existing differences found between cultivated eggplant and wild relatives, which might outcome as gene reservoirs for breeding in tolerance. Also, genetic diversity found in insular-adapted varieties constitutes a reservoir of alleles with potential relevance for adaptative responses. Finally, we present a novel MAGIC population in *Capsicum* that can be extensively used to provide previously unknown genetic information for future

breeding efforts. In conclusion, this work contributes with new available information for interesting agronomical traits and tolerance in commercial crops like eggplant and peppers, supporting future efforts in these species.

Evaluation of eggplant cultivated species and wild relatives for salinity tolerance

Plant materials adapted to diverse edaphoclimatic conditions are crucial for a more sustainable agriculture. Crop wild relatives (CWR) inhabit a wide range of different environments, and exhibit valuable performances for plant breeding, with unlimited potential for its direct use in agriculture (Davidar et al., 2015). Many populations have been built in eggplant incorporating CWRs with a cultivated background, such as advanced backcrosses (ABs), and introgression lines (ILs) (Prohens et al., 2017). These populations exploit the genetic variation embedded in CWRs and allows an easier development of improved varieties with especial attention to abiotic stresses. Also, the tolerance observed in many wild species, together with the increasing availability of genetic data can help in understanding the regulation pathways of important stresses.

The data presented in Chapter I suggest that each eggplant genotype responds to salinity with a specific modulation of its metabolism. All the species studied showed some tolerance to salinity, even in the case of cultivated *S. melongena*, indicating that this species is a good candidate for breeding ensuring their growth in more saline soils without losing fruit quality as mentioned in previous works (Brenes et al., 2020b; Plazas et al., 2016). In fact, Chapter II confirms that fruit quality is barely affected by salinity in eggplant cultivated varieties. Nevertheless, physiological responses analysed in *S. melongena, S. insanum,* and *S. dasyphyllum*, showed significant differences. Overall growth was reduced under salt stress for all genotypes, and they also seemed to accumulate antioxidant and protective metabolites, although the magnitude of each response was dependant on the genotype and the level of stress. These results agreed with the expression results, where *S. melongena* and *S. insanum* showed some common altered metabolic pathways, but each genotype had their own mechanism of response, with preferred routes to cope with the stress and unique regulations of these routes.

Moreover, a display of heterosis was observed in the interspecific hybrid *S. insanum* × *S. melongena* for phenotypical measurements like plant height or root length, and a similar behaviour of the hybrid with *S. insanum* for other physiological parameters. The expression study revealed shared responses in both the parental lines, and common responses between the hybrid and each one of the parental lines. The unique DEGs observed in the hybrid and absent in the parental lines, seem to be key in the imminent response to salt stress and could be related to the heterotic effect (Kumar et al., 2020). The applicability of these findings in hybrids of other CWRs with cultivated eggplants would broaden the knowledge about tolerance and its inheritance and enhance the applicability of CWRs into eggplant breeding.

The present results emphasise the role of proline accumulation and ion homeostasis as central mechanisms for salinity tolerance in eggplant. However, latest publications reveal the critical role of the Ascobate-Glutathione cycle in detoxifying ROS and mitigating oxidative stress under salt stress (Hasanuzzaman et al., 2021). Including this pathway in future evaluations would offer new information of the tolerance and adaptation mechanisms. Moreover, while treatments based on $NaCl^-$ salinity are easy to control and show high repetitiveness, the use of diluted seawater – comprising a more complex ion composition– would mimic better on-farm scenarios of stress enhancing ecological relevance of the study and applicability (Nissim et al., 2021).

Genetic diversity, importance for breeding and sustainable agriculture

Experimental populations and CWRs are arising as a powerful source of genetic variation for breeding efforts. However, in Chapter III we propose that cultivated landraces, maintained by farmers during centuries and constantly evolving together with the ecosystem can also be promising materials. They include new genetic background with interesting haplotypes, necessary to develop more resilient varieties. In this context, traditional varieties from the Balearic archipelago evaluated in Chapter III showed great phenotypic and genetic diversity both intra- and inter-variety. These results indicate the potential of these materials for widening peppers genetic background. Traditional cultivated varieties have been proposed as better plant starter materials for breeding compared to CWRs, avoiding some limitations of wild species like incompatibility barriers for hybridisation, fruit undesirable traits, lower production, prickliness, and lack of adaptation to cultivation systems (Manzur et al., 2015; Plazas et al., 2016; Rodríguez-Burruezo et al., 2009). Furthermore, although CWRs produce high contents of secondary metabolites which act as defence mechanisms, they have negative effects in the fruit flavour and overall quality (Luna-Ruiz, Nabhan, & Aguilar-Meléndez, 2018). Since landraces have been selected and improved by farmers during centuries for agronomic and fruit quality traits, using them as gene donors avoid detrimental effects. Finally, as they have been recollected in different local climatic conditions, growth in differential soils, with a wide range of horticultural practices and pathogens, they might host many interesting and useful genetic factors for breeding other materials facing the risks arising from climate change. Therefore, these materials are much more resilient to biotic and abiotic stresses and more prone to prevail under low input conditions (Fita et al., 2015).

On the matter, as Spain is a main centre of diversity for *C. annuum* due to the huge introduction of peppers from America, selection of pepper plants in different regions has led to a plethora of traditional varieties, landraces, ecotypes, and heirlooms, even representing some of the Spanish most relevant PDOs and PGIs (Gobierno de España, 2024; Rodríguez-Burruezo et al., 2016). These materials have been displaced by farmers in the last decades due to the massive cultivation of commercial hybrids. However, our results regarding Chapter III demonstrate that landraces still maintained in cultivation, such as those in the Balearic archipelago, can help partially mitigate the genetic erosion

seen in pepper commercial hybrids. This preservation is crucial to protecting future breeding potential and promoting a more sustainable agriculture.

Creating genetic diversity: MAGIC populations

Multi-parent experimental populations have been developed to create new recombinant genetic material and diversity for plant breeding projects (Huang et al., 2015). They combine a wide range of phenotypic and genetic variability of multiple founder lines, increasing recombination rate and reducing population structure (Arrones et al., 2020; Dell'Acqua et al., 2015; Scott et al., 2020). To our knowledge, this is the first MAGIC population developed in the world for *Capsicum.* Moreover, *C. chinense* was included as two founder lines to increased even more genetic variability of the offspring and introduce interesting traits of a different cultivated species. However, during the course of Chapter III, it was necessary to consider the complexity of genomes reorganization and incompatibly barriers. The inclusion of two different species in the initial intercross led to deleterious effects observed during the first crosses and the first two selfing generations. Even using the *C. chinense* genotypes as male parental to avoid maternal effect of this genotype, partial sterility occurred in the subsequent generations.

Nevertheless, after overcoming these initial difficulties, a collection of the MAGIC population, consisting of 200 individuals from each of the S3 and S4 generations was evaluated in Chapter III. Phenotypic data confirmed a high variability embedded in the population including new phenotypes and heterotic effect in some agronomical and fruit traits. Moreover, genetic markers confirmed a high degree of founder recombination, including the *C. chinense* genomes, and the potential of this population to find new candidate genes for interesting traits in peppers. This MAGIC population has proven to be valuable for dissecting the genetic basis of morphological and agronomic traits. In the following generations, a fine mapping of the QTLs found in this work might be performed to confirm the candidate genes and demonstrate the association using other biotechnological tools and plant transformation. Furthermore, because of its genetic recombination, this population could be tested for complex agronomical traits like yield and fruit quality traits like fruit pungency, volatile profile or antioxidant patterns. Also, additional investigation about tolerance to abiotic stresses in these eternal population can be of great interest for generating more resilient varieties used in a low input agriculture. Recent methodological advances allow GWAS to handle complex traits more effectively, taking into account the influence of environmental conditions on the expression of the genes (enviromics) and the influence of genes in multiple traits (pleiotropy) (Crossa et al., 2021; Guo, Li, & Wang, 2023; Jamil et al., 2020; Wolc & Dekkers, 2022). Applying these new approaches could improve the ability to detect genetic regions thereby enhancing the potential of the MAGIC population.

Advances in genetic and genomic resources

In the present work, the eggplant genome "67/3" was used for read alignment and mapping in the transcriptome analysis. Similarly, the CM334 genome was used in pepper for the GBS analysis. These reference genomes have been widely used as a standard; however, recent advances in genomics highlight the benefits of moving beyond a single-reference approach. For instance, pangenomes have already been developed in many Solanaceae species capturing structural and allelic variants. The construction of the tomato pangenome solved regions with incomplete linkage disequilibrium, revealed novel genes, and identified new alleles regulating fruit flavour (Gao et al., 2019; Zhou et al., 2022). Similarly, the eggplant pangenome enabled the identification of structural variants and orthologues corresponding to domestication traits (Barchi et al., 2021). In the case of *Capsicum,* pangenomes including both cultivated and wild species, uncovered significant genomic differences highlighting key regions involved in the evoluation and diversification of the genus (Liu et al., 2023).

Additionally, recent publications emphasise the relevance of newly developed super-pangenomes, which allowed the detection of diversity loss during domestication and provide a more comprehensive understanding of evolution and adaptation (He, Li, Qian, & Shang, 2025; Li et al., 2023). In future works, integrating the eggplant pangenome into transcriptomic analysis could enhance the depth and accuracy of genetic insights. Furthermore, the development of a pepper pangenome based on the founder lines of the MAGIC population would facilitate a more accurate genotype imputation and the discovery of novel alleles.

Concluding remarks and future perspectives

Materials and information captured in this thesis provide a basis for future genomic studies in eggplant and pepper. The evaluation of CWRs in eggplant, and landraces and the MAGIC population in peppers has demonstrated their potential as pre-breeding materials. We have also developed advanced materials that have broaden the genetic diversity of pepper. Moreover, identifying the genetic basis of important agronomic traits using the MAGIC pepper population can reduce the gap with other Solanaceae species with more developed tools like eggplant and tomato. Also, including two different species as founder lines in the MAGIC population has proved to be a good strategy to increase recombination and reduce population structure, key for searching QTLs and candidate genes associated to interesting traits. Our purpose with respect to the experimental population is performing a fine mapping in the detected QTL regions and select candidate genes for each interesting trait. In addition, the high homozygosity level calculated in the S3 generation indicate that next generations S5-S6 of the population can be characterise for a plethora of complex morpho-agronomic and fruit quality traits like yield, sugar content, and ascorbic acid (vitamin C) content and our goal is to identify genomic regions related to these traits.

In conclusion, this collection of eternal individuals is a promising material for evaluating their potential as donor of tolerance because of its diverse genetic background, aiming to find tolerance genetic markers in peppers for future uncertain climatic conditions. Altogether, the materials, knowledge and tools presented in this doctoral thesis will be of great relevance for the development of a new generation of eggplant and pepper commercial varieties adapted to climate change.

General Conclusions

1. The cultivated and wild relative eggplant species present different responses when salinity treatment is applied, indicating that evolution and adaptation to certain environments develop, in each genotype, a unique response to stress by activation and deactivation of metabolic pathways.

2. The interspecific *S. insanum* × *S. melongena* hybrid showed a different pattern regarding growth and production of antioxidant compounds compared to the parental lines, indicating the possibility of an unequal genetic recombination of both species that can generate heterosis in the progeny for salt tolerance.

3. Supporting these observations, gene expression analysis revealed that the molecular profile of the hybrid is closer to *S. melongena* than to the maternal line *S. insanum*, indicating the absence of maternal effects, but a trend in the progeny to resemble to the cultivated species of the intercross.

4. The morphological and physiological differences observed in the three genotypes correlated with the activation of specific expression pathways. Moreover, the specific biochemical responses observed in the hybrid agreed with the activation of genes involved in sugar metabolism to ensure plant nutrition despite the stress.

5. Altogether, wild relatives have proved to be very useful resources as gene reservoirs for establishing new breeding programmes improving salinity tolerance in eggplant.

6. Endemisms in pepper have shown great phenotypic diversity intra- and inter-variety, responding to the evolution and adaptation to the insular environments. These materials hold valuable haplotypes for broadening the genetic background of commercial varieties.

7. The first MAGIC population in *Capsicum* comprised by two different species, *C. annum* and *C. chinense,* has been developed and evaluated through the years, coming into de fifth generation of autopollination with huge prospects for genomic diversity analysis and gene discovery.

8. This population have major importance for three purposes: i) as a genetic resource with extreme variability among the individuals for developing and commercializing new pepper varieties; ii) as a biotechnological tool to understand genetic regulation of complex traits, and iii) to perform deep genome studies to perform gene discovery in interesting agronomical, quality, and tolerance traits.

9. High phenotypic variability was revealed in the third and fourth generation of autopollination. The genomic rearrangements have shortened the linkage disequilibrium, allowing the appearance of novel phenotypic combinations, including the appraising of new phenotypes in some qualitative traits and cases of heterosis in some quantitative traits.

10. With the GBS data obtained in this work, significant associations have been made between plant, flower, and fruit traits with genomic regions. Most of the traits showed several associated regions in different chromosomes, even traits previously considered simple or regulated by few genes, indicating a more complex genetic regulation than expected and the potential of this population.

11. The high homozygosity arisen in this third generation indicates that latter generations of this population can be used for fine mapping of genes associated to complex agronomical traits like yield or fruit quality. These traits might have more impact in the commercial production of peppers, being a great information for future breeding efforts in the industry.

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Supplementary Materials

Chapter II

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Supplementary table 1. Mineral correlations between fruit (f), root (r), and leaf (l). An asterisk indicates statistically significant differences with $p < 0.05$ and two asterisks indicates statistically significant differences with $p < 0.01$.

	Ca f	Fe f	K f	Mg f	Mn f	Na f	P f	Zn f	B l	Ca l	Fe l	K l	Mg l	Mn l	Na l	P l	Zn l
B f	0.54**	0.53**	0.47**	0.44**	0.49**	-0.08	-0.25	0.36*	-0.05	-0.2	-0.16	0.38*	-0.29	-0.33*	-0.24	0.32*	0.1
Ca f		0.62**	-0.01	0.67**	0.75**	0.19	-0.25	0.64**	-0.09	-0.25	0.12	0.3	-0.41*	-0.31*	-0.07	0.2*	0.26
Fe f			-0.02	0.41**	0.62**	0.32	-0.11	0.54**	-0.12	-0.26	0.2	0.33*	-0.35*	-0.32	-0.08	0.32	0.24
K f				0.62**	0.2	0.72**	0.01	0.22	-0.2	0	0.4**	0.26**	0.11	0.07	-0.17	0.22	0.19
Mg f					0.56**	0.18	0.03	0.33*	-0.23	-0.16	0.12	0.07	-0.34*	-0.38*	-0.02	0.09	0.53**
Mn f						0.12	0.12	0.53**	-0.08	0.21	0.18*	0.34*	-0.09	0.04	-0.14	0.32*	0.37*
Na f							0.06	0.31	-0.23	-0.14	0.33*	0.14	-0.05	-0.11	0.12	0.12	0.09
P f								0.01	0.15	0.42**	0.18	-0.16	0.59**	0.51**	-0.02	-0.1	0.34*
Zn f									0.06	-0.15	0.14	0.24	-0.23	-0.25	0.15	0	0.17
B l										0.6**	-0.34*	-0.46**	0.52**	0.4**	-0.1	-0.27	0.04
Ca l											-0.26	-0.3*	0.76**	0.74**	-0.26	-0.16	0.15
Fe l												0.29*	-0.06	-0.08	-0.1	0.3*	0.4**
K l													-0.23	0.01	-0.53**	0.54**	-0.02
Mg l														0.74**	-0.31*	-0.08	0.16
Mn l															-0.42**	-0.1	0.07
Na l																-0.21	-0.14
P l																	-0.02
Zn l																	
B r																	
Ca r																	
Fe r																	
K r																	
Mg r																	
Mn r																	
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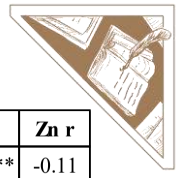
P r																	



	B r	Ca r	Fe r	K r	Mg r	Mn r	Na r	P r	Zn r
B f	0.46**	-0.11	0.02	0.16	0.25	0.14	-0.13	0.43**	-0.11
Ca f	0.22	-0.24	0.04	0.05	0.25	0.13	-0.09	0.33*	-0.13
Fe f	0.24	-0.26	0.01	0.27	0.29	-0.04	-0.24	0.15	0.01
K f	0.16	0.01	0.02	0.07	0.08	-0.15	0.21	-0.08	0.08
Mg f	0.09	-0.02	0.1	-0.04	0.2	0.28**	0.26	0.21	-0.2
Mn f	0.1	-0.05	-0.15	0.07	0.44**	-0.04	-0.06	0.37*	0.06
Na f	0.01	-0.14	0.13	-0.14	-0.04	-0.18	0.29	-0.27	0.16
P f	-0.37*	0.21	-0.02	-0.05	0.07	-0.07	0.21	-0.29	0.24
Zn f	0.21	-0.37*	-0.04	0.15	0.02	0.17	0.04	0.24	0.02
B l	0.1	0.01	-0.14	0.07	0.06	0.01	-0.08	-0.14	-0.13
Ca l	-0.03	0.21	-0.28*	-0.01	0.24	-0.28*	-0.06	-0.02	0.15
Fe l	-0.12	0.31*	0.14	-0.18	-0.07	0.02	-0.09	-0.07	0.41**
K l	0.23**	-0.1	-0.19	0.37**	0.17	-0.38**	-0.28*	0.44**	0.1
Mg l	-0.14	0.27*	-0.2	-0.02	0.09	-0.18	-0.03	-0.17	0.17
Mn l	-0.01	0.17	-0.25	0.15	0.35**	-0.33*	-0.27	0.05	0.18
Na l	-0.17	-0.15	0.2	-0.24	-0.32*	0.33**	0.49**	-0.27	-0.11
P l	-0.01	-0.06	-0.19	0.27*	0.15	-0.21	-0.13	0.18	0.05
Zn l	-0.01	0.2	-0.05	-0.1	0.06	-0.02	0.16	0.07	0.22
B r		0.16	0.26	0.43**	0.32*	-0.03	-0.18	0.62**	0.02
Ca r			0.24	-0.35*	0.11	0.16	-0.1	-0.07	0.47**
Fe r				-0.25	0	0.63**	0.04	0	-0.03
K r					0.54**	-0.38**	-0.3*	0.5**	-0.25
Mg r						-0.04	-0.38**	0.48**	-0.09
Mn r							0.23**	-0.08	-0.07
Na r								-0.21	-0.07
P r									0

Chapter III

Ortega-Albero, N., Barchi, L., Fita, A., Díaz, M., Martínez, F., Luna-Prohens, J. M., & Rodríguez-Burruezo, A. (2024). Genetic diversity, population structure, and phylogeny of insular Spanish pepper landraces (*Capsicum annuum* L.) through phenotyping and genotyping-by-sequencing. *Frontiers in Plant Science*, *15*, 1435427. doi.org/103389/fpls1435427

Supplementary table 1. List of studied accessions with corresponding code, genebank code, varietal type, and origin (I= Eivissa/Ibiza, M= Mallorca).

Acces	Code	Type	Ori	Acces	Code	Type	Ori
P1	PI-0152	Pebrera Blanca	I	P35	PI-0014	Citró de Matances	I
P2	PI-0153	Pebrera Blanca	I	P36	PI-0071	Citró de Matances	I
P3	PI-0155	Pebrera Blanca	I	P37	PI-0069	Citró de Matances	I
P4	PI-0156	Pebrera Blanca	I	P38	PI-0251	Citró de Matances	I
P5	PI-0157	Pebrera Blanca	I	P39	-	Citró de Matances	I
P6	PI-0159	Pebrera Blanca	I	P40	BGHZ5244	Citró de Matances	I
P7	PI-0160	Pebrera Blanca	I	P41	BGHZ3337	Citró de Matances	I
P8	PI-0161	Pebrera Blanca	I	P42	BGHZ5245/1	Citró de Matances	I
P9	PI-0162	Pebrera Blanca	I	P43	PI-0010/5	Citró de Matances	I
P10	PI-0163	Pebrera Blanca	I	P44	PI-0257	Citró de Matances	I
P11	PI-0164	Pebrera Blanca	I	P45	PI-0256	Citró de Matances	I
P12	PI-0165	Pebrera Blanca	I	P46	PI-0258	Citró de Matances	I
P13	PI-0166	Pebrera Blanca	I	P47	PI-0073	Citró de Matances	I
P14	PI-0167	Pebrera Blanca	I	P48	BGIB069*	Banya de cabra	M
P15	PI-0168	Pebrera Blanca	I	P49	BGIB086	Banya de cabra	M
P16	PI-0169	Pebrera Blanca	I	P50	BGIB085	Blau	M
P17	PI-0170	Pebrera Blanca	I	P51	BGIB166	Blau	M
P18	PI-0171	Pebrera Blanca	I	P52	BGIB229	Cirereta	M
P19	PI-0068	Pebrera Blanca	I	P53	BGIB137	D'envinagrar	M
P20	PI-0264	Pebrera Blanca	I	P54	BGIB068	Fulla d'olivera	M
P21	-	Pebrera Blanca	I	P55	BGIB152	Fulla d’olivera	M

P22	PI-0253	Pebrera Blanca	I	P56	BGIB160	Ros	M
P23	PI-0265	Pebrera Blanca	I	P57	BGIB222*	Ros	M
P24	PI-0263	Pebrera Blanca	I	P58	BGIB039	Ros gruixat	M
P25	PI-0250	Pebrera Blanca	I	P59	BGIB091	Ros gruixat	M
P26	PI-0249	Pebrera Blanca	I	P60	BGIB084*	Ros gruixat	M
P27	PI-0015/3	Citró de Matance:	I	P61	BGIB070	Ros prim	M
P28	PI0117/4A	Citró de Matance:	I	P62	BGIB087	Ros prim	M
P29	PI-0117/4B	Citró de Matance:	I	P63	BGIB195	Tap de cortí	M
P30	PI-0079A/2	Citró de Matance:	I	P64	BGIB022*	Tap de cortí	M
P31	PI-0208	Citró de Matance:	I	P65	BGIB110*	Tap de cortí	M
P32	-	Citró de Matance:	I	P66	BGIB128	Tap de cortí	M
P33	PI-0254	Citró de Matance:	I	P67	BGIB136	Tap de cortí	M
P34	PI-0255	Citró de Matance:	I				

Supplementary table 2. Phenotyping values (mean ± standard deviation, in traits where several measurements were possible) for the quantitative traits analysed in all the selected accessions and reference varieties. NA indicates missing data.

Accession	SL[1]	FL	FA	FB	FLE	FWI	FW	NL	PMV	PB)	SW
Pebrera Blanca											
P4	115	NA	NA	NA	NA	NA	NA	NA	80	80	0.82
P6	5	34.72 ± 1.3	35.66 ± 2.6	19.89 ± 3.7	127 ± 8	57 ± 6	179.54 ± 37.4	3.2 ± 0.4	64	64	0.69
P13	225	NA	NA	NA	NA	NA	NA	NA	72	72	0.64
P23	27	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.73
P25	17	36.89 ± 1.6	39.92 ± 2.9	24.99 ± 3.7	147 ± 18	43 ± 3	113.32 ± 20.3	3 ± 0.7	67	67	0.62
P26	NA	34.27 ± 1.7	33.68 ± 4	21.09 ± 2.9	118 ± 7	63 ± 12	162.13 ± 32.8	3.4 ± 0.5	85	85	0.76
Citró de matances											
P27	30	33.82 ± 1.9	35.43 ± 1.9	16.61 ± 2.4	110 ± 20	31 ± 2	32.1 ± 9.2	2.8 ± 0.8	NA	NA	0.84
P29	195	34.5 ± 1.7	34.54 ± 2.8	17.06 ± 2.3	78 ± 10	2.98 ± 2	27.99 ± 4.4	2	76	75	0.65
P32	235	32.83 ± 0.8	33.64 ± 3	14.89 ± 2.9	109 ± 27	3.38 ± 4	29.85 ± 13.9	2.2 ± 0.4	90	89	0.99
P33	19	33.04 ± 0.7	33.82 ± 2	15.28 ± 2.4	137 ± 9	3.02 ± 2	46.51 ± 9.7	2.6 ± 0.5	62	45	0.69
P34	22	34.23 ± 2.4	33.27 ± 3.1	16.8 ± 4.2	96 ± 8	3.6 ± 4	34.86 ± 15.1	2.6 ± 0.5	66	49	0.71
P42	285	34.49 ± 2.6	39.12 ± 2.2	21.93 ± 2.8	132 ± 11	3.04 ± 5	45.3 ± 20.6	3.2 ± 0.4	NA	NA	0.46
P44	555	38.99 ± 0.9	41.39 ± 1.5	21.17 ± 2.3	53 ± 3	1.54 ± 1	4.94 ± 0.5	2.2 ± 0.4	88	86	0.48
P45	57	41.26 ± 3	44.5 ± 1.2	25.9 ± 2.8	46 ± 4	10 ± 1	2.13 ± 0.3	2	NA	NA	0.44
P46	375	39.73 ± 1.2	43.24 ± 1.4	23.58 ± 2.5	19 ± 2	8 ± 1	0.63 ± 0.1	2.2 ± 0.4	61	61	0.28
Banya de Cabra											
X1	39	34.97 ± 2.3	37.37 ± 3.1	18.52 ± 3.2	107 ± 40	29 ± 4	30.85 ± 9	3.2 ± 0.4	78	78	0.69
P48	7	31.88 ± 2.2	31.32 ± 2.7	14 ± 3	191 ± 19	37 ± 4	65.94 ± 24.9	3.4 ± 0.5	NA	NA	0.67
P49	295	33.91 ± 3.3	33.66 ± 3.9	16.39 ± 3.1	163 ± 33	38 ± 4	62.26 ± 17.8	3.2 ± 0.4	86	86	0.79
Blau											
X2	38	NA	NA	NA	NA	NA	NA	NA	89	87	NA
P50	39	NA	NA	NA	NA	NA	NA	NA	16	16	NA
P51	345	NA	NA	NA	NA	NA	NA	NA	79	79	0.52
Cirereta											
P52	255	35.1 ± 1.7	40.16 ± 1.4	18.06 ± 1.5	102 ± 20	17 ± 4	12.79 ± 5.6	2.2 ± 0.4	72	72	0.42
D'Envinagrar											
X3	375	41.92 ± 1.9	39.93 ± 2.6	27.26 ± 3.6	79 ± 10	2.46 ± 2	14.82 ± 1.3	2.8 ± 0.4	NA	NA	0.42
P53	615	43.54 ± 2	44.99 ± 1.4	36.32 ± 3.4	91 ± 3	1.94 ± 2	12.76 ± 2.5	2.6 ± 0.5	78	78	0.57

[1] SL: stem length (mm), FL: fruit colour lightness, FA: fruit colour green/red, FB: fruit colour blue/yellow, FLE: fruit length (mm), FWI: fruit width (mm), FW: fruit weight (g), NL: number of locules, PMV: pollen morphological viability (%), PBV: pollen biological viability (%), SW: seed weight (g).

Accession	SL[1]	FL	FA	FB	FLE	FWI	FW	NL	PMV	PB)	SW
Fulla d'Olivera											
X4	23	40.22 ± 1.7	41.11 ± 2	22.45 ± 3.4	84 ± 16	14 ± 1	6.6 ± 2.5	2	82	81	0.52
X5	205	36.88 ± 1.1	38.97 ± 2.2	17.48 ± 2.5	101 ± 1.	14 ± 2	7.69 ± 2.5	2.8 ± 0.4	89	89	0.54
P54	20	31.57 ± 1.2	38.77 ± 1.3	16.53 ± 1.5	121 ± 0.5	18 ± 2	17.75 ± 3.5	2.4 ± 0.5	71	70	0.39
Ros											
X6	20	35.75 ± 2	36.07 ± 2.7	18.43 ± 2.7	103 ± 1.3	44 ± 6	84.14 ± 14.6	3.6 ± 0.5	94	94	0.68
P56	195	35.25 ± 2.2	38.78 ± 4.1	21.53 ± 3.7	123 ± 1.1	46 ± 3	96.62 ± 11.4	3.2 ± 0.8	82	82	0.6
P57	8.5	35.5 ± 1.2	39.82 ± 3.6	21.64 ± 4	152 ± 3.2	47 ± 8	132.09 ± 40	3 ± 0.7	82	82	0.62
Ros Gruixat											
X7	20	NA	NA	NA	NA	NA	NA	NA	68	67	0.71
P58	285	35.82 ± 2.1	37.59 ± 4.7	21.84 ± 4.9	148 ± 21	43 ±8	119.96 ± 28.7	2.6 ± 0.5	76	76	0.56
P59	NA	NA	NA	NA	NA	NA	NA	NA	62	59	0.49
P60	19	37.17 ± 3.7	39.63 ± 3.5	23.58 ± 5.4	140 ± 16	45	109.35 ± 22.8	2.4 ± 0.5	69	68	0.76
Ros Prim											
P61	215	37.61 ± 2.3	39.12 ± 3.1	22.38 ± 3.6	142 ± 12	44 ± 3	112.4 ± 8.9	2.8 ± 0.4	58	56	0.62
P62	22	33.88 ± 1.3	39.75 ± 2.2	22.7 ± 3.6	143 ± 29	48 ± 2	123.73 ± 30.4	3	43	43	0.69
Tap de Cortí											
P63	17	NA	NA	NA	NA	NA	NA	NA	83	82	NA
P64	16	32.66 ± 2.7	32.54 ± 2.3	14.15 ± 2.4	54 ± 5	2.68 ± 1	17.17 ± 1.5	2.8 ± 0.4	NA	NA	0.55
P65	205	32.93 ± 1.1	32.1 ± 2.4	12.88 ± 2.2	65 ± 5	2.86 ± 2	24.66 ± 1.7	2.4 ± 0.5	75	75	0.67
P66	18	33.78 ± 2	31.95 ± 2.6	14.12 ± 1.2	61 ± 2	2.9 ± 1	23.48 ± 2.2	2.6 ± 0.5	NA	NA	0.49
P67	205	33.84 ± 0.8	33.34 ± 1.8	14.83 ± 1.4	65 ± 3	2.84 ± 3	21.93 ± 4	2.4 ± 0.5	86	86	0.51
Reference accessions											
California Wonder	245	59.12 ± 3.3	-0.08 ± 2.5	56.24 ± 3	72 ± 4	62 ± 4	93.15 ± 8.1	3	7	5	0.78
Chile Serrano	85	41.93 ± 2.4	40.5 ± 1.1	26.95 ± 4	36 ± 4	13 ± 1	3.24 ± 1	2.4 ± 0.5	79	78	0.6
Bola	435	36.5 ± 1.3	35.59 ± 2.7	18.86 ± 1.5	33 ± 2	27 ± 3	14.61 ± 5.4	2.6 ± 0.5	39	26	0.7
Pasilla Bajío	485	28.03 ± 0.8	3.12 ± 1.1	3.73 ± 0.8	155 ± 12	29 ± 2	27.06 ± 7.7	2.2 ± 0.4	68	66	1.29
Serrano Criollo de Morelos	23	39.9 ± 2.6	39.83 ± 1.7	23.65 ± 2.4	101 ± 9	2 ± 1	16.52 ± 4.3	2.2 ± 0.4	88	83	0.74
Piquillo	25	36.13 ± 1.9	34.63 ± 3.5	17.2 ± 2.9	71 ± 9	41 ± 3	41.8 ± 9.3	2.6 ± 0.9	81	76	0.77

[1] SL: stem length (mm), FL: fruit colour lightness, FA: fruit colour green/red, FB: fruit colour blue/yellow, FLE: fruit length (mm), FWI: fruit width (mm), FW: fruit weight (g), NL: number of locules, PMV: pollen morphological viability (%), PBV: pollen biological viability (%), SW: seed weight (g).

Supplementary table 3. Observed (O) and expected (E) number of heterozygous sites, fixation index (F), and estimated heterozygosity values (Het, %) for all the studied accessions.

Accession	O	E	F	Het (%)
Pebrera Blanca				
PB_P1	6,588	72,475	0.91	0.55
PB_P2	9,127	72,463	0.87	0.76
PB_P3	8,898	72,479	0.88	0.74
PB_P4	9,067	72,465	0.87	0.76
PB_P5	7,470	72,482	0.90	0.62
PB_P6	11,611	72,473	0.84	0.97
PB_P7	8,942	72,463	0.88	0.75
PB_P8	8,603	72,477	0.88	0.72
PB_P9	8,708	72,477	0.88	0.73
PB_P10	8,128	72,470	0.89	0.68
PB_P11	9,620	72,473	0.87	0.80
PB_P12	7,696	72,472	0.89	0.64
PB_P13	10,927	72,486	0.85	0.91
PB_P14	7,096	72,485	0.90	0.59
PB_P15	8,139	72,472	0.89	0.68
PB_P16	7,507	72,481	0.90	0.63
PB_P17	6,605	72,497	0.91	0.55
PB_P18	11,201	72,448	0.85	0.94
PB_P19	12,753	72,461	0.82	1.07
PB_P20	11,766	72,468	0.84	0.98
PB_P21	9,144	72,476	0.87	0.76
PB_P22	11,697	72,469	0.84	0.98
PB_P23	13,603	72,453	0.81	1.14
PB_P24	11,225	72,471	0.85	0.94
PB_P25	9,775	72,470	0.87	0.82
PB_P26	12,747	72,459	0.82	1.06
Citró de Matances				
CdM_P27	11,445	72,465	0.84	0.96
CdM_P28	10,722	72,466	0.85	0.90
CdM_P29	10,067	72,470	0.86	0.84
CdM_P30	13,320	72,462	0.82	1.11
CdM_P31	9,012	72,479	0.88	0.75

Accession	O	E	F	Het (%)
Citró de Matances				
CdM_P38	7,649	72,498	0.89	0.64
CdM_P39	5,786	72,492	0.92	0.48
CdM_P41	4,917	72,500	0.93	0.41
CdM_P42	6,772	72,486	0.91	0.57
CdM_P43	4,584	72,503	0.94	0.38
CdM_P44	5,272	72,497	0.93	0.44
CdM_P45	5,779	72,492	0.92	0.48
CdM_P46	8,373	72,497	0.88	0.70
CdM_P47	4,798	72,499	0.93	0.40
Banya de Cabra				
BdC_P48	9,032	72,478	0.88	0.72
BdC_P49	10,097	72,478	0.86	1.03
Blau				
B_P50	9,247	72,472	0.87	0.84
B_P51	8,985	72,478	0.88	0.77
Cirereta				
C_P52	7,588	72,488	0.90	0.82
D'Envinagrar				
DE_P53	9,865	72,482	0.86	1.95
Fulla d'Olivera				
FO_P54	6,699	72,491	0.91	0.69
FO_P55	8,594	72,479	0.88	0.86
Ros				
R_P56	8,640	72,467	0.88	0.75
R_P57	10,812	72,468	0.85	0.74
Ros Gruixat				
RG_P58	12,384	72,474	0.83	0.75
RG_P59	9,157	72,483	0.87	0.77
RG_P60	9,166	72,471	0.87	0.90
Ros Prim				
RP_P61	9,179	72,471	0.87	0.72
RP_P62	8,824	72,472	0.88	0.76

CdM_P32	7,499	72,494	0.90	0.63
CdM_P33	6,807	72,486	0.91	0.57
CdM_P34	6,750	72,484	0.91	0.56
CdM_P35	8,971	72,491	0.88	0.75
CdM_P36	10,831	72,483	0.85	0.90
CdM_P37	6,108	72,496	0.92	0.51

Tap de Cortí				
TdC_P63	8,215	72,485	0.89	0.63
TdC_P64	10,288	72,474	0.86	0.56
TdC_P65	23,380	72,469	0.68	0.77
TdC_P66	11,042	72,479	0.85	0.92
TdC_P67	7,768	72,487	0.89	0.65

Ortega-Albero, N., Díaz-Riquelme, M., Gaccione, L., Barchi, L., Fita, A., & Rodríguez-Burruezo, A. (2025). First interspecific multi-parent advanced generation intercross (MAGIC) population in *Capsicum* peppers: development, phenotypic evaluation, genomic analysis, and prospects. *Horticulture Research*, *182,* doi.org/10.1093/hr/uhaf182

Supplementary table 1. Qualitative trait values for founder lines A: California wonder (*Capsicum annuum*), B: Ají Dulce (*C. chinense*), C: Chile Serrano (*C. annuum*), D: Ecu-994 (*C. chinense*), E: Bola (*C. annuum*), F: Serrano Criollo de Morelos (*C. annuum*), G: Piquillo (*C. annuum*), H: Pasilla Bajío (*C. annuum*), F_1 and $F_1 \times F_1$ hybrids; and phenotype distribution observed in the S3 and S4 progenies.

Trait abbreviation	Founder lines							
	A	B	C	D	E	F	G	H
SC[1]	Green	Green	Green	Green	Green	Green	Green	Green
AN	Light purple	Green	Light purple	Green	Purple	Light purple	Purple	Light purple
SP	Sparce	Sparce	Intermediate	Sparce	Sparce	Dense	Sparce	Sparce
LD	Dense	Intermediate	Intermediate	Dense	Intermediate	Dense	Sparce	Sparce
LC	Light green	Light green	Dark green	Green	Green	Green	Green	Green
LS	Deltoid	Ovate	Lanceolate	Lanceolate	Ovate	Lanceolate	Ovate	Lanceolate
NFA	1	2	1	2	1	1	1	1
FP	Pendant	Intermediate	Pendant	Intermediate	Pendant	Erect	Intermediate	Pendant
AC	Pale blue	Purple	Purple	Purple	Blue	Pale blue	Pale blue	Purple
FC	White	Purple	White	Purple	White	White	White	White
SE	Inserted	Exserted	Exserted	Same level	Inserted	Exserted	Same level	Exserted
CM	Intermediate	Enture	Dentate	Enture	Intermediate	Dentate	Enture	Dentate
CAC	Present	Present	Absent	Present	Absent	Absent	Present	Absent
AS	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
FCI	Light green	Green	Dark green	Light green	Green	Light green	Green	Dark green
FCM	Lemon-yellow	Light red	Red	Red	Red	Red	Dark red	Brown
FS	Blocky	Disc	Elongated	Triangular	Blocky	Elongated	Triangular	Elongated
FSPA	Lobate	Cordate	Acute	Cordate	Cordate	Cordate	Lobate	Acute
NB	Absent	Absent	Present	Absent	Absent	Absent	Absent	Absent
FSBE	Sunken	Blunt	Blunt	Pointed	Blunt	Pointed	Sunken	Pointed
FSC	Intermediate	Slightly corrugated	Slightly corrug.	Slightly corrug.	Slightly corrug.	Slightly corrug.	Slightly corrug.	Intermediate
PP	Persistent	Slight	Slight	Intermediate	Persistent	Intermediate	Persistent	Intermediate
FWC	Present	Present	Absent	Present	Present	Present	Present	Present

[1] SC: stem colour, AN: nodal anthocyanin, SP: stem pubescence, LD: leaf density, LC: leaf colour, LS: leaf shape, NFA: number of flowers per axil, FP: flower position, AC: anther colour, FC: filament colour, SE: stigma exsertion, CM: calyx margin, CAC: calyx annular constriction, AS: anthocyaninic spots, FCI: fruit colour intermediate, FCM: fruit colour mature, FS: fruit shape, FSPA: fruit shape pedicel attachment, NB: neck at fruit base, FSBE: fruit shape at blossom end, FSC: , fruit cross-section corrugation, PP: pedicel persistence, FWC: fruit wall consistency.

Trait abbreviation	Offsprings							
	A × B	C × D	E × F	G × H	AB × EF	CD × GH	S3 progeny	S4 progeny
SC	Green	Green	Green	Green	Green	Green	Green (83%)	Green (89%)
							Green with purple stripes (12%)	Green with purple stripes (7%)
							Purple (5%)	Purple (4%)
AN	Green	Purple	Light purple	Light purple	Green	Green	Green (26%)	Green (32%)
							Light purple (32%)	Light purple (26%)
							Purple (41%)	Purple (42%)
SP	Sparce	Intermediate	Intermediate	Sparce	Sparce	Sparce	Sparce (74%)	Sparce (67%)
							Intermediate (23%)	Intermediate (26%)
							Dense (3%)	Dense (7%)
LD	Intermediate	Intermediate	Dense	Intermediate	Intermediate	Intermediate	Sparce (17%)	Sparce (20%)
							Intermediate (55%)	Intermediate (59%)
							Dense (27%)	Dense (21%)
LC	Light green	Green	Green	Dark green	Green	Dark green	Light green (18%)	Light green (14%)
							Green (62%)	Green (65%)
							Dark green (19%)	Dark green (21%)
LS	Lanceolate	Lanceolate	Lanceolate	Ovate	Ovate	Ovate	Deltoid (17%)	Deltoid (14%)
							Ovate (41%)	Ovate (58%)
							Lanceolate (42%)	Lanceolate (28%)
NFA	1	1	1	1	1	1	1 (80%)	1 (85%)
							2 (20%)	2 (15%)
FP	Erect	Intermediate	Pendant	Pendant	Intermediate	Pendant	Pendant (35%)	Pendant (27%)
							Intermediate (50%)	Intermediate (43%)
							Erect (15%)	Erect (20%)
AC	Purple	Purple	Blue	Pale blue	Pale blue	Purple	Pale blue (25%)	Pale blue (22%)
							Blue (34%)	Blue (48%)
							Purple (41%)	Purple (30%)
FC	Purple	Purple	White	White	White	Light purple	White (80%)	White (80%)
							Yellow (1%)	Yellow (0%)
							Blue (1%)	Blue (1%)
							Light purple (6%)	Light purple (9%)
							Purple (13%)	Purple (10%)

Trait abbreviation	Offsprings							
	A × B	C × D	E × F	G × H	AB × EF	CD × GH	S3 progeny	S4 progeny
SE	Exserted	Exserted	Same level	Exserted	Same level	Exserted	Inserted (5%)	Inserted (4%)
							Same level (30%)	Same level (22%)
							Exserted (66%)	Exserted (72%)
CM	Intermediate	Intermediate	Intermediate	Dentate	Intermediate	Intermediate	Enture (21%)	Enture (12%)
							Intermediate (69%)	Intermediate (73%)
							Dentate (10%)	Dentate (15%)
CAC	Absent	Present	Absent	Present	Present	Present	Absent (78%)	Absent (82%)
							Present (22%)	Present (18%)
AS	Absent	Absent	Absent	Absent	Absent	Absent	Absent (98%)	Absent (95%)
							Present (2%)	Present (5%)
FCI	Green	Green	Light green	Dark green	Light green	Light green	Light green (13%)	Light green (15%)
							Green (59%)	Green (58%)
							Dark green (28%)	Dark green (27%)
FCM	Red	Red	Red	Dark red	Red	Red	Lemon-yellow (1%)	Lemon-yellow (1%)
							Pale orange-yellow (2%)	Pale orange-yellow (3%)
							Orange-yellow (3%)	Orange-yellow (2%)
							Pale orange (2%)	Pale orange (1%)
							Light red (5%)	Light red (5%)
							Red (63%)	Red (70%)
							Dark red (16%)	Dark red (12%)
							Purple (1%)	Purple (0%)
							Brown (3%)	Brown (4%)
							Black (3%)	Black (2%)

Trait abbreviation	Offsprings							
	A × B	C × D	E × F	G × H	AB × EF	CD × GH	S3 progeny	S4 progeny
FS	Triangular	Triangular	Triangular	Triangular	Elongate	Blocky	Elongate (20%) Almost round (8%) Triangular (27%) Blocky (46%)	Elongate (17%) Almost round (11%) Triangular (40%) Blocky (32%)
FSPA	Truncate	Acute	Cordate	Cordate	Cordate	Cordate	Acute (17%) Truncate (25%) Cordate (51%) Lobate (7%)	Acute (23%) Truncate (31%) Cordate (41%) Lobate (5%)
NB	Absent	Absent	Absent	Absent	Absent	Absent	Absent (99%) Present (1%)	Absent (99%) Present (1%)
FSBE	Blunt (50%) Sunken (50%)	Sunken	Blunt	Pointed	Blunt	Sunken	Pointed (12%) Blunt (43%) Sunken (45%)	Pointed (12%) Blunt (32%) Sunken (56%)
FSC	Slightly corrug (70%) Intermediate (20%) Corrugated (10%)	Slightly corrug	Slightly corrug	Slightly corrug (80%) Intermediate (20%)	Slightly corrug (20%) Intermediate (50%) Corrugated (30%)	Slightly corrug (60%) Intermediate (40%)	Slightly corrug (63%) Intermediate (27%) Corrugated (10%)	Slightly corrug (57%) Intermediate (37%) Corrugated (10%)
PP	Intermediate	Intermediate	Persistent	Persistent	Intermediate	Slight	Slight (20%) Intermediate (63%) Persistent (16%)	Slight (18%) Intermediate (56%) Persistent (32%)
FWC	Present	Present	Present	Present	Present	Absent	Absent (18%) Present (82%)	Absent (22%) Present (78%)

[1] SC: stem colour, AN: nodal anthocyanin, SP: stem pubescence, LD: leaf density, LC: leaf colour, LS: leaf shape, NFA: number of flowers per axil, FP: flower position, AC: anther colour, FC: filament colour, SE: stigma exsertion, CM: calyx margin, CAC: calyx annular constriction, AS: anthocyaninic spots, FCI: fruit colour intermediate, FCM: fruit colour mature, FS: fruit shape, FSPA: fruit shape pedicel attachment, NB: neck at fruit base, FSBE: fruit shape at blossom end, FSC: , fruit cross-section corrugation, PP: pedicel persistence, FWC: fruit wall consistency.

Supplementary table 2. Quantitative trait values (mean ± standard deviation) for founder lines A: California wonder (*Capsicum annuum*), B: Ají Dulce (*C. chinense*), C: Chile Serrano (*C. annuum*), D: Ecu-994 (*C. chinense*), E: Bola (*C. annuum*), F: Serrano Criollo de Morelos (*C. annuum*), G: Piquillo (*C. annuum*), H: Pasilla Bajío (*C. annuum*), F_1, $F_1 \times F_1$ hybrids, and the S3 and S4 progenies; and coefficient of variation (CV %) for the later generations.

	Founder lines							
Trait abbreviation	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
SL[1]	238.3 ± 25	570 ± 72	853.3 ± 100	380 ± 35	441.7 ± 7	272.5 ± 60	275 ± 47	395 ± 78
ILe	80 ± 21	138.8 ± 19	100 ± 21	95 ± 3	68.8 ± 5	103.8 ± 33	92.5 ± 10	100 ± 28
IN	12.5 ± 0.7	9.5 ± 0.7	17.5 ± 6	15.5 ± 5	13 ± 1	13 ± 2	10 ± 1	11.5 ± 2
LLe	158.5 ± 19	134.3 ± 10	81.5 ± 9	77 ± 5	134.3 ± 14	119.8 ± 13	121.5 ± 9	122.5 ± 8
LWi	79 ± 6	75.5 ± 7	32 ± 3	45.7 ± 4	76.3 ± 6	38 ± 3	67 ± 5	57.3 ± 7
LL	37.5 ± 1	38.9 ± 1	32.9 ± 2	34.5 ± 1	34.4 ± 0.5	32.1 ± 0.5	33.5 ± 1	30.6 ± 1
LA	-14.7 ± 1	-16.3 ± 1	-10.2 ± 1	-12.5 ± 0.4	-11.8 ± 1	-10.4 ± 1	-11.6 ± 1	-8.8 ± 2
LB	17.3 ± 1	21.5 ± 1	13.1 ± 2	14.1 ± 0.5	14.8 ± 1	13.9 ± 1	15.5 ± 2	11.5 ± 2
FL	61.2 ± 2	42.4 ± 2	41.5 ± 2	41.1 ± 2	36.5 ± 1	39.8 ± 2	35.2 ± 2	27.7 ± 1
FA	0.5 ± 3	38.4 ± 2	40.3 ± 1	39.9 ± 2	35.5 ± 2	38.8 ± 3	32.7 ± 5	3.1 ± 1
FB	56 ± 3	27.3 ± 3	25.9 ± 4	26.9 ± 3	18.3 ± 2	23.5 ± 3	15.9 ± 3	3.5 ± 1
FLe	71.3 ± 9	15.3 ± 2	36 ± 4	55.2 ± 3	32.5 ± 4	96.4 ± 11	77.1 ± 9	132.4 ± 33
FWi	63.6 ± 2	26.3 ± 4	12.6 ± 1	16.6 ± 3	26.4 ± 2	19.7 ± 2	38.7 ± 5	26.3 ± 4
FW	124 ± 20	3.8 ± 1	3.2 ± 1	5.9 ± 1	12.4 ± 4	15.4 ± 4	39.1 ± 12	20.4 ± 11
PL	41 ± 5	29 ± 3	35 ± 05	27 ± 3	34 ± 5	47 ± 10	55 ± 10	59 ± 5
PFB	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1 ± 0.1	0.9 ± 0.1
DFB	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.5 ± 0.01	0.9 ± 0.01	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
FST	1.3 ± 0.2	1.5 ± 0.1	1.1 ± 0.2	2.5 ± 0.4	1.4 ± 0.1	1.6 ± 0.2	1.4 ± 0.4	1.3 ± 0.1
NL	3.4 ± 0.5	2.5 ± 0.5	2.3 ± 0.5	2.9 ± 0.3	2.7 ± 0.5	2.3 ± 0.5	2.5 ± 0.7	2.2 ± 0.4
PMV	0.76 ± 0.05	0.41 ± 0.01	0.59 ± 0.01	0.31 ± 0.01	0.69 ± 0.01	0.68 ± 0.04	0.78 ± 0.03	1.3 ± 0.01
PBV	93.9 ± 1	94.1 ± 2	65.2 ± 9	22.7 ± 14	90.5 ± 7	90.8 ± 5	76.2 ± 17	88.1 ± 2
PGV	93.8 ± 1	94.1 ± 2	64.2 ± 8	22.5 ± 13	90.4 ± 8	88.2 ± 6	76.2 ± 16	88.1 ± 2
SW	23.7 ± 5	29.3 ± 8	55.4 ± 18	9.6 ± 13	43.8 ± 5	40.9 ± 12	9 ± 3	15.3 ± 7

[1] SL: stem length (mm), ILe: internode length (mm), IN: internode number, LLe: leaf length (mm), LWi: leaf width (mm), LL: leaf colour lightness, LA: leaf colour green/red, LB: leaf colour blue/yellow, FL: fruit colour lightness, FA: fruit colour green/red, FB: fruit colour blue/yellow, FLe: fruit length (mm), FWi: fruit width (mm), FW: fruit weight (g), PL: pedicel length (mm), PFB: proximal fruit blockiness, DFB: distal fruit blockiness, FST: fruit shape triangle, NL: number of locules, PMV: pollen morphological viability (%), PBV: pollen biological viability (%), SW: seed weight (g).

Trait abbreviation	Offsprings									
	A × B	C × D	E × F	G × H	AB × EF	CD × GH	S3 progeny		S4 progeny	
							Mean	CV	Mean	CV
SL	510 ± 158	820 ± 210	455 ± 87	410 ± 62	310 ± 42	395 ± 96	391.7 ± 167.8	42.8	370.2 ± 178.8	48.2
ILe	82.5 ± 25	167.5 ± 45	107.5 ± 33	117.5 ± 55	90 ± 23	185 ± 44	111.2 ± 64.5	57.9		
IN	18 ± 4	5 ± 2	16 ± 5	13 ± 4	15 ± 4	12 ± 4	15.8 ± 4.7	30.1		
LLe	148.5 ± 6	119.8 ± 4	125.8 ± 8	135.5 ± 6	131 ± 4	113.3 ± 12	109.1 ± 18.7	17.1		
LWi	73.8 ± 4	59 ± 3	62 ± 4	73.5 ± 4	60.5 ± 5	65.5 ± 5	56.3 ± 10.9	19.3		
LL	38.7 ± 0.5	33.9 ± 0.5	33.4 ± 0.5	29.3 ± 1	35 ± 1	30.6 ± 1	33.5 ± 2.9	8.6		
LA	-16.1 ± 0.3	-12.9 ± 1	-11.9 ± 1	-7.6 ± 1	-13.1 ± 1	-7.8 ± 1	-11.5 ± 2.7	23		
LB	20.4 ± 0.5	15.8 ± 1	15.3 ± 1	9.5 ± 2	17.7 ± 1	10.2 ± 1	14.7 ± 3.7	25.4		
FL	40.2 ± 2	40.6 ± 3	41.8 ± 3	36.7 ± 2	40.9 ± 2	40.2 ± 2	0.4 ± 7.9	19.7	37.4 ± 5.6	15
FA	35.8 ± 3	35.8 ± 3	39.2 ± 3	36.5 ± 3	37.5 ± 2	40.5 ± 4	33.9 ± 11.6	34.2	34.9 ± 8.4	23.8
FB	22.9 ± 4	27.1 ± 4	28.3 ± 5	19 ± 2	23.4 ± 3	26.4 ± 4	24.6 ± 13.4	54.6	21.8 ± 9.3	42.6
FLe	40 ± 8	35.2 ± 3	44.5 ± 11	104.1 ± 19	37.6 ± 5	36 ± 4	37.5 ± 15.8	42.2	35.8 ± 16.5	46.1
FWi	24.9 ± 4	12.4 ± 1	21.3 ± 5	26.9 ± 4	23.2 ± 2	17 ± 2	20.6 ± 6	29	20.3 ± 6.1	29.8
FW	9.4 ± 4	2.4 ± 0.6	7.7 ± 1	21.9 ± 6	8.8 ± 2	4 ± 1	7.6 ± 7.7	101.9	6.9 ± 9.1	132.9
PL	28 ± 4	32 ± 3	34 ± 5	64 ± 9	45 ± 5	34 ± 5	33 ± 12	36.3		
PFB	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	11		
DFB	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	7.9		
FST	1.4 ± 0.1	1.3 ± 0.1	1.6 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	11.5		
NL	2.5 ± 0.5	2.9 ± 0.3	2 ± 0	2 ± 0	2.8 ± 0.5	2.7 ± 0.5	2.6 ± 0.4	17.6	2.7 ± 0.5	18
PMV	0.4 ± 0.01	0.4 ± 0.01	0.7 ± 0.06	0.9 ± 0.03	0.6 ± 0.02	0.6 ± 0.01	0.7 ± 0.1	21	0.6 ± 0.2	28.5
PBV	6.7 ± 1	5.4 ± 0.9	85.2 ± 12	54.2 ± 9	53.4 ± 10	1 ± 0.01	40.5 ± 26.5	65.5	41.5 ± 28.7	69
PGV	5.4 ± 1	5.4 ± 0.8	84 ± 21	46.8 ± 13	51.8 ± 10	1 ± 0.01	33.2 ± 27.5	82.7	38.6 ± 29.5	76.3
SW	0 ± 0.1	0 ± 0.1	6.3 ± 1	3 ± 0.5	23.7 ± 4	0 ± 0.1	11.4 ± 14.5	126.8	6.4 ± 10.1	156.2

[1] SL: stem length (mm), ILe: internode length (mm), IN: internode number, LLe: leaf length (mm), LWi: leaf width (mm), LL: leaf colour lightness, LA: leaf colour green/red, LB: leaf colour blue/yellow, FL: fruit colour lightness, FA: fruit colour green/red, FB: fruit colour blue/yellow, FLe: fruit length (mm), FWi: fruit width (mm), FW: fruit weight (g), PL: pedicel length (mm), PFB: proximal fruit blockiness, DFB: distal fruit blockiness, FST: fruit shape triangle, NL: number of locules, PMV: pollen morphological viability (%), PBV: pollen biological viability (%), SW: seed weight (g).

Supplementary table 3. Significant QTLs, chromosome, QTL start, QTL end, FDR adjusted value, statistical model (B=Blink, M=MLMM), gene function, and metabolic pathway for stem colour, nodal anthocyanin, stem pubescence, filament colour, anthocyanin spot in the immature fruit, fruit colour at the immature stage, fruit colour at the mature stage, pedicel persistence, and fruit wall consistency.

Chr	QTL start	QTL end	FDR-val	Model	Gene function	Metabolic pathway
Stem colour						
10	200409946	200809947	4.44E-04	B	CPRD49 GDSL esterase/lipase	Lipid metabolism
10	200409946	200809947	4.44E-04	B	DEX1 Protein Defective in exine formation 1	Pollen development
10	200409946	200809947	4.44E-04	B	OPS Protein Octopus	Root and vascular development
Nodal anthocyanin						
10	91708986	92108987	5.29E-04	B	HSP17.5-M 17.5 kDa class I heat shock protein	Response to thermic shock
10	91708986	92108987	5.29E-04	B	PME46 pectinesterase/pectinesterase inhibitor 46	Root development
10	185672100	186072101	4.68E-03	M	RPA1C Replication protein A 70 kDa DNA-binding subunit C	DNA replication
10	185672100	186072101	4.68E-03	M	SBT6.1 Subtilisin-like protease SBT6.1	Cell development
10	185672100	186072101	4.68E-03	M	HEMH Ferrochelatase-2, chloroplastic	Hydrogen production
10	185672100	186072101	4.68E-03	M	CYCB2-4 Cyclin-B2-4	Cell development
10	185672100	186072101	4.68E-03	M	PBL7 serine/threonine-protein kinase	Protein modification
10	185672100	186072101	4.68E-03	M	RPL4A 60S ribosomal protein L4-1	Protein synthesis
10	185672100	186072101	4.68E-03	M	APRR7 Two-component response regulator-like	Response to temperature/circadian rhythm
10	185672100	186072101	4.68E-03	M	BTB/POZ domain-containing protein	Response to temperature/circadian rhythm
10	185672100	186072101	4.68E-03	M	TERC Thylakoid membrane protein, chloroplastic	Cell energy system
Stem pubescence						
02	144824842	145224843	4.27E-02	B	CYS4 Cysteine proteinase inhibitor 4	Response to general stress and general defence
02	144824842	145224843	4.27E-02	B	KIC Calcium-binding protein	Trichome morphogenesis
02	144824842	145224843	4.27E-02	B	Suberization-associated anionic peroxidase	General defence
02	144824842	145224843	4.27E-02	B	FAD-OXR Berberine bridge enzyme-like 22	Oxidative reactions

02	144824842	145224843	4.27E-02	B	Berberine bridge enzyme-like 23	Oxidative reactions
02	144824842	145224843	4.27E-02	B	G-type lectin S-receptor-like serine/threonine-protein kinase	Protein modification
02	144824842	145224843	4.27E-02	B	Pentatricopeptide repeat-containing protein	RNA modification process
10	232972943	233321800	4.19E-07	M	NSE4A	Chromosome integrity
10	232972943	233321800	4.19E-07	M	MOD1 Enoyl reductase, chloroplastic	Lipid metabolism
10	232972943	233321800	4.19E-07	M	Zinc finger CCCH domain-containing protein 14	Response to general stress
10	232972943	233321800	4.19E-07	M	GMPM1 18 kDa seed maturation protein	Response to abiotic stress
10	232972943	233321800	4.19E-07	M	LEA46 Late embryogenesis abundant protein 46	Response to abiotic stress
10	232972943	233321800	4.19E-07	M	R1B-17 Late blight resistance protein homolog	Pathogen defence
10	232972943	233321800	4.19E-07	M	R1A-3 Late blight resistance protein homolog	Pathogen defence
10	232972943	233321800	4.19E-07	M	Germin-like protein 2-2	Plant and seed development
10	232972943	233321800	4.19E-07	M	Disease resistance protein	General defence
10	232972943	233321800	4.19E-07	M	R1B-23 Late blight resistance protein homolog	Pathogen defence
10	232972943	233321800	4.19E-07	M	Germin-like protein 2-3	Plant and seed development
10	232972943	233321800	4.19E-07	M	R1A-10 Late blight resistance protein homolog	Pathogen defence
10	232972943	233321800	4.19E-07	M	Disease resistance protein	General defence
10	232972943	233321800	4.19E-07	M	PUB4 U-box domain-containing protein 4	Protein degradation and ROS scavenging
10	232972943	233321800	4.19E-07	M	Disease resistance protein	General defence
10	232972943	233321800	4.19E-07	M	Disease resistance protein	General defence
10	232972943	233321800	4.19E-07	M	Phosphatase 2C 43	Glucose metabolism
10	232972943	233321800	4.19E-07	M	RLP24 Ribosome biogenesis protein	Ribosome biogenesis
10	232972943	233321800	4.19E-07	M	LE25 Protein	Ion scavenger
Filament colour						
10	194182140	194582141	3.60E-04	M	ELC Protein	Cell development
10	194182140	194582141	3.60E-04	M	D7OMT Isoflavone 7-O-methyltransferase	Formononetin biosynthesis
10	194182140	194582141	3.60E-04	M	FBL11 BTB/POZ domain-containing protein	Phenol accumulation
10	194182140	194582141	3.60E-04	M	CTR1 Serine/threonine-protein kinase	Ethylene signalling
10	194182140	194582141	3.60E-04	M	HEMA1 Glutamyl-tRNA reductase 1, chloroplastic	Photosynthetic metabolism
10	194182140	194582141	3.60E-04	M	IQM3 IQ domain-containing protein	Root development
10	194182140	194582141	3.60E-04	M	FBL11 BTB/POZ domain-containing protein	Phenol accumulation

Anthocyanin spots

04	219650264	220050265	6.22E-05	B	R1A Late blight resistance protein	Pathogen defence
06	5264024	5664025	2.20E-02	B	R1B-14 Late blight resistance protein homolog	Pathogen defence
06	5264024	5664025	2.20E-02	B	R1B-13 Late blight resistance protein homolog	Pathogen defence
06	5264024	5664025	2.20E-02	B	R1A-4 Late blight resistance protein homolog	Pathogen defence
06	5264024	5664025	2.20E-02	B	R1B-8 Late blight resistance protein homolog	Pathogen defence
06	5264024	5664025	2.20E-02	B	R1A-3 Late blight resistance protein homolog	Pathogen defence
06	5264024	5664025	2.20E-02	B	R1A-10 Late blight resistance protein homolog	Pathogen defence
06	5264024	5664025	2.20E-02	B	COX2 Cytoome c oxidase subunit 2	Cell energy system

Fruit colour at intermediate stage

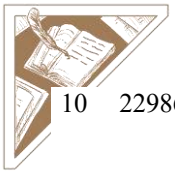
10	7699760	8099761	1.17E-04	B/M	LSH10 Protein light-dependent short hypocotyls 10	Glucosinolates biosynthesis
10	7699760	8099761	1.17E-04	B/M	PAB1 Proteasome subunit alpha type-2-A	Protein degradation
10	7699760	8099761	1.17E-04	B/M	MENG 2-phytyl-1,4-beta-naphthoquinone methyltransferase, chloroplastic	DNA modification
10	7699760	8099761	1.17E-04	B/M	TBL39 Protein trichome birefringence-like 39	Cell development
10	7699760	8099761	1.17E-04	B/M	OST3B dolichyl-diphosphooligosaccharide glycosyltransferase	Protein modification
10	7699760	8099761	1.17E-04	B/M	PSL4 Glucosidase 2 subunit beta	Response to microorganisms
10	7699760	8099761	1.17E-04	B/M	SD25 G-type lectin S-receptor-like serine/threonine kinase	Protein modification
10	7699760	8099761	1.17E-04	B/M	14-3-3-like protein B	Signalling
10	7699760	8099761	1.17E-04	B/M	GTE4 Transcription factor G	Transcription factor
10	7699760	8099761	1.17E-04	B/M	ACX3 Acyl-coenzyme A oxidase 3, peroxisomal	Lipid metabolism
10	20556647	20956648	6.96E-03	B	Endo-1,3;1,4-beta-D-glucanase	Abiotic stress response

Fruit colour at mature stage

01	4945937	5345938	1.38E-07	B	GT16 Xyloglucan-specific galacturonosyltransferase 1	Root development
01	4945937	5345938	1.38E-07	B/M	GSTT1 Glutathione S-transferase T1	Protein degradation
01	4945937	5345938	1.38E-07	B/M	HAL3A Phosphopantothenoylcysteine decarboxylase	Lipid metabolism

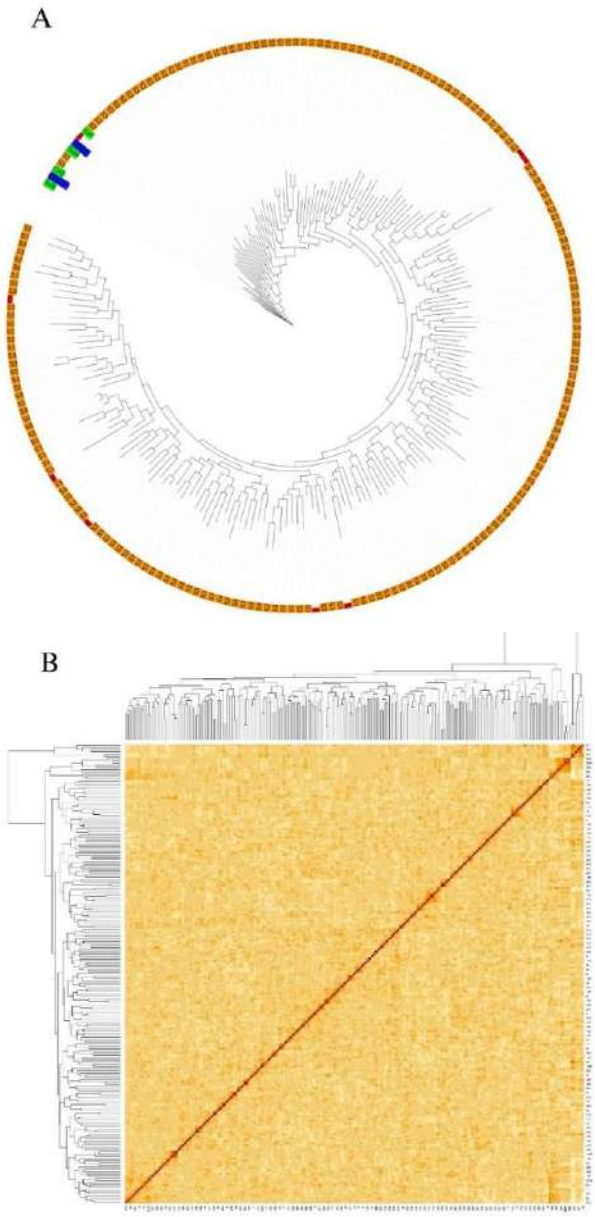
01	4945937	5345938	1.38E-07	B/M	RALFL5 Protein RALF-like 5	Hormone response and signalling
01	4945937	5345938	1.38E-07	B/M	Ethylene-responsive proteinase inhibitor 1	Fruit development and ripening
01	4945937	5345938	1.38E-07	B/M	OSML81 Osmotin-like protein	Abiotic stress response
01	4945937	5345938	1.38E-07	B/M	OSML13 Osmotin-like protein	Abiotic stress response
01	4945937	5345938	2.48E-03	B/M	OSML15 Osmotin-like protein	Abiotic stress response
01	4945937	5345938	2.48E-03	B/M	ERG3 Elicitor-responsive protein 3	Abiotic stress response
01	4945937	5345938	2.48E-03	B/M	MBD6 Methyl-CpG-binding domain-containing protein 6	Transcription factor
04	229216238	229616239	2.38E-03	B/M	CYP72A219 Cytoome P450	Cell energy system
04	229216238	229616239	2.38E-03	B/M	F-box protein	Transcription factor
04	229216238	229616239	2.38E-03	B/M	PCMP-E91 Pentatricopeptide repeat-containing protein	RNA modification process
04	229216238	229616239	2.38E-03	B/M	TPS7, alpha-trehalose-phosphate synthase 7	Glucose metabolism
04	229216238	229616239	2.38E-03	B/M	SCPL19 Serine carboxypeptidase-like 19	Protein degradation
04	229216238	229616239	2.38E-03	B/M	SCPL52 serine carboxypeptidase-like 52	Protein degradation
04	229216238	229616239	1.49E-02	M	SCPL17 Serine carboxypeptidase-like 17	Protein degradation
06	232345051	232745052	2.09E-08	B	MOCS2 Molybdopterin synthase catalytic subunit	Plant growth
06	232345051	232745052	2.09E-08	B	CDKC-1 Cyclin-dependent kinase C-1	Plant growth
06	232345051	232745052	2.09E-08	B	GAMYB Transcription factor	Flower and fruit development
06	232345051	232745052	2.09E-08	B	F-box/WD-40 repeat-containing protein	Transcription factor
06	232345051	232745052	2.09E-08	B	LTPG2 Non-specific lipid transfer protein GPI-anchored 2	Lipid metabolism
06	232345051	232745052	2.09E-08	B	SE Serrate RNA effector molecule	RNA modification process
06	232345051	232745052	2.09E-08	B	SRM1 Transcription factor SRM1	Transcription factor
06	232345051	232745052	2.09E-08	B	LSM8 Sm-like protein LSM8	RNA modification process
06	232345051	232745052	2.09E-08	B	Glucan endo-1,3-beta-glucosidase 14	Seed development
06	232345051	232745052	2.09E-08	B	EIN3 Protein ethylene insensitive 3	Signalling
06	232345051	232745052	2.09E-08	B	Xyl2 Beta-xylosidase/alpha-L-arabinofuranosidase 2	Protein degradation
06	232345051	232745052	2.09E-08	B	SMC3 Structural maintenance of omosomes protein 3	omosome structure
06	232345051	232745052	2.09E-08	B	FRS3 Protein far-1 related sequence 3	Plant growth

06	232345051	232745052	2.09E-08	B	S13-6 G2/mitotic-specific cyclin	Cell development
06	232929162	233329163	1.81E-10	B	BLH1 BEL1-like homeodomain protein 1	Embryo development
06	232929162	233329163	1.81E-10	B	PUB24 E3 ubiquitin-protein ligase	Response to microorganisms
06	232929162	233329163	1.81E-10	B	NTL8 NAC domain-containing protein 40	Response to general stress
06	232929162	233329163	1.81E-10	B	CYP89A9 Cytoome P450 89A9	Cell energy system
06	232929162	233329163	1.81E-10	B	PSAO Photosystem I subunit O	Cell energy system
06	232929162	233329163	1.81E-10	B	mRNA-decapping enzyme-like protein	Epidermal morphology
06	232929162	233329163	1.81E-10	B	CCS Capsanthin/capsorubin synthase, omoplastic	Carotenoid biosynthesis
06	232929162	233329163	1.81E-10	B	VAMP714 Vesicle-associated membrane protein 714	Hormone response and signalling
06	232929162	233329163	1.81E-10	B	FAF3 Protein fantastic four 3	Plant growth
06	232929162	233329163	1.81E-10	B	BAD1 Ankyrin repeat-containing protein	Plant growth
06	232929162	233329163	1.81E-10	B	FDX3 Ferredoxin-3, chloroplastic	Cell energy system
06	232929162	233329163	1.81E-10	B	Cytoome b-c1 complex subunit 9	Cell energy system
06	232929162	233329163	1.81E-10	B	FRI Protein frigida	Flower development and reproduction
06	232929162	233329163	1.81E-10	B	TGA9 Transcription factor	Flower development and reproduction
06	232929162	233329163	1.81E-10	B	LRX4 Leucine-rich repeat extensin-like protein 4	Cell wall development
Pedicel persistence						
10	228910007	229310008	3.86E-02	M	CYP96A15 Alkane hydroxylase MAH1	Cell energy system
10	228910007	229310008	3.86E-02	M	Miraculin	General defence
10	228910007	229310008	3.86E-02	M	PIP5K4 Phosphatidylinositol 4-phosphate 5-kinase	Pollen development
10	228910007	229310008	3.86E-02	M	Thioredoxin-like 1-1, chloroplastic	Cell energy system
10	228910007	229310008	3.86E-02	M	TGA-2.1 TGACG-sequence-specific DNA-binding protein	Carotenoid biosynthesis
10	228910007	229310008	3.86E-02	M	SMG7 Protein	Meiosis
10	228910007	229310008	3.86E-02	M	HD16 Casein kinase 1-like protein	Flower development and reproduction
10	229862715	230262716	1.83E-05	B	PYM Protein	Cell division
10	229862715	230262716	1.83E-05	B	SAP8 Zinc finger A20; AN1 domain-containing stress-associated protein 8	Response to general stress
10	229862715	230262716	1.83E-05	B	NEK6 Serine/threonine-protein kinase	Epidermal cell differentiation
10	229862715	230262716	1.83E-05	B	Vestitone reductase	Antioxidant isoflavone

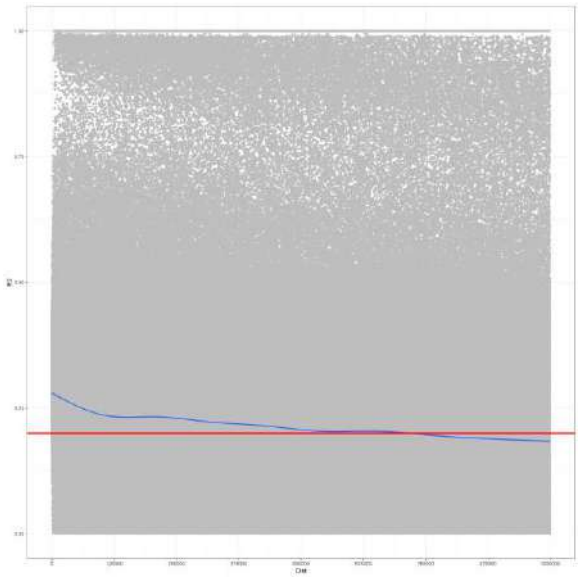


10	229862715	230262716	1.83E-05	B	CRP1 Pentatricopeptide repeat-containing, chloroplastic	Cell and plant development
10	229862715	230262716	1.83E-05	B	DREB2F Dehydration-responsive element-binding protein	Transcription factor
10	229862715	230262716	1.83E-05	B	Zinc finger CCCH domain-containing protein 30	RNA modification process
10	229862715	230262716	1.83E-05	B	REM4.2 Remorin 4.2	Response to abiotic stress
10	229862715	230262716	1.83E-05	B	SAP11 Zinc finger AN1; C2H2 domain-containing stress-associated protein 11	Response to general stress
10	229862715	230262716	1.83E-05	B	SMT1 Cycloartenol-C-24-methyltransferase	Lipid metabolism
10	229862715	230262716	1.83E-05	B	RRP45A Exosome complex component	RNA modification process
10	229862715	230262716	1.83E-05	B	TGA10 bZIP transcription factor	Response to abiotic stress
10	229862715	230262716	1.83E-05	B	DAO 2-oxoglutarate-dependent dioxygenase	Lipid metabolism
10	229862715	230262716	1.83E-05	B	NRPA1 DNA-directed RNA polymerase I subunit 1	DNA replication
10	229862715	230262716	1.83E-05	B	ZIP4 Zinc transporter 4, chloroplastic	Zinc absorption
10	229862715	230262716	1.83E-05	B	PCMP-H51 Pentatricopeptide -containing protein, chloroplastic	RNA modification process
10	229862715	230262716	1.83E-05	B	Polygalacturonase	Fruit development and ripening
Fruit wall consistency						
05	1634149	2034150	4.44E-02	M	G-type lectin S-receptor-like serine/threonine-protein kinase	Protein modification
05	1634149	2034150	4.44E-02	M	PGR3 Pentatricopeptide repeat-containing protein, chloroplastic/mitochondrial	RNA modification process
05	1634149	2034150	4.44E-02	M	MTACP2 Acyl carrier protein 2, mitochondrial	Lipid metabolism
05	1634149	2034150	4.44E-02	M	PGLP2 Phosphoglycolate phosphatase 2	Glucose metabolism
05	1634149	2034150	4.44E-02	M	EMB2654 Pentatricopeptide repeat-containing protein	RNA modification process
05	1634149	2034150	4.44E-02	M	CHX28 Cation/H(+) antiporter 28	Pollen development
05	1634149	2034150	4.44E-02	M	HSFB3 Heat stress transcription factor B-3	Response to abiotic stress
05	1634149	2034150	4.44E-02	M	CCMB cytoome c biogenesis, mitochondrial	Cell energy system
05	1634149	2034150	4.44E-02	M	RPL2 60S ribosomal protein L2, mitochondrial	DNA modification
05	1634149	2034150	4.44E-02	M	PIP5K1 Phosphatidylinositol 4-phosphate 5-kinase 1	Lipid metabolism

05	1634149	2034150	4.44E-02	M	VLN2 Villin-2	Cell development
05	1634149	2034150	4.44E-02	M	ABCA1 ABC transporter A family member 1	Lipid metabolism
10	229862715	230262716	2.24E-02	B/M	PYM Protein	Cell division
10	229862715	230262716	2.24E-02	B/M	SAP8 Zinc finger A20; AN1 domain-containing stress-associated protein 8	Response to general stress
10	229862715	230262716	2.24E-02	B/M	NEK6 Serine/threonine-protein kinase	Epidermal cell differentiation
10	229862715	230262716	2.24E-02	B/M	Vestitone reductase	Antioxidant isoflavone
10	229862715	230262716	2.24E-02	B/M	CRP1 Pentatricopeptide repeat-containing protein, chloroplastic	Cell development
10	229862715	230262716	2.24E-02	B/M	DREB2F Dehydration-responsive element-binding protein 2F	Transcription factor
10	229862715	230262716	2.24E-02	B/M	Zinc finger CCCH domain-containing protein 30	RNA modification process
10	229862715	230262716	2.24E-02	B/M	REM4.2 Remorin 4.2	Response to abiotic stress
10	229862715	230262716	2.24E-02	B/M	SAP11 Zinc finger AN1; C2H2 domain-containing stress-associated protein 11	Response to general stress
10	229862715	230262716	2.24E-02	B/M	SMT1 Cycloartenol-C-24-methyltransferase	Lipid metabolism
10	229862715	230262716	2.24E-02	B/M	RRP45A Exosome complex component	RNA modification process
10	229862715	230262716	2.24E-02	B/M	TGA10 bZIP transcription factor	Response to microorganisms
10	229862715	230262716	2.24E-02	B/M	DAO 2-oxoglutarate-dependent dioxygenase	Lipid metabolism
10	229862715	230262716	2.24E-02	B/M	NRPA1 DNA-directed RNA polymerase I subunit 1	DNA replication
10	229862715	230262716	2.24E-02	B/M	ZIP4 Zinc transporter 4, chloroplastic	Zinc absorption
10	229862715	230262716	2.24E-02	B/M	PCMP-H51 Pentatricopeptide repeat-containing protein, chloropl/mitochon	RNA modification process
10	229862715	230262716	2.24E-02	B/M	Polygalacturonase	Fruit development and ripening
12	249642713	250042714	3.10E-02	B	matK Maturase K	RNA modification process
12	249642713	250042714	3.10E-02	B	FAD12 Delta(12)-acyl-lipid-desaturase	Lipid metabolism
12	249642713	250042714	3.10E-02	B	Short-chain type dehydrogenase/reductase	Lipid metabolism
12	249642713	250042714	3.10E-02	B	PDR1 Pleiotropic drug resistance protein 1	Transcription factor
12	249642713	250042714	3.10E-02	B	AOP1.2 2-oxoglutarate-dependent dioxygenase	Glucosinolates biosynthesis
12	249642713	250042714	3.10E-02	B	AOP3 2-oxoglutarate-dependent dioxygenase	Glucosinolates biosynthesis



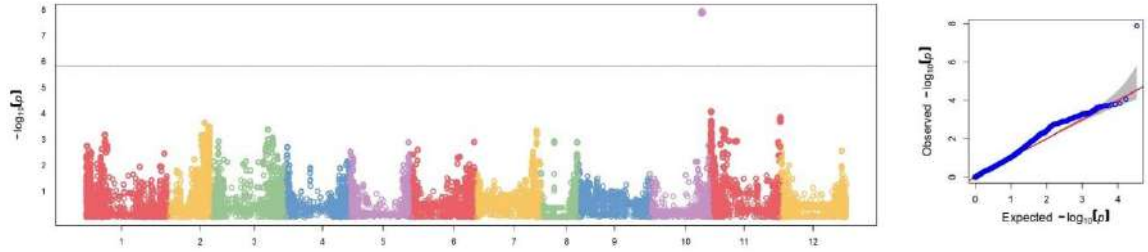
Supplementary figure 1. Population structure analysis for MAGIC population, including founder lines in red colour A: California wonder (*Capsicum annuum*), B: Ecu-994 (*C. chinense*), C: Chile Serrano (*C. annuum*), D: Ají dulce (*C. chinense*), E: Bola (*C. annuum*), F: Pasilla Bajío (*C. annuum*), G: Serrano Criollo de Morelos (*C. annuum*), H: Piquillo (*C. annuum*) (red), F_1 hybrids (green), $F_1 \times F_1$ hybrids (blue) and the 200 S3-individuals (orange) in A: maximum likelihood phylogenetic tree and B: kinship matrix.



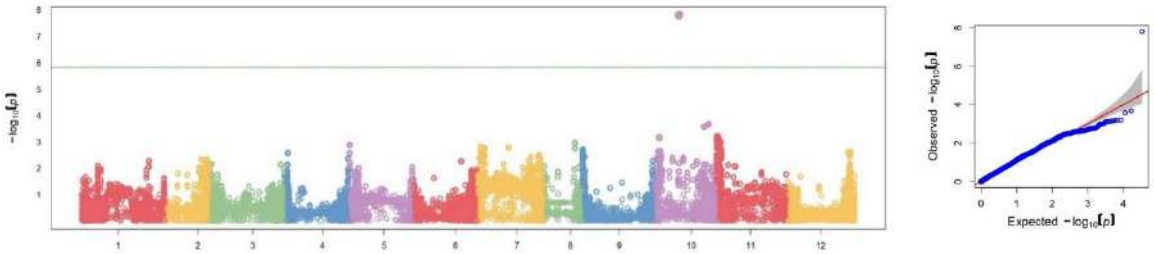
Supplementary figure 2. LD decay (r^2) in Kb off all SNPs pairs related to the physical distance of pairs of SNPs.

Supplementary figure 3. QQ plots and Manhattan plots built with the BLINK and MLMM statistical models for stem colour, nodal anthocyanin, stem pubescence, filament colour, anthocyanin stripes, fruit colour at intermediate stage, fruit colour at mature stage, pedicel persistence with the fruit, and fruit wall consistency. Horizontal lines in the Manhattan plots represent FDR significance threshold at $p = 0.05$.

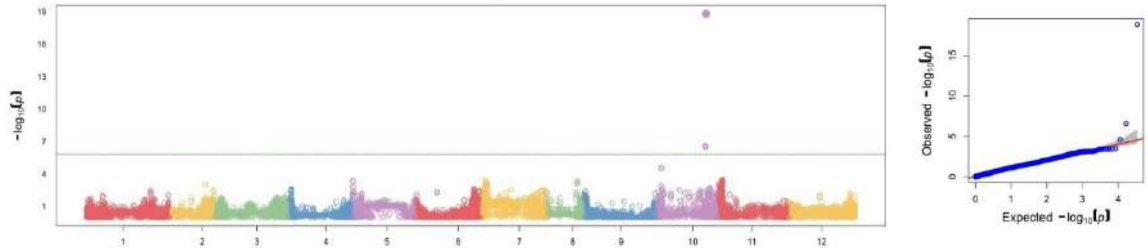
STEM COLOUR BLINK



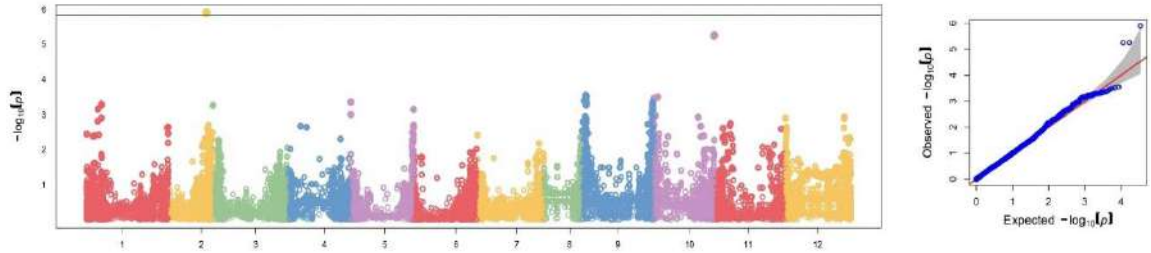
NODAL ANTHOCYANIN BLINK



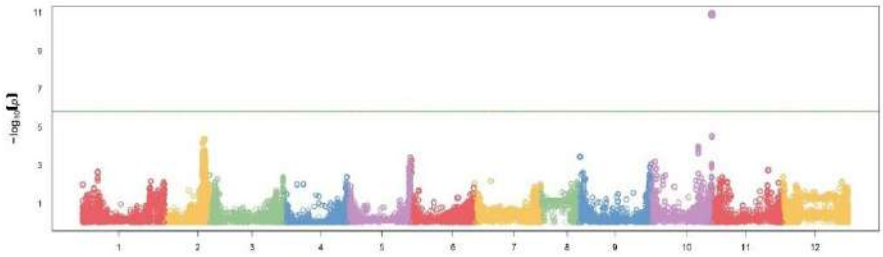
NODAL ANTHOCYANIN MLMM

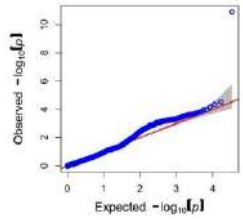


STEM PUBESCENCE BLINK

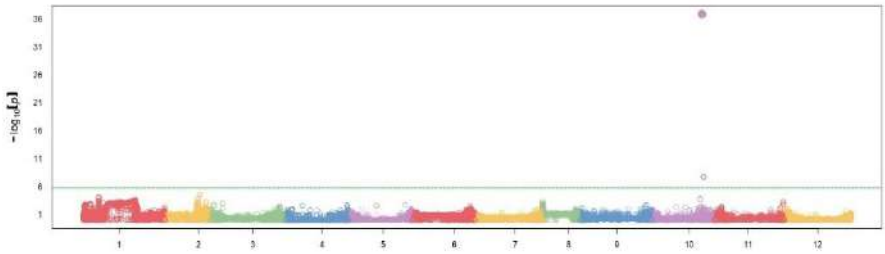


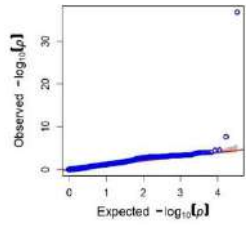
STEM PUBESCENCE MLMM



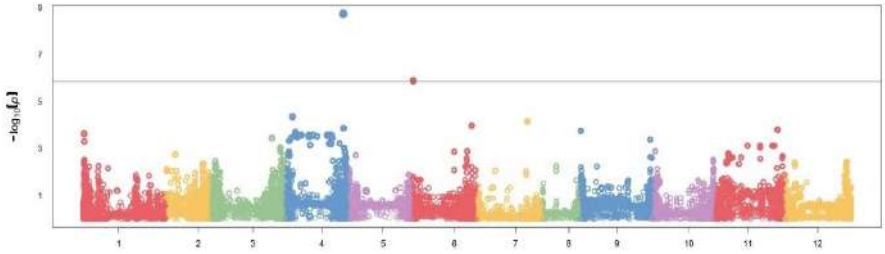


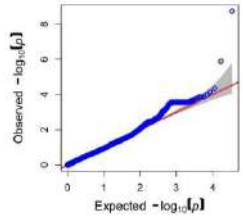
FILAMENT COLOUR MLMM



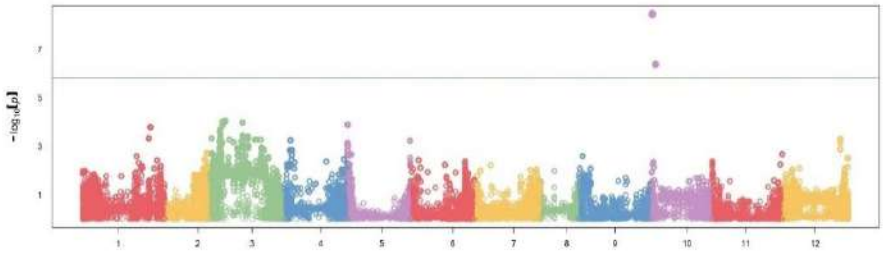


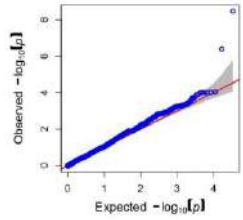
ANTHOCYANIN STRIPES BLINK



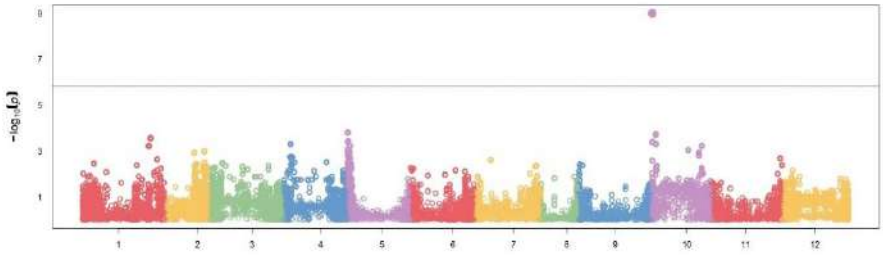


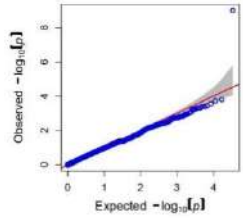
FRUIT COLOUR AT INTERMEDIATE STAGE BLINK



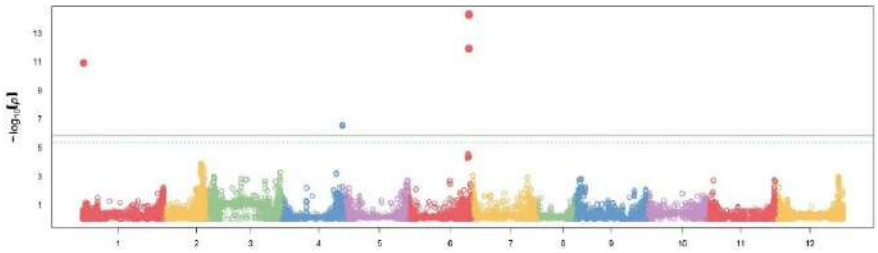


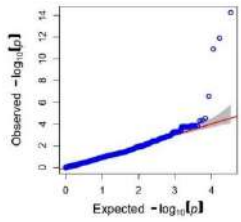
RUIT COLOUR AT INTERMEDIATE STAGE MLMM



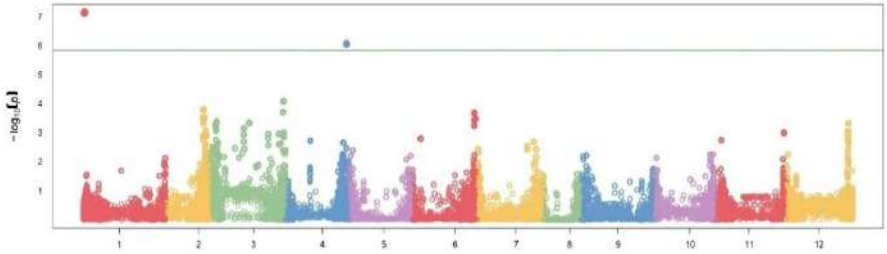


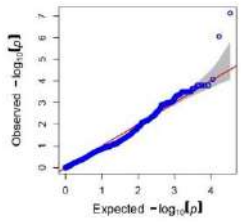
FRUIT COLOUR AT MATURE STAGE BLINK



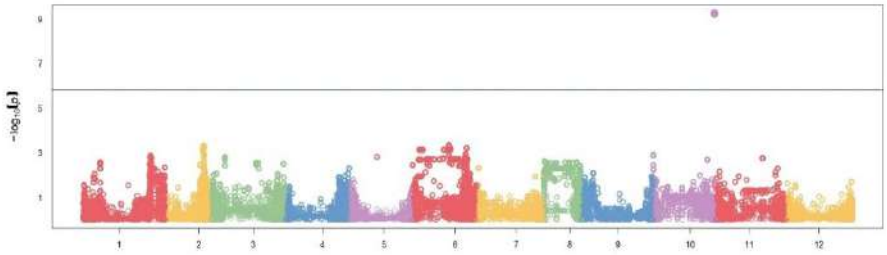


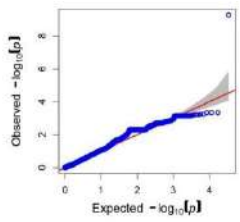
FRUIT COLOUR AT MATURE STAGE MLMM



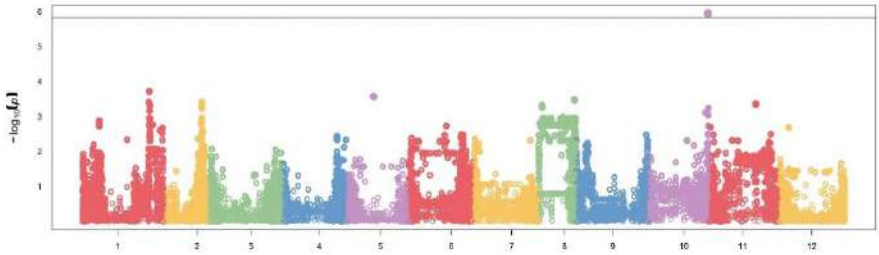


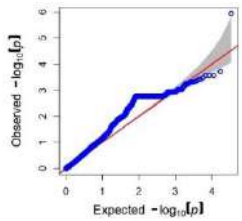
PEDICEL PERSISTENCE BLINK



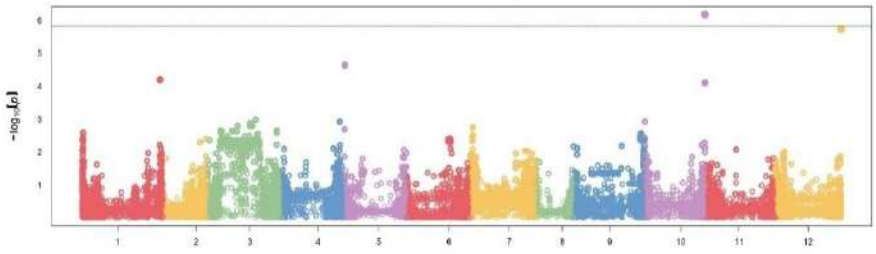


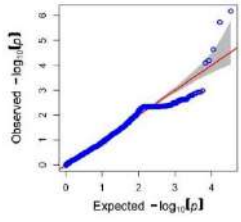
PEDICEL PERSISTENCE MLMM





FRUIT WALL CONSISTENCY BLINK





FRUIT WALL CONSISTENCY MLMM