DOCTORAL THESIS

Development of biotechnological tools for the genetic improvement of pepino (*Solanum muricatum*) and tree tomato (*S. betaceum*)

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Valencia, June 2022

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Programa de Doctorado en Biotecnología

TESIS DOCTORAL

Desarrollo de herramientas biotecnológicas para la mejora genética del pepino dulce (*Solanum muricatum*) y tomate de árbol (*S. betaceum*)

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TESI DOCTORAL

Desenvolupament d'eines biotecnològiques per a la millora genètica del cogombre dolç (*Solanum muricatum*) i tomata d'arbre (*S. betaceum*)

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Abstract

Pepino (*Solanum muricatum*) and tree tomato (*S. betaceum*) belong to the group of crops of the Solanaceae family. These two crops are native to South America and currently are grown in various countries with tropical, subtropical and Mediterranean climates. They have been underutilized for a long time and have become relevant only in recent years due to their high nutritional quality. Pepino exhibit significant levels of potassium, vitamin C and carotenoids and it is reported to present antioxidant, antidiabetic, anti-inflammatory and antitumor properties. Its fruits can be consumed both as a dessert or in salads. Tree tomato also highlights high content of bioactive compounds such as carotenoids, anthocyanins, flavonoids and vitamins. Severals products such as juices, jams, sauces and pharmaceutical products are made from its fruits.

Due to these crops have been introduced into new regions, where they may be exposed to biotic and abiotic stresses that can threaten their production, and since pepino is specially affected by water scarcity, a study was needed to determine the response of seven pepino cultivars to physiological and biochemical parameters to drought stress. This work can help develop selection and improvement programs that allow the generation of new varieties that are more tolerant to drought.

On the other hand, in countries with a Mediterranean climate, pepino is grown as a protected crop, applying the same agricultural techniques as other solanaceous plants such as tomato and pepper. These agricultural systems also provide optimal conditions for the

development of diseases such as *Fusarium oxysporum* f. sp. *lycopersici* (FOL), *Verticillium dahliae* (VE), pepino mosaic virus (PepMV) and tomato mosaic virus (ToMV), which could potentially cause great damage to pepino crops. For this reason, a study was performed to evaluate the response of a collection of pepino and their wild relatives against these four diseases, and find sources of resistance/tolerance to those pathogens

Although tree tomato is an important fruit crop due to its nutritional value and beneficial health effects, there is currently no publicly available genomic and transcriptomic information. Therefore, it was essential to sequence the transcriptome of two tree tomato cultivars with purple fruits (A21) and orange fruits (A23). These two cultivars have been widely used and cultivated commercially in countries of the Andean region such as Ecuador and Colombia. Obtaining the first tree tomato transcriptome has made it possible to perform a comparative study between tree tomato and its close species, tomato and potato, identify genes involved in the carotenoid biosynthesis pathway, and develop single nucleotide polymorphism (SNP) markers.

In general, this Doctoral Thesis provides relevant information on the response of pepino to various environmental stresses, which can be used for the development of new varieties of pepino resistant to multiple stresses. While in tree tomato, the development of genomic tools will accelerating up breeding programs.

Resumen

El pepino dulce (*Solanum muricatum*) y el tomate de árbol (*S. betaceum*) pertenecen al grupo de cultivos de la familia Solanaceae. Estos dos cultivos son originarios de América del Sur y actualmente se cultivan en varios países con climas tropicales, subtropicales y mediterráneos. Han sido infrautilizados durante mucho tiempo y han cobrado relevancia solo en los últimos años debido a su alta calidad nutricional. El pepino dulce exhibe niveles significativos de potasio, vitamina C y carotenoides y se informa que presenta propiedades antioxidantes, antidiabéticas, antiinflamatorias y antitumorales. Sus frutos se pueden consumir tanto como postre o en ensaladas. El tomate de árbol también destaca por su alto contenido en compuestos bioactivos como carotenoides, antocianinas, flavonoides y vitaminas. Varios productos como jugos, mermeladas, salsas y productos farmacéuticos son elaborados a partir de sus frutos.

Debido a que estos cultivos se han introducido en nuevas regiones, donde pueden estar expuestos a estreses bióticos y abióticos que pueden amenazar su producción, y dado que el pepino dulce se ve especialmente afectado por la escasez de agua, fue necesario realizar un estudio para determinar la respuesta de siete cultivares de pepino dulce a parámetros fisiológicos y bioquímicos al estrés por sequía. Este trabajo puede ayudar a desarrollar programas de selección y mejoramiento que permitan generar nuevas variedades más tolerantes a la sequía.

Por otro lado, en los países de clima mediterráneo, el pepino dulce se cultiva como cultivo protegido, aplicando las mismas técnicas agrícolas que otras solanáceas como el tomate y el pimiento. Estos sistemas agrícolas también brindan condiciones óptimas para el desarrollo de enfermedades como *Fusarium oxysporum* f. sp. lycopersici (FOL), *Verticillium dahliae* (VE), virus del mosaico del pepino (PepMV) y virus del mosaico del tomate (ToMV), que potencialmente podrían causar grandes daños a los cultivos de pepino dulce. Por tal motivo, se realizó un estudio para evaluar la respuesta de una colección de pepino dulce y sus parientes silvestres contra estas cuatro enfermedades, y encontrar fuentes de resistencia/tolerancia a estos patógenos.

Aunque el tomate de árbol es un cultivo frutal importante debido a su valor nutricional y efectos beneficiosos para la salud, actualmente no hay información genómica y transcriptómica disponible públicamente. Por lo tanto, fue fundamental secuenciar el transcriptoma de dos cultivares de tomate de árbol con frutos morados (A21) y frutos anaranjados (A23). Estos dos cultivares han sido ampliamente utilizados y cultivados comercialmente en países de la región andina como Ecuador y Colombia. La obtención del primer transcriptoma de tomate de árbol ha permitido realizar un estudio comparativo entre el tomate de árbol y sus especies cercanas, tomate y patata, identificar genes implicados en la ruta de biosíntesis de carotenoides y desarrollar marcadores de polimorfismo de nucleótido único (SNP).

En general, esta Tesis Doctoral aporta información relevante sobre la respuesta del pepino a diversos estreses ambientales, que puede ser utilizada para el desarrollo de nuevas variedades de pepino resistentes a múltiples estreses. Mientras que en tomate de árbol, el desarrollo de herramientas genómicas acelerará los programas de mejoramiento.

Resum

El cogombre dolç (*Solanum muricatum*) i tomata d'arbre (*S. betaceum*) pertanyen al grup de cultius de la família Solanaceae. Aquests dos cultius són originaris d'Amèrica del Sud i actualment es cultiven en diversos països amb climes tropicals, subtropicals i mediterranis. Han sigut infrautilitzats durant molt de temps i han cobrat rellevància només en els últims anys a causa de la seua alta qualitat nutricional. El cogombre dolç exhibeix nivells significatius de potassi, vitamina C i carotenoides i s'informa que presenta propietats antioxidants, antidiabètiques, antiinflamatòries i antitumorals. Els seus fruits es poden consumir tant com postres o en ensalades. La tomaca d'arbre també destaca pel seu alt contingut en compostos bioactivos com carotenoides, antocianinas, flavonoides i vitamines. Dels seus fruits s'elaboren diversos productes com a sucs, melmelades, salses i productes farmacèutics.

Pel fet que aquests cultius s'han introduït en noves regions on poden estar exposats a estressos biòtics i abiòtics que poden amenaçar la seua producció, atés que el cogombre es veu especialment afectat per l'escassetat d'aigua, va ser necessari realitzar un estudi per a determinar la resposta de set cultivars de cogombre dolç a paràmetres fisiològics i bioquímicos a l'estrés per sequera. Aquest treball pot ajudar a desenvolupar programes de selecció i millorament que permeten generar noves varietats més tolerants a la sequera.

D'altra banda, als països de clima mediterrani, el cogombre dolç es cultiva com a cultiu protegit, aplicant les mateixes tècniques agrícoles

que unes altres solanáceas com la tomaca i el pimentó. Aquests sistemes agrícoles també brinden condicions òptimes per al desenvolupament de malalties com *Fusarium oxysporum* f. sp. lycopersici (FOL), *Verticillium dahliae* (VE), virus del mosaic del cogombre (PepMV) i virus del mosaic de la tomaca (ToMV), que potencialment podrien causar grans danys als cultius de cogombre dolç. Per tal motiu, es va realitzar un estudi per a avaluar la resposta d'una col·lecció de cogombre dolç i els seus parents silvestres contra aquestes quatre malalties, i trobar fonts de resistència/tolerància a aquests patògens.

Encara que la tomaca d'arbre és un cultiu fruiter important a causa del seu valor nutricional i efectes beneficiosos per a la salut, actualment no hi ha informació genòmica i transcriptómica disponible públicament. Per tant, va ser fonamental seqüenciar el transcriptoma de dues cultivars de tomaca d'arbre amb fruits morats (A21) i fruits ataronjats (A23). Aquestes dues cultivars han sigut àmpliament utilitzats i cultivats comercialment en països de la regió andina com l'Equador i Colòmbia. L'obtenció del primer transcriptoma de tomaca d'arbre ha permés realitzar un estudi comparatiu entre la tomaca d'arbre i les seues espècies pròximes, tomaca i creïlla, identificar gens implicats en la ruta de biosíntesi de carotenoides i desenvolupar marcadors de polimorfisme de nucleòtid únic (SNP).

En general, aquesta Tesi Doctoral aporta informació rellevant sobre la resposta del cogombre a diversos estressos ambientals, que pot ser utilitzada per al desenvolupament de noves varietats de cogombre resistents a múltiples estressos. Mentre que en tomaca d'arbre, el desenvolupament d'eines genòmiques accelerarà els programes de millorament.

General Introduction

1 Biotic and abiotic stresses

A wide variety of environmental stresses adversely affect the growth, development, or productivity of crops (Semenov and Shewry, 2011; Cohen and Leach, 2019). Plants respond to stress by activating different processes that involve changes at the transcriptomic, cellular, physiological, biochemical and molecular levels (Atkinson and Urwin, 2012). Changes in environmental conditions are reflected in the stress affecting plants. However, exposure of plants to a specific stress leads to tolerance of that stress (Verma et al., 2013). Recent studies have indicated that plants respond differently to individual stresses when exposed to multiple stresses (Gull, 2019). Environmental stresses that affect plants are generally classified into two different types: abiotic and biotic stresses. Among the abiotic stress factors that have a great impact on agriculture are heat, cold, drought and salinity.

Drought stress is the environmental factor that has the most negative effects on crop productivity (Basu et al., 2016). The genotype influences the response of plants to drought stress that depends on the growth stage of the plant and other environmental factors (Fahad et al., 2017). On the other hand, biotic stresses include various pests and pathogens like viruses, fungi and bacteria (Pandey et al., 2017; Gull, 2019). Plants develop a specific cellular and molecular response system for each stress to prevent damage, but generally to the detriment of growth and yield (Rejeb et al., 2014). To overcome the threats of abiotic and biotic stress, plants have developed some mechanisms, which are activated by stimuli received from sensors located in various

cell compartments (Verma et al., 2013). This generates differential transcriptional changes in the plant to make it more tolerant to stress by triggering a response at the biochemical and physiological levels (Suzuki et al., 2014).

Global warming has adverse effects on plant growth due to higher temperatures also influence a wide range of pest and disease habitats, facilitating the emergence and spread of new races and biotypes (Etesami and Jeong, 2018). Due to the constant increase of multiple abiotic and biotic stresses globally, the expansion of new crops in new regions may be restrained by these stresses. Therefore, there is an urgent need to select and develop resistant varieties that can tolerate multiple stresses (Atkinson and Urwin, 2012; Nelson et al., 2018).

2 The Solanaceae family in the Andean region

The Andean region has a great diversity of Solanaceae comprising about 60% of the family diversity, being very variable between South American countries (Palchetti et al., 2020). The Solanaceae family contains 98 genera and comprises about 2,800 species (Dupin et al., 2017). Their distribution extends to all continents except Antarctica, with a preference for warm to tropical zones, which inhabits many heterogeneous environments and is subject to natural mutation (Palchetti et al., 2020). Solanaceae have been reported among the 12 most diverse families, being the genus *Solanum* L. with around 1,500 species distributed worldwide, the largest and most diverse genus within Solanaceae. (Knapp and Peralta, 2016; Ulloa et al., 2017).

The Andean region is considered one of the main centers of origin and diversity of many Solanaceae (Olmstead, 2013), some of them economically very important, such as tomato *(S. lycopersicum* L.) and potato (*S. tuberosum* L.), while others locally important as pepino (*S. muricatum* Aiton), tree tomato (*S. betaceum* Cav.), naranjilla (*S. quitoense* Lam.) or cocona (*S. sessiliflorum* Dunal), have been underutilized and are not well known in commercial markets.

Some native crops were marginalized due to the introduction of crops from other regions during the European colonization or by local crops that caught the attention of the colonisers (National Research Council, 1989; Galluzzi and López Noriega, 2014). In addition, the new agricultural production systems initiated from the green revolution had an impact on the marginalization of these crops, focusing on a few such as wheat (*Triticum aestivum* L.), maize (Zea mays L.) or rice (*Oryza sativa*) (Pingali, 2017). Nowadays, several native plants of South America are considered minor, underutilized or neglected and are categorized within a group of plant species known as "NUS" (neglected and underutilized species). There are many reasons today for promoting a greater use of underutilized species in agricultural activities, the overarching justification for the development and safeguarding of these species is certainly their close link that binds these resources and food security and climate change.

3 Pepino

The pepino (*S. muricatum*) Aiton is an underutilized Andean fruit crop, phylogenetically related to tomato and potato (Prohens et al., 1996). The pepino is a diploid species with 2n=2x=24 chromosomes known as pepino dulce, melon pear, or sweet cucumber, grown throughout the tropics, subtropics and Mediterranean climates (Contreras and Gonz, 2016). Pepino is an annual herbaceous plant, highly branched, evergreen, with a woody stem that can grow up to 1.2 m in height (Lim, 2013) (Figure 1A). The leaves are green, simple or pinnate and elliptical-lanceolate with strigose or glabrous laminae and folioles (Figure 1 B). The flowers are pentamerous with violet petals and whitish margins that are larger than the stamens. The anthers are yellow and five in number, with a length between three and five millimetres. The calyx persists on the fruits (Figure 1 C). The fruits are soft, ovoid to ellipsoid to subspherical in shape, 5 to 20 cm long, green, creamy white or yellow with purple streaks. (Figure 1 D) and yellowish inside, with a pleasant aroma and flavor. Some varieties are parthenocarpic and can be eaten in salads (Prohens et al., 2002a).

Figure. 1. Pepino plants at Pallatanga, Ecuador (A), flowers (B), fruits (D)

3.1 Taxonomy

The pepino is a member of the Solanaceae family and was originally described as *S. variegatum*, due to the characteristic veining of the fruits (Ruiz and Pavón, 1957). In the 18th century, this name was modified to *S. muricatum* by William Aiton, of the Royal Botanic Garden, Kew, in London (Aiton, 1814). Another term by which this

species has been known was *S. guatemalense*, especially in North America, because this plant was first introduced to the United States from Guatemalan materials. (Wickson, 1889).

Table 1. *S. muricatum* taxonomic classification

Kingdom	***Plantae***
Division	Magnoliophyta
Class	Magnoliopsida
Order	Solanales
Family	Solanaceae
Genus	Solanum
Subgenre	Potatoe
Section	Basarthrum
Species	Solanum muricatum Aiton

3.2 Relationships of wild pepino relatives

Solanum section *Basarthrum* (Solanaceae) includes the cultivated species (*S. muricatum*) and 22 additional wild relatives. Within the *Basarthrum* section, it is the only member of the Muricata series, but it is closely related to a set of wild species, belonging to the Caripensa series. It is within this series where we find the species most likely involved in the origin of the pepino: *S. caripense*, *S. tabanoense*, *S. basendopogon*, *S. cochoae*. Among these, *S. caripense* appears to be the most probable pepino ancestor since it is easy to obtain interspecific hybrids between the two species that are usually fertile (Blanca et al., 2007).

3.3 Origin and domestication

Pepino is a species native to South America and was domesticated by farmers of the Andean region since pre-Hispanic times (Prohens et al., 1996). The exact place where this domestication took place is unknown, although it seems clear that it was in the valleys and highlands, from 500 to 3,500 m above sea level (from southern Colombia to southern Peru), currently including the countries of Colombia, Peru and Ecuador (Anderson et al., 1996). The representations of the pepino in the pre-Columbian cultures of the ancient societies of Ecuador and Peru show the importance of this crop in food security and in the cultural heritage of those countries.

3.4 Geographical distribution

The pepino originally spread to several countries in the Andean region, from Ecuador to Bolivia and Peru. Later, pepino was introduced in Mexico and also in other Central American countries with tropical and subtropical climates, during the first half of the 17th century (Cobo, 1956). The dispersal routes of pepino in Europe are known as a result of the botanical expedition to the kingdoms of Peru and Chile carried out by Ruiz and Pavón at the end of the 18th century (Prohens et al., 1996). During this expedition, several shipments of plant material were made to the Botanical Garden of Madrid in 1785 and to that of Tenerife. Probably in this same expedition of Ruiz and Pavón plants were sent to the court of the king of France and from there to the Kew Gardens, where Aiton gave pepino the scientific name by which it is currently known (Aiton, 1789). Then pepino was spread to Russia, England,

Italy, and the Netherlands during the first half of the 20th century (Baccarini, 1908; Nanetti, 1912; Bukasov, 1930; Casella, 1955). In the United States, a material from Guatemala was introduced and adapted to Florida and California areas at the end of the 19th century, while the pepino was grown in Abyssinia (Ethiopia) in the first half of this century (Van der Slikke, 1951). In 1952, it was introduced in Morocco, where a commercial plantation was carried out, which was intended to supply the markets of Agadir, France and England (Chapot, 1955). In 1906, the pepino was introduced in New Zealand (Cossio, 1988), and in the 1930s it was cultivated by the famous nurseryman Hayward R. Wright, appearing in some commercial catalogues (Morley-Bunker, 1983).

3.5 Genetic diversity

Various natural, as well as breeding activities such as domestication, natural intercrossing, mutation, selection and hybridization, created broad genetic diversity of this crop in Andean region and other areas (Prohens et al., 1996). In the region of origin of the pepino, there are no different names for the different cultivars, or they have been lost. However, cultivar classes predominate depending on each country. In Ecuador, there are two types of cultivars: i) cultivars with large fruits and globose shapes, where the background color of the immature fruit is green and with a sparse veining with well-defined purple bands, and ii) cultivars with smaller and more elongated shapes, sometimes almost cylindrical, where the color of the immature fruits is almost white and

the purple veining is more abundant, sometimes occupying a high percentage of the fruit with bands less well defined.

In Peru, fruits with heart shapes predominate:

Morado rayado: the leaves are dark green, suberect branches and ovoid fruits of different sizes, very sweet yellow pulp, much appreciated.

Oreja de burro: the leaves are light green, straight and long branches, whitish fruits with few spots and medium-large size, the pulp is also whitish and less sweet.

Two types are grown in Chile (Coquimbo, La Serena):

Oval or heart-shaped cultivars, with rounded ends, with little veining.

Elongated cultivars, with a pointed apical end, creamy in color with many purple streaks.

On the other hand, different commercial varieties have been developed outside the region of origin: New Zealand (Asca, Kawi, El Camino), Australia (Pepino Gold, Wayfarer Special, Temptation , Golden Spendour, Naragold, and Colossal) and Spain (Sweet Long, Sweet Round, Puzol, Turia, Valencia) through selection and breeding.

3.6 Physicochemical and bioactive properties

The most important organic constituents of the pepino fruit are water (92%) and carbohydrates (7%) (Di Scala et al., 2011). In another analysis, it was found that the nutrient content of pepino fruit was protein 0.93%, moisture 93.80%, ash 0.46%, oil 0.05%, sugars 4.48%,

of which glucose 34.6%, fructose 43.2%, sucrose 22.2% (Yalçin, 2010). In the ripe fruit, the sugar content is the main component of the dry matter (7.03%). In addition, pepino contains several vitamins such as niacin, thiamin, riboflavin and ascorbic acid (vitamin C), which have antioxidant activity associated with the detoxification of oxygen species (Shathish and Guruvayoorappan, 2014). The major mineral constituents include nitrogen, potassium, phosphorus, magnesium, calcium and sodium (Redgwell and Turner, 1986) Table 2. Other microelements are iron, manganese, copper, and zinc.

Table 2. Proximate analysis of ripe pepino fruits

Compound	Values per 100 g
Dry weight (g)	6.8-8.2
Protein (g)	0.10-0.13
Lipids and pigments (mg)	24.6-44.4
Soluble sugars (g)	4.9-6.4
Starch (mg)	20.0-90.0
Cellulose (mg)	154-220
Hemicellulose (mg)	40.1-53.6
Pectin (mg)	26.7-34.5
Vitamin C	46.0-68.8
Non-volatile organic acids (mg)	119-153
Free amino acids (mg)	52-70
Nitrogen (mg)	23-30
Phosphorus (mg)	10.7-12.3
Potassium (mg)	115-123
Sulfur (mg)	3.0-4.0
Calcium (mg)	2.3-3.0
Magnesium (mg)	5.3-6.1
Sodium (mg)	0.76-2.30
Iron (mg)	0.20-0.31
Manganese (mg)	0.06-0.07
Zinc (mg)	0.02-0.05
Copper (mg)	0.02-0.03
Boron (mg)	0.03-0.05

The pepino fruit is characterized by being aromatic, it synthesizes a high number of volatile compounds including amino acids, lipids and carotenoids. Among the main volatile compounds detected are terpenes, aldehydes, alcohols and esters and other exotic notes that contribute to aroma profiles such as mesifuran, lactones and β-damascenone (Contreras et al., 2017). About 30 volatile compounds were found in three cucumber cultivars, of which the majority were 24 esters, alcohols, aldehydes and ketones (Shiota et al., 1988; Ruiz-Beviá et al. 2002; Rodríguez-Burruezo et al., 2004).

3.7 Bioactive compounds and their health benefits

Pepino fruits have an important antioxidant activity due to their high content of phenols, flavonoids and carotenoids, which influence the yellow pigmentation of the pulp (Sudha et al., 2012). The phenolics present in pepino are flavonoids, myricetin, naringenin, quercetin, rutin and hydroxycinnamic acid derivatives (Hsu et al., 2011; Herraiz, et al., 2016a). Pepino fruits also exhibit antidiabetic potential due to their high content of ascorbic acid and total flavonoids (Hsu et al. 2011). Furthermore, pepino extracts possess selective activity on a wide range of human tumor cell lines such as prostate, liver, breast, ovarian, stomach, colon, and lung cancer cells. (Ren and Tang, 1999).

3.8 Drought tolerance

Pepino is a superficially rooted crop (20.0 - 30.8 cm) (Pacheco et al., 2021a), therefore it requires a frequent supply of water throughout the growing period. The critical phases for pepino water supply are flowering, fruit set and fruit development. The frequency of irrigation in pepino will depend mainly on the type of soil, if it is light, it will require more frequent contributions than if it is clayey (Prohens et al., 1997). Drought stress affects the physiological characteristics of pepino in innumerable ways. Duman et al. (2015) studied the response of cultivar ('Miski') to drought stress in various characters such as relative water content, biochemical changes and proline content. In this work, it was shown that relative water content, photosynthetic pigments chlorophyll a, chlorophyll b and carotenoids, and total chlorophyll decreased, while total phenolic compounds and proline levels increased significantly as a consequence of drought.

After exposure to drought stress, many different types of genes in pepino are differentially expressed. A recent study has been carried out to reveal the genes involved in the tolerance of *S. muricatum* to drought. Yang et al. (2021) identified 71 NAC genes, which were divided into seven subfamilies. In root tissues, gene expression levels were high compared to leaf and stem tissues, which were relatively similar. A high degree of homology was observed between the amino acid sequences of NACs from Solanaceae, and NACs from *S. muricatum* strongly aligned with NACs from tomato, potato, pepper, and tobacco.

3.9 Salinity

Pepino cultivars may differ in their sensitivity to salinity stress. Several studies have shown that pepino shows greater earliness between 9 and 16 days when exposed to saline conditions, while the content of soluble solids increases around 25% compared to the control (Pluda et al., 1993; Prohens et al., 2002b). In addition, there was a 3% to 5% weight loss and a slight firmness reduction, but those changes did not affect the visual appearance and acceptance of the fruits. The observed changes were influenced by the genotype-environment interaction during the stage of growth and development of the fruit. Therefore, pepino can be successfully grown in salt-affected soils.

3.10 Pests and diseases

In several countries outside the region of origin, pepino is grown under the protected cultivation system, using production systems similar to commercial crops such as tomatoes and peppers (Rodríguez-Burruezo et al., 2011). Under this protected cultivation system, the pepino is affected by several types of pests and diseases, which are the prominent limiting factors for its production. The most important pests are:

The red spider mite (Tetranychus urticae), difficult to control in greenhouses during the hot season.

- Whiteflies (Trialeurodes vaporarium, Benisia tabaci), which mainly affect greenhouse crops.

- Aphids (various species).

- The potato beetle (Leptinotarsa decemlineata).

- The miner flies (Liriomyza trifolii, Tuta absoluta).

Regarding diseases, the most common are the following:

- Fusarium oxysporum f. sp. lycopersici is a soil-borne pathogen that causes Fusarium wilt, affects a wide variety of horticultural crops, and can cause high losses (Mandal et al., 2009). The symptoms of the attack appear first as a slight yellowing of the leaves, wilting and defoliation of the plants, and finally death of the host plant (Nirmaladevi et al., 2016).

- Verticillium dahliae is a fungus that causes vascular wilt particularly in dicotyledonous plant species (Acharya et al., 2020). In tomato and pepino, the symptoms appear with chlorosis and necrotic lesions, reduced growth, yield and death of the plant. (Karagiannidis et al., 2002).

- Tomato wilt virus (TSWV) disease is widely distributed, mainly in tropical and Mediterranean environments. The main host plants include tomato, pepper, lettuce, potato, and tobacco (Qi et al., 2021). In pepino, it produces symptoms similar to those of tomato plants, such as necrosis, chlorosis, and dark brown spots that affect the leaves, stems, and fruits, although there is no apparent decrease in production.

- Pepino mosaic virus (PepMV). This virus was first described in 1980 in Peru (Jones et al., 1980), causes significant yield and quality losses in tomato production (Souiri et al., 2017). The most common

symptoms on pepino include yellow mosaic and chlorosis on leaves (Hasiów-Jaroszewska et al., 2011).

- Tomato mosaic virus (ToMV), is the cause of a serious disease in tomato, but it also affects other plants of the Solanaceae family (Pérez-Benlloch et al., 2001). Pepino and tomato plants infected with ToMV develop mosaic and necrotic lesions on leaves (Bae et al., 2019; Leiva-Brondo, et al., 2006).

The selection and characterization of resistant and tolerant pepino and wild relatives accessions to cope with biotic and abiotic stresses is a vital requirement for using these accessions in future pepino breeding programs. In this thesis, the evaluation of the response to water deficit and the differences amongst seven pepino cultivars under three water stress treatments have been determined. In addition, the resistance behaviour of these accessions and the wild relatives against Fusarium, Verticillium, PepMV and ToMV were screened.

4 Tree tomato

Tree tomato (*Solanum betaceum* Cav.), also known as tamarillo, is an important Andean fruit crop very related to other Solanaceae such as tomato and potato (Olmstead et al., 2008; Särkinen et al., 2013). The tree tomato is a diploid plant with 2n = 24 chromosomes, although triploid and tetraploid individuals have been spontaneously identified in commercial orchards (Pringle and Murray, 1992; Acosta-Quezada et al., 2016).

The tree tomato is a tree that grows mainly in height between two and four meters. (Figure 2A). The consistency of the stem and branches is semi-woody and fragile and the bark is grayish-green in color. It is usually divided into three branches at a height range between 1 and 1.50 m (Figure 2A). The leaves are evergreen, alternate, simple and with the entire edge, the beam is colored dark green while the underside is lighter green (Figure 2A). The main stem leaves have between 22 to 34 cm length and 21 to 28 cm width in plants in production (Acosta-Quezada et al., 2011). The flowers are small (1 cm in diameter), hermaphrodite and have five petals that are cream-white, pink-white, grouped in scorpioid cymes and are fragrant (Pringle et al., 1991; Ramírez and Kallarackal, 2019) (Figure 2B). The flower has a staminal cone with five yellow bilocular anther stamens, above the cone the pistil protrudes. In each cyme there are up to 50 flowers, of which three to six manage to set, forming the fruits and reaching physiological maturity (Pringle et al., 1991; Ramírez and Kallarackal, 2019). Tree tomato cultivars in particular conditions are classified into self-compatible and self-incompatible cultivars. The flowers are pollinated with the help of the wind, or mainly with insects that act as vectors, with bees having the highest incidence (Pringle et al., 1991; Ramírez and Kallarackal, 2019). The fruit (4 - 8 cm long and 1.3 - 1.5 cm in length/width ratio) is ellipsoidal or ovoid in shape (Figure 2 C). The epicarp is smooth, varies between genotype and can be yellow, orange, red or purple, with dark longitudinal stripes. The mesocarp presents a sweet semi-acid taste, generally yellow, orange, or purple in color and has two locules (Acosta-Quezada et al., 2011; 2012; 2016). The seeds

are flattened, round, 2.0 to 4.0 mm in diameter, yellowish-white in color and are found inside the fruit surrounded by the pulp of the fruit. The number of seeds per fruit differs between varieties in a range of 294 to 382, constituting the main form of propagation (Acosta-Quezada et al., 2011).

Figure. 2. Tree tomato cultivars at Riobamba, Ecuador (A), flowers (B), immature fruit (C).

4.1 Taxonomy

The tree tomato or tamarillo belongs to the large Solanaceae family, and was initially named as *S. betaceum* by Cavanilles in 1799 based on a plant grown in the Royal Botanical Garden of Madrid. However, Sendtner moved it to the genus Cyphomandra and named the tree tomato *Cyphomandra betacea* (Cav.) Sendtn, as a genus different from Solanum. Later molecular studies, carried out by Bohs (1995), demonstrated that the genus Cyphomandra is deeply nested within the genus Solanum and the tree tomato was transferred back to the original name *Solanum betaceum*.

Table 3. *Solanum betaceum* taxonomic classification

Kingdom	***Plantae***
Division	Angiospermae
Class	Magnoliopsida
Subclass	Asteridae
Order	Solanales
Family	Solanaceae
Genus	Solanum
Subgenre	Cyphomandra
Section	Pachyphylla
Species	Solanum betaceum Cav.

4.2 Relationships of wild tree tomato relatives

The cultivated tree tomato, *S. betaceum*, is closely related to 3 wild species *S. maternum*, *S. unilobum*, and *S. roseum* (Bohs, 1994; 2007). Interspecific hybridizations are possible between the cultivated tree tomato genotypes and the wild relatives (S. *betaceum* x *S. roseum*, *S. betaceum* x *S. unilobum* y *S. betaceum* x *S. maternum*). The possibility of developing hybrids with wild relatives is an important strategy to incorporate useful genes into the genetic background of the tree tomato (Bohs, 1994; Bohs and Nelson, 1997). The tree tomato is morphologically very similar to *S. maternum*, suggesting that this one may be considered the wild ancestor of the cultivated tree tomato (Bohs and Nelson, 1997).

4.3 Origin and domestication

The precise origin of domestication is unknown, but it is probably native to southern Bolivia where it is common to find wild species in their natural state (Bohs, 1989; Ramírez and Kallarackal, 2019). Thus, taking advantage of the great genetic variability present in the region of origin, Andean farmers domesticated the tree tomato. Peru and Ecuador can be considered the center of domestication of this crop due to the high number of genotypes found (Acosta-Quezada et al., 2012).

4.4 Geographical distribution

According to Bohs (1989), the tree tomato has been spread around the world (Figure 4). Tree tomato was found in eastern South America, Buenos Aires, around 1849 (Miers., et al 1849). In Latin America, the tree tomato was introduced in Mexico and then to Central America, either spontaneously or deliberately during human migrations..

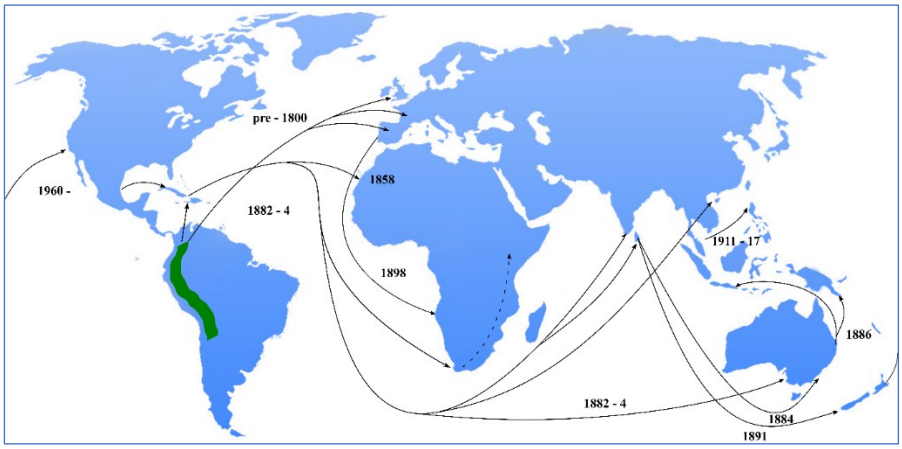

Figure 4. Distribution of tree tomato. Image adapted from Bohs (1989)

Tree tomato spread throughout the Caribbean (Jamaica and Cuba) by the 19th century, around the year 1884 (Morris, 1884; Roig and Mesa, 1965) and was present in the minor Antilles (on the island of Martinique) before the year 1900. In 1948, this species was also introduced in Puerto Rico (Hume and Winters, 1949). Cultivation in

the United States began in the 1886s in Florida and 1890s in California. On the other hand, in Europe, it was introduced according to the description of Cavanilles (1979) from a plant that was cultivated in the botanical garden of Madrid, and then spread to England, France and Germany (Willdenow, 1809; Dunal, 1813). In the mid-nineteenth century the tree tomato spread from southern Europe to Egypt (Morris, 1884), while by the year 1880, seeds of this species were transported from Jamaica to South Africa, India, Sri Lanka, Hong Kong and Australia (Morris, 1884). By 1886 tree tomato was distributed from the European colonies in Southeast Asia (Burkill, 1966) and in 1899 cultures were reported in China (Hooker, 1899). In the Philippines, it would have been introduced as of 1911 (Wester, 1924). From 1922, after the "Boer War", its seeds were transported from the Cape of Good Hope to Kenya and Tanzania. At the end of the 19th century, the tree tomato was brought from Porto (Portugal) to Southwest Africa (Warburg, 1903).

In 1891, the tree tomato was introduced to New Zealand from materials from India, being today the largest producer of tree tomato in the world (Fletcher, 1979). New Zealand mainly exports to Japan, US, Hong Kong, Australia, and Singapore. Colombia, Peru and Ecuador are the countries of the Andean region where the tree tomato is cultivated extensively and the largest commercial production is obtained (Schotsmans et al., 2011).

4.5 Genetic diversity

Tree tomato cultivars are distinguished by the colour, size and shape of the fruit. In the Andean region, three types of fruits are known, differentiated by the color of the skin when ripe: red, yellow and purple.

Red: Red-orange skin, with light longitudinal greenish-brown stripes, oval shape. It weighs between 50 and 80 g. Orange pulp, its flavor is more acidic than that of yellow-type fruits.

Yellow: Intense yellow skin with not very noticeable vertical greenish brown stripes and oval shape. It weighs approximately 50 to 70 g. Yellow-orange pulp and its flavor, it is less acidic than that of red and purple fruits.

Purple: It is also known as “blackberry tomato”. The fruits are round or oval, having an intense dark red skin, It weighs between 60 and 100 g. The pulp is orange, although the mucilage is purple so the juice is also purple, being the flavor more acidic than that of the yellow fruits. This type is the most common in New Zealand plantations, where it was obtained by selection in the 1920s, which is why in certain regions of South America it is known as “neozelandés”.

According to León et al. 2004, in Ecuador there are five types of cultivars, which are distinguished by characteristics of the fruit at the complete physiological maturity: color of the skin, color of the mucilage that covers the seeds and size.

Orange-pointed: Orange skin, pulp and mucilage. Fruits reach a length of 6.8 cm, a diameter of 4.6 cm and about 75 g in weight.

Orange-round: Orange-colored skin, pulp and mucilage, similar to the orange-pointed type, but smaller in size and weight than the previous one. The length and diameter are 5.5 and 4.7 cm, respectively, and it weighs about 70 g.

Orange-giant: skin, pulp and mucilage of orange color, but size and weight greater than the rest of orange types, with a length of 7 cm and about 120 g in weight.

Purple-New Zealand: Dark red skin, orange flesh and dark red or purple mucilage, with a length of 6.4 cm and a diameter of 4.6 cm, weighing about 85 g.

Purple-giant: Dark red skin (the same as Neozean purple), orange pulp and dark red or purple mucilage, with a length of 8 cm and a diameter of 5.8 cm. It weighs about 120 g.

In addition, due to the many selection and improvement programs of tree tomato, the following cultivars have been obtained:

Purple group: ‘Holmes’, ‘Kaitaia’, ‘Rothamer’, ‘Ruby Red’ and ‘Mulligan’.

Red group: ‘Andys Sweet Red’, ‘Ecuadorian Orange’, ‘Oratia Red’, ‘Secombes Red’, ‘Solid Gold’, ‘Red Beam’, ‘Red Beau’, ‘Red Delight’ and ‘Laird’s Large’.

Yellow group: ‘Egmont Gold’, ‘Goldmine’, ‘Inca Gold’ and ‘Amber’.

4.6 Physicochemical and bioactive properties

The tree tomato is considered a functional food due to its interesting nutritional value that comprises 4% proteins, 1% carbohydrates, 11% dietary fibre, 1% unsaturated fat, minerals (Iron, Phosphorus, Calcium, Magnesium, Potassium, Zinc), and Vitamin A (Table 4). The concentration of dietary fibre is generally high (more than 4 g/100 g FW) (Lister et al., 2005) similar to an apple or even kiwifruit analysed in the same way (Table 2). Tree tomato is an excellent source of vitamin C (25–35 g/100 g FW), vitamin B6, vitamin E and vitamin A (2,475 IU/100 g edible portion). Tree tomato presents a high amount of pro-vitamin A and carotenoids compared to orange, tomato, kiwifruit, and apple (Table 4) (Lister et al., 2005). Furthermore, tree tomato also contains calcium, phosphorus, copper, iron, magnesium, zinc and a high content of potassium, which is similar in concentration to that of the banana (Vasco et al., 2009; Acosta-Quezada et al., 2015;).

Flavor is the most appreciated quality characteristic of tree tomatoes and has a great influence on purchase choices and consumer acceptance. Approximately, 70 volatile compounds of different chemical nature have been described in tree tomato, out of these, only, three volatiles (ethyl hexanoate, methyl hexanoate and terpinene-4-ol) were common in all tree tomato cultivars from three countries (Diep et al., 2020a). Finally, the flavor in the tree tomato can be affected by several factors such as genotype, geographical, climatic and environmental.

Table 4. Nutritional composition of different tree tomato cultivars from New Zealand (per 100 g FW). FW = Fresh Weight; GAE = Gallic acid equivalent; TEAC = Trolox equivalent antioxidant capacity, % DI (Daily Intakes) are shown in brackets (Table from Diep et al., 2020).

Component/100 g	Yellow tree tomato	Red tree tomato	Kiwifruit	Banana	Tomato	Orange	Apple	Strawberry
Moisture (%)	86.3	86.1	83.8	74.9	94.5	86.8	85.56	90.95
Energy (kJ)	139 (2%)	165 (2%)	241	371	74	197	218	136
Protein (g)	1.9 (4%)	2 (4%)	1.06	1.09	0.88	0.94	0.26	0.67
Fat (g)	0.5 (1%)	0.4 (1%)	0.44	0.33	0.2	0.12	0.17	0.3
Dietary fibre (g)	3.2 (11%)	3.3 (11%)	3.0	2.6	1.2	2.4	2.4	2.0
Available carbohydrate (g)	3.7 (1%)	3.8 (1%)	9.1	20.8	2.7	8.5	10.8	6.6
Total sugars (g)	3.4 (4%)	3.5 (4%)	9.0	12.2	2.63	8.5	10.5	6.5
Fructose (g)	0.9	0.9	4.35	4.85	-	-	-	2.44
Glucose (g)	0.8	0.8	4.11	4.98	-	-	-	1.99
Sucrose (g)	1.6	1.7	0.15	2.39	-	-	-	0.47
Vitamin A, retinol equivalent (µg)	127	190	4	3	42	11	3	1
Vitamin B (mg)	0.38 (24%)	0.2 (12%)	0.063	0.4	0.046	0.06	0.041	0.047
Vitamin C (mg)	31 (78%)	29.8 (74%)	93	8.7	14	53.2	4.6	58.8
Vitamin E (mg)	1.9 (19%)	1.94 (19%)	1.46	0.1	0.54	0.18	0.18	0.29
Folate (µg)	4 (2%)	4 (2%)	25	20	15	30	3	24
Calcium (mg)	11 (1%)	11 (1%)	34	5	13	40	6	16
Copper (mg)	0.06 (2%)	0.05 (2%)	0.13	0.078	0.059	0.045	0.027	0.048
Iron (mg)	0.44 (4%)	0.57 (5%)	0.31	0.26	0.36	0.1	0.12	0.41
Magnesium (mg)	20 (6%)	21 (6%)	17	27	11	10	5	13
Manganese (mg)	0.185 (4%)	0.114 (2%)	0.098	0.27	0.114	0.025	0.035	0.386
Phosphorus (mg)	40 (4%)	39 (4%)	34	22	24	14	11	24
Potassium (mg)	292	321	312	358	237	181	107	154
Zinc (mg)	0.17 (1%)	0.15 (1%)	0.14	0.15	0.07	0.07	0.04	0.14
Total phenolics (mg GAE/100 g FW)	117	191	258.55	120	425b	39	187	240
Total anthocyanins (mg/100 g FW)	0	82	-	0	-	0	0	28-70
Antioxidant activity (µmol TEAC/100 g FW)	1002	1659	800	64	-	874	500	1850

4.7 Bioactive compounds and their health benefits

In an comprehensive review, Diep et al. (2020a) have reported that the tree tomato exhibits a high amount of bioactive compounds, flavonoids, phenols, carotenoids and anthocyanins. These compounds have antioxidant properties which makes them beneficial for human health. Up to now, more than 42 bioactive compounds have been found in three tomato, of these 15 are phenolics, 20 carotenoids and 7 anthocyanins.

Phenolics and carotenoids are the main bioactive compounds present in tree tomato. Among the phenolics present in tree tomato fruit, flavanol, flavanone, phenolic glycosides are present in large amounts while the carotenoids are β-cryptoxanthin, β-carotene, zeaxanthin, antheraxanthin and lutein (Diep et al., 2020b). Interestingly, the global content of total carotenoids in the tree tomato is similar or even higher than that of other fruits such as mango, kiwi, passion fruit, persimmon, jackfruit and orange (Isabelle et al., 2010).

Anthocyanins are members of the flavonoid group. They have coloring and antioxidant properties, the latter help prevent cardiovascular and neuronal diseases, cancer, diabetes, inflammation (Yousuf et al., 2016). The main anthocyanins reported in tree tomato are delphinidin and cyanidin, which are major responsible for the characteristic purple-red colour (Espin et al., 2016; Osorio et al., 2012).

Different studies have revealed that the tree tomato has a higher antioxidant activity compared to fruits known as kiwifruit and grape, and a higher phenolic content than other fruits rich in phenolic contents: grape, apple, plum, pineapple, persimmon and the cherries (Table 1) (Fu et al., 2011; Espin et al., 2016).

4.8 Biotechnology and genomics

In the last decade, several studies focusing on using biotechnology tools on tree tomato breeding have been carried out. Important achievements are expected soon since the tree tomato is amenable to tissue, cell and protoplast culture, the ploidy level can be easily manipulated, and the plants can be vegetatively propagated.

4.8.1 *In vitro* culture

There are many reports on tree tomato *in vitro* culture with different biotechnological applications such as micropropagation through axillary shoot proliferation, regeneration from different explants (including cotyledons, hypocotyls, leaves, stem sections) via organogenesis, somatic embryogenesis, virus-free plants, and genetic transformation (Patiño Torres et al., 2007; Correia et al., 2012a; 2012b; 2018; Criollo et al., 2016;). Among the first successful tissue culture techniques reported in tree tomato there are micropropagation by proliferation of axillary shoots (Cohen and Elliot, 1979), and plant regeneration via organogenesis from explants of leaves and protoplasts were (Guimarães et al., 1996;).

Guimarães et al., (1996) also reported the regeneration of tree tomato plants through shoot organogenesis and somatic embryogenesis from explants of hypocotyls, cotyledons, and protoplasts in vitro. On the other hand, several tests were carried out on contaminated tree tomato plants to eliminate viruses. In this work, the tips of the shoots were subjected to thermotherapy for several periods, showing the results that the plants obtained by this method were virus-free.

Recently, our laboratory reported the obtention of tetraploid tree tomato through *in vitro* polyploidization. In this work, the culture medium SIM used to regenerate plants promoted both the induction of callogenesis and the elongation of adventitious tetraploid shoots. The induction rate reached 26.7% of the explants treated. Among the regenerated plants we detected tetraploid plants, with 12 autotetraploid plants having been obtained so far (Pacheco et al., 2019).

4.8.2 Genetic transformation and genome editing

Initial research has focused upon genes that might solve traditional problems in agriculture, such as viral diseases. In tree tomato, this approach has been applied to generate transgenic plants resistant to tamarillo mosaic virus (TaMV) (Cohen et al., 2000).

Successful Agrobacterium-mediated transformation of tree tomato was first reported by Atkinson and Gardner (1993).

In this study, leaf disks of tree tomato plants were transformed using the avirulent *Agrobacterium tumefaciens* strain LBA4404, which harbored the binary vector pKIWI110. The results revealed a high transformation efficiency in all regenerated plants that showed resistance to kanamycin and expressed the reporter gene for β-d-glucuronidase (gusA). Several recent studies reported on new applications of genetic transformation in tree tomato plants, Cruz and Tomé, (2007) used the avirulent strain LBA4404 Agrobacterium to insert a plasmid with the nptII-resistant marker, while Correia (2011) conducted studies of functional genomics, in which the gene encoding NEP25 was silenced.

Nowadays, there is interest in using genetic engineering technology to develop new varieties that are more tolerant or resistant to biotic and abiotic stresses, which are often difficult to achieve by classical breeding methods. On the other hand, genome editing by nucleases targeting a specific site in the genome provides new strategies for plant breeding. Currently, due to its high efficiency and simplicity, the clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR/Cas9) system is the potential tool for manipulation and improvement of new and important traits in plants. CRISPR has been used successfully in various crop species (Tian et al., 2021), including horticulture crops such as potato (Wang et al., 2015; Nadakuduti et al., 2019;), tomato (Brooks et al., 2014; Danilo et al., 2019; Pan et al., 2016), and cabbage (Lawrenson et al., 2015);

tree crop species such as orange (Jia and Nian, 2014), apple, (Nishitani et al., 2016), pear (Charrier et al., 2019) and banana (Tripathi et al., 2020). However, the way to obtain genome-edited plants requires the availability of well-annotated and assembled reference genome and transcriptome sequences where sequence information is a prerequisite. In addition, the transformation and regeneration protocols are also a basic requirement for successful genome editing. With the availability of the tree tomato transcriptome sequence (Pacheco et al., 2021b), the CRISPR technique may allow the exploration of gene functions and the improvement of the characteristics of tree tomato varieties by modifying specific genes.

4.8.3 Next-generation sequencing (NGS)

Nowadays, several methods are available to reveal the information of the sequences of the genetic code. Among the best known methods, the classic dideoxy method, developed by Friedrich Sanger in 1970, stands out. This method uses an enzymatic reaction known as sequencing by synthesis. DNA polymerase synthesizes and sequences a DNA fragment and adds modified and labeled dideoxynucleotide triphosphates (ddNTPs) resulting in detectable chain termination and thus allowing identification of the DNA (Sanger et al., 1977). On the other hand, next-generation sequencing (NGS) technologies such as pyrosequencing is an alternative to the conventional Sanger method, based on real-time monitoring of DNA synthesis. In this

methodology the four deoxynucleotide triphosphates (dNTPs) are added separately during DNA sequencing which are controlled by luminescence and only the incorporated nucleotides cause a signal. Pyrosequencing has been used successfully for both whole genome sequencing and genotyping of thousands of single nucleotide polymorphisms (SNPs) in a large number of samples. (Marsh, 2007; Slatko et al., 2018).

Due to the advancement of NGS, the reduction of time and cost, this technology has been applied in whole genome sequencing, target sequencing, transcriptome, epigenome, molecular markers, gene discovery and small RNA sequencing (Zhou et al., 2010). Other applications of NGS include metagenomic studies to characterize microbial diversity by analyzing environmental and clinical samples, including soil, water, sediment, and intestinal content. (Prayogo et al., 2020). Furthermore, high-throughput NGS technologies have been successfully applied to the *de novo* assembly transcriptome and gene expression profiling. This makes it possible to quantify and detect the levels of gene expression under different conditions and between different types of cells or tissues. (Finotello and Di Camillo, 2015).

NGS technologies have revolutionized the field of transcriptomics, facilitating the study of gene expression in both model plants and crops that do not have a reference genome. (Huang et al., 2016; Ward et al., 2012). To date,

transcriptomes have been sequenced for hundreds of plant species, including plants from neglected species. (Xia et al., 2011).

This thesis presents an exhaustive analysis of the first transcriptome of two tree tomato cultivars. *De novo* sequencing, assembly, functional annotation, detection of intra and interspecific SNP molecular markers, identification of putative genes involved in the carotenoid biosynthesis pathway have been carried out.

Objectives

The following Doctoral Thesis is focused on the development of biotechnological tools for the genetic improvement of *S. muricatum* and *S. betaceum*, presenting two main objectives:

1. Selection of tolerant or resistant varieties to improve biotic and abiotic stress tolerance in pepino.
2. Development of biotechnological tools for the improvement of the tree tomato.

To achieve these objectives, the structure of the work has been divided into three chapters that encompass the three articles presented:

1. Moderate and severe water stress effects on morphological and biochemical traits in a set of pepino (*Solanum muricatum*) cultivars
2. Screening of pepino (*Solanum muricatum*) and wild relatives against four major tomato diseases threatening its expansion in the Mediterranean region
3. De novo transcriptome assembly and cromprehensvive annotation of two tree tomato cultivars (*Solanum betaceum* Cav.) with different fruit color

Chapter 1

Moderate and severe water stress effects on morphological and biochemical traits in a set of pepino (*Solanum muricatum*) cultivars

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PhD candidate contribution

Juan Pacheco had a main role in the following activities: conceived and designed the research, performed the experiments, analyzed the results, wrote the manuscript, and was responsible for the verification of the paper.

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1 Abstract

The pepino (*Solanum muricatum*) is a neglected crop from the Andean region with potential for expansion to many areas of the world. However, there is a lack of studies in pepino related to its response to water stress. In this study, we have subjected plantlets of seven pepino cultivars (Mur1-Mur7) to three treatments consisting of a fully irrigated control (C), a moderate water stress (WS-M), and a severe water stress (WS-S). Thirty-one traits related to growth, photosynthetic pigments, mono and divalent ions, osmolytes and antioxidants were measured. Significant differences were found among cultivars for most traits. The WS-M treatment did not affect most growth and biochemical parameters, while large differences with respect to the control were observed with the WS-S treatment. In general, the WS-S treatment induced an inhibition of the growth parameters, mainly the reduction of the fresh weight of leaves, stems and roots, as well as their water content. A principal component analysis (PCA) performed on the relative values of growth traits, together with the ANOVA for the traits for which significant interaction cultivar × treatment was detected, showed that cultivars Mur2 and Mur4 are the most tolerant to water stress. Although no clear-cut differences were observed among cultivars, the water-stressed plants of Mur2 and Mur4 displayed less variation with respect to the control than the other cultivars for the physiological and biochemical traits measured. Overall, photosynthetic pigments, malondialdehyde and total flavonoids decreased under severe

water stress, while proline, Na^+ and K^+ contents increased significantly. The results obtained provide relevant information on the response to drought of pepino and have allowed identifying two cultivars better adapted to water stress that could be useful in breeding pepino for drought tolerance.

Keywords: *Solanum muricatum*; Drought; Water stress; Photosynthetic pigments; Ions; Osmolytes; Antioxidant compounds

2 Introduction

The pepino (*Solanum muricatum* Aiton) is a neglected solanaceous crop from the Andean region with great potential, both for domestic markets and as an emerging crop in other regions of the world (Gurung et al., 2016). The pepino is a diploid (2n = 2x = 24), grown for its edible fruits and displays a great morphological variability amongst cultivars for fruit weight, shape and colour (Anderson et al., 1996; Herraiz et al., 2016). Pepino fruits have a high water content (92% of fresh weight) and are low in calories (250 kcal/kg) (Adrián Rodríguez-Burruezo et al., 2011b). At maturity, it has a characteristic mild sweet flavour and intense fruity aroma (Prohens et al., 2005). The pepino fruit is usually eaten as fresh juicy fruit, although some cultivars are used in vegetable salads due to their higher acidity content and herbaceous flavour (Prohens et al., 2002). Different studies found that pepino displays antioxidant, antidiabetic, anti-inflammatory and antitumor properties (Hsu et al., 2011; 2018; Shathish and Guruvayoorappan, 2014; Sudha et al., 2011; Virani et al., 2020; Wang et al., 2019; Yue et al., 2019, 2020). One of the most interesting features of pepino is its close phylogenetic relationship with the major crops potato and tomato (Särkinen et al., 2013; Spooner et al., 1993).

The pepino has traditionally been grown in the Andean zone in temperate climates and generally in the absence of drought stress (Prohens et al., 1996). However, its cultivation has been

introduced in Mediterranean-type areas where the availability of water is a limiting factor, which is likely to be aggravated by climate change. Until now, not many studies have been performed on the response of pepino to drought (Duman and Sivaci, 2015). However, several studies exist on its performance under salinity conditions (Pluda et al., 1993; 2019; Prohens et al., 2003).

Determining the biochemical responses of pepino plants against drought stress is of great relevance for the development of cultivation techniques and for the selection and breeding programmes that allow a better crop management and the development of varieties with greater tolerance to drought (Fang and Xiong, 2015; Fita et al., 2015). However, to our knowledge, the biochemical responses of pepino to drought stress and the intraspecific variation in these responses have not yet been studied. Consequently, there is no information on biochemical tolerance markers that can be used as predictors of drought tolerance in pepino.

Metabolites and enzymes involved in the general responses of plants to water deficit are suitable candidates to be used as biochemical markers to assess the relative degree of drought tolerance of different cultivars. They include photosynthetic pigments, such as chlorophylls and carotenoids, which often decrease in drought-stressed plants, accompanying the inhibition of photosynthesis generally observed under stress (Batra et al., 2014; Kumar et al., 2017a; Reis et al., 2020; Szekely-Varga et al.,

2020). Also, different inorganic and organic osmolytes accumulate in plant cells to maintain the cell turgor pressure under stress conditions, such as drought or salinity, that cause cell dehydration (Seki et al., 2007; Singh et al., 2017; Al Hassan et al., 2016). Abiotic stress also induces, directly or as a secondary effect, oxidative stress in plants, which can be quantified by measuring the levels of specific markers (Del Rio et al., 2005; Kar, 2011). As a defence against oxidative stress, plants activate antioxidant systems; therefore, increases in the specific activities of antioxidant enzymes and/or the concentrations of antioxidant compounds are frequently observed in drought-stressed plants (Das and Roychoudhury, 2014; Kozminska et al., 2019; Plazas et al., 2019).

In this work, we have evaluated the response to water stress in seven pepino cultivars subjected to three different treatments under controlled greenhouse conditions: well-watered plants (control) and two degrees of water stress (reduction or complete withholding of irrigation). Once the treatments were finished, the plants were evaluated for growth parameters and photosynthetic pigments (chlorophyll a, chlorophyll b and total carotenoids) levels. Mono (Na^+, K^+, Cl^-) and divalent (Ca^{2+}) ion contents were measured in roots, stems and leaves, and leaf concentrations of proline (Pro) and total soluble sugar (TSS) (common plant osmolytes), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) (oxidative stress biomarkers), and total phenolic

compounds (TPC) and total flavonoids (TF) (representative antioxidant compounds) were also quantified. The final objective of this work was to determine the responses to water deficit in pepino and to evaluate the possible differences amongst varieties in these responses. These results will provide relevant information to better understand the drought-tolerance mechanisms in this species and may allow the identification of biochemical markers for the selection of cultivars more tolerant to this abiotic stress.

3 Materials and methods

3.1 Plant material and experimental design

The pepino cultivar '37-A' (Mur1), originating from Ecuador, and the improved varieties 'Sweet Round' (Mur2), 'Valencia' (Mur3), 'Turia' (Mur4), 'El Camino' (Mur5), 'Sweet Long' (Mur6), and 'Puzol' (Mur7), developed through different breeding programmes in Spain and New Zealand (Murray et al., 1992; Prohens et al., 2002; 2004; Rodríguez-Burruezo et al., 2004), were used for this study. These seven cultivars were selected based on their agronomic interest and genetic, phenotypic and composition diversity (Blanca et al., 2007; Herraiz et al., 2015; 2016) (Supplementary Data S1).

All the cultivars are maintained at the Solanaceae breeding laboratory at the COMAV, Universitat Politècnica de València (UPV; Spain). Pepino cultivars were vegetatively propagated *in*

vitro and, after acclimatization, were transplanted to individual thermoformed pots (with a diameter in the upper part of 14.5 cm and 1.3 L capacity) containing commercial growing substrate N3 (Klasmann-Deilmann, Saterland, Germany). The plants were grown in a benched greenhouse with controlled environmental conditions. During the experiment, temperatures ranged between 17ºC and 30ºC, and humidity between 50% and 80%. After an initial period of three weeks in which the plants were watered to field capacity three times a week on Monday, Wednesday and Friday (starting the watering on a Wednesday) and when the plants reached the phenological stage 19 (nine or more leaves on the main shoot unfolded) of the specific pepino BBCH (Biologische Bundesanstalt, Bundessortenamt, CHemische Industrie) scale (Herraiz et al., 2015), three watering treatments were applied: control (C), moderate water stress (WS-M), and severe water stress (WS-S). Control and WS-M plants were irrigated with water (300 and 100 mL per pot, respectively) three times a week. Runoff water was freely allowed through the holes in the bottom of the pots, although for the WS-M plants no runoff was observed. The WS-S water stress treatment consisted of the complete withholding of irrigation during the entire treatment period. Treatments were carried out for 19 days, with five replicates per cultivar and treatment arranged in a completely randomized design in the same greenhouse. The moisture of the substrate (% vol) was measured at the start of the experiment and at each irrigation date, just before the irrigation, with a WET-2

sensor (Delta-T Devices, Cambridge, United Kingdom). Traits measured in the plants at the end of the experiment are indicated in Table 1.

Table 1. List of the 31 traits with abbreviations and units used for the morphoagronomic and biochemical characterization measured in the seven pepino cultivars assessed in this study.

Code	Trait	Scale/Unit
Growth		
NL	Number of leaves	unit
SL	Stem length	cm
SD	Stem diameter	cm
RL	Root length	cm
LFW	Leaf fresh weight	g
SFW	Stem fresh weight	g
RFW	Root fresh weight	g
LWC	Leaf water content	%
SWC	Stem water content	%
RWC	Root water content	%
Photosynthtetic pigments		
Chl a	Chlorophyll a	mg g^{-1} DW
Chl b	Chlorophyll b	mg g^{-1} DW
Caro	Carotenoids	mg g^{-1} DW
Mono and divalent ions		
Na^+l	Sodium concentration in leaves	µmol g^{-1} DW
Na^+s	Sodium concentration in stems	µmol g^{-1} DW
Na^+r	Sodium concentration in roots	µmol g^{-1} DW
K^+l	Potassium concentration in leaves	µmol g^{-1} DW
K^+s	Potassium concentration in stems	µmol g^{-1} DW

Continued

K^+r	Potassium concentration in roots	µmol g^{-1} DW
Cl^-l	Chlorine concentration in leaves	µmol g^{-1} DW
Cl^-s	Chlorine concentration in stems	µmol g^{-1} DW
Cl^-r	Chlorine concentration in roots	µmol g^{-1} DW
$Ca^{2+}l$	Calcium concentration in leaves	µmol g^{-1} DW
$Ca^{2+}s$	Calcium concentration in stems	µmol g^{-1} DW
$Ca^{2+}r$	Calcium concentration in roots	µmol g^{-1} DW
Osmolytes		
Pro	Proline	µmol. g^{-1} DW
TSS	Total soluble sugars	mg eq. glucose g^{-1} D
Antioxidants		
MDA	Malondialdehyde	nmol g^{-1} DW
H_2O_2	Hydrogen peroxide	µmol g^{-1} DW
TPC	Total phenolic compounds	mg eq. GA g^{-1} DW
TF	Total flavonoids	mg eq. C g^{-1} DW

3.2 Growth parameters

The number of leaves (NL), stem length (SL), stem diameter (SD), and root length (RL) were measured at the end of the treatments (Table 1). Immediately after the experiment was finished, leaves, stems and roots were collected separately and weighed for obtaining fresh weight (LFW, SFW, and RFW, respectively). A fraction of the fresh material was stored at -80 ºC, and samples of the three organs were dried for 72 h in an oven

at 65 ºC until a constant weight was achieved and then weighed again to calculate the dry weight (DW) of leaves, stems and roots (LDW, SDW and RDW, respectively). Water content percentage of each plant part (LWC, SWC and RWC), was calculated as follows (Gil et al., 2014): WC (%) = [(FW - DW/FW] × 100.

3.3 Photosynthetic pigments contents

Chlorophylls a and b (Chl a, Chl b) and total carotenoids (Caro) were determined following the protocols described by Lichtenthaler and Welburn (1983). To extract the pigments, 0.05 g of fresh leaf material was ground in 1 mL of ice-cold 80% (v/v) acetone and mixed. After centrifuging for 15 min at 13,300 *g* and 4 ºC, the supernatant was collected and its absorbance was measured at 663, 646, and 470 nm. Chl a, Chl b, and Caro concentrations were calculated following Lichtenthaler and Welburn (1983) equations and expressed as mg g^{-1} DW. Determination of photosynthetic pigments, as well as all other UV/visible spectrophotometric assays described below, were carried out using a UV-1600PC spectrophotometer (VWR, Shanghai, China).

3.4 Ion content measurements

Contents of mono (Na^+, K^+, Cl^-) and divalent (Ca^{2+}) ions in leaves, stem and roots were determined according to Weimberg (1987), from 0.05 g of ground dry plant material mixed with 15 mL of deionised water. The samples were incubated at 95 ºC for

15 min in a water bath, cooled to room temperature and filtered through a 0.45 µm nylon filter (Gelman, NY, USA). Na^+, K^+ and Ca^{2+} concentrations were quantified with a PFP7 flame photometer (Burlington, VT, USA) and Cl^- with a chloride analyser (Sherwood, Cambridge, UK).

3.5 Osmolyte quantification

Proline (Pro) was extracted from 0.05 g dry leaf material with 2 mL of a 3% (w/v) aqueous sulphosalicylic acid solution and was quantified according to Bates et al. (1973). The extract was subsequently mixed with acid ninhydrin solution, incubated for 1 h at 95 ºC, cooled on ice and then extracted with two volumes of toluene. Absorbance of the organic phase was measured at 520 nm using toluene as a blank. Reaction mixtures containing known amounts of Pro were run in parallel to obtain a standard curve. Pro concentration was expressed as µmol g^{-1} DW.

Total soluble sugars (TSS) contents were quantified following the method of Dubois, et al. (1956), mixing 0.05 g of fresh leaf material with 3 ml of 80% (v/v) methanol on a rocker shaker for 24 h. The extract was recovered by centrifugation, concentrated sulphuric acid and 5% phenol were added to the supernatant and the absorbance was measured at 490 nm. TSS contents were expressed as equivalents of glucose, used as the standard (mg eq. glucose g^{-1} DW).

3.6 Oxidative stress biomarkers and antioxidant compounds

Malondialdehyde (MDA) content was determined following the method of Hodges et al. (1999;), with some modifications (Taulavuori et al., 2001), using the same 80% methanol extracts prepared for TSS quantification. The samples were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA), (or with 20% TCA without TBA for the controls), and then incubated at 95 ºC for 20 min. After stopping the reaction by cooling the samples on ice and centrifugation at 13,300 *g* for 10 min at 4 ºC, the supernatant absorbance was measured at 532 nm. MDA concentration was calculated using the equations described in Taulavuori et al. (2001) subtracting the non-specific absorbance at 600 and 440 nm.

The hydrogen peroxide content (H_2O_2) was determined according to a previously published method (Loreto and Velikova, 2001). H_2O_2 was extracted from 0.05 g fresh leaf material with a 0.1% (w/v) trichloroacetic acid (TCA) aqueous solution, followed by centrifuging the extract at 13,300 *g*. The supernatant was thoroughly mixed with one volume of 10 mM potassium phosphate buffer (pH 7) and two volumes of 1 M KI. The absorbance of the sample was recorded at 390 nm. H_2O_2 concentrations were expressed as μmol g^{-1} DW.

Total phenolic compounds (TPC) were quantified in leaf methanol extracts by their reaction with sodium bicarbonate and

the Folin–Ciocalteu reagent (Blainski et al., 2013). After 90 min of incubation at room temperature in the dark, the absorbance of the samples was measured at 765 nm. TPC concentrations were expressed as equivalents of gallic acid (GA), used as the standard (mg eq. GA g^{-1} DW).

Total flavonoids (TF) were measured by the method described by Zhishen et al. (1999), based on the nitration with $NaNO_2$ of aromatic rings carrying a catechol group, followed by reaction with $AlCl_3$ at alkaline pH. Absorbance was measured at 510 nm, and the concentration of flavonoids was expressed in equivalents of the standard catechin (C) (mg eq. C g^{-1} DW).

3.7 Data analyses

Statistical analysis was performed using a two factorial ANOVA, with cultivar and water stress treatments as main effects for all the parameters. Interactions between the effects (cultivar × treatment) were also analysed. The significance of differences ($p<0.05$) was assessed with Student-Newman-Keuls multiple range tests. For traits in which no interaction was observed, the main effects of the cultivar and treatment are presented in tables, whereas for those traits for which the interaction cultivar × treatment was significant, figures displaying the interaction are also included. To identify the most tolerant cultivars, for each cultivar the relative mean values of the WS-M and WS-S treatments in relation to the control were calculated. Subsequently, a principal component analysis (PCA) was

performed on these data using two R packages: FactoMineR (Lê et al., 2008) to compute PCA, and factoextra package (Kassambara, 2015) for extracting and visualising the results.

4 Results

4.1 Substrate moisture analysis

The moisture of the substrate in the pots showed the expected oscillations, according to the watering schedule. For the control plants, it was maintained at high levels during the 19 days of the treatment, with an average value of 61.6% at the end of the experiment (Figure 1). In contrast, for the WS treatments, the substrate moisture level suffered a sharp decrease during the first week, with a more pronounced reduction in the WS-S treatment, as compared to the WS-M treatment. After the water stress treatments, average moisture values of the substrate for WS-S and WS-M were of 7.8% and 18.8%, respectively. Within each treatment, all cultivars showed a similar pattern of temporal evolution of the pot substrate moisture (Figure 1).

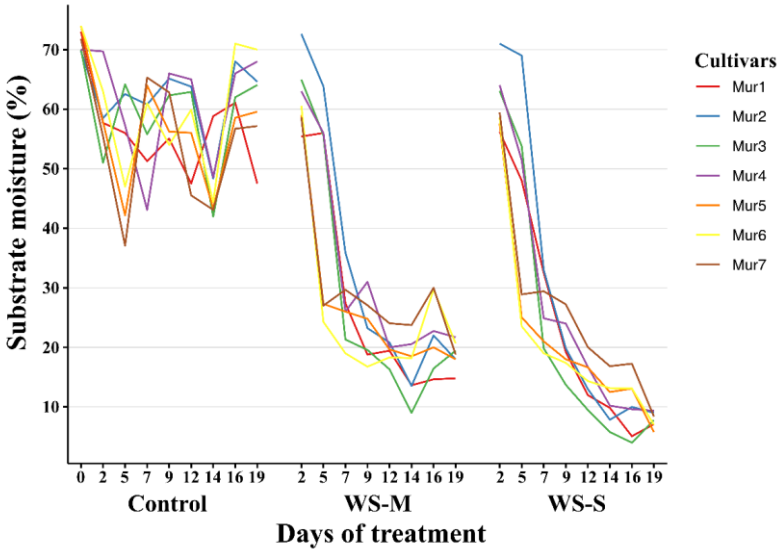

Figure 1. Average percentage of substrate moisture measured every two or three days for the control, moderate water stress (WS-M) and severe water stress (WS-S) treatments during the 19 days of the experiment.

4.2 Analysis of variance

The ANOVA revealed significant differences for both, cultivar and treatment main factors (Table 2). Out of the 31 traits analysed, 25 displayed significant differences for the cultivar effect and 17 for the treatment effect. For growth parameters, significant differences among cultivars were observed for all traits, except for the water content in leaves (LWC), stems (SWC), and roots (RWC). Similarly, differences between water stress treatments were highly significant for all growth

parameters, except for the number of leaves (NL) and the stem length (SL) (Table 2).

Table 2. Two-way factorial ANOVA (F-values) for the traits measured in seven pepino cultivars under three drought stress treatments.

Trait	Cultivar	Treatment	Cultivar × treatment
Growth			
NL	16.65^{***}	3.09^{ns}	1.90^{*}
SL	9.73^{***}	2.16^{ns}	0.71^{ns}
SD	19.12^{***}	40.79^{***}	2.48^{**}
RL	9.52^{***}	10.95^{***}	1.30^{ns}
LFW	10.34^{***}	61.63^{***}	2.23^{*}
SFW	6.44^{***}	26.89^{***}	1.47^{ns}
RFW	11.19^{***}	49.69^{***}	1.50^{ns}
LWC	1.00^{ns}	36.05^{**}	1.01^{ns}
SWC	0.96^{ns}	29.04^{***}	0.65^{ns}
RWC	1.78^{ns}	72.26^{***}	0.99^{ns}
Photosynthtetic pigments			
Chl a	3.28^{**}	6.03^{**}	1.30^{ns}
Chl b	4.10^{***}	5.17^{***}	1.56^{ns}
Caro	2.41^{*}	22.87^{***}	1.00^{ns}
Mono and divalent ions			
Na^{+}l	7.75^{***}	0.43^{ns}	0.61^{ns}
Na^{+}s	16.88^{***}	2.04^{ns}	1.15^{ns}
Na^{+}r	1.59^{ns}	5.29^{**}	1.55^{ns}
K^{+}l	19.33^{***}	0.15^{ns}	0.72^{ns}
K^{+}s	11.80^{***}	13.78^{***}	4.08^{***}
K^{+}r	4.08^{**}	24.45^{***}	1.96^{*}
Cl^{-}l	1.51^{ns}	0.77^{ns}	1.21^{ns}
Cl^{-}s	9.16^{***}	1.67^{ns}	2.03*
Cl^{-}r	5.68^{***}	3.03^{ns}	1.01^{ns}
Ca^{2+}l	26.39^{***}	1.46^{ns}	0.56^{ns}
Ca^{2+}s	4.68^{***}	2.39^{ns}	1.69^{ns}

Coninued

$Ca^{2+}r$	2.52*	1.15^{ns}	1.81^{ns}
Osmolytes			
Pro	4.78^{***}	8.27^{***}	1.95^{*}
TSS	12.92^{***}	0.20^{ns}	0.98^{ns}
Antioxidants			
MDA	2.30^{*}	10.50^{***}	0.48^{ns}
H_2O_2	2.05^{ns}	3.03^{ns}	1.46^{ns}
TPC	6.10^{***}	1.40^{ns}	1.80^{ns}
TF	76.82^{***}	11.53^{***}	8.37^{***}

^{ns}, *, **, *** indicate non-significant or significant at $p < 0.05$, 0.01, and 0.001, respectively.

For biochemical traits, significant differences between cultivars were observed for all parameters, except for the concentrations of Na^+ in roots, Cl^- in leaves, and H_2O_2. Contrarily, no significant differences were observed for most traits for the 'treatment' factor, except for Na^+ and K^+ in roots, K^+ in stems, proline (Pro), malondialdehyde (MDA), and total flavonoids (TF), which were found to be significant (Table 2). Significant differences were also found for the interactions between the 'cultivar' and 'treatment' factors, for three growth (NL, SD, and LFW) and five biochemical (K^+ in stems, K^+ and Cl^- in roots, Pro, and TF) traits.

4.3 Growth traits and identification of tolerant accessions

The results of the analysis of the mean effects on growth parameters of the factors 'cultivar' and 'treatment' are shown in Table 3. The number of leaves (NL) at the end of the experiment varied greatly in the seven selected cultivars, ranging from 16.8

leaves in Mur6 to 41.1 in Mur3, whereas no significant differences were observed between treatments (Table 3). Significant differences between cultivars were also observed for both stem parameters (SL and SD), being Mur2 and Mur6 the cultivars that registered the longest and the shortest stems, respectively; on the other hand, Mur1 to Mur5 had the broadest stem diameter and Mur7 the thinnest one (Table 3). Stem diameter (SD), but not stem length (SL), exhibited notable differences between water stress treatments. In this way, the average reductions of SD with respect to the control were 8.4% for WS-M and 33.4% for WS-S. Significant differences were found for root length between cultivars and also between treatments. Cultivars, Mur1, Mur2 and Mur3 had on average longer roots than those of the rest of the cultivars. The WS-M treatment resulted in significantly longer roots than the control and WS-S plants, with no differences between these latter groups (Table 3).

Fresh weight of leaves, stem and roots displayed some significant differences among cultivars, as well as between treatments (Table 3). For most cultivars, only small, generally non-significant differences were observed in the fresh weights of the three organs, with some exceptions; for example, LFW was significantly higher in Mur3 than in all other cultivars, whereas Mur6 showed the lowest LFW, SFW and RFW values. On the other hand, considerable water stress-induced effects were observed for the

WS-S treatment, leading to an average FW reduction of 64.5% in leaves, 56.9% in stems, and 73.5% in roots, compared to the corresponding controls; however, no significant differences were observed between the control and WS-M treatments.

Table 3. Mean effect of cultivar and treatment and the average standard error (SE) from the analysis of variance for growth and photosynthetic pigment traits in seven cultivars of pepino (Mur1 to Mur7) subjected to three drought stress treatments (Control; moderate water stress WS-M; severe water stress, WS-S). Different lowercase letters denote significant means differences within cultivar or treatments according to the Student-Newman-Keuls multiple range test ($p < 0.05$).

Factor	NL (n)	SL (cm)	SD (cm)	RL (cm)	LFW (g)	SFW (g)	RFW (g)	LWC (%)	SWC (%)	RWC (%)	Chl a (mg g-1 DW)	Chl b (mg g-1 DW)	Caro (mg g-1 DW)
Cultivar													
Mur1	24.5 bc	22.1 c	4.46 c	30.8 b	9.54 b	3.53 c	2.27 d	73.2 a	77.6 a	70.3 a	7.35 a	1.74 a	0.74 a
Mur2	23.7 bc	26.2 d	4.31 c	34.2 b	8.63 b	3.07 c	2.04 cd	84.1 a	84.3 a	76.3 a	16.07 b	6.19 b	0.66 a
Mur3	41.1 d	21.4 bc	4.49 c	32.9 b	13.49 c	2.98 c	2.35 d	80.3 a	79.3 a	70.6 a	17.02 b	6.53 b	0.66 a
Mur4	30.1 c	19.1 bc	3.40 c	23.0 a	9.05 b	2.79 c	1.59 bc	76.1 a	76.4 a	73.6 a	10.57 ab	4.45 ab	0.69 a
Mur5	25.8 bc	17.6 ab	4.09 c	24.2 a	7.40 b	1.73 ab	1.23 ab	76.4 a	76.7 a	65.4 a	11.88 ab	4.43 ab	1.03 b
Mur6	16.8 a	15.1 a	2.93 ab	20.0 a	4.70 a	1.39 a	0.77 a	72.3 a	76.2 a	65.1 a	11.14 ab	4.32 ab	0.62 a
Mur7	20.9 ab	19.7 bc	2.57 a	23.8 a	9.46 b	2.52 bc	0.97 a	80.2 a	80.7 a	68.7 a	16.45 b	6.38 b	0.68 a
SE	1.94	1.14	0.18	1.79	0.84	0.30	0.19	4.26	2.98	3.01	2.06	0.86	0.09
Treatment													
Control	27.6 a	21.1 a	4.36 c	24.3 a	11.37 b	3.25 b	2.23 b	86.6 b	84.7 b	83.2 c	14.81 b	5.20b	0.92 b
WS-M	27.2 a	20.4 a	3.99 b	31.5 b	11.29 b	3.06 b	1.99 b	87.4 b	84.7 b	75.4 b	14.85 b	5.94b	0.86 b
WS-S	23.6 a	19.0 a	2.90 a	25.2 a	4.03 a	1.40 a	0.59 a	58.5 a	66.8 a	51.4 a	9.13 a	3.45a	0.41 a
SE	1.27	0.75	0.12	1.18	0.55	0.20	0.13	2.79	1.95	1.97	1.35	0.57	0.06

The water content in leaves, stems and roots did not differ significantly between the seven cultivars (Table 3). The WS-S treatment had a strong impact on the water content of leaves and stems, with a 32.4% and 21.0% reduction, respectively, comparing to the corresponding controls. The WS-M treatment, on the other hand, only caused a significant decrease in the water content of roots, amounting to 9.4% of the control.

The effects of the WS treatments on those growth parameters for which a significant cultivar × treatment interaction was observed in the ANOVA, namely NL, SD and LFW (Table 2), are shown in Figure 2 for all cultivars. Regarding NL, no significant differences between the control and the water stress treatments (WS-M and WS-S) were observed in any of the cultivars except in Mur3, for which both, moderate and severe water deficit resulted in a substantial reduction of leaf number (up to 36.4%) (Figure 2A). For the stem diameter (SD), the WS-M treatment promoted a significant reduction only in Mur1 (13.8% of the control) and Mur7 (30.6%), whereas the WS-S treatment had a strong effect in all cultivars, particularly in Mur5 with a reduction of more than 50% of the control, except in Mur3 and Mur4 (Figure 2B). Finally, leaf fresh weight (LFW) did not vary significantly in any of the seven cultivars, when comparing the well-watered controls and the plants subjected to the moderate water stress treatment. On the other hand, LFW decreased in most cultivars, in relation to the corresponding control, under WS-S

conditions; the strongest reduction was observed in Mur5 (78.5%), followed by Mur1 (75.8%), Mur3 (71.9%) and Mur7 (70.2%). Mur2 and Mur4 were the only cultivars which did not display significant differences for LFW between the control and the WS-S treatments (Figure 2C).

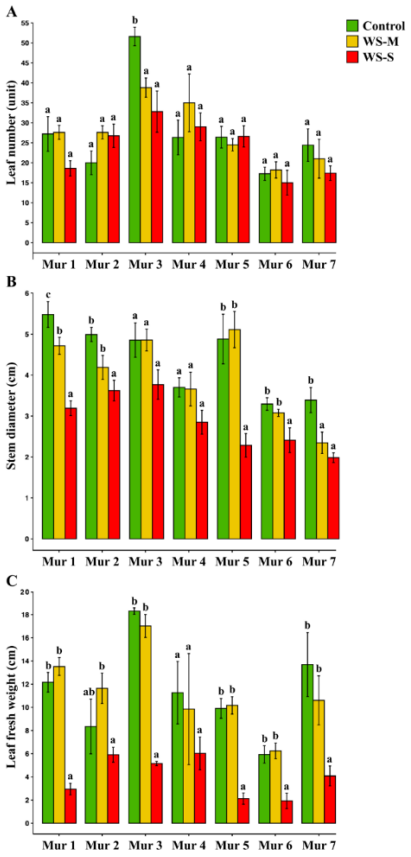

Figure 2. Growth parameters that exhibited significant cultivar × treatment interactions. (A) number of leaves, (B) stem diameter and (C) leaf fresh weight in seven pepino cultivars after 19 days of treatment as mean values with SE (n = 5) for the control (green bars), moderate water stress (WS-M) (yellow bars), and severe water stress (WS-S) (red bars) treatments. Different lowercase letters above the bars indicate significant differences between treatments for each cultivar, according to the Student-Newman-Keuls multiple range test ($p < 0.05$).

The principal component analysis (PCA) for plant growth and water content traits, which allows the combined study of all traits in a single multivariate analysis, discriminated tolerant and sensitive cultivars. The first and second components (PC1 and PC2) performed on the relative values of growth and water content traits of the WS-M and WS-S treatments (expressed as percentages of the corresponding controls) accounted, respectively, for 69.1% and 14.9% of the total variation (Figure 3A). The variables that most contributed to the PC1 were those related to the water content and the fresh weight of the three tissues measured (leaf, stem and root), as well as the stem diameter (SD), which displayed high negative correlations ($r<-0.75$) with the PC1 (Figure 3A). Regarding PC2, the number of leaves (NL) and stem length (SL) were negatively correlated with this component and displayed the highest absolute values ($r<-0.60$) for the correlation with PC2 (Figure 3A). The PC1 clearly separated the two treatments, with the WS-S treatment being positively correlated with PC1, while the WS-M treatment was negatively correlated with PC1 (Figure 3B). The two cultivars of the WS-S treatment with the lowest values for the PC1 (i.e., associated with the smallest reduction of fresh weight and water content) were Mur2 and Mur4. Regarding PC2, these two latter cultivars were also associated with the lowest reduction of the number of leaves (NL) and stem length (SL) under both, WS-M and WS-S treatments. The PCA data, together with the ANOVA analyses for the traits for which significant interaction cultivar ×

treatment was detected, indicates a greater tolerance to water stress of cultivars Mur2 and Mur4.

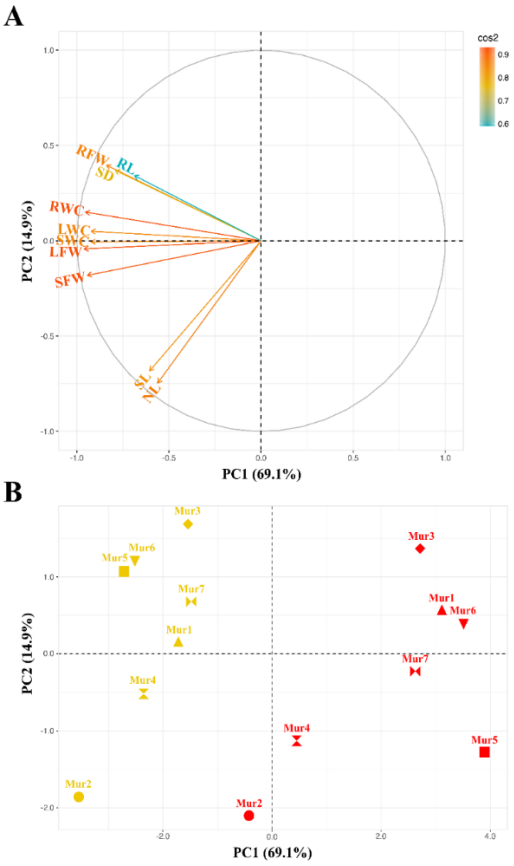

Figure 3. Principal component analysis (PCA) similarities based on the characterization of the values of 10 growth-related traits, expressed as percentages of the corresponding controls, of seven cultivars of pepino under moderate (WS-M) and severe (WS-S) water stress treatments. A)

The variable correlation plot indicates the relationships between variables and the PC1 and PC2. Variables that are close to the circumference are more correlated to the first two PCs and those that are grouped together are positively correlated among them. B) Graph of cultivars under moderate water stress (WS-M) (yellow symbols), and severe water stress (WS-S) (red symbols) treatments. Each symbol represents one cultivar.

4.4 Photosynthetic Pigments

Regarding photosynthetic pigments (chlorophyll a, chlorophyll b and total carotenoids), significant differences were observed between the cultivars (Table 3). Mean Chl a and Chl b contents were lowest in Mur1 (7.35 and 1.74 mg g^{-1} DW, respectively) and highest in Mur3 (17.02 and 6.53 mg g^{-1} DW). Carotenoids concentrations in Mur5 were significantly higher than in the rest of cultivars. Regarding water stress treatments, no significant differences in the contents of the three pigments were observed between the control and the WS-M treatment, whereas the WS-S treatment resulted in significant reductions of their concentrations: 38.3%, 33.6% and 55.4% with respect to the corresponding controls, for Chl a, Chl b and Caro, respectively (Table 3).

4.5 Ion Accumulation

The mean concentrations of the monovalent (Na^+, K^+, Cl^-) and divalent (Ca^{2+}) ions in leaves, stems and roots generally varied

between cultivars, with some exceptions. For example, the contents of Na^+ in roots or Cl^- in leaves did not differ significantly in the seven selected cultivars; on the other hand, Mur1 showed Ca^{2+} concentrations in roots significantly lower than in all other cultivars, whereas the highest levels of this cation in leaves were measured in Mur2 (Table 4). Despite differences among specific cultivars, some common trends were maintained; most important, the concentrations of all ions were much higher in the aerial part of the plants than in the roots, and in all cases (except Ca^{2+} in Mur1) the ions accumulated predominantly in the stems, reaching levels ranging from 1.7 to 3.2-fold (for Na^+), 2.2 to 4.5-fold (for K^+), 4 to 7-fold (for Cl^-) or 3.3 to 15-fold (for Ca^{2+}) higher than in the roots, depending on the cultivar (Table 4).

When considering the main effects of the water stress treatments on the mean contents of the different ions in leaves, stems and roots, under moderate water stress conditions (WS-M treatment) no significant differences with the controls were found for any of the ions, in any organ, except for K^+ in roots (1.3-fold higher than in the well-watered control). Regarding the WS-S treatment, significant differences were only observed in roots for Na^+ (1.3-fold higher than in the control) and K^+ (1.7-fold) (Table 4).

Table 4. Mean effect of cultivar and treatment and the average standard error (SE) from the analysis of variance for mono- and divalent ion contents (µmol g^{-1} DW) in stem (s), leaves (l) and roots (r) in seven cultivars of pepino (Mur1 to Mur7) subjected to three drought stress treatments (Control; moderate water stress WS-M; severe water stress, WS-S). Different lowercase letters denote significant means differences within cultivars or treatments according to the Student-Newman-Keuls multiple range test ($p < 0.05$).

Factor	Na^+l	Na^+s	Na^+r	K^+l	K^+s	K^+r	Cl^-l	Cl^-s	Cl^-r	$Ca^{2+}l$	$Ca^{2+}s$	$Ca^{2+}r$
Cultivar												
Mur1	1,432 a	3,534 b	1,088 a	629 ab	2,007 c	544 ab	2,254 a	7,034 ab	1,007 ab	75.6 a	108.5 b	6.9 a
Mur2	2,069 ab	4,340 c	1,345 a	786 b	1,358 a	606 b	1,699 a	4,836 a	1,198 b	181.6 b	88.6 ab	17.7 b
Mur3	1,771 ab	3,609 b	1,224 a	777 b	1,300 a	464 ab	1,738 a	4,025 a	1,040 ab	48.9 a	68.0 a	15.6 b
Mur4	1,652 a	2,319 a	1,271 a	617 ab	1,742 bc	389 a	1,955 a	4,836 a	989 ab	49.0 a	77.5 a	22.5 b
Mur5	1,637 a	2,372 a	1,419 a	503 a	1,801 bc	482 ab	1,721 a	5,110 a	1,023 ab	67.9 a	107.9 b	19.3 b
Mur6	2,069 bc	2,355 a	1,106 a	1,065 c	1,571 ab	387 a	1,616 a	4,318 a	699 a	75.7 a	90.6 ab	20.0 b
Mur7	2,320 c	2,270 a	1,298 a	1,166 c	1,399 a	439 a	2,520 a	4,044 a	899 ab	83.7 a	77.1 a	23.1 b
SE	108	209	96	54.51	80	40	279	359	94	24.1	7.5	2.4
Treatment												
Control	1,832 a	2,915 a	1,094 a	805 a	1,430 a	350 a	1,758 a	4,663 a	937 a	79.5 a	88.3 a	20.2 a
WS-M	1,751 a	2,812 a	1,270 ab	777 a	1,549 a	459 b	1,949 a	4,874 a	946 a	62.8 a	81.1 a	19.3 a
WS-S	1,827 a	3,187 a	1,386 b	793 a	1,812 b	610 c	2,080 a	5,260 a	1,055 a	108.0 a	96.0 a	15.0 a
SE	71	137	63	35.73	52	26	183	235	62	15.8	4.9	1.6

The effects of the WS treatments on the concentration of ions for which a significant cultivar × treatment interaction was observed in the ANOVA (K^+ in stems and roots, and Cl^- in stems, see Table 2) are shown in Figure 4. In the WS-M treatment, K^+ contents in stem increased significantly over the control only in Mur5 (1.6-fold) (Figure 4A); for the WS-S treatment, several cultivars showed a significant increase of K^+ concentrations in the stem: Mur5 (2.1-fold over the control), followed by Mur7 (1.6-fold), and Mur6 and Mur3 (ca. 1.3-fold) (Figure 4A). Under moderate water stress conditions, the concentration of K^+ in the root increased significantly in Mur2 (2.5-fold) and Mur3 (1.6-fold) (Figure 4B). Under WS-S treatment, in addition to Mur2 (ca. 3-fold increase) and Mur3 (1.9-fold), Mur6 also showed a significant increase in K^+ stem contents, approximately 1.3-fold over the well-watered control (Figure 4B). For Cl^- contents in the stem, the only significant differences were observed in the WS-M treatment for cultivars Mur1 and Mur2, which accumulated 1.2 and 1.4-fold more Cl^- than the control, respectively (Figure 4C).

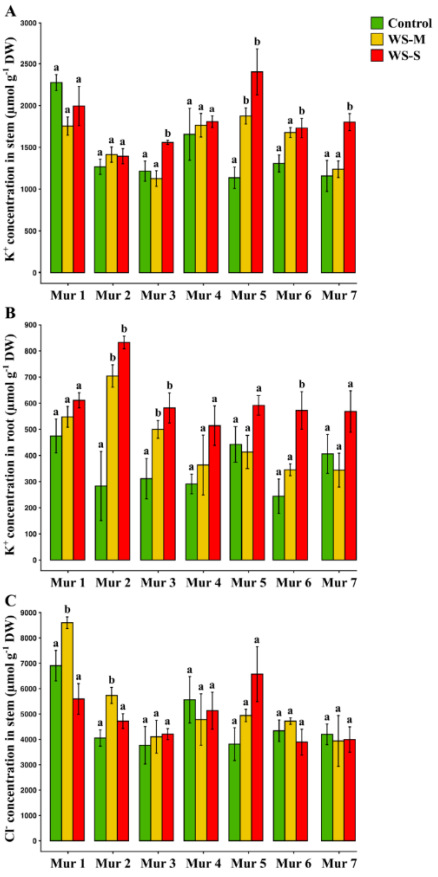

Figure 4. Ions that exhibited cultivar × treatment interactions. (A) Potassium concentration in stem, (B) potassium concentration in root and (C) chlorine concentration in stem in seven pepino cultivars after 19 days of treatment as mean values with SE (n = 5) for the control (green bars), moderate water stress (WS-M) (yellow bars), and severe water stress (WS-S) (red bars) treatments. Different lowercase letters above the bars indicate significant differences between treatments for each cultivar, according to the Student-Newman-Keuls multiple range test ($p < 0.05$).

4.6 Osmolytes, oxidative stress markers and antioxidants

The main effects of the cultivar and treatment factors on the mean values of the analyzed osmolytes, oxidative stress markers and non-enzymatic antioxidants are shown in Table 5. Proline (Pro) was found highly variable in the seven cultivars, ranging from 14.8 μmol g^{-1} DW in Mur3 to 50.5 μmol g^{-1} DW in Mur1, which represents a 3.4-fold difference (Table 5). Average Pro levels varied significantly in the WS-S treatment, compared to the control plants, with an increase of 1.8-fold increase, approximately, whereas no differences were observed in the moderate water stress treatment (Table 5). Total soluble sugar (TSS) concentrations also displayed considerable differences between cultivars, from 54.86 (in Mur1) to 283.66 (in Mur2) mg eq. glucose g^{-1} DW, with intermediate values in the rest of cultivars; however, contrary to Pro, no significant differences were found between the control and water stress treatments (Table 5). MDA concentrations also varied between cultivars, but only about 2-fold, with the minimum value measured in Mur3 and the maximum one in Mur2; MDA contents decreased significantly, by more than 50% of the control, in the WS-S treatment (Table 5). Regarding hydrogen peroxide (H_2O_2) concentrations, no significant differences were detected, either between cultivars or when comparing the control and water stress treatments (Table 5). Significant differences were observed between cultivars for total phenolic compounds (TPC), with Mur6 displaying the highest concentrations and Mur2 and Mur3 the lowest; on the other hand, no significant differences were found for TPC between the

different treatments (Table 5). Finally, total flavonoids (TF) contents did not vary in the pepino cultivars, except for Mur3, which showed a value significantly higher than all others, whereas a significant decrease of TF levels, of about 50% of the control, was detected only in the WS-S treatment (Table 5).

Two of the above traits, Pro and TF contents, showed significant cultivar × treatment interactions (Table 2). Average Pro concentrations increased significantly over the control only in the WS-S treatment, and only for Mur6 (ca. 3.7-fold) and Mur7 (ca. 2.6-fold) (Figure 5A). For TF, no significant differences were observed between the controls and the two WS treatments in most cultivars. However, in cultivar Mur3, which showed a mean control TF concentration about 10-fold higher than those of the remaining cultivars, severe WS conditions resulted in a reduction in TF contents of 65.7% of the control (Figure 5B).

Table 5. Mean effect of cultivar and treatment and the average standard error (SE) for osmolytes and antioxidants in seven cultivars of pepino (Mur1 to Mur7) subjected to three drought stress treatments (Control; moderate water stress WS-M; severe water stress, WS-S). Different lowercase letters denote significant means differences within cultivars and treatments according to the Student-Newman-Keuls multiple range test ($p < 0.05$). GA: gallic acid; C: catechin.

Factor	Pro (µmol. g-1 DW)	TSS. (mg eq. glucose g-1 DW)	MDA (nmol g^{-1} DW)	H_2O_2 (µmol g^{-1} DW)	TPC. (mg eq. GA g^{-1} DW)	TF (mg eq. C g^{-1} DW)
Cultivar						
Mur1	50.51 c	54.9 a	517.6 ab	68.06 a	15.76 bc	1.62 a
Mur2	37.15 abc	283.7 c	553.6 b	31.02 a	9.05 a	3.44 a
Mur3	14.80 a	220.7 bc	257.0 a	75.00 a	7.38 a	15.61 b
Mur4	24.19 ab	223.5 bc	519.0 ab	93.35 a	12.60 abc	1.37 a
Mur5	44.06 bc	233.9 bc	522.3 ab	49.93 a	10.98 ab	1.64 a
Mur6	25.79 ab	198.1 b	492.3 ab	75.03 a	17.47 c	1.40 a
Mur7	30.63 abc	192.2 b	445.5 ab	73.84 a	12.85 abc	1.35 a
SE	5.70	20.30	66.71	13.97	1.46	0.62
Treatment						
Control	25.34 a	203.8 a	583.3 b	81.10 a	10.97 a	4.22 b
WS-M	27.28 a	205.2 a	520.2 b	66.23 a	13.03 a	4.86 b
WS-S	44.72 b	194.3 a	313.9 a	50.71 a	12.90 a	2.25 a
SE	3.74	13.6	43.71	9.15	0.96	0.41

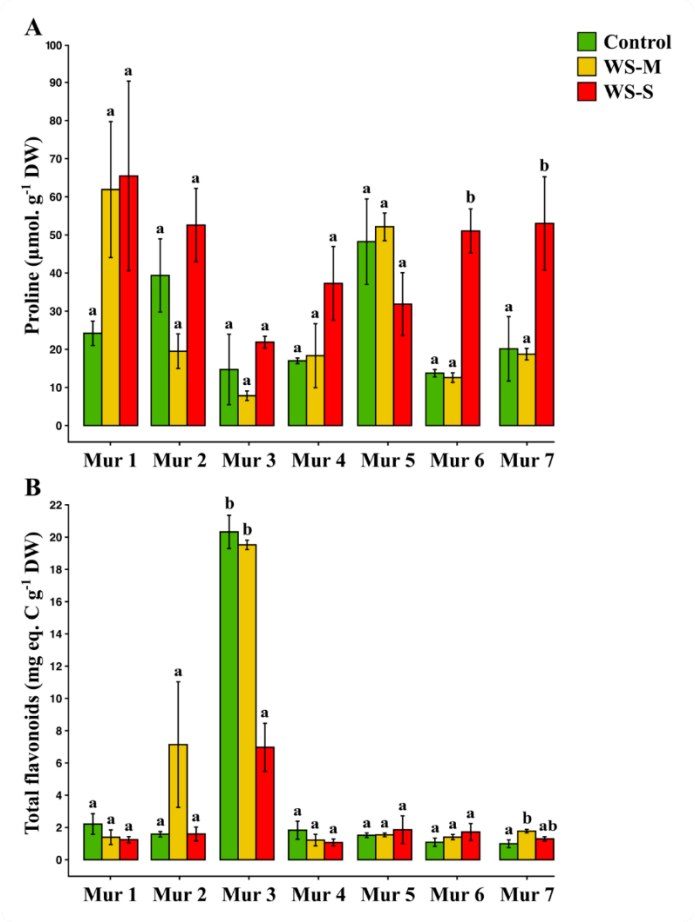

Figure 5. Osmolytes and antioxidants that exhibited cultivar × treatment interactions. (A) Proline and (B) total flavonoids in stem in seven pepino cultivar after 19 days of treatment as mean values with SE (n = 5) for the control (green bars), moderate water stress (WS-M) (yellow bars), and severe water stress (WS-S) (red bars) treatments. Different lowercase letters above the bars indicate significant differences between treatments for each cultivar, according to the Student-Newman-Keuls multiple range test ($p < 0.05$).

5 Discussion

The pepino is a crop with a wide potential for expansion (Kumar et al., 2017b). However, many aspects related to the improvement of cultural practices and its tolerance to stresses, including drought, remain to be elucidated. The expansion of the crop to new areas outside the Andean region (Gurung et al., 2016), as well as the threat of climate change in its region of origin (Buytaert et al., 2010), makes it likely that stress due to drought will become more common in pepino cultivation in the near future. However, very little information is available on pepino responses to drought (Duman and Sivaci, 2015).

Evaluation of diversity for tolerance to drought within pepino genotypes may allow detecting sources of tolerance and could help to identify the most relevant mechanisms of response to water stress in this species. In other crops related to pepino, such as tomato and eggplant, diversity has been observed for tolerance to drought (Plazas et al., 2019; Raja et al., 2020). The work presented here represents the first systematic study of this type in pepino, assessing the effects of water stress on growth and biochemical responses in different pepino cultivars. There is a previously published report (Duman and Sivaci, 2015), which used a similar approach but was much more limited in scope, focused on a single cultivar (‘Miski’), and characterised a narrower range of drought-induced responses. However, even though our study assessed more growth and biochemical parameters, it confirmed some trends observed in Duman and Sivaci (2015), like a decrease in water content, chlorophylls and carotenoids, and an

increase in proline. In contrast, we did not observe a significative increase in the total phenolic compounds and MDA compared with the control; it should be mentioned, however, that the latter responses were observed only after persistent severe drought conditions (Duman and Sivaci, 2015).

In this work, we have found quantitative differences in growth and biochemical parameters, among pepino cultivars, both in control and drought-stressed plants, thus confirming at the physiological and molecular levels the already known high phenotypic and genetic diversity of pepino (Blanca et al., 2007; Herraiz et al., 2016). This opens the door to the exploitation of this diversity for selecting and breeding more drought-tolerant pepino varieties. In our study, in general, no inhibition of growth was observed under moderate water stress conditions (from 16.7 to 26.4% average percentage of substrate moisture from day 7), as no significant differences with the controls (from 46.9% to 71.7% average percentage of substrate moisture) were detected in most measured growth parameters. This led to hypothesise that pepino, in comparison with other crops, is moderately tolerant to drought and therefore lower amount of water can be applied without affecting severely the plant development. An interesting exception refers to root length, which increased significantly in the WS-M treatment; this response appears to mimic the behaviour of the plants in nature, where drought may induce root growth, as roots search for deeper and wetter layers of the soil (Kano et al., 2011). Similarly, with very few exceptions, the moderate water stress treatment did not affect

the contents of the determined biochemical variables, including photosynthetic pigments, ions, osmolytes, oxidative stress biomarkers and antioxidant compounds. On the contrary, the severe stress treatment (WS-S) induced significant differences in several of these biochemical parameters and caused a clear inhibition of growth, mostly reflected in the reduction of the fresh weight of leaves, stems and roots – a reduction partly due to dehydration of the three organs, as a decrease in their water content percentages was also observed. Therefore, as pepino seems to be relatively resistant to moderate water deficit conditions, it is likely that improvements in the water use efficiency of this crop can be achieved with proper irrigation management (Hatfield & Dold, 2019). Also, the analysis of the effects of drought on growth traits in the different cultivars led to the identification of two of them, Mur2 and Mur4, as more drought-tolerant than the rest of accessions, opening the way to the establishment of breeding programmes for tolerance to drought in pepino. The 'Sweet Round' cultivar (Mur2) was developed for being introduced in the Mediterranean climates, showing high productivity (around 30 and up to 67.5 t ha^{-1}), good tolerance to salinity, high levels of soluble solids (10.4%) and ascorbic acid (26 mg 100 g^{-1}) and an excellent flavour, texture and intensive scent. At commercial maturity, on average, fruit weights around 215 g and show yellow flesh and shiny golden-yellow purplish-striped skin, and is consumed mostly as a dessert (J. J. Ruiz et al., 1997), Supplementary Data S1). Contrarily to 'Sweet Round', which is more adapted to protected cultivation, the 'Turia' cultivar (Mur4) has shown good performance in a wide range of cultivations

and environments. Also, it is mostly consumed in salads for its herbaceous-green aroma, firm flesh and medium soluble solids content (7-8º Brix) (Rodríguez-Burruezo et al., 2004). 'Turia' is highly productive (between 50 and 70 t ha^{-1}) and vigorous, and was the first pepino cultivar tolerant to tomato mosaic virus (ToMV), one of the main diseases affecting this crop. Phenotypically, 'Turia' has oval golden purple-striped fruits weighing around 250-350 g and with yellow flesh (Supplementary Data S1). Ascorbic acid is also high, with values between 25 and 35 mg 100 g^{-1}. Both cultivars were developed at the Universitat Politècnica de Valencia.

The general responses of pepino to water deficit treatments, namely, inhibition of growth and degradation of photosynthetic pigments, are shared by other nightshade species, such as tomato and eggplant (Plazas et al., 2019; Raja et al., 2020), and by many other vegetable crops (Abid et al., 2018; Chmielewska et al., 2016; G. Zhou et al., 2018). What is not a general response to water stress in crop species, is the accumulation of monovalent ions, Na^+ and Cl^- (and, to a lesser extent, also K^+) to very high levels in the roots, mostly considering that the plants were grown under low salinity conditions; mean Na^+ and K^+ concentrations increased significantly in response to the water stress treatment. Furthermore, ion concentrations were even higher in the aerial parts of the plants, accumulating predominantly in the stems rather than in the leaves. This points to the presence in pepino of mechanisms for the active uptake by the roots and transport to the aboveground organs of these ions, which could contribute to cellular

osmotic adjustment under water stress conditions and, therefore, to the (relative) drought tolerance of this species. The stem could act as a 'buffer' organ, limiting the transport of the toxic ions to leaf cells. The use of ions, such as Na^+ and Cl^-, as 'inorganic osmolytes' is a general mechanism largely contributing to salt tolerance in dicotyledonous halophytes (Flowers et al., 1977; Flowers and Colmer, 2008) but, in some cases, it has also been observed as a response to drought in drought-tolerant species (Xi et al., 2018). Typical glycophytes, on the contrary, tend to block their transport from the roots to the leaves in response to salt stress (Munns and Tester, 2008). Regarding the divalent cation Ca^{2+}, its participation in multiple stress signalling pathways is well established (Tuteja and Mahajan, 2007; Bose et al., 2011) and could also be involved in drought tolerance mechanisms in pepino, as it accumulates to relatively high levels in the leaves, by active transport from the roots. However, if this is the case, those mechanisms should be constitutive since the water deficit treatments did not induce a significant increase in the average Ca^{2+} concentrations in the plants.

Proline is one of the most common plant osmolytes, accumulating in many species in response to different abiotic stress conditions, including drought; in addition to its role in osmotic adjustment, proline may participate in stress tolerance mechanisms as an osmoprotectant, by the direct stabilisation of proteins and macromolecular structures, as a ROS-scavenger and/or a signalling molecule (Szabados and Savouré, 2010; Akram et al., 2018). In the present study, proline contents

increased in pepino cultivars under severe drought stress conditions. Similar findings have already been reported in many species of different families, such as tomato (Al Hassan et al., 2015; Raja et al., 2020), beans (Morosan et al., 2017), barley (Dbira et al., 2018), Norway spruce (Schiop et al., 2017), or different cultivars of ornamental species of the genus *Tagetes* (Cicevan et al., 2016), to give only a few examples. Since no positive correlation between the increment of proline levels and the relative drought tolerance of the pepino cultivars has been established, it is not clear whether proline is directly involved in the mechanisms of tolerance. In any case, proline could be a useful biochemical marker of water stress in this species, as it has been demonstrated in *Phaseolus vulgaris* cultivars (Arteaga et al., 2020).

Soluble sugars are also functional osmolytes in many different plant species (e.g., Gil et al., 2013; Al Hassan et al., 2016; Plesa et al., 2019). It is, however, unlikely that these compounds play any relevant role in pepino responses to drought, as no significant changes in TSS levels were detected in the water-stressed plants, as compared with the controls. Similarly, the stress treatments did not induce an increase in the concentrations of the tested oxidative stress markers, MDA and H_2O_2; in fact, MDA levels even decreased under severe WS. These data indicate that, under the specific conditions used in our experiments, there was no induction of oxidative stress as a secondary effect of the applied water deficit. Consequently, we also did not detect an increase in the levels of antioxidant compounds.

6 Conclusions

In our experimental conditions, pepino has shown to be relatively tolerant to moderate drought conditions even though it is affected by severe water stress, which was reflected in inhibition of growth, degradation of photosynthetic pigments and changes in several biochemical parameters. More intermediate water stress conditions between the two tested in this study will help to further adjust the water optimum requirements for pepino and study its physiological and biochemical response under drought stress. All tested pepino cultivars responded to water deficit in the same way, qualitatively, as should be expected for closely related genotypes, but with quantitative differences that allowed identifying two specific cultivars, Mur2 and Mur4, as relatively more tolerant to drought. Even though further studies will be required to elucidate the mechanisms of water stress tolerance in pepino, the active uptake of monovalent ions (Na^+, Cl^-, K^+) and their accumulation to very high concentrations in the aboveground organs of the plants, may be involved in those mechanisms, contributing to cellular osmotic adjustment under stress. The differences observed among cultivars in tolerance to water stress and the associated biochemical responses observed are relevant for the selection and breeding of more drought tolerant pepino cultivars.

7 Statements

Supplementary data

Supplementary Data S1: Pictures of leaves and fruits of the seven pepino cultivars assessed in this study, Mur1 (A and H), Mur2 (B and I), Mur3 (C and J), Mur4 (D and K), Mur6 (E and L), Mur6 (F and M), Mur7 (G and N).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 2

Screening of pepino (*Solanum muricatum*) and wild relatives against four major tomato diseases threatening its expansion in the Mediterranean region

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PhD candidate contribution

Juan Pacheco. had a main role in the following activities: conceived and designed the research, performed the experiments, analyzed the results, wrote the manuscript, and was responsible for the verification of the paper.

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1 SUMMARY

The pepino (*Solanum muricatum*) is an Andean vegetable crop closely related to tomato. In the last decades it has been introduced in the Mediterranean region and other parts of the world as a potential new crop. However, several tomato major pathogens may threaten the expansion of pepino cultivation. We identified *Fusarium oxysporum f. sp. lycopersici* (FOL), *Verticillium dahliae* (VE), pepino mosaic virus (PepMV), and tomato mosaic virus (ToMV) as four of the most likely pathogens to cause damage to pepino crops in Mediterranean climates. In order to evaluate the response of the pepino genepool against these pathogens, as well as to identify sources of tolerance, we inoculated six accessions of cultivated pepino, nine accessions of seven pepino wild relatives, and one interspecific hybrid with FOL, VE, PepMV and ToMV and followed its symptomatology for 30 d (FOL and VE) or 60 d (PepMV and ToMV). ELISA tests were also performed for PepMV and ToMV. Susceptible tomato materials were used as controls. The pepino genepool displayed fewer symptoms than susceptible tomato controls after inoculation with FOL, with most accessions being tolerant or resistant. Regarding VE, a wide variation of values for the symptoms index (SI) was observed, with three cultivated pepino accessions displaying tolerance. For PepMV a wide variation for SI was also observed, with one accession of *S. caripense* being resistant, and several accessions of pepino and other wild relatives displaying different degrees of tolerance. PepMV absorbance values obtained by ELISA tests followed a pattern similar to that of SI. For ToMV no resistances were found, although two wild accessions and the

interspecific hybrid displayed low values for the SI and were considered as moderately tolerant. ELISA tests against ToMV revealed that the virus replicated well in all materials. None of the accessions evaluated displayed resistance or high levels tolerance to the four pathogens, but some of them were complementary for resistance or high levels of tolerance. Although the interspecific hybrid tested was not resistant to any of the pathogens, it was tolerant to FOL and PepMV and moderately tolerant to VE and ToMV. A multivariate hierarchical clustering revealed similar patterns among accessions in the response to the two fungal diseases (FOL and VE) on one side and to the two viral ones (PepMV and ToMV) on the other. The information generated in this study has allowed identifying materials within the pepino genepool for the development of multi-resistant pepino cultivars to major diseases threatening its expansion in the Mediterranean region.

Keywords: DAS-ELISA, *Fusarium oxysporum* f. sp. *lycopersici*, inoculation, PepMV, *Solanum muricatum*, ToMV, *Fusarium oxysporum* f. sp. *lycopersici*; *Verticillium dahliae*.

2 Introduction

The pepino (*Solanum muricatum* Aiton), also known as “pepino dulce" or "sweet cucumber”, is a vegetatively propagated vegetable crop native to the Andean region grown for its fruits (Prohens et al., 1996). Pepino fruits are fleshy, typically of a golden yellow color with purple stripes, and can be consumed as a fresh table fruit in the case of cultivars that have more aromatic and sweet fruits, or as a vegetable in salads, for cultivars with less sweet and more acid fruits (Rodríguez-Burruezo et al., 2011). Although pepino cultivation has been mainly restricted to the Andean region, in the last decades there has been a growing interest in several countries from the Mediterranean region, as well as in China, Japan, New Zealand, or the USA, in introducing pepino as a new vegetable crop (Rodríguez-Burruezo et al., 2011; Herraiz et al., 2015a; Gurung et al., 2016; Kim et al., 2017). However, the introduction of pepino in other countries outside its region of origin is threatened due to susceptibility to pests and diseases of tomato (Nuez & Ruiz, 1996), which is phylogenetically closely related to pepino (Herraiz et al., 2015a, 2016a; Särkinen et al., 2013).

In the Mediterranean region, pepino is mostly grown as a greenhouse crop, following agricultural practices similar to those of tomato (Prohens et al., 1999; Rodríguez-Burruezo et al., 2011). Under these protected cultivation conditions, we have identified two fungal and two viral pathogens that affect tomato (Lahoz et al., 2015), namely *Fusarium oxysporum* f. sp. *lycopersici* (FOL), *Verticillium dahliae* (VE), pepino mosaic virus (PepMV), and tomato mosaic virus

(ToMV), that potentially could cause significant damage to pepino crops (Ge et al., 2012; Jones et al., 1980; Nuez & Ruiz, 1996; Pérez-Benlloch et al., 2001). Although late blight (*Phytophthora infestans*) is a serious disease of pepino in its region of origin (Adler et al., 2002), in the Mediterranean area is infrequent in tomato (Lahoz et al., 2015), probably because most of its cultivation, like that of pepino (Rodríguez-Burruezo et al., 2011), is under controlled greenhouse conditions that do not favour its spread.

Fusarium wilt caused by *Fusarium oxysporum* is one of the most devastating fungal diseases of tomato and pepino (Nuez & Ruiz, 1996; Mandal et al., 2009). It is soil-borne and affects both greenhouse and open field cultivation in temperate vegetable production areas through irrigation water and contaminated farm equipment (Maurya et al., 2019). In tomato, FOL directly penetrates roots and colonizes vascular tissue (Srinivas et al., 2019), causing yellowing of the leaves and wilting of the plants, which can lead to a complete loss of production (Nirmaladevi et al., 2016). Under wet conditions, white, pink or orange fungal growth can be seen on the surface of the affected stems (Ajilogba & Babalola, 2013).

Verticillium wilt is caused by VE, a fungal pathogen that affects a wide range of solanaceous hosts (Inderbitzin & Subbarao, 2014; Klosterman et al., 2009), responsible for serious economic losses both in the greenhouse and in open field cultivations (Gayoso et al., 2010). This pathogenic fungus infects roots and then invades the xylem (Hu et al., 2019), causing in tomato and eggplant symptoms of vascular

discoloration, wilting and yellow-bronze leaf spots, with reduction of growth, yield and fruit quality, and eventually plant death (Karagiannidis et al., 2002). The pathogen spreads especially by irrigation and infested seeds and locally from field to field through crop management practices (Baroudy et al., 2018; Carroll et al., 2018).

PepMV, a potexvirus that was first isolated from infected pepino plants in 1980 (Jones et al., 1980), causes important losses worldwide in tomato production, especially in Europe and North America (Souiri et al., 2017). The symptoms in pepino include yellow mosaic in young leaves (Jones et al., 1980), while in tomato are very diverse, and may occur in the form of fruit discoloration, chlorosis and yellow angular leaf spots, severe leaf mosaics and occasionally leaf or stem necrosis (Hanssen & Thomma, 2010; Hasiów-Jaroszewska & Komorowska, 2013; Sempere et al., 2016; Soler et al., 2011). PepMV is transmitted mechanically with high efficiency, mainly during cultural pruning and fruit harvesting practices through contaminated tools and clothing (Hasiów-Jaroszewska et al., 2010). In addition, low rates of transmission have been reported by bumblebees, seeds, vegetative propagation and the soil-borne fungus *Olpidium virulentus* (Alfaro-Fernández et al., 2010; Córdoba-Sellés et al., 2007; Schwarz et al., 2010; Shipp et al., 2008; Van der Vlugt & Stijger, 2009).

ToMV, a member of the genus *Tobamovirus* (Adams et al., 2009), has a wide host range including members of the Solanaceae family such as tomato and pepino, undermining their yield and fruit quality (Ge et al., 2012; Leiva-Brondo et al., 2006; Pérez-Benlloch et

al., 2001; Ullah et al., 2019). Symptoms of infected plants, both in pepino and tomato, include local lesions, systemic mosaics on leaves, mottling, malformation, necrosis, and fern-leaf symptoms (Bae et al., 2019; Chitra et al., 1999; Leiva-Brondo et al., 2006; Park & Cha, 2002; Pérez-Benlloch et al., 2001). ToMV is efficiently transmitted through mechanical inoculation, grafting, and infested seed (Ghodoum Parizipour & Keshavarz-Tohid, 2020; Soler et al., 2010).

In tomato, decades of breeding programs, have allowed the identification of QTLs, sources of genetic resistance and major genes, either in the cultivated species or in wild relatives, to *Fusarium*, *Verticillium* and ToMV. These achievements have allowed the development of modern varieties with effective protection against these diseases (Lee et al., 2015). Resistant rootstocks are also commonly used in tomato for resistance to FOL and VE (King et al., 2010). However, so far, no effective resistance against PepMV has been incorporated in tomato (Pechinger et al., 2019), although some sources of resistance have been identified in the wild tomato *S. lycopersicoides* (Soler et al., 2011) and in tomato accessions 11R.412000 and 11R.446400 (US patent US9637757B2). In pepino, several accessions resistant to ToMV have been described, although its genetic control has not been determined so far (Leiva-Brondo, Prohens, et al., 2006; Pérez-Benlloch et al., 2001).

The evaluation of the response of pepino to these four diseases and the search for sources of resistance or tolerance is of great relevance for the development of new cultivars of pepino in the

Mediterranean region. In particular, the identification of accessions with multiple resistances or tolerances would facilitate the improvement of breeding programmes to develop new pepino cultivars with multiple resistances and/or tolerances to these major diseases. For the purpose of this paper, we considered a plant as resistant if it did not display symptoms, and as a tolerant if it had mild symptoms without a significant effect on development (Atibalentja et al., 1997; Reis et al., 2004). In this work, we evaluate the response of a collection of cultivated and wild related pepino accessions to FOL, VE, PepMV, and ToMV with the aim of evaluating the threat represented by them for pepino in Mediterranean regions and identifying new sources of variation for breeding to these diseases.

3 Materials and methods

3.1 Plant materials and growing conditions

Six clonal accessions of the cultivated pepino (*Solanum muricatum*), including local Andean varieties and modern cultivars from different locations, were selected for their genetic and phenotypic diversity and for their breeding interest (Herraiz et al., 2015a; 2016b; Prohens et al., 2002; Rodríguez-Burruezo et al., 2004a) (Table 1). In addition, nine clones from seven species of pepino wild relatives (*Solanum* section *Basarthrum*) from Central and South America, plus one interspecific hybrid between cultivated pepino and a wild relative (*S. muricatum* x *S. caripense*), were chosen to represent the wild genepool diversity of pepino (Blanca et al., 2007). Finally, two

accessions of tomato (*S. lycopersicum*) were included in the study as susceptible controls for the biotic stresses assessed (Table 1).

Table 1. Accessions and controls assessed in this study and their respective study code, species, geographical origin and number of plants evaluated for each pathogen.

Accession	Code	Species	Origin	FOL	VE	PepMV	ToMV
				n	n	n	n
Cultivated							
37-A	Mur1	*S. muricatum*	Ecuador	10	5	5	10
Sweet Round	Mur2	*S. muricatum*	Spain	10	10	9	10
Valencia	Mur3	*S. muricatum*	Spain	10	10	5	7
OV-8	Mur4	*S. muricatum*	Chile	9	3	8	6
Virú	Mur5	*S. muricatum*	Peru	10	12	10	10
Vetas Verdes	Mur6	*S. muricatum*	Ecuador	10	12	5	6
Wild relatives							
EC-40	Car1	*S. caripense*	Ecuador	7	7	9	10
PI-243342	Car2	*S. caripense*	Ecuador	6	3	10	10
BIRMS1034	Car3	*S. caripense*	Ecuador	7	7	9	10
E-34	Trac	*S. trachycarpum*	Ecuador	10	10	5	10
E-80	Cati	*S. catilliflorum*	Peru	10	8	6	10
E-62	Perl	*S. perlongysttilum*	Peru	10	7	7	10
PT-084	Base	*S.basendopogon*	Peru	10	7	10	10
BIRMS1975	Cane	*S. canense*	Panama	7	6	6	10
BIRMS1978	Frax	*S. fraxinifolium*	Costa Rica	3	5	10	10
Interspecific hybrid							
F1 (Sweet Long x EC-40)	Hb	*S. muricatum* x *S. caripense*	Spain	7	11	9	10
Susceptible controls							
Mallorquin	MLL	*S. lycopersicum*	Spain	10	10	18	18
Valenciano	VLC	*S. lycopersicum*	Spain	10	9	-	-

All materials came from the Germplasm Bank of the Universitat Politècnica de València and from the collection of the authors. Pepino clones were vegetatively propagated *in vitro* from one individual mother plant per clone (Çavuşoğlu, 2013), whereas wild relatives were germinated from seeds following the protocol of Ranil et al. (2015) and one individual per accession was clonally propagated *in vitro* using the same protocol than for pepino. After acclimatization in a climatic chamber with 16 h light (25 °C) / 8 h dark (18 °C) and relative humidity of 65% to 95% (day and night) regime, the clones were transplanted in the same climatic chamber to 8 × 8 × 6 cm polyethylene pots filled with Neuhaus N3 substrate (Klasmann-Dellmann GmbH, Geeste, Germany). Simultaneously, tomato accessions were germinated and seedlings were maintained in a climatic chamber until transplanting to pots. For each pathogen inoculation experiment, the plants were distributed according to a completely randomized design, with each plant constituting a replicate. The number of plants tested per accession for inoculation with each of the four pathogens varied between 3 to 12 depending on plantlets availability (Table 1). In addition, one plant per accession was kept as a mock-inoculated control. These controls were kept separated in the same climatic chamber to avoid infection with the evaluated pathogens. The pathogens were evaluated one at a time to avoid cross-contaminations.

3.2 Pathogen preparation, inoculation and disease symptoms assessment

Inoculation with *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and *Verticillium dahliae* (VE) was performed, respectively, with FOL race 2 and VE race 0 isolates provided by Variety and Seed Study and Control Group (GEVES, Beaucouzé, France) and were cultured on Potato Dextrose Agar medium (PDA; Scharlab, Barcelona, Spain) at 24º C for 10 d for FOL and 26 ºC for 25 d for VE. Spores of FOL and VE were collected from the PDA culture by flooding the medium surface with 10 mL of sterile distilled water followed by gently scraping with a loop. Spore solutions were then filtered through a sterile gauze in order to remove the hyphae and concentrations were measured using a Neubauer Chamber (Celeromics Technologies, Valencia, Spain). At the stage of four true-leaves, which corresponds to the phenological stage 104 of the specific pepino BBCH (Biologische Bundesanstalt, Bundessortenamt, CHemische Industrie) scale (Herraiz et al., 2015b), seedlings were carefully uprooted from the germination trays and the root system was washed with tap water. For inoculation with FOL or VE, 2 cm of the apical part of the root system were excised with a pair of scissors, and dipped for 3 h (FOL) or 12 h (VE), respectively, in a 150 mL conidial solution of 1.0×10^7 spores/mL. Disease severity in each inoculated plant was evaluated at 7, 15, 21 and 30 DAI (Days After Inoculation) according to a symptoms index (SI) based on a numerical scale from 0 to 4, where 0 = absence of symptoms; 0.5 = mild symptoms; 1 = moderate symptoms; 2 =

severe symptoms; 3 = very severe symptoms; 4 = dead plant (Atibalentja et al., 1997; Reis et al., 2004).

Inoculation with pepino mosaic virus (PepMV) and tomato mosaic virus (ToMV) was performed, respectively, with PepMV isolate PV-0750 provided by Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), which was obtained from tomato-infected plants collected in the region of Almeria (Spain), and ToMV race 0 provided by GEVES. Inoculum of PepMV and ToMV were prepared using a 1:10 (w:v) proportion of infected tomato leaves and inoculation buffer. The inoculation buffer was prepared using the procedure described by Figàs et al. (2017). The solution was homogenised by macerating 1 g of virus-infected leaves of tomato with 10 mL of inoculation buffer using a mortar and a pestle. Carborundum 1% (w:v) (VWR International S.A.S, Pennsylvania, USA) and 1% (w:v) of activated charcoal (Scharlab, Barcelona, Spain) were added to the solution. PepMV and ToMV were mechanically inoculated rubbing the leaves with a cotton-bud stick, previously dipped in the inoculum when plantlets had four true leaves, stage 104 of the specific pepino BBCH scale (Herraiz et al., 2015b). All true leaves were inoculated. Mock-inoculation on the non-inoculated control plant was performed using only inoculation buffer and carborundum. Disease severity was visually scored for each individual plant at 15, 30, 45, and 60 days after inoculation (DAI) following a severity scale for the symptoms index (SI): 0 = absence of symptoms; 0.5 = mild symptoms consisting mild

mosaic, plant recovered in the apical leaves; 1 = moderate symptoms characterized by intensification of first symptoms, and mottling on leaves; 2 = severe yellow mosaic and mottling on leaves; 3 = very severe mottling and necrotic lesions on stems 4 = plant death. Double Antibody Sandwich - Enzyme-Linked Immunosorbent Assay (DAS-ELISA) was performed on young new leaves of each plant to evaluate the presence and level of virus accumulation. PepMV and ToMV antibodies and their enzyme conjugate were supplied by Loewe Biochemica (Sauerlach, Germany). The absorbance of the serologic reaction was measured at 405 nm with a Bio-Rad iMark 550 microplate reader (Bio-Rad Laboratories, Hercules, California, USA). A sample was considered infected (positive) when the absorbance was higher than the average absorbance of the mock-inoculated controls plus three times their standard deviation, representing a final threshold value of 0.174 for PepMV and 0.123 for ToMV. Samples were considered to be non-infected (negative) when the absorbance value was below these thresholds.

Disease severity was used to discriminate the accessions in four reaction classes depending on the mean maximum symptoms index (MMSI), which was obtained by averaging the maximum value for the symptoms index (SI) of each plant at any of the dates in which symptoms were evaluated. Plants with a MMSI = 0 were considered to be resistant (R); those with a $0 < MMSI \leq 0.5$ were considered to be tolerant (T); those with $0.5 < MMSI \leq 1.0$ were considered to be

moderately tolerant (MT); while those with MMSI > 1.0 were considered to be susceptible (S).

3.3 Data analyses

For each combination of accession and disease, the mean and standard error (SE) of the MMSI was calculated. For the two viral diseases, the mean and SE of mean maximum absorbance (MMA) and viral accumulation index, were also calculated. MMA values were obtained by averaging the maximum value for the absorbance of each plant at any of the dates in which symptoms were evaluated, while viral accumulation index values were obtained from the quotient between the viral accumulation of each accession and that of the control, which had a standardized value of 1.00. Pearson linear correlations for MMSI for the four diseases and MMA for PepMV and ToMV were calculated. A hierarchical clustering multivariate analysis using all data from the four pathogens was performed using the package "gplot" as an enhanced version or its basic function stats in R (Warnes et al., 2016). Genotypes were divided into different clusters using Ward's hierarchical clustering method (Kamble, 2010), and the patterns of their disease traits were shown in colors in the heatmap.

4 Results

4.1 Symptoms evolution

4.1.1 Fusarium oxysporum f. sp. lycopersici (FOL)

Mild symptoms of chlorosis were observed at 7 DAI on the inoculated leaves of the tomato susceptible control plants, with SI

values of 0.11 for MLL and of 0.65 for VLC (Supplementary data S1). Symptoms became more severe at 15 DAI with values of the SI of 0.94 for MLL and 1.75 for VLC, and increased progressively (Figure 1). At 30 DAI, all inoculated susceptible control plants developed severe disease symptoms, reaching values for SI of 3.10 for MLL and 3.80 for VLC (Figure 2C and Table 2), indicating a high infectivity of the inoculum used and the effectiveness of mechanical inoculation.

Regarding the cultivated *S. muricatum* materials, at 7 DAI, mild symptoms were observed in all accessions, except in Mur2, which did not display any symptoms (Figure 1 and Supplementary Data S1). However, from 15 DAI until the end of the experiment at 30 DAI no more symptoms were observed in any of the *S. muricatum* accessions (Figure 1). Thus, except Mur6, which had an MMSI of 1.15, the pepino clones could be considered as resistant (in the case of Mur2) or tolerant (in the case of the other five accessions) to FOL race 2 (Table 2).

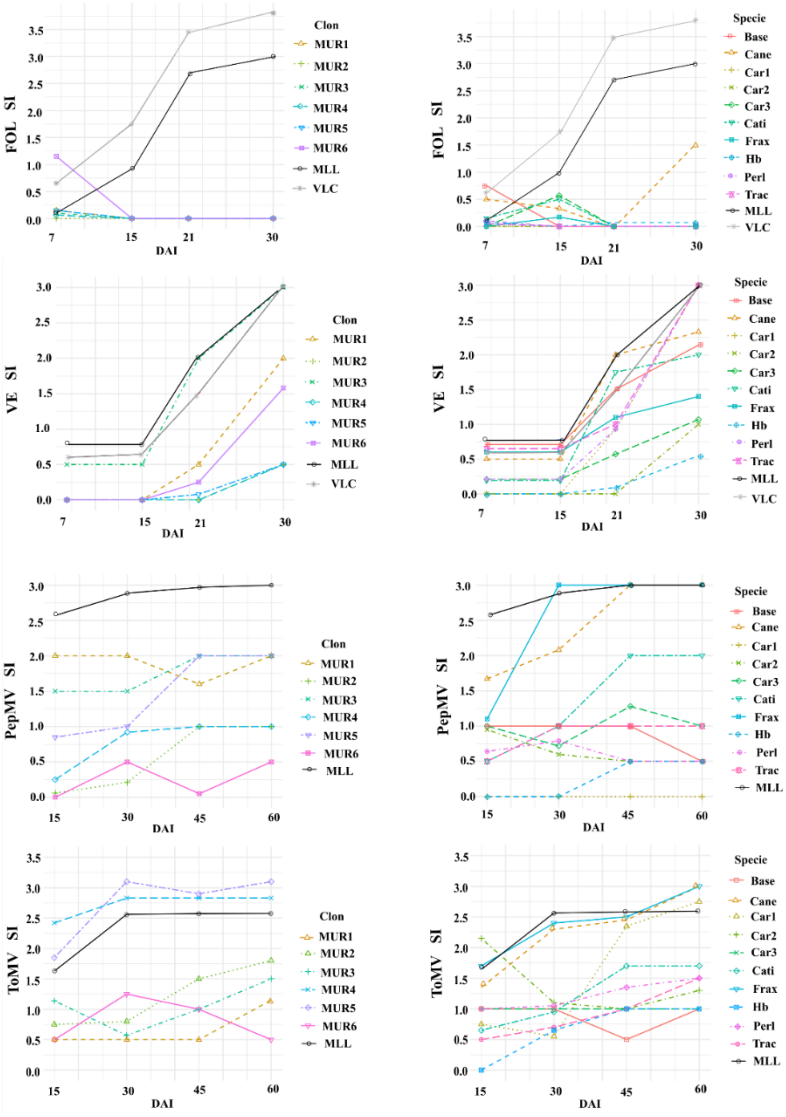

Figure 1. Evolution of the average symptoms index (SI) at 7, 15, 21, 30, 45 and 60 days of cultivated pepino (left) and wild relatives (right) accessions plus tomato controls (MLL and VLC) after inoculation with FOL, VE, PepMV and ToMV.

Six out of the ten clones of pepino wild relatives showed mild symptoms of FOL infection at 7 DAI, with SI ranging from 0.05 of Trac to 0.75 of Base (Supplementary data S1). However, just two of them (Cane with SI of 0.33 and Cati with 0.5), plus Car3 (0.57) and Frax (0.17), which were asymptomatic at 7 DAI, exhibited symptoms at 15 DAI. From 21 DAI until the end of the experiment, only Cane continued exhibiting disease symptoms (Figure 2B). Two out of three *S. caripense* accessions, Car1 and Car2, did not develop any symptoms throughout the experiment (Table 2 and Figure 2A). Similarly, the Trac, Cati, Perl, Frax accessions and the hybrid Hb displayed only slight symptoms in responses to FOL infection, with MMSI values ranging from 0.05 to 0.5 (Table 2), and they could be considered as tolerant. Finally, Cari3 and Bas exhibited moderate disease severity, with MMSI values ranging from 0.57 to 0.75, indicating moderate tolerance against FOL, while Cane with MMSI of 1.5 was classified as susceptible (Table 2).

4.1.2 Verticillium dahliae (VE)

Plants of the susceptible tomato controls exhibited moderate symptoms at 7 and 15 DAI, with SI values of 0.78 for MLL and 0.60 for VLC (Supplementary data S1), showing the typical symptoms of the disease, consisting of leaf chlorosis stem yellowing (Figure 2F). The intensity of symptoms increased with time with SI from 2.00 and 1.50 for MLL and VLC, respectively at 21 DAI, to 3.00 for both tomato accessions at 30 DAI (Figure 1). The MMSI values of 3.00 for controls confirmed that the VE infection was correctly made (Table 2).

The response of the accessions tested of pepino and wild relatives' genotypes varied considerably. Symptoms of VE on pepino clones appeared 7 DAI after the inoculation, except for Mur3 that exhibited slight symptoms (SI=0.50) at this date (Figure 1 and Supplementary Data S1). At 21 DAI, also Mur5 (0.08), Mur6 (0.25) and Mur1 (0.50) showed light symptoms while those of Mur3 substantially increased (2.00). At the end of the experiment, all clones reached higher symptoms levels, with MMSI values ranging from 0.50 to 3.00 (Figure 1 and Table 2). Based on the records, three of them (Mur2, Mur4 and Mur5) could be considered as tolerant, being Mur2 and Mur4 the most promising since they showed mild symptoms (0.50) only at 30 DAI (Figure 2D and Supplementary data S1). The rest of the accessions exhibited severe symptoms, ranging from 1.58 of Mur6 to 3.00 of Mur3, indicating higher susceptibility to VE race 0 (Figure 2E and Table 2). Regarding pepino wild relatives, they exhibited mild symptoms at 7 and 15 DAI, while only Car1, Car2 and Hb showed no symptoms (Supplementary data S1). At 21 DAI, all the accessions displayed a considerable increase in the severity of the symptoms, reaching MMSI values between 1.07 and 3.00 at the end of the experiment (Table 2 and Figure 1).

Table 2. Mean maximum symptoms index (MMSI, ± SE) for symptoms severity registered at any of the dates where measurements were performed (7, 15, 21, 30) days for FOL and VE and (15, 30, 45, 60) days for PepMV and ToMV, percentage of plants with symptoms and reaction classification for the pathogens evaluated in this study. Resistant (R), tolerant (T), moderately tolerant (MT), and susceptible (S).

Accession code	FOL			VE			PepMV			ToMV		
	MMSI	% of plants with symptoms	Reaction	MMSI	% of plants with symptoms	Reaction	MMSI	% of plants with symptoms	Reaction	MMSI	% of plants with symptoms	Reaction
Cultivated												
Mur1	**0.15 ± 0.08**	100	**T**	2.00 ± 0.00	100	S	2.00 ± 0.00	100	S	1.15 ± 0.08	100	S
Mur2	**0.00 ± 0.00**	**0**	**R**	**0.50 ± 0.00**	100	**T**	**1.00 ± 0.00**	100	**MT**	1.80 ± 0.08	100	S
Mur3	**0.10 ± 0.07**	100	**T**	3.00 ± 0.00	100	S	2.00 ± 0.00	100	S	1.57 ± 0.07	100	S
Mur4	**0.06 ± 0.06**	100	**T**	**0.50 ± 0.00**	100	**T**	**1.00 ± 0.00**	100	**MT**	2.92 ± 0.69	100	S
Mur5	**0.15 ± 0.08**	100	**T**	**0.50 ± 0.00**	100	**T**	2.00 ± 0.00	100	S	3.15 ± 0.24	100	S
Mur6	1.15 ± 0.25	100	S	1.58 ± 0.15	100	S	**0.50 ± 0.00**	100	**T**	1.25 ± 0.11	100	S
Wild relatives												
Car1	**0.00 ± 0.00**	**0**	**R**	3.00 ± 0.00	100	S	**0.00 ± 0.00**	0	**R**	2.75 ± 0.08	100	S
Car2	**0.00 ± 0.00**	**0**	**R**	**1.00 ± 0.17**	100	**MT**	**0.95 ± 0.27**	100	**MT**	2.30 ± 0.23	100	S
Car3	**0.57 ± 0.57**	100	**MT**	1.07 ± 0.17	100	S	1.28 ± 0.09	100	S	1.00 ± 0.00	100	**MT**
Trac	**0.05 ± 0.05**	100	**T**	3.00 ± 0.00	100	S	**1.00 ± 0.00**	100	**MT**	1.50 ± 0.00	100	S
Cati	**0.50 ± 0.00**	100	**T**	2.00 ± 0.00	100	S	2.00 ± 0.00	100	S	1.70 ± 0.08	100	S
Perl	**0.10 ± 0.07**	100	**T**	3.00 ± 0.00	100	S	**0.79 ± 0.18**	100	**MT**	1.50 ± 0.00	100	S
Base	**0.75 ± 0.08**	100	**MT**	2.14 ± 0.26	100	S	**1.00 ± 0.00**	100	**MT**	1.00 ± 0.00	100	**MT**
Cane	1.50 ± 0.00	100	S	2.33 ± 0.21	100	S	3.00 ± 0.00	100	S	3.00 ± 0.00	100	S
Frax	**0.17 ± 0.17**	100	**T**	1.40 ± 0.22	100	S	3.00 ± 0.00	100	S	3.00 ± 0.00	100	S
Hb	**0.06 ± 0.06**	100	**T**	**0.55 ± 0.08**	100	**MT**	**0.50 ± 0.00**	100	**T**	1.00 ± 0.00	100	**MT**
Susceptible controls												
MLL	3.80 ± 0.20	100	S	3.00 ± 0.00	100	S	3.03 ± 0.07	100	S	2.69 ± 0.09	100	S
VLC	3.10 ± 0.46	100	S	3.00 ± 0.00	100	S	-	-	-	-	-	-

None of the wild accessions performed better than the best pepino clones. In fact, the best performance was of Hb (0.55 at 30 DAI), which is a hybrid between "Sweet Long", a pepino clone not included in this study, and Car1 that exhibited one of the worst results (MMSI of 3.00). However, the *S. caripense* accession Car2 displayed the lowest symptoms among the wild relatives, with 1.00 MMSI values, and could be considered as moderately tolerant against VE race 0 (Table 2). The rest of the wild accessions displayed moderate to severe symptoms and therefore were classified as susceptible (Table 2).

4.1.3 Pepino mosaic virus (PepMV)

The susceptible tomato control MLL showed severe symptoms (2.58) already at 15 DAI and increased progressively during all the experiment (Figure 1 and Supplementary Data S1). At 60 DAI all inoculated susceptible control plants developed severe mosaic in leaves (Figure 2I) with MMSI values of 3.03 (Table 2). The serological analyses of the plants indicated that MLL had high virus titre, with MMA values of 3.08 (Table 3 and Supplementary Data S2). Both symptoms and viral accumulation displayed high levels throughout the experiment (Figures 1 and 3). Therefore, also for this pathogen, the inoculum and inoculation were successful.

The behaviour of the tested cultivated pepino clones varied considerably among the different accessions. While some clones at 15 DAI exhibited no symptoms (Mur6) or very light

ones, such as Mur 2 (0.06) and Mur4 (0.25), the rest showed mild (Mur 5 with 0.85) or severe symptoms (Mur3 with 1.50 and Mur1 with 2.00) (Figure 1 and Supplementary Data S1). After increasing at 30 DAI, the symptoms generally reached their maximum at 45 DAI and maintained stable until the end of the experiment (Figure 1). The lowest MMSI was found in Mur6 with a value of 0.50, followed by Mur2 and Mur 4 with a value of 1.00 and finally Mur3 and Mur5 with the highest MMSI (2.00) (Table 2). These symptoms results followed the same patterns of the MMA values (Supplementary Data S2). In this way, Mur6 absorbance levels were the lowest with an MMA value of 0.30, followed by Mur2 (1.43), Mur4 (1.61) and Mur3 (1.99), and finally by Mur1 (2.28) and Mur5 (2.54) (Table 3). Taking account all these data and also the normalized ones, using the susceptible control for the viral accumulation index (Table 3), we could consider Mur6 as tolerant, and Mur2 and Mur4 as moderately tolerant to PepMV (Figure 2 and Table 2).

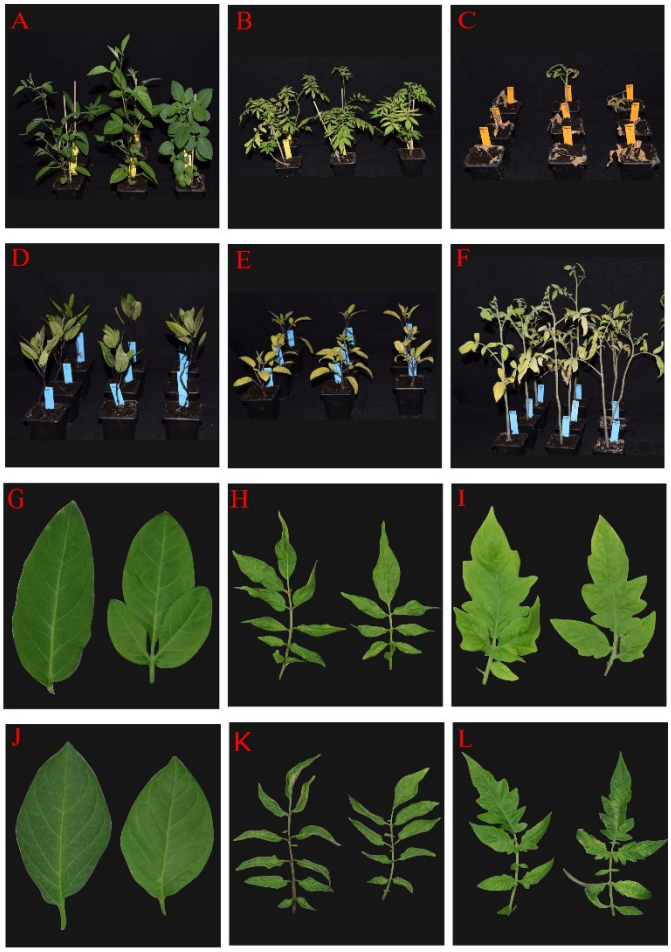

Figure 2. Foliar symptoms in plants at the end of each experiment. A, B, C; plants infected with FOL, Car2 accession plants showing no damage (A), generalized chlorosis in leaves of the Cane accession (B), dead plants of the tomato susceptible control MLL (C). D, E, F; plants infected with VE, Mur5 accession plants showing no damage (D), follicular chlorosis in leaves of the Mur3 accession (E), generalized chlorosis in tomato plants of the susceptible control VLC (F). G, H, I; plants infected

with PepMV, Mur6 accession leaves showing no damage (G), crushing in leaves of the Frax accession (H), mild chlorosis and crushing in leaves of the susceptible tomato control MLL (I). J, K, L; plants infected with ToMV, Hb accession leaves showing no damage (J), generalized severe curling and chlorosis at the ends of the leaves of the Frax accession (K), generalized curling and chlorosis in tomato leaves of the susceptible control MLL (L).

Wild relatives exhibited a wide range of performance after infection with PepMV. First symptoms, from light to mild, were registered already at 15 DAI, except for Car1 and Hb (Figure 1) and similar to pepino clones they generally reached their higher values around 45 DAI (Supplementary data S1). Accession Car1 and its hybrid Hb displayed a good response against this pathogen. In this way, Car1 did not exhibit any symptoms during the test and could be considered as a resistant accession, while Hb showed mild symptoms only at 45 and 60 DAI with MMSI of 0.5 and was classified as tolerant (Table 2). Other accessions that presented moderate symptoms and low MMSI values were Perl (0.79), Car2 (0.95), Trac and Base (1.00), being moderately tolerant to PepMV (Table 2). Overall, the progression of absorbance values (Figure 3 and Supplementary Data S2) and MMA values (Table 3) were consistent with those of the progression of symptoms (Figure 1 and Supplementary Data S1) and MMSI values (Table 2).

Table 3. Mean maximum absorbance (MMA, ± SE) at any of the dates where measurements were performed (15, 30, 45, 60) days for PepMV and ToMV, viral accumulation index and total percent of plants with systemic infection measured with DAS-ELISA for the viruses PepMV and ToMV after mechanical inoculation. Samples were considered infected (positive) when absorbance was greater than the threshold value of 0.174 for PepMV and 0.123 for ToMV.

Accession code	PepMV			ToMV		
	MMA	Viral accumulation index	Total % of plants with systemic infection	MMA	Viral accumulation index	Total % of plants with systemic infection
Cultivated						
Mur1	2.28 ± 0.55	0.74	100	1.27 ± 0.07	0.68	100
Mur2	1.43 ± 0.37	0.47	100	1.12 ± 0.15	0.60	100
Mur3	1.99 ± 0.48	0.65	100	1.15 ± 0.07	0.62	100
Mur4	1.61 ± 0.42	0.52	100	1.11 ± 0.34	0.60	100
Mur5	2.54 ± 0.40	0.82	100	1.08 ± 0.23	0.58	100
Mur6	0.30 ± 0.10	0.10	100	1.44 ± 0.04	0.77	100
Wild relatives						
Car1	0.28 ± 0.04	0.09	100	1.54 ± 0.21	0.83	100
Car2	1.51 ± 0.45	0.49	100	2.04 ± 0.07	1.10	100
Car3	0.44 ± 0.10	0.14	100	1.85 ± 0.10	0.99	100
Trac	1.63 ± 0.51	0.53	100	1.76 ± 0.08	0.94	100
Cati	3.22 ± 0.14	1.04	100	2.18 ± 0.07	1.17	100
Perl	1.63 ± 0.50	0.53	100	2.18 ± 0.06	1.17	100
Base	0.60 ± 0.16	0.19	100	1.10 ± 0.08	0.59	100
Cane	3.50 ± 0.00	1.14	100	2.54 ± 0.02	1.37	100
Frax	3.44 ± 0.06	1.12	100	2.55 ± 0.12	1.37	100
Hb	0.41 ± 0.07	0.20	100	2.06 ± 0.08	1.11	100
Susceptible controls						
MLL	3.08 ± 0.34	1.00	100	1.86 ± 0.08	1.00	100

4.1.4 Tomato mosaic virus (ToMV)

The susceptible tomato control MLL showed the characteristic symptoms of ToMV infection, with light and dark green leaf mosaic, mottling and deformation of leaves (Figure 2L). At 15 DAI moderate symptoms were observed, becoming severe from 30 DAI on until the end of the experiment (Figure 1 and Supplementary Data S1). The absorbance values were high at 15 DAI but decreased slightly at the end of the experiment with a MMA of 1.86 (Figure 3 and Supplementary Data S2). Again, this indicates that the conditions and the inoculation method were adequate.

All pepino clones showed symptoms at 15 DAI, ranging from light (Mur1 and Mur6 with 0.50) to severe (Mur4 with 2.42) (Supplementary Data S1). However, while some accessions were able to avoid the onset of more severe symptoms, others like Mur4 (2.92) and Mur5 (3.15) reached MMSI values higher than the tomato control MML (Figure 1 and Table 2). Nevertheless, all pepino clones reached MMSI values higher than 1.00 and therefore were considered susceptible to ToMV (Table 2). The large differences recorded for symptoms were not observed for the absorbance and the viral accumulation index (Table 3 and Supplementary Data S2). During all the experiment, the difference in the absorbance values among pepino clones was limited and MMA values ranged from 1.08 of Mur5 to 1.44 of Mur6 (Table 3 and Supplementary Data S3). All wild relatives,

except Hb which did not display symptoms (Figure 2), showed from mild (Trac with 0.50, Cati with 0.65 and Car1 with 0.75) to moderate symptoms (Car2 with 2.15) at 15 DAI (Supplementary Data S1). Symptoms became more severe after 30 DAI and at the end of the experiments Car1 (2.75), Car2 (2.30), Cane and Frax (3.00) recorded higher MMSI values than the control MLL (Figure 1 and Table 2). Better performances were observed for Car3, Base and Hb (MMSI at 1.00) and they were considered as moderately tolerant (Table 2). However, except Base, these accessions were not the ones that registered lower absorbance values along the experiment (Figure 3 and Supplementary Data S2), confirming that pepino wild relatives exhibit different symptoms severity at similar viral concentrations. Also, some wild relatives Car1, Car2 and Trac displayed similar symptoms severity and absorbance than the control MLL and therefore are considered as susceptible.

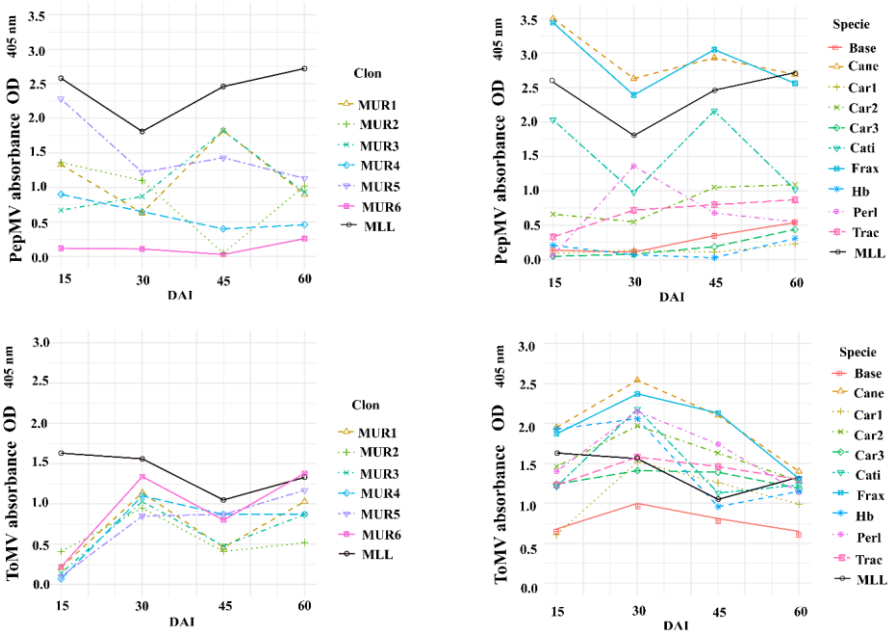

Figure 3. Evolution of mean absorbances of cultivated pepino (left) and wild relatives (right) accessions plus a tomato control (MLL) at 15, 30, 45 and 60 days after inoculation regarding PepMV and ToMV mechanical inoculation. Samples were considered infected (positive) when absorbance was greater than the threshold value of 0.174 for PepMV and 0.123 for ToMV.

4.2 Hierarchical clustering analysis

Unsupervised hierarchical clustering of disease traits and accessions revealed several clusters (Figure 4). Clustering for traits revealed two major clusters. Cluster I is formed by the MMSI of both fungal diseases (FOL-MMSI and VE-MMSI), while cluster II grouped tightly MMSI and MMA of PepMV. ToMV-MMSI and ToMV-MMA were placed in two separated

branches. The heatmap is in agreement with the disease traits correlation values observed (Supplementary Data S3). Genotypes were also grouped into two major clusters (Figure 4). Cluster A included the wild accessions Cati, Frax and Cane, which exhibited high values for MMSI and MMA. Cluster B is the most heterogenous and is subdivided into two sub-clusters. The sub-cluster (a) included Car3 and Hb on one branch and Base and Mur6 in another branch, with these accessions generally displaying a good performance against the two viruses, except for Car3 and the hybrid Hb for ToMV-MMA. On the contrary, these two accessions displayed a good performance against VE (Figure 4). The sub-cluster (b) is divided in turn in three groups, being the first one comprised by Car2, Mur2, Mur4 and Mur5 that shared good response to FOL-MMSI, VE-MMSI and ToMV-MMA (except Car2). The second one, which included Trac, Perl, Mur1 and Mur3 shared good behavior for FOL-MMSI and ToMV-MMSI but severe symptomatology for VE-MMSI. Finally, the third group is formed only by Car1 which showed good response to all the traits, except for VE-MMSI and ToMV-MMSI.

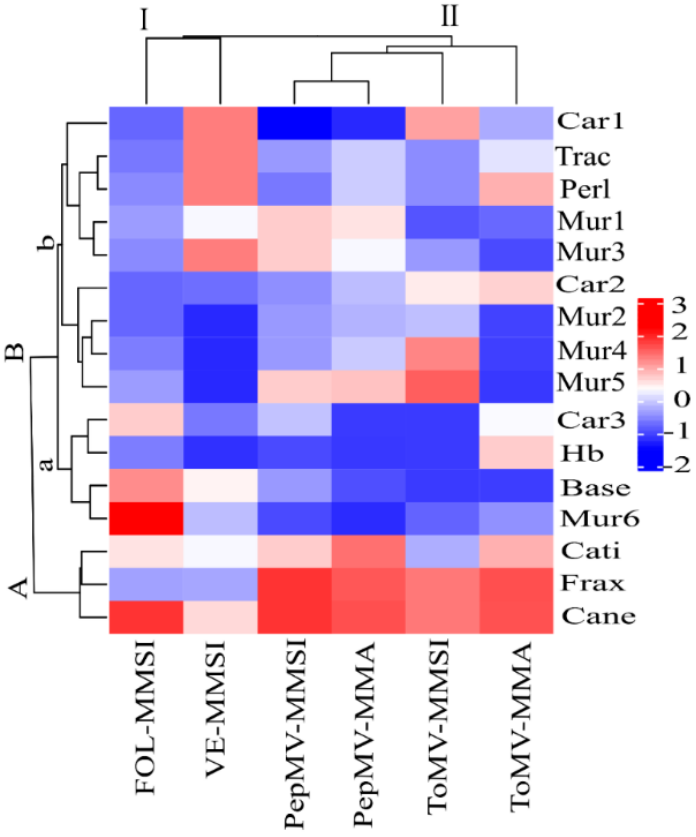

Figure 4. Heatmap of genotypes and disease traits mean maximum symptoms index (MMSI), mean maximum absorbance (MMA) in plants infected with FOL, VE, PepMV and ToMV. The colors of the clusters indicate the severity of the disease, the blue being the least severe, the white having an intermediate value and the red the most severe.

5 Discussion

The success of a new vegetable crop such as pepino in regions where it is being introduced depends largely, among many other factors, on the availability of resistant varieties (Nelson et al., 2018). In the Mediterranean region, tomato and other solanaceous crops are widely cultivated and therefore their pests and diseases can difficult the introduction of pepino in this region (Nuez & Ruiz, 1996; Hanssen and Lapidot, 2012; Lee et al., 2015). Among the most threatening diseases, we have identified four tomato pathogens that, for their efficient mode of transmission and wide distribution (Lahoz et al., 2015; Janssen et al., 2018), are especially threatening in the case of pepino, which is phylogenetically closely related to tomato (Herraiz et al., 2015, 2016b; Särkinen et al., 2013).

In order to increase the likelihood to find stable and multiple disease resistance sources, in addition to cultivated clones, we have also selected pepino wild relatives, due to their greater diversity and for having been demonstrated their usefulness for improving pepino quality through introgression breeding (Blanca et al., 2007; Herraiz et al., 2015a; Rodríguez-Burruezo et al., 2011). All of the pepino wild relatives selected for this study, with the exception of *S. canense* and *S. fraxinifolium*, are cross-compatible with the cultivated pepino, producing fertile interspecific hybrids (Prohens et al., 2003; Rodríguez-Burruezo et al., 2011), and so, suitable to transfer the

desired traits from the wild to the cultivated background. By incorporating wild species in the materials screened, we tried to mimic the approach used in tomato, where the majority of the disease resistance genes incorporated nowadays in the high performing tomato commercial cultivars come from its wild genepool (Kaushal et al., 2020). In this way, resistance genes, *I-2* and *Ve-2*, found respectively in the *S. lycopersicum* × *S. pimpinellifolium* hybrid PI126915 and in *S. lycopersicum* accession Peru Wild, that confers resistance against FOL race 2 and VE (Lee et al., 2015; Stall & Walter 1965) have been introgressed to modern commercial tomato varieties. Regarding *Tm-1*, *Tm-2* and *Tm-2²* genes, that confer resistance against ToMV, was originally identified from *S. habrochaites* (*Tm-1)*PI126445 and in an *S. peruvianum* (*Tm-2* and *Tm-2²*) (Lanfermeijer et al., 2005; Lee et al., 2015).

Surprisingly, in this study, some of the pepino cultivated clones have revealed as sources of variation for the disease resistance of the pathogens screened that were as good as or even better than the wild ones, which suggests that, unlike other traits such as soluble solids content (Prohens et al., 2005; Herraiz et al., 2015a), cultivated materials may be of great interest as sources of resistance in pepino breeding. This may facilitate developing new pepino resistant cultivars, since using cultivated clones instead of wild relatives would drastically reduce the linkage drag of undesired traits typical of interspecific crosses (Prohens et al., 2017).

The results indicate that in cultivated pepino germplasm resistant or tolerant accessions to *Fusarium* and *Verticillium* can be identified, so it may be possible to select and develop varieties resistant or tolerant to these pathogens by using the diversity present in the cultivated species. Furthermore, given the phylogenetic closeness to tomato (Blanca et al., 2007; Herraiz, Blanca, et al., 2016; Spooner et al., 1993), pepino resistant clones could be tested as potential tomato rootstocks against these pathogens (H. Singh et al., 2017).

Regarding the viruses screened, one wild accession from *S. caripense* (Car1) has been resistant to PepMV. This is in contrast to tomato, where no complete resistance has been found yet to PepMV (Pechinger et al., 2019). The fact that viable somatic hybrids between tomato and pepino have been obtained (Sakomoto & Taguchi, 1991), may represent a way to transfer the resistance from *S. caripense* accession Car1 to tomato. However, as in tomato, some cultivated accessions and wild materials have shown different degrees of tolerance to PepMV (Soler et al., 2011). In the case of ToMV, no resistance has been found in the evaluated cultivated and wild materials selected for this study, so we suggest resorting to other materials that have previously been identified as resistant (Leiva-Brondo, Prohens, et al., 2006), and which in fact have already been used to develop a ToMV resistant cultivar (Rodríguez-Burruezo et al., 2004b). Our results also show that while symptomatology and virus titer are well correlated in the case of PepMV suggesting, that greater

multiplication of the virus is associated to more severe symptoms, for ToMV they are not correlated. These results indicate that for ToMV there may be different mechanisms of tolerance to ToMV infection, as already suggested by Pérez-Benlloch et al. (2001) and Leiva-Brondo et al. (2006).

Interestingly, the hybrid with *S. caripense* has shown a general good performance against all diseases, indicating that it may be a good material for introgression breeding for resistance or tolerance to multiple diseases. Also, given that hybrids of solanaceous crops generally are heterotic for vigor traits (Kumari et al., 2020), this hybrid might be of interest for being used as rootstock (Spanò et al., 2020). The moderate resistance of the hybrid suggests incomplete dominance for the resistance or tolerance to the pathogens assessed, although further studies with segregating populations are needed to confirm the genetic control of these phenotypes. Although we did not find any cultivated pepino accession of resistant or tolerant to all pathogens, some of them (Mur2, Mur4 and Mur6) have shown good behavior against all four, so they could be interesting candidate materials to start breeding programs. Simultaneously, it will be worth investigating if the broad spectrum of tolerance of some materials to more than one pathogen has a common genetic cause or is provided by the combination of multiple genes, which often occur in clusters (Wiesner-Hanks & Nelson, 2016).

However, further studies will be required to dissect the genetic patterns of the tolerance and resistance identified in the materials screened. By linkage analysis and synteny, it may be possible to find out if the pepino genomic regions involved in the defence mechanisms are syntenic with the tomato ones and if the genes are orthologs and conserved in *Solanum* crops (Rinaldi et al., 2016). The results obtained have made it possible to identify materials with tolerance or resistance to some of the main potential pepino pathogens in Mediterranean climates. It is worth pointing out that even though the results presented here came from single experiments for each of the four pathogens, they interaction of each of them with pepino constitute pathosystems characterized by an efficient infection of their easy transmission in the host by the pathogen. Therefore, the likelihood of identifying false-positive resistant plants due to the lack of infection is considered as low. However, in future studies, new pathogens strains and races should be tested in order to investigate if the tolerances and resistances found are broad or strain/race specific.

Our results suggest that by hybridizing materials that complement to each other for resistance or tolerance for the four diseases, it may be possible to develop multi-resistant varieties of pepino. These materials can contribute to the development of multi-resistant varieties for pepino and consequently to the expansion of this crop in Mediterranean regions.

6 Statements

Conflict of interest

The authors have no conflict of interest to declare.

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Chapter 3

De novo Transcriptome Assembly and Comprehensive Annotation of Two Tree Tomato Cultivars (*Solanum betaceum* Cav.) with Different Fruit Color

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PhD candidate contribution

Juan Pacheco. had a main role in the following activities: conceived and designed the research, performed the experiments, analyzed the results, wrote the manuscript, and was responsible for the verification of the paper.

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1 Abstract:

The tree tomato (*Solanum betaceum* Cav.) is an underutilized fruit crop native to the Andean region and phylogenetically related to the tomato and potato. Tree tomato fruits have a high amount of nutrients and bioactive compounds. However, so far there are no studies at the genome or transcriptome level for this species. We performed a *de novo* assembly and transcriptome annotation for purple-fruited (A21) and an orange-fruited (A23) accessions. A total of 174,252 (A21) and 194,417 (A23) transcripts were assembled with an average length of 851 and 849 bp. A total of 34,636 (A21) and 36,224 (A23) transcripts showed a significant similarity to known proteins. Among the annotated unigenes, 22,096 (A21) and 23,095 (A23) were assigned to the Gene Ontology (GO) term and 14,035 (A21) and 14,540 (A23) were found to have Clusters of Orthologous Group (COG) term classifications. Furthermore, 22,096 (A21) and 23,095 (A23) transcripts were assigned to 155 and 161 (A23) KEGG pathways. The carotenoid biosynthetic process GO terms were significantly enriched in the purple-fruited accession A21. Finally, 68,647 intraspecific single-nucleotide variations (SNVs) and almost 2 million interspecific SNVs were identified. The results of this study provide a wealth of genomic data for the genetic improvement of the tree tomato.

Keywords: *de novo* transcriptome assembly; emerging crop; functional annotation; molecular markers; RNA-Seq; Solanaceae; *Solanum betaceum*; structural annotation

2 Introduction

The tree tomato or tamarillo (*Solanum betaceum* Cav.) is a Solanaceae crop native to the Andean region [1,2]. The tree tomato is phylogenetically related to the potato (*S. tuberosum* L.) and tomato (*S. lycopersicum* L.), forming part of the same clade [3]. The tree tomato plant develops into a small tree, even though some cultivars can grow up to four meters in height, with a fast-growing, shallow root system and simultaneous reproductive and vegetative development [4]. In recent years, the tree tomato has caught the attention of growers and the industry due to its attractive, fleshy, edible fruits, which can be consumed either in salads or as a dessert fruit, or processed for making jams, yogurts, juices, or alcoholic beverages, among others [5]. It has developed from being a neglected crop, with a local interest in subsistence farms [6], into a promising fruit crop, having been introduced in several countries of Oceania, Southeast Asia, Europe and Africa [7]. Aside from South American countries, New Zealand is the largest producer and exporter of the tree tomato, where the marketable word, "tamarillo", was coined from the Maori term "tama", meaning leadership, combined with the Spanish word, "Amarillo", meaning yellow, or the word, "Tomatillo", meaning small tomato [8].

The interest in the tree tomato also lies in the high amounts of antioxidants, vitamins and carotenoids present in the fruit. The standard servings of tree tomato provide 67–75% of the recommended dietary intake (RDI) of ascorbic acid, 16–23% RDI

of α-tocopherol and 9-20% RDI of β-carotene [8]. However, the phytochemical profile of the tree tomato varies among cultivars and environmental conditions [8,9]. The main cultivar groups (orange, orange-pointed, purple, red, and red conical) are differentiated by the fruit colour and shape, with different ranges of morphological and genetic variation among them [6,10]. Despite the great potential of tree tomato as a new major fruit crop, there are no high-throughput genetic or genomic studies conducted for this species. Recent advances in RNA next-generation sequencing (RNA-seq) and bioinformatics resources facilitate transcriptomic studies, even for non-model plant species where reference genomes are not available [11,12]. In fact, RNA-Seq is successfully and increasingly performed to decipher the plant transcriptome of neglected plant species [13]. Nevertheless, RNA sequencing offers many other interesting features such as the evaluation of gene expression, polymorphism discovery, small RNA profiling, phylogenomics, and splice variant discovery, among others [14].

In this study, we performed the transcriptome sequencing and assembly of two tree tomato accessions with different fruit colors (purple and orange) followed by their comprehensive structural and functional annotation. In addition, intraspecific polymorphisms between the two cultivars and interspecific ones with tomato and potato were identified. The transcriptomes and the information generated in the present study will be a useful

resource for further genomic and molecular studies and will be a key genomic tool in assisting tree tomato breeding programmes.

3 Materials and Methods

3.1.1 Plant Material

The study was carried out in 2019 at the the Universitat Politècnica de València (UPV). A purple-fruited tree tomato accession (A21, with purple epicarp and mean fruit weight of 108.8 g) and an orange-fruited tree tomato accession (A23, with orange epicarp a mean fruit weight of 75.1 g) [6] (Figure 1), obtained from the UPV germplasm bank, were used for the present study. Seeds from each accession were germinated following the protocol of Ranil et al., (2015) [15]. Subsequently, the plants were grown in a greenhouse at UPV, Spain (GPS coordinates: latitude, 39° 28′ 55” N; longitude, 0° 20′11” W; 7 m above sea level). From each accession, tissues were sampled from several young leaves and flower buds and pools were made for each tissue and accession. Unfortunately, the two accessions did not set fruit under greenhouse conditions at our latitude, and thus fruit tissues were not used for the transcriptome assembly. All samples collected were immediately frozen in liquid nitrogen and stored at −80 °C for later use.

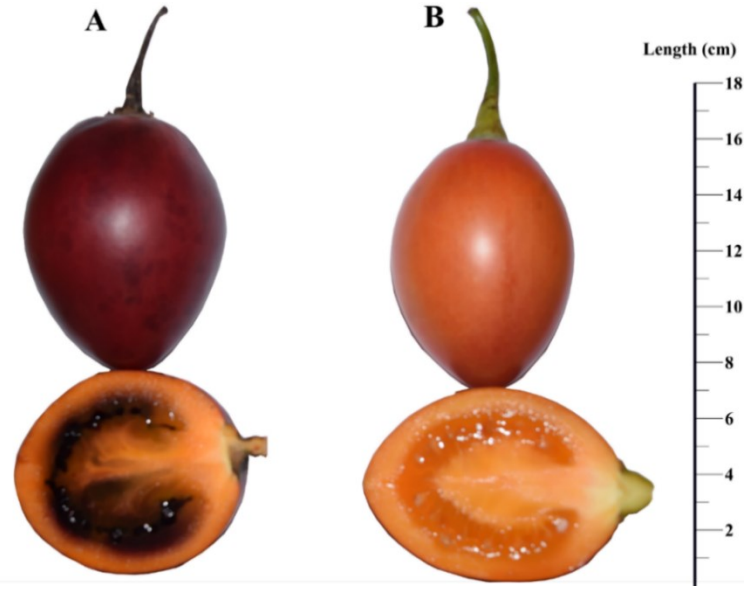

Figure 1. Fruits of tree tomato accessions A21 (**A**) and A23 (**B**).

3.2 RNA Extraction, Library Construction and RNA Sequencing

Total RNA was isolated from each tissue using the Mini spin kit (Macherey-Nage, Dueren, Germany). RNA integrity was determined by 1.0% (*w*/*v*) agarose gel electrophoresis and RNA quantification was performed by Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). From each accession, tissues were sampled from several young leaves and flower buds and pools were made for each tissue and accession. A total of 2 μg of RNA for each pool was sent to Novogene (Cambridge, UK) for library preparation and sequencing. The cDNA paired-end libraries of 150 bp (250~300 bp insert size) libraries were constructed according to Illumina's instructions. The mRNA of

each sample was purified from the total RNA by using Sera-mag Magnetic Oligo (dT), then fragmented into short fragments using the fragmentation buffer. Using these fragments as templates, the first strand of cDNA was synthesized. The second strand of cDNA was synthesized using the buffer containing dNTPs, RNase H, and DNA polymerase I. Short fragments (200 ± 20 bp) were connected to the sequencing adapters and suitable fragments were excised from an agarose gel using a gel extraction kit. Then, the library was sequenced using the Illumina Hiseq-2000 sequencer. The raw reads data are available at NCBI Sequence Read Archive (SRA) with accession number SRR15258852 (A21) and SRR15258851 (A23), within the bioproject number PRJNA749599, available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA749599 http://www.ncbi.nlm.nih.gov.

3.3 DNA Sequence Processing and de novo Transcriptome Assembly

The quality of reads was assessed using FastQC v0.11.8 [16]. The adapter sequences, low-quality reads (Phred score <30) and reads with an average length of less than 135 bp were trimmed using Trimmomatic v0.36 [17]. The two accessions were assembled separately using Trinity software v2.10 [18] with a default k-mer size of 25. Identical or near-identical contigs were clustered into a single contig by CD-HIT-EST tool v 4.8.1 [19] with an identity of more than 80%. The quality and completeness

of the assemblies were first evaluated with Bowtie2 v2.3.2 [20] for assessing the number of paired-end reads that were present in the assembled transcripts, then the Ex90N50 transcript contig length (the contig N50 value based on the set of transcripts representing 90% of the expression data) was computed using contig ExN50 statistic.pl script bundled with Trinity. Finally, the completeness of the assemblies was evaluated using BUSCO v4.1.1 [21,22] using a set of eukaryotic genes as a database (https://busco-data.ezlab.org/v5/data/lineages/eukaryota_odb10.2020-09-10.tar.gz) (accessed on 10 August 2020).

3.4 Structural and Functional Annotation

Gene open reading frames (ORFs) were predicted using Transdecoder v5.5.0 (http://transdecoder.sourceforge.net/) using the assembled unitranscripts as input. After the ORFs were extracted from the assembly, redundant contigs with over 90% identity were eliminated using CD-HIT-EST. Functional annotation of the assembled transcripts was conducted using OmicsBox software v 1.4.11 [23] and the Trinotate v3.2.1 pipeline (https://trinotate.github.io/) [24]. Both nucleotide transcripts and protein sequences were blasted against the UniProtKB/Swiss-Prot database (uniprot_sprot.trinotate_v2.0.pep.gz), using NCBI-BLASTx and BLASTp v2.10.1+ (-evalue 1e−3 -max_target_seqs 1 -outfmt 6).

Functional domains were identified using the Pfam domain database (Pfam-A.hmm.gz), which used HMMER v3.3.1 [25]. Potential signal peptides were identified using the SignalP v4.1 tool [26]. The OmicBox program (https://www.biobam.com/omicsbox/) was used to further annotate the transcripts using the functional annotation feature of Blast2GO software to predict Gene Ontology (GO) terms, EC Enzyme Code, identify potential KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, and orthology relationships using the eggNOG v5.0 databases [23,27,28]. GO enrichment analysis was performed using the "topGO" Bioconductor package (http://www.bioconductor.org/packages/release/bioc/html/topGO.html) (accessed on 5 September 2020).

3.5 Single-Nucleotide Variations (SNVs)

For the intraspecific SNVs, clean reads of each accession were mapped separately to the A23 assembled transcriptome which acted as a reference using BWA v0.7.17 [29], while for interspecific SNVs the reads were mapped against the reference genome of tomato (Heinz 1706 version SL4.0) [30] and potato (DM 1-3 516 R44 v6.1) [31]. Subsequently, SAMtools v1.10 [32] was used to convert SAM to BAM format while duplicate reads were removed from respective alignment sequences using Picard-tools v2.23.8 (http://picard.sourceforge.net) (accessed on 20 January 2021). Variants were called by FreeBayes v1.3.4 [33] to

identify intra- and interspecific polymorphisms that were filtered using VcfFilter v0.2 (https://github.com/biopet/vcffilter) (accessed on 20 January 2021) based on a minQualScore of 30, minTotalDepth of 40 and a minSampleDepth of 20. Finally, the variant impact effects were predicted using SnpEff v5.0 [34].

4 Results

4.1 Transcriptome Sequencing and Assembly

The RNA sequencing of the two tree tomato accessions yielded 100,919,310 (14.68 Gb) and 113,802,281 (15.84 Gb) raw paired-end reads for A21 and A23, respectively (Table 1). After the initial trimming and stringent quality filtering to remove adapters and low-quality data, 38,411,167 (4.25 Gb) clean paired-end reads were obtained for A21 and 54,474,055 (5.97 Gb) for A23 (Table 1). The two cohorts of clean reads were assembled independently into transcriptomes using Trinity. For the A21 accession, the assembled transcriptome consisted of 174,252 transcripts and spanned 148,352,996 bp, with an average transcript length of 851.37 bp (Table 1). The N50 value was 1494 bp and the GC content of 38.8% (Table 1). On the other hand, the A23 accession was assembled in 194,417 transcripts with a total length of 165,074,290 bp and an average length of 849.07 bp (Table 1). The N50 value for the latter was 1503 bp and the GC content of 38.6% (Table 1). The assembled sequence lengths ranged from the 200 bp cut-off value to a maximum transcript

length of 17,046 bp for A21 and 16,865 bp for A23 (Table 1). The majority of the assembled sequences were in the ranges of 200 bp to 500 bp and 501 to 1000 bp.

Table 1. Summary of raw and clean reads statistics before and after processing, de novo assemblies, and BUSCO completeness for tree tomato accessions A21 and A23.

	Accessions	
Statistics	**A21**	**A23**
Total raw reads	100,919,310	113,802,281
Total raw reads data size (Gb)	14.68	15.84
G/C (%)	42.2	42.2
Total clean reads	38,411,167	54,474,055
Total clean reads data size (Gb)	4.25	5.97
Number of transcripts	174,252	194,417
Total nucleotide length	148,352,996	165,074,290
Average transcript length	851.37	849.07
Maximum transcript length	17,046	16,865
N50	1494	1503
G/C (%)	38.8	38.6
Overall alignment rate (%)	99.09	99.21
BUSCO (%)	98.4	98.8

To evaluate the quality of the assemblies, the clean reads were mapped back to the final assembled transcriptome. The overall alignment rates using the alignment software Bowtie2 were 99.09% for A21 and 99.21% for A23 (Table1). BUSCO was employed to evaluate the accuracy and completeness of our transcriptome assembly, gene set, and transcripts. When comparing the set of genes with the genome, we found that the

proportion of complete BUSCO was 98.4% for A21 and 98.8% for A23, which indicated that the integrity of the whole transcriptome was very good (Table 1).

4.2 Structural and Functional Annotation

TransDecoder software was used to identify the open reading frames (ORFs) of the unitranscripts assembled and their associated functions, predicting 27,441 ORFs and 34,636 potential proteins for the A21 and 28,336 ORFs and 36,224 potential proteins for A23 (Table 2).

Table 2. Overview of the functional annotation by homology of transcriptomes for tree tomato accessions A21 and A23.

Statistics	Accessions	
	A21	**A23**
Predicted ORFs	27,441	28,336
Predicted proteins	34,636	36,224
sprot_Top_BLASTX_hit	57,422	60,772
sprot_Top_BLASTP_hit	24,311	25,054
Pfam	22,954	23,637
SignalP	1623	1745
TmHMM	6899	7216
GO terms	196,800	204,090
EC numbers	15,828	16,668
Kegg	14,035	14,540

Subsequently, the unique transcripts and the putative proteins identified were annotated by performing Blast searches against several databases using the Trinotate pipeline. A total of 57,422 (33%) and 60,772 unigenes (31.3%) displayed a significant homology when Blastx was performed and 24,311 (14.0%) and 25,054 protein sequences (12.3%) when Blastp searches were performed against the UniProtKB/Swiss-Prot database (cut-off E-value of 1e-3) for A21 and A23, respectively (Table 2). Furthermore, 22,954 and 23,637 unique Pfam protein motifs, 1623 and 1,745 protein sequences with signal peptides (SignalP), and 6899 and 7216 transcripts with at least one transmembrane domain (TmHMM) were predicted for A21 and A23, respectively (Table 2). The species distribution showed that most sequences exhibited a high similarity mainly to those of *Arabidopsis thaliana* (L.) Heynh. (17,602 for A21 and 18,117 for A23), *Oryza sativa* L. japonica group (1039 and 1066), *Nicotiana tabacum* L. (613 and 653), *S. lycopersicum* (487 and 486) and *S. tuberosum* (267 and 276) (Figure 2).

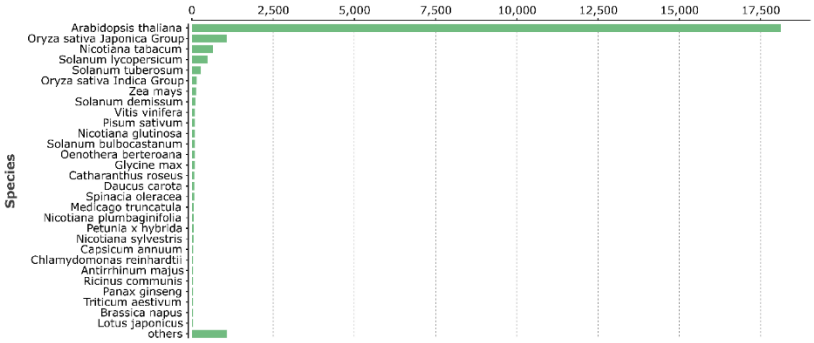

Figure 2. Top species distribution of annotated unigenes for tree tomato accessions A21 and A23.

GO-based functional classification for *A21 and A23* transcriptomes assemblies retrieved a total of 196,800 GO terms for A21 and 204,090 for A23 from 22,096 and 23,095 transcripts, respectively (Table 2). The largest number of GO terms (75.2%) was annotated in sequences with a length between 100 and 500 bp (Figure 3).

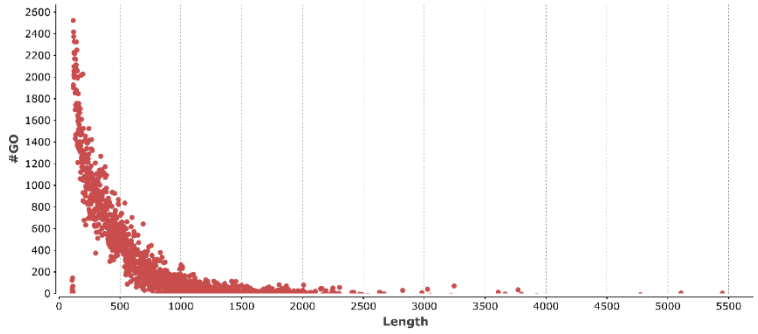

Figure 3. Numbers of GO terms relative to sequence length in the transcriptomes of tree tomato accessions A21 and A23.

Both assemblies had a similar GO distribution for each category; four to nine terms in biological process (BP), three to nine in molecular function (MF) and four to eight in cellular components (CC) category (Figure 4). The GO levels that ranged between 5 and 15, were 88.9% for biological processes, 69.8% for molecular function and 88.2% for cellular components, indicating that the precision of the annotation was accurate (Figure 4) and that a broad diversity of genes was sampled in our transcriptomes.

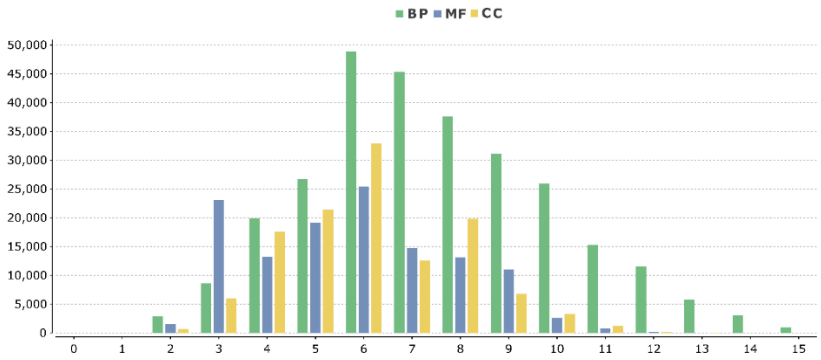

Figure 4. GO level distribution in each category for the annotated tree tomato unigenes. X axis represents the GO level and Y axis the number of annotated unigenes. BP = Biological Process, MF = Molecular Function, CC = Cellular Component.

Among all the GO terms extracted, 137,333 (69.8%) for A21 and 140,193 (68.7%) for A23 were assigned to the biological process category, 35,153 (17.9%) and 38,464 (18.9%) to the

molecular function class and 24,314 (12.4%) and 25,233 (12.5%) to the cellular components, respectively (Figure 5).

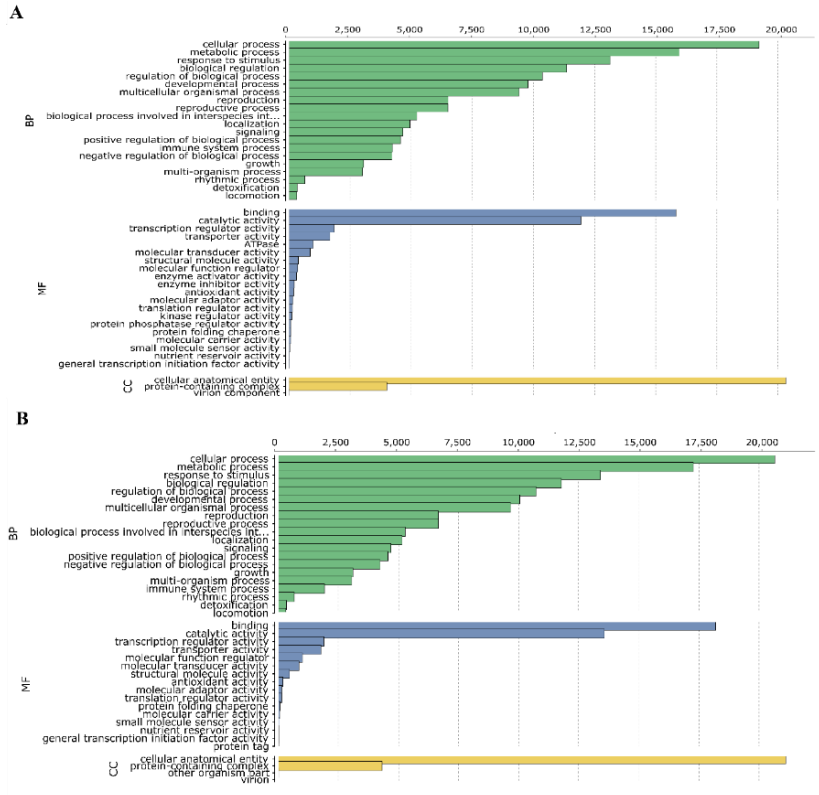

Figure 5. Gene ontology (GO) functional classification of tree tomato A21 (**A**) and A23 (**B**) transcriptomes. Histograms of transcripts annotated to specific GO categories; BP = biological process, MF = molecular functions and CC = cellular components and are represented by green, blue, and yellow bars, respectively.

For the biological process category, the top three subcategories were the cellular process with 19,220 (14.0%) sequences for A21 and 20,660 (14.8%) for A23, the metabolic

process with 15,954 (11.6%) and 17,273 (12.3%) and the response to stimulus with 13,134 (9.6%) and 13,400 (9.6%) sequences (Figure 5). For the molecular function category, the vast majority of sequences belonged to two subcategories: binding sequences (15,824; 45% for A21 and 18,204; 47.3% for A23) and catalytic activity (11,940; 34% and 13,566; 35.3%) (Figure 5). Finally, for the cellular component category, most sequences were classified into two sub-categories: cellular anatomical entity (20,315 sequences; 83.6% and 21,109; 83%) and the protein-containing complex (4315; 16.4% and 3998; 17%) (Figure 5). For A21, the GO term enrichment analysis indicated significant GO terms associated with a defense response (GO:0006952), proteolysis (GO:0006508), cellular and lipid metabolic processes (GO:0006629, GO:0044255), carotenoid metabolic processes (GO:0016116), and carotenoid biosynthetic processes (GO:0016117) (Figure 6, Supplementary Table S1). Different to A21, the significantly enriched GO terms of A23 were protein localization (GO:0008104), root morphogenesis (GO:0010015), root development (GO:0048364), post-embryonic plant organ morphogenesis (GO:0090697), the regulation of catabolic process (GO:0009894), the regulation of the cellular catabolic process (GO:0031329) (Figure 6, Supplementary Table S1).

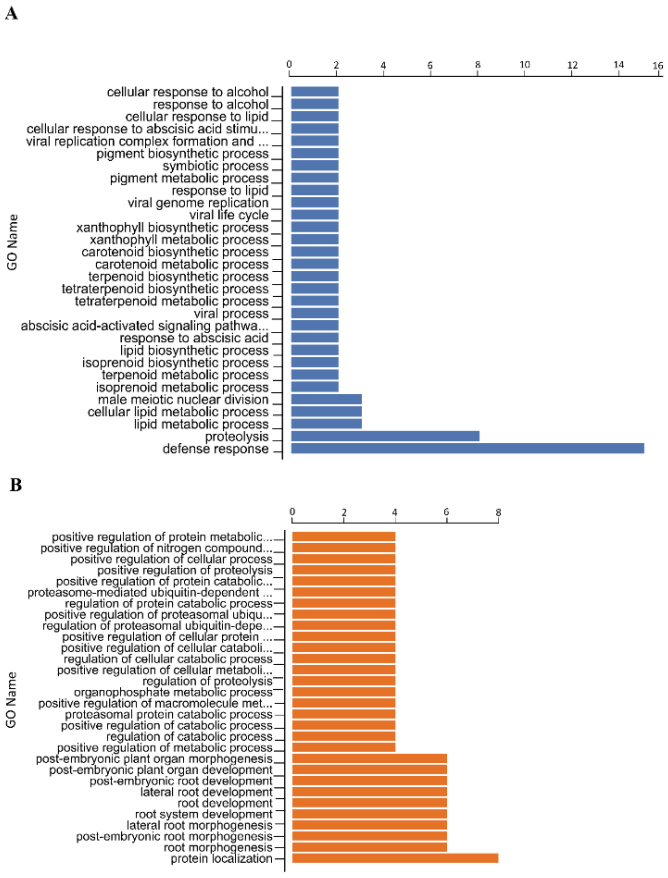

Figure 6. GO enrichment analysis in tree tomato A21 (A) and A23 (B) transcriptomes.

Several candidate regulatory genes of the carotenoid biosynthetic pathway were identified in the assembled transcriptomes from *S. lycopersicum* and *A. thaliana*. The protein query sequences used for mining the transcriptomic data were the *S. lycopersicum* prolycopene isomerase (*CRTISO*), 9-cis-epoxycarotenoid dioxygenase (*NCED1*), lycopene epsilon cyclase (*Lcy-e*), neoxanthin synthase (*NSY*) and the *A. thaliana*

protein ORANGE (*OR*) (Supplementary Table S2). All of them were found to be expressed in both cultivars, where *CRTISO* and *Lcy-e* homologues exhibited a high identity (96%), followed by *NCED1* with 95%, *NSY* with 93%, and finally, *OR*, which showed a higher identity in A23 (74%) than A21 (71%) (Supplementary Table S2).

The enzyme commission (EC) numbers were assigned to 15,828 for the A21 and 16,668 for the A23 unigenes (Table 2). The most represented enzymes were hydrolases (5489 unigenes in A21 and 5562 in A23), transferases (5430 and 5857), oxidoreductases (1766 and 2031) and translocases (1708 and 1680) (Figure 7). Other enzyme classes such as lyases, isomerases, and ligases were represented to a lesser degree.

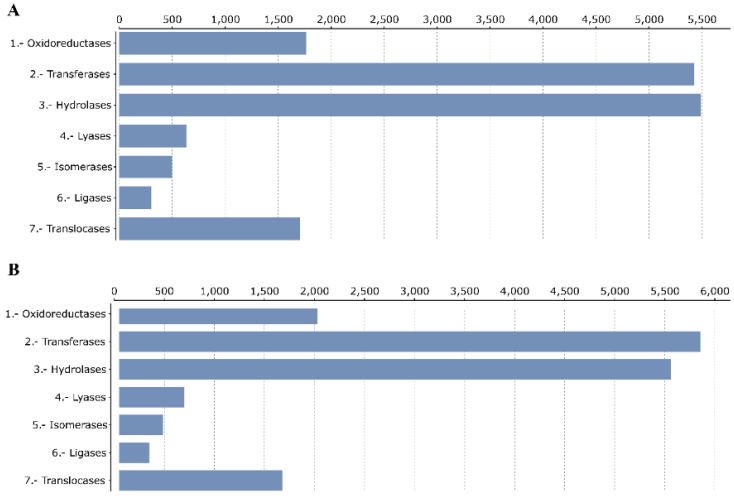

Figure 7. Number of unigenes for each enzyme commission (EC) category for tree tomato A21 (A) and A23 (B) transcriptomes.

KEGG analysis was performed to identify the potential mechanisms and pathways represented in the identified unigenes. A total of 14,035 unigenes for A21 and 14,540 unigenes for A23 were assigned to the 155 and 161 KEGG pathways, respectively (Table 2). The most represented pathways in terms of the number of homologous transcripts were purine metabolism (map00230, 58 sequences), cysteine and methionine metabolism (map00270, 58 sequences), amino sugar and nucleotide sugar metabolism (map00520, 46 sequences), terpenoid backbone biosynthesis (map00900, 33 sequences), drug metabolism (map00983, 20 sequences), flavonoid biosynthesis (map00941, 17 sequences), and carotenoid biosynthesis (map00906, 17 sequences).

4.3 COG Classification

A Cluster Orthologous Group (COG) is defined as a cluster of three or more homologous sequences that diverge from the same speciation event. Orthologous groups were functionally annotated using the EggNog (evolutionary genealogy of genes: Non-supervised Orthologous Groups) database. In total 97,437 for the A21 and 99,471 for the A23 GO were assigned to 14,530 and 14,928 unique sequences, respectively (Figure 8). The largest group is represented by the cluster for cellular processes and signaling (CPS) (6311; 21.4% and 6443; 21%), followed by metabolism (MB) (6052; 20.5% and 6,417; 20.9%), information storage and processing (ISP) (6040; 20.4% and 6,396; 20.9%) (Figure 8). Within the CPS category, the largest proportion was

assigned to signal transduction mechanisms (T) (2359 for A21 and 2392 for A23) and post-translational modification, protein turnover, and chaperones (O) (2051 and 2104). Within the MB category, the largest proportion was assigned to amino acid transport and metabolism (E) (1232 and 1,342), and carbohydrate transport and metabolism (G) (1249 and 1314), and within the ISP category, the majority were assigned to replication, recombination, repair (L) (2043 and 2254), and transcription (K) (1997 and 2043) (Figure 8).

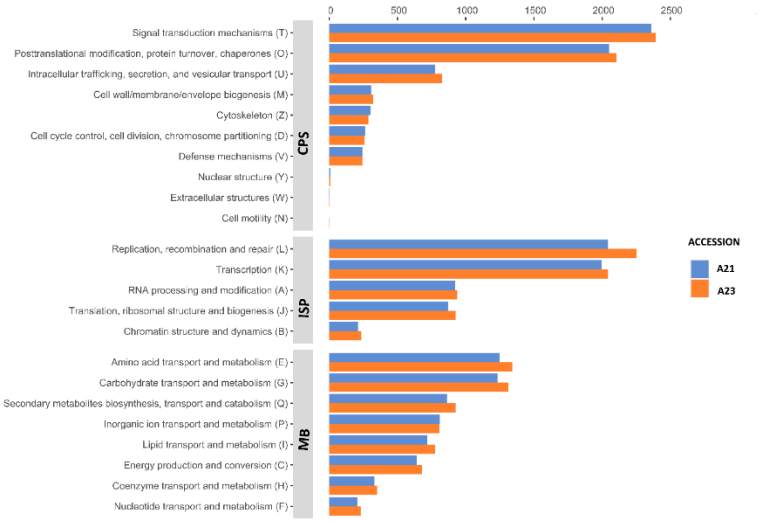

Figure 8. COG categories in the transcriptomes of tree tomato accessions A21 and A23.

4.4 Identification and Characterizacion of SNVs

Intra- and interspecific polymorphisms were identified in both accessions and between the tomato and potato genomes. The number of intraspecific SNVs was significantly higher in the A23 (49,530) than in the A21 accession (19,117) (Table 3). Of these, 14,837 (77.6%) in A21 and 38,183 (77.1%) in A23 were SNPs, 3283 (17.2%) and 8213 (16.6%) were multiple-nucleotide polymorphisms (MNP), 767 (4%) and 2391 (4.8%) were InDels, and 227 (1.2%) and 726 (1.5%) were multiple-nucleotide and an InDel (MIXED) (Table 3). Among the SNPs, the number of transitions (10,687 in A21 and 25,925 in A23) was higher than the number of transversions (5758 and 13,810), with a transition/transversion (Ts/Tv) ratio of 1.86 and 1.88, respectively (Supplementary Table S2). For transition substitution, the most abundant were C/T (17.4% in A21 and 17% A23), followed by G/A (16.8% and 16.6%), A/G (15.7% and 16.1%), and T/C (14.2% and 15.4%) (Supplementary Table S3). In the case of the transversion substitution, the frequency of occurrence of the SNPs was A/T, (6.2% and 5.4%) followed by T/A (5.6% and 5.4%), G/T (4.6% and 4.8%), C/A (4.3% and 4.5%), A/C (4.1% and 4.3%), G/C (3.0% and 2.9%) and C/G (2.7% and 2.6%) (Supplementary Table S3). The average genomic SNPs and InDels variation frequency were 1 in 242 bp in A21 and 1 in 204 bp in A23. In both accessions, the number and proportion of heterozygous variants were higher (74% in A21

and 79% in A23) than the homozygous variants (Supplementary Table S3).

Table 3. Polymorphism statistics for the tree tomato A21 and A23 transcriptomes.

Statistics	SNPs	MNP	INDELs	MIXED	Total SNVs
SNVs intraspecific variations					
A21	14,837	3283	767	227	19,117
A23	38,183	8213	2391	726	49,530
SNVs interspecific variations					
A21 and *S. tuberosum*	619,626	174,982	28,2835	23,115	1,973,023
A23 and *S. tuberosum*	805,997	242,484	42,142	36,352	
A21 and *S. lycopersicum*	624,503	194,857	23,407	20,788	1,809,264
A23 and *S. lycopersicum*	684,775	218,205	27,102	24,627	

The vast majority of the variants (12,095; 50.4% in A21 to 38,632; 61.3% in A23), classified according to SNPeff, were predicted as a "modifier", i.e., the variants were located in intergenic or intronic regions, or in an exon from a non-coding transcript, which indicates that there is no evidence of their impact or that their predictions are difficult to assess (Supplementary Table S4). The second most abundant impact effects predicted were "low" (6507; 27.1% and 11,732; 18.7%), which were mostly harmless variants or unlikely to change

protein behaviour (Supplementary Table S4). The third ones were those predicted as having "moderate" impact effects (5139; 21.4% and 11,637; 18.6%), i.e., nondisruptive variants, such as codon insertion/deletion or codon substitution, which might change protein effectiveness (Supplementary Table S4). Finally, the less abundant impact class corresponded to the "high" variation effects (262; 1.1% and 808; 1.3%), which were considered to have a disruptive impact on the protein-like truncation or loss of function caused by exon deletion/deletion (Supplementary Table S4). The top variant categories were in the exon regions (48% for A21 and 37% for A23), intergenic regions (21% and 30%), 3' UTR variant (17%), 5' UTR variant (14% and 16%) and the synonymous variant (25% and 17%) (Supplementary Table S5). Regarding the effects on protein function, on average, 58% of the variants in A21 and 52% in A23 were predicted to produce a silent effect (41% and 47%), a missense impact (1%) and a nonsense protein product (Supplementary Table S5).

Regarding the interspecific SNVs, the highest number of SNVs were identified with potato (1,973,023) and a little less with tomato (1,809,264). Of those, 1,425,623 (72.3%) with potato and 1,309,278 (72.0%) with tomato were SNPs; 417,416 (21.2%) and 413,062 (22.7%) were MNP; 70,427 (3.6%) and 50,509 (2.8%) were InDels; and 59,507 (3.0%) and 45,415 (2.5%) were MIXED

(Table 3). The accession A23 exhibited a higher number of interspecific variants than A21 (Table 3). In contrast to the intraspecific SNVs, the proportion of homozygous variants was higher (over 95%) than the heterozygous ones. Considerable differences were observed in the average number of polymorphisms among the chromosomes, with differences of over two-fold between chromosome 1 (259,267 in potato and 237,098 in tomato) and chromosome 12 (127,595 and 113,202) in both accessions (Table 4).

Table 4. Chromosome distribution of tree tomato variants with potato (*S. tuberosum*) and tomato (*S. lycopersicum*).

	Species	
Chromosome	***S. tuberosum***	***S. lycopersicum***
1	259,267	237,496
2	200,827	90,558
3	205,561	92,045
4	176,113	78,104
5	133,442	59,508
6	164,694	72,583
7	152,942	67,774
8	140,397	62,569
9	148,610	64,651
10	130,071	58,183
11	133,138	59,040
12	127,595	54,634

The impact of 3,186,724 SNVs (58.7%) in potato and 2,095,805 (59.9%) in tomato was classified as a "modifier"; 1,192,042 (21.9%) and 728,209 (22.3%) were classified as "low"; 1,029,629 (19%) and 611,869 (17.5%) were classified as "moderate"; and the impact of the remaining 23,696 (0.4%) and 12,268 (0.4%) SNPs were classified as "high" (Supplementary Table S4). The majority of variant categories were in the exons (39% to 43%), downstream gene variant intergenic regions (25% and 28%), upstream gene variant (15% and 19%), 3' UTR variant (5% and 8%), intron variant (2% and 6%), intergenic region (2% and 3%), and the 5' UTR variant (2% and 3%).

We further analyzed the sequences of the candidate genes that played an important role in the carotenoids biosynthesis, identifying a total of 1548 SNVs in the two cultivars assessed when compared to the tomato reference genome (Table 5). Of them, 478 SNPs were found in the coding region of the prolycopene isomerase (*CRTISO*) gene, 372 in 9-cis-epoxycarotenoid dioxygenase (*NCED1*), 194 in lycopene epsilon cyclase (*Lcy-e*), 164 in neoxanthin synthase (*NSY*), and 340 in protein ORANGE (OR) (Table 3). The impact of the majority of variants (42.2%) was classified as a "modifier", 31.6% as "low", 17.9% as "moderate" and 1.9% as "high". Regarding the effects on protein function, on average, 51% of the variants were synonymous mutations, while the remaining variants were missense mutations.

Table 5. Single-nucleotide variations (SNVs) identified in candidate genes of the carotenoids biosynthesis.

Statistics	A21	A23	Total SNVs
CRTISO	233	245	478
NCED1	174	198	372
Lcy-e	91	103	194
NSY	79	85	164
OR	139	201	340

5 Discussion

Although tree tomato is one of the most promising fruit crops in the Mediterranean and temperate regions [4], its genomic landscape has not yet been explored yet. Other unexploited crops similar to the tree tomato, such as the cape gooseberry (*Physalis peruviana L.)* and amaranth *(Amaranthus cruentus* L.), have greatly benefited from genomic studies, which have fostered the dissection of multiple agronomic traits and breeding programs [35–37]. In this study, we conducted the de novo transcriptome assembly of two tree tomato cultivars to provide useful genomic data for the improvement of this unexploited but emerging crop. Through RNA sequencing, a total of 174,252 (for A21) and 194,417 (for A23) transcripts were assembled from 38 and 54 million filtered reads and with an average length of 851 and 849 bp, respectively. The number of transcripts of these accessions was slightly higher than those obtained in previous transcriptome studies in other related Solanaceae species such as tomato, potato

or pepino *(Solanum muricatum* Aiton*)* [14,38,39], but it was similar to others obtained in plant species of the same family, such as *S. commersonii* Dunal and *S. aculeatissimum* [40,41], suggesting the high quality and reliability of our assemblies. Furthermore, the assembly and annotation completeness was quantitatively confirmed by the high percentage values (>98%) of the BUSCO assessment, values that were comparable or even higher than those of other recent *Solanum* transcriptomes, which exhibited values of 97% for *S. tuberosum* and 93% for *S. chilense* [42,43].

The functional annotation of the assembled unigenes is essential for understanding the role of the represented genes [44]. Even though the number of protein-coding genes is unknown in tree tomato, the prediction of the potential ORFs (27,441 in A21 and 28,336 in A23) and proteins (34,636 in A21 and 36,224 in A23) was in agreement with those observed for protein-coding genes in other *Solanum* species, such as tomato (35,535), potato (39,290), eggplant (*S. melongena* L.) (30,630 and 34,231) [45–47]. Similarly, signal peptides, transmembrane and Pfam domains were assigned to around 5%, 20%, and 65% of the identified proteins, respectively. These percentages were higher than those obtained in other plant species of the Solanaceae family such as *S. trilobatum* and *S. sisymbriifolium* [48,49]. The GO annotation revealed that unigenes could be categorized into three major functional categories: biological processes (68%), molecular functions (18%) and cellular components (12%). The top two

subcategories were the cellular and metabolic processes in the biological processes, binding, and catalytic activity of the molecular function; and the cellular anatomical entity and protein-containing complex in the cellular component, which suggests that many novel genes involved in metabolic activities could play important roles during the growth and development stages of the plant.

The KEGG annotation allows for the functional analysis and interpretation of transcriptomic data and exhibits how the assembled transcripts are integrated into metabolic pathways and biological systems [50]. A total of 155 pathways in A21 and 161 in A23 involving 14,035 and 14,540 unigenes were annotated, including pathways of great interest that could be used to improve the quality of breeding programs for the breeding of tree tomato such as purine metabolism, drug metabolism, terpenoid backbone biosynthesis, and the biosynthesis of flavonoid and carotenoids. The increased accumulation of flavonoids and carotenoids in fruit crops improves their commercial and health values [51]. Among the biological features, the most renowned property of flavonoids and carotenoids is their antioxidant effects, which are often much higher than those of vitamin E and vitamin C [52,53]. Our transcriptional results confirmed the presence of known genes and enzymes in pathways related to the synthesis of flavonoids and carotenoids. These results are in agreement with previous studies that reported the tree tomato as an abundant source of carotenoids, anthocyanins, flavonoids, and phenolic compounds

and has higher antioxidant activity than other antioxidant-rich fruits such as kiwifruit or grape [7]. The carotenoid concentration in tree tomato may be under the control of several genes that are associated with the structure and function of the genes in the carotenoid pathway. In the accession A21, our data showed that the carotenoid biosynthetic process GO terms were significantly enriched. This is in agreement with the previous results of [54,55] who reported that the purple cultivar had higher levels of carotenoids compared to the yellow or orange cultivars. Our results suggested that the flavonoid and carotenoid biosynthesis pathway-related genes were well conserved in the tree tomato when compared with the tomato [56]. The sequence variants in these genes among tree tomato varieties could be used as functional markers for marker-assisted breeding to obtain new varieties of tree tomato with improved nutritional values.

We obtained a total of 68,647 SNVs between both accessions, suggesting a high level of polymorphisms for tree tomato. The SNVs reported here were higher than the cohorts identified in other transcriptomic studies of Solanaceae, such as the 17,000 SNVs found in tomato [39]; however, in the case of potato a similar number of SNPs, 69,011, were reported [57]. The A21 accession exhibited a higher number of SNVs and, interestingly, the vast majority of the detected variants were heterozygous. The latter might be due to the fact that, even though some tree tomato cultivars are considered self-compatible and

autogamous, the flowers are frequently visited by pollinator insects, which can lead to cross-pollination [4]. The annotated SNP effects located in the exon and intergenic regions and a transition to transversion ratio of 1.86 agree with previous findings in tomato and eggplant [39,58]. On the other hand, the number of interspecific variants detected with potato was significantly higher than those of tomato, confirming that tree tomato is phylogenetically closer to the latter [59]. The data also indicated that the differences in variant number between the tree tomato and its closely related species were evident, particularly for chromosomes 1 and 12, which were highly related to the physical length between them. Regarding the SVNs found in the candidate genes involved in the carotenoids biosynthesis pathway, our results showed that the *CRTISO* gene exhibited the highest number of SNPs, which could be due to mutations in its coding sequence. The carotenoid analysis in tomato ripe fruits showed that a mutation in CRTISO leads to a prolycopene accumulation instead of all-trans-lycopene compounds, resulting in a fruit color change from red to orange [60]. In addition, most of the SNVs within the genes involved in carotenoid metabolism resulted in synonymous substitutions. These results were consistent with previous studies in tomato [61] where protein expression and protein folding may be influenced by synonymous SNPs as they are involved in regulating microRNA-mediated genes [62,63]. Hence, the synonymous SNPs identified in the tree

tomato cultivars in this work may have potential functional significance in carotenoid biosynthesis.

The identification of the intraspecific and interspecific variants will foster several applications including genetic mapping, genotype identification, marker-assisted selection, breeding, comparative genomics, and understanding the genetic control of adaptive traits in the tree tomato [64].

6 Conclusions

In this work, we assembled high-quality transcriptome sequences of two tree tomato cultivars, a fruit crop closely related to tomato and potato, with great potential in subtropical regions. The comprehensive annotation provided extensive and detailed information that facilitates the dissection of traits of agronomic interest, such as the content in bioactive compounds or the response to stresses, among others. In addition, this is the first study in tree tomato where a high number of polymorphisms have been identified, both intraspecifically and with closely related species that could be used in genetic diversity analysis, qualitative and quantitative trait mapping, and breeding programs in tree tomato. This information constitutes a valuable resource for tree tomato breeding programs and genetic diversity studies and will help in the enhancement of tree tomato and its successful introduction in other regions and countries.

7 Statements

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: GO enrichment analysis in A21 and A23, Table S2: Identification of regulatory genes of the carotenoid biosynthetic pathway in A21 and A23 from *Solanum lycopersicum* and *Arabidopsis thaliana,* Table S3: Number of transitions/transversions, variant rate, and homozygous and heterozygous variants, Table S4: Number of predicted effects by impact, Table S5: Percentage of effects by region and functional class.

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Conflicts of Interest: The authors declare no conflict of interest.

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General Discussion

Today, our food systems depend on a small number of plant species, three of which rice, corn and wheat are responsible for more than 50% of our energy intake and are called “staple crops”. However, we ignore the enormous diversity of neglected and underutilized crop species (NUS) that present an enormous opportunity for food and nutrition security and the health of the world's population. NUS also could help agriculture adapt to climate change by improving genetic diversity and the resilience of agroecosystems in the face of increasing environmental stresses and offering farmers and researchers opportunities to improve crops that can be grown in harsh climatic conditions, such as drought, saline soils, poor soil fertility and biotic stress. Among those underutilized crop groups are pepino and tree tomato, these species are highly valued for their various nutritional and health benefits.

Therefore, the development or introduction of new crop varieties adapted to different growing areas and climate-resilient is a key strategy for improving food security and adaptation to climate change.

In this context, assessing and selecting pepino germplasm in search of new desirable characteristics to face climate change, such as the one studied in this thesis, could promote the improvement and expansion of this underutilized species in other regions of the world. On the other hand, bioinformatics tools

could be exploited to use the information from genomic and genetic studies for the improvement of the tree tomato.

Water stress is one of the main limitations for agriculture. In recent decades, global warming has been aggravating this situation in most agricultural regions. Therefore, it is essential to study the biological mechanisms and processes that allow plants to overcome the effects of water deficit. In fact, under prolonged drought, plants show a wide range of adaptations, at different levels of their organization, which varies from species to species and even within species.

In the first chapter of this Thesis, we described the strategies used by pepino to adapt to water stress conditions at the physiological and biochemical levels.

In general, during moderate water deficit pepino has been shown to be tolerant, with no significant changes in growth and biochemical parameters compared to control plants. While severe water stress affects several growth characteristics that was observed in the reduction of fresh weight of leaves, stems and roots, water content, degradation of photosynthetic pigments and changes in several biochemical parameters. These results are in accordance with previous research on pepino (Duman and Sivaci, 2015).

Since pepino is tolerant under conditions of moderate water deficit, there are opportunities for substantial improvements in the water use efficiency of this crop to be achieved using different irrigation techniques without affecting plant development (Hatfield and Dold, 2019). Responses to water stress of all pepino cultivars were qualitatively similar, as would be expected from closely related genotypes, however, selection of drought tolerant cultivars using quantitative methods such as principal component analysis of growth characteristics discriminated between Mur2 and Mur4 cultivars as the most drought tolerant. The increase observed in the content of ions, fundamentally Na^+, Cl^-, K^+ in response to water stress, has as a consequence the protection of pepino plants from the effects caused by water stress, which could be one of the mechanisms used for water stress tolerance to drought, since these osmolytes contribute to the cellular osmotic adjustment of plants under stress (Flowers and Colmer, 2008). The drought-tolerant cultivars identified in this study could be useful to develop cultivars with higher tolerance to drought.

Biotic stress is a major factor causing yield losses in most crops in all agricultural areas of the world. Pests and diseases can reduce up to 100% of the yield in vegetables. In addition, climate change is generating a higher incidence of insects, pests, diseases and weeds in crop production, especially in tropical and Mediterranean regions.

The increasing of climate change will become more relevant to some biotic stresses and may threaten the expansion of new emerging crops such as pepino. In this sense, the development of pepino cultivars with stable and long-lasting resistance to several pests and diseases that affect other solanaceous crops can help the introduction of pepino in other regions.

In the second chapter of this Thesis, the response of a collection of cultivated pepino and wild related species to four main diseases *Fusarium*, *Verticillium*, PepMV and ToMV that can affect pepino was evaluated in order to identify tolerant or resistant genotypes to these diseases.

The screening of cultivated pepino and wild related species revealed that there is a wide variation for the resistance and tolerance against *Fusarium*, *Verticillium*, PepMV and ToMV. Symptoms varied greatly among susceptible and resistant/tolerant accessions, indicating a reduced disease progression in resistant/tolerant accessions.

According to the incidence of the disease, most of the cultivated pepino accessions and wild relatives were tolerant or resistant to *Fusarium*, Mur2 accession of cultivated pepino did not display symptoms during the entire trial, so it should be considered highly resistant to *Fusarium* race 2. Regarding the wild relatives of pepino, the Car1 and Car2 (*S. caripense*) accessions had a good performance and no symptoms were

observed in all the phases of the experiment, so they could also be considered resistant. In the *Verticilium* trial, the cultivated pepino genotypes exhibited a better response to the disease compared to the wild relatives, the Mur2, Mur4 and Mur5 accessions presenting mild symptoms that could be considered tolerant. These results suggest that cultivated pepino could be used in breeding programs instead of wild species, avoiding the incorporation of undesirable traits of wild species because of the linkage drag. (Prohens et al., 2017).

On the other hand, the results indicate that when plants of different genotypes were inoculated with PepMV, only the wild accesion Car1 was resistant to the infection, while the cultivated Mur6 was tolerant. These results were confirmed by ELISA assays that were similar to those of the symptoms. None of the plants inoculated with ToMV were resistant, in contrast with the work reported by Leiva-Brondo et al. (2006) who found resistance in pepino plants.

Broadly, our results show that the cultivated pepino accessions Mur2, Mur4 and the hybrid with *S. caripense* have been shown to be tolerant and moderately tolerant to the four diseases, indicating that they may be good materials to introduce multiple resistance genes against these pathogens in othert elite commercial varieties.

"Next-generation sequencing" (NGS) has evolved over the last two decades, greatly contributing to the development and advancement of new insights obtained by analyzing large-scale data. Omics-level data derived from whole-genome analyses has led to substantial improvements in quality and performance of plant breeding, being able to analyzes and obtain large-scale data at multiple levels derived from the analyses of complete genomes, proteomes, and transcriptomes.

In the third chapter of this Thesis, we sequenced and assembled the transcriptome of two tree tomato accessions. Specifically, we focused on the analysis and identification of unigenes, their annotation and the identification of a wide set of molecular markers.

Since the tree tomato genome is not yet available, de novo transcriptome assembly is the most pragmatic way to retrieve reliable and informative data for a plethora of applications. Using the Illumina sequencing platform, the transcriptome of two tree tomato accessions with purple fruits (A21) and orange fruits (A23) were assembled. A total 38,411,167 clean reads were obtained for A21 and 54,474,055 for A23 and two transcriptomes of (4.25 Gb) and (5.97 Gb) respectively. The assembled transcriptome consisted of 174,252 transcripts for A21 and 194,417 for A23, with an average transcript length of 851.37 and 849.07 bp. The GC content analysis revealed that tree tomato transcripts have GC content similar to that of other Solanaceae

such as eggplant and pepino (Gramazio et al., 2016; Herraiz et al., 2016). The GC content analysis of DNA sequences from an organism's genome provides useful information about gene structure and regulation, thermostability, and evolution (Thanki et al., 2014; Zhang et al., 2004).

Tree tomato transcripts were annotated by searching various databases. At the protein level, more than 31% showed significant similarity to predicted unigenes/proteins from other sequenced vegetable or plant genomes. In addition, functional categorization based on GO terms revealed the conservation of genes involved in various biological processes in tree tomato. Another aspect of this work was to assign transcripts to different metabolic pathways to identify candidate genes related to traits of interest. In our tree tomato transcriptome database, we identified genes encoding putative enzymes involved in carotenoid biosynthetic pathways. Comparative analysis between assembled transcriptomes of *S. lycopersicum* and *A. thaliana* showed that the expression levels of prolycopene isomerase (CRTISO), 9-cis-epoxycarotenoid dioxygenase (NCED1), lycopene epsilon cyclase (Lcy-e), neoxanthine synthase (NSY) and ORANGE (OR) were more abundant than other genes involved in carotenoid biosynthesis pathway, indicating that the transcriptional regulation of these genes could be important for the accumulation of carotenoid content in tree tomato.

To to develop sets of markers that can be easily used in tree tomato breeding programs, intra and interspecific variants were detected in both accessions and between tomato and potato genomes. A total of 68,647 single nucleotide variations (SNVs) were identified in silico between both accessions, while the number of interspecific SNVs was almost 2 million.

The results obtained in this Doctoral Thesis may be of great interest for the improvement of these underutilized crops, mainly to develop crops adapted to both biotic and abiotic environmental stress conditions, in addition, information at the genomic level will facilitate the understanding of the genetic mechanisms and molecular related to characters of agronomic interest.

Conclusions

From the results obtained in each of the chapters that make up this doctoral thesis, we can draw the general conclusions listed below.

1. Moderate and severe water stress affects several growth and biochemical traits, compared to control plants, being more significant for plants under conditions of severe water stress.
2. Severe water stress causes a dramatic loss of photosynthetic pigments, malondialdehyde, and total flavonoids, while an increase in proline, Na^+, and K^+ contents was observed.
3. Screening of drought tolerant pepino cultivars using growth and biochemical parameters identified the Mur2 and Mur4 cultivars as the most drought tolerant.
4. Some cultivated pepino accessions and wild relatives showed a high level of resistance/tolerance against *Fusarium* and *Verticilium*.
5. Our data suggest that several accessions of the cultivated species of pepino showed a better performance than wild relatives in the search for sources of resistance/tolerance to some of the main potential pepino pathogens.
6. The only three cultivated pepino accessions Mur2, Mur4 and Mur6 and the hybrid were classified as tolerant and moderately tolerant to all diseases.
7. The tree tomato transcriptome is the first genomic resource to provide a large collection of assembled and functionally annotated sequences.

8. The large number of SNPs detected are important data for tree tomato genetic improvement through marker-assisted breeding.
9. Some of the genes identified in this work provide candidates for genes related to the carotenoid biosynthesis pathway, being a valuable resource for quality improvement of tree tomato.
10. The results obtained in this Thesis have made it possible to identify materials that can be used to develop pepino varieties that are tolerant or resistant to biotic and abiotic stresses. The developed genomic tools will be useful to deepen the knowledge of the molecular mechanisms of the tree tomato.

References (Introduction and General Discussion)

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