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ROLE OF THE ROOTSTOCK IN INFLUENCING FRUIT QUALITY AND TOLERANCE TO SALT STRESS IN PIGMENTED CITRUS SPECIES

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Research highlights

- Flavonoids and phenolic acids were identified in eleven blood orange cultivars and 15 phenolic compounds were individually identified and quantified.
- The influence of ten rootstocks on fruit metabolic profile and antioxidant activity of Tarocco Sciré blood orange was assessed, and 24 phenolic compounds were individually identified and quantified.
- Rootstock influence on the evolution of bioactive compounds of blood orange Tarocco Sciré during maturation was evaluated and a positive correlation between rootstock and the regulation of fruit-quality related genes in the scion was found.
- The influence of eleven rootstocks in combination with Mandared on yield precocity, fruit drop, cumulative production, yield efficiency and fruit quality, including anthocyanin accumulation, was detected.
- Morpho-physiological, enzymatic and hormonal evaluation of eight rootstocks under different levels of salt stress was evaluated. Overall, Furr had a tolerance response similar to *C. macrophylla* and *C. volkameriana.*

Abstract

Horticulture is a key sector for the Italian and Mediterranean agricultural economy and citrus, with a world production of 157 million tons, is ranked as the first fruit crop in the world in terms of production. The use of grafting in citriculture played a key role in the success of citrus industries worldwide. In fact, rootstock has a strong influence on many agronomical traits such as: yield, fruit quality, tree size, and can also play a pivotal role in conferring tolerance to abiotic (i.e. calcareous soils, salinity, flooding, cold,) and biotic (Phytophthora, CTV, nematodes, *Diaprepes/Phytophthora* complex) constraints. For this reason, the choice of the most suitable rootstock for a specific growing area (thus, taking into consideration pedoclimatic conditions, crop management, scion adopted, fruit destination,) is a crucial aspect for the set-up of novel productive orchards. Furthermore, the rootstock can be effective to limit the environmental stresses, as it is able to modulate the plant response showing a decisive influence on plant growth, flowering, and fruiting.

In the present thesis, a multidisciplinary approach has been adopted to evaluate the effect of citrus rootstocks on the fruit quality and on the resistance to abiotic stress on pigmented citrus species. As for the fruit quality, the biochemical profile of eleven pigmented varieties, widespread in the Mediterranean basin, was studied to get novel insights on the phenols involved in pigmented fruits, with particular emphasis to anthocyanins, responsible for the red colour in the peel and the pulp. Furthermore, different scion/rootstock combinations have been studied, as well as their interaction with the vegetative/productive aspects and the qualitative traits, focusing also to biochemical and molecular insights during ripening process. The performance of new rootstocks under high salt stress levels was also evaluated through the analysis of the morpho-physiological, enzymatic and hormonal responses.

Overall, the results of this thesis will provide valuable information for researchers to understand the role of rootstock in

influencing several agronomical traits of pigmented orange and for breeders to get a complete overview of the performances of novel and widely spread rootstocks.

Keywords: rootstock, phenols, anthocyanins, pigmented citrus, salt stress, physiology, enzymatic activity, hormonal quantification

Riassunto

L’agricoltura è uno dei principali settori dell'economia del Mediterraneo e italiana e gli agrumi sono la prima coltura frutticola in termini di produzione mondiale con 157 milioni di tonnellate. Il portinnesto ha svolto per centinaia di anni un ruolo determinante sulla produzione, sulla qualità dei frutti e sullo sviluppo della pianta. Inoltre, il soggetto può influire sulla resistenza a numerose avversità abiotiche (ad esempio, suoli calcarei, salinità dell’acqua, inondazioni, freddo) e biotiche (presenza di malattie come *Phytophthora*, CTV, nematodi, complesso *Diaprepes*/Phytophthora, HLB), limitando anche gli stress ambientali e modulando la risposta fisiologica della pianta, influenzando in modo determinante la crescita, la fioritura e la fruttificazione. Per tale ragione, risulta risolutivo identificare il portinnesto che meglio si adatti alle diverse condizioni pedo-climatiche in combinazione con le principali specie e varietà dell’agrumicoltura in ambiente mediterraneo.

Nella presente tesi è stato adottato un approccio multidisciplinare per valutare l'effetto dei portinnesti sulla qualità dei frutti e sulla resistenza agli stress abiotici in combinazione con agrumi pigmentati. Per quanto riguarda la qualità dei frutti, è stato studiato il profilo biochimico di undici varietà pigmentate, diffuse nel bacino del Mediterraneo, per ottenere nuove conoscenze sui fenoli presenti nei frutti pigmentati, con particolare attenzione agli antociani, responsabili del colore rosso della buccia e della polpa. Sono state inoltre studiate diverse combinazioni nesto/portinnesto, nonché la loro interazione con gli aspetti vegeto/produttivi e le caratteristiche qualitative, approfondendo i risvolti biochimici e molecolari durante il processo di maturazione. La performance di nuovi portinnesti sottoposti ad elevati livelli di stress salino è stata valutata anche attraverso l'analisi della risposta morfofisiologica, enzimatica e ormonale.

Nel complesso, i risultati di questa tesi forniranno preziose informazioni ai ricercatori per comprendere il ruolo del portinnesto

nell'influenzare diversi tratti agronomici delle varietà pigmentate e per ottenere una panoramica completa sulle performance di portinnesti nuovi e di altri ampiamente diffusi.

Parole chiave: portinnesti, fenoli, antociani, agrumi pigmentati, stress salino, fisiologia, attività enzimatica, quantificazione ormonale

Chapter 1 - General Introduction

Origin and economic importance of citrus fruit in the world

Citrus is one of the most important horticultural crops worldwide. The genus Citrus is composed by many species of economic interest such as, in order of world harvested area: sweet orange, mandarin and mandarin-like hybrids, lemon and lime, grapefruit. Fruits of the above-mentioned citrus species are priced for fresh consumption and as processed products, thanks also to their high nutritive value (Gmitter et al., 2012).

Despite consolidated scientific reports evidenced that citrus species originated in the subtropical and tropical regions of Asia, only recently a genomic, phylogenic, and biogeographical analysis (Wu et al., 2018) showed that the centre of origin of the genus Citrus is likely the southeast foothills of the Himalayas (Figure 1.1).

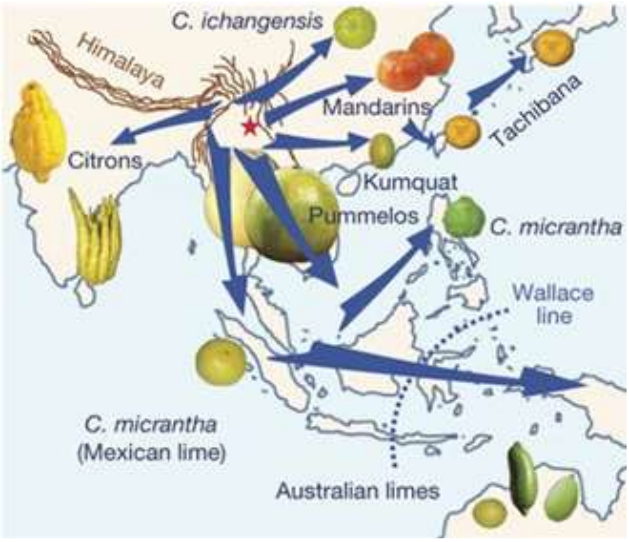


Figure 1.1 Proposed origin of citrus and ancient dispersal routes (modified by Wu et al., 2018)

Although Italy is the 12th producing country in the world, citrus species are well appreciated by consumers, thanks in particular to both the occurrence of rather unique pedoclimatic conditions and a deep cultivation knowledge resulting in peculiar citrus productions such as: pigmented orange, bergamot or citron. Orange and mandarin-like are the most important citrus commodities and they are marketed either as fresh fruit and processed into juice products. World citrus production in 2019 was 157 millions tons (figure 1.2) and China is the first country for production (43 million tons), followed by Brazil and India (19 and 14 million tons, respectively) (FAOSTAT, 2021).

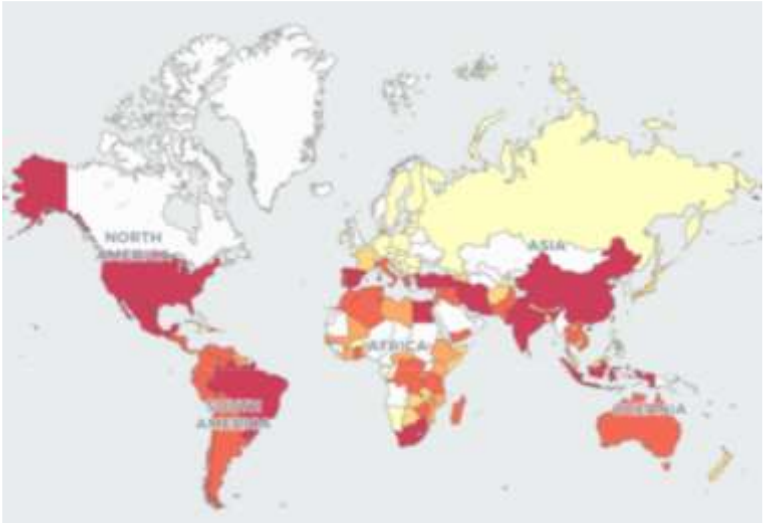


Figure 1.2. Total citrus production in the world in 2019 (FAOSTAT report, 2021)

In 2019 Italy produced 2.8 million tons of citrus and it is the 12th producing country in the world, and confirm its ranking position for orange (2.0 million tons). Among all citrus fruits, oranges are widely cultivated in different parts of the world (figure 1.3). Brazil is

the first producing country (17 million tons), followed by China and India (10.4 and 9.5 million tons, respectively).

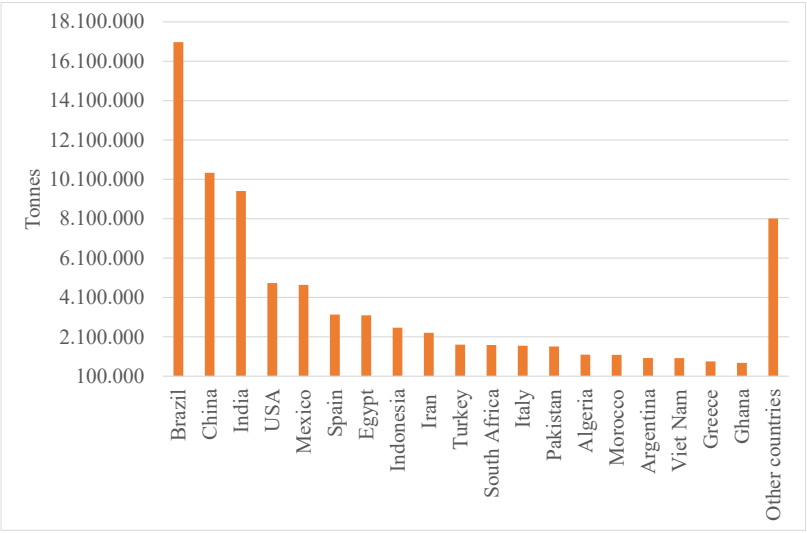


Figure 1.3. Total production of oranges in 2019 in the world (FAOSTAT report, 2021)

As for mandarin and mandarin-like hybrids, such as tangerines, clementines and satsumas, China is the first world producer (19.7 million tons), since it produces more than 50% of mandarins and mandarin-like hybrids (figure 1.4). Spain produces 1.8 million tons, followed by Turkey that produces 1.4 million tons. Italy is the 8th producing country in the world and accounts for the 2% of the world production.

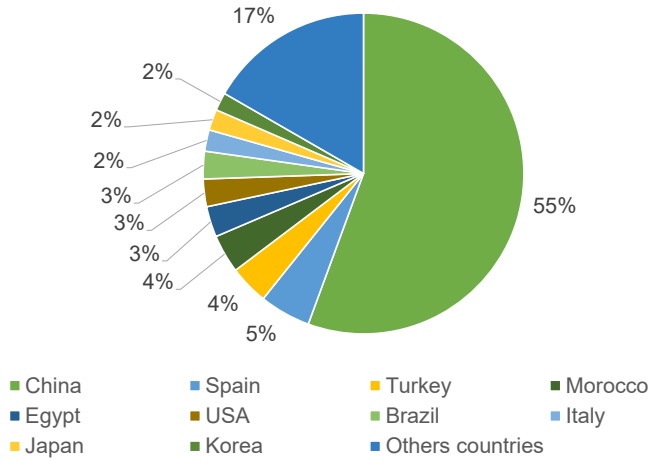


Figure 1.4. Production (%) of mandarins and mandarin-like hybrids in 2019 in the world (FAOSTAT report, 2021)

Italian orange varieties are well appreciated by consumers: in fact, the last years experienced a general increase in orange production that passed from 17,106,669 q in 2019 to 20.244.983 q in 2021 (ISTAT 2021). In recent years, an increasing interest in blood orange due to their antioxidant potential has occurred (Galvano et al., 2004) representing nearly one third of Italian orange production (figure 1.5). This is confirmed by a decrease in navel orange production, counterbalanced by an increase in blood orange production (figure 5). Half of the Italian orange cultivation is located in Sicily and in this region, blood orange represents the 70% of the whole orange production (Continella et al., 2018).

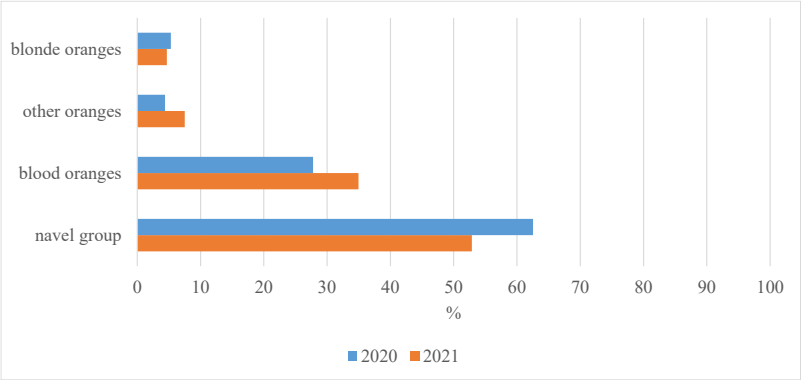


Figure 1.5. Percentage of orange production in Italy (ISTAT report 2021)

History and origin of blood orange

When blood oranges were introduced in Europe, they were maintained in historical gardens and used mainly as ornamentals. Blood orange probably originated either in China or in the Southern Mediterranean region, probably Malta or Sicily (Grosso et al., 2013) to spread later in the whole Mediterranean basin thanks to the red colouration of the peel and of the flesh. This trait is due to high content of anthocyanins, a water-soluble polyphenolic compounds which play an important nutraceutical role thanks to their antioxidant activity (Habibi et al., 2020).

Giovanni Battista Ferrari (1646), a Jesuit with knowledge on horticulture, mentioned red oranges in '*Hesperides'*, and in the book '*Hesperides sive de malorum aureorum cultura et usu'*, He illustrated and described 3 varieties of citron, 25 of lemon and 15 of orange. Juan de Loureiro (1790) reported about a *Citrus nobilis* that was '*intus & foris rubra'* (red inside and outside). Several authors described different varieties of citrus fruits with the red and purple flesh (Gallesio, 1811), but the first illustration of blood oranges was made by Antonio Risso and Pierre Antoine Poiteau (1815-1822). They

described at least 7 pigmented citrus orange genotypes differing in shape, shade of the red colour and distribution of the pigmentation in the flesh. Casella (1935) distinguished blood oranges into different varieties describing the most diffused: Sanguinello, Moro and Tarocco. Some neglected clones were also reported, such as Sanguinello Vaccaro, Tarocchino and Ovaletto sanguigno. Hodgson (1967) suggested that blood oranges derived from three independent lines: Doppio Sanguigno or Maltaise Sanguine in Italy (that is Sicily), Doblefina in Spain and Shamouti orange referred to as Shamouti Blood or Palestinian Blood Jaffa Orange. The origin of Shamouti orange is widely discussed as Hodgson (1967) reported that it derived from a grafting with Maltaise Sanguine, while Spiegel-Roy (1979) stated that it was a chimera. Yuan and colleagues (2008) claimed that Jingxian, discovered in 1958, was the only blood orange of Chinese origin. Recently, Butelli and colleagues (2012) proposed an independent origin of the Jingxian variety due to some differences in Ruby locus compared to Mediterranean blood varieties. They also reported that Doblefina derived by Sanguinelli, that also had a common origin with Sicilian blood oranges.

Pomological characteristics and qualitative traits of blood orange

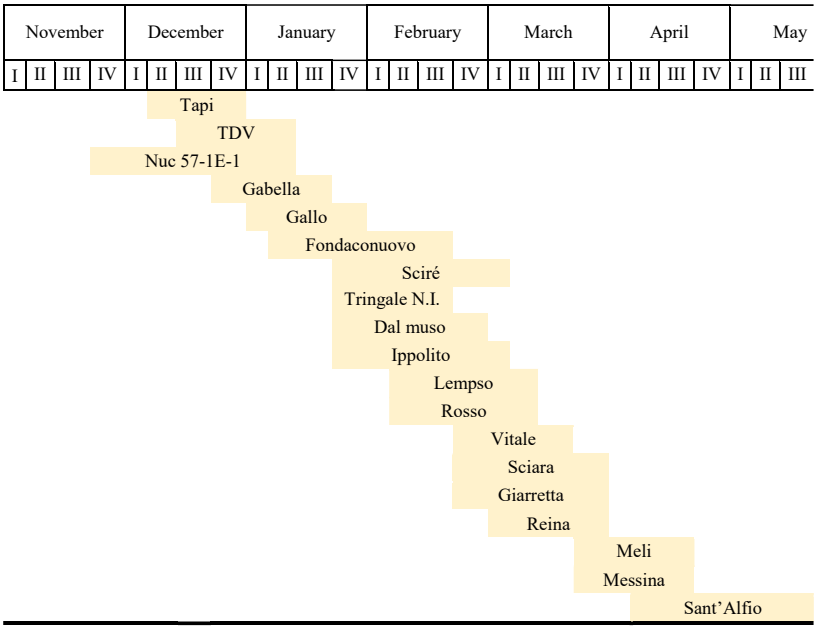
In recent years, an increasing interest in plant antioxidants has occurred because of the potential anticarcinogenic and cardioprotective actions mediated by their biochemical properties (Galvano et al., 2004). Due to the growing interest in antioxidant properties, the importance of blood oranges (*Citrus sinensis* L. Osbeck) is growing in the Mediterranean citrus production area.

Nowadays, blood orange is mostly cultivated in Mediterranean region, specifically Sicily (southern Italy), Spain, Morocco, Algeria and Tunisia. In Sicily the cultivation of blood orange in citrus industry lays back to the beginning of the XXth century, and until the 1950s,

the most cultivated blood orange variety was Sanguinello, followed by Tarocco and Moro. In the last decades, Tarocco became the most cultivated blood orange, its cultivation relies on about 20 different linesclones (Chen et al., 2008). Recently, consumption of Tarocco orange has increased as fresh fruit for the easy peeling trait and for the optimal sweet-acid ratio (Rapisarda & Russo, 2000). For these reasons, numerous researchers selected several lines derived from old Tarocco varieties (Tribulato and La Rosa, 1994). and previous research (La Rosa, 2012) distinguished Tarocco lines according to the ripening period (table 1): Nucellar 57-1E-1, Tapi and TDV are those characterized by the earliest ripening season. Despite the occurrence of such early variety, most of the production is concentrated in a period spanning from February to March, a period in which the most widely spread varieties (Rosso, Ippolito and Lempso) are harvested. Such varieties are particularly priced for the colour of the flesh. Among the late ripening varieties, the most popular are Meli, Messina and Sant'Alfio.

In addition to the varieties of blood oranges reported in table 1.1, other cultivars are of particular interest for their wide cultivation and/or particular morphological characteristics, such as Tarocco Francofonte, that has a distinctive obovate shape, with medium sized neck and an orange colour at maturity (often in January). Tarocco Dal muso is a natural mutation of Tarocco Comune, and it has a distinctive pronounced neck (Saunt et al.,2000). Tarocco Sciré is the most widely spread line in the last twenty years. It has medium-large size and spherical shape with medium thickness of peel (La Rosa, 2012). Caruso et al. (2016) provided insights on the pomological and qualitative trait of blood orange, according to their ripening stage (early ripening: harvested in middle December) and late ripening (harvested in May).

Table 1.1. Ripening calendar of the most popular Tarocco orange lines (La Rosa, 2012)



They classified Tarocco Liscio and Tarocco Piatto Magrantino as early clones and found a higher TSS/acidity ratio than standard in early selections, such as Tarocco TDV. Regarding the presence of seed, they recorded a higher value in Ovaletto Sanguigno and Vaccaro than in Tarocco lines. Beyond the harvest period, among the qualitative aspects, shape and weight are important for fruit commercialization. Sanguigno and its clones has a smaller fruit size compared to Tarocco and Moro cvs. Caruso and colleagues (2016) investigated on Tarocco clones, and they evidenced the highest solid soluble content in Tarocco 571E1 foglie piccolo PB. The juice acidity ranged from 1.67 g 100 mL^{-1} in Tarocco Rosso mutato to 0.1 g 100 mL^{-1} in Tarocco Ferreri, an acidless mutation.

Concerning peel and juice colour, Caruso et al. (2016) showed a different behavior of the accumulation of anthocyanins; clones of Sanguinello had less anthocyanin content in the flesh respect to its peel, meanwhile Doppio Sanguigno and Conzamustu had more anthocyanin in the peel than its pulp. Previous studies reported a higher anthocyanin content in fruit of Moro compared to Tarocco and Sanguinello lines (Rapisarda et al., 2000; Rapisarda et al., 2009), meanwhile other investigations quantified more anthocyanins content in Tarocco Ganci Pedagaggi and in the two nucellar selections of Tarocco 571E1 (PA and PB), compared with Moro cultivar (Caruso et al., 2016). Among the other Mediterranean countries, Spain is interested in the production of blood orange. Bono (1991) reported that Doblefina, also called Ovale Sangre, Sanguina oval or Rojo Oval, was the most cultivated due to its high productivity. Other widespread varieties were Entrefina, Washington Sanguina and Sanguinelli that were originated by natural mutations of Doblefina. Sanguinelli variety is currently largely cultivated due its very intense pigmentation and it is very well pigmented in the peel, but not in the juice. The fruit has an oval shape and a small-medium size, and it is characterized by a weak pigmentation of the flesh and a high juice content and less acidity than Doble Fina. The shape of Doble Fina varies from round to oval and it is harvested in January. Doble Fina, Entrefina and Murtera are characterized by a low juice pigmentation, but by being very rich in phenolic compounds. Maltaise demi Sanguine has a medium shape and it is predominantly orange in the peel and in the flesh: it is harvested in February when it has a high solid soluble content (Saunt et al.,2000).

Phenols compound in blood orange

Nowadays, consumers are increasingly aware of the healthy feature of some fruit commodities as testified by the sharp increase in demand for nutraceutical foods, known also as superfoods. Several

studies highlighted the high nutraceutical properties of sweet orange, especially for what concerns the antioxidant efficiency of orange juices due to the phenol components, whereas ascorbic acid seems to play a minor role (Rapisarda et al., 1999). Blood orange contains elevated quantities of various antioxidant compounds such as phenols, flavanones, anthocyanins, hydroxycinnamic acids, and ascorbic acid. In particular, polyphenols are characterized by the presence of more than one phenol unit, as shown in figure 1.6.

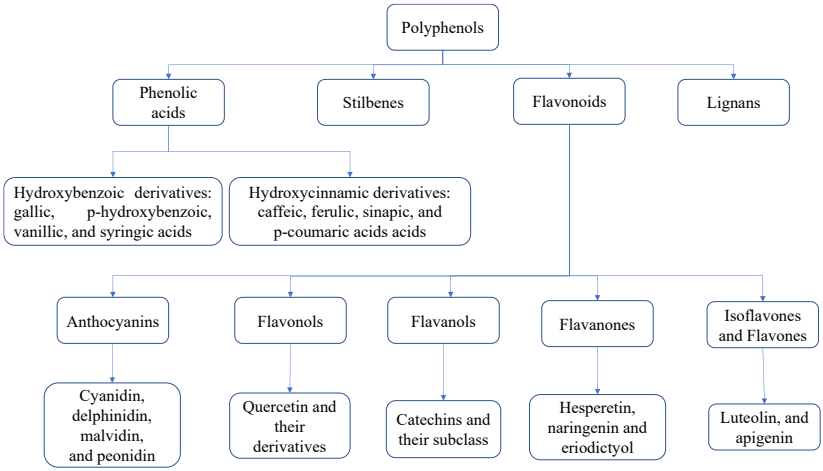


Figure 1.6. Main polyphenols present in blood orange (modified from Durazzo et al., 2019)

Main flavonoids in blood orange

Several researches (outlined in table 1.2) investigated the phenolic composition of blood oranges and how the phenols compounds are affected by the ripening stage (Rapisarda et al., 2003; Ordóñez-Díaz et al., 2020; Multari et al., 2020).

Gattuso et al. (2007) reported hesperidin, followed by narirutin and didymin, as the most abundant flavanone-O-glycosides identified and quantified in sweet orange, present in both blood and blonde

oranges, even if a higher content has been found in blood orange varieties (Sanguinello, Moro, and Tarocco) compared with blonde ones (Navel, Valencia, and Ovale) (Proteggente, et al., 2003). Also, Morales et al. (2021) highlighted that hesperidin was the predominant flavanone in Tarocco Rosso and Moro, followed by narirutin and didymin, as confirmed by previous studies (Barreca et al., 2014; Cebadera-Miranda et al., 2019). They also focused the effect of the harvest period and of the rootstock on the biosynthesis of flavanones and detected that their content was significantly affected in Moro in relation to both rootstock and harvest time. Ordóñez-Díaz et al. (2020) detected hesperidin, narirutin, vicenin-2 and didymin in cultivar Salustiana and Sanguinelli and, accordingly with previous studies (Giuffrè et al., 2017; Lado et al., 2018), hesperidin was the main flavanone.

Anthocyanins are a group of phenolic compounds responsible for the colour of the peel and of the juice of pigmented citrus, especially in blood oranges. Intensity of the colour of the anthocyanins is dependent on the number of hydroxyl and methoxyl groups; if more hydroxyl groups are present, then the colour goes toward a more bluish shade; redness is increased by an higher concentration of methoxyl groups (Heredia et al., 1998 He et al., 2010). Several authors provided a quantification, and a characterization of the different anthocyanins present in blood oranges (table 2). The major anthocyanins belong to the group of delphinidin, petunidin, malvidin, cyanidin, peonidin and pelargonidin (Giusti et al., 1999; Kahkonen et al., 2003). Several studies have shown that cyanidin-3-glucoside is the main component (Maccarrone et al., 1998; Kafkas, et al., 2009). Recently, Morales et al. (2021) identified four individual anthocyanins: delphinidin 3-glucoside, cyanidin 3-glucoside, cyanidin 3-(6″malonyl)-glucoside and cyanidin3-(6″-dioxalyl)-glucoside in Moro and Tarocco Rosso. Another research (Ordóñez-Díaz et al., 2020) studied the anthocyanin quantification on Salustiana and Sanguinelli and observed that

anthocyanin profile was characterized by glycosides of cyanidin and glycosides of delphinidin and peonidin; in particular, they quantified cyanidin-3-O-(6”-malonyl) glucoside, cyanide 3-O-glucoside and cyanide 3-O-rutinoside.

Blood orange production was restricted to certain citrus areas because anthocyanin synthesis is related to cold temperatures. Different research focused on the evolution of the pigmentation during cold storage (Rapisarda et al., 2001; Crifò et al., 2001; Carmona et al., 2019). Crifò and colleagues (2001) reported an anthocyanin increase at either 4 or 8 °C in blood orange fruits. Recently, Carmona and coll., (2019) showed that storage at 9 °C was more effective than 4 °C for the synthesis of anthocyanins. Pannitteri et al. (2017) investigated on different cold treatments to increase pigmentation on Tarocco Sant’Alfio blood orange, finding an increase of anthocyanin accumulation at 4 °C compared to fruit maintained at 20 °C.

Anthocyanin biosynthesis is known to be activated as a response to thermal stress, but further studies are necessary to identify the most suitable temperature to activate its biosynthesis and increase anthocyanin accumulation.

Main phenolic acids in blood orange

Hydroxycinnamic acids represent an important group of compounds that derive from the general phenylpropanoid pathway and blood oranges contain more hydroxycinnamic acids than blonde ones (Rapisarda et al., 1998). The difference in the distribution of hydroxycinnamic acids is variety-dependent, and the concentration of this compound allow to identify their role as suitable markers for Italian blood orange juices (Rapisarda et al., 1998). Regarding the individual profile, Kelebek and coll. (2008) reported that ferulic acid was the most dominant hydroxycinnamic acids in Moro and Sanguinello juices, followed by sinapic acid, chlorogenic, coumaric, caffeic acids. Nevertheless, Morales and coll. (2021) reported that

chlorogenic acid was the most dominant hydroxycinnamic acid, followed by ferulic and sinapic acid in Moro and Tarocco Rosso. Moreover, they reported that the concentration of these hydroxycinamic acids was affected by harvest period and rootstock; indeed, only the interaction between these two factors was significant for chlorogenic acid.

The evolution of phenol compounds during maturation in blood orange

Several authors investigated on the evolution of phenolic compounds, especially anthocyanin, during maturation. Rapisarda and coll. (2003) studied the behavior of Omo-31, hybrid of Oroval clementine and Moro orange, compared to its parents at different stages of maturity: the evolution of total flavanone glycosides concentration during ripening was determined and Moro was the richest in anthocyanin content with only two anthocyanins, cyanidin 3-glucoside and cyanidin 3-(6’’-malonylglucoside).

Cebadera-Miranda et al. (2019) analyzed some blood oranges widespread in Mediterranean basin, Sanguinelli, Tarocco Rosso and Tarocco Ippolito, collected in three different periods. They identified ten different compounds: seven cyanidin derivatives and three delphinidin derivatives, and the most abundant was cyanidin 3-(6″-malonylglucoside) and cyanidin 3-glucoside; Sanguinelli presented the highest concentration in anthocyanin compounds, although all varieties showed similar values in the total anthocyanins content and also flavonoids were affected by maturity stages. Multari and coll. (2020) studied the variations in phenolic compounds of Tarocco in different tissues and stages of maturity highlighting a decrease in the flavedo during maturation.

Table 1.2. List of the main studies carried out on polyphenols in blood oranges from 1996 to 2021

Phenolic compound	Reference	Aim of the work
Anthocyanins	Cebadera-Miranda et al. (2019); Ordóñez-Díaz et al., (2020); Morales et al. (2021a)	effect of maturation
	Maccarone et al. (1998); Rapisarda et al. (2000); Kahkonen et al., 2003; Rapisarda et al. (2001); Lee (2002); Dugo et al. (2003); Hillebrand et al. (2004); Caruso et al. (2016); Cebadera-Miranda et al. (2019)	quantification and characterization
	Rapisarda et al. (2001); Crifò et al., 2001; Pannitteri et al. (2017); Carmona et al. (2021)	temperature effects
Flavanones	Rapisarda et al., (2003); Ordóñez-Díaz et al., (2020); Multari et al., (2020)	effect of maturation
	Horowitz & Gentili (1977); Rapisarda et al. (1999); Nogata et al. (2006); Gattuso et al. (2007); Kelebek et al. (2008); Barreca et al., 2014; Letaief et al. (2016); Cebadera-Miranda et al., 2019; Morales et al. (2021a)	quantification and characterization
	Rapisarda et al. (1999); Rapisarda et al. (2009)	analysis of the antioxidant potential
Hydroxycinnamic acids	Multari et al. (2020), Ordóñez-Díaz et al., (2020)	effect of maturation
	Rapisarda et al. (1998); Fallico et al. (1996); Rapisarda et al. (2001); Kelebek et al., (2008); Morales et al. (2021a)	quantification and characterization

Rootstock in citriculture

In citriculture, an important aspect to consider is the rootstock onto which a specific cultivar is grafted, because it may influence several tree growth and development aspects, including yield, fruit quality and tolerance to stress caused by biotic and abiotic factors (Filho et al., 2007). Furthermore, rootstocks can modify the citrus response to a range of environmental stresses, affecting tree growth, flowering and fruiting.

It is difficult to find an ideal rootstock and the success of citrus rootstock is related to its tolerance to abiotic and biotic stresses, such as conditions of soil, climate, insects and disease. In the meantime, it still must produce high yields of quality fruits. In this context, the main objective for breeding programs is to find a rootstock that is resistant to biotic and abiotic factors. Evolution of the genetic improvement of the rootstock

Rootstocks influence the plant yield, the quality of the fruits and the tree architecture, and can also affect the tolerance to abiotic (i.e. calcareous soils, salinity, flooding, cold temperatures, etc.) and biotic (*Phytophthora*, CTV, nematodes, Diaprepes/*Phytophthora* complex, HLB, etc.) stresses. Until the mid-1800s, citrus was grown mainly from seeds, even if grafting was already common in the Mediterranean area, and some examples of sweet orange budded on sour orange were present in Florida (Wutscher, 1979). The advent of the *Phytophthora* (the first report was registered in the Azores islands in the 1842), a pathogen causing serious damages to the root system of most of the economically important citrus species, contributed to the widespread of the practice of employing Phytophthora-resistant rootstocks (Chapot, 1975). In this context, sour orange (*Citrus aurantium* L.) was the main rootstock used in most of the citrus growing area, until the arrival of CTV. It positively influenced the qualitative traits of orange and mandarin, such as yields, pre-harvest drop, and fruit size. The

rusticity of sour orange (natural hybrid of pummelo and mandarin) permitted to adapt to several range of soil types; in fact it was used for citrus growing on moist, heavy soil, and it was tolerant to calcareous soils (Forner-Giner et al., 2020). The use of sour orange as rootstock was rapidly abandoned due to the spread, a century later, of a new epidemic disease, the citrus tristeza virus (CTV) (Castle et al., 2010). In the last few years, several natural hybrids have been used as rootstocks. In Brazil, sour orange was largely replaced by Rangpur lime (*Citrus limonia* (L.) Osbeck) thanks to its high productivity and drought-tolerance (Castle et al., 2010). The same rootstock was largely employed in South Africa, especially in combination with lemon, and in Florida in combination with sweet orange (Castle et al., 2010). In China, Japan and other cold producing areas, trifoliate orange (*Poncirus trifoliata*) is employed due to its resistance to cold temperatures (Forner-Giner et al., 2020).

Cleopatra mandarin (*C. reshnii* Hort ex Tan.) has been widely used as rootstock for mandarin, mandarin-like varieties, and grapefruit, meanwhile Volkamer lemon (*Citrus volkameriana*), a natural hybrid originated in Philippines, and macrophylla (*Citrus macrophylla*, also known as Alemow) were widely used in combination with varieties characterized by acid-fruit production (Castle et al., 2010).

Nowadays genetic improvement uses trifoliate orange as a pollen parent in breeding plans focused on selecting new rootstocks. The result of several genetic improvement programs led to the production of numerous citrumelos, citranges and citrandarins (Hutchison, 1974). The inheritance of cold-hardiness, nematode resistance, and high fruit quality, typically of trifoliate orange, were among the objectives (Forner-Giner et al., 2020).

Prior to HLB becoming endemic, citranges (*C. sinensis* (L.) Obseck x *P. trifoliata* (L.) Raf.) and citrumelos (*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.) hybrids became the prevalent rootstocks in

many citrus production areas. Among the main citranges, Carrizo and Troyer, that originated in 1909 in California (Savage et Gardner, 1965), are the most used in the main citrus growing countries. They are originated with the aim to obtain a sweet orange cultivar resistant to cold hardiness, but without success as the fruit is not edible. Only much later, Carrizo and Troyer emerged as the top commercial rootstocks and they spread in several citrus producing countries (Castle et al., 2010). Troyer was the first rootstock used, especially in California, while in Florida Swingle citrumelo and Carrizo citrange were the most widely planted rootstock (Forner-Giner et al., 2020). In California and Spain, among citrange, Carrizo and C35 (*C. sinensis*cv Ruby x *P. trifoliata*), a citrange developed by the University of California in 1951 and released in 1986 (Cameron et Soost, 1986), were used for fresh fruit scions (Forner-Giner et al., 2020). The University of California, at Riverside, released in 2009 Bitters, Carpenter, and Furr (also known as C22, C54 and C57 respectively). All three rootstocks were obtained by the cross of Sunki mandarin × *Swingle trifoliate* orange (Siebert et al., 2010). In Texas, Bitters was mostly used in combination with grapefruit, replacing sour orange (Louzada et al., 2008).

In 1974, Forner and colleagues, at the IVIA research centre located in Valencia, began a program to breed citrus rootstocks by hybridization. They selected more than 500 hybrids and they released some new rootstocks such as Forner-Alcaide 5 (*Citrus reshni* Hort. ex Tan. × *P. trifoliata* (L.) Raf.). It became very popular due to its high adaptability to Spanish environmental conditions and thanks to the high productivity and good fruit quality that it confers to the scion (Forner-Giner et al. 2003).

Other promising new hybrid citrus rootstocks are US-802, US-812, US-897, and US-942. They were released by the U.S. Department of Agriculture (USDA) between 2001 and 2010 (Bowman et al., 2016); and are widely used in new citrus plantations in Florida.

Recently, some new rootstocks, namely US-1279, US-1281, US-1282, US-1283, and US-1284, were released showing both improved tolerance to HLB and high fruit quality (Bowman et McCollum, 2015). In Italy, the Council for Research in Agriculture and the Agricultural Economic Analysis (CREA-OFA) of Acireale (CT) started in 1968 a breeding program for rootstocks developing and evaluating 257 hybrids. Among all, F6P13 and F6P12 (*C. latipes*. x *P. trifoliata*) stand out for their agronomical performances. In particular, F6P12, patented in 2014, showed promising characteristics in terms of yield and fruit quality (Recupero et al., 2009). In South Africa, at the Subtropical Fruit Research Institute (CSFRI), X639, a hybrid of Cleopatra mandarin and *P. trifoliata* was released in early 1950s (Von Broembsen, 1985). It is becoming widespread in South Africa for the tolerance both to salinity (Syvertsen, 2012) and to high pH soil levels (Castle and Baldwin 2006).

Agronomic and qualitative traits of the main citrus rootstocks

Rootstock is reported to significantly affect fruit quality such as external (size, rind thickness, peel colour) and internal (juice content and colour, pH, total soluble solids) traits. Colour is an important parameter in citrus fruit quality which greatly influences consumer acceptance (Lado et al., 2014). Previous researches studied the influence of the rootstock on juice and peel colour (Continella et al., 2018; Morales et al., 2021a). Another fundamental parameter used as a ripening index is the sugar-acid ratio. Kunwar and coll. (2021) investigated on different sexual and somatic hybrids grafted with Hamlin sweet orange, evidencing the importance of total soluble solids and juice percentage, as these qualitative parameters were influenced by the rootstocks adopted.

Among citranges clones, Carrizo and Troyer, both had a high response to biotic and abiotic adversity, although a higher tolerance of Carrizo for burrowing nematode was reported (Forner-Giner et al.,

2020). Analysing the different agronomic traits, both citranges are moderately vigorous and they are capable to adapt to a wide range of soil types, even if they have poor salt tolerance and are sensitive to calcareous soils. For what concerns cold stress, previous researches reported that both rootstocks show a moderate cold-tolerance (Hodgson, 1967; Castle et al. 1993), even if, field experience indicates that Carrizo rootstock is generally not as cold resistant as sour orange, Cleopatra or Swingle citrumelo (Forner-Giner et al., 2020).

Another recently introduced citrange, C35, was very studied due to its good performance in combination with a wide range of varieties such as navels, Valencias, pigmented oranges, grapefruit, mandarin and mandarin-like (Continella et al., 2018; Caruso et al., 2020; Forner-Giner et al., 2020), meanwhile other studies reported an incompatibility with Clemenules and Fukumoto (Castle et al., 2016; Roose, 2014). C35 determined a 25–30% reduction of the tree size, and a high-yield efficiency (kg of fruit for each cubic meter of canopy volume), keeping high fruit quality traits (Forner-Giner et al., 2020).

Plants grafted on Swingle citrumelo has a high vigour and productivity. It shows resistance to loam and sandy-loam soils but perform poorly in heavy and calcareous ones. Sacaton citrumelo is highly productive and has a good performance in combination with Valencia (Castle and Stover, 2000; Roose, 2014).

Cleopatra mandarin is a widely used rootstock, though it has low yields in the first years. It has been shown that scions grafted onto Cleopatra show fruit with good internal quality, but fruit size is usually small to intermediate. It is a cold-tolerant rootstock and it well adapt to several types of soils (Saunt, 1990).

Plants grafted on macrophylla are extremely precocious and grow vigorously, indeed it is often used in combination with lemon as produce poor fruit quality due to the low content in total soluble solids and less juice percentage. Trees grafted on macrophylla are very sensitive to cold damage, even if it has a good salt tolerance and it

adapt well to sandy and sandy loam soils, while it does not grow well in heavy soil (Roose 2014; Saunt,1990).

Trees grafted on Volkameriana are often vigorous and show high yield, but with poor fruit quality due to the low soluble solids/acid ratio. Volkameriana does not grow well in heavy soil, while it well adapts to sandy, sandy loam and calcareous soils and it shows fair salt tolerance (Saunt 1990; Castle et al., 1993; Roose 2014). Among emerging rootstocks, Forner–Alcaide 5 (FA5) results to be a productive rootstock and induce good fruit quality (Forner-Giner et al. 2003; Forner et al. 2003). Indeed, FA5 is tolerant to calcareous soils (Forner et al. 2003; González-Mas et al. 2009) and to salinity (Forner-Giner et al. 2009; Lopez-Climent et al. 2008), like Cleopatra mandarin. It is more tolerant to iron chlorosis than Carrizo and has a good resistance in water stress and flooding. Navel oranges, satsumas and clementines grafted on FA5 show higher productivity than when grafted on other rootstocks, such as Carrizo citrange, and induced good fruit size and quality (Forner-Giner et al., 2020).

Regarding California's citrandarin, previous study carried out in Texas reported Bitters as very tolerant to high pH, calcareous soils, and saline conditions, like C146, hybrid with the same parents (Louzada et al. 2008). Continella and coll. (2018) reported that trees grafted on Bitters were highly productive with a reduced development of the tree and, when grafted onto pigmented citrus cultivars, Bitters influenced positively juice anthocyanin content (Continella et al., 2018; Caruso et al., 2020). Carpenter induces good fruit quality on late navels reducing the size of the trees as well, on the other hand, Furr produced medium to large trees, characterized by good yields and it is moderately tolerant to calcareous soil. Among the rootstocks released by the U.S. Department of Agriculture (USDA), US-812 is highly productive and induces a moderate-sized tree. Forner-Giner and colleagues (2020) reported that Valencia trees grafted onto US-812 are characterized by good tolerance to high alkalinity soils (pH 8.1–8.3).

Bowman and colleagues (2016) reported that US-942 increases production considerably, and US-802 and US-897 induced good productivity per tree size. US-897 rootstock is largely considered for its semi-dwarfing effect on scions and salinity tolerance (Syvertsen 2012). Castle and Baldwin (2006) reported that X639 rootstock coupled tolerance to high pH soil levels and salinity and good fruit quality and yields. Among all hybrid released by CREA-OFA of Acireale, F6P12 was evaluated in combination with Washington navel orange, SRA 92 clementine, and Tarocco blood orange, showing high cumulative yield and good fruit quality in all the scions tested (Recupero et al., 2009).

Influence of rootstock on phenol content

Several research investigated on nutritional quality of citrus fruit, focusing on phenols composition, especially flavonoids, anthocyanins and hydroxycinnamic acids for mandarin and blood orange (Rapisarda et al., 1998; Sdiri et al., 2019; Morales et al., 2021a; Morales et al., 2021b).

Rootstock influence in anthocyanin content

Many authors demonstrated the valuable role of anthocyanins on the organoleptic features of pigmented citrus and the relation between rootstock and anthocyanin has been largely investigated. Although anthocyanins synthesis is regulated genetically, pigmentation process is dependent on environmental conditions such as cold temperatures, and it is influenced by the rootstock, as several authors reported (Reforgiato Recupero et al. 1999, Butelli et al., 2012; Continella et al., 2018). Reforgiato Recupero and colleagues (1999) reported an increase on anthocyanin content in fruits of Tarocco 571E1 grafted on Flying Dragon trifoliate orange with respect to other rootstocks. Continella and colleagues (2018) reported that Bitters conferred great quantitative of anthocyanin in combination with

Tarocco Sciré, while Swingle citrumelo induced a low level of pigmentation in the fruit flesh. Other study showed that *C. volkameriana* induced low levels of pigmentation in fruit pulp (Morales et al., 2021a). Regarding citrange, Continella and colleagues (2018) found a different performance on Troyer and Carrizo grafted with blood orange. The influence of rootstock on anthocyanin content has been extensively studied in the last twenty years due to the increasing interest to blood orange. Several studies investigated the anthocyanin content in Moro and in Tarocco lines. Rapisarda and colleagues (2000; 2009) reported a significantly higher accumulation of anthocyanin in Moro compared to Tarocco and Sanguinello varieties in plants grafted onto sour orange and Carrizo citrange. In general, Moro was deeper red than Tarocco Rosso, due to higher content of red pigments, as previously reported (Kafkas et al., 2009). Indeed, Morales and colleagues (2021a) recently investigated the interaction with harvesting period of Tarocco Rosso and Moro on different rootstocks, finding significant interaction between rootstocks, harvest, and variety. The rootstock, in addition to influencing the total content of anthocyanins, acts on the individual pigments; in fact, Morales and colleagues (2021a) showed that individual pigments, such as cyanidin 3-(6″malonyl)-glucoside and cyanidin 3-(6″-dioxalyl)-glucoside, were affected by the rootstock. In particular they found the lowest content of these two pigments in C35, *C. macrohpylla* and Swingle citrumelo, meanwhile high cyanidin 3-O-glucoside and delphinidin 3-O-glucoside content was found in Forner-Alcaide 5 and Forner-Alcaide 13. Forner-Alcaide 5 also showed excellent results in combination with Sanguinelli. Ordóñez-Díaz and colleagues (2020) observed that Forner-Alcaide 5 and Cleopatra mandarin had the highest concentrations of the main cyanidin derivatives, including cyanidin-3-O-(6”-malonyl) glucoside, cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside; meanwhile, they found a high 3,5-O-diglucoside cyanidin content in Carrizo, *C.*

volkameriana. Furthermore, they observed different anthocyanin profiles between the two harvesting years studied; these differences may be due to the cold addiction (Continella et al., 2018).

Rootstock influence in flavonoids content

Citrus fruits are a rich source of flavonoids with a direct role in scavenging reactive oxygen species, which can counteract lipid oxidation and so antioxidant enzyme activity (Zhou et al., 2016; Sdiri et al., 2019; Morales et al., 2021b). There are few studies on the effect of rootstock on flavonoids in citrus fruit, especially in blood orange. Morales and colleagues (2021b) focused on the main flavonoid composition (hesperidin, narirutin and dydimin) in Tango mandarin: they showed that rootstock did not influence the hesperidin content, meanwhile narirutin and didymin were affected by Forner-Alcaide 5. Ordóñez-Díaz and colleagues (2020) studied the effect of rootstock on phenolic profile in Salustiana and Sanguinelli. They quantified hesperidin, narirutin, vicenin-2 and didymin and, in agreement with other study (Gattuso et al., 2007), hesperidin was the main flavanone contained in the juice. Its content was strongly influenced by Forner-Alcaide 5, followed by Cleopatra mandarin in fruits of Sanguinelli. Narirutin and vicenin-2 were higher in Forner-Alcaide 5 and Carrizo citrange in both cultivars. They also showed that in fruits of Salustiana didymin was quantified with higher concentrations in Cleopatra mandarin than in other rootstocks. This confirm that the rootstocks and the changes in the profile of secondary metabolites, including flavonoids, depended on scion/rootstock interactions (Feng et al., 2018; Ordóñez-Díaz et al., 2020). Morales and colleagues (2021a) quantified hesperidin, narirutin and didymin in fruits of Moro and Tarocco Rosso during different stages of harvest. During the samplings, a significant increase for all components in Moro was observed, except in the fruits grafted with Forner-Alcaide 5 that exhibited the maximum of hesperidin at the first and second harvest

and then dropped. At the third stage the maximum level of narirutin was achieved by C35, meanwhile in Forner-Alcaide 13 the lowest was reported. Concerning didymin, C35 achieved one of the highest values, while the lowest was observed in both Swingle citrumelo and Forner-Alcaide 13. They evidenced a different behaviour of phenolic profile in Tarocco Rosso grafted with the same rootstocks. At the first stage, the highest value of hesperidin was found in Swingle citrumelo, meanwhile Forner-Alcaide 13 showed the lowest. The naringin content increased at the second harvest to then decrease in the third, excepted for fruits of C35 that did not change with harvest showing the lowest values. At the third harvest, the content of didymin had the highest values in Carrizo citrange, followed by Swingle citrumelo and Cleopatra mandarin.

Rootstock influence in hydroxycinnamic acids content

The strong influence of rootstock on secondary metabolites such as the hydroxycinnamic acids in blood orange was studied (Rapisarda et al., 1998; Kelebek et al., 2008). Morales et al. (2021a) investigated on chlorogenic, ferulic, sinapic, caffeic and ρ-coumaric acids in Moro and Tarocco Rosso grafted on different rootstocks at different ripening stage. At the first harvest, fruit of Moro had the highest values of chlorogenic acid in Forner-Alcaide 5, meanwhile in Forner-Alcaide 13 was found the lowest values. The content of chlorogenic acid increased in all Moro/rootstock combination, except in fruits from Forner-Alcaide 5. *C. macrohpylla* negatively affected the ferulic acid content evidencing the lowest values. Concerning sinapic acid, Swingle citrumelo had the highest value, while the lowest was in fruits of C35 and Cleopatra mandarin, especially at the first harvest. In Tarocco Rosso they observed major differences among rootstocks. The lowest chlorogenic acid content was present in C35, followed by Forner-Alcaide 13, Carrizo citrange and Swingle citrumelo. Forner-Alcaide 5, Cleopatra mandarin, *C. macrohpylla* and

C. volkameriana reported maximum content of chlorogenic acid.

There are few studies on the variations of the single metabolites in blood orange as a function of rootstock genotype. These data need to be further corroborated by studies of rootstocks in combination with various blood oranges, to identify the metabolic behaviour of the different scion/rootstock combinations.

Rootstock behaviour under salt stress

Citrus trees are affected by several abiotic stresses that limits plant growth and productivity (Boyer, 1982). Among these, today salinity is a widespread problem, although in the past this problem appeared in arid and semiarid areas where rain was not enough to wash the salts of the soil. Although there are important differences between species and varieties; citrus are considered a salt-sensitive crop because suffer physiological disturbances such as ion-specific toxicity, nutrient imbalances, altered gas exchange parameters, and adverse water relations, leading to stunted growth. Simpson and colleagues (2014) focused on the concentration of salt in irrigation water, and they reported that in citrus when concentration of Na+ ions exceed 1500 ppm or 25 mM, plants are stressed by salinity. Moreover, value of Cl- concentration higher than 355 ppm prevented citrus plant growth. It is important to consider that there is an interaction between variety and rootstock, and the response of trees to salinity depends on the individual behaviour of each of the component parts, as well as on the possible interactions scion/rootstock that may occur (Forner-Giner, et al., 2020). In this context, the choice of the appropriate rootstock is a crucial aspect to obtain the maximum yield in citrus crops, both in saline and other conditions.

Concentration of salt ions (chloride and sodium) in rootstock

The rootstock has been considered sensitive to salinity due to the accumulation of excessive concentrations of Cl^- in the leaves,

which implies a high absorption of this ion by the roots, as well as its efficient translocation in the aerial part of the plant (Cooper 1962; Grieve and Walker 1983), while the tolerance to salinity in citrus plants was related to the ability to limit the uptake and/or the transport to the shoots (Walker et al. 1983; Zekri and Parsons 1992). The Cl^- and Na^+ exclusion mechanisms in the different rootstocks (Camara et al., 2004) and/or compartmentation at the tissue and cell levels play an important role in ion exclusion (Balal et al., 2012; Rodriguez-Gamir et al., 2012), although the mechanism remains largely unclear. Several researches investigated the physiological response of rootstocks to salt. Sykes (1992) reported that the capacity to exclude Cl^- and Na^+ ion in citrus rootstocks was heritable and can be transmitted to progenies; indeed, Storey et Walker (1998) found Na^+ accumulation was lower compared to the accumulation of Cl^- and it is influenced by the morphology of the root system. Definitely, Syvertsen et Garcia-Sanchez (2014) reported that Cl^- was more toxic than Na^+, although several physiological mechanisms, such as leaf transpiration, ion sequestration in root tissues and/or compartmentation at the tissue and cell levels are involved in toxic ion exclusion.

Among commercial citrus rootstocks, salt tolerant genotypes limited the translocation of the toxic Cl^- and Na^+ ions in the leaves and, by closing the stomata, they can also reduce the transpiration of the leaves (Nieves et al., 1991). Among the tolerant rootstocks, sour orange, *C. macrohpylla* and rough lemon were considered good Cl^- and Na^+ excluders. Sykes (1985, 1992) reported that Rangpur lime and Cleopatra mandarin were also good Cl^- excluders, meanwhile other studies confirmed that *P. trifoliata* and its hybrids, such as citrange Carrizo and Swingle citrumelo, were Na^+ excluders (Nieves et al., 1991; Storey et Walker, 1999; Munns et Tester, 2008; Gonzalez et al., 2012). Regarding root accumulation, Gonzalez and colleagues (2012) observed the ability of Swingle citrumelo roots to maintain lower levels of Na^+ in the leaves compared to the root system of Rough

lemon. They reported that the sequestration of Na^+ in root tissue vacuoles and its immobilization by cell walls were a key mechanism of salt tolerance.

Physiological responses

Citrus is a salt-sensitive crop, which, even at moderate salinities, suffers physiological disturbances such as ion toxicity, mineral discrepancies, physiological troubles. These aspects could strongly limit plant growth due to osmotic stress and nutritional imbalances (Storey and Walker, 1999; Syvertsen et Garcia-Sanchez, 2014). In fact, the salts dissolved in the nutrient solution exert an osmotic effect that reduces the availability of free water in the plant. The role of rootstock against these limiting constraints was deeply investigated with the aim of knowing the most suitable genotypes in relation to the different limiting factors that affect a given area of production (González-Mas et al., 2009). Nevertheless, current understanding of the physiological basis underlying the rootstock effect on the entire tree is still limited (Webster, 2004; Jones, 2012). The primary effects of salinity in citrus are stomatal closure, reduced CO_2 diffusion, decreased net photosynthesis and increased ion accumulation (Levy et Syvertsen, 2004; García-Sánchez et Syvertsen, 2006; Brumós et al., 2010). The osmotic stress could increase abscisic acid (ABA), ethylene production and leaf abscission (Syvertsen et Garcia-Sanchez, 2014). All this could result in reductions of root and shoot growth and yield, reduced leaf area, and produces chlorotic and necrotic patches on leaves (Netondo et al., 2004; Boman et al., 2005; Pérez-Tornero et al., 2009).

Some studies reported that physiological mechanisms leading to stress tolerance are aimed at minimizing the damaging effects of stress to tissues, such as stomatal closure to reduce transpiration (Arbona et al., 2017). Previously research suggested that the rootstock regulate tree physiology, especially the translocation of minerals, plant growth

regulators, carbohydrates, and water uptake (Castle, 1995; Webster, 1995). Lopez-Climent and colleagues (2007) observed different citrus rootstocks under salt stress and investigated the stability of the photosynthetic machinery with respect to the relative salt tolerance. They selected three sensitive genotypes (Carrizo and C35 citranges, Citrumelo CPB 4475) together with the moderately salt tolerant genotype Cleopatra mandarin and Forner-Alcaide 5. After 3 month the treatment with 60 mM NaCl, visible toxicity symptoms in the leaves of Citrumelo was reported. In Carrizo and C35 a rapid increase in leaf damages after 45 days was found, while in Forner-Alcaide 5 leaf damage increased after 60 days of treatment. A correlation between photosynthesis and stomatal closure was observed, reporting that stomatal closure decrease in Forner-Alcaide 5 and Cleopatra mandarin.

More recent studies have analysed the behaviour of new genotypes (Aparicio-Durán et al., 2021) under salt stress. They observed a different response on X639 that did not show significant differences in growth among all the treatments, meanwhile in other citrus rootstocks a reduction in height with increasing salt concentration was found. Other research showed that X639 and US942 has a high ability to adapt to salt (Adams et al., 2019; Aparicio-Durán et al., 2021). However, Syvertsen and Bandaranayake (2012) reported that Cleopatra mandarin was a good salt tolerant rootstock, compared with X639 and US-897 that are largely affected by salt stress.

Effect of salinity on antioxidant activities and osmolyte concentration of rootstock

Few research investigated on the responses of the antioxidant system in roots and leaves, combined with measurements of toxic ion (Cl^- and Na^+), osmolytes concentrations, and physiological parameters in response to rootstock (Shahid et al., 2019). Previous research

reported that citrus plant under salt stress, can modulate the osmotic pressure through the synthesis of several solutes such as proline, sugars, and organic acids (Lima-Costa et al., 2008). This result was confirmed by Dichio and colleagues (2009) that studied the behaviour of compatible solutes like proline and non-structural sugar compounds, describing that these compounds played an important role through osmotic adjustment which helps plant tissues to maintain their essential water status. In fact, it was demonstrated that sugar levels tend to increase in salt-sensitive genotypes rather than in the resistant ones. Anjum et al. (2008) analysed the concentrations of sucrose, glucose, and fructose in Cleopatra mandarin and Troyer citrange showing a decline in sugars concentration in leaves of Cleopatra and in both leaves and roots of Troyer, with increasing levels of salinity stress.

Shahid and colleagues (2019) described that proline and glycinebetaine protected the cells from dehydration caused by salinity stress having a significant role in osmotic adjustment in the cytosol of both root and leaf cells. They observed that Kinnow mandarin grafted on salt tolerant rootstocks (Rangpur lime and Rubidoux trifoliate) had the highest accumulation of these organic osmolytes in leaves and roots in response to salt stress. Gill and Tuteja (2010) explored the role of reactive oxygen species (ROS) related with stress tolerance in crop plants. They showed that antioxidant activity of the cell reduced toxicity level of ROS converting them into water and oxygen. Hence, plants with high antioxidant activities show greater tolerance to salt stress and a low rate of ROS formation. Recently researches (Andre et al. 2013; Shahid et al., 2019; Khalid et al., 2020) described that superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were a group of very important antioxidant enzymes that play a major antioxidant defensive role, eliminating $O2^{-}$ from tissues. Shahid and colleagues (2019) reported that Kinnow mandarin grafted on Rangpur lime and Rubidoux trifoliate had higher H_2O_2 scavenging potential

than citrange Carrizo and Sacaton citrumelo, which were not tolerant.

Oxidative stress was primarily affected by the rootstock response under salt stress and is often measured by lipid peroxidation (LPO) which was estimated by measuring malondialdehyde (MDA) concentration. The use of salt-tolerant rootstock influenced the formation of ROS in the scion; in fact, rootstock as Rangpur lime and Rubidoux, reported as salt-tolerant, had less formation of ROS in both leaves and roots, due to a higher ROS-scavenging capacity and a higher accumulation of osmolytes (proline and GB) that limited the root-to-shoot movement of toxic ions (Shahid et al., 2019). This result has also been confirmed by other studies showing a direct correlation between stress increase and high concentration of MDA in leaves and roots (Xie et al., 2021).

Effect of salinity on hormone imbalance

Plant hormones play an active role in influencing plant growth and in regulating different aspects of plants, including vegetative and reproductive growth and signal transduction pathways (Talat et al. 2020; Qureshi et al., 2021). Davies (2004) described the role of hormones in several physiological processes: auxins are produced by the shoots and influence the plant vigour, cytokinins and gibberellins regulate bud growth and dormancy phase and are produced by the roots. Cytokinins travel through root meristems through xylem upward enhancing flowering and fruit set (Young, 1989). As previously reported (Gonzalez-Mas et al. 2009), the physiological performance of scion is related to rootstock with effects on endogenous plant hormones, leaf gas exchange, and photosynthetic pigments, influencing tree vigour and crop production. Abscisic acid (ABA) is a key regulator of many plant responses to environmental stresses (Hubbard et al. 2010), although other plant hormones have important roles in abiotic stress responses as well. Gibberellins (GAs), ethylene (ET), salicylic acid (SA), jasmonic acid (JA) interplay with

ABA at different levels, and GAs regulate vegetative and reproductive growth and development (Claeys et al. 2014; Schwechheimer and Willige, 2009; Wang and Irving, 2011). Several studies demonstrated that SA also play an important role in plant defence responses interacting with GAs (Kang et al. 2014; Miura and Tada 2014). Previous research focused on the behaviour of Cleopatra mandarin under extreme conditions, such as water stress, and they showed that 1-aminocyclopropane-1-carboxylic acid (ACC) in roots drastically arrested xylem flow, although no increase of ACC and ethylene in leaves and leaf abscission was observed. They also explained that the rootstock was salt-tolerant due to the restore of the normal xylem fluid flow after rehydration and the ACC transport to the shoots, where it was oxidized to ethylene, triggering leaf abscission (Tudela and Primo-Millo, 1992).

Balfagón et al. (2019) investigated salt and heat stress response on Carrizo rootstock, and reported that ABA increased in response to high salinity stress. They also reported that JA increased strongly in leaf of Carrizo in plants subjected to salt stress combined with heat stress. Zandalinas and colleagues (2018) investigated on Cleopatra and Carrizo under heat stress, and they confirmed that Cleopatra mandarin was a sensitive genotype due to the high malondialdehyde (MDA) accumulation that indicate a stronger oxidative damage.

Scope and thesis outline

This thesis provides novel insights on the biochemical features of blood orange fruits in relation to the rootstock genotype; the investigation focused on the agronomical effects and on the qualitative fruit characteristics affected by the rootstock. With this purpose, a multidisciplinary approach was adopted, in order to evaluate the bioactive compounds profile and the physiological effect of the graft combination in blood orange.

In **Chapter 2** the phytochemical profile of 11 blood oranges was analysed for two years to evaluate and characterize the main blood orange cultivars spread in the Mediterranean basin. The deepest red peel and juice was found in Sanguinelli, followed by Tarocco Rosso and Moro. Moro showed the greatest levels of antioxidant activity. Regarding phenolic compounds, p-coumaric acid was found as the main hydroxycinnamic acid in all cultivars, and the highest amounts of cyanidin-3-O-glucoside and cyanidin-3-(6''-malonyl)-glucoside were found in Moro.

In **Chapter 3** the influence of 10 rootstocks on fruit qualitative characteristics of Tarocco Sciré blood orange was evaluated for two years. Individual and amount of the phenolic compounds (anthocyanins, flavanones, flavones and hydroxycinnamic acids) and their antioxidant potential were investigated. The study was conducted for two consecutive years, in which the influence of both rootstock and environmental temperatures (and their interaction) was evaluated to explain the metabolic profile of the juice.

In **Chapter 4** rootstock influence on the evolution of bioactive compounds of blood orange Tarocco Sciré during maturation was evaluated. The investigation aimed also to determine if any rootstock

effects are related to changes in the expressions of some key quality trait genes in the scion. The evolution of peel colour, anthocyanin content, sugars, acids and vitamin C was investigated and a positive correlation between rootstock and the regulation of fruit-quality related genes in the scion was found.

In **Chapter 5** the influence of 11 rootstocks on yield and fruit quality (including pigmentation) of Mandared, a pigmented mandarin hybrid, was evaluated. Differences in terms of yield precocity, fruit drop percentage, cumulative production and yield efficiency were observed. Regarding qualitative traits, no statistical difference was found in sugars and acidity, meanwhile an influence of the rootstock in the anthocyanin content was detected.

In **Chapter 6** the behaviour of new citrus rootstock (C35, Bitters, Carpenter and Furr) compared with others spread in Mediterranean basin (Carrizo citrange, Citrumelo Swingle, *Citrus volkameriana*and *Citrus macrophylla*) was evaluated under salinity stress. The objective of this study was to investigate the effect of different salinity levels on physiological and morphological features. The performance of the rootstocks was also evaluated through lipid peroxidation, estimated by measuring the concentrations of malondialdehyde, free proline content of leaves, antioxidant activity (APX, SOD, CAT) and hormone analyses.

Finally, in **Chapter 7** results of the experimental investigations are integrated to outline the overall conclusions referring to the role of the rootstock on the biosynthesis of bioactive compounds in blood orange. The effect of the rootstock in citrus crops has been highlighted, evidencing the importance of choosing the most suitable one to gain the agronomic benefits conferred to the whole tree. In addition, the role of the rootstock in order to obtain a high quality fruit product for

pomological and organoleptic traits and with recognized nutraceutical characteristics in blood orange was determined.

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Chapter 2 - Bioactive compounds, antioxidant activity and fruit quality evaluation of eleven blood orange cultivars

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Abstract

BACKGROUND: Blood oranges are grown increasingly in Europe for fresh consumption because of their special taste and excellent nutraceutical properties that confer the status of a functional food. The health benefits are associated with the range of additional bioactive compounds they contain with respect to blonde oranges.

RESULTS: We analysed the physicochemical properties and the levels of organic acids, sugars and antioxidants in eleven blood orange cultivars which represent the most representative cultivars of blood oranges widespread in the Mediterranean basin. In particular, we examined the levels of the phenols, flavonoids and anthocyanins present in these cultivars at harvest maturity.

The physicochemical, antioxidant and colour properties differ significantly among these cultivars. The deepest red peel and juice was found in Sanguinelli, followed by Tarocco Rosso and Moro. High-performance liquid chromatography with refractive index detector analysis revealed sucrose as the main sugar in all these cultivars, followed by fructose and glucose. Citric acid was the dominant organic acid, followed by malic acid and ascorbic acid. Moro showed the greatest levels of antioxidant activity. As regarding the phenolic composition, we found p-coumaric acid as the main hydroxycinnamic acid in all cultivars, with maximum amounts in Moro and Sanguinelli. The highest amounts of cyanidin-3-O-glucoside and cyanidin-3-(6"-malonyl)-glucoside were found in Moro, whose juice was of deepest red colour.

CONCLUSION: It was assessed the phenolic composition and the antioxidant activity of the eleven cultivars. Results showed that Moro was the one with the highest content of polyphenols and level of antioxidant activity, followed by Sanguinelli.

Introduction

Sweet oranges (*Citrus sinensis* L. Osbeck) are one of the most important citrus fruits. Total world production of sweet oranges is around 79 million tons (FAOSTAT, 2019). The two major groups of sweet oranges are blonde oranges, which are widespread in almost all citrus-producing countries, and blood oranges, which are grown in the relatively few regions and where the climate favours the synthesis of the red pigment. The red colour of blood oranges is due to the high levels of the water-soluble pigment, anthocyanin, which belongs to the larger family of flavonoids. The amounts of these pigments, both in the peel and in the flesh, depend on a range of factors including cultivar, rootstock, maturity, region of cultivation and environment

(Lo Piero, 2015; Lana et al., 2021). Of the latter, the thermal excursions between night and day are thought to be a crucial factor in stimulating anthocyanin synthesis (Pannitteri et al., 2017; Continella et al., 2018; Habibi et al., 2020). For these reasons, the pigmented cultivars, the so-called blood oranges, are cultivated mainly in Italy and, especially, in a Sicilian production district of almost 40,000 ha characterised by a Protected Geographical Indication (GPI). These fruits are mostly consumed fresh. The most important Italian blood orange cultivars are Tarocco and Moro. The first is characterised by having a number of clones with different maturation periods. It is consumed as a fresh fruit and favoured for its easy peelability and high levels of sugar/acid ratio. Moro is the most deep red in colour and its juice offers some anti-inflammatory effects due to the high levels of certain bioactive compounds (Grosso et al., 2013) including flavonoids that are important in the human diet (Proteggente et al., 2003).

In Spain, red oranges are cultivated on 952 ha and represent about 0.7% of total sweet orange production there, while more than 72.3% is represented by early and late navels (MAPA, 2019). The most common blood orange is cv. Sanguinelli, a spontaneous mutation of cv. Doble Fina. Although Sanguinelli and Moro are the most colourful, there have been a number of pigmented cultivars grown in Spain, including cv. Doble Fina and its natural mutations, cv. Entrefina and the very similar cv. Murtera. Both are characterised by a lower juice pigmentation but by being very rich in phenolic compounds (Porra et al., 2014). The cultivar Maltaise demi Sanguine is of unknown origin and it is grown extensively in Tunisia and, to a lesser extent, in Morocco (Saunt et al., 2000).

Recently, increased interest has been shown in blood oranges as a good source of natural antioxidants and bioactive compounds such as polyphenols (flavonoids, anthocyanins, hydroxycinnamic acids)

and higher amounts of ascorbic acid to respect to blonde oranges (Rapisarda et al., 2009; Cebadera-Miranda et al., 2019). Antioxidants play important roles in the human diet (Comert et al., 2018). In particular, epidemiological studies show their antioxidant and anti-inflammatory activities have beneficial effects on human health, lowering the risk of cardiovascular diseases, diabetes and cancers (Wang et al., 2008; Comert et al., 2018).

In this context, this study characterises 11 cultivars of blood orange, grown under the same environmental conditions, with respect to their contents of phytochemical compounds and bioactive properties.

Materials and methods

Cultivars and fruit sampling

Fruits of the blood orange cultivars were collected from the principal citrus germplasm bank of Spain (latitude 39º 35' 22.6''N x 0º 23' 41.0''W, Coord UTM, ETRS 89 Huso 30, X: 723678 Y: 4385379) located at the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Valencia, Spain. The study included the fruits of 11 cultivars of blood orange: Doble Fina, Entrefina, Maltaise Blonde, Maltaise demi sanguine, Moro, Murtera, Sanguinelli, Tarocco Comune, Tarocco Messina, Tarocco Rosso and Washington Sanguine. All cultivars were grafted on the same hybrid rootstock Carrizo citrange.

For each cultivar, three replicates samples, each consisting of 10 fruits (n=30) were harvested over two seasons (2017 and 2018). The fruit were hand-harvested at physiological maturity, to ensure best flavour and colour, and immediately transported under well-ventilated conditions to the laboratory.

Physical and chemical determinations

Once in the laboratory, 30 oranges per cultivar were selected for analytical determination. Fruits of three subsamples per cultivar (each of 10 fruits) were cut in half and carefully hand-squeezed in a commercial juicer. The fresh juices were centrifuged at 13.000 g force for 20 min (Sigma 3-18K, Germany).

Fruit weights were determined with a digital balance Sartorius (model BL-600, 0.01 g). Fruit size (equatorial diameters and length) and pulp thickness were measured with an electronic digital slide gauge Mitutoyo (model CD-15 DC, England, 0.01 mm).

Total soluble solids (TSS) contents were measured with a digital refractometer Atago N1 (Atago Co. Ltd., model N-1, Tokyo, Japan, 0.2 °Brix) at 20ºC with values being expressed as ºBrix. The titratable acidity (TA) was determined using an automatic titration device (877 Titrino plus, Metrohm ion analyses CH9101, Herisau, Switzerland) with 0.1 N NaOH up to pH 8.1, and results expressed as g of citric acid L^{-1}, since this is the dominant organic acid in oranges. Once the TSS and TA contents had been assessed, the maturity index (MI) was calculated as the TSS/TA ratio. Results are shown as mean values ± SE.

Colour determinations were made of both the rind (n=120) and juice (n=12) according to the Commission Internationale de l' Éclairage (CIE) and expressed as L*, a*, b*: L* (brightness or lightness; 0 = black, 100 = white), a* (-a* = greenness, +a* = redness) and b* (-b* = blueness, +b* = yellowness). These values were then used to calculate Hue angle degree [Hº* = arctang (b*/a*)], where 0º = red-purple; 90º = yellow, 180º = bluish-green and 270º = blue and Chroma [C* = (a*2 + b*2)]1/2, indicate of the colour intensity or saturation. As suggested in previous studies (McGuire, 1992), hue angle (Hº*) and chroma (C*) have been accepted as the more intuitively understandable colour variables. Colour index (CI) was

calculated using the following formula CI = 1000 a*/L* b* (Jimenez-Cuesta et al., 1981). Colour variables were measured using a Minolta C-300 Chroma Meter (Minolta Corp., Osaka, Japan) coupled to a Minolta DP-301 data processor.

Determination of sugars and organic acids

Individual organic acids and sugars were also determined using three juice samples for each cultivar as described in previous research (Legua et al., 2013). Briefly, 1 mL of the centrifuged juice was passed through a 0.45-µm Millipore filter and injected into a Hewlett-Packard series 1100 (Wilmington Del., U.S.A.) high-performance liquid chromatography (HPLC). The elution system consisted of 0.1% phosphoric acid with a flow rate of 0.5 mL min^{-1}. Organic acids were separated on a Supelcogel TM C-610H column (30 cm×7.8 mm i.d., Supelco, Bellefonte, Pa., USA) and Supelguard column (5 cm×4.6 mm, Supelco, Inc.), and detected using a diode-array detector set at 210 nm. For the sugar analyses, the same HPLC equipment, elution system, flow rate and columns were used. The detection of sugars was carried out using a refractive index detector (HP 1100, G1362A). Standard curves for pure standards of organic acids (oxalic, citric, malic, quinic, and ascorbic acids) and for sugars (glucose, fructose, and sucrose) (Sigma, Poole, Dorset, UK) were used for quantification. Results for both organic acids and sugars are expressed as concentrations g kg^{-1} fresh weight (FW). Sugars and organic acids were determined in triplicate.

Total polyphenol content (TPC) and Total antioxidant activity (TAA)

Total polyphenol content (TPC) was quantified using Folin–Ciocalteu reagent (Singleton et al., 1999). Briefly, for each sample, 2 g of flesh tissue was homogenised in 5 mL of MeOH/water (80:20 v/v)

+ 2 mM NaF and centrifuged at 13.000 g force for 20 min. Absorption was measured at 760 nm using a spectrophotometer (ThermoSpectronic Heγios, UK). Results (mean ± SD) are expressed as mg of gallic acid 100 g^{-1} FW equivalent.

Total antioxidant activity was quantified as described by previous study (Legua et al., 2011). This procedure allows determination of both the hydrophilic and lipophilic TAA in the same extraction. Briefly, for each subsample, 5 g of flesh tissues were homogenised in 5 mL of 50 mM phosphate buffer pH = 7.8 and 3 mL of ethyl acetate, then centrifuged at 10000 x g for 15 min at 4 ºC. The upper fraction was used for TAA due to lipophilic compounds (L-TAA) and the lower one for TAA due to hydrophilic compounds (H-TAA). In both cases, TAA was determined in triplicate for each extract using an enzyme system composed of the chromophore 2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the horseradish peroxidase enzyme and its oxidant substrate (hydrogen peroxide), in which ABTS•+ radicals are generated and monitored at 730 nm. The decrease in absorbance after adding the extract was proportional to the TAA of the sample. A calibration curve was obtained using Trolox ((R)-(+)-6-hydroxy-2,5,7,8-tetramethyl-croman-2-carboxylic acid) (0 to 20 nmol) from Sigma (Madrid, Spain), and results (means ± SD) are expressed as mg of Trolox equivalent 100 g^{-1} FW.

HPLC-DAD-ESI-MSn analyses: identification and quantification of phenolic compounds

Three samples of each of the eleven cultivars (n= 33) were frozen in liquid nitrogen, to be later freeze-dried in an Alpha 2–4 freeze drier (Christ Alpha 2–4; Braun Biotech, Osterode am Harz, Germany) for 24 h at a pressure reduction of 0.220 mbar. The temperature in the drying chamber was −25 °C, while the heating plate

reached 15 °C. Samples of 50 mg of freeze-dried powder were mixed with 2 mL of 80% aqueous methanol acidified with formic acid (1%), vortexed and sonicated for 1 min at room temperature. The resulting heterogeneous mixture was centrifuged at 900 g force for 4 min and the supernatant passed through a 0.45 μm PTFE filter (Waters, Milford, USA) prior to injection into the chromatograph system.

Chromatographic analyses were carried out on an Agilent 1100 series HPLC-ESI-DAD-MSn Ion Trap (Waldbronn, Germany). This HPLC system with DAD detector series 1100 was coupled to a mass spectrometer equipped with an ion trap and an electrospray ionisation interface (ESI). The capillary temperature and voltage for the ESI source were set at 350ºC and 3500 V, respectively. The collision-induced fragmentation occurred inside the ion trap using helium as the collision gas and a collision energy of 50%. The mass range for the precursor ions (MS) and their subsequent fragmentations (MS-MS) was from 100 to 1000 m / z and the data were acquired in negative ionisation mode, where the deprotonation of the molecules was observed. A reverse-phase Agilent Pursuit XRs 5 C18 column was used (250 × 4.6 mm i.d. and particle size 5 μm, Waldbronn, Germany). Water/formic acid (95:5, v/v) and acetonitrile were used as the mobile phases A and B, respectively, with a flow rate of 0.8 mL/min. The gradient started with 5% of solvent B, reaching 60% solvent B at 37 min, and 98% at 40 min, which was maintained up to 2 min. The injection volume was 10 μL. Chromatographic comparison with analytical standards, absorbance spectra and mass spectra, using MSn data (not shown) were used to identify compounds. Flavanones and flavone C-glucosides were monitored and quantified at 280 and 330 nm, whereas anthocyanins at 520.

Cyanidin 3-O- glucoside, chlorogenic acid, p-coumaric acid were purchased from Sigma (Sigma-Aldrich., Milan, Italy), whilst naringin, hesperidin and vitexin were from Extrasynthese (Lyon,

France).

All analyses were carried out in triplicate; results are reported in milligram of compound per liter (mg L^{-1}) of juice.

Statistical Analyses

A basic descriptive statistical analysis was followed by an analysis of variance test for mean comparisons. The method used to discriminate among the means (Multiple Range Test) was Fisher's Least Significant Difference (LSD) procedure at a 95.0% confidence level. A principal component analysis (PCA) was performed. All statistical analyses were carried out using Statistica 6.0 (Statsoft, Tulsa, USA).

Results

Physicochemical properties

In table 2.1 are listed the physicochemical properties, size, weight, soluble solids and titratable acidity. Maltaise demi Sanguine, Murtera and Sanguinelli showed significantly lower fruit weights (108, 113 and 113 g, respectively), while Tarocco Rosso (212 g), followed by Maltaise Blonde (197 g), Moro (196 g) and Washington Sanguine (189 g), had higher mean fruit weight. Fruit equatorial diameters and height ranged between 58-74 mm and 64-75 mm, respectively.

The thickest rind was in Moro, Entrefina, Maltaise demi Sanguine and Washington Sanguine, while the thinnest was in Tarocco Comune and Tarocco Messina (2.5 and 3.2 mm, respectively). Concerning the values of TSS and TA, fruits of Tarocco Comune showed the highest TSS content (15.03 °Brix), while Tarocco Messina fruits recorded the lowest TSS (11.33 °Brix). At the end of sampling, all the pigmented cultivars registered values of total acidity between 11 and 14 g citric acid L^{-1}.

Table 2.1. Physicochemical properties of blood oranges

	Fruit weight (g)	Equatorial diameter (mm)	Fruit height (mm)	Rind thickness (mm)	TSS (° Brix)	TA (g citric acid L^{-1})	MI (TSS/TA)
Doble Fina	158 ± 5.2 d	64.3 ± 2.1 d	73.2 ± 2.3 ab	3.6 ± 0.6 bc	12.45 ± 0.09 f	14.17 ± 0.22 a	8.78 ± 0.08 e
Entrefina	137 ± 6.8 e	65.5 ± 3.3 cd	62.0 ± 3.5 cd	4.4 ± 1.2 a	12.17 ± 0.06 g	13.94 ± 0.19 ab	8.73 ± 0.08 e
Maltaise Blonde	197 ± 20.5 b	70.0 ± 7.3 b	73.5 ± 6.5 ab	3.4 ± 1.3 bc	13.00 ± 0.10 c	11.99 ± 0.20 e	10.84 ± 0.15 b
Maltaise demi Sanguine	108 ± 4.6 f	59.8 ± 2.5 e	58.8 ± 2.6 d	4.0 ± 0.8 ab	12.63 ± 0.08 e	12.24 ± 0.25 de	10.32 ± 0.16 c
Moro	196 ± 11.7 b	70.5 ± 4.2 b	72.9 ± 3.9 ab	4.6 ± 0.6 a	12.80 ± 0.10 d	13.38 ± 0.29 c	9.57 ± 0.14 d
Murtera	113 ± 4.6 f	58.7 ± 2.4 e	60.8 ± 3.3 cd	3.2 ± 0.8 c	12.93 ± 0.06 cd	11.95 ± 0.17 e	10.82 ± 0.19 b
Sanguinelli	113 ± 3.3 f	58.0 ± 1.7 e	64.1 ± 3.7 c	3.6 ± 1.0 bc	12.03 ± 0.15 g	12.68 ± 0.52 d	9.50 ± 0.26 d
Tarocco Comune	138 ± 6.0 e	63.5 ± 2.8 d	60.5 ± 3.2 d	2.5 ± 0.6 d	15.03 ± 0.06 a	13.47 ± 0.54 bc	11.16 ± 0.43 ab
Tarocco Messina	174 ± 10.0 c	66.5 ± 3.8 cd	75.5 ± 4.4 a	3.2 ± 1.0 cd	11.33 ± 0.14 h	12.23 ± 0.12 de	9.26 ± 0.19 d
Tarocco Rosso	212 ± 12.6 a	74.5 ± 4.4 a	70.1 ± 2.4 b	3.5 ± 1.2 bc	12.13 ± 0.06 g	10.75 ± 0.33 f	11.30 ± 0.40 a
Washington Sanguine	189 ± 7.6 b	67.8 ± 2.7 bc	72.2 ± 7.0 ab	4.0 ± 0.6 ab	13.90 ± 0.10 b	13.60 ± 0.30 bc	10.22 ± 0.15 c

Data are the mean ± SD (n = 60). Values followed by the same lowercase letter, within the same column, are not significant different according to Fisher's LSD procedure at 95% confidence level.

External and juice colour

Regarding rind and juice colour, all properties are summarised in Table 2.2. The H° values were lower in the peels of Sanguinelli, Tarocco Rosso, Murtera and Moro, while the highest were in the peels of Maltaise Blonde and Tarocco Messina. The high Citrus Colour Index (CCI) values observed in fruits of Sanguinelli confirmed the deep red colour. Maltaise blonde showed the lowest CCI value. The highest value of a* was found in Tarocco Rosso (13.83) and Tarocco Messina (13.67), instead the lowest values were in Doble Fina (7.54) and Maltaise Blonde (7.42).

Based on H° and CCI, the juice of Moro was of deepest colour (Figure 2.1), followed by Sanguinelli and Tarocco Rosso. Several cultivars showed pale red juice with values not significantly different from one another (Doble Fina, Entrefina, Maltaise Blonde, Maltaise demi Sanguine, Murtera, Washington Sanguine and Tarocco Comune).

Table 2.2. Peel and juice colour of blood oranges

Parameter[a]	L*				a*				b*				C*				Hº				CCI			
Rind colour*																								
Doble Fina	59.63	±	2.08	b	27.89	±	1.57	ef	39.72	±	3.29	d	48.57	±	3.01	ef	54.84	±	2.46	bc	11.88	±	1.46	de
Entrefina	59.39	±	1.04	b	33.09	±	1.21	a	39.92	±	1.76	d	51.88	±	1.26	cd	50.33	±	1.91	de	14.01	±	1.20	d
Maltaise Blonde	65.63	±	2.20	a	31.33	±	1.30	b	66.40	±	2.83	a	73.45	±	2.19	a	64.70	±	1.73	a	7.24	±	0.83	g
Maltaise demi Sanguine	58.77	±	2.37	b	30.78	±	1.30	bc	37.82	±	4.78	de	48.87	±	3.59	ef	50.62	±	4.06	de	14.18	±	2.71	cd
Moro	54.62	±	2.17	c	30.51	±	1.67	bc	34.90	±	5.68	ef	46.47	±	4.76	fg	49.49	±	4.41	ef	16.48	±	3.29	bc
Murtera	53.84	±	2.98	c	29.57	±	1.51	cd	32.78	±	4.70	f	44.26	±	3.64	g	47.70	±	4.32	ef	17.24	±	3.52	b
Sanguinelli	50.37	±	3.35	d	27.34	±	1.66	f	25.22	±	5.52	b	37.34	±	4.67	h	42.15	±	5.13	g	22.63	±	5.66	a
Tarocco Comune	59.54	±	1.78	b	29.15	±	1.13	de	46.02	±	2.77	c	54.51	±	2.04	c	57.59	±	2.30	b	10.72	±	1.24	ef
Tarocco Messina	63.70	±	1.49	a	28.08	±	1.87	ef	52.19	±	2.19	b	59.31	±	1.37	b	61.69	±	2.45	a	8.50	±	1.07	fg
Tarocco Rosso	54.49	±	3.07	c	30.99	±	1.86	bc	32.08	±	4.52	f	44.75	±	2.96	g	45.78	±	5.09	fg	18.31	±	4.45	b
Washington Sanguine	55.35	±	4.04	c	29.68	±	1.42	cd	39.68	±	6.61	d	49.70	±	5.50	de	52.79	±	4.60	cd	14.07	±	3.41	cd
Juice colour**																								
Doble Fina	43.16	±	3.08	d	7.54	±	1.33	d	22.39	±	2.86	d	23.69	±	2.46	d	71.03	±	4.88	a	8.21	±	2.87	d
Entrefina	47.84	±	1.62	a	9.21	±	1.10	c	32.01	±	2.02	a	33.31	±	2.20	a	73.99	±	1.17	a	6.02	±	0.58	d
Maltaise Blonde	43.13	±	0.97	cd	7.42	±	1.08	d	25.11	±	2.10	c	26.19	±	2.30	c	73.59	±	1.08	a	6.83	±	0.44	d
Maltaise demi Sanguine	45.90	±	1.32	b	8.60	±	1.04	cd	28.68	±	1.37	b	29.96	±	1.38	b	73.30	±	1.95	a	6.56	±	0.92	d
Moro	30.38	±	2.40	g	11.27	±	3.81	b	9.22	±	2.61	g	14.84	±	3.55	e	39.95	±	11.48	d	47.75	±	37.27	a
Murtera	45.10	±	2.91	bc	10.83	±	1.28	b	28.70	±	2.24	b	30.72	±	1.97	b	69.23	±	3.14	a	8.52	±	1.80	d
Sanguinelli	34.30	±	2.91	f	9.09	±	2.45	c	11.92	±	3.00	f	15.34	±	1.86	e	52.13	±	13.04	c	26.04	±	15.81	b
Tarocco Comune	49.29	±	3.21	a	11.69	±	1.68	b	32.17	±	4.19	a	34.26	±	4.25	a	69.95	±	2.61	a	7.50	±	1.55	d
Tarocco Messina	41.87	±	2.81	de	13.67	±	1.70	a	26.01	±	3.26	c	29.50	±	2.46	b	61.96	±	5.49	b	13.14	±	4.34	cd
Tarocco Rosso	36.41	±	0.93	f	13.83	±	1.17	a	17.22	±	1.21	e	22.11	±	1.10	d	51.23	±	3.32	c	22.23	±	3.23	bc
Washington Sanguine	40.89	±	1.61	e	8.22	±	1.42	cd	21.95	±	1.37	d	23.47	±	1.45	d	69.52	±	3.31	a	9.22	±	1.91	d

Data are the mean ± SD. *n = 120; **n = 12. Values followed by the same lowercase letter, within the same column, are not significant different according to Fisher's LSD procedure at 95.0% confidence level.

[a] L*, lightness; *a**, green/red coordinate; *b**, blue/yellow coordinate; *C**, chroma; *H°**, hue angle; CI, colour index.

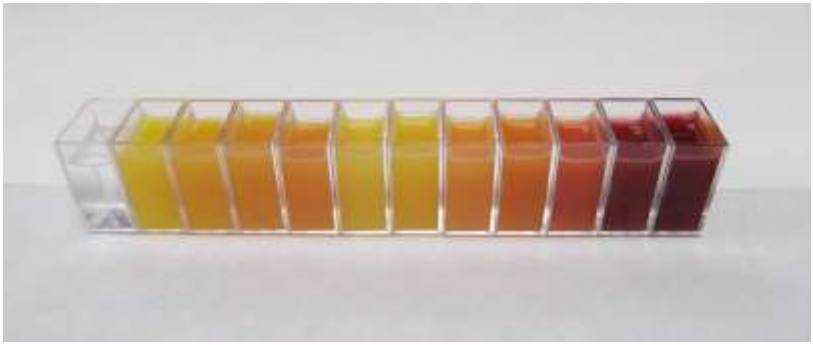

Figure 2.1. Juice of the eleven cultivars analysed: from left to right, distilled water, Entrefina, Murtera, Washington Sanguine, Doble Fina, Maltaise Blonde, Maltaise demi sanguine, Tarocco Comune, Tarocco Messina, Tarocco Rosso, Sanguinelli and Moro

Organic acids and sugars

Marked differences were found in organic acid composition (Table 2.3), in which citric acid was the main organic acid, followed by malic and ascorbic acids. Doble Fina and Sanguinelli showed the highest concentrations of citric acid (20.5 and 18.1 g kg^{-1}, respectively), Maltaise demi Sanguine (9.6 g kg^{-1}) the lowest. But these were not significantly different in Murtera (17.5 g kg^{-1}), Moro (17.3 g kg^{-1}), Washington Sanguine (17.2 g kg^{-1}), Maltaise Blonde (16.7 g kg^{-1}) and the three Tarocco's clones (ranging between 16.6 and 16.9 g kg^{-1}).

The vitamin C content, that is calculated as ascorbic acid, had the highest levels in Entrefina and Sanguinelli (0.49 and 0.50 g kg^{-1}, respectively). There were no significant differences between ascorbic acid levels in Doble Fina, Maltaise Blonde, Maltaise demi Sanguine, Moro or Washington Sanguine.

Sucrose was the main sugar in all cultivars, followed by fructose and by glucose (Table 2.3). The highest concentrations of sucrose were found in Maltaise demi Sanguine, Maltaise Blonde and

Washington Sanguine (59.4, 58.5 and 58.4 g kg^{-1}, respectively). Tarocco Comune (34.3 g kg^{-1}), Sanguinelli (33.5 g kg^{-1}) and Murtera (33.4 g kg^{-1}) showed the largest contents of fructose, while Tarocco Messina had the lowest fructose content (22.2 g kg^{-1}). Entrefina, Maltaise Blonde, Maltaise demi Sanguine, Tarocco Comune and Moro had the highest levels of glucose, ranging from 22.6 to 21.7 g kg^{-1}, while the lowest level was in Tarocco Messina (14.6 g kg^{-1}).

Table 2.3. Organic acids and sugars of blood orange juice (g kg^{-1} FW)

Parameter	Malic acid		Ascorbic acid		Citric acid		Fructose		Glucose		Sucrose	
Doble Fina	2.01 ± 0.13	ef	0.35 ± 0.01	c	20.49 ± 0.48	a	30.66 ± 0.73	bc	20.40 ± 0.45	bc	50.60 ± 0.51	d
Entrefina	2.37 ± 0.24	b	0.49 ± 0.03	a	13.39 ± 0.29	bc	32.40 ± 1.90	ab	22.20 ± 1.60	a	53.10 ± 1.05	bcd
Maltaise Blonde	1.96 ± 0.03	f	0.36 ± 0.02	c	16.65 ± 0.072	ab	29.26 ± 0.92	cd	20.30 ± 0.36	bc	58.46 ± 0.95	a
Maltaise demi Sanguine	2.03 ± 0.04	def	0.38 ± 0.03	c	9.60 ± 0.80	c	32.60 ± 1.34	ab	22.56 ± 0.92	a	59.43 ± 1.35	a
Moro	2.25 ± 0.06	bc	0.37 ± 0.02	c	17.33 ± 0.41	ab	32.70 ± 0.85	ab	22.33 ± 0.60	a	51.83 ± 1.19	cd
Murtera	2.20 ± 0.02	bcde	0.43 ± 0.01	b	17.49 ± 0.33	ab	33.43 ± 1.43	a	22.43 ± 0.66	a	53.73 ± 1.07	bc
Sanguinelli	2.23 ± 0.19	bcd	0.50 ± 0.04	a	18.12 ± 0.31	a	33.46 ± 2.65	a	21.73 ± 1.69	ab	53.10 ± 1.66	bcd
Tarocco Comune	2.06 ± 0.07	cdef	0.40 ± 0.01	ab	16.64 ± 0.53	ab	34.26 ± 1.07	a	22.66 ± 0.57	a	54.46 ± 1.01	b
Tarocco Messina	2.01 ± 0.09	ef	0.38 ± 0.02	bc	16.89 ± 0.68	ab	22.20 ± 1.05	e	14.60 ± 0.30	d	51.36 ± 1.00	cd
Tarocco Rosso	2.72 ± 0.11	a	0.38 ± 0.02	bc	16.92 ± 0.162	bc	27.73 ± 0.47	d	18.26 ± 0.32	c	52.70 ± 0.30	bcd
Washington Sanguine	2.39 ± 0.15	b	0.35 ± 0.03	c	17.18 ± 0.77	ab	32.06 ± 1.97	ab	21.80 ± 1.40	ab	58.43 ± 3.62	a

Data are the mean ± SD (n = 6). Values followed by the same lowercase letter, within the same column, are not significant different according to Fisher's LSD procedure at 95% confidence level.

Polyphenols and total antioxidant activity

Total polyphenols (TPC) and the antioxidant activity (TAA) of the 11 blood oranges were listed Table 4. The TPC ranged between 117.26 and 241.91 mg of gallic acid 100 g^{-1}. Maximum phenolic contents were recorded in the juices of Tarocco Messina, Tarocco Rosso and Doble Fina (241.9, 236.34 and 222.02 mg gallic acid 100 g^{-1}, respectively). Tarocco Comune showed the lowest values (117.26) together with Maltaise demi Sanguine and Maltaise Blonde (131.51 and 133.59, mg gallic acid 100 g^{-1}, respectively).

Our records of hydrophilic antioxidant activity (H-TAA) were significantly higher than those of lipophilic antioxidant activity (L-TAA) (Table 2.4). Moro showed the highest level (129.75 mg Trolox 100 g^{-1}). No significant differences were found between levels in Doble Fina, Tarocco Messina, Washington Sanguine, Tarocco Comune, Entrefina, Maltaise demi Sanguine and Maltaise Blonde, the latter two showing the lowest antioxidant activities (75.47 and 75.22 mg Trolox 100 g^{-1}, respectively). The results of lipophilic total antioxidant activity among the 11 cultivars were not significantly different.

Table 2.4. Polyphenols and TAA of blood orange juice

Parametera	TPC (mg gallic acid 100 g^{-1})	H-TAA (mg Trolox 100 g^{-2})	L-TAA (mg Trolox 100 g^{-3})
Doble Fina	222.02 ± 11.78 a	89.72 ± 5.92 bcde	1.35 ± 0.05 a
Entrefina	163.10 ± 24.21 cd	82.12 ± 2.41 cde	1.55 ± 0.18 a
Maltaise Blonde	133.59 ± 1.56 ef	75.22 ± 8.49 e	1.35 ± 0.20 a
Maltaise demi Sanguine	131.51 ± 12.90 ef	75.47 ± 1.11 de	1.41 ± 0.23 a
Moro	188.89 ± 8.80 bc	129.75 ± 23.79 a	1.43 ± 0.10 a
Murtera	192.58 ± 4.53 b	96.87 ± 11.98 bc	1.52 ± 0.28 a
Sanguinelli	157.64 ± 19.94 de	104.38 ± 5.43 b	1.52 ± 0.05 a
Tarocco Comune	117.26 ± 17.16 f	82.18 ± 4.59 cde	1.38 ± 0.13 a
Tarocco Messina	241.91 ± 19.45 a	85.08 ± 8.07 cde	1.55 ± 0.28 a
Tarocco Rosso	236.34 ± 25.30 a	91.29 ± 4.46 bcd	1.35 ± 0.13 a
Washington Sanguine	168.57 ± 4.30 bcd	84.43 ± 5.33 cde	1.29 ± 0.10 a

Data are the mean ± SD (n = 6). Values followed by the same lowercase letter, within the same column, are not significant different according to Fisher's LSD procedure at 95.0% confidence level.
TPC, total polyphenols content; H-TAA, hydrophilic total antioxidant activity; L-TAA, lipophilic total antioxidant activity.

Total anthocyanins, total hydroxycinnamic and phenolic composition

Total anthocyanin content appeared in Figure 2.2. The highest level of total anthocyanins was in Moro (133.10 mg L^{-1}). Next was Sanguinelli that had less than half of this amount (45.59 mg L^{-1}). No significant differences were observed between the Tarocco cultivars.

Flavanones, flavones and flavonols varied significantly from 325.97 mg L^{-1} to 841.63 mg L^{-1}. Entrefina was lowest in this matrix of chemical markers, followed by Maltaise Blonde, Maltaise demi Sanguine and Doble Fina, whose values were all below 400 mg L^{-1}. The highest values, greater than 800 mg L^{-1}, were found in Tarocco Comune, followed by Moro. Considering the hydroxycinnamic acids the highest level was again in Moro (82.43 mg L^{-1}), while the lowest in Maltaise Blonde (39.96 mg L^{-1}).

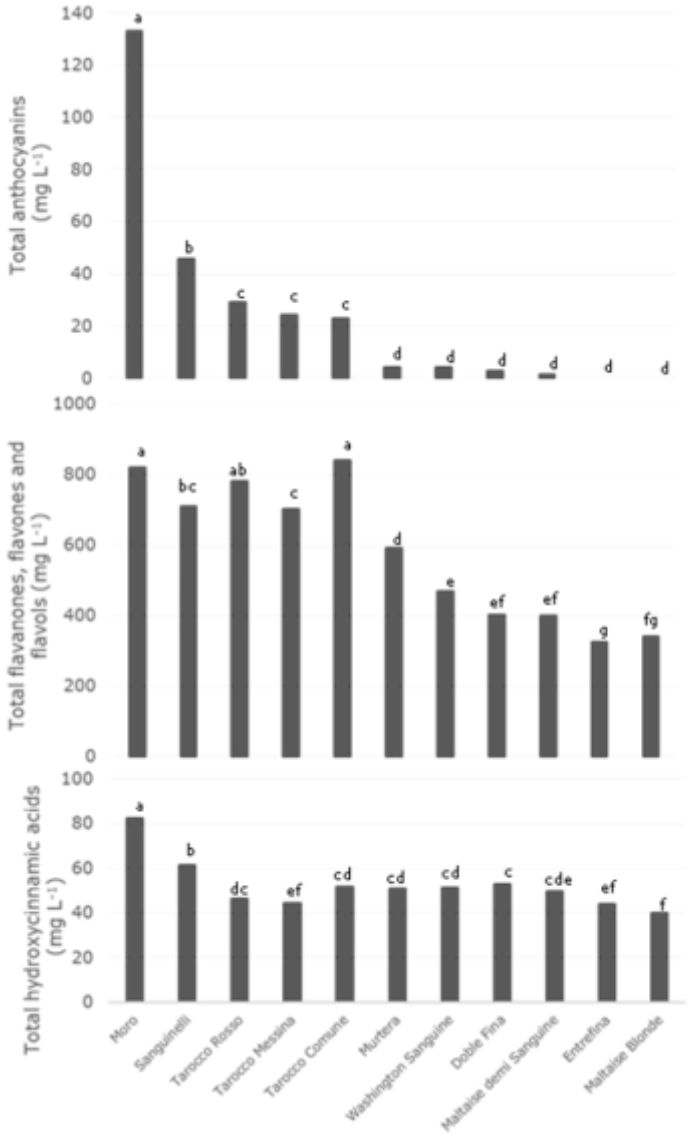

Figure 2.2. Content of anthocyanins, flavanones and flavones, and

hydroxycinnamic acids of the eleven blood orange juice

Data are the mean ± SD (n = 6). Values followed by the same letter, within the same row, are not significant different according to Fisher's LSD procedure at 95% confidence level.

Individual phenolics in blood orange juice

The different subclasses of phenolic compounds found in blood oranges juices are listed in Table 2.5. We identified eight phenolic acids, with p-coumaric acid as main compound in all cultivars; Moro (35.82 mg L^{-1}), and Sanguinelli (27.95 mg L^{-1}) were the juices richer in this compound. Concerning ferulic acid, the highest content was found again in Moro and Sanguinelli. Another major phenolic component was P-coumaroylquinic acid found in Moro (5.26 mg L^{-1}), Tarocco Rosso (4.06 mg L^{-1}), Maltaise Blonde (4.24 mg L^{-1}) and Tarocco Comune (3.94 mg L-1). The content of P-coumaroylquinic acid was lowest in Tarocco Messina (1.71 mg L^{-1}).

Among the flavonoids found in the eleven cultivars examined here, the predominant one was the hesperidin, a flavanone glycoside. In our results the highest values, greater than 500 mg L^{-1}, were found in Tarocco's clones Comune, followed by Moro and Sanguinelli. Entrefina, Maltaise Blonde, Maltaise demi Sanguine, Washington Sanguine and Doble Fina showed moderate values of hesperidin, in the range between 198 and 266 mg L^{-1}. Naringin, another flavanone glycoside, showed the lowest content in Maltaise Blonde and Doble Fina, and the highest in Moro and Sanguinelli.

Among all cultivars, the lowest juice concentrations of anthocyanins was found in Doble Fina, while Moro, which had the deepest red juice colour, had the highest contents of cyanidin-3-O-glucoside and cyanidin-3-(6"-malonyl)-glucoside.

The two-year mean of the entire set of parameters measured during the experiment was evaluated through a Principal Component Analysis (PCA). The first two principal components accounted for

68.39% of the total explained variance. PC1 (51.64% of variance explained) was highly correlated to cyanidin-3-O-glucoside, cyanidin-3-(6"-malonyl)-glucoside, naringin and rutin (Figure 2.3). PC2 (16.75% of variance explained) was positively correlated mostly to some hydroxycinnamic acids (ferulolyl hexose,), meanwhile the p-coumaric acid and ferulic acid were negatively correlated to the genotypes.

Considering the first two principal components, Moro was the cultivar which showed far the highest negative score (-7.2), related with anthocyanins, naringin and rutin, followed by Sanguinelli, Tarocco Rosso and Tarocco Comune (-2.0, -1.2 and -1.0, respectively). Conversely, Maltaise blonde and Entrefina showed the highest scores (3.0 and 2.8, respectively).

Table 2.5. Content (mg L^{-1} of juice) of individual phenolic subclasses in blood orange juice

Compounds	Peak no.	Rt (min)	MW	Doble Fina	Entrefina	Maltaise Blonde	Maltaise demi Sanguine	Moro	Murtera	Sanguinelli	Tarocco Comune	Tarocco Messina	Tarocco Rosso	Washington Sanguine
Hydroxycinnamic acids														
Feruloyl hexose	1	13.6	356	2.5 ± 0.04 e	4.68 ± 0.07 b	3.59 ± 0.02 c	4.88 ± 0.03 b	5.08 ± 0.21 b	2.85 ± 0.02 de	3.58 ± 0.13 c	4.92 ± 0.08 b	4.69 ± 0.01 b	6.11 ± 0.36 a	3.02 ± 0.06 d
Feruloyl quinic acid 1	2	14.7	368	2.65 ± 0.07 d	1.23 ± 0.05 e	1.61 ± 0.08 e	2.5 ± 0.07 d	4.99 ± 0.02 a	3.19 ± 0.03 cd	2.77 ± 0.01 d	3.58 ± 0.46 bc	3.02 ± 0.6 cd	4.14 ± 0.4 b	2.69 ± 0.05 d
P-coumaroyl quinic acid	3	16.0	338	3.46 ± 0.74 bc	3.14 ± 0.24 bcd	4.24 ± 1.33 ab	2.27 ± 0.18 cd	5.26 ± 0.25 a	2.25 ± 0.02 cd	3.26 ± 0.15 bc	3.94 ± 0.25 ab	1.71 ± 0.41 d	4.06 ± 0.18 ab	2.32 ± 0.18 cd
Feruloyl quinic acid 2	4	16.3	368	7.58 ± 1.06 cd	5.41 ± 0.74 e	6.99 ± 0.25 cde	9.56 ± 0.15 ab	8.52 ± 0.44 abc	8.02 ± 0.08 bcd	6.86 ± 0.16 de	9.89 ± 0.28 a	7.81 ± 0.86 cd	9.74 ± 0.61 a	7.32 ± 0.3 cd
Feruloyl quinic acid 3	5	17.9	368	3.24 ± 0.02 b	6.33 ± 0.5 a	2.42 ± 0.88 bc	1.16 ± 0.04 c	7.27 ± 0.22 a	1.88 ± 0.03 c	2.46 ± 0.17 bc	3.35 ± 1 b	1.64 ± 0.14 c	1.37 ± 0.14 c	1.92 ± 0.04 c
Sinapic acid	6	18.5	224	5.6 ± 0.25 cd	7.35 ± 0.46 b	4.62 ± 0.37 d	6.98 ± 0.01 bc	10.3 ± 1 a	4.72 ± 0.07 d	8.05 ± 0.35 b	7.23 ± 0.24 b	4.71 ± 1.09 d	7.72 ± 0.51 b	5.3 ± 0.07 d
P-coumaric acid	7	21.2	164	23.18 ± 1.05 c	14.78 ± 0.11 ef	14.43 ± 0.27 f	18.41 ± 0.27 d	35.82 ± 2.42 a	22.97 ± 0.14 c	27.95 ± 0.93 b	16.29 ± 1.42 def	17.84 ± 0.23 de	13.2 ± 0.98 f	25.31 ± 1.07 bc
Ferulic acid	8	21.4	194	7.01 ± 0.11 def	5.75 ± 0.13 ef	5.64 ± 0.26 f	8.65 ± 0.41 bc	10.25 ± 0.87 a	7.78 ± 0.21 cd	10.03 ± 0.37 ab	7.17 ± 0.19 de	7.59 ± 1.11 cd	6.09 ± 0.43 ef	6.53 ± 0.11 def
Flavanones, flavones and flavonols														
Vicenin 2	9	22.1	594	114.24 ± 2.47 b	66.17 ± 1.02 e	83.68 ± 1.27 c	72.68 ± 0.65 ed	120.38 ± 2.07 ab	116.8 ± 1.67 b	129.5 ± 0.75 a	117.32 ± 1.55 b	117.22 ± 10.72 b	80.63 ± 2.86 cd	128.62 ± 0.87 a
Isorhamnetin-3-O-rutinoside	10	31.0	624	2.37 ± 0.01 c	1.52 ± 0.04 d	1.61 ± 0.15 d	1.8 ± 0.16 d	3.12 ± 0.17 b	3.28 ± 0.04 b	2.74 ± 0.01 c	1.78 ± 0.24 d	3.75 ± 0.14 a	3.26 ± 0.11 b	2.5 ± 0.04 c
Naringin	11	30.2	580	43.8 ± 1.52 h	57.64 ± 1.51 fg	36.44 ± 0.19 h	55.85 ± 0.79 g	196.1 ± 2.36 a	85.89 ± 1.4 c	107.64 ± 5.09 b	85.46 ± 1.12 c	78.51 ± 4.68 cd	71.66 ± 6.21 ed	65.7 ± 1.85 ef
Rutin	12	27.2	610	4.07 ± 0.06 cd	2.28 ± 0.04 f	2.86 ± 0.01 e	2.77 ± 0.07 e	7.3 ± 0.1 a	4.24 ± 0.05 c	5.02 ± 0.12 b	3.89 ± 0.19 cd	3.92 ± 0.18 cd	4.76 ± 0.26 b	3.82 ± 0.02 d
Hesperidin	13	32.0	610	238.22 ± 8.02 d	198.34 ± 9.7 d	214.86 ± 2.15 d	65.05 ± 24.11 d	491.83 ± 62.1 b	380.18 ± 23.94 c	465.54 ± 11.26 b	633.17 ± 16.78 a	499.31 ± 8.12 b	621.67 ± 6.91 a	266.75 ± 23.9 d
Anthocyanins														
Cyanidin-3-O-glucoside	14	18.7	449	1.18 ± 0.1 d	0 ± 0 d	0 ± 0 d	0.49 ± 0.08 d	69.43 ± 2.17 a	1.41 ± 0.06 d	20.95 ± 0.99 b	8.68 ± 0.22 c	8.28 ± 1.48 c	10.21 ± 1.68 c	1.8 ± 0.05 d
Cyanidin-3-(6''-malonyl)-glucoside	15	22.7	535	1.65 ± 0.11 e	0 ± 0 e	0 ± 0 e	0.88 ± 0.13 e	63.47 ± 1.61 a	2.75 ± 0.06 e	24.63 ± 1.54 b	14.24 ± 0.01 d	16.11 ± 1.81 cd	18.84 ± 2.35 c	2.26 ± 0.01 e

Data are the mean ± SD (n = 6). Values followed by the lowercase same letter, within the same column, are not significant different according to Fisher's LSD procedure at 95% confidence level.

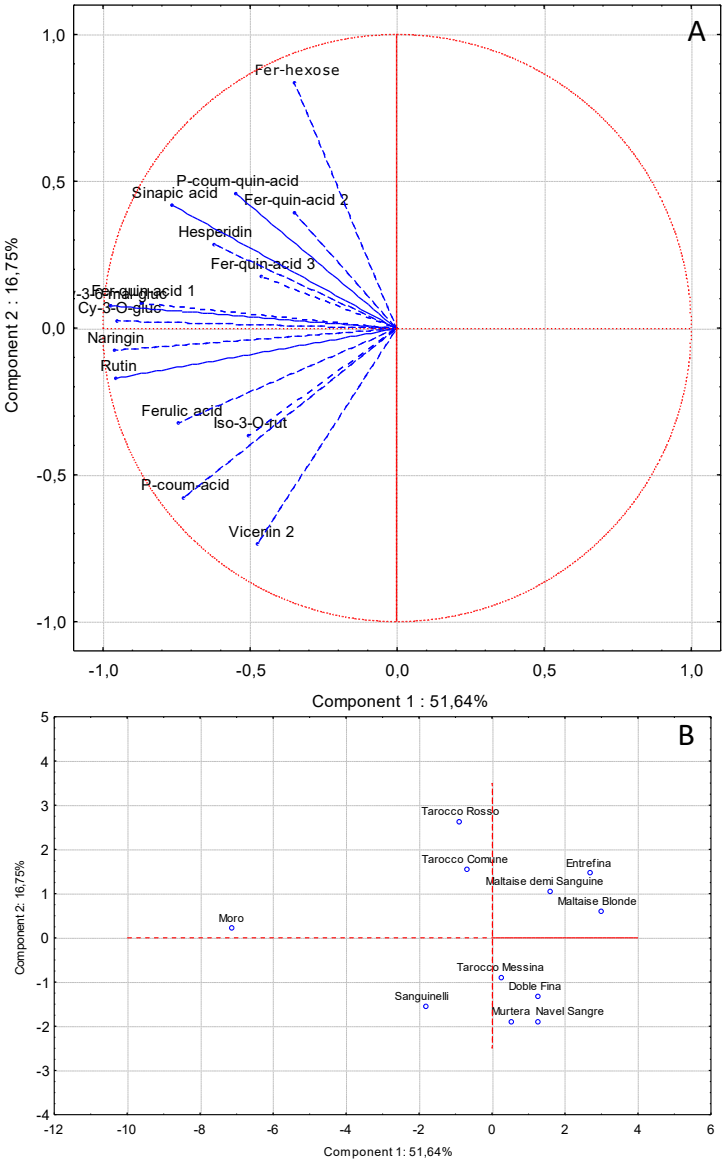

Figure 2.3. Principal component analysis of the individual phenolics in blood

orange juice of the eleven cultivars (average of two years). Data set: eleven cultivars (Doble Fina, Entrefina, Maltaise Blonde, Maltaise demi Sanguine, Moro, Murtera, Sanguinelli, Tarocco Comune, Tarocco Messina, Tarocco Rosso, Washington Sanguine); fifteen phenolic compounds (Hydroxycinnamic acids: Feruloyl hexose, Feruloyl quinic acid 1, P-coumaroyl quinic acid, Feruloyl quinic acid 2, Feruloyl quinic acid 3, Sinapic acid, P-coumaric acid, Ferulic acid. Flavanones, flavones and flavonols: Vicenin 2, Isorhamnetin-3-O-rutinoside, Naringin, Rutin, Hesperidin. Anthocyanins: Cyanidin-3-O-glucoside, Cyanidin-3-(6''-malonyl)-glucoside)

Discussion

Physicochemical properties

Considering fruit weight parameters, Tarocco clones confirmed to be among the ones with higher values (Caruso et al., 2016), with the exception of the old line Tarocco Comune that, indeed, is no longer cultivated in modern citriculture. Conversely, Sanguinelli is characterized by a small size of the fruit which limits its use for the fresh market, being instead preferred for processing (Ordonez-Diaz et al., 2020). Some of the cultivars were more or less isodiametric (e.g., Maltaise demi Sanguine, Tarocco Comune, Murtera and Entrefina) and others were more elongated. Moro had the thickest rind and, indeed, it is preferred for transport to distant markets for its greater resistance to mechanical damage (Caruso et al., 2016). Tarocco Comune and Tarocco Messina were the ones with the thinnest rind: this aligns with the easy peelability of the Tarocco cultivars (Rapisarda et al., 2000), a usual feature of mandarin fruits (Caruso et al., 2020).

The values of TSS and TA are the most relevant determinants of juice quality. We observed significant differences between the cultivars evaluated. Fruits of Tarocco Comune showed the highest TSS content (15.03 °Brix), while Tarocco Messina fruits, usually considered a late-maturing cultivar, recorded the lowest TSS (11.33 °Brix). The TSS values, of both blonde and red orange cultivars, are usually reported to range between 11 and 15 °Brix, depending on maturity, environmental conditions and agronomical practices (Legua et al., 2011; Lado et al., 2018). All the pigmented cultivars registered values of total acidity between 11 and 14 g citric acid L^{-1}, and the characteristic taste of the juice show a high acidity, markedly different from the blonde orange varieties. At physiological maturity, the latter normally reach values well below 10 g citric acid L^{-1} (Legua et al., 2011; Hervalejo et al., 2015), so ensuring sweetness.

This is confirmed by the TSS/TA ratio, an important indicator of commercial and sensory ripeness. This is used widely for citrus fruits, as it helps define their characteristic flavour. The European Commission determined the maturity requirements for orange fruits and, among these requirements, the minimum sugar/acid ratio for commercialisation is 6.5, regardless of cultivar, or of fruit type (blood or navel orange) (Anon, 2011). Here, the maturity indices of all the pigmented cultivars fell within the range 8.7 to 11.3, but did not achieve the higher values recorded in some blonde oranges (Legua et al., 2011; Hervalejo et al., 2015). This is because, during ripening, the acids decrease more slowly in the blood oranges than in the blonde ones (Rapirarda et al., 2000).

External and juice colour of blood oranges

The activation of the anthocyanin biosynthetic pathway in the rind and flesh of blood oranges is one of the major differences distinguishing these cultivars from blonde ones (Butelli et al., 2012). The deepness of the red colour is strongly attractive to consumers. Fruit pigmentation is also increasingly appreciated for its high nutraceutical value due to the presence of bioactive compounds (Dugo et al., 2003; De Pascual and Sanchez-Ballesta, 2008). Peel and flesh colour are not linked, so strong peel colour did not necessarily correspond to strong flesh colour, confirming previous results (Cebadera-Miranda et al., 2019). Evaluating H° and CCI values, the cultivars that showed the deepest red colour were Sanguinelli for the rind and Moro for the juice, accordingly with previous researches (Porras et al., 2014; Cebadera-Miranda et al., 2019; Morales et al., 2021).

Generally, observing all the colour parameters evaluated (L, a*, b*, C*, H°, CCI), the CCI seems to be most useful for distinguishing between these cultivars, more so than H° that is commonly used for

colour characterisation and considered an objective and reliable parameter to evaluate the maturity indices of citrus fruits (Rapisarda et al., 2000; Lado et al., 2014). Also, the external colour in red oranges, has been used widely as a good indicator of fruit maturity. Examining the values of both the peel and juice colours, it is evident that the intensity of the red colour is different as the anthocyanin biosynthesis pathways are not the same (Lado et al., 2014; Lo Cicero et al., 2016; Chaves-Silvaab et al., 2018). Furthermore, in the Tarocco clones, there is a frequent lack of correspondence between the intensities of the peel and flesh pigmentations (Rapisarda et al., 2000).

Organic acids and sugars

Accumulation of soluble sugars and decline in acid content (mainly citric acid) in blood oranges are typical changes taking place in the pulp during maturation of citrus fruits. The sweetness of an orange depends on the sugars, which are the major components of the juice. The organic acid composition is also important because it influences the sensory properties of the juice (Legua et al., 2014). Blood oranges are generally more acid than blonde ones at harvest (Rapisarda et al., 2000; Lado et al., 2018). The main organic acid found in the oranges under study was citric acid, followed by malic acid and ascorbic acids. Juices from Moro and Sanguinelli showed the highest acidic content, as broadly reported (Ordonez-Diaz et al., 2020; Morales et al., 2021).

The quality of a citrus fruit is expressed through the levels of a range of chemical compounds including vitamins, mineral elements and phenolics that have important antioxidant activity (Zhuo et al., 2016). Among the vitamins, vitamin C is calculated as ascorbic acid and it is present in high amounts in the fresh juice (Cebadera-Miranda et al., 2019). Blood orange is a rich source of vitamin C; indeed, levels in Moro and Tarocco have higher concentrations than many blonde

orange varieties, ranging from 0.50 to 0.80 g kg^{-1} of juice (Rapisarda et al., 1999). In our results values ranged from 0.35 to 0.50 g kg^{-1}, quite similar to what reported in previous researches (Ordonez-Diaz et al., 2020).

As mentioned, the sweetness of orange juice depends on the levels of the common sugars - sucrose, glucose and fructose. Taken together, these account for about 80% of the total soluble solids in orange juice and the ratio sucrose:glucose:fructose is generally about 2:1:1.38 The glucose:fructose ratio is a key indicator for determining the authenticity of citrus juices and it is generally higher than 0.85. In most other fruits, the level of glucose exceeds that of fructose but oranges contain glucose and fructose in nearly equal quantities and, sometimes, fructose can be slightly more concentrated than glucose as also found in our study (Kelebek et al., 2011). Our results were similar to, and consistent with, those reported by other authors (Sicari et al., 2016).

Polyphenols and total antioxidant activity

Citrus fruits are rich in phenolic acids and flavonoids, two major groups of natural antioxidants to which most of their functional properties are due. The variability in flavonoid composition among fruits is mainly attributable to genotype (Hunlun et al., 2019). These secondary metabolites participate in various functions in the plant; particularly in the fruit they are associated with colour, sensory characteristics (flavour, astringency, hardness), nutritional characteristics, and antioxidant activity (Simon-Grao et al., 2014). In our study, the total phenolic content ranged between 117.26 and 241.91 mg of gallic acid 100 g^{-1}. Even if Tarocco clones were the cultivars with the highest TPC content, generally our results were lower to those reported in previous researches (Rapisarda et al., 2009; Letaief et al., 2016).

Antioxidant activity of different foods has been thoroughly investigated due to their counteracting oxidation processes that prevent the chronic diseases related to oxidative stress in human body (Modica et al., 2021). Different types of antioxidant compounds, such as ascorbic acid, flavonoids and phenolic acids, were thought to be natural sources in horticultural products. For this reason, the ABTS assay was used to evaluate the antioxidant capacity of both lipophilic and hydrophilic antioxidants, including flavonoids (flavones, flavanones and flavonols) and phenolic acids (among all, ferulic acid and p-coumaric acid).[43] Previous researches on blood oranges assessed that the higher amount of anthocyanins play an important role on the antioxidant activity of the juice (Ordonez-Diaz et al., 2020; Reche et al., 2021).

Hydrophilic antioxidant activity was far more prevalent than lipophilic antioxidant activity, as reported in other researches (Kelebek et al., 2008; Legua et al., 2013). In accordance with previous results,[48] Moro showed the highest level, followed by Sanguinelli; contrarily, other studies40 reported higher levels in Tarocco's clones than in Moro.

Total anthocyanins, total hydroxycinnamic and phenolic composition

Anthocyanins synthetized and accumulated in blood oranges impart their distinctive purple-red colouration to both the peel and juice (Lo Piero, 2015; Morales et al., 2021). In line with other studies (Rapisarda et al., 2009; Pereira-Caro et al., 2014), we found the highest level of total anthocyanins in Moro, followed by Sanguinelli. No significant differences were observed between the Tarocco cultivars, while lower levels were found in the remaining cultivars. The different levels of these pigments recorded in this study confirm their accumulation process is genetically driven rather than

environmental, as all the abiotic conditions (the aerial and soil microenvironments) were identical in our collection area.

Oranges and other citrus fruits have high concentrations of phenolics and flavonoids, mainly linked to the flavanones group (Rapisarda et al., 1998). The total phenolic composition in citrus fruit may range from about 300 to 1200 mg L^{-1}, and depends on the species, growing season, ripening, and environmental factors (Kelebek et al., 2011).

In the present study, the total phenolic compound ranged between 326 and 842 and the highest content of total flavanones, flavones and flavonols was in Moro, followed by Tarocco comune, as reported in other works (Rapisarda et al., 1999; Fallico et al., 2017).

Previous studies51 showed hydroxycinnamic acids were distinct for the blood orange cultivars, being much more abundant in the blood than in the blonde orange juices. We found Moro had the greatest amount of total hydroxycinnamic acids, which falls in line with several authors (Pereira-Caro et al., 2014; Morales et al., 2021), with decreasing concentrations in Sanguinelli.

Individual phenolics compounds

The citrus species contain multiple classes of polyphenols, each possessing a spectrum of chemical behaviours and bioactivities. The hydroxycinnamic acids represent an important group of compounds that derive from the general phenylpropanoid pathway (Morales et al., 2021). In this work, eight phenolic acids were assessed, and p-coumaric acid was the main hydroxycinnamic acid identified in all cultivars, followed by ferulic and sinapic acid. These data are in contrast to what reported in literature (Gattuso et al., 2007; Fallico et al., 2017), where ferulic or chlorogenic (Morales et al., 2021) acids were found to be the most dominant hydroxycinnamic acid in some blood orange cultivars. In our study, the highest content of ferulic acid

was found in Moro and Sanguinelli. P-coumaroylquinic acid was found in high amount in Moro and Tarocco's clones, with the exception of Tarocco Messina. This may have been because the latter is a very late-maturing cultivar.

The presence of naringin and hesperidin in blood oranges has been found by previous studies (Fallico et al., 2017; Morales et al., 2021). The predominant flavonoid found in the eleven cultivars here evaluated was hesperidin, in line with previous researches (Barreca et al., 2014; Barreca et al., 2016; Cebadera-Miranda et al., 2019). In a previous investigation on blood oranges (Arena et al., 2001), Sanguinello and Tarocco presented a similar profile to the one obtained in the current study, in which Tarocco showed greater amount than Moro. Naringin is confirmed to be present in higher amount in Moro to respect to Tarocco cultivars (Morales et al., 2021). Anthocyanin content, as mentioned, is related to internal fruit colour. As previously reported (Rapisarda et al., 2009), cyanidin 3-glucoside and cyanidin 3-(6''-malonylglucoside) were predominant in blood orange, especially in Moro. We found the lowest concentrations of anthocyanins in juices from Doble Fina, Entrefina, Maltaise Blonde, Maltaise demi Sanguine, Murtera and Washington Sanguine, while Moro, as expected, had the highest contents of cyanidin-3-O-glucoside and cyanidin-3-(6"-malonyl)-glucoside. The content of all these phenolic compounds is well known to depend strongly on both species and cultivar (Ballistreri et al., 2013; Todaro et al., 2016; Reche et al., 2021).

Previous researches (Kelebek et al., 2008; Reche et al., 2021) observed a positive trend for the increase of most bioactive compounds in blood orange, such as organic acids (ascorbic acid, malic acid, and citric acid), phenolic compounds (didymin, narirutin, and vicenin-2), anthocyanin compounds (cyanidin 3-O-glucoside and cyanidin 3-O-(6''-acetyl) glucoside) and antioxidant activity.

The PCA analysis permitted to highlight the influence of the genotype on the biosynthesis of the anthocyanins and of the other phenolics. Being Moro and Sanguinelli the varieties with the highest content of phenols, the results herein obtained point out the strong influence of the cultivars on the classes of polyphenols studied.

Conclusions

Blood oranges are an excellent source of natural antioxidants and bioactive compounds such as phenols, flavonoids, anthocyanins and ascorbic acid. In Spain, their cultivation represents only a relatively small part of total citrus production. Nevertheless, consumer interest in blood oranges is increasing because of their perceived valuable nutraceutical properties. Hence, a nutraceutical characterisation of the main blood orange cultivars is timely, especially one that compares the autochthonous Spanish cultivars with several others that are more widespread elsewhere in the Mediterranean basin. In this study we analysed the colour of both the peel and the juice, making comparisons using different colour index parameters. Moro was the darkest red orange cultivar, followed by Sanguinelli. Maturity index was another important parameter related to the qualitative characteristics of citrus fruit and is linked to fruit ripeness. Tarocco Rosso had the highest value due to its low citric acid content. Results show that the juice of Moro has the greatest levels of total flavonoids, total phenolics and total anthocyanins, followed by Sanguinelli.

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Chapter 3 - Influence of rootstock genotype on individual metabolic responses and antioxidant potential of blood orange cv. Tarocco Scirè

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Abstract

In fruit crops, tree rootstock plays a relevant role in tolerance of biotic and abiotic factors, various qualitative traits and biochemical profiles of the juice. In this work, the fruit quality characteristics of sweet orange Tarocco Sciré grafted onto ten rootstocks were evaluated. Analyses were performed on the fruit juice, coupling the quantification of phenolic compounds (anthocyanins, flavanones, flavones and hydroxycinnamic acids) through hyphenated chromatographic techniques and the assessment of antioxidant potential (measured in vitro). The study was conducted for two consecutive years in which the different minimum temperatures recorded during fruit ripening determined a higher accumulation of

polyphenols, especially anthocyanins (63.6%), and higher levels of antioxidant activity (48.92) in the colder year. The analyses allowed the identification and quantification of 24 phenolic compounds (6 anthocyanins, 5 flavanones, 1 flavone and 12 hydroxycinnamic acids). The results highlighted a significant effect of both rootstocks and the environment (and their interaction) on the metabolic profile of the juice, with low temperatures being directly involved in juice pigmentation and significant differences among the rootstocks within each year of analysis. In addition to temperature, a significant effect of rootstocks on the metabolic profile of the juice was revealed, with C35 and Bitters being the most effective in determining anthocyanin accumulation in the fruit of the grafted variety.

Altogether, the data presented shed light on the significant role exerted by the rootstock on fruit quality, providing useful insights to guide the selection of rootstocks in novel implants.

Introduction

Citrus fruits are highly appreciated for their unique flavour (both in terms of taste and aroma), as well as for their high nutraceutical value, by consumers worldwide (Pannitteri et al., 2017). Citrus production takes advantage of the agronomical practice of grafting, often used to confer higher tolerance to biotic and/or abiotic stress to the scion. An increasing wealth of data highlights the strong influence of rootstocks on traits of economic relevance, such as fruit yield, fruit size, juice quality, fruit maturation and postharvest performance (Bowman et al., 2016; Forner et al., 2020; Rodriguez-Gamir et al., 2010). Citrus production has historically relied on the use of sour orange (Citrus aurantium L.) as rootstock due to its high resistance to several biotic stresses (especially root rot), abiotic stresses (including drought, salinity, calcareous soils, and mineral deficiency), and good productivity in a wide range of pedoclimatic

conditions (Moreno et al., 2008). Mediterranean citriculture is seriously threatened by the spread of citrus tristeza virus (CTV) (Davino et al., 2003) because sour orange shows high susceptibility to the virus. Considering this, new plantings have been established, using mostly citranges [*Citrus sinensis* (L.) Osb × *Poncirus trifoliata* (L.) Raf.] and other *Poncirus*-derived intergeneric hybrids (i.e., 'Swingle' citrumelo) that are tolerant to CTV. However, these rootstocks are susceptible to various environmental and abiotic stress conditions and/or pathogens, and the search for new rootstocks is important in many citrus-producing countries.

Citrus fruits are rich in phenolic compounds in significant quantities. Polyphenols are plant secondary metabolites derived from the shikimate and acetate pathways. The term polyphenol includes several subclasses of molecules that are grouped according to their structure as flavones, flavanones, anthocyanins, stilbenes, chalcones, coumarins, tannins, lignans, and many others (Dewick, 2002). These compounds play pivotal roles in plants, as they may act as phytoalexins, antioxidants, and antifeedants and are involved in plant pigmentation, reproductive processes and protection from UV light. As a component of the food matrix, polyphenols affect the colour, sensory, and nutritional properties of fruits and vegetables and exert a primary role in protection against several degenerative pathologies in humans (Manach et al., 2004; Heimler et al., 2017). Moreover, polyphenol content in food is remarkably influenced by genetic and environmental factors such as climate, soil, and agronomic practices (Siracusa and Ruberto, 2014), including rootstock selection (Continella et al., 2018; Caruso et al., 2020). In this context, the accumulation of anthocyanin in several blood oranges has been investigated (Rapisarda and Giuffrida, 1994; Butelli et al., 2012; Incesu et al., 2013; Lo Piero, 2015; Caruso et al., 2016); very recently, Morales et al. (2021a) pointed out the strong influence of 8 different

rootstocks on phenolics (four anthocyanins, three flavanones and five hydroxycinnamic acids) in the juices of Moro and Tarocco Rosso oranges.

Although the antioxidant potential of citrus species has been extensively investigated (Zhuo et al., 2016; Cömert et Gökmen, 2017), few examples have been reported regarding the effect of rootstocks on the antioxidant activity of blood oranges. Recently, Ordonez et al. (2020) analysed the impact of four different citrus rootstocks on the antioxidant activity of Salustiana and Sanguinelli cultivars. The aim of the present work was to investigate the role of rootstock in influencing polyphenol biosynthesis and accumulation in citrus fruit. We performed evaluations over two harvest years to consider the effects of autumn and winter temperature patterns, the metabolic profile and the antioxidant potential (DPPH and ABTS) of a pigmented sweet orange variety, Tarocco Scirè, grafted on ten different rootstocks, including some recently released rootstocks (Federici et al., 2009) and others that are currently widely used in the Mediterranean basin.

Material and methods

Plant material

The experimental field was established in 2010 in Catania, south Italy (37°17'04.3"N; 14°53'17.3"E; 57 a.s.l.) as a complete randomized block with ten replications per rootstock. Tree spacing was 5 x 3 m, and plants were subjected to standard cultural practices: pruning every two years, drip irrigation system, fertilizer formula NPK 2–1–1.5, and integrated weed management.

The analyses were performed on Tarocco Scirè sweet orange. This variety was grafted onto ten different rootstocks: Bitters (C22), Carpenter (C54) and Furr (C57) citrandarins (hybrids of *Sunki mandarin* × *Swingle trifoliate* orange released by the University of

California Riverside in 2009), F6P12® and F6P13 (hybrids of Citrus latipes and *Poncirus trifoliata* released by CREA-ACM in 2014), Troyer, Carrizo and C35 citranges, Swingle citrumelo, and Severinia [*Severinia buxifolia* (Poir.) Ten.].

Sampling

Fifteen fruits per plant were collected at commercial maturity (first half of March) in 2017 (year I) and 2018 (year II) from three trees per rootstock (Fig. 3.1). The experimental plot consisted of ten trees per rootstock, which were divided into three biological replicates, each including 3 or 4 trees. We collected 30 fruits from the 4 cardinal points for each biological replicate. Then, three juice samples obtained from the pooled juice of 30 fruits per replicate were used for chemical analyses. Juice was extracted using a domestic squeezer (Citrus Juicer JE290, Kenwood, UK), filtered before analysis, and then used for chromatographic analyses. The juice was stored at -80 °C in 50 mL disposable plastic centrifuge tubes until use.

Chemicals

All solvents and reagents used in this study were high purity laboratory solvents from VWR (Milan, Italy); HPLC grade water and acetonitrile were also obtained from VWR. Cyanidin 3-O-glucoside, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid and sinapic acid were purchased from Sigma (Sigma–Aldrich., Milan, Italy), while eriocitrin, neoeriocitrin, narirutin, hesperidin, didymin and vitexin were purchased from Extrasynthese (Lyon, France).

ABTS+ [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] and DPPH (2,2-diphenyl-1-picrylhydrazyl) standards were purchased from Sigma (Sigma–Aldrich., Milan, Italy).

Identification and quantification of Tarocco Scirè biochemical markers via HPLC/DAD and HPLC/ESI/MS analyses

Flavonoids (anthocyanins, flavanones, flavones) and hydroxycinnamic acid derivatives were determined in Tarocco Scirè juice obtained from the different grafting combinations.

Small juice samples (2 mL) were placed in 15 mL plastic sample tubes, and 100 µL of formic acid (98%) was added. Samples were sonicated (DU-32, Argolab) for 5 min and then centrifuged (5417R Eppendorf, Osterode, Germany) at 4000 rpm for 15 min to separate the solid portion of the juices. One of the clear supernatants was transferred into 2 mL HPLC amber vials and immediately analysed. Chromatographic analyses were carried out on an Ultimate3000 UHPLC-focused instrument equipped with a binary high-pressure pump, a photodiode array detector, a thermostatted column compartment and an automated sample injector (Thermo Fisher Scientific, Inc., Milan, Italy). Collected data were processed through a Chromeleon Chromatography Information Management System v. 6.80. Chromatographic runs were all carried out using a reverse-phase column (Gemini C18, 250 x 4.6 mm, 5 µm particle size, Phenomenex Italia s.r.l., Bologna, Italy) equipped with a guard column (Gemini C18 4 x 3.0 mm, 5 µm particle size, Phenomenex Italia s.r.l., Bologna, Italy). Tarocco juice polyphenols were eluted with the following gradient of B (2,5% formic acid in acetonitrile) in A (2,5% formic acid in water): 0 min: 10% B; 20 min: 35% B; 25 min: 10% B. The solvent flow rate was 1 mL/min, the temperature was kept at 25 °C, and the injector volume selected was 40 µL. DAD acquisitions were all performed according to Pannitteri et al. (2017).

To unambiguously identify the chromatographic signals and/or to confirm peak assignments, a series of HPLC/ESI/MS analyses were performed on a selected number of representative samples. In this case, aliquots (5 mL) of the centrifuged juices were freeze dried

(Lyoquest-85, Telstar Italy, Legnano, Milan, Italy), redissolved in 2 mL of HPLC grade water and transferred into 2 mL HPLC amber vials ready for ESI/MS analyses. Chromatographic analyses were performed using the same conditions described above, while ESI mass spectra were acquired by a Thermo Scientific Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Inc., Milan, Italy) using a heated electrospray ionization (HESI II) interface. LC/ESI/MS settings and mass spectra acquisition were conducted according to our previous work (Pannitteri et al., 2017). All analyses were carried out in triplicate; the results are reported in milligrams (mg) of compound per litre (L) of juice.

Antioxidant activity ($ABTS^+$ and $DPPH^•$ methods) determination

The antioxidant activity was determined by two different methods: $ABTS^+$ [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] according to Re et al., 1999 and the DPPH radical method (2,2-diphenyl-1-picrylhydrazyl) according to Brand-Williams et al., 1995. The ABTS+ and DPPH• methods were used only for 9 rootstocks since trees grafted on *Severinia buxifolia* produced small amounts of fruits. A methanol extract was prepared using 1 mL of each sample juice sample mixed with 10 mL of MeOH/water (80:20, v/v) +1% HCl, and the mixture was sonicated at 20 °C for 15 min and left for 24 h at 4 °C. Then, the extract was again sonicated for 15 min and centrifuged at 15000 rpm for 10 min. The radical scavenging activity was evaluated using the $DPPH^•$ radical (2,2-diphenyl-1-picrylhydrazyl) method and the $ABTS^+$ [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation method. The decrease in absorbance of all samples was measured in a UV-visible spectrophotometer (Helios Gamma model, UVG 1002E; Helios, Cambridge, UK) at 515 nm and 730 nm for $DPPH^•$ and $ABTS^+$,

respectively. A calibration curve was generated with Trolox ((R)-(+)-6-hydroxy-2,5,7,8-tetramethyl-croman-2-carboxylic acid) (0 to 20 nmol) from Sigma (Madrid, Spain), and the results were expressed as mmol of Trolox equivalent per kg of fresh weight (mmol TE kg-1 FW).

Statistical analysis

Analysis of variance (ANOVA) was carried out using the 'stat' package of R software. A basic descriptive statistical analysis was followed by an analysis of variance test for mean comparisons. The method used to discriminate among the means (multiple range test) was Fisher's least significant difference (LSD) procedure at a 95.0% confidence level. Principal component analysis (PCA) was calculated using the 'prcomp' function embedded in the 'stat' R package. PCA results were displayed using the R package 'factoextra'.

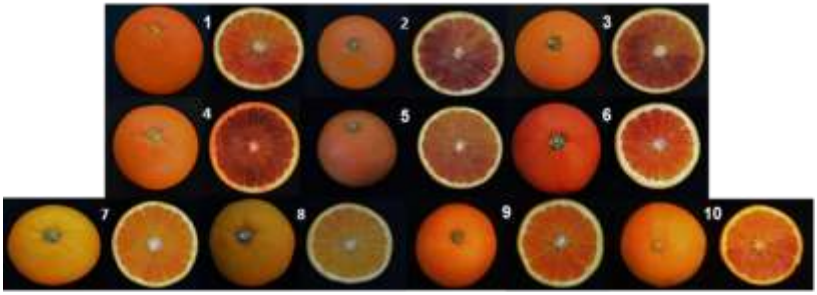

Figure 3.1. Fruits of Tarocco Sciré cultivar harvested at maturity grafted onto 10 rootstocks. From left to right: Carrizo citrange (1), Troyer citrange (2), C35 (3), Bitters (4), Carpenter (5), Furr (6), Swingle citrumelo (7), Severinia buxifolia (8), F6P12 (9), F6P13 (10)

Results and discussion

Tarocco Scirè metabolic profile characterization

Peak assignments were performed by evaluating the UV-vis and mass spectral data collected; metabolite identification was confirmed with coinjections of pure analytical standards when available and corroborated with existing literature reports (Fabroni et al., 2016). Fig. S3.1 and Table 3.1 report the results obtained: of a total of 24 compounds identified, five metabolites, marked F1-F5, belong to the subclass of flavanones; compounds F6 belong to flavones, compounds C1-C12 belong to the subclass of hydroxycinnamic acids, and six compounds belong to the subclass of anthocyanins (A1-A6). Flavanones are considered Citrus genus markers, and in sweet orange, they almost entirely occur as eriodictyol and naringenin glycosides, with glycosylation taking place at position 7 either by rutinose or neohesperidose moiety (Siracusa and Ruberto, 2014). Hesperidin (hesperetin 7-O- rutinoside) and narirutin (naringenin 7-O- rutinoside) are broadly reported as the main flavanones present in orange juices (Barreca et al., 2016; Rapisarda et al., 2009); our results agreed with the literature, as we found hesperidin (F4) as the main compound in all the samples analysed (see Fig. S3.2), followed by narirutin (F3) and dydimin (F5). Eriocitrin and its isomer neoeriocitrin (F1 and F2, respectively) were the other flavanones detected, even in smaller amounts. Regarding hydroxycinnamic acids and their derivatives, similar to what was reported in Pannitteri et al. (2017), we tentatively identified and quantified two caffeic acid derivatives (caffeoyl-hexose C1 and chlorogenic acid C5), four p-coumaric acid-based metabolites (C2, C4, C7 including the free acid C11) and free sinapic acid C10. Ferulic acid is undoubtedly the most represented hydroxycinnamic acid with four different derivatives (hexoside C3, quinic acid derivatives C6, C8 and C9, and finally the free form C12). The C-

glycosylated flavone vitexin (F6) has been identified as the sole representative of its subclass. The presence of anthocyanins is a particular feature of blood orange cultivars that has been extensively reported (Hillebrand et al., 2004); in Tarocco Scirè, six different pigments A1-A6 were detected (Table 3.1 and Fig. S3.1), and more specifically, two delphinidin derivatives (A1, A3) and a peonidin derivative A6 were detected. The cyanidin nucleus dominated the anthocyanin profiles with three derivatives A2, A4 and A5; A4 (cyanidin 3-O-(6‘’malonyl) glucoside) was the main compound detected within its subclass in all the samples analysed. Anthocyanin profiles correspond to those described by other authors in Tarocco orange (Liang et al., 2011; Barreca et al., 2016). Cebadera-Miranda et al. (2019) identified seven cyanidin derivatives and three delphinidin derivatives. The most frequent anthocyanin compounds recognized in blood orange were the same as those observed in this work (A1-A6) plus cyanidin 3-O-sophoroside (Dugo, et al., 2003; Fabroni et al., 2016; Hillebrand et al., 2004; Kelebek et al., 2008).

Table 3.1. Peak list and diagnostics, as obtained through HPLC/DAD and HPLC/ESI-MS analyses, for Tarocco Scirè orange juice biochemical markers. Peak letters and numbers refer to Figure S1

Compound code	Rt, min[a]	Compound identification	λmax, nm[b]	MW	MS (ESI^+/ESI^-) data, m/z[c]
Anthocyanins - 520 nm					
A1	7.00	delphinidin 3-O-glucoside	524, 320sh, 278	465	465 $(M)^{+}$*
A2	7.92	cyanidin 3-O-glucoside[d]	515, 278	449	449 $(M)^{+}$, 287*
A3	9.62	delphinidin 3-O-(6"- malonyl)glucoside	520, 328, 284	551	551 $(M)^{+}$*
A4	10.68	cyanidin 3-O-(6"- malonyl)glucoside	517, 330, 279	535	535 $(M)^{+}$, 449*, 287
A5	11.16	cyanidin 3-O-(6"- dioxalyl)glucoside	517, 278	593	593 $(M)^{+}$, 449*, 287
A6	12.69	peonidin 3-O-(6"- malonyl)glucoside	518, 330, 278	549	549 $(M)^{+}$, 463*, 301
Flavanones and flavones - 280 nm					
F1	12.08	eriocitrin[d]	328, 283	596	595 $(M-H)^{-}$
F2	13.97	neoeriocitrin[d]	328, 284	596	595 $(M-H)^{-}$
F3	15.41	narirutin[d]	329, 284	580	579 $(M-H)^{-}$,433*, 271
F4	16.57	hesperidin[d]	326, 284	610	609 $(M-H)^{-}$ *, 463, 301
F5	21.43	didymin[d]	328,283	594	593 $(M-H)^{-}$
F6	10.19	vitexin[d]	338, 270	432	431 $(M-H)^{-}$ *,311
Hydroxycinnamic acids - 330 nm					
C1	5.43	caffeoyl-hexose	328, 300sh	342	341 $(M-H)^{-}$*, 179
C2	6.04	p-coumaroylquinic acid 1[e]	312	338	337 $(M-H)^{-}$*, 191
C3	6.73	feruloyl-hexose	326, 300sh	356	355 $(M-H)^{-}$
C4	7.17	p-coumaroylquinic acid 2[e]	312	338	337 $(M-H)^{-}$, 191*
C5	8.05	chlorogenic (3- caffeoylquinic) acid[d]	325, 300sh	354	353 $(M-H)^{-}$*, 191
C6	8.67	feruloylquinic acid 1[e]	323, 300sh	368	367 $(M-H)^{-}$, 191*
C7	8.85	p-coumaroylquinic acid 3[e]	312	338	337 $(M-H)^{-}$, 191*
C8	9.62	feruloylquinic acid 2[e]	322, 300sh	368	367 $(M-H)^{-}$*, 191
C9	10.02	feruloylquinic acid 3[e]	323, 300sh	368	367 $(M-H)^{-}$, 191*
C10	12.31	sinapic acid[d]	323	224	223 $(M-H)^{-}$
C11	14.26	p-coumaric acid [d]	311	164	163 $(M-H)^{-}$
C12	15.23	ferulic acid [d]	323, 298sh	194	193 $(M-H)^{-}$

[a] as average of 10 rootstocks x 3 replicates x 2 years = 60 analytical measurements; [b] from HPLC; [c] main peaks marked with an asterisk; [d] co-injection with pure analytical standards; [e] correct isomer not determined

Total and individual metabolic changes in Tarocco Scirè juice depending on the rootstock genotype

The total anthocyanin contents (mg/L) of the different Tarocco Scirè/rootstock combinations in year I are reported in Table 3.2. Anthocyanin content largely varied among rootstock genotypes, with C35 being the most performant (15.7 mg anthocyanins/L juice), followed by Furr and Bitters (14.6 mg/L and 14.3 mg/L, respectively); F6P13, Troyer and Carpenter, even with lower contents (12.4, 12.0 and 11.8, respectively), were not significantly dissimilar from the previous genotypes. Overall, anthocyanins showed a higher variability than the other compound classes investigated. The variability of anthocyanin content with rootstock genotype is rather high, with C35 being the most performant rootstock among those tested (15.7 mg anthocyanins/L juice), followed by Furr and Bitters (14.6 mg/L and 14.3 mg/L, respectively). Morales et al. (2021a) observed high anthocyanin contents in Moro in combination with C35 during the first and second harvests occurring in early and late February, detecting a marked decline at the third harvest in late March.

In our study, based only on the anthocyanin profile, the rootstock/scion combination with the lowest pigment content was indeed that with *Severinia buxifolia* (2.2 mg anthocyanins per litre). In contrast, Morales et al. (2021a) observed the lowest anthocyanin contents in Tarocco Rosso blood orange grafted onto C35, Citrus macrophylla and Swingle citrumelo.

The rootstock effect on secondary metabolic profiles has been investigated in different fruit tree crops. Tavarini et al. (2011) demonstrated that preharvest factors such as rootstock genotype may influence peach fruit quality, especially in terms of anthocyanin amount. Regarding grapes, Rezazad Bari et al. (2021) and Rumbaugh et al. (2021) detected a significant influence of rootstocks on the content of grape polyphenols, particularly anthocyanins.

As shown in Fig. S3.3, the contents of individual anthocyanins displayed almost the same variations towards all of the rootstocks used, with the sole difference being values corresponding to rootstock Carpenter. Interestingly, this rootstock differentiates anthocyanins A1 and A3 (delphinidin derivatives) from compounds A2, A4 and A5 (cyanidin derivatives). A behaviour similar to that registered for delphinidin derivatives was observed for metabolite A6, the sole peonidin-based pigment detected (see Fig. S3.3). Previous works present in the literature on different matrices reported dissimilarities in cyanidin- and delphinidin-based pigments depending on geographical variations (Lätti et al., 2008); this phenomenon is biochemically supported, as delphinidin and cyanidin, albeit originating from the same biosynthetic route, seem to differentiate in the B-ring, the precursor coming from the shikimate pathway (see Fig. S3.4).

Unlike anthocyanins, flavonoids F1-F6 and hydroxycinnamic acids C1-C12 did not show substantial changes among the Tarocco Scirè/rootstock combinations (Table 3.2). The total flavonoid content peaked at 174.7 mg/L juice with the genotype Bitters, followed by Carpenter (166.8 mg/L) and C35 (159.4 mg/L), while the lowest value was registered for rootstock genotype citrumelo (125.5 mg/L). When considering the individual metabolite responses within this subclass, a difference emerged between eriodictyol-based flavanones (F1, F2 and F4) and narigenin-based metabolites F3 and F5, and a similar rootstock-dependent trend was observed (Fig. S3.2). The study of the biosynthetic pathway of these compounds again offered a possible explanation for the differences registered; in fact, as observed for anthocyanins, narigenin and eriodictyol present slight differences in their biosynthesis, evidenced again in their B-ring derived from p-coumaroyl-CoA and caffeoyl-CoA, respectively (Fig. S3.4). Accordingly, the influence of the rootstock on some flavonoids, such

as naringin, hesperidin, and neohesperidin, was assessed in some blood orange cultivars (Hammati et al., 2018). Flavone vitexin (F6) was indeed the least variant compound among flavonoids (Fig. S3.2). Hydroxycinnamic acid derivative contents ranged from 86.4 mg/L (lowest value, genotype Carrizo) to 123 mg/L (rootstock genotype F6P13, Table 3.2); this rootstock generated a peak in the contents of all hydroxycinnamic acids involved excluding sinapic acid C10 (Fig. S3.5). As already mentioned, no significant rootstock-driven differences were observed within this subclass, with the sole exception of rootstock F6P13 (see Fig. S3.5).

Recent studies have identified and characterized several structural genes that are involved in the process of biosynthesis of metabolites. Anthocyanin biosynthetic genes are regulated by a complex system of transcription that was thoroughly studied by Butelli et al. (2012) and Lo Piero (2015). Little is known about the effects of rootstock at the molecular level; however, recent advancements have shown that the use of a rootstock can affect scion gene regulation (Liu et al., 2017). Moreover, it seems that scion gene expression might be induced by the movement of proteins and small RNA through the grafting point (Tzarfati et al., 2013; Wu et al., 2019).

Table 3.2. Content (mg L^{-1}) of Tarocco Scirè juice anthocyanins, flavanones and flavones and hydroxycinnamic acids measured on fruits on different rootstocks in year I and II. P value resulting from the two-way analysis of variance (ANOVA) considering rootstock, year and their interaction as fixed effect

rootstock genotype	Total anthocyanins (mg L^{-1})			Total flavanones/flavones (mg L^{-1})			Total hydroxycinnamic acids (mg L^{-1})		
	year I[a]	year II	variation %	year I	year II	variation %	year I	year II	variation %
Carrizo	8.1 bc	4.8 abcd	−40.0	132 de	186 bc	40.6	86.4 b	82.6 b	−4.5
Troyer	12.0 ab	6.7 a	−45.0	154 abcd	204 b	32.5	87.2 b	94.0 b	−3.2
Ferr	14.6 a	8.1 ab	−45.1	144 cde	176 cde	22.6	93.9 b	77.0 b	−17.1
Citrumelo	8.4 bc	1.7 ef	−82.0	126 e	160 e	27.2	91.5 b	85.6 b	−6.5
Carpenter	11.8 ab	4.0 cdefg	−66.1	167 ab	183 bc	11.2	96.3 b	86.2 b	−10.5
PdP13	12.4 ab	3.6 cdef	−70.6	135 de	186 cd	37.3	123 a	90.8 a	−26.2
S. bonifacio	2.2 d	0.7 f	−69.4	133 de	275 a	107.3	94.6 b	83.6 b	−11.7
PdP12	6.9 cd	3.6 bcde	−39.0	151 bcd	191 bc	26.7	88.8 b	84.0 b	−5.4
CiS	15.7 a	3.2 def	−85.7	150 abc	173 de	0.0	92.6 b	78.7 b	−15.1
Bitters	14.3 a	6.0 ab	−58.5	179 a	174 cde	−2.4	91.5 b	33.0 b	−6.0
mean	10.7 ± 4.2	3.7 ± 1.9	−63.6	145 ± 16	191 ± 32	31.4	95.6 ± 10.2	84.6 ± 4.9	−10.9
ANOVA factors	*p value*								
rootstock	*< 0.0001*			*< 0.0001*			*< 0.0001*		
year	*< 0.0001*			*< 0.0001*			*< 0.0001*		
*rootstock*year*	*0.002*			*< 0.0001*			*0.007*		

[a] Values followed by the same letter, within the same column, are not significant different according to Fisher's Least Significant Difference (LSD) procedure at 95.0 % confidence level.

Metabolic changes in the Tarocco Scirè/rootstock combinations over two years of cultivation

The variations in anthocyanins, flavanones and flavones, and hydroxycinnamic acids over two years are reported in Table 3.2. For all three classes of metabolites, significant differences between years and among the rootstocks tested were detected. The significance of the rootstock effect ranged from 1.3-8 (total flavanones/flavones) to 9.33-7 (total anthocyanins), while the year effect showed a p value ranging from 2.4-6 (total hydroxycinnamic acids) to values lower than 2-16 (total flavanones/flavones). When the interaction of rootstock and year was investigated, the p value ranged from 6.1-11 (total flavanones/flavones) to 0.007 (total hydroxycinnamic acids). As observed for rootstock genotype, total anthocyanin content was greatly affected by minimum temperature, as explained below in the text; their relative values showed significant differences between the two years of observation, being significantly reduced in the second year: a 63.6% mean decrease with a maximum value for the rootstock

genotype C35 (85.7%) and a minimum for F6P12, for which a 39.8% decrease was observed.

As already mentioned, there are many induction factors in anthocyanin biosynthesis and accumulation, including environmental factors such as photoinduction, osmotic induction, deficiencies in nitrogen and phosphorous, low pH, wounding, and pathogen infections (Chalker-Scott, 1999). In particular, for blood orange varieties, it is known that a wide day-night thermal range is required to maximize colour formation (Maccarone et al., 1983; Butelli et al., 2012; Lo Piero, 2015). Furthermore, several authors shed light on the role of temperature in the activation of anthocyanin biosynthesis and their accumulation in both the peel and pulp postharvest, which could be relevant to the effects of temperature observed before harvest (Pannitteri et al., 2017; Fabroni et al., 2020). It has been reported that storage at either 4 or 8 °C is a viable option to increase anthocyanin content in blood orange fruit (Rapisarda et al., 2001; Crifò et al., 2012). Recently, Carmona et al. (2017) showed that storage at 9 °C is more effective than storage at 4 °C in enhancing anthocyanin production and thus fruit colour in blood oranges.

In our study, there was a different temperature regime in the field. In particular, according to analysis of the air temperatures (T) recorded during the ripening period (1 December−15 March), the hours below 9 °C did not differ significantly among years (941 in 2016-17 vs. 865 in 2017-18). Nevertheless, year 1 registered a higher frequency of hours with temperatures below 6 °C compared to the second year (454 vs. 259, respectively). The second year was characterized by high temperatures during the monitored period, which likely determined a severe fall in anthocyanin accumulation for all rootstocks studied compared to the first year in which cooler climatic conditions occurred, determining higher pigmentation levels, in agreement with what was observed by Continella et al. (2018).

Regarding individual pigment contributions, in year II, anthocyanins A1-A6 decreased in all rootstocks, and no difference emerged between their profiles (Fig. S3.6). Based on what was observed, it appears that differences in accumulation for different anthocyanins are increased when a wide day-night thermal range occurs during autumn and winter, with more effective minimum temperatures below 6 °C. These findings further support the generally accepted assumption that pigment biosynthesis and accumulation, performed by extremely complex enzymatic networks, is a multiple-step process (Lo Piero, 2015; Carmona et al., 2019).

The hydroxycinnamic acid derivative content was rather stable over the two years (see Table 3.2 and individual contributions in supplementary material, Fig. S3.7). In contrast to what was observed for anthocyanins, a general increase in flavonoids was observed; a mean value of 31.4% over 10 rootstock genotypes with a maximum obtained for Severinia (107% increase) and a minimum registered for genotype C35 (8.7%), the sole exception being genotype Bitters which maintained almost the same value (Table 3.2). Regarding individual flavonoids, also in year II, a similar trend with respect to rootstock genotype was observed for naringenin-based metabolites F3 and F5, with flavone vitexin (F6) showing the lowest variation in the two years (Fig. S3.8). The different trend observed between anthocyanin and colourless flavonoid contents is in accordance with the findings of Crifó et al. (2012) and Lo Piero (2015).

Determination of antioxidant potential of different Tarocco Scirè/rootstock combinations

Bioactive compounds and antioxidant activity in citrus have been previously investigated during preharvest (Di Matteo et al., 2021), processing (Lo Scalzo et al., 2004) and storage (Habibi et al., 2020). It is well known that the use of rootstocks markedly influences

the antioxidant activity of citrus fruits (Aguilar-Hernández et al., 2020; Morales et al., 2021b), but few studies have been reported on blood orange in combination with rootstocks (Ordonez-Diaz et al., 2020).

Furthermore, different components in plant extracts contribute unequally to their total antioxidant ability; in fact, the antioxidant capacity depends on plant extracts and their chemical composition (Zou et al., 2016).

DPPH measurements showed a wide variability in the antioxidant activity between rootstocks with Furr/Tarocco Scirè and Swingle citrumelo/Tarocco Scirè characterized by the highest and lowest values, respectively, in the first year (3.59 mmol and 1,42 TE kg^{-1} FW) (Table 3.3). In the second year, the antioxidant activity of the orange juice sample decreased, except for Carpenter and Swingle citrumelo, both of which showed similar values between the years. $ABTS^{+}$ analyses were remarkably affected by the rootstock in the first year. Troyer/Tarocco Sciré showed the highest value (4.27 mmol TE kg^{-1} FW), followed by Furr (3.83 mmol TE kg^{-1} FW) and F6P13 (3.46 mmol TE kg-1 FW) (Table 3.3). On the other hand, Swingle citrumelo/Tarocco Sciré showed the lowest value, even if it was not significantly different from that of Carrizo, Bitters and C35. During the second year, antioxidant activity decreased in all rootstocks, with values ranging from 1.12 (Furr) to 1.40 (F6P12) mmol TE kg^{-1} FW, and no significant differences among combinations were detected (Table 3.3).

When the interaction of rootstock and year was investigated, the p value was significant for both $ABTS^{+}$ (<2-16) and $DPPH^{\bullet}$ (4.29-16).

Antioxidant activity was related to the fact that higher concentrations of both anthocyanins and hydroxycinnamic acids were assessed in the first year of observation. Indeed, the decrease in antioxidant activity was dependent on many factors, including

environmental conditions. Differences in the scion/rootstock interaction were observed in antioxidant activity in different species (Forcada et al., 2019; Ordóñez-Díaz et al., 2020; Aguilar-Hernández et al., 2020). The different methods for determining the antioxidant properties of the juice were incomparable, as already highlighted in previous studies (Zou et al., 2016); however, both methodologies are considered reliable for the determination of antioxidant potential, and under our conditions, the influence of the different rootstocks on the biosynthesis and accumulation of antioxidant-related compounds was clearly evident.

Principal component analysis (PCA)

To further dissect the differences among the rootstocks tested in terms of the production of metabolites, a multifactorial analysis was conducted on the two years of the data. For the phenotyping carried out in 2017 (harvest year I) (Fig. 3.2A), the first two principal components explained 64.5% of the cumulative phenotypic variability (Dim1 = 34.9%, Dim2 = 29.6%). While Dim1 was mainly associated with the quantity of metabolites produced (with Dim1 > 0 associated with lower production as observed for F6P12, Citrumelo and Carrizo, Table 3.2), Dim2 allowed a more precise differentiation according to the different classes of metabolites. Rootstocks characterized by high production of anthocyanins, such as C35, Bitters, Furr and Carpenter, clustered in the upper-left quadrant (Dim1<0 and Dim2 > 0), while samples showing higher synthesis of hydroxycinnamic acids were plotted in the lower-left quadrant (Dim1<0 and Dim2 < 0). A more complex pattern was observed for the loading projections of the flavanones and flavones, with vitexin and eriocitrin pointing towards the left and down, respectively, and the remaining four pointing towards the upper part of the graph (Fig. 3.2 A, Table 3.2). For the analysis performed in 2018 (harvest year II), the first two principal

components explained 64% of the total phenotypic variability. Similar to what was observed in 2017, accessions showing high accumulation of metabolites were characterized by negative Dim1 values (Fig. 3.2B and Table 3.2). Moreover, in the second year, the loadings related to the anthocyanins showed high consistency, with all projected towards the same PCA quadrant (lower-left), while flavanones, flavones, and hydroxycinnamic acids pointed mainly towards the upper-left quadrant (Fig. 3.2B). An exception is represented by eriocitrin, which is characterized by an orthogonal projection compared to the other five flavanones and flavones. The high positive correlation among the six anthocyanin components was further confirmed by the heatmap depicted in Fig. 3.3, with correlations ranging from 0.91 (delphinidin 3-O-glucoside and peonidin 3-O-(6”- malonyl) glucoside) to 0.99 (delphinidin 3-O-(6”- malonyl) glucoside and cyanidin 3-O-(6”- malonyl) glucoside). Fig. 3.3 shows the occurrence of a negative correlation between anthocyanins and most flavanones, flavones, and hydroxycinnamic acids.

Table 3.3. Content (mmol TE kg^{-1} FW) of $DPPH^{\bullet}$ and $ABTS^{+}$ measured in Tarocco Sciré juices from fruits grown on different rootstocks in year I and II. P value resulting from the two-way analysis of variance (ANOVA) considering rootstock, year and their interaction as fixed effect

	DPPH (mmol TE kg^{-1} FW)			ABTS$^{\cdot-}$ (mmol TE kg^{-1} FW)		
Rootstock genotype	year I	year II	variation %	year I	year II	variation %
Bitter	2.63 a	1.70 ab	-36.45	2.16 fg	1.38 a	-36.17
C35	2.37 d	1.81 a	-23.72	2.23 f	1.32 a	-41.43
Carpentin	1.62 I	1.69 ab	4.06	2.54 e	1.36 a	-47.30
Carrizo	2.24 de	1.64 ab	-26.89	2.08 gh	1.39 a	-33.12
Citrumelo	1.46 g	1.47 b	0.04	1.94 h	1.35 a	-30.44
RP113	3.20 a	1.66 ab	-24.50	2.27 d	1.40 a	-51.34
RP153	3.43 b	1.76 ab	-48.41	3.46 c	1.37 a	-60.35
Ruzi	3.59 a	1.60 ab	-53.41	3.53 b	1.12 a	-70.67
Troyer	2.10 e	1.79 ab	-14.93	4.27 a	1.39 a	-66.48
mean	2.23 ± 1.04	1.68 ± 0.10	34.24 ± 20.24	2.83 ± 0.84	1.33 ± 0.08	-46.92 ± 15.18
ANOVA factors	*p value*					
rootstock	< 0.0001			< 0.0001		
year	< 0.0001			< 0.0001		
rootstock * year	< 0.0001			< 0.0001		

Values followed by the same letter, within the same column, are not significant different according to Fisher's Least Significant Difference (LSD) procedure at 95.0 % confidence level.

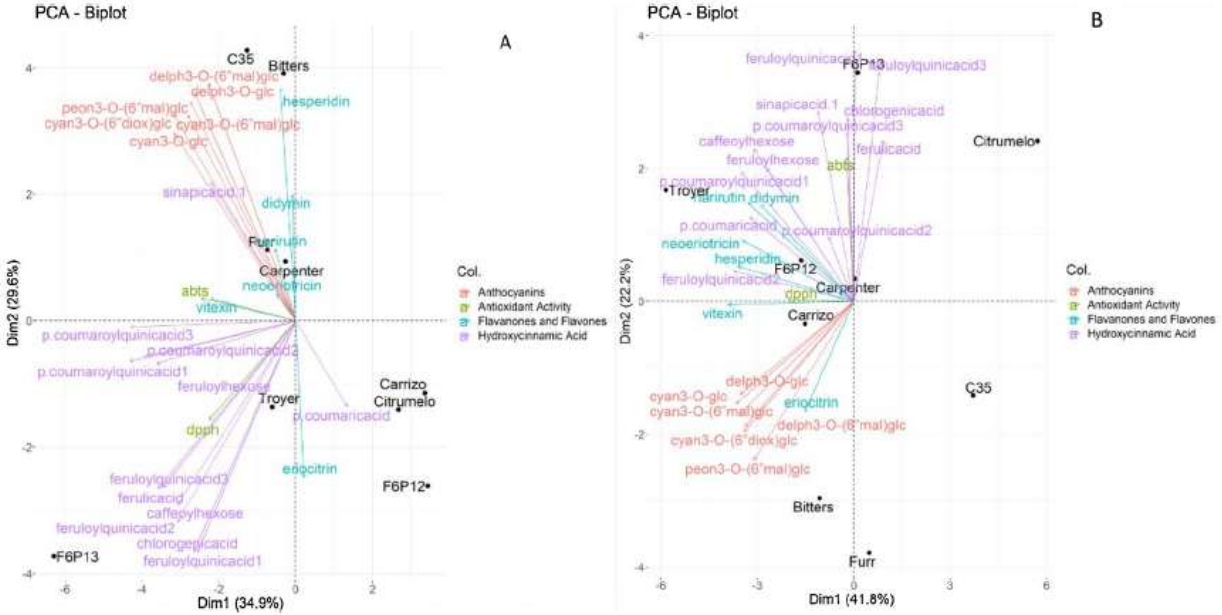

Figure 3.2. Principal component analysis (PCA) of the 26 parameters analysed in year I (A) and year II (B). Phenol compounds, antioxidant activity and rootstocks are coloured as categories as specified in the figure legend

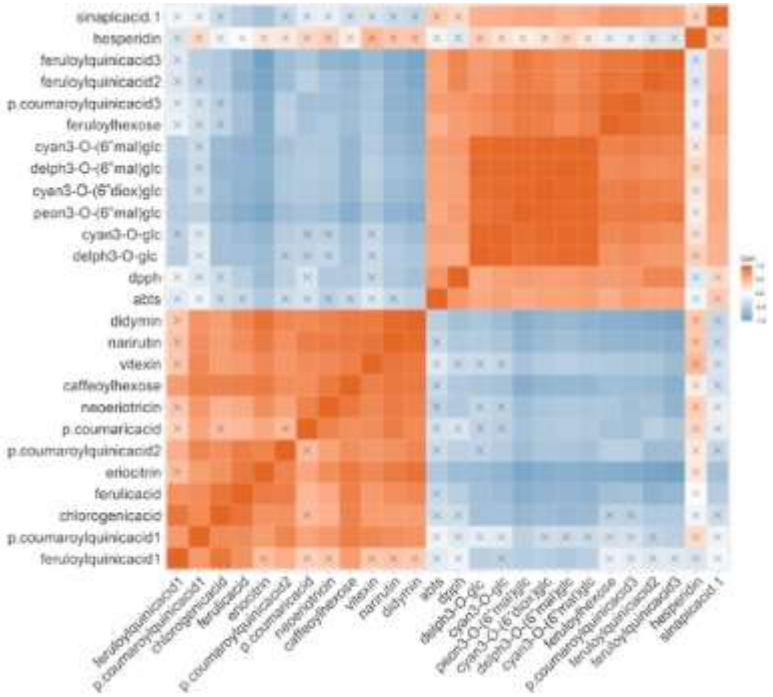

Figure 3.3. Heatmap of the pairwise correlations between individual phenolic compounds and antioxidant activity (26 traits in total). Colors reflect the correlation level between two traits ranging from dark red (positive correlation) to white (no correlation) and dark blue (negative correlation). Correlations values exceeding the significance threshold level (p value > 0.05) were crossed (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conclusions

In this work, individual metabolite variations in Tarocco Scirè pigmented orange were investigated as a function of the rootstock genotype used and in relation to the minimum temperature changes over two years; the results showed significant differences in accumulation both between subclasses and within each compound, and such differences can be ascribed to biosynthetic factors. The effect of the environment and, specifically, of the low temperatures, on juice pigmentation has been confirmed: in fact, total anthocyanin content estimation revealed a substantial difference between the two years of study, with a double-fold increase in the colder year. As expected, the different methods used to determine the antioxidant potential of Tarocco Sciré juice gave incomparable results but provided a reliable indication of the behaviour of the different rootstocks/scion combinations. More data seem to be necessary to achieve unequivocal interpretation of the antioxidant activity of these products.

Overall, the rootstocks C35, Bitters, Carpenter and Furr were the most interesting for pigmented oranges under the tested conditions, as they positively enhanced fruit pulp anthocyanin content. On the other hand, some other rootstocks were considered unsatisfactory for further evaluation because their effect on qualitative fruit parameters was substandard.

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Chapter 4 - Molecular Insights into the Effects of Rootstocks on Maturation of Blood Oranges

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Abstract

Rootstock choice has important effects on the horticultural and pathological traits of the citrus cultivars. Thus, the scion/rootstock combination can affect tree vigour, nutrition, and stress resistance; it can also have positive influences on the fruit quality traits. Although the study of rootstock effects has been a relevant research topic in citrus for many years, the main body of such study has been conducted at the biochemical level, while little effort has been directed to the determination of the rootstock influences at the molecular level. A comparative study of three combinations of scion and rootstock shows a positive correlation between the regulation of the fruit quality-related genes and the accumulations of bioactive compounds, as well

as with acid degradation. Monitoring the anthocyanin accumulation during ripening shows the scion/rootstock combination can increase anthocyanin synthesis in the fruit, as well as vitamin C accumulation and acid degradation. Our results show that the rootstock genotype can exert important influences on citrus fruit quality by affecting gene expression in the scion. New insights into the molecular interactions between scion and rootstock may help unravel the systems through which rootstocks exert their influences on the regulatory networks in the scion, so as to influence relevant agronomic traits. This information should result in an improved rootstock breeding selection and definition of scion/rootstock combinations to enhance fruit quality traits.

Introduction

Although a very ancient practise, grafting remains a widespread and almost indispensable technique in modern fruit culture. The use of rootstocks offers several advantages, such as better adaptation to limiting soil conditions, improved tolerance to temperature and water stress, and, especially, improved resistance/tolerance to pests and diseases. The relationship between rootstock and the scion can strongly modify the performance of the whole plant combination (Gainza et al., 2015). The main fruit quality traits are highly dependent on the scion genotype, even though some of them can also be strongly influenced by rootstock genotype. Thus, a particular scion, in combination with different rootstocks, can lead to very different results, in terms of fruit size, shape, pigmentation, juice content, and soluble solid concentration (Forner-Giner et al., 2003). For this reason, the rootstock choice is a key first step in the development of a productive and profitable orchard (Castle et al., 1995). Although, it is well known that some rootstocks can provide fruits of better internal quality than others, the current understanding suggests the rootstock

influences on fruit flavour depend on specific interactions established between a particular scion cultivar and the rootstock itself (Lin et al., 2015). Evidence for the influence of rootstock on fruit qualitative traits in the Citrus species have been investigated extensively (Legua et al., 2014; Cano-Lamadrid et al., 2018). Thus, the trifoliate orange rootstocks (*Poncirus trifoliata*L. Raf.) enhances fruit quality and anthocyanin content, while the Carrizo citrange rootstock [*C. sinensis* (L.) Osb. cv. Washington navel *P. trifoliata* (L.) Raf.] (widespread in the USA and Europe) confer good internal qualitative traits. The *C. volkameriana* rootstock produces very vigorous and productive trees, but the fruit quality is poor. Meanwhile, Swingle citrumelo [*C. paradisi* (Macfadyen) x *P. trifoliata* (L.) Raf.] is highly productive but induces low fruit acidity and performs poorly in heavy and calcareous soils (Forner-Giner et al., 2020). Fruit colour is the first characteristic perceived by consumers and, therefore, is one of the key factors influencing a fruit's aesthetic value and market appreciation. In the case of blood oranges, the red pigments are also associated with perceived health benefits. The changes in colour that take place during ripening are related to the synthesis and degradation of three main classes of compounds: chlorophylls, carotenoids, and anthocyanins (Rodrigo et al., 2013).

Chlorophylls are the predominant pigments in the green skin of an unripe citrus fruit, while the yellow–orange colour, typical of a mature fruit, is due to the carotenoids. The synthesis of anthocyanins is limited to the blood orange group and its hybrids, which are responsible for the purple–red colour of both their flesh and skin (Rodrigo et al., 2013; Lo Piero, 2015). The pigmentation is regulated by a number of factors, including environmental conditions, cultivation practices, water, and nutrient availability; it also depends on both the maturity of fruit (Morales et al., 2020) and can be affected by the postharvest storage temperature (Carmona et al., 2020). As in

other foods, due to their antioxidant properties, anthocyanins protect from oxidative stress and help prevent cardiovascular disease and cancer (Grosso et al., 2013; Pojer et al., 2013).

The use of different citrus rootstocks can significantly influence the timing of fruit development, ripening, and harvesting of pigmented fruits, as well as modifying their final chemical composition (Caruso et al., 2020). Ordóñez-Díaz et al. (2020) drew attention to the strong influence of rootstock on the composition of some bioactive compounds developed during fruit maturation in the cvs. Salustiana and Tarocco. The maturation process influences total phenol content and increases the concentrations of anthocyanins at harvest. Indeed, the increase in bioactive compounds during maturation has also been observed inblood oranges (Morales et al., 2020; Cebadera-Miranda et al., 2019). Most of the genes encoding the enzymes that are involved in anthocyanin synthesis have been identified and characterised in a range of species, including in citrus species. Phenylalanine ammonia-lyase (PAL) is considered a key gene in anthocyanin metabolism because phenylalanine is the main precursor of these pigments. The first enzyme of the anthocyanin synthetic pathway is chalcone synthase (CHS), which is also involved in the production of other phenols. In the next steps, dihydroflavonol 4-reductase (DFR) reduces the dihydroflavonols to leucoanthocyanidins; these colourless compounds are then transformed into coloured anthocyanins by anthocyanidin synthase (ANS). The last step of synthesis is driven by UDPglucose-flavonoid glucosyl transferase (UFGT), which increases the stability and water solubility of the recently produced anthocyanins by adding a glucose molecule in the 3-OH position. The anthocyanin synthesis genes are regulated by a complex system of transcription factors, referred to as the MBW complex, of which Ruby is the R2R3 MYB activator in citrus fruits (Butelli et al., 2012; Lo Piero, 2015). The taste of citrus flesh is highly dependent on its sugar

and acid content and, in particular, on the ratio between total soluble solids (TSS) and titratable acidity (TA), which has a strong effect on the perception of sweetness and sourness (Benjamin et al., 2013). This is the reason the ratio TSS/TA is considered one of the most important fruit quality traits and the most common parameter used to define the ripening index in some citrus fruit, such as oranges or mandarins. In citrus spp., sugars accumulate in juice sacs during maturation, while organic acids are gradually degraded. Blood oranges are generally more acidic than blonde ones at harvest. This can mean that they do not reach an adequate sugar/acid ratio when fully ripe, with a consequent loss of consumer acceptance. For this reason, the control of fruit acidity is of considerable economic relevance (Terol et al., 2010; Lado et al., 2014). Recent studies have identified and characterised a number of structural genes involved in the synthesis and catabolism of organic acids in citrus flesh. Among these, citrate synthase (CS) participates in the synthesis of citrate, while isocitrate dehydrogenase (IDH) and ATP citrate lyase (ACL) encode the enzymes involved in citrate utilization (Guo et al., 2016). The transcription of the sucrose phosphate synthase (SPS) gene has been pointed out as one of the major factors regulating sucrose synthesis and storage in mature fruit. The enzyme produced by this gene catalyses the conversion of fructose-6-phosphate and UDP glucose to sucrose-6-phosphate and is responsible for sucrose synthesis in plants (Komatsu et al., 1999; Langenkamper et al., 2002).

Citrus fruits are a great source of vitamin C for humans, who are not able to synthesise it themselves and, thus, are obliged to meet their requirement through a daily dietary intake. L-ascorbic acid (AsA) is produced in large amounts in plants, where it protects tissues from oxidative damage, related to numerous biotic and abiotic stresses (Giovannoni et al., 2007). Due to its free-radical scavenging activity and role as a cofactor in many chemical reactions, AsA is also an

essential component of the human diet. The reaction catalysed by the enzyme encoded by GDP-L-galactose phosphorylase (GGP) is considered the first committed step of the main pathway, as well as the most important of AsA synthesis. A second key gene in AsA synthesis is D-galacturonate reductase (GalUR), since it regulates one of the alternative routes for AsA synthesis [27]. Blood orange is a rich source of vitamin C; indeed, the Tarocco clones have higher concentrations than many blonde orange varieties, ranging from 500 to 800 mg/L of juice (Arena et al., 2001; Rapisarda et al., 2008), similar to those observed in the Moro and Sanguinello cultivars (Rapisarda et al., 1999). Nevertheless, it is still unclear how rootstocks can exert their influences on fruit quality parameters, such as anthocyanins, sugars, acids, and vitamin C. In this study we attempt to determine if any rootstock effects are related to changes in the expressions of the key quality trait genes in the scion. With this aim, we carried out metabolic studies and gene expression analyses on fruits of the blood orange selection (*C. sinensis* (L.) Osb. cv Tarocco Sciré), grafted on to one of three different rootstocks.

Materials and Methods

Plant Material

Fruits of Tarocco Sciré sweet orange were harvested from nine-year-old trees, grafted on one of three different rootstocks: (1) Carrizo citrange (CAR) [*C. sinensis* (L.) Osb. cv. Washington navel x *P. trifoliata* (L.) Raf.], (2) Bitters (BIT), or (3) Furr (FUR) [*C. sunki* Hort. ex Tan. *P. trifoliata* (L.) Raf.]. Plants were cultivated in an experimental field, located in Lentini (37 17004" N, 14 53016" E, Syracuse, Italy), and subjected to standard cultural practice. Three biological replicates for each of the scion/rootstock combinations were selected in a randomised block design. Samples were taken every 30 d at mid-month, from November 2018 to March 2019, at five maturity

stages: 184 days after full bloom (DAFB), 214, 245, 276, and 304 DAFB. These timings ranged from the onset of ripening to the fully mature stage (Figure 4.1A). For each block, 28 randomised fruits were collected from 9 trees and quickly transferred to the laboratory. Fruit juice was extracted with a commercial juice extractor (Kenwood Citrus Juicer JE290, Havant, UK), filtered, quick-frozen in liquid nitrogen, and stored at -80 °C until processing.

Biochemical Analysis

HPLC/DAD and HPLC/ESI/MS analyses were used to quantify total anthocyanin content (TAC) for each scion/rootstock combination. All solvents and reagents used were high purity laboratory solvents from VWR (Milan, Italy); HPLC grade water and acetonitrile were also obtained from VWR. Cyanidin 3-O- glucoside was purchased from Sigma (Sigma-Aldrich, Milan, Italy). Small samples (2 mL) of the juices were placed in 15 mL plastic sample tubes, and 100 L of formic acid (98%) was added. Samples were sonicated for 5 min, then centrifuged (5417R Eppendorf, Osterode, Germany) at 800 g force for 15 min to separate the solid portion of the juice. Next, 1 mL of the clear supernatant was transferred to a 2 mL HPLC amber vial and immediately analyzed. Chromatographic analyses were carried out on an Ultimate3000 UHPLC focused instrument (equipped with a binary high-pressure pump), photodiode array detector, thermostatted column compartment, and automated sample injector (Thermo Fisher Scientific, Inc., Milan, Italy). The chromatographic column, elution program, and DAD acquisition parameters used were the same as previously reported (Pannitteri et al., 2017). Collected data were processed through a Chromeleon Chromatography Information Management System v. 6.80. The results are expressed as mg of cyanidin-3-glucoside equivalents per litre. The TSS content was measured using a digital refractometer

(Atago Co., Ltd., model PR-32, Tokyo, Japan) and expressed as Brix. According to the AOAC method (AOAC, 1995), the TA was determined by potentiometric titration (Hach, TitraLab AT1000 Series) of the juice using 0.1 N NaOH at pH over 8.1, results are expressed as g L^{-1} of citric acid equivalent. Vitamin C (L-ascorbic acid, AsA) was determined using an automatic titration apparatus (702 SM Titrino, Metrohm, Herisau, Switzerland) with 0.001 M I2, and the results are expressed as mg L^{-1}. Ripening index (RI) represents the ratio between TSS and TA. Juice colour was recorded with a Minolta CR-400 chroma meter (Minolta Corp., Osaka, Japan), as described by Caruso et al. (2020). The results were expressed as citrus colour index (CCI = a*1000/L*b), a maturation index widely used in the citrus industry (DOGV, 2006).

Gene Expression Analysis

The plant material used for total RNA extraction was the same as that used for metabolic study. Total RNA was isolated from 3 mL of juice, as described by Butelli and colleagues (Butelli et al., 2019), and subsequently treated with RNAse-free DNAase (DNase treatment and removal, Ambion, Madrid, Spain). Total RNA concentration and purity were assessed using a spectrophotometer (NanoDrop-2000, Thermo Scientific, Wilmington, NC, USA). The absence of DNA was checked by gel electrophoresis; cDNA was synthesised from 1-g of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Inchinnan, UK), following the procedure indicated by the manufacturer. Quantitative real-time polymerase chain reaction (qPCR) was carried out on a Rotor- Gene Q thermocycler (Qiagen, Hilden, Germany) in 10 uL total reaction volume containing 1 x PCR buffer II, 2 mM MgCl2, 0.2 mM dNTPs, 0.3 M of forward and reverse primers (Table 4.1) (Life Technologies, Inchinnan, UK), 1.5 M SYTO9 (Life Technologies, Inchinnan, UK),

20 ng of cDNA, and 1U of MyTaq DNA polymerase (Bioline, London, UK). Thermal cycling conditions included a pre-incubation at 95 C for 5 min, followed by 35 cycles at 95 °C for 10 s for denaturation and 60 °C for 60 s for a single annealing-elongation step. The DDCt method was used to normalise the raw Ct data, the elongation factor 1-alpha (EF-1) was chosen as reference gene, while CAR gene expression of November was taken as calibrator. The results are the average of three independent biological sample replicates.

The qPCR Data and Statistical Analysis

The outputs of the different analyses were processed and visualised using R software (R core team, Foundation for Statistical Computing 2016, Vienna, Austria). The bar plots were produced through the base package. The qPCR data were normalised using the 'HTqPCR' package (Dvinge et al., 2009) and the outputs were visualised using the 'heatmap30 package (Zhao et al., 2014). The ANOVA tests were conducted with the 'aov' function of the base package, and a Tukey test was applied to the results. Samples had previously been submitted to the Shapiro–Wilk test to check the normality of their distribution. Significant differences in metabolic data were represented on plots by the letters a, b, and c (p value < 0.01).

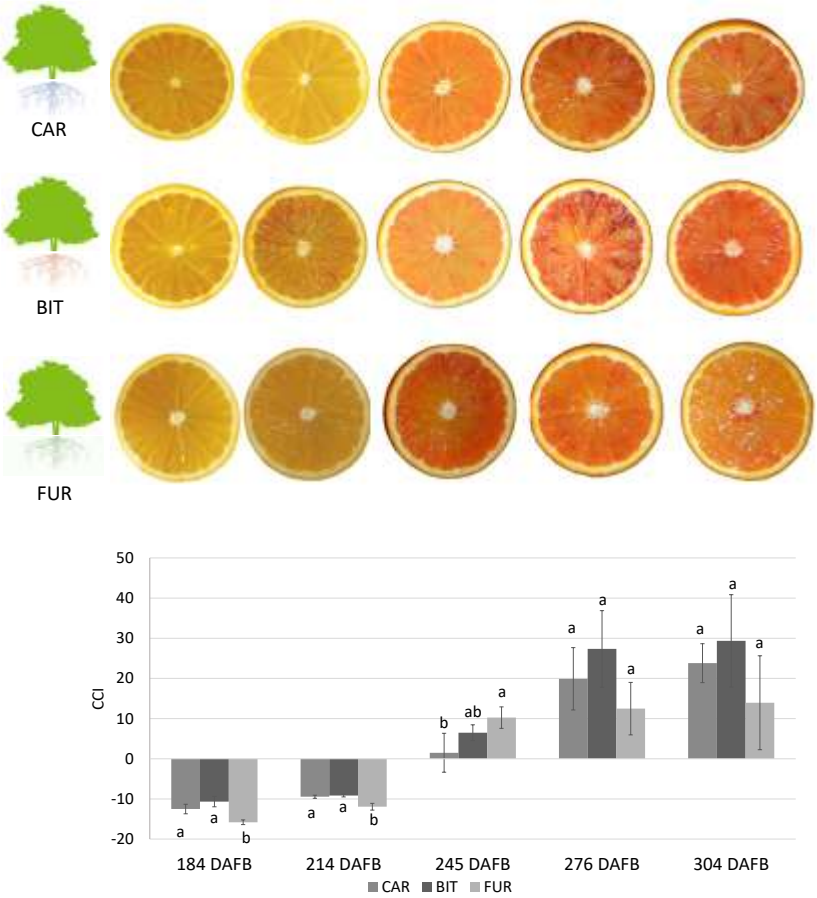

Figure 4.1. (A) Flesh colour of the sweet orange Tarocco Scirè grafted on the rootstocks Carrizo (CAR), Bitters (BIT) and Furr (FUR) during the different harvest dates (DAFB: days after full bloom). (B) Citrus Colour index (CCI) of Tarocco Scirè juice measured at the different harvest dates (n= 84: mean values of 28 fruits x 3 replicates). Vertical bars indicate standard deviation. Statistically significant differences by the two-way analysis of variance (P value <0.01) are represented by different letters

Table 4.1. List of the primers used for qPCR analysis

Accession number	Gene name	Forward 5'-3'	Reverse 5'-3'	Reference
XM_006481431.3	PAL	GATTTGAGACATTTGGAGGA	ATGGATGAAGCTCTCCACTA	
XM_006420545.2	CHS	TCTATCGACGGGCATCTTC	TGCCTCGGTTAGGCTTTT	Lo Piero et al. 2005
NM_001288931.1	DFR	GCTGTTCGTGCTACTGTTC	GGCTAAATCGGCTTTCCATA	
XM_025097974.1	ANS	GGGTGACTGCTAAATGTGTT	CAAGTCCCCTGTGAAGAATA	
NM_001320060.1	UFGT	TCTTCAGCACTCCGCAATC	TCCATCGGATACGTCGTAAG	
NM_001288889.1	R2R3Myb	ACAATCCACCCCGTCTGATC	CTGGCCTGCTTCAATGACTC	
XM_006483335.2	SPS	TTGTAACTAGCACCCGACAGG	CAACCATACGAGGCATAAACC	Wang et al. 2015
XM_006480234.2	ACL	GATACTGTTGGAGACTTGGG	GCTCTCTTACGACCATCAGG	Guo et al. 2016
XM_006494513.2	NADP-IDH1	GAAAATTGGGGATTGGGATT	CAACAGAGGTGCAGCTCAAA	Guo et al. 2016
XM_006482744.2	CS	GGTGCCCCCAATATTAACAA	AGAGCTCGGTCCCATATCAA	Guo et al. 2016
XM_006474957.2	GGP	TACCAAAGTGGGGCAAGAAG	TGGCAACAACACTTGGAGAA	Alos et al.2014
XM_006492225.2	GalUR-12	CCCAGGTTTCTTTGAGGTGGGTTTATC	TACTGTGGAATTTGTTCGATCTTTTGCAGC	Alos et al.2014
AY498567	EF	CACCACCCCCAAGTACTC	GTTGTCACCCTCGAAACC	

Results

Biochemical Analysis

The citrus colour index (CCI) of the juice is dependent on the stage of ripening. At 184 and 214 DAFB, the value of CCI was negative because of the green appearance (Figure 4.1B). The lowest values were in FUR, 15.81 (184 DAFB) and 11.94 (214 DAFB), while BIT and CAR were almost of the same colour level (Figure 1A). Juice colour changed progressively from yellow/orange to deep orange/reddish at 245 DAFB for FUR, which showed the highest value (10.25). Nevertheless, at full ripening (304 DAFB), FUR (13.95) had a lower value than for either BIT (29.37) or CAR (23.80). The TSS values increased progressively until January (245 DAFB) in BIT

and FUR, subsequently they maintained quite constant values; CAR was the only one in which the sugar content increased from January (245 DAFB) to March (304 DAFB) (Figure 4.2A). No differences among the three rootstocks were recorded for TSS at 304 DAFB, even though statistical differences indicated a lower content of TSS in the fruits of FUR during November (184 DAFB) (9.7) and December (214 DAFB) (10.53). At the end of maturation, the pH values in the blood orange samples were between 3.57 (BIT) and 3.88 (CAR). Marked differences were found in TA, which was consistently higher in FUR than in the other two rootstocks (Figure 4.2B). Although TA values were similar in CAR and BIT, the TA of CAR decreased from 245 to 304 DAFB, while in BIT, the TA was relatively constant. Even in this case, the largest changes in metabolite reduction were observed at 245 DAFB, when TA decreased substantially in all samples. In CAR, there was a simultaneous decrease of TA and an increase of TSS during the last three stages of ripening, resulting in the higher TSS/TA values, while MI showed slight changes in FUR and BIT during 276 and 304 DAFB (Figure 4.2C). Total anthocyanin content appeared at the second month of sampling (214 DAFB) in all three scion/rootstock combinations, even though TAC was not clearly detectable colourimetrically (Figure 4.1A). The TAC content was significantly higher in BIT at 214 DAFB, but it increased in January (245 DAFB) and again in February (276 DAFB), then maintaining these values until March (304 DAFB). The rootstock reaching the highest concentration during the last three months was BIT, which accumulated significantly higher amounts of anthocyanins after 245 DAFB (Figure 4.3A). Meanwhile, FUR showed the lowest values, compared to the other two rootstocks, except in January (245 DAFB), when the lowest anthocyanins content was in CAR. The only rootstock showing a significant increment between 276 and 304 DAFB was CAR. The vitamin C content decreased slightly during maturation,

reaching a lowest value in 304 DAFB (Figure 4.3B). Among the samples, the lowest AsA content was recorded in CAR, where levels were consistently lower than in BIT and FUR. The variations between these last two rootstocks were not statistically significant, except in 276 DAFB, when BIT was significantly higher than FUR.

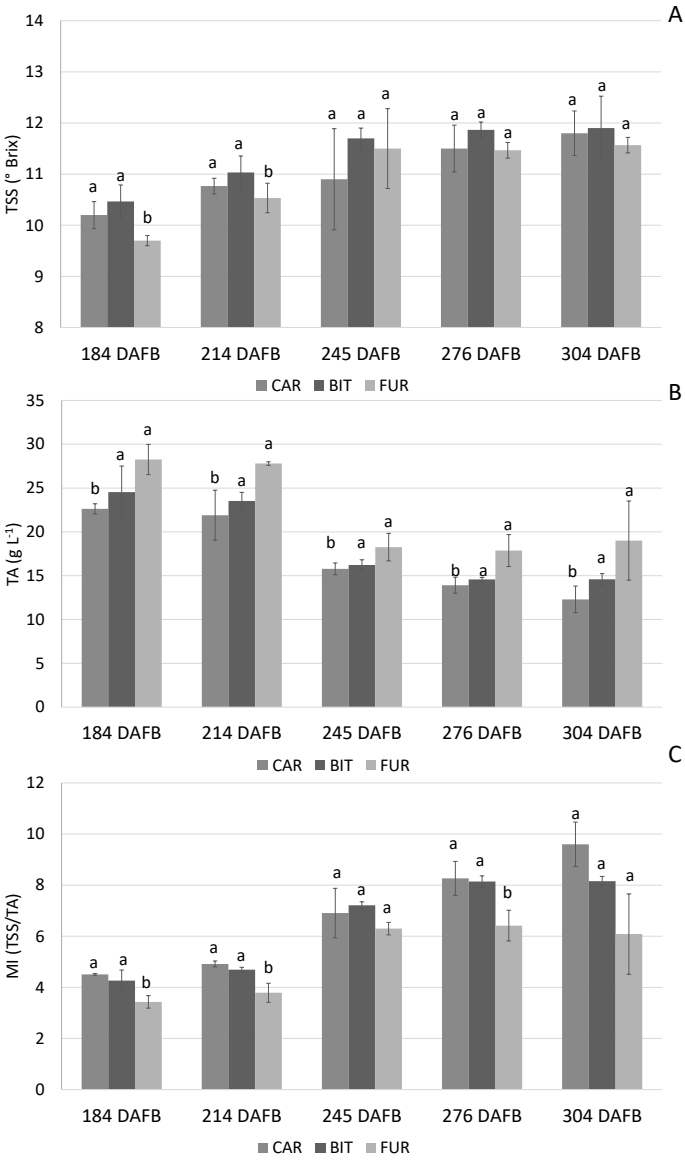

Figure 4.2. Evolution of biochemical data recorded on juice of the sweet orange

Tarocco Sciré grafted onto Carrizo (CAR), Bitters (BIT) or Furr (FUR) rootstocks during the different harvest dates (DAFB: days after full bloom). (A) TSS, total soluble solids (°Brix). (B) TA, titratable acidity (g L-1). (C) Maturity index expressed as total soluble solids/titratable acidity (TSS/TA). Vertical bars indicate standard deviation. Statistically significant differences by the two-way analysis of variance (P value <0.01) are represented by different letters

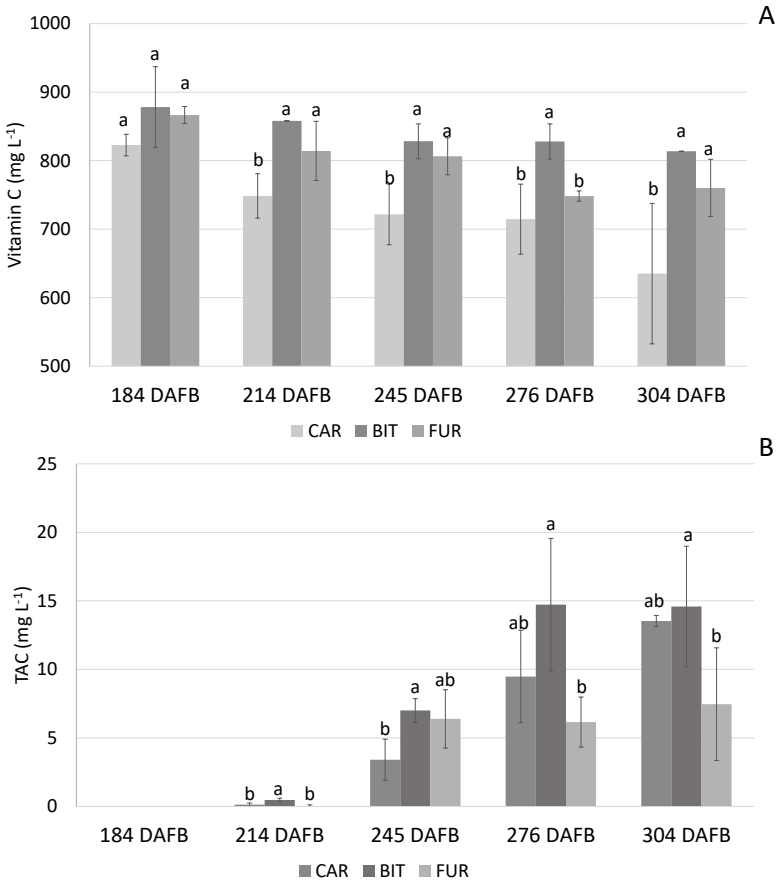

Figure 4.3. Pattern of biochemical data recorded on juice of sweet orange Tarocco Sciré grafted onto Carrizo (CAR), Bitters (BIT) or Furr (FUR)

rootstocks during the different harvest dates (DAFB: days after full bloom). (A) TAC, total anthocyanin content (mg L-1). (B) Vitamin C (mg L-1). Vertical bars indicate standard deviation. Statistically significant differences by the two-way analysis of variance (P value <0.01) are represented by different letters

Gene Expression Analysis

Transcription of PAL was clearly higher in BIT than in the other two rootstocks, at all inspection times, except for 245 and 276 DAFB, when the values for FUR and CAR were very similar to the one for BIT (Figure 4.4). Similarly, CHS expression reached a high for BIT at 214 DAFB and in FUR at 245 DAFB. At 214 and 276 DAFB, CAR values were significantly lower than those for BIT. The highest transcription levels of DFR were recorded at 214 DAFB for BIT and at 276 DAFB for FUR. The expression of this gene was not significantly different in the two rootstocks at 184 and 304 DAFB but was more elevated at 214 and 276 DAFB for BIT and at 245 DAFB for FUR. In comparison to CAR, DFR showed increased transcription for BIT at 214 DAFB, but the differences (245, 276, and 304 DAFB) were not significant. Anthocyanidin synthase was highly expressed during 214 and 245 DAFB for both BIT and FUR; for BIT, the peak of expression was anticipated at 214 DAFB, whereas in FUR the highest FC value was reached at 245 DAFB. It is worth noting that the expressions of ANS were generally lower for CAR than for BIT and FUR, and the highest FC value was reached only at 276 DAFB. Transcription of UFGT was relatively constant in all three rootstocks, slight differences were found exclusively at 184 DAFB, when the accumulations of gene transcripts for CAR and FUR were higher than for BIT, and at 276 DAFB, when they were higher than for either BIT or FUR. The only differences in Ruby expression were recorded at 276 DAFB, when CAR showed a higher accumulation of mRNA than for BIT at 276 DAFB.

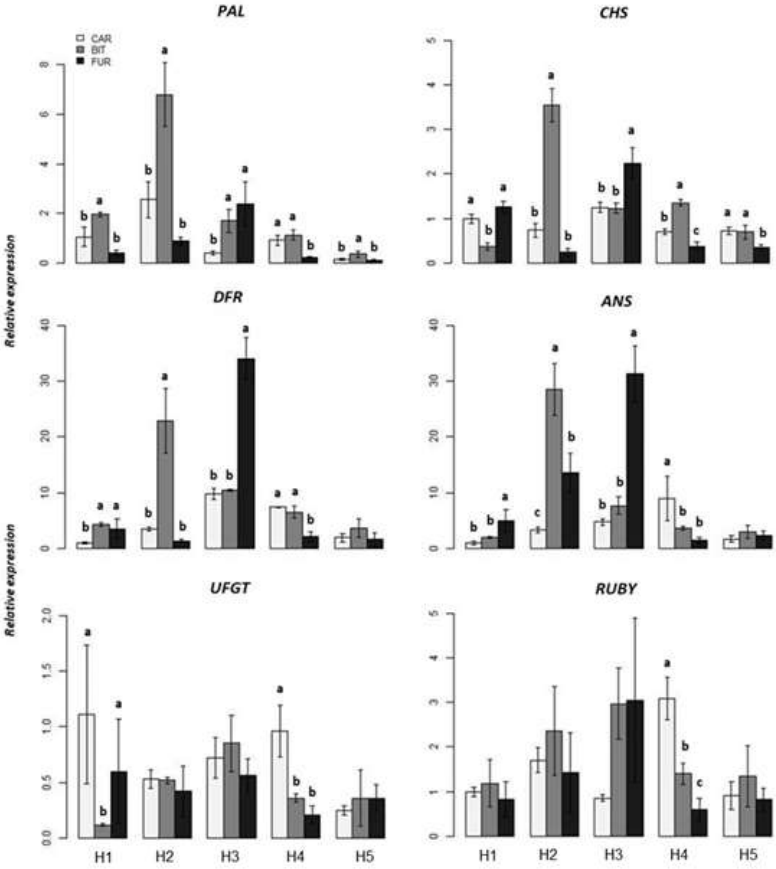

Figure 4.4. Expression of genes involved in anthocyanin synthesis. Histograms of qPCR data (FC, fold change) detected in juice of 'Tarocco Sciré' grafted on Carrizo (CAR), Bitters (BIT) or Furr (FUR) rootstocks at five sampling stages. PAL, Phenylalanine ammonia-lyase. CHS, chalcone synthase. DFR, dihydroflavonol 4-reductase. ANS, anthocyanidin synthase. UFGT, UDPglucose-flavonoid glucosyl transferase. RUBY, R2R3Myb. Vertical bars indicate standard deviations. Statistically significant differences are represented by letters a,b,c (p value<0.01)

The expression of SPS showed no significant differences among the rootstocks during the whole process of ripening, except at 214 DAFB, when the gene was more strongly expressed for CAR than for the other two rootstocks (Figure 4.5).

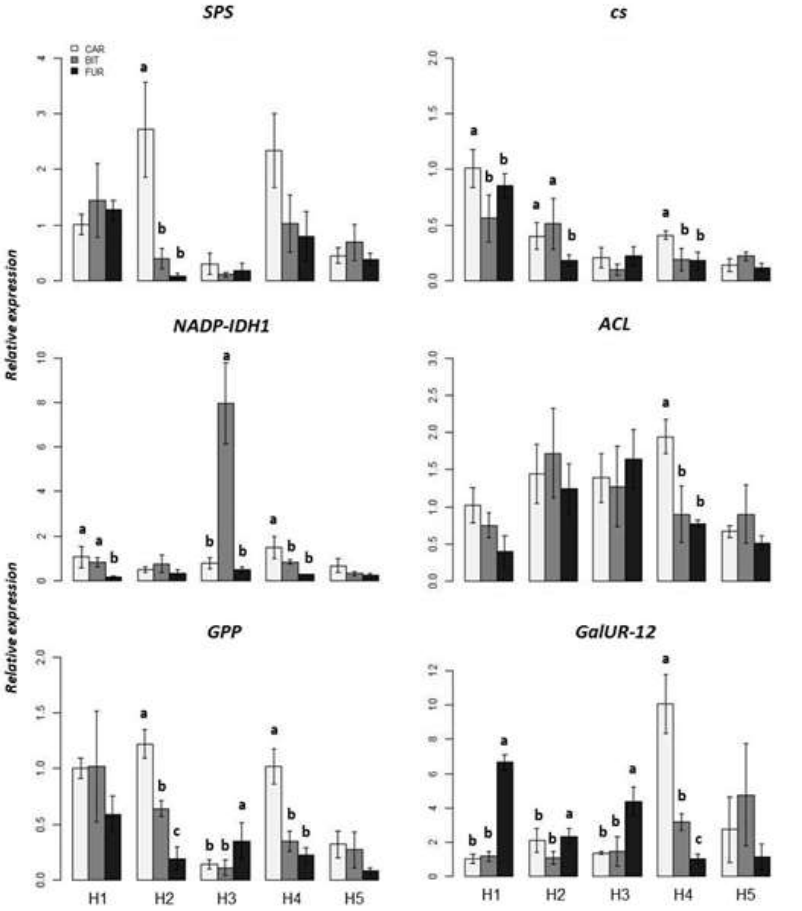

Figure 4.5. Histograms of qPCR data (FC, fold change) detected in juice of 'Tarocco Sciré' grafted onto Carrizo (CAR), Bitters (BIT) or Furr (FUR)

rootstocks at the five sampling stages. SPS, sucrose phosphate synthase. CS, citrate synthase. NADP-IDH1, isocitrate dehydrogenase. ACL, ATP citrate lyase. GGP, GDP-L-galactose phosphorylase. GalUR-12, D-galacturonate reductase. Vertical bars indicate standard deviation. Statistically significant differences are represented by letters a,b,c (p value<0.01)

For FUR, GPP was upregulated at 245 DAFB compared with either CAR or BIT. At 214 DAFB and 276 DAFB, the highest FC values were for CAR and the lowest were for FUR. Early upregulation of GalUR-12 was evident for FUR from the first stages of development. Although GalUR-12 transcription was lower for BIT and CAR than for FUR at 184 DAFB, 214 DAFB and 245 DAFB, but it increased significantly at 276 DAFB and 304 DAFB. The highest peak of expression was for FUR at 184 DAFB, before the FC value dropped at 214 DAFB but was still higher than for BIT. At 245 DAFB GalUR-12 was upregulated for FUR compared with for BIT and CAR, but at 276 DAFB it was the CAR rootstock that showed the highest regulation of the gene while FUR showed the lowest values of the three rootstocks.

Discussion

Several studies have documented the increase in anthocyanin content of pigmented oranges during ripening (Barbagallo et al., 2007; Cebadera-Miranda et al., 2019) but few in blood orange, as a function of rootstock (Ordonez-Diaz et al., 2020). A recent study demonstrates that some recently released rootstocks are better than others, with respect to the production of fruit with high levels of pigmentation. These enhance the synthesis and accumulation of anthocyanins in the flesh of citrus fruit (Continella et al., 2018). By monitoring anthocyanin accumulation during fruit ripening, our results seem to support these findings, since the metabolic data indicates clear differences in fruit anthocyanin content among the three rootstocks

tested. Total anthocyanin content was at almost high levels by the third sampling (245 DAFB) for all three scion/rootstock combinations; though, for the rootstock BIT, a higher content was already significantly evident at the second sampling (214 DAFB). Positive CCI values were recorded only in January (245 DAFB), when TAC settled to about 5 mg L^{-1} in all three samplings. The anthocyanin accumulation is especially relevant inside the juice sacs adherent to the segments, and particularly so in the ones nearest the albedo (Figure 1); the influence of rootstock was first clearly evident colourimetrically in the early stages of synthesis (Figure 4.1). The rootstock CAR was the only one that increased the increment of TAC from 245 to 304 DAFB, while the two citrandarins (BIT and FUR) seemed to be more precocious, already achieving their maximum anthocyanin levels at 276 DAFB. The fruits of CAR seemed to synthesise anthocyanins later on, reaching the highest values at 304 DAFB. Meanwhile, BIT showed intense pigmentation from January (245 DAFB), the month in which anthocyanin was most markedly accumulated. According to our earlier results (Ordonez-Diaz et al., 2020), the concentration of anthocyanins increased progressively during the sampling period. These authors detected the influence of rootstock on the accumulation of anthocyanins in the juice of the blood orange cultivar Sanguinelli. They noted that FA5 and Cleopatra rootstocks were the ones with the highest total content, while Carrizo citrange slightly enhanced the pigmentation, and Volkameriana scarcely had any effect. Also, Morales et al. (2020) observed similar anthocyanin contents in the fruits of cv. Tarocco Rosso, assessing FA13, FA5, and Cleopatra mandarin as the rootstocks that most determined increases in pigmentation, while Carrizo just affected it and *C. macrohpylla*; Swingle citrumelo showed the least effects. The higher TAC recorded in BIT (Figure 4.3A) matched an early up-regulation of anthocyanin synthetic gene (Figure 4.4). qPCR data

showed that PAL, CHS, DFR and ANS showed their highest peaks of expression in BIT at 214 DAFB, while FUR, DFR and ANS showed highest expressions at 245 DAFB, a month later than for BIT (Figure 4.4). In pistachio, it has been found that rootstock can influence the activity of PAL, which plays a pivotal role in the production of phenolic compounds, flavonoid, and anthocyanin (Nadernejad et al., 2013). While, in Arabidopsis a mutation of CHS led to a phenotype with flavonoid synthesis deficiency (Shirlet et al., 1995). A key gene for synthesis of anthocyanin is DFR because its transcription is generally not detected in blonde oranges (Lo Piero et al., 2006) and it is highly up-regulated in the flesh of pigmented citrus fruits (Catalano et al., 2020). Both DFR and ANS were generally downregulated for CAR compared to for BIT or FUR and seemed to reach highest expression only at 276 DAFB, two months later than for BIT and one month later than for FUR (Figure 4.4). This delayed activation of gene transcription may explain why for CAR, fruit anthocyanin accumulation began later than for BIT or FUR. Indeed, both DFR and ANS are part of the downstream gene of then pathway and, for this reason, are called late synthetic genes (LBGs). These are the opposite of the early synthetic genes (EBGs) such as PAL and CHS, which encode for precursors in common with several other pathways. The LBGs encode specifically for enzymes involved in the synthesis of anthocyanins (Xu et al., 2015). Although fruits for FUR reached a higher TAC earlier than for CAR, by the end of ripening FUR had the lowest TAC of the three rootstocks (Figure 4.2A). Unlike for BIT, where the expression of anthocyanin synthetic genes decreased after the peak at 214 DAFB, for FUR, the transcription of these genes dropped suddenly at 276 DAFB (Figure 4.4). The amount of pigment accumulated in the citrus pulp tissues was positively correlated with the expressions of the anthocyanin synthetic genes (Cotroneo et al., 2006). The lower TAC in the ripe fruit for FUR may, therefore, be

connected with the early downregulation of these genes. The accumulation of anthocyanins in citrus fruit pulp is regulated not only by the structural genes just discussed, but also by Ruby, a transcriptional factor belonging to the MYB family (Butelli et al., 2012). The transcription of this gene is activated in blood orange by the insertion of a retrotransposon called Tcs1, which is cold-dependent and for this reason is also responsible for temperature-dependent anthocyanin synthesis (Lo Piero et al., 2005; Butelli et al., 2012; Carmona et al., 2017). During our study, no remarkable differences in Ruby expression were detected among the three rootstocks (Figure 4.4). During maturation, juice pH showed an increase in all three rootstocks, as also reported in other studies with citrus fruits (Di Matteo et al., 2021). Our results are similar to those reported for blood orange cultivars, such as Tarocco Rosso and Tarocco Ippolito (Cebadera-Miranda et al., 2019). Sugars are important components of the chemical composition of blood oranges and their profiles depend on cultivar, harvest time and environmental conditions. Several authors have studied the influence of rootstock on the sugar profiles of citrus fruits (Legua et al., 2014). Morales et al. (2020) investigated rootstock effects on fruit quality of the blood oranges Moro and Tarocco Rosso: the FA5 rootstock showed the highest levels of TSS in fruits of both cultivars during ripening. This contrasts with those grafted on Carrizo citrange where TSS scarcely increased. In our study, CAR showed a late increase in TSS and a decrease in TA, compared with the other two citrandarin rootstocks. This confirms a delay in the whole ripening process, with a lag of about a month. Also, Domingues et al. (2021) reported both higher and anticipated TSS and maturation index (MI) of fruits of Valencia grafted onto some citrandarins (US–852 and IPEACS–256), like BIT and FUR, with respect to some other rootstocks (such as some citrumelo clones). Values obtained during the evolution of TSS and TA at full maturation

stage agreed with those reported by Abdelaali et al. (2018), Continella et al. (2018), and Cebadera-Miranda et al. (2019). Since no large differences were recorded in the TSS content in the final stages of maturation (276 and 304 DAFB), the TSS/TA ratio (MI) was more influenced by TA (Figure 4.2C). A slightly lower content of TSS was found for FUR at 184 and 214 DAFB (Figure 4.2B), but it was not possible to correlate this with changes in SPS transcription (Figure 4.5). The regulatory network that controls sugar accumulation is strictly related to sink strength, the competitive ability of fruits to attract assimilates, compared with other plant organs (Komatsu et al., 1999). Thus, the lower values assumed for FUR during 184 and 214 DAFB may be related to other factors involved in sugar accumulation, not to SPS expression. Although TA was consistently higher for FUR than for BIT or CAR (Figure 4.2C), CS was generally downregulated for FUR (Figure 4.5). The acidity of citrus fruits largely depends on citrate accumulation, since 90% of the organic acids contained in the juice vesicles is represented by citrates. However, the reduction of acidity taking place during fruit ripening seems to be due to increased catabolism of citrate rather than to its reduced synthesis (Cercos et a., 2006). As suggested in several studies, the main regulator of citrate content is a group of genes related to the citrate degradation pathways and not CS, which is involved in the synthesis of these compounds (Chen et al., 2013; Hu et al., 2014; Lin et al., 2015; Guo et al., 2016). One of the key genes involved in citric acid catabolism is NADP-ID184 DAFB and its upregulation has been related to reduced accumulation of citrate (Guo et al., 2016). Clear downregulation of this gene was noted for FUR, in comparison to BIT or CAR (Figure 4.5). It is possible to speculate that the higher TA values recorded for FUR (Figure 4.2C) are related to a lower rate of citrate degradation. A second important gene participating in acidity reduction is ACL, which catalyses the conversion of citrate to oxaloacetate. Although

this gene plays a fundamental role in citric acid utilization (Hu et al., 2014), no noteworthy changes of its transcription were observed during our study. Citrus fruits are beneficial in human nutrition because of their antioxidant activity and high concentration of ascorbic acid that is influenced both by the stage of ripening and by rootstock (Arena et al., 2001; Morales et al., 2021). Previous studies with blood orange have reported concentrations of ascorbic acid in Moro and Sanguinello above 500 mg/L, with higher contents than in blonde orange cultivars (Mondello et al., 2000; Arena et al., 2001; Rapisarda et al., 2001). Influences of rootstock on vitamin C content have been reported by Morales et al. (2021), where mandarins have the highest levels of ascorbic acid in January, but this decreases by the end of February. Rootstock strongly influenced the total amount of vitamin C, with FA5 showing higher values than for Carrizo citrange. Accordingly, the same behaviour has also been observed on Tarocco Sciré: vitamin C decreases during maturation, with BIT showing the highest vitamin C content at full maturation in March (304 DAFB). In recent years, the regulation of AsA has been subjected to in-depth study because of its known positive effects on both plant and human health. Accumulation of AsA depends on the balance between its synthesis and oxidation rates, which are characteristics of each genotype and tissue. The regulation of GPP has been strongly related to AsA concentration in the tissues of several plants (Mellidou et al., 2017). However, the steadily rising content of AsA for FUR (Figure 4.3B) may not be related to the transcription of this gene, since it was generally downregulated for FUR compared with for CAR or BIT (Figure 4.5). More likely, the higher level of AsA for FUR was caused by an early upregulation of GalUR-12. The expression of the gene for CAR and BIT increased from 276 to 304 DAFB, while for FUR the transcription of GalUR-12 was quite high from 184 to 245 DAFB, before a decreasing trend was initiated (Figure 4.5). GalUR-12 is

highly expressed in citrus fruits and represents the rate-limiting step of the galacturonate pathway, an alternative synthetic route for AsA accumulation to the main L-Galactose pathway. However, recent evidences suggest the GalUR genes may be the main ones responsible for the high accumulation of vitamin C in citrus fruit (Xu et al., 2013). Despite it being well-documented that grafting can affect fruit organoleptic and nutritive qualities such as the content of bioactive compounds (Sharma et al., 2015; Suriano et al., 2016), it is still unclear how rootstocks exert their influence on these characteristics. The results discussed here suggest that the performances of grafted trees are related to more specific interactions between scion and rootstock and not simply to factors such as plant water and nutrient status or crop yield (Castle, 1995). The higher accumulation of anthocyanins for BIT and of AsA for FUR was connected to the upregulation of genes encoding for the key enzymes involved in the synthesis of these metabolites. While the higher level of acidity recorded for FUR was correlated with the downregulation of a gene that activates the degradation of citrates. Not much is known about the effects of rootstock at the molecular level; however, recent advances have shown that the use of a rootstock can affect scion gene regulation (Liu et al., 2014; Bennici et al., 2021). Moreover, it seems that scion gene expression may be induced by the movement of proteins and small RNA across the graft junction (Tzarfati et al., 2013; Wu et al., 2019). In conclusion, on the basis of our results, it is reasonable to assert that a rootstock plays a fundamental role in the control of the gene regulatory networks of the citrus scion that are involved in the fruit quality traits.

Conclusions

This comparative study compared three combinations of scion/rootstock and indicates a positive correlation between rootstock and the regulation of fruit-quality related genes in the scion and the accumulation of bioactive compounds in the fruit. Monitoring the anthocyanin accumulation during fruit development and ripening shows certain combination of scion and rootstock can enhance the synthesis of these compounds, as well as of vitamin C accumulation and acid degradation. Ripening index and CCI are consistent indicators of fruit maturation in blood oranges. Among the rootstocks evaluated citrandarins, Bitters and Furr were effective in causing earlier maturation of fruits. It is possible to conclude that rootstock genotype can exert important influences on citrus fruit quality by affecting gene expression in the scion.

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Chapter 5 - Rootstocks Influence Yield Precocity, Productivity, and Pre-Harvest Fruit Drop of Mandared Pigmented Mandarin

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Abstract

Citrus fruit quality and scion productivity are influenced by the choice of rootstock. We aimed to evaluate the e ect of rootstocks on yield and fruit quality of Mandared, a triploid pigmented mandarin. To do so, we established a rootstock field trial on a high pH soil (8.6) in which Mandared was grafted onto 11 rootstocks. These included some standard rootstocks, such as trifoliate orange ((*Poncirus trifoliata* (L.) Raf.), Troyer citrange (*Citrus sinensis* (L.) Osb. X *P. trifoliata*), Swingle citrumelo (*Citrus paradisi* Macf. X *P. trifoliata*), and C35 citrange (*C. sinensis*X *P. trifoliata*), as well as new releases from the Council for Agricultural Research and Economics (CREA, Acireale, Italy) and the University of California Riverside (UCR). The cumulative yield was measured over five consecutive years, while fruit quality was analyzed for two years. The

trees on C35, C57 (*Citrus sunki* Hort. ex. Tan. X *P. trifoliata*), and C22 (*C. sunki* X *P. trifoliata*), started to set fruits one year earlier than the others. The trees on C57 provided some of the highest cumulative yields and canopy volumes. The production of Mandared grafted onto C57 was double that of Mandared grafted onto Troyer, while Mandared grafted onto C35 and C22 resulted in the best yield efficiency. The trees on Swingle and C57 significantly reduced the pre-harvest fruit drop, to which Mandared is particularly sensitive. However, grafting Mandared onto Swingle resulted in the highest variation among replicates, probably due to its high sensitivity to iron chlorosis. Most of the fruit quality parameters, such as fruit size, total soluble solids (TSS), and acidity were not significantly different among the rootstock treatments. However, fruits produced by Mandared grafted onto C22 had one of the highest rates of anthocyanin accumulation. The results indicate that C57, C35, and C22 were the most suitable rootstocks for Mandared in South-Eastern Sicily.

Introduction

Rootstocks are known to affect the performance of many traits of different citrus varieties, including tolerance to biotic and abiotic stresses, fruit quality and size, productivity, ripening period, and yield precocity (Castle, 2010; Bowman et al., 2020). Recently, an effect of rootstocks on anthocyanin pigmentation of blood oranges was also observed (Continella et al., 2018). Therefore, in the case of pigmented citrus cultivars, an appropriate rootstock choice is essential to produce high quality fruits.

Italy is the second largest European producer of citrus fruits (FAOSTAT, 2020). Most of the citrus fruits produced in Italy are intended for fresh sale at markets, and therefore fruit quality is of great importance for the Italian citrus fruit production industry. Until the early 2000s, most of the Italian citriculture was almost exclusively

based on the use of sour orange as a rootstock, which conferred high quality to the scion cultivars. After the outbreak of citrus tristeza virus (CTV) (Davino et al., 2003), new plantings have been established, mostly using Troyer and Carrizo citranges (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata*), and Swingle citrumelo (Citrus paradisi Macf. × *P. trifoliata*). However, these rootstocks are known to have low tolerance to high pH calcareous soils, which are typical of many growing areas of Southern Italy. As such, growers prefer to use Volkamer lemon (*Citrus volkameriana* Pasq.) or Alemow (*Citrus macrophylla* Wester), which guarantee high yield but an overall low fruit quality and low anthocyanin pigmentation (Barry et al., 2020). New rootstocks are therefore needed to confer high productivity and high fruit quality to citrus fruits grown under suboptimal pedological conditions.

Recently, many breeding programs around the world have released new rootstocks (Bowman et al., 2020; Forner-Giner et al., 2020). Among these, the University of California Riverside (UCR) released three Sunki mandarin (*Citrus sunki* Hort. ex Tan.) × trifoliate orange (*P. trifoliata*) hybrid rootstocks, namely "Bitters" (C22), "Carpenter" (C54), and "Furr" (C57). These three rootstocks showed good performance in many trials in terms of yield efficiency (C22), productivity (C54 and C57), and high (C22) or moderate (C54 and C57) tolerance to calcareous soils (Kupper et al., 2010). The Council for Agricultural Research and Economics (CREA, Italy) released three *Citrus latipes* (Swing.) Tan. × *P. trifoliata* hybrid rootstocks (F5P12, F6P12, and F6P13) that showed high cumulative yield in combination with "TDV" Tarocco blood orange, "Washington" Navel orange, and "SRA92" Clementine (*Citrus clementina* Hort. ex Tan.) (Reforgiato Recupero et al., 2009).

Mandared (Figure 5.1) is a triploid hybrid between "Nules" Clementine and tetraploid Tarocco orange (Reforgiato Recupero et al.,

2009).

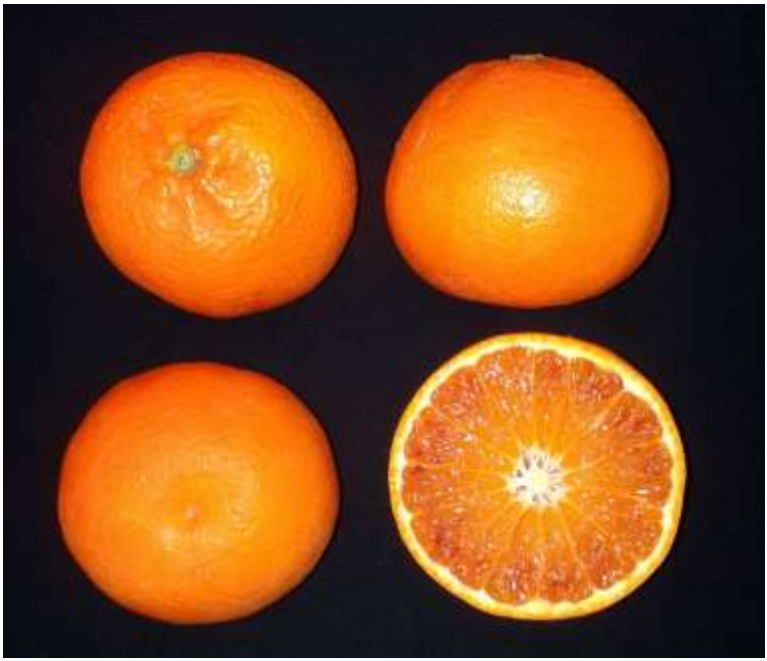

Figure 5.1. Fruits of "Mandared" (Citrus clementina 2 x × *Citrus sinensis* 4 x)

Mandared was released by CREA in 2004 and is cultivated or under evaluation in different citrus producing countries. It ripens from mid-January to mid-February in the northern hemisphere. Mandared has unique pomological features among seedless mandarins, including its pulp pigmentation, which has attracted the interest of many growers. It is a mid to large size mandarin, with an oblate shape and a thin and smooth rind, and an acidic taste resembling that of blood oranges (Barry et al., 2020). In suitable environments, the flavor of Mandared fruits reaches a good balance between sugars and acids. However, after almost a decade since the establishment of the first Mandared commercial plantings, some disadvantages have become

evident. These include: A delay in the first production compared to other citrus varieties; low productivity; thorniness, which causes damage to fruit rinds, especially in windy areas; the smooth and thin rind, which does not protect fruits from damage; and a high incidence of pre-harvest fruit drop, which is probably the largest problem (G. Russo, personal communication). Mandared fruits tend to fall before they reach commercial maturity, with a high percentage of fruit loss compared to other citrus cultivars, especially in suboptimal climatic conditions (frost, dry winds, etc.).

Here we describe the performance of Mandared triploid mandarin grafted onto 11 rootstocks and evaluated during five consecutive years. The trial was performed: (i) To assess the influence of the rootstock on Mandared fruit quality (including pigmentation) and productivity; and (ii) to search for the most suitable CTV-tolerant rootstocks that can be used as alternatives to sour orange for the establishment of Mandared orchards under the soil and climate conditions of South-Eastern Sicily.

Materials and Methods

Plant Material and Trial Management

The trial was conducted at the CREA experimental farm of Palazzelli (Siracusa, Italy) (37° 20′ N, 14° 53′ E, 48 m a.s.l.), with trees spaced at 6 × 4 m. Mandared (*C. clementina* × *C. sinensis*) budsticks were grafted onto 11 two-year old rootstocks (for details, see Table 5.1) in the spring of 2009.

Table 5.1. List of rootstocks used in the field trial

Rootstock Name	Botanical Species	Abbreviation
"Bitters" (C22) citrandarin	*Citrus sunki* Hort. ex. Tan. × *Poncirus trifoliata*	C22
"C35" citrange	*Citrus sinensis* (L.) Osb. × *P. trifoliata* (L.) Raf.	C35
"Carpenter" (C54) citrandarin	*C. sunki* × *P. trifoliata*	C54
"Furr" (C57) citrandarin	*C. sunki* × *P. trifoliata*	C57
"Swingle" citrumelo	*Citrus paradisi* Macf. × *P. trifoliata*	Swingle
68IG26-C1F6-P12	*Citrus latipes* (Swing.) Tan. × *P. trifoliata*	F6P12
68IG26-C1F6-P13	*C. latipes* × *P. trifoliata*	F6P13
Poncirus trifoliata "Serra"	*P. trifoliata*	P. trifoliata
Severinia buxifolia	*Severinia buxifolia* (Poir.) Ten.	S. buxifolia
"Troyer" citrange	*C. sinensis* × *P. trifoliata*	Troyer
Flying dragon	*P. trifoliata*	F. dragon

The trial was established in July 2010 in a completely randomized design using 10 single tree replicates for each rootstock. Soil conditions were as described in Caruso et al. (2016), with pH 8.6 and active lime ranging from 23.5 to 38.9 g kg−1 in different parts of the experimental plot.

Plants were grown under conventional cultural practices. Fruits were treated with Spinosad (Spintor Fly) every 10 days from September to mid-November to control Mediterranean fruit fly (*Ceratitis capitata*). Moreover, fruits were treated once with 20 ppm 3,5,6-trichloro-2-pyridyl-oxyacetic acid (Tryclopir) at color break (mid-November), and a month later with 15 ppm 2, 4-dichlorophenoxyacetic acid (2, 4-D), to prevent or reduce fruit drop.

Sampling

Fruits were sampled every second half of January from 2015 to 2019. To measure the yield, the production of each replicate tree was harvested and weighed, and all fruits were counted. Average fruit weight was calculated by dividing the total sample weight by the number of fruits per tree. Due to the sensitivity of Mandared to pre-harvest fruit drop, two rounds of fruit drop counts were performed every year before harvest (10 days and one day before, respectively).

The fruit drop rate was calculated as the ratio between the number of fruits that dropped before harvest and the total number of fruits, and multiplying by 100. Cumulative fruit drop did not include the 2015 harvest since not all rootstocks produced fruits in 2015. Dropped fruits were included in the yield. Canopy volume was approximated as one half prolate spheroid, as suggested by Turrel [12], with V = 4/6πh(d/2)2, where h is tree height, and d is tree diameter. Yield efficiency of the canopy was calculated as the ratio of the cumulative yield to the canopy volume in the spring of 2018.

Fruit Quality

Samples of 15 fruits per plant were collected in 2017 and 2019 from three representative trees per rootstock to evaluate the rootstock effect on fruit quality traits. Specifically, we evaluated the following parameters: juice yield, peel color index (CI); total soluble solids (TSS); titratable acidity (TA); and total anthocyanin concentration.

Juice was extracted from the fruits using a domestic squeezer (Citrus Juicer JE290, Kenwood, UK) and filtered before analysis. Three juice samples, from the pooled juice of five fruits from three replicates per rootstock combination, were used for chemical analyses. Juice yield was calculated as follows: (Juice weight/fruit weight) × 100. TSS were measured using a digital refractometer (Atago CO., LTD, model PR-32 α, Tokyo, Japan), with the results expressed as °Brix. TA, expressed as the percentage of anhydrous citric acid, was determined using a potentiometric titration (Hach, TitraLab AT1000 Series) of the juice with 0.1 N NaOH beyond pH 8.1, according to the AOAC method [13] with the results expressed as g L−1 of citric acid equivalent. Ripening index (RI) was calculated as the ratio between TSS and TA.

Peel color was recorded on two opposite points of the equatorial region for each fruit using a Minolta CR-400 chroma-meter (Minolta

Corp., Osaka, Japan). The CI, which is widely used in the citrus industry as a maturation index, was calculated following the method described by Jimenez-Cuesta et al. (1978), and was determined as 1000 a*/L*b*, where L* is lightness, a* is the red–green component, and b* is the yellow–blue component. Juice total anthocyanin concentration was measured using a Nanodrop (NanoDrop 2000, Thermo Scientific) spectrophotometer (at 510 and 700 nm) using the pH differential method (1968), and was expressed as cyanidin 3-glucoside equivalents (mg L^{-1}).

Data Analysis

Comparisons of means were performed using one-way analysis of variance (ANOVA) with Tukey tests and the confidence level was at 95%, with rootstock genotypes as fixed effects. Statistical analysis was performed using the R Commander (2005) package or R software, version 3.6.1. A radar chart was generated using Poltly chart studio (https://chart-studio.plot.ly) to summarize the productive performance of the analyzed rootstocks. In particular, cumulative yield, yield efficiency, and cumulative fruit drop were independently rescaled within the range (0, 1) using maximum and minimum normalization on the basis of the best (1) and the worst (0) performance for each parameter.

Results

In the period of time between the establishment and the end of the field trial, some replicates died or showed poor growth. Specifically, six Mandared plants grafted onto F. dragon, five plants grafted onto *S. buxifolia*, and one plant grafted onto F6P13 died during the first 3–4 years after planting. The remaining Mandared plants grafted onto F. dragon did poorly in terms of production during all

years of the trial, and for this reason were excluded from further analysis.

Moreover, some non-representative plants were not considered in the statistical analysis. We excluded one C22, one F6P12, and two Swingle replicates, since the productivity of Mandared grafted onto these rootstocks, and their canopy volume were extremely low compared to the other replicates. This might be due to the presence of off-types that were not recognized during plant propagation. The statistical analysis regarding cumulative production, yield efficiency, percent fruit drop, and quality traits were performed using the rest of the replicates.

Iron chlorosis symptoms were observed on trees on F. Dragon, *P. trifoliata*, and Swingle, but not in the rest of the plants. Most of the plants started to set fruits in 2015 (five years after planting), with significant differences among trees grafted onto different rootstocks. A shorter non-productive period was observed in Mandared grafted onto C35, C57, and C22, with means of 11.7, 9.6, and 8.8, respectively. Mandared onto C54 produced 5.2 kg of fruits per tree, while the trees on the other rootstocks had very low (around 1 kg of fruit produced per plant or less) or no fruit production in 2015 (Table 5.2).

Table 5.2. Yield of Mandared grafted onto 10 rootstocks calculated from 2015 to 2019. Different letters indicate significantly different means according to Tukey's test at $p < 0.05$

Rootstock	Yield (kg Per Tree)				
	2015	2016	2017	2018	2019
C22	8.8 ab	48.9 ac	39.1 ab	40.7 ab	45.3 ab
C35	11.7 a	71.9 a	34.2 ac	43.1 a	40.6 ab
C54	5.2 ac	49.3 ac	46.6 ab	43.1 a	51.0 ab
C57	9.6 a	68.2 ab	46.8 ab	49.0 a	51.5 ab
F6 P12	1.1 c	45.6 bcd	25.5 bc	37.6 abc	40.9 ab
F6P13	0.8 c	20.5 def	35.9 ac	29.6 ad	56.6 a
P.trifoliata	0.4 c	14.0 ef	43.2 ab	8.5 d	39.0 ab
S. buxifolia	0.1 c	2.8 f	9.0 c	12.0 cd	24.9 ab
Swingle	1.2 bc	38.3 ce	51.6 a	31.3 ad	59.8 a
Troyer	0.9 c	12.3 ef	54.5 a	18.5 bd	25.3 b

Cumulative production between 2015 and 2019 (Figure 5.2) was the highest in Mandared grafted onto C57 (225 kg), followed by Mandared grafted onto C35 (202 kg), and Mandared grafted onto C54 (195 kg).

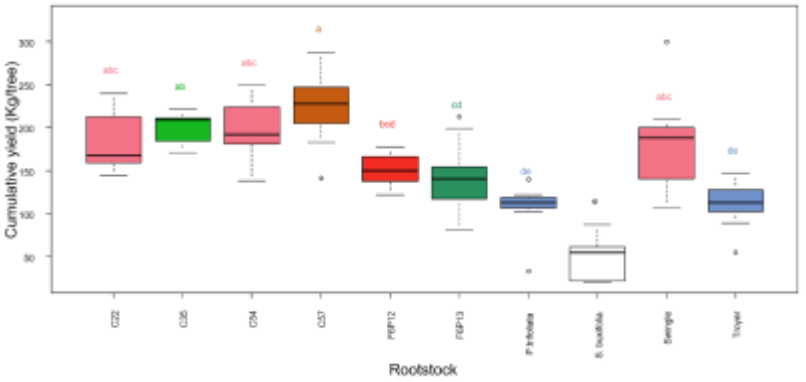

Figure 5.2. Cumulative yield of Mandared grafted onto 10 rootstocks, calculated from 2015 to 2019. Different letters indicate significantly different means according to Tukey's test at p< 0.05. Different colors of the boxes indicate different significance groups

The lowest cumulative production was observed in trees on *S. buxifolia*. Interestingly, trees on C57 had double the cumulative yield of trees on Troyer, which was included in the trial as a standard rootstock. Mandared grafted onto Swingle showed high productivity (182 kg of fruit produced), but also the highest standard deviation (65 kg), indicating a high variability among replicates.

Canopy volume was significantly affected by the rootstock (Figure 5.3). The highest canopy volume was observed for Mandared grafted onto C57 (27.3 m^3), while the lowest was observed for Mandared grafted onto *P. trifoliata* (12.8 m^3), indicating the poor adaptability of trifoliate orange to the pedological conditions of the trial.

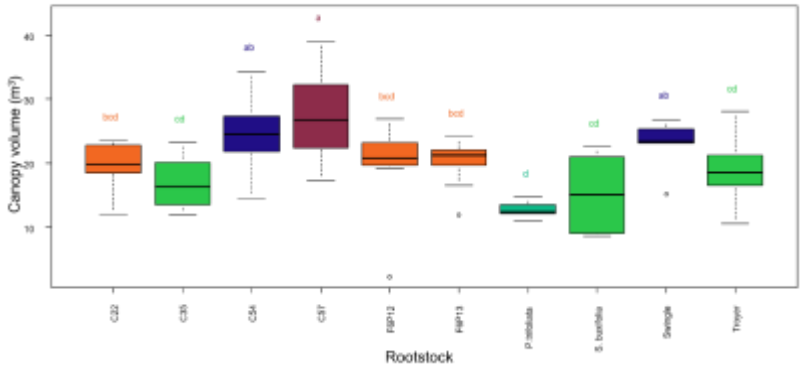

Figure 5.3. Canopy volume of Mandared grafted onto 10 rootstocks, recorded in spring 2018. Different letters indicate significantly different means according to Tukey's test at p< 0.05. Different colors of the boxes indicate different significance groups

The scatterplot in Figure 5.4 shows the relationship between cumulative yield and canopy volume of each replicate tree. Most replicates of the best performing rootstocks fall above the regression line.

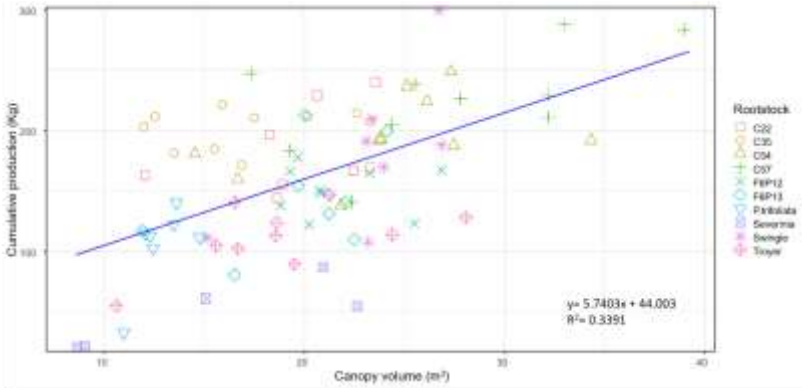

Figure 5.4. Scatterplot with regression line showing the relationships between canopy volume and cumulative production of the single replicate trees

Mandared grafted onto C35 was the most efficient (12.4 kg/m^3; Figure 5.5) followed by Mandared grafted onto C22 (9.5 kg/m^3), while Mandared grafted onto *S. buxifolia* was the least efficient (3.1 kg/m^3).

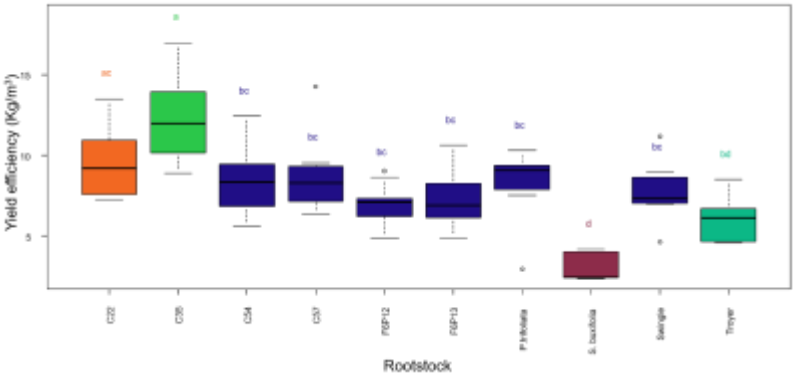

Figure 5.5. Yield efficiency of Mandared grafted onto 10 rootstocks. Different letters indicate significantly different means according to Tukey's test at p< 0.05. Different colors of the boxes indicate different significance groups

The differences in fruit weights among rootstocks were not statistically significant (Figure 5.6). The average fruit weight was highest for trees on C35 and Swingle (183.6 and 183.9 g, respectively).

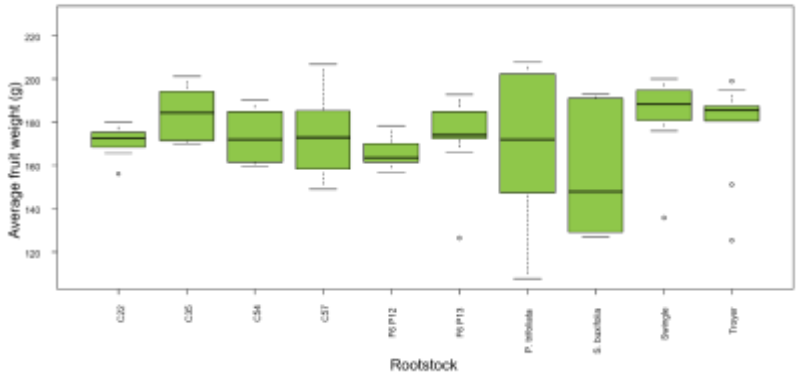

Figure 5.6. Fruit weight of Mandared grafted onto 10 rootstocks

Despite the use of triclopyr and 2,4-D, we observed a marked pre-harvest fruit drop. This was partly due to thorn damage and Mediterranean fruit fly attacks, but mostly attributable to the cultivar characteristics. However, the percentage of fruit drop varied among rootstocks and among years (Figure S5.1). Huge differences were found in the percentage of fruit drop of Mandared grafted onto different rootstocks (Figure 5.7).

The trees on Swingle showed the highest ability to retain fruits on the tree, with an average of fruit drop of 20.0%, followed by the trees on C57, with an average fruit drop of 34.6%. Intermediate fruit drop rates were observed in Mandared grafted onto C54 (42.5%), C22 (43.1%), and C35 (51.0%), while the highest rates were found in Mandared grafted onto *P. trifoliata* (72.4%) and Troyer (77.1%).

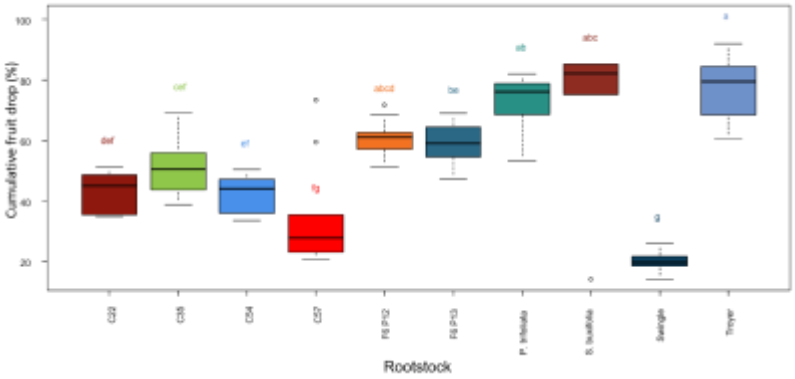

Figure 5.7. Percentage of fruit drop in Mandared grafted onto 10 rootstocks, recorded between 2016 and 2019. Different letters indicate significantly different means according to Tukey's test at $p < 0.05$. Different colors of the boxes indicate different significance groups

Regarding the rates of fruit drop in different years, we observed particularly severe events in 2017 and 2019, associated with two frost events that occurred a few days before harvest. Specifically, in early January 2017, we recorded two consecutive nights when temperatures were below 0 °C for at least 8 h, and minimum temperatures reached −4 °C and −3 °C for approximately 1 h, respectively. In 2017, the average fruit loss was close to 100% in Mandared grafted onto *P. trifoliata* and Troyer, 82% in Mandared grafted onto F6P12 and F6P13, and 73% in Mandared grafted onto C35, while the average fruit loss in trees on Swingle was less than 30%. A similar event occurred in 2019, with the minimum temperature reaching −2.5 °C for one night. In 2019, pre-harvest fruit drop in Mandared grafted onto Swingle was approximately 32%, while it was 76% in trees on C35, and fruit loss close to 100% was observed in Mandared grafted onto Troyer, *P. trifoliata* , F6P12, and F6P13. In 2016 and 2018 there were no frost event before harvest, and rates of fruit drop were less severe

(Supplemental Figure 5.1). However, the trees on Swingle and C57 always showed the best performance with regards to reducing the fruit drop rate. Fruit quality was analyzed in January 2017 and 2019 (Table 5.3).

Table 5.3. Qualitative analysis on Mandared fruits sampled in 2017 and 2019. Different letters indicate significant differences among rootstocks in each year as determined by Tukey multiple range test ($p \leq 0.05$)

Rootstock	TSS (°Brix)		Acidity (g L−1)		TSS/Acidity		Juice %		Anthocyanins (mg L−1)		Peel Color Index	
	2017	2019	2017	2019	2017	2019	2017	2019	2017	2019	2017	2019
C22	11.97	12.07	14.93	13.34	8.03	9.32	56.67	57.24 ab	10.47 a	9.88	8.59 a	6.61
C35	12.07	10.53	15.70	14.62	7.70	7.24	56.33	56.49 ab	3.16 b	10.23	7.56 b	6.43
C54	10.87	11.03	13.97	13.93	7.80	7.95	54.67	59.87 a	3.79 ab	5.26	7.74 ab	7.66
C57	11.00	11.57	14.70	13.16	7.47	8.86	59.40	56.07 ab	3.44 ab	4.87	7.48 b	6.46
Swingle	11.43	12.27	15.33	15.76	7.47	7.09	60.20	56.66 ab	3.87 ab	7.21	7.72 ab	5.83
F6P12	10.80	12.27	14.73	12.33	7.40	9.97	52.53	54.72 b	6.8 ab	10.52	7.89 ab	6.52
F6P13	11.40	10.73	14.97	15.27	7.63	7.19	55.50	54.92 ab	4.96 ab	4.19	8.07 ab	8.56
P. trifoliata	10.80	10.13	15.47	12.56	7.03	8.06	54.13	54.27 b	1.88 a	7.77	7.49 b	6.37
S. buxifolia	11.57	10.40	16.10	11.95	7.20	8.73	49.13	58.38 ab	5.60 ab	9.16	8.24 ab	6.23
Troyer	11.90	11.70	15.23	13.40	7.83	8.74	58.43	59.30 ab	5.07 ab	5.87	7.92 ab	6.18

Fruits were carefully selected and fruits that were damaged due to low temperatures were discarded. TSS ranged from 10.13 (Mandared grafted onto *P. trifoliata*) to 12.27 °Brix (Mandared grafted onto F6P12 and Swingle), and acidity ranged from 11.95 to 16.10 g L^{-1}. However, differences related to TSS and acidity were not statistically different (Tukey multiple range test; $p > 0.05$). The lack of significance might be due to an insufficient number of sampled fruits and/or replicate trees considered in the analysis. Significant differences related to anthocyanin accumulation, peel color index, and juice percentage were observed in one sampling year. Specifically, significant differences were found in juice percentage in 2019, and in anthocyanins and peel color index in 2017. In 2019, the TSS/acidity ratio was usually higher than in 2017. In 2019, the highest average TSS/acidity ratios were observed in Mandared grafted onto F6P12, C22, and C57.

Discussion

Effect of Rootstocks on Yield Precocity, Productivity, and Fruit Quality

In our study we evaluated the productivity of Mandared triploid mandarins in combination with 11 rootstocks during the first five years of production. The scion grafted onto different rootstocks showed significant differences in terms of yield precocity, cumulative production, and yield efficiency. Some fruit traits also varied among rootstocks, but only in one of the two sampling years in which fruit quality was analyzed.

Mandared grafted onto the rootstocks of the UCR breeding program (in particular C22, C57, and C35) started to set fruits one year earlier than Mandared grafted onto the other rootstocks. This result was previously reported by Continella et al. (2018), who performed a similar trial in combination with 'Scirè' Tarocco blood orange. The four UCR rootstocks were among the most productive. Moreover, C35 and C22 showed high yield efficiencies. The good performance of the new UCR rootstocks was previously reported in studies performed in the United States (Louzada et al., 2008; Castle et al., 2011), and these rootstocks appeared promising for use in the Mediterranean area (Continella et al., 2018). The trees on Troyer, which is among the most diffused rootstocks worldwide (Bowman et al., 2020) did not perform well, with about a half of the productivity the trees on C57. This clearly indicates that low productivities sometimes encountered in new citrus varieties may be corrected by choosing the appropriate rootstock. Mandared grafted onto the CREA hybrids (F6P12 and F6P13), which in previous trials showed high productivity (Kupper et al., 2010), were not among the most productive rootstocks. Moreover, the trees on F6P12 and F6P13 delayed their first production, so it is not advisable to use these rootstocks in combination with Mandared.

The trees on Swingle showed high productivity, but also had the highest variation among replicates. This variability may be attributable to the relatively high levels of active lime in some areas of the experimental farm at which our study was conducted. Swingle is known to be sensitive to iron chlorosis (Hutchinson et al., 1974) when active lime levels are around or above 4% (Kupper et al., 2010; Caruso et al., 2016), and iron chlorosis might have affected productivity.

Regarding fruit quality traits, statistically significant differences related to TSS and acidity were not observed. Mandared showed a level of TSS comparable to other mandarin cultivars but a higher acidity. The Mandared high acidity was already reported in a previous study (Reforgiato Recupero et al., 2005) and confers a tart taste similar to that of Tarocco blood orange, which is the male parent of this hybrid. Some fruit traits, such as anthocyanin concentration and juice percentage also differed among rootstocks, at least in one sampling year. Anthocyanin accumulation is known to be a critical trait for high quality fruits. The rate of anthocyanin accumulation is rather complex and variable among pigmented cultivars (Caruso et al., 2016). In addition to the genotype, this trait is highly affected by the environmental (low temperature during winter) and culture conditions, as well as by the rootstock (Continella et al., 2018). In our study, fruits of Mandared grafted onto C22 had the highest anthocyanin accumulation in 2017, and had a similar anthocyanin accumulation in 2019, while the year-to-year variability of the Mandared grafted onto the other rootstocks was higher. It would be worthwhile to investigate the rate of anthocyanin accumulation of Mandared grafted onto C22 in the following years, since a rootstock conferring high and stable anthocyanin accumulation would be desirable for the citrus industry. It will be also important to perform future investigations on a larger number of fruit samples during several years to clarify the influence of the rootstock on pigmented mandarin quality.

Influence of Rootstocks on Pre-Harvest Fruit Drop

Pre-harvest fruit drop is a phenomenon that is exacerbated by biotic and abiotic stresses (Albrigo et al., 2015; Tedeo et al., 2015) and is influenced by the scion genotype. Blood oranges, but also mandarin hybrids such as "Kinnow", are known to be particularly sensitive to pre-harvest fruit drop (Dettori et al., 1992; Nawaz et al., 2008). Fruit drop is one of the main disadvantages of Mandared. This characteristic is typical of Tarocco, which is the tetraploid pollen parent of Mandared. In Mandared, we noticed that the tendency to fruit drop is even stronger than in Tarocco, especially in the case of frost events.

An effect of the rootstock on pre-harvest fruit drop has been observed in previous studies (Sharma et al., 1999; Albrecht et al., 2016). In the present trial we noticed a strong effect of the rootstock on rates of fruit drop, which was particularly significant in cases of frost events occurring before harvest. In particular, Mandared grafted onto Swingle and C57 showed the best performance, and both might be suggested to reduce pre-harvest fruit drop in Mandared, whilst maintaining high productivity and fruit quality.

In conclusion, C57, C35, and C22 were the best performing rootstocks in our conditions (Figure 5.8).

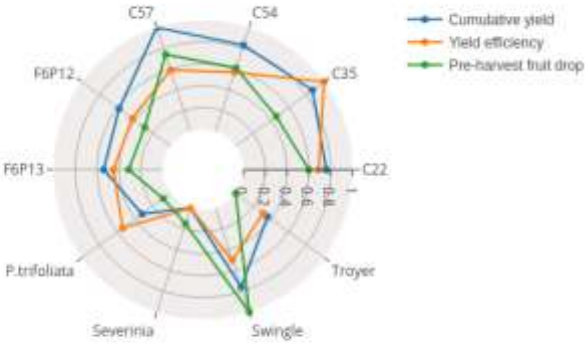

Figure 5.8. Radar chart showing the yield performance of the 10 rootstocks in combination with Mandared. Data were rescaled independently, normalizing the best performance to 1 and the worst performance to 0 for each parameter

Mandared onto these three rootstocks started to set fruits one year earlier than the others. The trees grafted onto C35 showed some of the highest production efficiencies, and started to produce fruit one year earlier than Mandared grafted onto standard rootstocks such as Troyer and Swingle. These results have implications for the cultivar profitability. The trees on Swingle had the strongest ability to avoid pre-harvest fruit drop, but also the highest variability among replicates. Mandared grafted onto C57 showed a significant reduction in rates of fruit drop, and resulted in one of the highest cumulative production. Mandared grafted onto C22 showed yield precocity and high yield efficiency, comparable to that of Mandared grafted onto C35. The reduced canopy volume of trees on C22 has been observed in previous studies (Siebert et al., 2010; Continella et al., 2018), and could be an advantage for new plantings with higher densities. In previous studies, C22 has been shown to be particularly tolerant to

high pH and soils with a high percentage of active lime (Louzada et al., 2008) as well as being tolerant to conditions of high salinity (Simpson et al., 2015). We tested this rootstock in a soil with moderate levels of active lime, where trees on F. dragon, *P. trifoliata*, and Swingle showed iron chlorosis symptoms. However, the conditions at our field site were not amongst the most extreme in terms of active lime percentages. Due to its overall good performance, and due to the need for alternatives to sour orange in high pH soils, it would be worthwhile to test C22 in soils with higher levels of active lime, which are often found in Italy and other Mediterranean citrus producing countries, such as Spain and Turkey. Taken together, the above results provide essential new information for the selection of rootstocks for new citrus orchards.

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Chapter 6 - Citrus rootstocks response to salt stress: evaluation of physiological, antioxidant and hormonal activity

Abstract

In many citrus growing areas several abiotic factors (such as drought, salinity, temperature stress) play an active role in influencing the fruit quality and yield. These abiotic stresses are particularly effective during summer, characterized by high temperatures and low water availability; therefore, the evaluation and identification of 'climate resilient' rootstocks coupling high tolerance to abiotic stress and good productive features represent a fundamental step. The aim of the research is the evaluation of 8 CTV-tolerant rootstocks subjected to salt stress: some are of those were recently released (Bitters, Carpenter and Furr) and showed good agronomical performance, while others were chosen for their sensitivity or tolerance to salt stress (C35 and Carrizo citrange, *Citrus macrophylla*, *Citrus volkameriana* and Swingle citrumelo).

Citrus rootstocks were irrigated with two levels of water salinity: mild (30 mM NaCl) and high (60 mM NaCl) salt stress in comparison with a control. A phenotyping approach was carried out, coupling morphological and physiological assessments with the evaluation of the antioxidant activity and the quantification of the proline and phytohormones in leaves. Results showed a similar osmotic response in leaf of *C. macrohpylla* and *C. volkameriana* and a high level of MDA in citrange rootstocks. The antioxidant activity and hormonal content revealed that Furr had a good aptitude to salt stress, confirming *C. macrohpylla* and *C. volkameriana* as salt-resistant rootstocks.

Introduction

Citrus crops are widely cultivated in Mediterranean basin, a region characterized by hot and dry summers often leading to annual drought stress conditions. In particular, such environmental conditions can lead to an accumulation of salts in the soil due to the low quality of irrigation water, characterized by excessive concentrations of soluble salts ($Cl^-$and/or Na^+) and with an electrical conductivity superior to 3 dS m^{-1} (Garcia-Sanchez et al., 2002; Syvertsen et al., 2014). High salinity in the root zone causes an osmotic effect, followed by a specific toxicity, derived from the accumulation of saline ions in plant tissues (Garcia-Sanchez et al., 2002; Arbona, et al., 2013). High concentration of ions in irrigation water lead to nutritional imbalances and toxicity effects on shoot. Several works highlighted that ion accumulation in plant tissue is dependent both by rootstock and scion (Garcia-Sanchez et al., 2002; García-Sánchez et al., 2006; Alam et al., 2020). Different species of citrus has a different tolerance level, that can be determined by their capacity to exclude toxicity ion, such as Na^+ and Cl^-. The concentration of these elements in citrus plant hampers several physiological and biochemical processes in the plant such as growth, tree architecture, fruit quality, etc. Accumulation of toxic ions in plant tissues also affects negatively hydraulic conductivity in plant, whereas in leaves, especially in chloroplast, causes a reduction in stomatal conductance, net assimilation of CO_2, transpiration rate, and determine a reduction of chlorophyll content, directly affecting photosynthetic activities, thus yield (Garcia-Sanchez et al., 2002; Moya et al. 2003; Alam et al., 2020). A growth reduction in Sunburst mandarin grafted onto Carrizo irrigated with salt water was reported, meanwhile leaf area was higher in plants grafted on Cleopatra. A general increase of Cl^- and Na^+ was observed in leaves and roots of both scion-rootstock combinations, but salinity induced

higher reductions in stomatal conductance (g_s), net assimilation of CO_2 and water use efficiency in Carrizo citrange compared to Cleopatra mandarin (Garcia-Sanchez et al., 2002). Several studies reported that Cleopatra mandarin and sour orange had greater salt tolerance than Swingle citrumelo and Carrizo citrange (Zekri and Parsons,1989; 1990). Simpson et al. (2014; 2015) compared the response to salt stress of Olinda Valencia orange grafted with different rootstocks, observing that C22 and C146 rootstocks were more tolerant to saline irrigation than the sour orange. A recent work by Aparicio-Durán and colleagues (2021) investigated on the capacity of tolerant citrus rootstocks of excluding Cl^- and Na^+ at 4 salt concentrations (25, 50 and 75 mM of NaCl compared to the control). Among the evaluated rootstocks, X639 was characterized by a similar plant growth under all salt treatments, whereas US942 and US897 reduced their growth only at the highest salt concentration.

The physiological effect of toxicity ion might be modulated by phytohormones, such as abscisic acid (ABA) and ethylene (Gomez-Cadenas et al., 1998; Arbona et al. 2006). Phytohormones regulates several aspects of plant growth playing an important role in the response of plants to abiotic stress (Peleg and Blumwald, 2011). Among them, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) are known to play a key role in plant tolerance against abiotic stress. Previous study investigated the behavior of Carrizo citrange under salt stress (60mM NaCl) in combination with high temperatures showing that ABA, JA and SA increased in response to high salinity (Balfagón et al., 2019). In addition, hormone accumulation seems to be responsible for the observed stomatal closure. Under salt stress the plant produces osmolytes (i.e.proline, glycine betaine, etc.) that are involved in maintaining the osmotic pressure. In addition, several oxidant-scavenging enzymes to protect the cellular systems from reactive oxygen species (ROS) are produced.

Among these, the most widely known are superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), whose increase under stress condition is used to estimate the salt tolerance (Khalid et al., 2021). Previous research found an increase on antioxidant activity in Kinnow mandarin grafted on tolerant rootstocks, such as Rangpur lime and Rubidoux, compared to scions grafted onto the sensitive Carrizo citrange and Sacaton citrumelo (Shahid et al., 2019). The increase in SOD, CAT and POD activity, as well as of antioxidant such as ascorbate peroxide (APx) and glutathione reductase (GR), at increasing salt concentration has been confirmed (Shahid et al., 2019). Generally, the response of citrus species to saline conditions is linked to the rootstock and their ability to exclude Cl^- and/or Na^+ from the plant tissue.

The objective of this study is to compare the behavior of new citrus rootstock (C35, Bitters, Carpenter and Furr) compared with others spread in Mediterranean basin (Carrizo citrange, Swingle Citrumelo, *Citrus volkameriana* and *Citrus macrophylla*) under two levels of salinity stress (30 and 60 mM NaCl). To this extent, morpho-physiological parameters were monitored, and the enzymatic and hormonal activities were measured to gain an evaluation of the rootstocks response to salt stress.

Material and methods

Plant material

Rootstocks of eight genotypes were used: Carrizo citrange (*Citrus sinensis* cv. Washington navel x *Poncirus trifoliata*), Citrus macrophylla, Citrus volkameriana, Swingle citrumelo (Citrus paradisi Macf. × *P. trifoliata* [L.] Raf.), selected for their sensitivity or tolerance to salt stress; C35 [*Citrus sinensis* (L.) Osb. cv. 'Ruby' x *Poncirus trifoliata* (L.) Raf.], Bitters (C22), Carpenter (C54), Furr (C57) (all three are hybrids of Citrus sunki x *Poncirus trifoliata*,

recently released by the University of Riverside, California (Federici et al., 2009), that were chosen among the most promising rootstocks. Seeds were obtained from the experimental farm of University of Catania and were disinfected for 10 min in a 1% sodium hypochloride solution. Then, seeds were rinsed in water, immersed in a 1% 8-hydroxyquinoline for 30 sec, dried, and stored at 4 °C until use. Seeds were sown into premoistened substrate composed by peat, coconut fibre, sand, and perlite (50;25:20:5) and maintained in greenhouse (mean temperatures were 27 °C, whereas relative humidity was 66%). During growth, plants were irrigated and fertilized as needed, in accordance with standard nursery practices to optimize growth and avoid inhibition of plant growth. After 90 days plants were transferred to plastic pots (Ø 20 cm) and watered twice per week with Hoagland solution (Hoagland and Arnon, 1950) until the beginning of the experiment.

Treatments

Ten one-year-old plants, homogeneous in size, were carefully selected for each rootstock per treatment. Plants were exposed to various salinity levels (control, 30 and 60 mM NaCl) by dissolving the specific quantities of pure sodium chloride to irrigation water (9 mM). Twice a week the same water amount was supplied for all treatments calculated by means of gravimetric water loss from 185 to 262 day of the year (DOY).

Stress assessment

To dissect the response of the eight rootstocks to salt stress, physiological and morphological parameters were monitored, while enzymatic and hormonal activities were measured at the end of the trial.

Physiological determinations

Gas exchange measurements, chlorophyll fluorescence and water potential were measured from the beginning of the trial every 15 days. Leaf transpiration (E, mmol H2O $m^{-2}s^{-1}$), photosynthesis (A, µmol CO_2 $m^{-2}s^{-1}$) and stomatal conductance (gs, µmol CO_2 $m^{-2}s^{-1}$) were measured with LCi, ADC Bioscientific Ltd., Hoddesdon, UK. Measurements were made on clear sunshine hours between 8:00 h and 10:00 h (solar time) on fully developed leaves (3 replicates per treatment for each rootstock).

Xylem water potential (Ψ, MPa) was measured using a pressure chamber (PMS 600, PMS Instruments, Corvallis, OR, USA). Ψ was measured on 3 shoots for each rootstock per treatment between 8:00 h and 10:00 h (solar time).

To account for the fluctuations in tree water status, Ψ values were integrated over the monitored period. Thus, the area of the trapezoid below the measured Ψ values was calculated using the following equation:

$$\text{Cumulative } \Psi = (\Psi t_1 + \Psi t_2) + (t_2 - t_1)/2$$

where Ψt_1 and Ψt_2 are the Ψ values at the two dates of the measurement interval ($t_2 - t_1$). Integrated Ψ values were obtained by dividing the cumulative Ψ by the total number of days between each sampling interval.

Chlorophyll fluorescence was measured using Handy Pea (Hansatech Instruments Ltd Narborough, United Kingdom) on the same leaves of the other physiological assessments. Leaves were first dark-acclimated with a "leaf clip" for at least 30 min to inhibit all light-dependent reactions by completely oxidizing PSII electron acceptor molecules. The Fv/Fm ratio, used to express the chlorophyll a fluorescence, was calculated according to Schreiber et al. (1986).

Morphological analysis and total chlorophyll content

Immediately after the end of the experiment, 10 plant for each rootstock per treatment were transported to the laboratory for morphological analysis. Leaf area (cm^2) and length of the roots (cm) were determined. For the analysis on leaf, shoot and root biomass, the plants were washed with distilled water and dried with filter paper. The fresh weights (FW) of the roots and shoots were recorded, and samples were placed in an oven at 70 °C for four days to determine the dry weight (DW). Shoot/root ratio was determined as the ratio of shoot and leaf dry weight (g) and root dry weight (g). The specific leaf area (SLA) was determined as the ratio of the leaf area (cm2) to the leaf dry weight (g). The total leaf area (TLA) of the tree was calculated by multiplying SLA with the total leaf dry mass of the tree. The SRL was determined as the ratio of the fibrous root length (m) to its dry weight (g).

The chlorophyll content of the leaves were assessed following the procedure described by Inskeep and Bloom (1985), by extracting 20 mg of the ground material with N,N-dimethylformamide and measuring the absorbance at 664.5 and 647 nm. The following equation was used for the determination of total chlorophyll content:

$$\text{Total Chl} = 17.90\ A_{647} + 8.08\ A_{664.5}$$

Antioxidant enzyme activities (APX, CAT, SOD)

At the end of the trial, leaves were harvested from ten plants and were crushed in liquid nitrogen to extract proteins. Plant tissues (0.5 g) were homogenized in 5 mL of potassium phosphate buffer 50 mM (pH 7.8), 1 mM EDTA, 1mM DTT, 1% PVP w/v, and 1 mM PMSF. Samples were filtered with 3 layers of gauze, and centrifuged at 15,000 rpm for 30 min at 4 °C. The resulting supernatant was recovered, and the total proteins were precipitated with solid $(NH_4)_2SO_4$ at 55% of saturation. Enzymatic activities were performed by using the total

protein extract from leaves. Enzymatic aliquots were centrifuged at 13,000 rpm for 30 min at 4 °C, the supernatant was discarded, and the pellet was dissolved in 4 mL 50mM sodium-phosphate buffer (pH 7.0) containing 1 mM EDTA, 1% (w/v) PVP-40 (w/v) and 1 mM PMSF (Donahue et al., 1997). Ascorbate peroxidase (APX, EC 1.11.1.11) was measured according to Ushimaru et al. (1997) by assessing the decrease in absorbance at 290 nm, defining one unit (U) of APX equal to 1 mmol mL^{-1} ascorbate oxidized min^{-1} at 20 °C. Catalase (CAT, EC 1.11.1.6) was determined as described by Aebi (1984), by measuring spectrophotometrically (240 nm) the decomposition of H2O2. To avoid a rapid decrease of the initial velocity of the reaction, the assay was conducted using low concentrations of H_2O_2 (<0.05 M). The amount of enzyme able to decompose 1 mmol of H_2O_2 per minute represents one enzyme unit (U). Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) according to Masia (1998). One unit of activity (U) was defined as the amount of enzyme that would inhibit 50% of NBT photoreduction at 560 nm. The total protein content was routinely determined by the Bradford (1976) method, using BSA as a standard curve.

Determination of proline and MDA

Proline was determined spectrophotometrically (520 nm) following the ninhydrin method as reported in Puglisi et al. (2019). Proline was determined spectrophotometrically following the ninhydrin method of Bates et al. (1973) modified by Khedr et al. (2003). Briefly, the frozen citrus leaves (1 g) were homogenized in 3% aqueous sulphosalicylic acid and the residues were removed by centrifugation at 12,000× g for 10 min. The supernatant (1 mL) was mixed with 1 mL of glacial acetic acid and ninhydrin reagent in a 1:1 (v/v) ratio. The reaction mixture was incubated at 100 ◦C for 1 h. After

extraction with toluene, the absorbance of the organic phase was read at a wavelength of 520 nm, using toluene as a blank. The proline concentration was determined from a standard curve using D-proline.

The malondialdehyde (MDA) content was determined was calculated by Heath and Packer (1968) modified.

$$MDA\ equivalents\ nmol\ mL^{-1} = (A532 - A600/155000) \cdot 106$$

532 nm is the absorbance peak of the TBA (Thiobarbituric Acid) - MDA complex

600 nm is the correction factor for non-specific turbidity

155000 the molar extinction coefficient of MDA

Hormone analyses

Hormone extraction was carried out with 0.15 mg of freeze-dried leaves. They were extracted in 1 ml of ultrapure water after spiking with 25 ug of ISTD solution (ABA-d6, DHJA, IAA-d5, 13C6SA), in a ball mill (MillMix20; Domel, Železniki, Slovenia). After centrifugation at 4000 g force and 4 °C for 10 min, supernatants were recovered and pH-adjusted to 3 with 30% acetic acid. The water extract was partitioned twice against 2 ml of diethyl ether and the organic layer recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac; Jouan, Saint Herblain Cedex, France). Once dried, the residue was resuspended in a 9:10 H_2O:MeOH solution by gentle sonication. The resulting solution was filtered through 0.22 μm polytetrafluoroethylene membrane syringe filters (Albet S.A., Barcelona, Spain), diluted 1:4 and directly injected into an ultraperformance LC system (Acquity SDS; Waters Corp., Milford, MA) connected online to a TQS triple quadrupole mass spectrometer (Micromass, Manchester, UK) through an orthogonal Z-spray electrospray ion source. Chromatographic separations were carried out on a reversed-phase C18 column (Luna Omega Polar C18, 50 × 2.1 mm, 1.8-μm particle size; Phenomenex, Torrance, CA, USA) using a

acetonitrile: water (both supplemented with formic acid at a concentration of 0.1%) gradient at a flow rate of 300 µl min^{-1}. Hormones were detected following their specific precursor-to-product ion transition and quantitated using an external calibration performed with injection of standard solutions of known amount.

Statistical analysis

Statistical analyses were performed using STATISTICA 6.0 (Statsoft Inc., Tulsa, OK) and used to test the significance of each variable ($P \leq 0.05$). A basic descriptive statistical analysis was followed by an analysis of variance test for mean comparisons. The method used to discriminate among the means (Multiple Range Test) was Fisher's Least Significant Difference (LSD) procedure at a 95.0% confidence level.

Results

Overall results highlighted a wide variability among the rootstocks tested in response to the two levels of imposed stress. Results are detailed in the following paragraphs.

Physiological determinations

During the experimental trial, net photosynthesis (A) generally showed a progressive reduction at the increase of the salinity levels, especially in Bitters and *C. volkameriana*. At 30 Mm NaCl, A increased significantly with respect to control in C35 (+ 100%) and *C. macrohpylla* (+100%); also Carrizo citrange and Swingle citrumelo showed an increase in A values, (+ 58.1% and + 50.2%, respectively) (fig. 6.1), while a decrease was recorded in *C. volkameriana* (- 59.1%), in Carpenter (- 42.8%) and in Bitters (- 84.6%). Photosynthesis of Furr and Bitters decreased at 60 mM NaCl, (-89.9% and -84.6%, respectively), while, Carpenter showed the lowest increase (+ 14.3%).

The transpiration rate (E) and stomatal conductance (g_s) were also modified by the salinity levels. At 262° DOY, the reduction of A value in Bitters and *C. volkameriana* was also confirmed by the reduction of E (-66.6% and -70.1%, respectively), as reported in figure 6.2. The highest increase was found in Swingle citrumelo and C35 (Fig. 6.1), while Furr had a lower increase (+ 8.1%). Regarding gs, during the salt treatment, a progressively decrease was recorded in all rootstocks especially in the hottest days (fig. 6.3). At 185° DOY, 199° DOY and 233° DOY $g_s$decreased sharply in *C. macrohpylla* at 30 Mm NaCl, even if a great increase with respect to control was recorded at the end of the trial (DOY 244 and 262). At 262 °DOY, Bitters, Furr and *C. volkameriana* showed a greater decrease in g_s value than the other rootstocks.

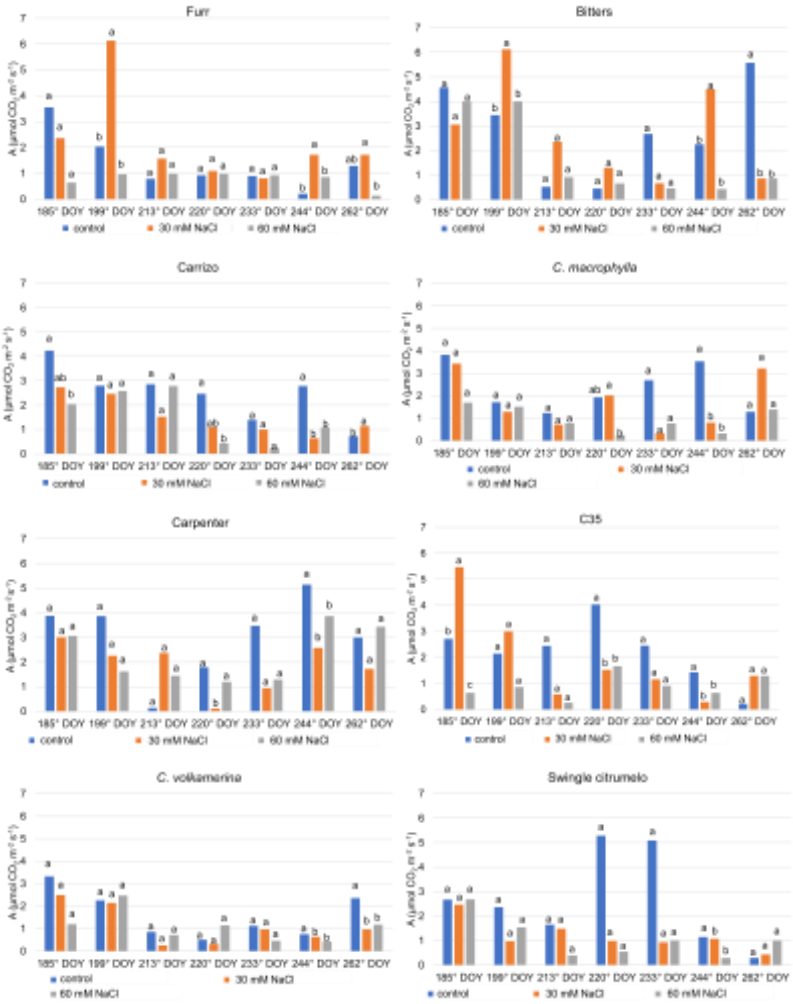

Figure 6.1. Monitoring of net photosynthesis (A, µmol CO_2 m^{-2} s^{-1}) in the different rootstocks under saline stress. Data are mean of values (n=3); different letters indicate statistically significant differences (p≤0.05) for each date

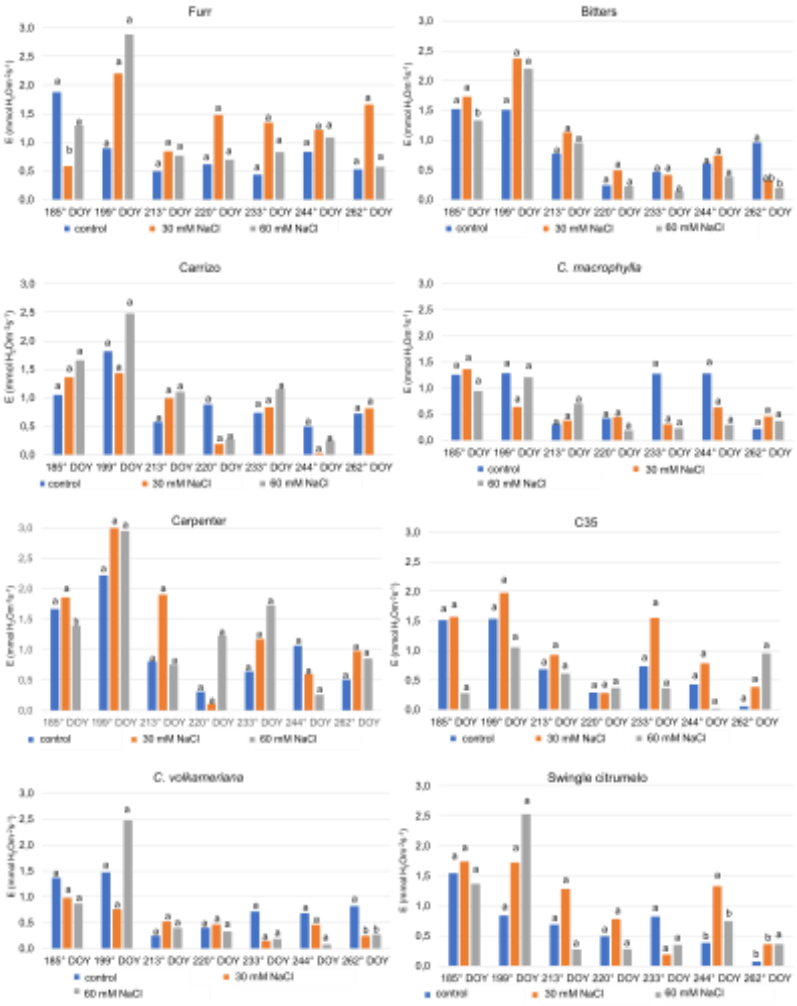

Figure 6.2. Monitoring of transpiration (E, mmol H_2O m^{-2} s^{-1}) in the different rootstocks under saline stress. Data are mean of values (n=3); different letters indicate statistically significant differences (p≤0.05) for each date

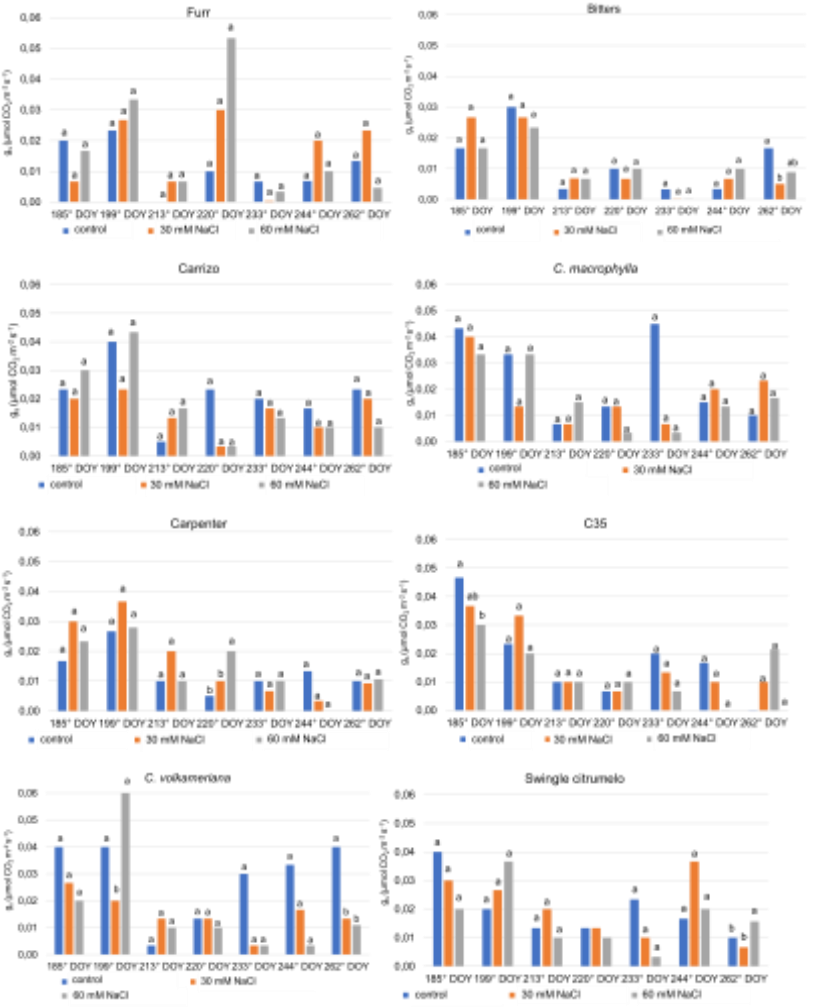

Figure 6.3. Monitoring of stomatal conductance (g_s, µmol CO_2 m^{-2} s^{-1}) in the different rootstocks under saline stress. Data are mean of values (n=3); different letters indicate statistically significant differences (p≤0.05) for each date

The maximum quantum yield of PSII (Fv/Fm) in dark-adapted leaves is shown in figure 4. At the end of salt treatment, Fv/Fm in Citrumelo and *C. volkameriana* at 30 mM NaCl was reduced by 2.0% and 4.13%, respectively, with respect to control. This salt stress increased Fv/Fm in Carpenter (0.6%), *C. macrohpylla*, Carrizo (1.26%), Bitters (3.6%) and Furr (6.0%) with respect to control. At 60 mM NaCl, C35, Bitters, *C. macrohpylla* and Swingle Citrumelo showed a decrease in Fv/Fm (-20.0%, -7.5%, -2.0%, -0.8%, respectively) than the control.

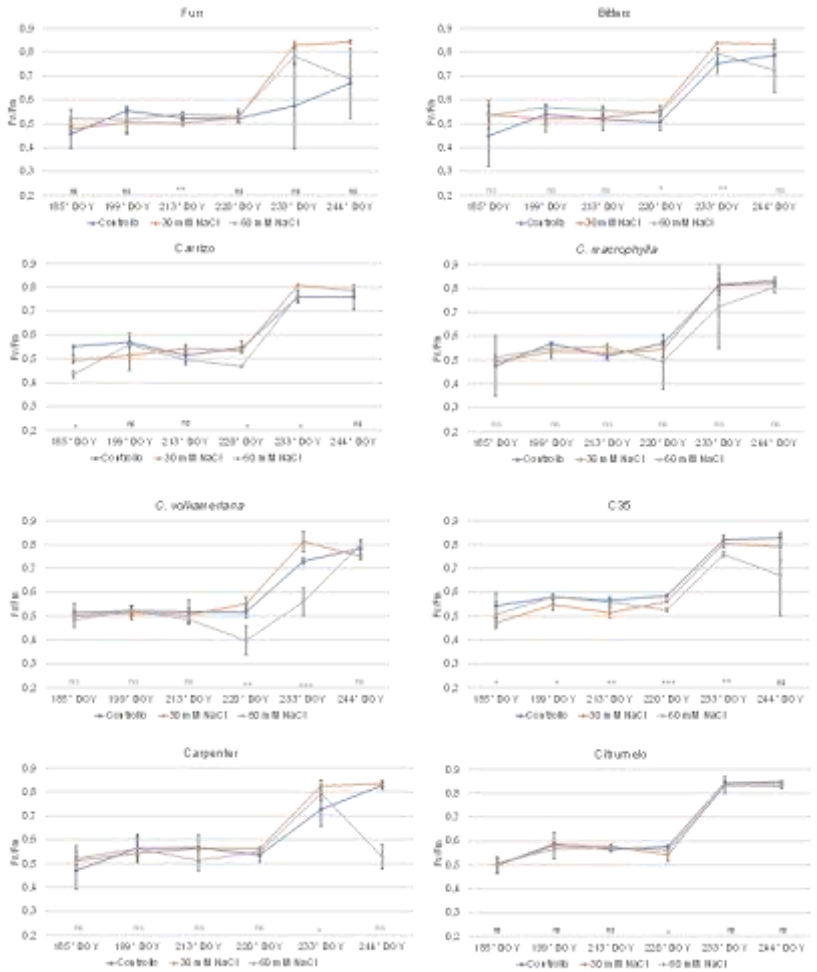

Figure 6.4. Monitoring of maximum quantum yield of PSII (Fv/Fm) in the different rootstocks under saline stress. Data are mean values (n=3) ± standard error. *** p<0.001, ** p<0.01, * p<0.05, ns=not significant.

Xylem water potential generally increased in all the studied genotypes under salt stress (figure 6.5). At the end of the trial, Carrizo

citrange (31.8%), C35 (30%) and Swingle citrumelo (29.4%) had a significant increase with respect to the control at 30 mM NaCl, even if no statistical difference was found. The lowest increase was recorded in Bitters and Carpenter (6.2% and 6.5%, respectively). At 60 mM, Swingle citrumelo had the highest increase (79.4%), followed by C35 (53.6%) and *C. macrohpylla* (68.2%). However, in C35 no statistical difference was found, meanwhile the values registered for *C. macrohpylla* during 60 mM NaCl were statistically different from those registered for 30 mM NaCl and the control.

Some remarkable differences were recorded between genotypes at the end of the trial; at 30 mM NaCl and 60 mM NaCl, Furr was statistically different compared to the control, and a growth of 27% was recorded for the two stress levels. Even though Bitters and Carpenter recorded an increase of 10.4% and 16.3% with increasing salt, no statistical difference was observed between the value.

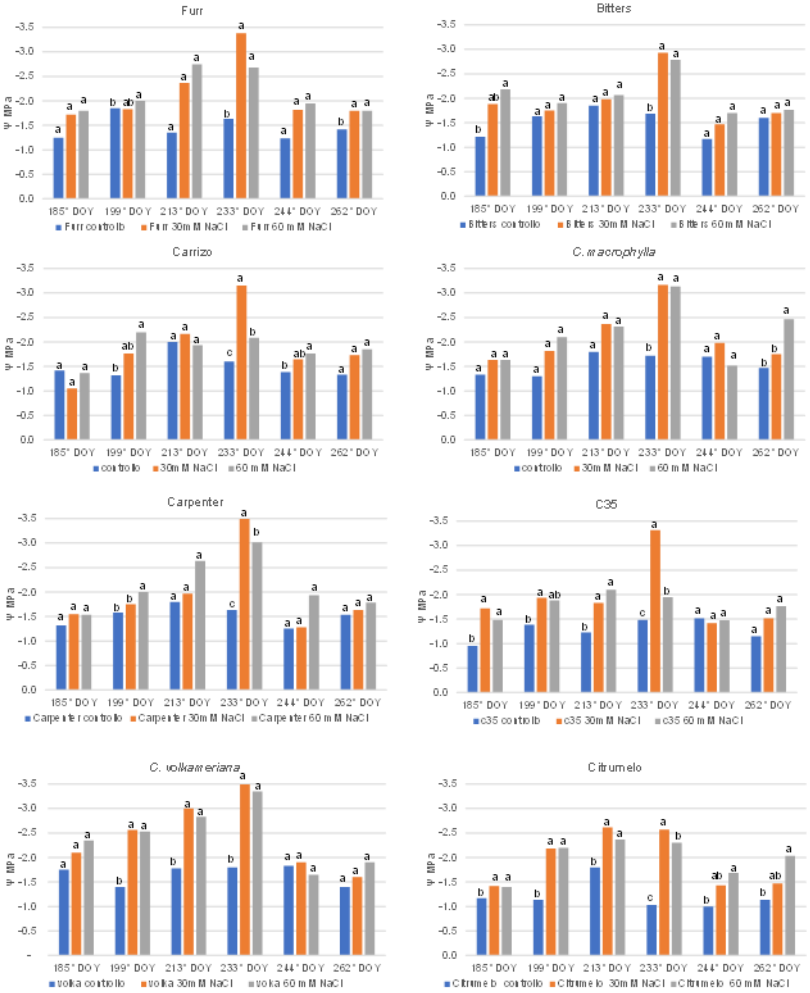

Figure 6.5. Monitoring of xylem water potential (Ψ, MPa) in the different rootstocks under saline stress. Data are mean of value (n=3); different letters indicate statistically significant differences (p≤0.05) for each date

Cumulative xylem water potential (Ψ, MPa*d) evidenced both in 30 and 60 mM NaCl a different response between the rootstocks and also compared to the control: the rootstocks with lowest values in the control were *C. volkameriana* and *C. macrohpylla* (–107 and –100 MPa*d, respectively).

At the end of the trial at 30 mM, cumulative Ψ values were –162, –140 and –138 MPa*d for *C. volkameriana*, Furr, and *C. macrohpylla*, respectively, while the rootstocks with less negative values were Bitters (–125 MPa*d), Carrizo (–126 MPa*d) Carpenter and C35 (both –127 MPa*d). At high salinity levels (60 mM NaCl) cumulative Ψ values were –159, –141 and –140 MPa*d for *C. volkameriana*, Furr, and *C. macrohpylla*.

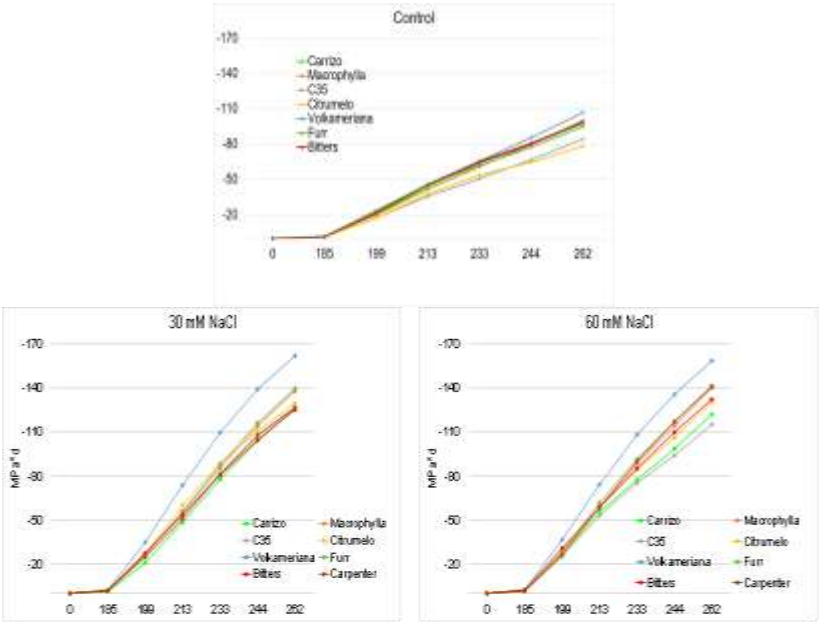

Figure 6.6. Monitoring of cumulative xylem water potential (Ψ, MPa*d) integrated with fortnight intervals in the different rootstocks investigated

Morphological analysis and total chlorophyll content

At the end of the experiment, all rootstocks showed a reduction in the specific leaf area (figure 6.8). Carrizo citrange showed the highest reduction (- 91.4%) than the control at 30 mM NaCl, while Bitters the lowest (- 59.8%), followed by Carpenter and *C. volkameriana*
(-60.8% and - 60.9%, respectively). At 60 mM NaCl, *C. volkameriana* reduced the specific leaf area by 80.9% compared to the control, meanwhile the highest reduction was recorded in Furr (- 97.7%). Regarding specific root length at 30 mM NaCl, Carpenter showed the highest reduction (-36%) compared to the control, while Furr displayed the highest increase of specific root length (58.8%) and root/shoot ratio (49.3%). At 60 mM NaCl, Carpenter showed the highest reduction in specific root length (-38.2%), with similar values at 30 mM NaCl. Salt stress significantly reduced root length of C35 in both salt stress levels (- 28% and - 32.2%). A decrease in root length of plant of *C. macrohpylla* (-16.1%), Carpenter (-20.5%) and *C. volkameriana* (-6.7%) was found, meanwhile Carrizo and Furr recorded an increase of root length at 30 mM NaCl (10.5% and 8.8%). All rootstocks had a decrease of leaf area under salt stress except Bitters at 30 mM NaCl where leaf area increased (14.2%). A general decrease in total chlorophyll was found with increasing salt stress. At 30 mM NaCl, the maximum decrease was found in Carrizo (-37.8%), although no statistical differences were observed between the control and 60 mM NaCl values. An increase was recorded in C35, Furr and Carpenter (71.6%, 42.3% and 13.7%, respectively). At 60 mM NaCl, *C. macrohpylla* had the highest value of total chlorophyll content (27.1 µg cm^{-2}), although no statistical difference with the control was observed.

Salt stress for 3 months affected the growth and influenced, in particular, the defoliation of the rootstocks studied. At the end of the

experiment, it is worth noting that 30 mM NaCl stressed plants presented a higher number of leaves in Bitters, Carpenter, Furr and Citrumelo when compared to the relative control plants. Moreover, a decrement was observed for *C. volkameriana*, Carrizo, C35, *C. macrohpylla* (-42.1%, -41.7%, -22.6% and -9.3%). A particular response was observed in Bitters that more than doubled the number of leaves at 30 mM NaCl, while a little increase was found at 60 mM NaCl. Furr and Bitters were the only rootstocks that had an increment at 60 mM NaCl and Furr showed an increase in leaves at both salinity levels (39.2% at 30 mM NaCl and 31.2% at 60 mM NaCl. At 60 mM NaCl Carrizo had the highest decrement due to the leaf fall (-93%), followed by *C. volkameriana* and C35 (-84% and -79%, respectively).

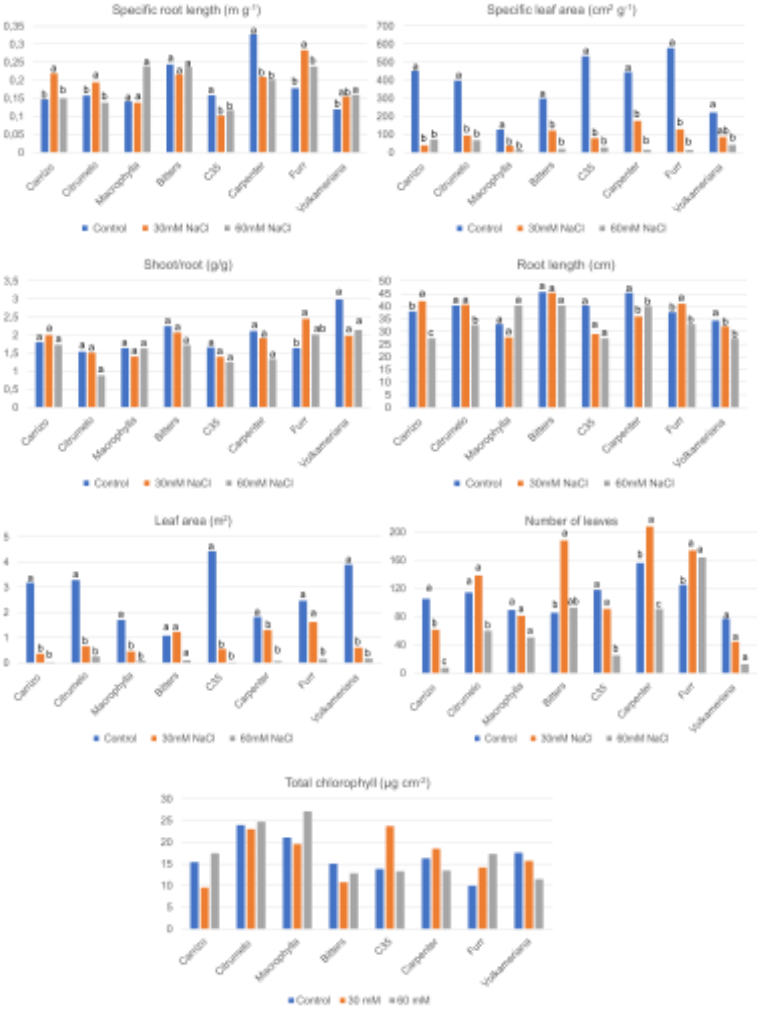

Figure 6.8. Morphological characteristics and total chlorophyll content of rootstocks during salt stress treatment. Data are mean of values (n=3); different letters indicate statistically significant differences (p≤0.05) for each date

Antioxidant enzyme activities (APX, CAT, SOD)

Exposure to salinity increased the antioxidant activities (figure 6.9). The activity of APX increased more in C35 at 30 mM NaCl and in *C. macrohpylla* at 60 mM NaCl (208.9 and 135.4 µmol of ascorbic acid/mg of protein min, respectively). A decrease was recorded in Bitters rootstock at both salinity levels (-115.5 and -92.0 µmol of ascorbic acid/mg of protein min, respectively). In the 30 mM NaCl treatment the highest value of CAT was observed in Swingle citrumelo (11.8 mmol of H_2O_2/mg of protein min), followed by C35 (4.0 mmol of H_2O_2/ mg of protein min). A little increase was recorded in Bitters (2.14 mmol of H_2O_2/mg of protein min) at 30 mM NaCl with respect to the control (2.0 mmol of H_2O_2/mg of protein min). At 60 mM NaCl Carrizo citrange showed the highest value of CAT (4.68 mmol of H_2O_2/mg of protein min). Regarding SOD activity a decrease was recorded in C35 (- 1.6%), citrumelo (-47.4 %) and Carpenter (-83%) at the highest salt treatment. A high increment of SOD was observed in Bitters at 30 mM NaCl (+39.7%), meanwhile it decreased at 60 mM NaCl (-83.2%).

Determination of proline and MDA

Exposure to salinity significantly increased the content of proline only at 30 mM NaCl in both citranges, *C. macrohpylla*, Carpenter, *C. volkameriana* and Furr. At 60 mM NaCl the highest proline contents were observed in Swingle citrumelo (13.9 mol g^{-1}), followed by Bitters, *C. macrohpylla* and *C. volkameriana* (8.2, 6.4, and 4.3 mol g^{-1}, respectively). The salt-susceptible rootstocks, such as C35 (3.1 mg g^{-1}) and Carrizo citrange (2.0 mg g^{-1}) showed the highest value of MDA at 30 mM NaCl and 30 mM NaCl, respectively. A progressive increase at increasing salt levels was found in Bitters and *C. macrohpylla*, even if these differences were not significant with respect to the relative controls.

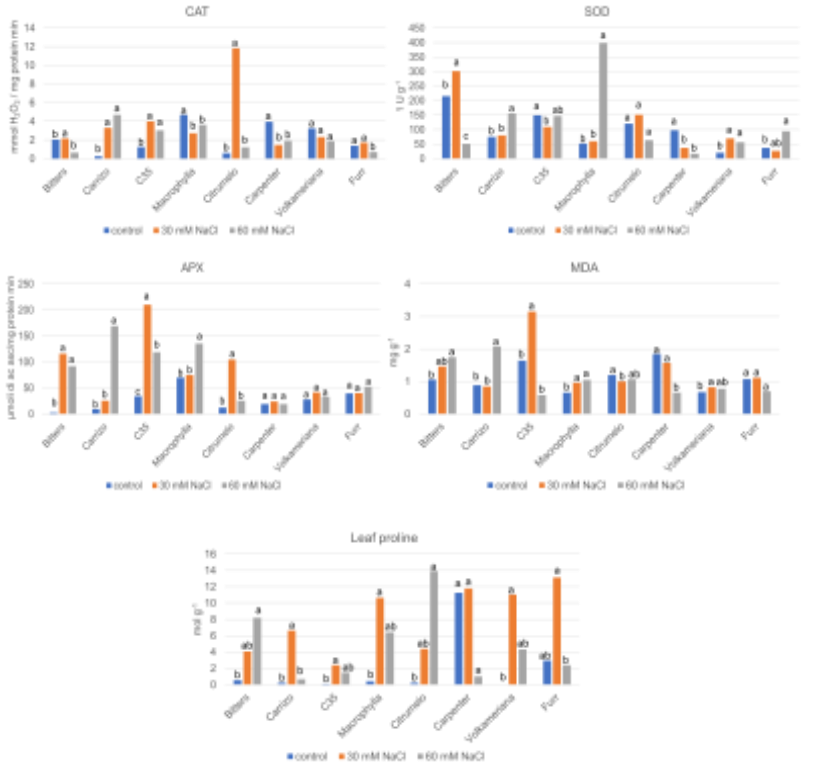

Figure 6.9. Measurements of osmolyte in the leaves and activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and malondialdehyde (MDA) in rootstocks during salt stress treatment. Data are mean of values (n=6); different letters indicate statistically significant differences ($p \leq 0.05$) for each date

Hormone analyses

Leaf concentration of ABA and of the product of its catabolism, PA, were analyzed (figure 6.10). At increasing salinity, the amount of ABA increased in leaves reaching statistical differences at 60 mM NaCl in Swingle citrumelo, *C. macrohpylla* and *C. volkameriana*. The highest values of ABA being recorded in C35 at 60 mM NaCl, although no statistical differences were found between control and 30 mM NaCl treatments. In general, ABA increased in salt-stressed plants, except for Bitters, Carpenter and Furr.

Regarding PA, the lowest values were recorded in *C. volkameriana* at 30 mM NaCl and in *C. macrohpylla* at 60 mM NaCl. The latter had a reduction of 37.9% with respect to control, meanwhile in *C. volkameriana* a reduction (- 24.5%) was found.

Leaf concentrations of JA and of the conjugate JA-lle were measured in the rootstocks leaves. JA levels increased in Furr (59.8%), Carrizo (24.9%) and C35 citranges (22.3%) at 30 mM NaCl. At both salinity levels a significant reduction of JA-lle in Carpenter (4.5% and 4.8%, respectively) was found, respect to controls. Conversely to ABA, JA levels decreased upon salt stress, whereas JA-lle did not show any significant variation except for C35 and Furr which showed a slight increase and Carpenter which exhibited a slight decrease.

No clear trend was found for SA, Swingle citrumelo, *C. macrohpylla* and *C. volkameriana* showed a significant accumulation under the highest NaCl concentration, but C35 showed a high accumulation of this hormone at 30 mM NaCl and much lower at 60 mM NaCl. The inactive form of SA, SAG was higher in those genotypes which did not show any significant variation of the free form, whereas it was not different in *C. volkameriana*, Swingle citrumelo and *C. macrohpylla*, probably related that the glycosylation pathway was active in the former. In general, no differences in IAA content were recorded for most citrus rootstocks analyzed, except for

Carrizo citrange, Bitters and Furr, which accumulated IAA in response to salt stress to a different extent. Carrizo citrange accumulated the highest levels upon the two salinity levels, whereas Bitters and Furr showed a significant increase of IAA only at 60 mM NaCl.

Figure 6.10. Quantification of hormones contents in rootstocks during salt stress treatment. Data are mean of values (n=6); different letters indicate statistically significant differences (p≤0.05) for each date

Discussion

Physiological determinations

Our results highlighted that the assimilation of CO_2 and stomatal conductance decreased at increasing levels of salt concentrations confirming that these physiological responses are rootstock-dependent, probably due to ions accumulation in plant tissue (Lloyd et al 1990; Garcia-Sanchez et al., 2002). Salt tolerant rootstock limit the translocation of the toxic ions into the leaves, and the closing of stomata reduce leaf transpiration, allowing the acclimation of the plant to the osmotic stress in the root zone (Syvertsen and Smith, 1983; Nieves et al., 1991). Previous studies reported that g_s lowered concomitantly with A (Syvertsen and Garcia-Sanchez, 2014). After all, during salt stress decrease in leaf area and changes in chlorophyll fluorescence, indicating a possible impairment of the electron transport chain (Lopez-Climent et al., 2008). In the present work, E value under saline stress decreased after 1 month in *C. macrophylla*, Carpenter and *C. volkameriana*. This behavior probably indicates some tolerance to the saline stress due to a faster osmotic adjustment in the tolerant rootstocks, as reported in other work (Moya et al, 2003). At 233° DOY, stomatal closure (g_s) was also observed in *C. macrophylla*, Carpenter and *C. volkameriana*, and it was probably due to ABA regulation (Gòmez- Cadenas et al., 1998).

The primary processes affected by ionic and osmotic stress is photosynthesis. Our results indicated a reduction in the photosynthetic values in Carrizo citrange in response to salt stress. Our results confirmed the high salt-sensitivity of Carrizo rootstock, as also reported by Perez et al. (2007), due to its capacity to accumulate excessive levels of toxic ions into the leaf, reaching toxicity levels and severely deregulating the photosynthetic apparatus (García-Sánchez et al., 2002).

Salinity at 60 mM NaCl affected fluorescence determining a decrease of Fv/Fm values in all rootstocks, except in *C. volkameriana* and Furr. These results showed that salt stress negatively affects the maximum PSII efficiency, as confirmed by Lopez-Climent et al., 2008. Rootstocks could modulate ions uptake based on nutrient availability, and they could also regulate Cl^- transport from root to shoot (Garcia-Sanchez et al., 2000) by reducing the Cl^- content in the root xylem (Brumos et al., 2010). In our trial, xylem water potential increased during the treatment in all rootstocks. This result could be explained because the salt tolerance in some genotypes seems to be related to the imbalance between Cl^-, Na^+ and K^+ ions. In particular, an increase in the absorption of K^+ and a higher transport speed can delay damage to plants, as observed among the salt-sensitive and tolerant Carrizo citrange and Cleopatra mandarin (Brumos et al. 2009).

Morphological analysis and total chlorophyll content

High salinity in irrigation water can reduce water uptake and tree growth causing nutritional imbalances and toxicity effects of Cl- and Na+. In our experiment, the highest reduction in growth was registered for plants subjected to 60 mM NaCl. Furthermore, the accumulation of toxic ions in salt stressed leaves was related to a reduction in leaf chlorophyll content for all rootstocks excepted for C35, Carpenter and Furr. A general decrease was found in leaf area and specific leaf area in all rootstocks at 60 mM NaCl. However, Bitters showed a slight increase in leaf area at 30 mM NaCl, probably inheriting the salt tolerance from Sunki mandarin parent. In fact, Bitters, Carpenter and Furr are a cross of Sunki mandarin with Swingle trifoliate orange, and another author reported that Sunki mandarin is highly tolerant to salt stress (Spiegel-Roy and Goldschmidt 1996).

Previous study reported that the decrease of leaf area of Carrizo

citrange was related to reduction in leaf gas exchange parameters due to a high accumulation of Cl^- and Na^+ in leaves (Garcıa-Sanchez et al., 2002). Regarding root length, a decrement in all rootstocks at 60 mM NaCl was observed, except for *C. macrophylla*, that also showed an increase of shoot/root ratio due to its high vigor. This result is in agreement with a study carried out on lemons grafted on *C. macrophylla* showing a greater vegetative growth development under salt stress compared to other rootstocks (Nieves et al., 1990). Differences in rootstock development in response to salinity can be associated with the growth characteristics of rootstocks. In our study, specific root length increased with increasing saline stress in *C. volkamerina*, Furr and Carrizo. Previous results reported that citrange Carrizo and Cleopatra mandarin are slow-growing rootstocks, whereas sour orange and *C. macrophylla* grow vigorously; this is reflected in the inherent partition of biomass between shoots and roots (Ruiz et al., 1997). In fact, regarding shoot/root ratio, *C. macrophylla*, Carpenter, C35 and Bitters value decreased with increasing salt stress because the root biomass was lower respect to the biomass of the shoot. Ruiz et al. (1997) reported that sour orange and *C. macrophylla* favored a high biomass in shoot, thus ensuring increased light interception, and consequently, increased growth. Our result confirmed that Carrizo citrange had a little increase (10.7%) in root/shoot ratio because it invested relatively more biomass in roots (Ruiz et al., 1997). Regarding the number of leaves, a decrement was observed in Carrizo, *C. macrophylla*, C35 and *C. volkameriana* with increasing salt concentrations, as previously showed by Moya et al. (2003). The high defoliation rate is probably due to the Cl^- absorption that is then translocated to the leaves by transpiration stream, and the high ionic concentration is likely the responsible of the high necrosis and defoliation rates.

Antioxidant enzyme activities (APX, CAT, SOD)

Under salt stress citrus plants increase antioxidative enzyme activities and production of bioactive compounds, providing a higher tolerance, which allows also to estimate the salt tolerance of a genotype (Ashraf et al., 2001). Regarding antioxidant activity, Hadian-Deljou and colleagues (2020) showed an increase of SOD activity at increasing salinity. On the contrary, our results showed similar values of enzymatic activities (CAT, APX and SOD) to those registered in the control, except for Furr at 60 mM NaCl, in which an increase of SOD activity was found.

Determination of proline and MDA

Plants under salt stress usually show a reduction in stomatal conductance, net photosynthesis, transpiration. In parallel, salt stress is associated with an increase in accumulation of carbohydrates (Arbona et al., 2005; García-Sánchez and Syvertsen, 2006) and of ROS, both important indicators of stress in plants (Mittler, 2017). To evaluate the cell membrane damage following salt stress, MDA contents were measured, as it represents one of the final products produced by the peroxidation of polyunsaturated fatty acids of membrane cell. Previous results reported high levels of H_2O_2 and MDA in highly stress sensitive plants (Demiral and Türkan, 2005; Hussain et al., 2018; Oustric et al., 2018). Khalid and coll. (2020) showed that *C. volkameriana* (2x) was more sensitive to salt stress than the tolerant tetraploid genotype with higher H_2O_2 and MDA content in leaf and root tissues. Our result confirmed high levels of MDA in salt sensitive rootstocks, such as C35 and Carrizo citranges, both at 30 and 60 mM NaCl. As reported by other studies, salt-tolerant rootstocks had the highest accumulation of organic osmolytes, such as proline in leaves and roots (Shahid, et al. 2019). These results highlighted the different behavior of citrus rootstock under salt stress

and confirmed the results obtained by other authors regarding the influence of rootstock to salinity response (Shahid, et al. 2019). In agreement with other study (Vives-Peris et al., 2017), *C. macrophylla* showed a higher quantity of proline than Carrizo citrange. Indeed, an increment of leaf proline was observed with increasing salt stress in Bitters, *C. volkameriana* and Furr.

Hormone analyses

Salinity determines an accumulation of ions that affect plant physiological responses causing osmotic stress, ion toxicity and nutritional imbalances. The physiological response of salinity may be modulated by hormones and among all, ABA mediated several responses of salt stress, such as proline accumulation, stomatal closure and growth inhibition (Gómez-Cadenas et al., 1998). It was demonstrated that ABA treatment under salt stress has a protective role in citrus plant, because it could reduce ethylene release and leaf abscission (Gomez Cadenas et al.,2002). Also, previous studies demonstrated the role of PA, ABA catabolite, that likely emerged in plants as a signaling molecule that improved plant physiology, environmental adaptation, and development (Balfagón et al., 2019). Previous research reported that SA and JA enhanced salinity stress tolerance (Khan et al. 2012; Ibrahim et al. 2018). The Ja precursor, 12-oxo-phytodienoic acid (OPDA), plays a decisive role in stomatal closure and it is related to plant tolerance (Kazan 2015). Under abiotic stress, SA plays an important role in protecting photosystem II (PSII) complex from an increase of ROS (Wang et al. 2010). In addition, SA and JA confer tolerance in Arabidopsis with increasing temperature (Clarke et al. 2009). Furthermore, the union of SA and methyl jasmonate (MeJA) mitigate abiotic stresses because they could control several biochemical and physiological traits (Khan et al. 2012). Results obtained in this work highlighted the different performance of

each genotype under salt conditions. Carrizo citrange had the highest value of ABA level in leaf at 30 mM NaCl, while C35 citrange had the highest value at 60 mM NaCl. This response was probably influenced by stomatal opening and regulated by high temperatures, as previously reported by Balfagon et al. (2019).

Regarding the accumulation of JA and JA-lle, the results confirmed their importance in salt stress, as reported by Balfagon et al. (2019). The decrease of JA concentration in Swingle citrumelo, *C. macrophylla* and *C. volkameriana*, well known as salt tolerant rootstocks, were related to some physiological mechanisms which involved the exclusion of toxic ions (Na^+ and Cl^-). In agreement to other study, SA was significantly accumulated in response to salt stress (Balfagon et al. 2019). Regarding IAA concentration, Carrizo citrange showed an increase of its content, while a slight decrease was found in *C. macrophylla*, in agreement with root and shoot tissue contents investigated by Vives-Peris and colleagues (2017). On the other hand, the pattern of accumulation of phytohormone in leaf depends on the rootstock genotype, although phytohormone is related to abiotic stress response, because it is described as important mediators in several biochemical processes (Gómez-Cadenas et al. 2015).

Conclusions

In conclusion, our study investigated the effect of salinity on citrus rootstock combining physiological and morphological analysis with the assessment of the antioxidant activity and the hormone quantification. Indeed, morphological measurements in plants subjected to salt stress pointed out the effects of salinity depending on the genotype. Our results showed that Furr had a similar response to *C. macrophylla* and *C. volkameriana*, that showed more rapid osmotic adjustments under salt conditions. A growth reduction was observed in all genotypes at 60 mM NaCl, especially with regards to leaf area and specific leaf area, apart from Bitters. The two citranges rootstocks were found to be sensitive to salt stress confirming high levels of MDA and the hormonal trend in Swingle citrumelo, *C. macrophylla* and *C. volkameriana*, confirmed their resistance to salt. Overall, Furr had a tolerance response similar to *C. macrophylla* and *C. volkameriana*.

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Chapter 7 - General Conclusions

In citriculture, as well as for several other tree crops, rootstock plays a fundamental role for its active role such as: physical support, absorption of water and nutrients, biosynthesis of plant growth regulators (phytormones), and physicochemical interaction with the surrounding soil. Grafting also played an important role in spreading the cultivation of a species in areas initially considered unsuitable due to environmental and/or biotic constraints. There is no ideal rootstock for all pedoclimatic conditions and scions; therefore, insights on the rootstock performances under different environments and/or in combination with different accessions are essential to guide the choice of the scion/rootstock combinations that best suit a given growing area. Indeed, rootstocks influence the physiology of plants, including traits of economic relevance such as fruit yield, production, fruit size, percentage of juice and juice quality, tree vigor, and resistance against biotic and abiotic stresses (such as cold, alkalinity, drought, salt, and flooding).

In this context, eleven cultivars of blood orange, grown under the same environmental conditions, were characterised, and compared with respect to their contents of phytochemical compounds and bioactive properties. The study focused on the influence of the genotype on the synthesis of phenolic compounds and, in particular of anthocyanins which are responsible for the red colour of the peel and the juice. Furthermore, after a pomological characterization of blood oranges, the influence of phenolic compounds on antioxidant activity was evaluated. The main fruit quality traits are highly dependent on the scion genotype, even if rootstocks improve qualitative performance. For this reason, the evolution of metabolic profile of blood orange during maturation was investigated to understand if any rootstock effects are related to changes in key expressions genes related to fruit quality. This comparative study indicates a positive

correlation between rootstock and the regulation of fruit-quality related genes in the scion and the accumulation of bioactive compounds in the fruit. We also investigated the influence of the rootstocks on metabolic profile, especially anthocyanin, and antioxidant activity and their interaction with low temperature. The results showed that the low temperature affects the accumulation of anthocyanins, although a decisive role is played by the rootstock.

Rootstock influence several qualitative traits, such as yield and productivity as shown in Mandared, a pigmented mandarin, grafted onto 11 rootstocks, but it can also play an active role in conferring resistance to salt stress as shown in the last chapter. In detail, the behaviour of rootstocks under salt stress has been assessed evaluating the performance of novel and spread ones through the analysis of the morpho-physiological, enzymatic and hormonal responses.

Altogether, the experimental evidences provided in this thesis underline the pivotal role exerted by the rootstock in influencing traits of agronomical interest. An understanding of the role of the rootstock in different environment and/or in combination with different scions will provide useful information for the set-up of novel productive orchards and toward the identification of the physiological and genetic mechanism underlying traits of interest.

Annexes

Papers published on Journals

Modica G., Siracusa L., Ruberto G., Di Guardo M., La Malfa S., Gentile A., Continella A. 2022. L'accumulo di polifenoli dipende dal portinnesto e dalle temperature invernali. Rivista di Frutticoltura e di Ortofloricoltura, 1: 22-25.

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Lana G.*, Modica G.*, Las Casas G., Siracusa L., La Malfa S., Gentile A., Sicilia A., Distefano G., Continella A. (2021). Molecular Insights into the Effects of Rootstocks on Maturation of Blood Oranges. Horticulturae, 7(11): 468. https://doi.org/10.3390/horticulturae7110468

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Abstracts of conference proceedings

Modica G., Arcidiacono F., Massimino Cocuzza G.E., Catara V., Tribulato A., La Malfa S., Continella A. 2021. Development of sustainable control strategies for citrus under the threat of climate change for the prevention of HLB entry into the EU: the Italian experience (LIFE18 CCA/ES/001109). In: III International Organic Fruit Symposium and I International Organic Vegetable Symposium. Book of abstract p.82, Catania, 14-17 dicembre 2021.

Oliveri C., Modica G., Bella P., Cirvilleri G., Continella A., Catara V. 2021. Preliminary evaluation of a zinc and copper compound complexed with citric-acid hydracids for the control of *Plenodomus tracheiphilus* causal agent of Citrus Mal secco disease. In: III International Organic Fruit Symposium and I International Organic Vegetable Symposium. Book of abstract p.81, Catania, 14-17 dicembre 2021.

Di Guardo M., Farneti B., Khomenko I., Modica G., Mosca A., Distefano G., Bianco L., Troggio M., Sottile F., La Malfa S., Biasioli F., Gentile A. 2021. Caratterizzazione genetica di collezione di mandorlo ed analisi del profilo aromatico del seme, allo stato fresco e dopo tostatura. In: XIII Giornate Scientifiche SOI. Acta Italus Hortus vol. 26, p. 94, Firenze: Società di Ortoflrofrutticoltura Italiana (SOI), Catania, 22-23 Giugno 2021.

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Modica G. 2020. A multidisciplinary approach for citrus rootstock evaluation. "Higher education for sustainable food production: abstract of 2st Joint Meeting of Agriculture-oriented PhD programs at UNICT, UNIFG AND UNIUD: Catania, Sicilia, 14-16 September (2020) Book of abstract edited by A. Priolo p. 22.

Modica G. 2019. A multidisciplinary approach for citrus rootstock evaluation. "Higher education for sustainable food production: abstract of 1st Joint Meeting of Agriculture-oriented PhD programs at UNICT, UNIFG AND UNIUD: Catania - Salina, Italy, 17th-21st June [2019] Book of abstract edited by A. Priolo and S. Tortorici. – Vol. 15 p. 44 - QUADERNI CSEI Catania III serie, giugno 2019.

Continella A., Pannitteri C., Modica G., La Malfa S., Legua P., Distefano G., Nicolosi E., Gentile A. 2018. Valutazione vegeto-produttiva di nuovi portinnesti e influenza sulla pigmentazione dei frutti di arancia rossa. In: XII Giornate Scientifiche SOI. Acta Italus Hortus vol. 23, p. 58, Firenze: Società di Ortoflrofrutticoltura Italiana (SOI), ISBN: 978-88-905628-3-9, Bologna, 19-22 giugno 2018.

Research stages in national or international universities and/or research institutions

- Research at activity the Edmund Mach Foundation at S. Michele all'Adige (TN, Italy). From 18-03-2019 to 18-04-2019.

The activity was aimed at a characterization of several pigmented citrus varieties and of a cultivar collection of Italian and foreign almonds. By using mass spectrometry (GC-PTR-ToF-MS), the main primary and secondary metabolites and volatile compounds were identified and quantified.

- Research activity at the the Istituto di Chimica Biomolecolare del CNR (Catania, Italy). From 2-12-2020 to 30-03-2021.

During this period, the research was focused on targeted primary and secondary metabolites on citrus fruits. In particular, I investigated on phenolic compounds (anthocyanins, flavanones, flavones and hydroxycinnamic acids), sugars and organic acids through hyphenated chromatographic techniques. Also, biochemical markers in citrus fruits were identified and quantified by HPLC / DAD and HPLC / ESI-MS analyses.

- Research activity at the Ecophysiology and Biotechnology laboratory of the Department of Agricultural Sciences and the Natural Environment of University Jaume I, Castellon de la Plana, Spagna. From 19-04-2021 to 21-06-2021 and from 11-10-2021 to 11-12-2021.

The research was focused on citrus tolerance responses under abiotic stresses. In particular, plant hormones and primary and secondary metabolites on tissue plants subjected to water and salt stress were analyzed by LC/MS.

Attendance to congresses and workshops

Attendance to III International Organic Fruit Symposium and I International Organic Vegetable Symposium – Catania (Italy), 14-17 December 2021.

Attendance in the XIII Scientific days SOI Catania (Italy), 22-23 June 2021.

Attendance to the Joint workshop of the Agriculture-oriented PhD programs UNICT, UNIFG and UNIUD, Catania (Italy), 14-16 September 2020.

Attendance to the Joint workshop with PhD students from Foggia and Udine - University of Catania, 17 June 2019, Island of Salina (Italy), 18-21 June 2019.

Attendance to courses

Biometry and data analysis course, Department of Agriculture, Food and Environment, University of Catania. 18-22 November 2019 (6 ECTS).

Phytochemistry - Department of Drug sciences, University of Catania. - Degree Course in Applied Pharmaceutical Sciences. February - June 2020 (6 ECTS).

Ecophysiology in fruit crops - Department of Agriculture, Food and Environment, University of Catania. - Degree Course in Agricultural Science and Technology. February - June 2020 (6 ECTS).

Managing biological data with Excel (advanced) and R, Department of Agriculture, Food and Environment, University of Catania. 11-22 February 2019 (6 ECTS).

Word processing, Department of Agriculture, Food and Environment, University of Catania. July 2019 (1 ECTS).

Supplementary figures to chapter 3

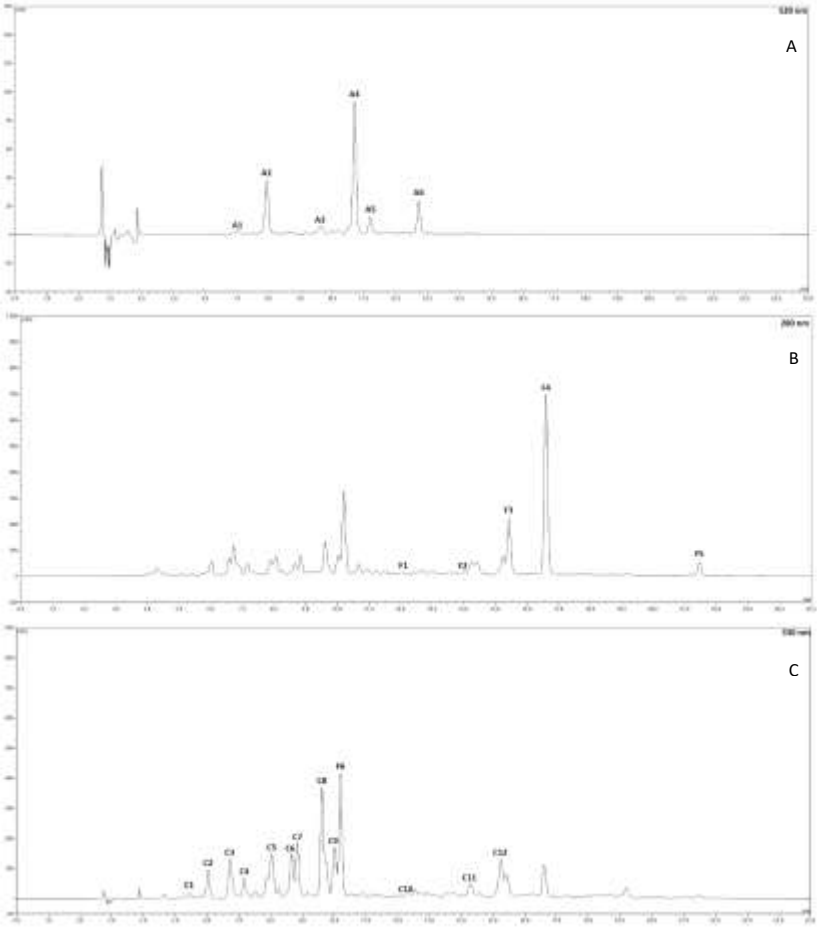
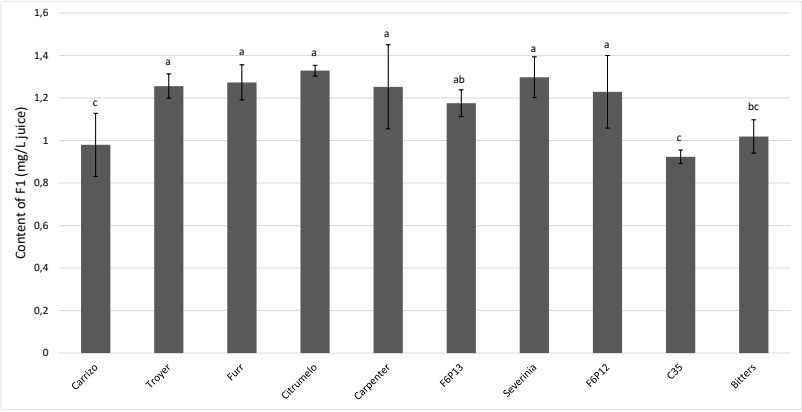


Figure S3.1. HPLC-DAD chromatograms, visualized at 520 nm (A) for anthocyanins, 280 nm (B) for flavanones and 330 nm (C) for flavones and hydroxycinnamic acid, of a representative sample of Tarocco Scirè blood orange juice. Peak letters and numbers refer to text and are listed in Table 1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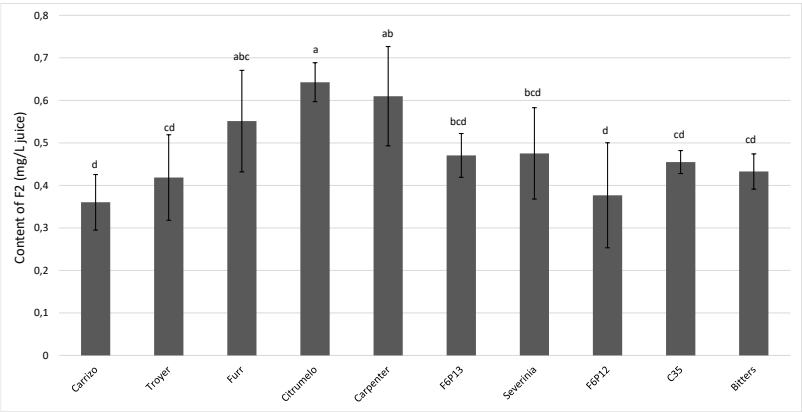
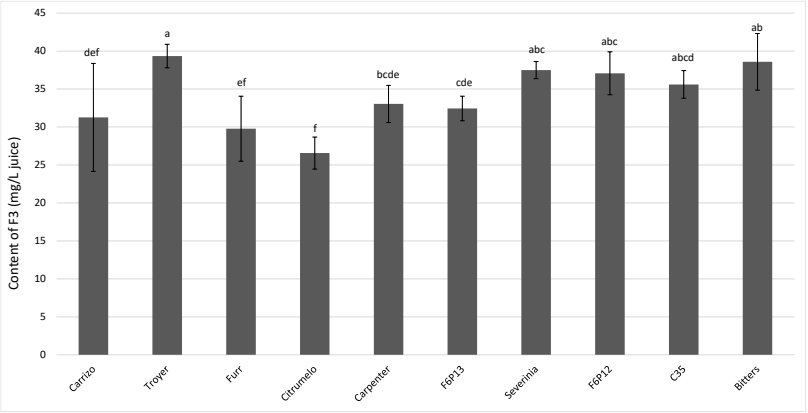
1,4
1,2
1
0,8
0,6
0,4
0,2
0
c
a
a
a
a
ab
a
a
c
bc
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of F2 (mg/L juice)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
d
cd
abc
a
ab
bcd
bcd
d
cd
cd
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of F3 (mg/L juice)
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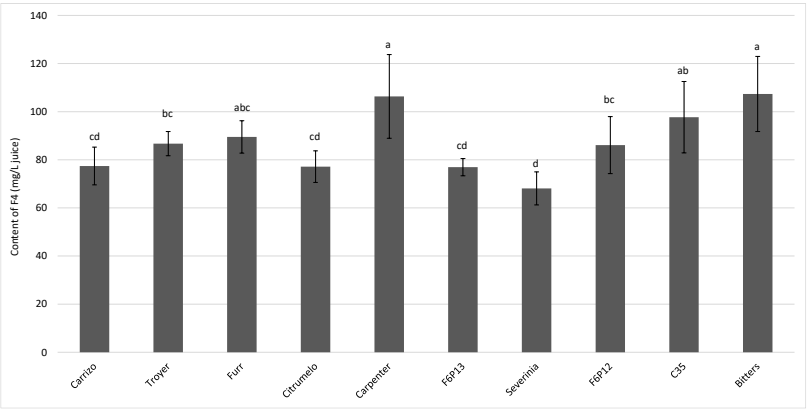
40
35
30
25
20
15
10
5
0
def
a
ef
f
bcde
cde
abc
abc
abcd
ab
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severnia
F6P12
C35
Bitters


Content of F4 (mg/L juice)
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120
100
80
60
40
20
0
cd
bc
abc
cd
a
cd
d
bc
ab
a
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severnia
F6P12
C35
Bitters

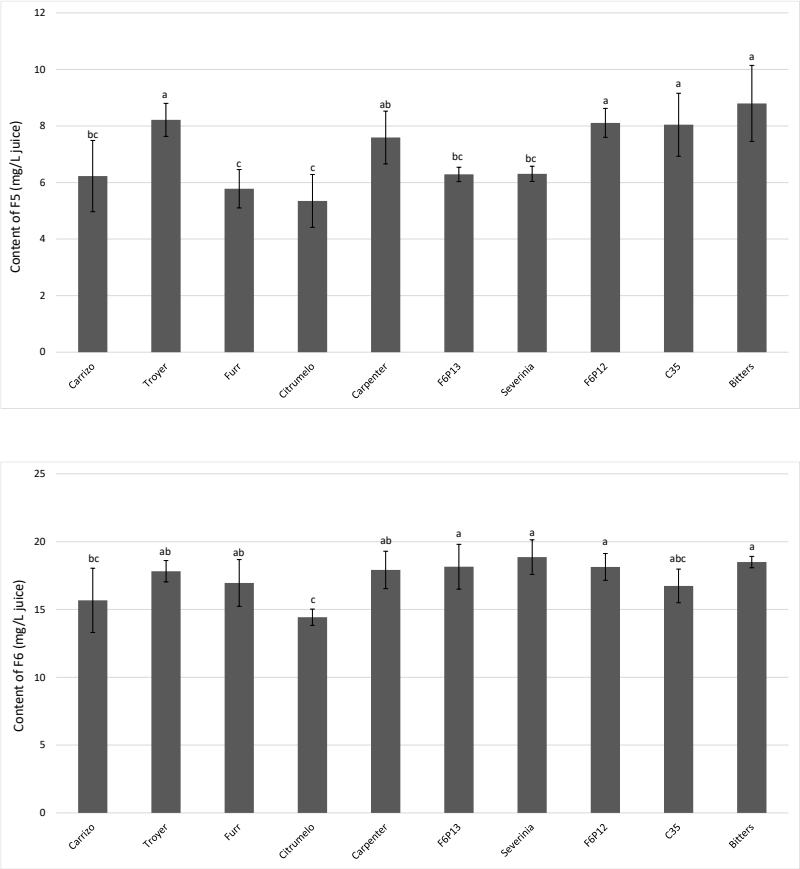


Figure S3.2. Tarocco Scire individual flavonoids variations depending on rootstock genotype during year I. Peak codes F1-F6 refer to Table 1. Values followed by the same letter are not significant different according to Fisher's Least Significant Difference (LSD) procedure at 95.0 % confidence level

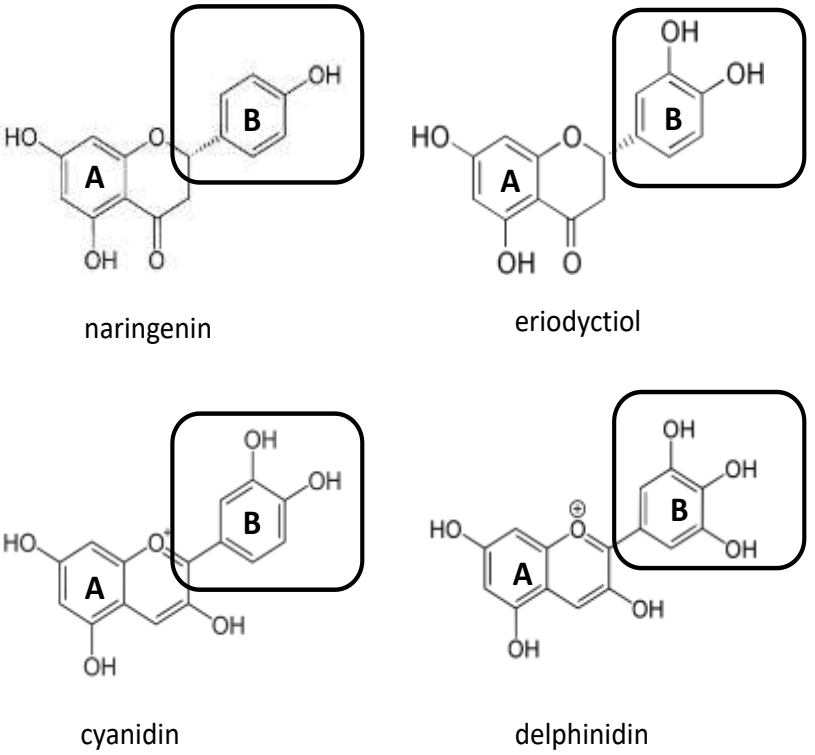
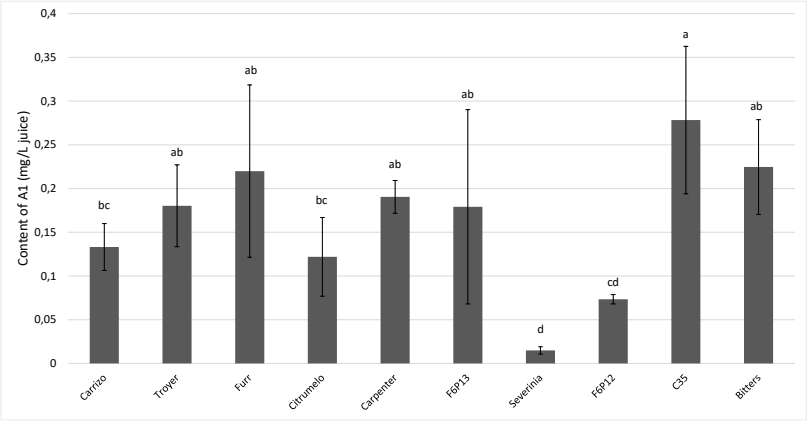


Figure S3.3. Chemical structures of flavanones naringenin and eriodyctiol (above) and anthocyanins cyanidin and delphinidin (below); B rings coming from shikimate pathway are highlighted. See text for details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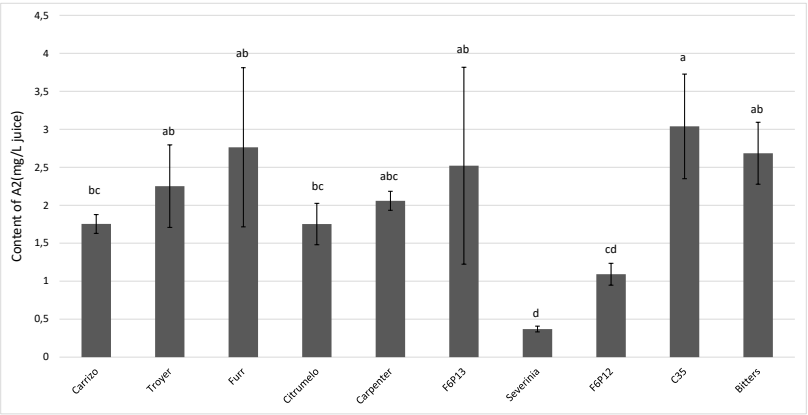
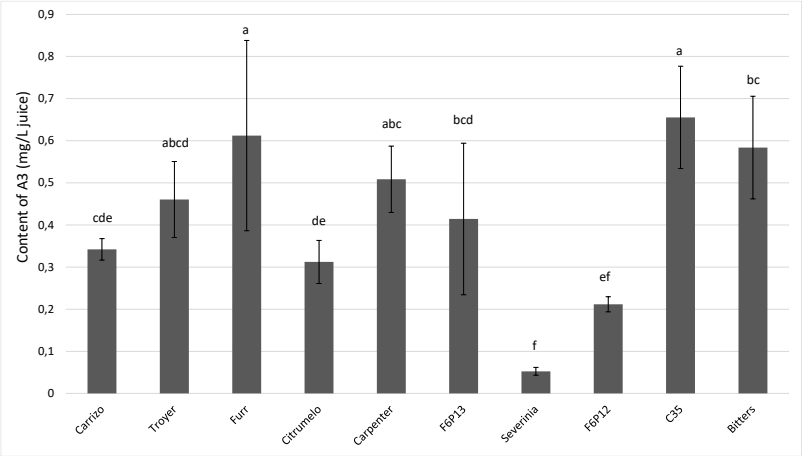
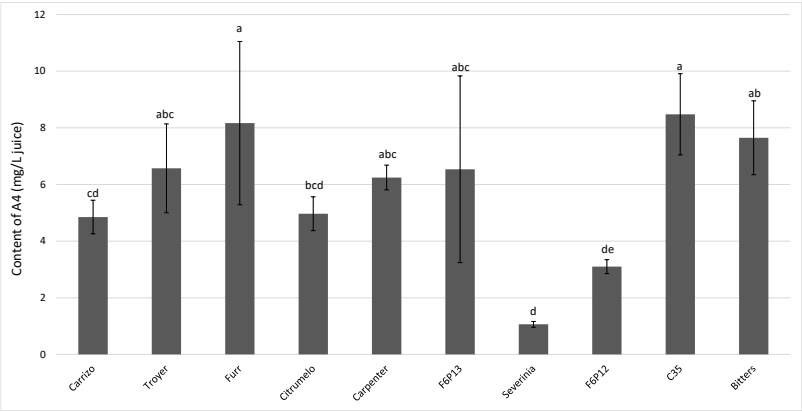
0,3
0,25
0,2
0,15
0,1
0,05
0
bc
ab
ab
bc
ab
ab
d
cd
a
ab
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of A2(mg/L juice)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
bc
ab
ab
bc
abc
ab
d
cd
a
ab
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of A3 (mg/L juice)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
cde
abcd
a
de
abc
bcd
f
ef
a
bc
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of A4 (mg/L juice)
12
10
8
6
4
2
0
cd
abc
a
bcd
abc
abc
d
de
a
ab
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters

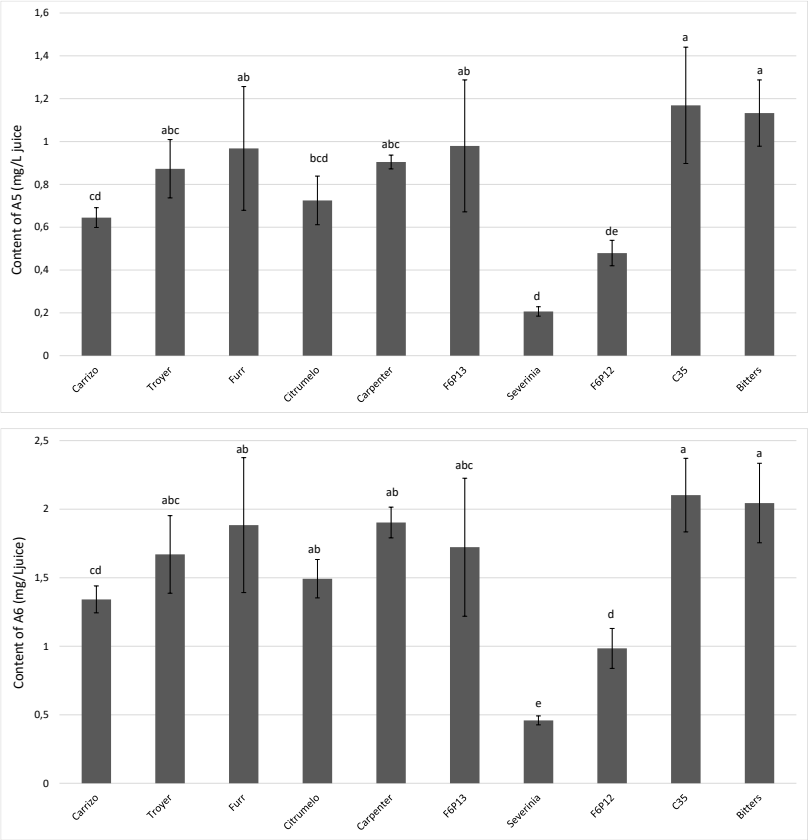
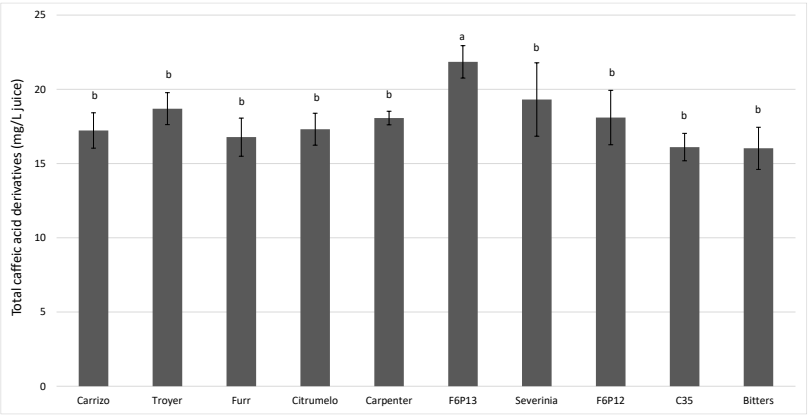
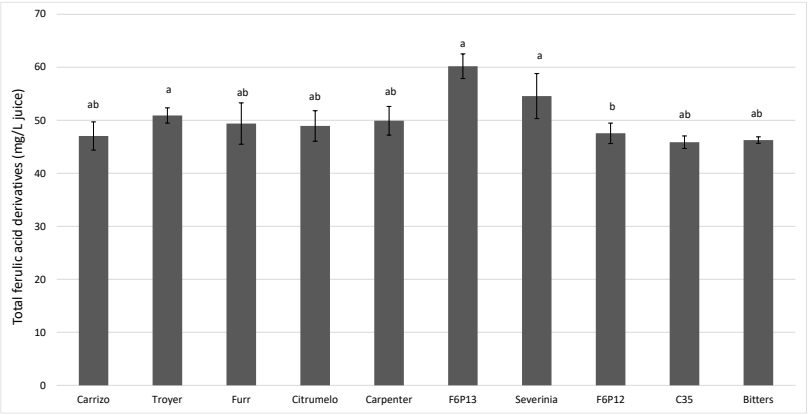
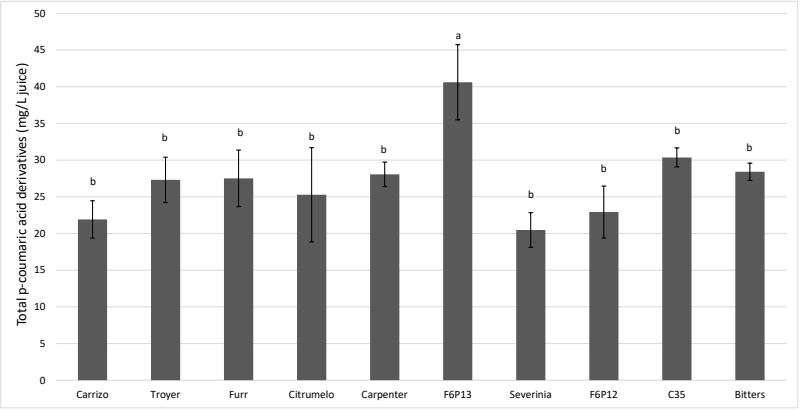


Figure S3.4. Tarocco Scire individual anthocyanin variations depending on rootstock genotype during year I. Peak codes A1-A6 refer to Table 1 . Values followed by the same letter are not significant different according to Fisher's Least Significant Difference (LSD) procedure at 95.0 % confidence level


Total caffeic acid derivatives (mg/L juice)
0
5
10
15
20
25
b
b
b
b
b
a
b
b
b
b
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Total ferulic acid derivatives (mg/L juice)
0
10
20
30
40
50
60
70
ab
a
ab
ab
ab
a
a
b
ab
ab
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Total p-coumaric acid derivatives (mg/L juice)
0
5
10
15
20
25
30
35
40
45
50
b
b
b
b
b
a
b
b
b
b
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters

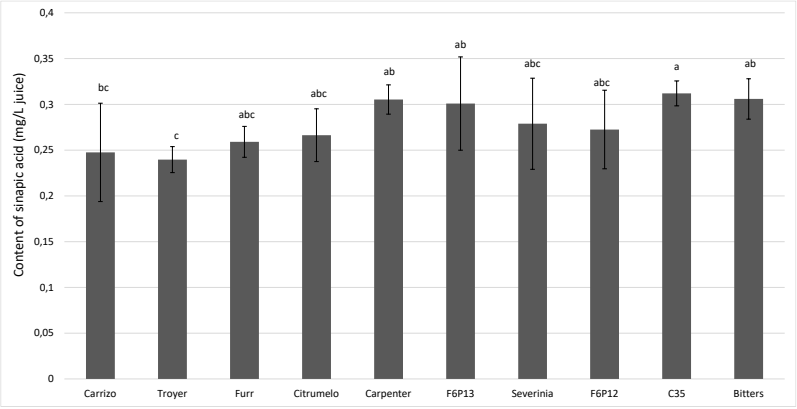
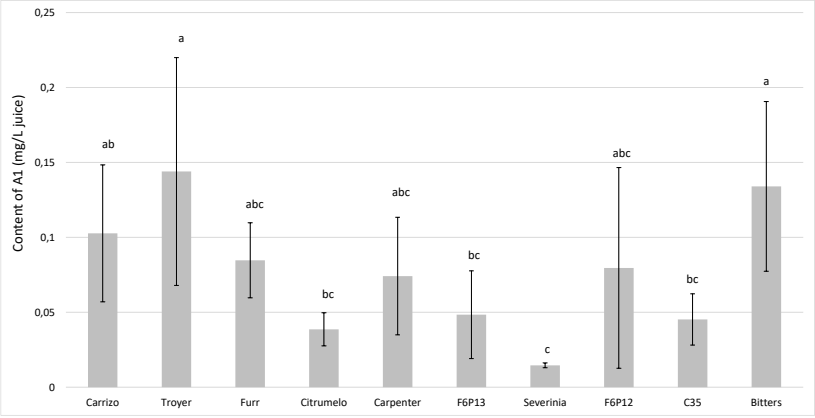
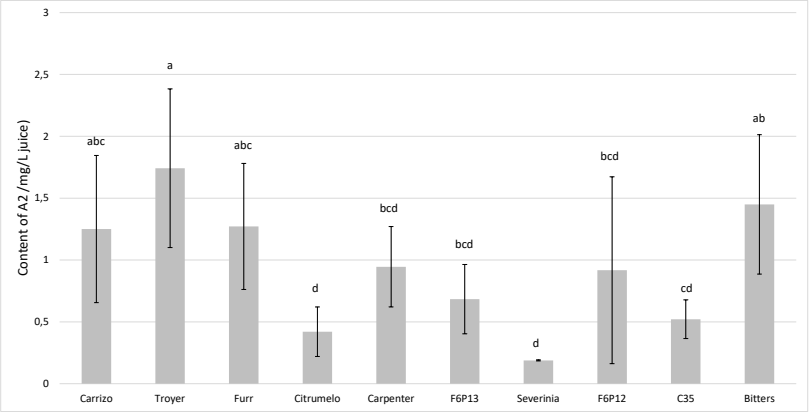
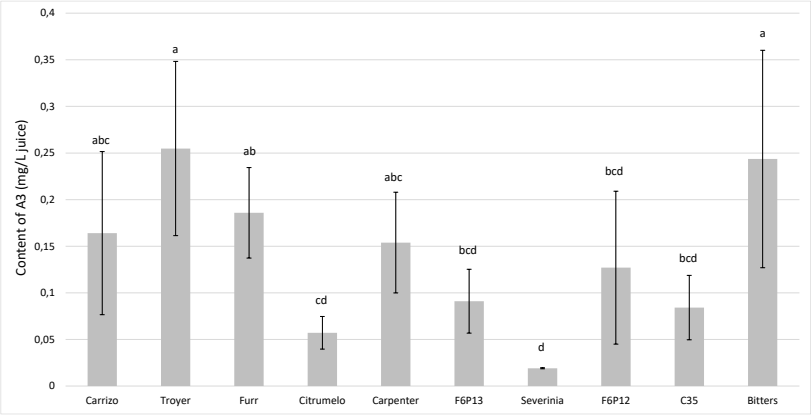
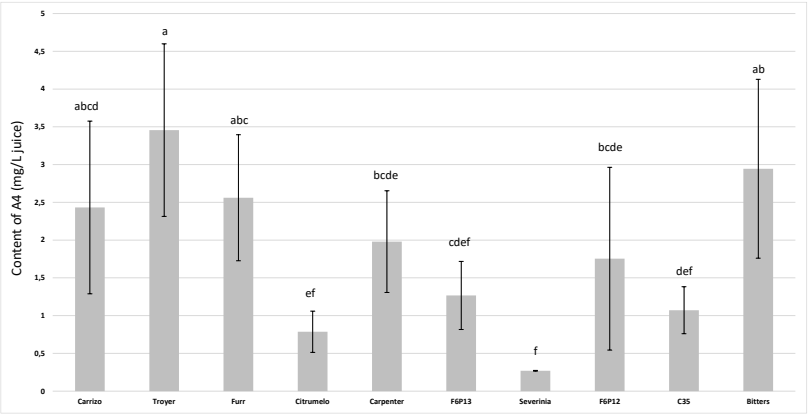


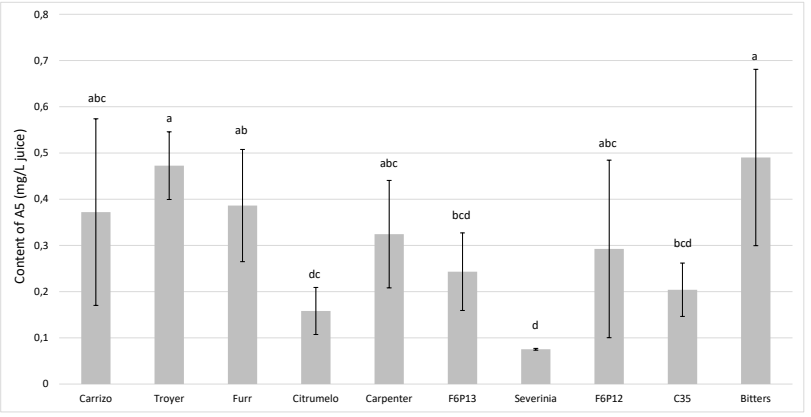
Figure S3.5. Tarocco Scire hydroxycinnamic acids and derivatives' variations depending on rootstock genotype during year I. Metabolites are grouped according to the corresponding hydroxycinnamic acid (see Table 1 for peak codes C1-C12): caffeic acid derivatives= C1+C5; ferulic acid derivatives = C3+C6+C8+C9+C12; p-coumaric acid derivatives = C2+C4+C7+C11; sinapic acid = C10.
Values followed by the same letter are not significant different according to Fisher's Least Significant Difference (LSD) procedure at 95.0 % confidence level


Content of A1 (mg/L juice)
0,25
0,2
0,15
0,1
0,05
0
ab
a
abc
bc
abc
bc
c
abc
bc
a
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of A2 /mg/L juice)
3
2,5
2
1,5
1
0,5
0
abc
a
abc
d
bcd
bcd
d
bcd
cd
ab
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of A3 (mg/L juice)
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
abc
a
ab
cd
abc
bcd
d
bcd
bcd
a
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of A4 (mg/L juice)
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
abcd
a
abc
ef
bcde
cdef
f
bcde
def
ab
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of A5 (mg/L juice)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
abc
a
ab
dc
abc
bcd
d
abc
bcd
a
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters

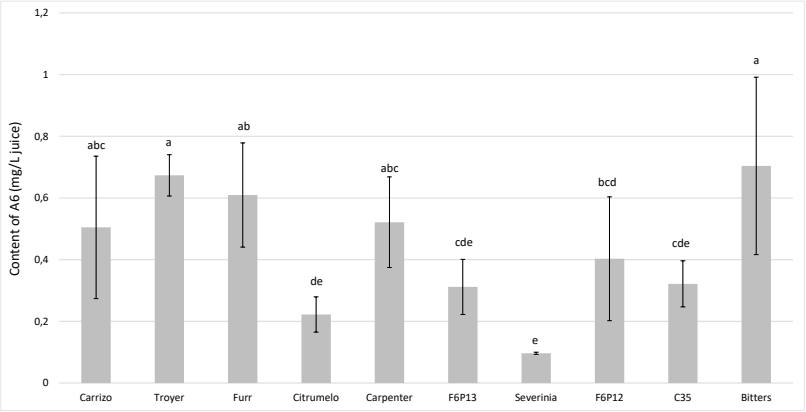
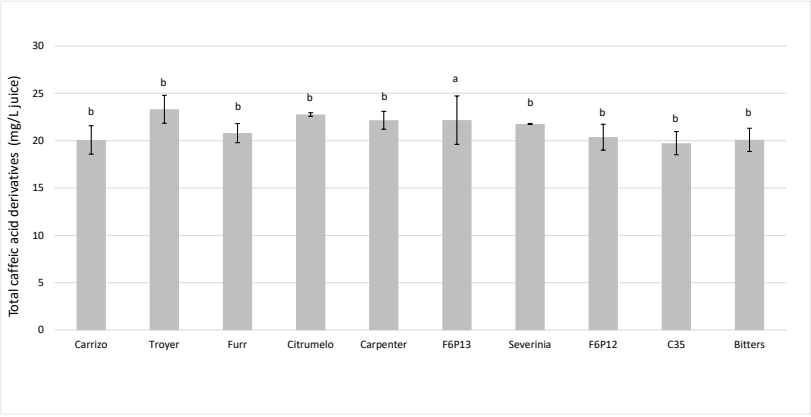
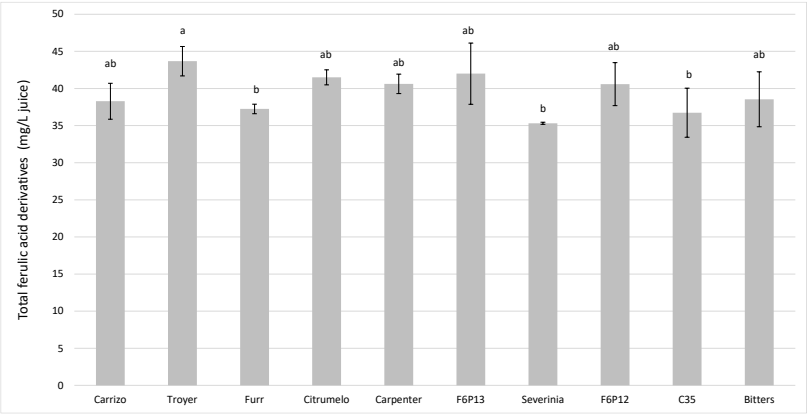
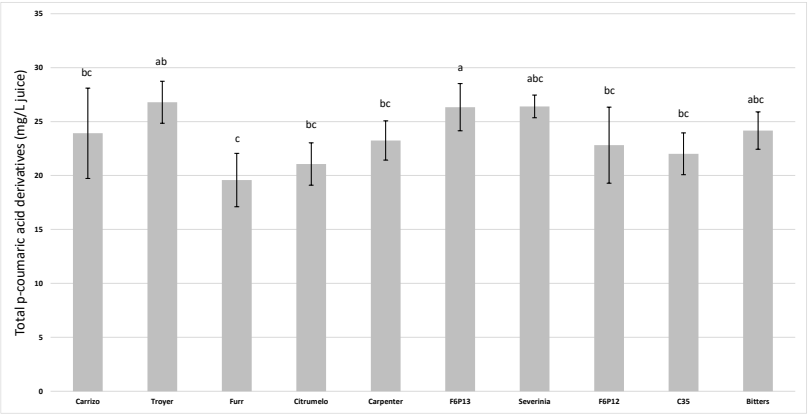


Figure S3.6. Tarocco Scire individual anthocyanin variations depending on rootstock genotype during year II. Peak codes A1-A6 refer to Table 1. Values followed by the same letter are not significant different according to Fisher's Least Significant Difference (LSD) procedure at 95.0 % confidence level


Total caffeic acid derivatives (mg/L juice)
30
25
20
15
10
5
0
b
b
b
b
b
a
b
b
b
b
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Total ferulic acid derivatives (mg/L juice)
50
45
40
35
30
25
20
15
10
5
0
ab
a
b
ab
ab
ab
b
ab
b
ab
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Total p-coumaric acid derivatives (mg/L juice)
35
30
25
20
15
10
5
0
bc
ab
c
bc
bc
a
abc
bc
bc
abc
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters

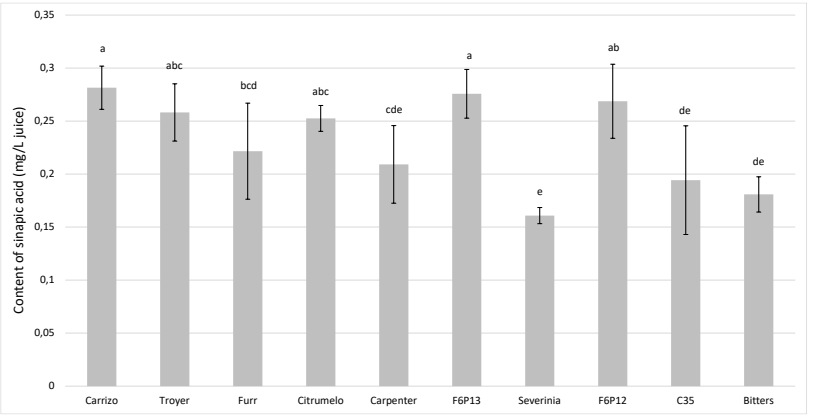
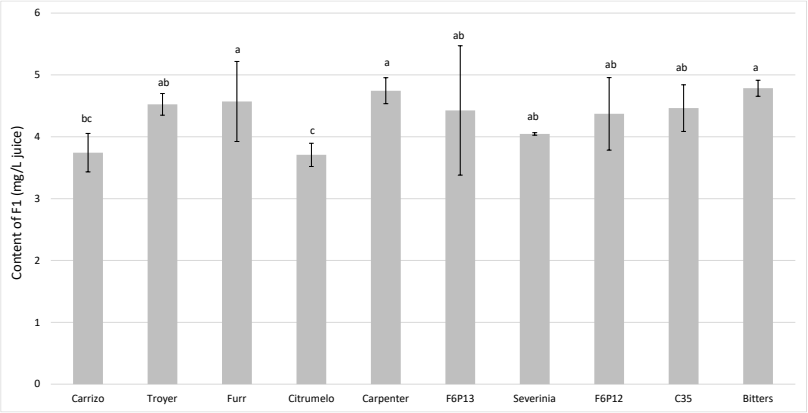
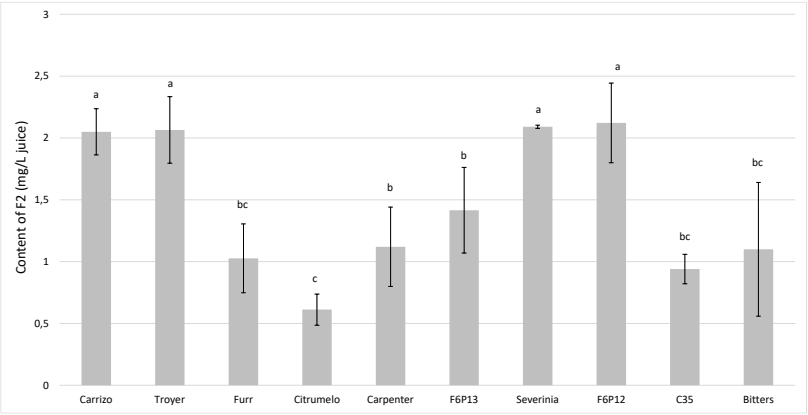
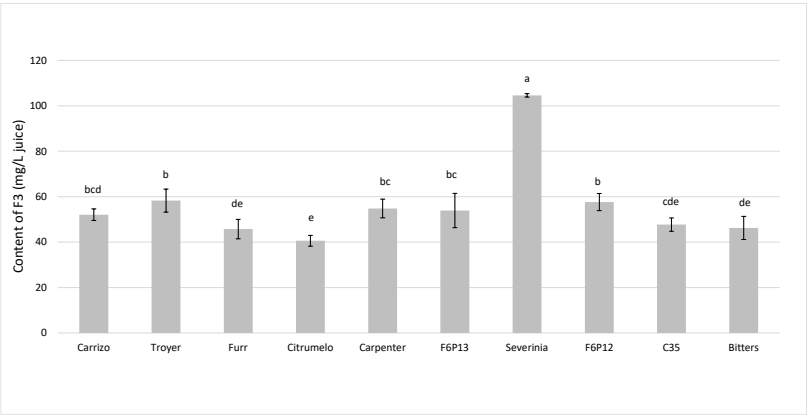
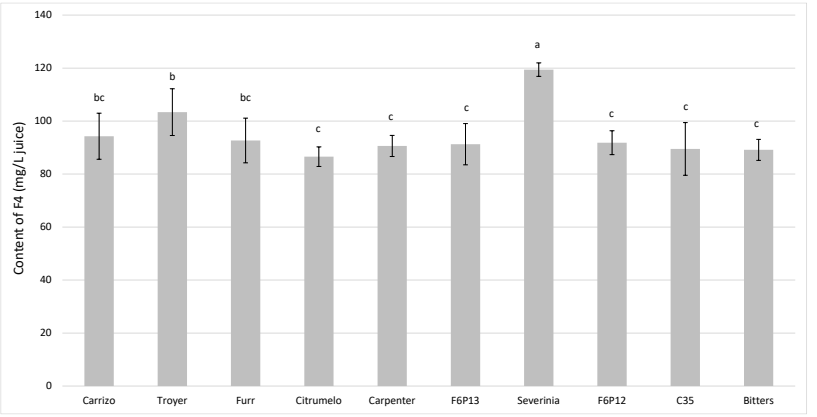


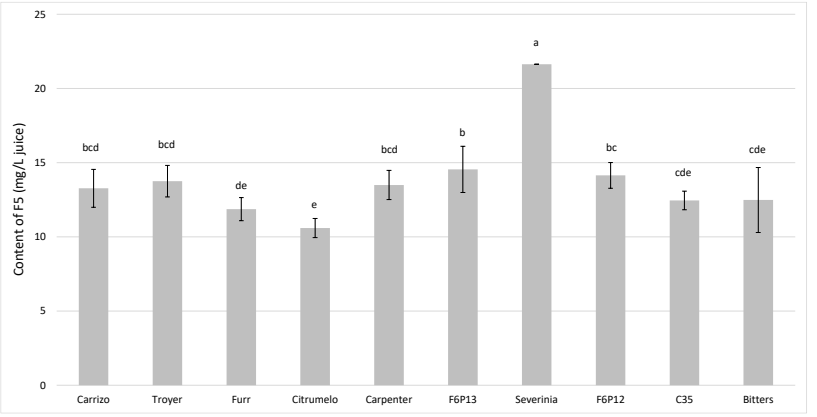
Figure S3.7. Tarocco Scire hydroxycinnamic acids and derivatives' variations depending on rootstock genotype during year II. Metabolites are grouped according to the corresponding hydroxycinnamic acid (see Table 1 for peak codes C1-C12): caffeic acid derivatives= C1+C5; ferulic acid derivatives = C3+C6+C8+C9+C12; p-coumaric acid derivatives = C2+C4+C7+C11; sinapic acid = C10. Values followed by the same letter are not significant different according to Fisher's Least Significant Difference (LSD) procedure at 95.0 % confidence level


Content of F1 (mg/L juice)
0
1
2
3
4
5
6
bc
ab
a
c
a
ab
ab
ab
a
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of F2 (mg/L juice)
0
0,5
1
1,5
2
2,5
3
a
a
bc
c
b
b
a
a
bc
bc
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of F3 (mg/L juice)
0
20
40
60
80
100
120
bcd
b
de
e
bc
bc
a
b
cde
de
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of F4 (mg/L juice)
140
120
100
80
60
40
20
0
bc
b
bc
c
c
c
a
c
c
c
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters


Content of F5 (mg/L juice)
25
20
15
10
5
0
bcd
bcd
de
e
bcd
b
a
bc
cde
cde
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters

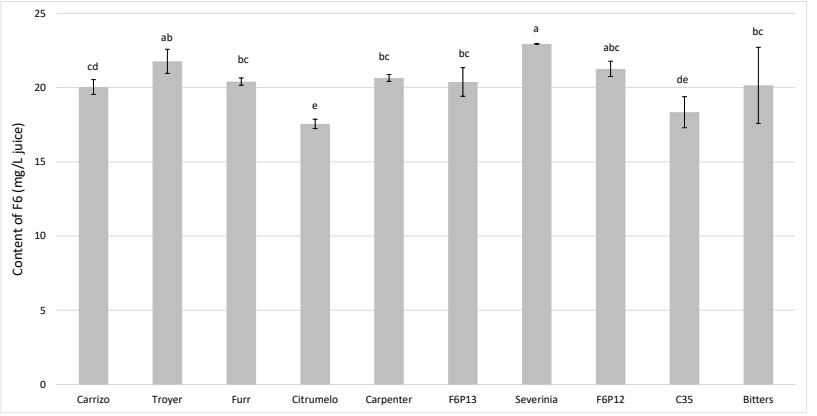

Content of F6 (mg/L juice)
25
20
15
10
5
0
cd
ab
bc
e
bc
bc
a
abc
de
bc
Carrizo
Troyer
Furr
Citrumelo
Carpenter
F6P13
Severinia
F6P12
C35
Bitters

Figure S3.8. Tarocco Scire individual flavonoids variations depending on rootstock genotype during year II. Peak codes F1-F6 refer to table 1. Values followed by the same letter are not significant different according to Fisher's Least Significant Difference (LSD) procedure at 95.0 % confidence level

Supplementary figures to chapter 5

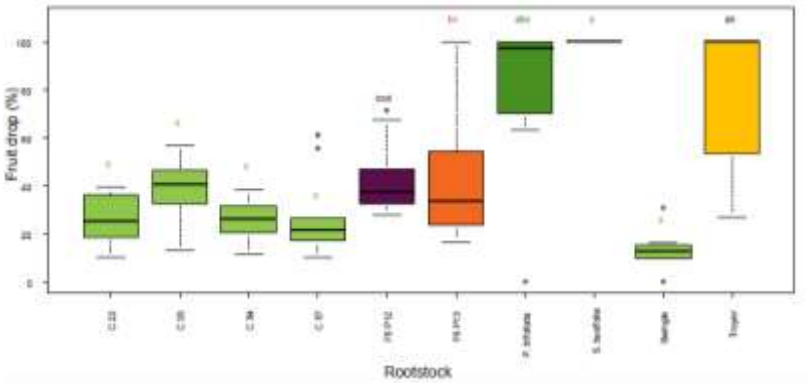


Figure S5.1. Percentage of fruit drop recorded in 2016

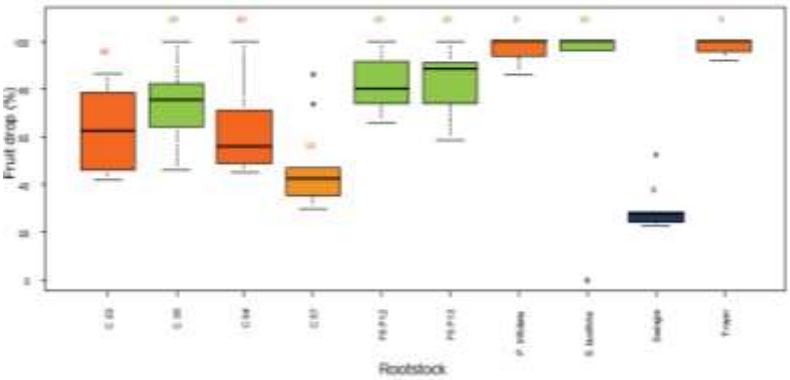


Figure S5.2. Percentage of fruit drop recorded in 2017

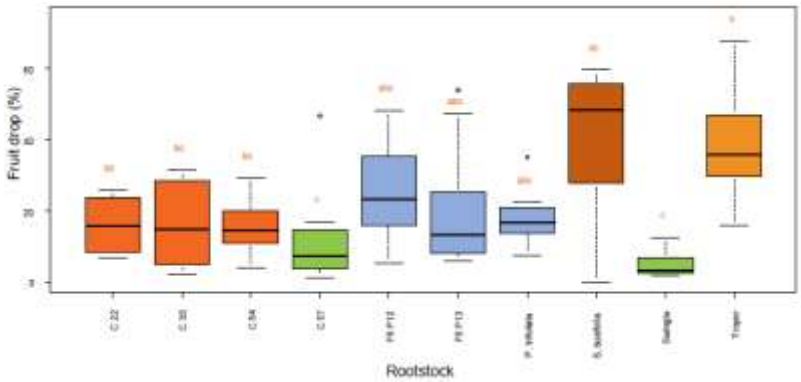


Figure S5.3. Percentage of fruit drop recorded in 2018

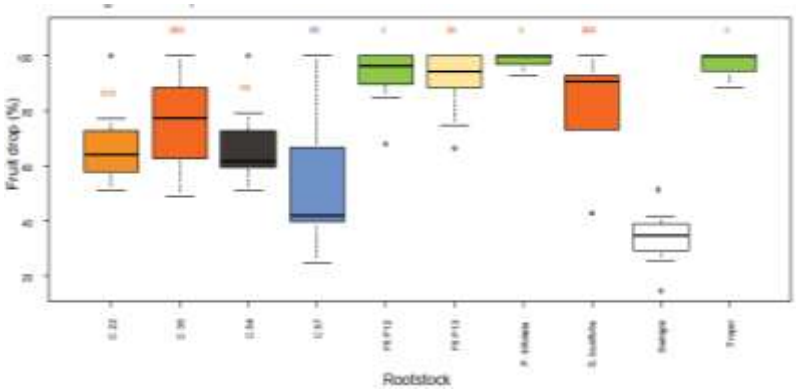


Figure S5.4. Percentage of fruit drop recorded in 2019